Tulasi Satyanarayana
Jennifer Littlechild
Yutaka Kawarabayasi *Editors*

Thermophilic Microbes in Environmental and Industrial Biotechnology

Biotechnology of Thermophiles

Second Edition

Springer

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Foreword

Not long ago, thermophilic microbes and their hot lifestyle had been seen as pretty rare secondary adaptations of mesophiles to the hot environment. In first cultivation experiments, hot environments yielded only very few thermophilic bacteria. Therefore, hot environments seemed to harbor only a very limited microbial diversity. However, during the last five decades, more and more extreme thermophiles had been discovered. Among those were hyperthermophiles which grow even optimally at pasteurization temperatures (80°C and above) and which were unable to make a living at 60°C or below. In addition, the groundbreaking discovery of the phylogenetic relationships of microbes by Carl Woese had changed our view of thermophiles in the history of life: Thermophiles clearly came before mesophiles. They are present in almost all groups of bacteria and archaea and represent an enormous phylogenetic diversity. In the ssrRNA-based tree of life, some phylogenetic lineages with very deep branching points (e.g., the Thermoproteales) even appear to be covered exclusively by thermophiles. Moreover, direct studies of ssrRNA genes within the hot environments similar to moderate environments revealed an enormous microbial complexity and phylogenetic diversity. By the extraordinary heat resistance and sturdiness of the cells including their components and by their complex metabolic abilities, thermophiles appear to be a "treasure chamber" for basic research and for biotechnological applications in the future.

The purpose of this new book "*Thermophilic Microbes in Environmental and Industrial Biotechnology*" edited by the world-renowned experts Tulasi Satyanarayana, Jennifer Littlechild, and Yutaka Kawarabayasi is to present an elaborate overview about all fields of thermophiles research and their potential for biotechnological applications. The first part is dedicated to thermophiles in the environment in general and provides important insights into the diversity and function of microbes and their viruses in different hot environments. This overview covers even less-studied biotopes like hot Artesian springs, self-heated compost, geothermal power plants, and hot radioactive waste water. The second part deals with genomics, metagenomics, and biotechnology in thermophilic bacteria and archaea. It includes genome analyses and novel ways of enzyme discovery, comparative genomics, host-vector systems in bacteria and archaea, functional studies of molecular chaperones,

the heterologous production of thermostable proteins, and metagenomic approaches to discover thermostable enzymes in environmental samples. In addition, a broad section of this part is addressed to various groups of thermophilic enzymes, their production and applications in biotechnology. All important enzymes are discussed in great detail. Furthermore, a chapter with insightful basic studies to increase protein thermostability is included. This book is unique by its concept and fills a gap in the literature about modern biotechnology. Therefore, it should be present in any biotechnology laboratory in the future. Congratulations to the authors about their great book and I wish them every success in their future

Karl O. Stetter
Professor of Microbiology
Germany

Preface

It is now very well known that microbial life can thrive in the upper as well as lower temperature limits that are known to be compatible with life. Temperature provides a series of challenges, from structural devastation due to ice crystal formation at one extreme to the denaturation of biomolecules and cell components at the other. A wide variety of microorganisms have, however, been discovered that can overcome these challenges. The thermophiles, the so-called heat-loving organisms, tolerate high temperatures and also usually require elevated temperatures for their growth and survival. Thermophilic organisms able to grow around 60°C have been known for over 90 years, while hyperthermophilic organisms able to grow over 80°C have been recognized only three decades ago. The currently known upper temperature limit for life is 121°C. Within the past few decades, a great diversity of thermophilic microbes have been isolated that exist in both natural and artificial hot environments. The microbial diversity of these habitats as analyzed by molecular biology techniques has indicated that the diversity of thermophilic organisms extends much further than those species already isolated. There are a large number of metagenomic projects being undertaken to look at complete microbial and viral communities in these environments.

An international effort has been made to understand the biology, evolution, role in the environment, physiology, and biochemistry of thermostable enzymes. These proteins have many applications industrially for "white biotechnology" including the generation of bioenergy. Their use is also essential in molecular biology for gene amplification using PCR.

This publication is an attempt towards understanding various aspects of thermophilic organisms. In order to review the progress made in thermophile biology and their biotechnological applications, we have collected a selection of chapters from the experts in the field. We wish to acknowledge the contribution of the authors who have written reviews included in this book and the publishers, Springer.

Tulasi Satyanarayana
Jennifer Littlechild
Yutaka Kawarabayasi
Editors

About the Editors

Dr. Tulasi Satyanarayana is now a Professor at the Department of Microbiology, University of Delhi South Campus, New Delhi, India. After completing M.Sc. and Ph.D. at the University of Saugar (India), he had postdoctoral stints at the University of Bhopal and in France. In 1988, he joined the Department of Microbiology, University of Delhi South Campus as Associate Professor and became Professor in 1998. During this period, his research efforts have been focused on understanding the diversity of yeasts and thermophilic fungi and bacteria, their enzymes and potential applications, heterotrophic carbon sequestration and metagenomics, and cloning and expression of yeast and bacterial genes encoding industrial enzymes. He has published over 170 scientific papers and reviews and edited five books. He is a fellow of the National Academy of Agricultural Sciences, Association of Microbiologists of India, Mycological Society of India and Biotech Research Society of India and a recipient of Dr. G.B. Manjrekar award of the Association of Microbiologists of India in 2003 and Dr. V.S. Agnihotrudu Memorial award of MSI in 2009 for his distinguished contributions. Dr. Satyanarayana is an Editor for Indian Journal of Microbiology and a member of the editorial board of Bioresource Technology and of Indian Journal of Biotechnology. He has over 38 years of research and teaching experience and published over 150 scientific papers and reviews in journals and books.

Dr. Jennifer Littlechild is Professor of Biological Chemistry and Director of the Exeter Biocatalysis Centre located in the Henry Wellcome Building for Biocatalysis. After completing her Ph.D. in the Biophysics Laboratory, Kings College, London University, UK, she had a postdoctoral stint at the Department of Biochemistry, Princeton University, USA. In 1975, she became a group leader at the Max Planck Institute for Molecular Genetics in Berlin, Germany. In 1980, she returned to Bristol University (UK) and in 1991 to Exeter with a Wellcome Trust University Award. Her current research focus is on the structural and mechanistic characterisation of the C–C bond forming enzymes transketolase and aldolase, vanadium haloperoxidases, Baeyer-Villiger monooxygenases, aminoacylases, novel esterases and lipases, gamma lactamases, alcohol dehydrogenases, dehalogenases, transaminases and

other enzymes from thermophilic bacteria and archaea. She has published over 150 papers in peer reviewed journals.

Dr. Kawarabayasi is now a Professor in the Faculty of Agriculture, Kyushu University, Fukuoka, Japan, working on functional genomics of extremophiles. After completing B.Sc. at Shizuoka University (Shizuoka, Japan), Yutaka Kawarabayasi entered the Graduate School of Science at Kyoto University. After obtaining his Ph.D. in 1997, he started his academic career at the Tohoku University. He participated in the establishment of the Kazusa DNA Research Institute (Kisaradu, Japan), and after 3 years, he joined the genomic project of thermophilic archaea at the National Institute of Technology and Evaluation (Tokyo, Japan). After the publication of entire genomic data of three thermophilic archaea (*Pyrococcus horikoshii* OT3, *Aeropyrum pernix* K1 and *Sulfolobus tokodaii* strain7), he concentrated on the post genome analysis at the National Institute of Advanced Industrial Science and Technology (Tsukuba, Japan). His focus has been on the carbohydrate metabolic enzymes in thermophilic archaea, and he has continued his research on genome-based identification of novel enzymes or activities. He has published over 115 scientific papers and reviews. During his tenure at Tsukuba in 2004, he was awarded a visiting professorship at the University of California, Berkley.

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Part I
Thermophiles in the Environment

Chapter 1
Diversity of Hot Environments and Thermophilic Microbes

Deepika Mehta and Tulasi Satyanarayana

Abstract The existence of life at high temperatures is quite fascinating. At both ends of the temperature range compatible with life, only microorganisms are capable of growth and survival. A great variety of microbes survives and grows at such elevated temperatures. Many thermophilic microbial genera have been isolated from man-made (acid mine effluents, biological wastes and waste treatment plants, and self-heated compost piles) and natural (volcanic areas, geothermal areas, terrestrial fumaroles, terrestrial hot springs, deep-sea hydrothermal vents, geothermally heated oil and petroleum reserves, sun-heated soils/sediments) thermal habitats throughout the world. Both culture-dependent and culture-independent approaches have been employed for understanding the diversity of microbes in hot environments. These organisms not only tolerate such high temperatures but also usually require these for their growth and survival and are known as thermophiles/thermophilic microbes, which include a wide variety of prokaryotes (bacteria and archaea) as well as eukaryotes. Interest in their diversity, ecology, and physiology and biochemistry has increased enormously during the past few decades. These organisms have evolved several structural and chemical adaptations that allow them to survive and grow at elevated temperatures.

Keywords Thermophile • Natural hot habitats • Man-made hot habitats • Hot water springs • Deep-sea hydrothermal vents • Acid mine drainage

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1.1 Introduction

Of enormous range of temperatures known (from 0 to approx. 3×10^9 K), only a minute fraction is compatible with life. It is now well known that microbial life can prevail or thrive in the upper as well as lower temperature limits that are known to be compatible with life. Temperature creates a series of challenges, from structural devastation due to ice crystal formation at one extreme to the denaturation of biomolecules and cell components at the other. A wide variety of microorganisms have, however, been discovered that can overcome these challenges. One such group of microorganisms includes thermophiles. The word "thermophile" has been derived from two Greek words "thermotita" (meaning heat) and "philia" (meaning love). Thermophiles are heat-loving organisms, which not only tolerate high temperatures but also usually require these for their growth and survival. A thermophile as defined by Brock (1978) is "an organism capable of living at temperatures at or near the maximum for the taxonomic group of which it is a part." Temperatures for their growth range from 50°C to as high as 121°C, the temperature used for sterilization in autoclaves. These microorganisms have been classified into moderate thermophiles, extreme thermophiles, and hyperthermophiles (Table 1.1).

The currently known upper temperature limit for life is 121°C (Kashefi and Lovley 2003) that corresponds to the maximum temperature limit for the growth and survival of nonphotosynthetic prokaryotes. The photosynthetic prokaryotes are unable to grow at such high temperatures or even closer and show the upper temperature limit of 70–73°C. Thermophilic prokaryotes have been known for over 90 years, while hyperthermophilic prokaryotes have been recognized only three decades ago (Brock 1978; Stetter et al. 1981; Zillig et al. 1981; Stetter 1982). The upper temperature limit for eukaryotic microorganisms is even lower, approximately 60–62°C (Tansey and Brock 1972), at which only a few species of fungi can grow. The upper temperature limits for eukaryotic algae and protozoa are slightly lower (55–60°C). Figure 1.1 presents temperature limits of growth for different taxonomic groups.

Within the past few decades, a great diversity of microorganisms has been discovered that exist in hot environments, both natural and man-made. This chapter deals with the diversity of thermophilic microorganisms and their environments and adaptations, which allow them to survive and grow at elevated temperatures. Although many thermophiles belonging to phylogenetically distinct groups have been cultured and are being used for various biotechnological applications, an unanticipated diversity of thermophilic species within high-temperature environments is evident by direct microscopic examination, 16S rDNA amplification, and other culture-independent techniques. This indicates that there are many more thermophiles that are yet to be cultivated.

1.2 Habitats with Elevated Temperatures

Habitats for the occurrence of thermophiles may be natural or man-made.

Table 1.1 Classification of thermophilic microorganisms

Category	Temperature optima (°C)	Examples
Moderate thermophile	40–60°C	*Tepidibacter*, *Clostridium*, *Exiguobacterium*, *Caminibacter*, *Lebetimonas*, *Hydrogenimonas*, *Nautilia*, *Desulfonauticus*, *Sulfurivirga*, *Caminicella*, *Vulcanibacillus*, *Marinotoga*, *Caldithrix*, *Sulfobacillus*, *Acidimicrobium*, *Hydrogenobacter*, *Thermoplasma*, *Mahella*, *Thermoanaerobacter*, *Desulfovibrio*
Extreme thermophile	60–85°C	*Methanocaldococcus*, *Thermococcus*, *Palaeococcus*, *Methanotorris*, *Aeropyrum*, *Thermovibrio*, *Methanothermococcus*, *Thermosipho*, *Caloranaerobacter*, *Thermodesulfobacterium*, *Thermodesulfatator*, *Deferribacter*, *Thermosipho*, *Desulfurobacterium*, *Persephonella*, *Kosmotoga*, *Rhodothermus*, *Desulfurobacterium*, *Balnearium*, *Acidianus*, *Thermovibrio*, *Marinithermus*, *Oceanithermus*, *Petrotoga*, *Vulcanithermus*, *Carboxydobrachium*, *Thermaerobacter*, *Thermosulfidibacter*, *Metallosphaera*
Hyperthermophile	>85°C	*Geogemma*, *Archaeoglobus*, *Methanopyrus*, *Pyrococcus*, *Sulfolobus*, *Thermoproteus*, *Methanothermus*, *Acidianus*, *Ignisphaera*, *Ignicoccus*, *Geoglobus*

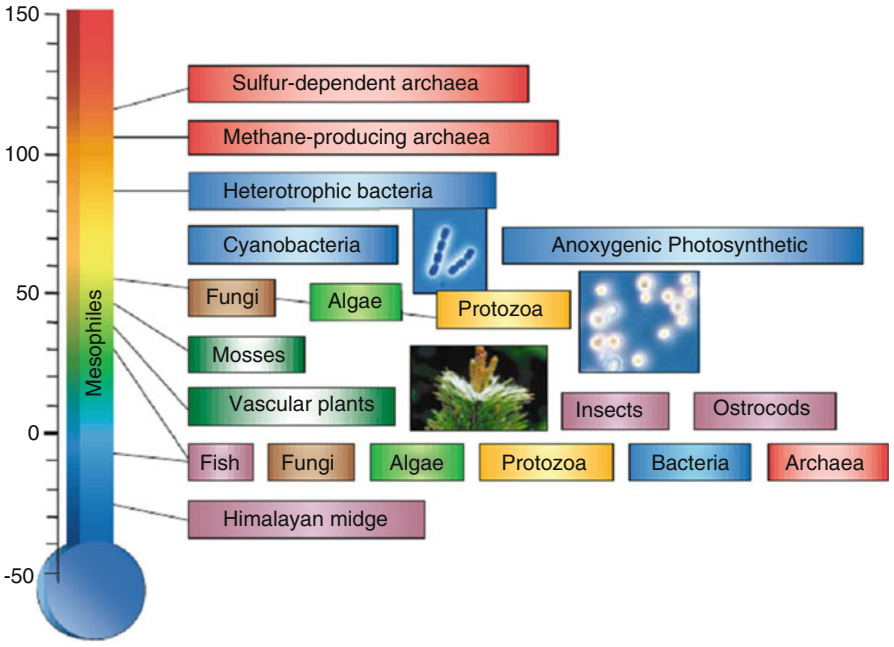

Fig. 1.1 Temperature limits for different taxonomic groups (Rothschild and Mancinelli 2001)

1.2.1 Natural Thermophilic Habitats

Natural biotopes for the occurrence of thermophilic microorganisms are distributed worldwide. These are relatively high-temperature habitats, as compared to those which are anthropogenic or man-made. These may be terrestrial or marine in origin. The most remarkable ones that harbor a considerable variety of thermophilic microorganisms include terrestrial geothermal and volcanic areas and deep-sea hydrothermal vents (submarine hydrothermal vents). Most of the currently known extreme thermophiles and hyperthermophiles have been recovered from these regions by culture-dependent as well culture-independent approaches. Geothermal and volcanic areas include terrestrial fumaroles (e.g., solfataras), terrestrial hot springs, and geysers. Others natural biotopes include geothermally heated oil and petroleum reservoirs and sun-heated soils/sediments.

1.2.1.1 Geothermal and Volcanic Areas

One of the most important natural biotopes of thermophiles includes geothermal and volcanic areas. These are worldwide in distribution and associated primarily with tectonically active zones where major crustal movements of the Earth occur. It is here in these areas where tectonic plates are highly converging or diverging. In the terrestrial zones, deep-seated magmatic materials are thrust close to the Earth's surface and serve as heat sources. As a result, groundwater percolating into the Earth becomes intensely heated but does not boil because of lithostatic pressure. When the percolating fluid reaches a sufficiently high temperature, the pressure generated forces the fluid through pores and fissures back to the surface of the Earth. These may lead to the formation of fumaroles, hot springs (or thermal springs), and geysers. These thermal habitats are concentrated in the small areas called thermal basins. In general, a thermal basin has geysers and/or flowing springs at the bottom of the hill, sulfur- and iron-rich non- (or weakly) flowing springs at the flanks of the hill, and fumaroles on the highest grounds. Highly mineralized geysers and geothermal waters at the bottom of a thermal basin have extensive water flow, neutral to alkaline pH, and constant temperature. Major components include bicarbonates and chlorides. The springs have progressively less flow and become less mineralized and more acidic because of the presence of sulfates (solfataras), as we move upslope. The temperatures are variable in these due to changes in steam sources beneath them.

Terrestrial Fumaroles and Solfataras

A fumarole (also known as steam vent) is an opening in the Earth's crust, often in the volcanic areas, which emits steam and gases such as carbon dioxide, sulfur dioxide, hydrochloric acid, and hydrogen sulfide. It has so little water that it all flashes into steam before reaching the surface. Fumaroles may occur along tiny cracks or

long fissures and on the surfaces of lava flows. Solfatara refers to fumaroles that emit sulfurous gases. Solfataras are found within the solfataric fields. Solfataric fields consist of soils, mud pots, and surface waters (sulfur-rich springs), heated by volcanic exhalations from the magma chambers located below them. They may have temperatures up to 100°C.

The chemical composition of solfataric fields is very variable and depends on the surrounding geology. Solfataras can be mildly acidic to slightly alkaline (pH 5–8) or extremely acidic, with pH values below 1.0. Acidic condition occurs due to the production of sulfuric acid (H_2SO_4) from the biological oxidation of H_2S and S°. Such hot and sulfur-rich environments are found throughout the world, including Italy, Iceland, New Zealand, and Yellowstone National Park in Wyoming (USA). Many of them are rich in iron minerals like ferric hydroxides and pyrite. Steam within the solfataric exhalations is mainly responsible for the heat transfer. In addition, they may contain CO_2; variable amounts of H_2, CH_4, N_2, and CO; and traces of nitrate and ammonia. Less common compounds like magnetite and the arsenic minerals may be present at some sites, for example, at Uzon caldera, Kamchatka, in Russia.

Diversity of Thermophilic Microorganisms in Solfataric Fields

Solfataric fields are colonized by a variety of acidophilic moderate and extreme thermophiles. Thermophilic members of bacteria and archaea have been recovered from solfataric habitats that include the species of *Pyrobaculum*, *Sulfolobus*, *Thermofilum*, *Methanothermus*, *Thermoproteus*, *Acidianus*, *Aciditerrimonas*, *Desulfotomaculum*, *Picrophilus*, *Alicyclobacillus*, *Thermoanaerobacter*, and *Thermogymnomonas*. *Thermoproteus neutrophilus*, *Thermoproteus tenax*, and *Pyrobaculum islandicum* are chemolithoautotrophs that reduce S° by H_2 anaerobically. While *Pyrobaculum aerophilum* grows anaerobically by the reduction of nitrate by H_2 and on H_2 and O_2, under microaerophilic conditions (Völkl et al. 1993). Strains of *Pyrobaculum organotrophum*, *Thermoproteus uzoniensis*, and *Thermofilum* are obligate heterotrophs growing on organic substrates by sulfur respiration. *Thermoproteus tenax* and *Pyrobaculum islandicum* are facultative heterotrophic sulfur respirers. Members of the genus *Sulfolobus* have been isolated from solfataric fields and acidophilic mud springs. *Sulfolobus acidocaldarius* has been isolated from Yellowstone National Park (the first hyperthermophilic microorganism isolated) (Brock et al. 1972) and *Sulfolobus solfataricus* strain P2, from a solfataric field near Naples, Pisciarelli, Italy (Zillig et al. 1980). *Sulfolobus* strains are hyperthermophilic crenarchaea that grow aerobically at optimum temperature and pH of 75–80°C and 2.5–3.5, respectively. *S. solfataricus* grows on a variety of different carbon sources like tryptone, various sugars, and amino acids. *Acidianus*, unlike *Sulfolobus*, can grow both aerobically as well as anaerobically. Under aerobic conditions, it oxidizes S° to H_2SO_4, with O_2 as electron acceptor. Anaerobically, it uses S° as an electron acceptor, H_2 as electron donor, and forming H_2S as the product (Segerer et al. 1986; Plumb et al. 2007). *Desulfotomaculum solfataricum*, isolated from hot solfataric fields of Iceland, uses sulfate, sulfite, or thiosulfate as electron

acceptors. The optimum temperature and pH for growth are 60°C and pH 7.3, respectively (Goorissen et al. 2003). The aerobic heterotrophs *Picrophilus oshimae* and *Picrophilus torridus*, isolated from Japanese soils permeated with solfataric gases, show optimal growth at pH 0.7 and 60°C (Schleper et al. 1995). The photosynthetic green alga, *Cyanidium*, that grows up to 55°C has been found to grow in the walls of the solfataric springs (Brock 1978). In Tengchong solfataric region, acidothermophilic sulfur oxidizers such as *Acidianus tengchongensis* and *Sulfolobus tengchongensis* have been found to play important roles in sulfur oxidation (He et al. 2004; Xiang et al. 2003). Most of the knowledge on the diversity studies of solfataras is based on cultivation studies. Using DNA-based culture-independent technique, up to 65 different phylotypes of crenarchaeotes have been described from one solfatara (Kvist et al. 2005; Siering et al. 2006). In most of the studies, many and often most sequences are only distantly related to cultivated relatives. In a study of a solfatara in Pisciarelli (Naples, Italy), 42% of the 201 analyzed clones have been found to cluster within the I.1b group crenarchaeota (Kvist et al. 2005). Kvist et al. (2007) analyzed genetic diversity of archaea by the analysis of amplified 16S rRNA genes and terminal restriction fragment length polymorphism (t-RFLP) from solfataras at the thermal active area of Hverageroi (Iceland). It has been reported that 18.0% of the total number of clones belonged to group I.1b Crenarchaeota, which was represented by only mesophilic species till now. Many other novel thermophilic isolates have been described by Plumb et al. (2007), Lee et al. (2007), and Itoh et al. (2007, 2011) from different solfataric fields. A list of thermophilic microorganisms isolated from solfataric fields is presented in Table 1.2.

Terrestrial Hot Springs

A hot spring is a spring that is produced by the emergence of geothermally heated groundwater from the Earth's crust. Geothermal systems are present in many geological settings and not necessarily related to volcanism. The water arising from a hot spring is either heated by geothermal heat, that is, heat from the Earth's interior, in a nonvolcanic area, or by coming in contact with magma (molten rock). In the nonvolcanic area, groundwater that percolates deeply into the Earth's crust comes in contact with the rocks that get heated as a result of geothermal gradient. While in the volcanic areas that are the tectonically active zones, high-temperature gradient near magma may cause water to be heated enough that it becomes superheated. The pressure is generated that forces the water to the Earth's surface through pores and fissures within the Earth's crust, where it can issue as a hot spring (thermal springs) or geysers. As the hot fluid passes up through the Earth's surface, minerals dissolve from the adjoining areas, thus accounting for extensive mineralization. Hot-spring waters usually show high concentrations of many elements and can be highly supersaturated with a variety of minerals. These usually include H_2S, CO_2, low-molecular-weight organic compounds, CH_4, H_2, NH_3, and trace elements (Brock 1978). The chemical interaction with the reservoir rocks and the rock forming minerals along

Table 1.2 List of thermophiles isolated from solfataric fields

Genus/species	T_{opt} (°C)	pH	Reference
Sulfolobus acidocaldarius	75	1–5	Brock et al. (1972)
Thermoproteus tenax	80	2.5–3.5	Zillig et al. (1980)
Sulfolobus solfataricus	88	2.5–6	Zillig et al. (1981)
Desulfurococcus mobilis	85	6	Zillig et al. (1982)
Thermofilum pendens	88	4–6.5	Zillig et al. (1983)
Acidianus infernus	88	1.5–5	Segerer et al. (1986)
Thermoproteus neutrophilus	88	6–7	Stetter (1986)
Methanothermus sociabilis	88	5.5–7.5	Lauerer et al. (1986)
Acidianus ambivalens	80	2.5	Segerer et al. (1986)
Pyrobaculum islandicum	100	5–7	Huber et al. (1987)
Pyrobaculum organotrophum	102	6	Huber et al. (1987)
Pyrobaculum aerophilum	100	5.8–9	Völkl et al. (1993)
Picrophilus oshimae	60	0.7	Schleper et al. (1995)
Picrophilus torridus	60	0.7	Schleper et al. (1995)
Desulfotomaculum solfataricum	60	7.3	Goorissen et al. (2003)
Acidianus tengchongensis	70	2.5	He et al. (2004)
Acidianus sulfidivorans	74	0.8–1.4	Plumb et al. (2007)
Thermoanaerobacter sulfurigignens	63–67	5–6.5	Lee et al. (2007)
Thermogymnomonas acidicola	60	1.8–4	Itoh et al. (2007)
Aciditerrimonas ferrireducens	50	2–4.5	Itoh et al. (2011)

the ascent path affects the composition of the hot water, which may be highly acidic or alkaline. Their temperature ranges from boiling or superheated at their sources to ambient temperature at their shores. Hot springs are present in many countries throughout the world. Countries that are renowned for their hot springs include Iceland, New Zealand, Chile, and Japan, but there are interesting and unique hot springs at many other places as well. The Yellowstone National Park (Wyoming, USA) has one of the highest numbers of hot springs in the world. Some examples of hot springs are presented in Tables 1.3 and 1.4.

Some examples of hot-spring sites in India are Ganeshpuri (Vajreshwari), Manikaran (Himachal Pradesh), Bendru Theertha (Karnataka), Chavalpani (Mahadeo Hills of Madhya Pradesh), Surya Kund (Bihar), Phurchachu (Reshi, Sikkim), Taptapani (near Berhampur), Atri (near Bhubaneswar), Tarabalo (Nayagarh District) of Orissa, Bakreshwar (West Bengal), and Tulsishyam (Gujarat).

A special type of hot spring is the geyser, where water and steam are ejected episodically through a vent. If the water is mixed with mud and clay, it is called a mud pot. Geysers exist in only a few places on Earth, so they are a fairly rare phenomenon. Generally all geyser field sites are located near active volcanic areas. Generally, surface water moves down to an average depth of around 2,000 m (6,600 ft) where it contacts hot rocks. The boiling of the pressurized water results in the geyser effect of hot water and steam spraying out of the geyser's surface vent. About a 1,000 known geysers exist worldwide, roughly half of which are in Yellowstone National Park, Wyoming, United States. Yellowstone Park is a lava plateau of an

Table 1.3 List of novel thermophilic bacteria isolated from hot springs

Genus/species	Hot-spring location	T_{opt} (°C)	Comments	References
Anaerobranca horikoshii	Yellowstone National Park	57	Alkalitolerant	Engle et al. (1995)
Thermothrix azorensis	Sao Miguel Island	76–78	S° oxidizer	Odintsova et al. (1996)
Meiothermus cerberus	Iceland	55	–	Chung et al. (1997)
Moorella glycerini	Yellowstone National Park	58	Homoacetogen	Slobodkin et al. (1997)
Porphyrobacter tepidarius	Brackish hot spring	40–48	Photosynthetic	Slobodkin et al. (1997)
Thermoterrabacterium ferrireducens	Yellowstone National Park	65	Iron reducer	Slobodkin et al. (1997)
Streptomyces thermogriseus	Yunnan	65–68	–	Xu et al. (1998)
Caldicellulosiruptor kristjanssonii	Iceland	78	Cellulolytic	Bredholt et al. (1999)
Hydrogenobacter subterraneus	Oita Prefecture, Japan	78	Heterotroph	Takai et al. (2001)
Hydrogenophilus thermoluteolus	Yellowstone National Park	63	H_2 oxidizer	Stohr et al. (2001)
Thermoanaerobacter tengcongensis	China	75	Anaerobe	Xue et al. (2001)
Caloramator viterbensis	Bagnaccio, Italy	58	Glycerol fermenter	Seyfried et al. (2002)
Carboxydocella thermautotrophica	Kamchatka Peninsula, Russia	58	CO utilizer	Sokolova et al. (2002)
Meiothermus rosaceus	Tengchong hot springs, Taiwan	55–60	Heterotroph	Chen et al. (2002)
Pseudoxanthomonas broegbernensis	Chi-ban hot springs, Taiwan	50–60	N_2O producing	Chen et al. (2002)
Roseiflexus castenholzii	Japanese hot spring	50	Photosynthetic	Hanada et al. (2002)
Microvirga subterranea	Great Artesian Basin, Australia	41	–	Kanso and Patel (2003)
Alicyclobacillus vulcanalis	Coso hot springs, USA	55	Acidophile	Simbahan et al. (2004)
Hydrogenivirga caldilitoris	Kagoshima Prefecture, Japan	75	H_2 and S° oxidizer	Nakagawa et al. (2004)
Phenylobacterium lituiforme	Great Artesian Basin	40–41	Facultative anaerobe	Kanso and Patel (2004)
Rubrobacter taiwanensis	Lushan hot springs, Taiwan	60	Radiation resistant	Chen et al. (2004)
Sulfurihydrogenibium azorense	São Miguel Island, Portugal	68	Microaerophile	Aguiar et al. (2004)
Thermosinus carboxydivorans	Yellowstone National Park	60	CO oxidizer	Sokolova et al. (2004)
Lebetimonas acidiphila	TOTO caldera, Mariana Arc	50	H_2 oxidizer	Takai et al. (2005)
Methylothermus thermalis	Japan	57–59	Methanotroph	Tsubota et al. (2005)
Paenibacillus assamensis	Assam, India	–	–	Saha et al. (2005)
Silanimonas lenta	Baekdu Mountain in Korea	47	Alkaliphile	Lee et al. (2005)
Sulfurihydrogenibium yellowstonense	Yellowstone National Park, USA	70	Facultative heterotroph	Nakagawa et al. (2005a)

Thermincola carboxydiphila	Lake Baikal region	55	Carboxydotroph	Sokolova et al. (2005)
Thiobacter subterraneus	Hot spring at Hishikari mine, Japan	50–55	Chemolithoautotroph	Hirayama et al. (2005)
Caldalkalibacillus thermarum	China	60	Alkaliphile	Xue et al. (2006)
Carboxydocella sporoproducens	Karymsky Lake, Kamchatka, Russia	60	CO-utilizing anaerobe	Slepova et al. (2006)
Cohnella laeviribosi	Volcanic pond, Likupang	45	Ribose assimilator	Cho et al. (2007)
Fervidobacterium changbaicum	Changbai Mountains, China	75–80	Obligately aerobe	Cai et al. (2007)
Geosporobacter subterraneus	Paris Basin, France	42	Chemoorganotroph	Klouche et al. (2007)
Planifilum yunnanense	Yunnan, China	60	–	Zhang et al. (2007)
Thermoanaerobacter pseudethanolicus	Yellowstone National Park	–	Heterotrophic anaerobe	Onyenwoke et al. (2007)
Ammonifex thiophilus	Kamchatka, Russia	75	Chemolithotroph	Miroshnichenko et al. (2008b)
Anoxybacillus bogrovensis	Dolni Bogrov, Bulgaria	65	Alkalitolerant	Atanassova et al. (2008)
Caldicellulosiruptor hydrothermalis	Kamchatka, Russia	65	Cellulose degrader	Miroshnichenko et al. (2008a)
Calditerrivibrio nitroreducens	Yumata, Nagano, Japan	55	Nitrate reducer	Iino et al. (2008)
Desulfotomaculum hydrothermale	Tunisia	55	Sulfate reducer	Haouari et al. (2008a)
Elioraea tepidiphila	São Miguel, Azores	45–50	Strict aerobe	Albuquerque et al. (2008)
Sulfurihydrogenibium kristjanssonii	Hveragerdi	68	H_2 and S° oxidizer	Flores et al. (2008)
Sulfurihydrogenibium rodmanii	Uzon caldera, Kamchatka	75	S° oxidizer	O'Neill et al. (2008)
Thermoanaerobacter uzonensis	Uzon caldera, Kamchatka, Russia	61	Anaerobic heterotroph	Wagner et al. (2008)
Thermodesulfovibrio hydrogeniphilus	Tunisian hot spring	65	Sulfate reducer	Haouari et al. (2008b)
Thiofaba tepidiphila	Fukushima Prefecture, Japan	45	S° oxidizer	Mori and Suzuki (2008)
Venenivibrio stagnispumantis	Waiotapu, New Zealand	70	H_2 oxidizer	Hetzer et al. (2008)
Caldanaerovirga acetigignens	Black Rock Desert, NV, USA	62–66	Xylanolytic alkaliphile	Wagner et al. (2009)
Carboxydothermus siderophilus	Kamchatka Peninsula, Russia	65	Hydrogenogenic	Slepova et al. (2009)
Fervidicola ferrireducens	Great Artesian Basin	70	Anaerobe	Ogg and Patel (2009a)
Thermotalea metallivorans	Great Artesian Basin, Australia	50	S° reducer	Ogg and Patel (2009b)
Thermus islandicus	Torfajokull, South Iceland	65	Mixotrophic S° oxidizer	Bjornsdottir et al. (2009)
Anoxybacillus tengchongensis	Tengchong and Eryuan, China	50	Alkalitolerant	Zhang et al. (2010, 2011)

(continued)

Table 1.3 (continued)

Genus/species	Hot-spring location	T_{opt} (°C)	Comments	References
Caldanaerobacter uzonensis	Uzon caldera, Kamchatka, Russia	68–70	Heterotroph	Kozina et al. (2010)
Caldilinea tarbellica	France	55	Filamentous anaerobe	Grégoire et al. (2011)
Thermus arciformis	Laibin	70	Aerobe	Zhang et al. (2010, 2011)
Chelatococcus sambhunathii	–	42	Aerobe	Panday and Das (2010)
Fervidicella metallireducens	Great Artesian Basin, Australia	50	Anaerobe	Ogg and Patel (2010b)
Hydrogenophilus islandicus	Graendalur, Iceland	55	H_2 oxidizer	Vésteinsdóttir et al. (2011)
Marinitoga litoralis	Coastal spring, near Indian Ocean	60	Organotroph	Postec et al. (2010)
Caloramator mitchellensis	Great Artesian Basin, Australia	55	Anaerobe	Ogg and Patel (2011a)
Ignavibacterium album	Yumata, Nagano, Japan	45	Chemoheterotroph	Iino et al. (2010)
Thermovenabulum gondwanense	Great Artesian Basin, Australia	65	Fe-reducing anaerobe	Ogg and Patel (2010)
Thiomonas islandica	Graendalur, Iceland	45	H_2 and S° oxidizer	Vésteinsdóttir et al. (2011)
Caloramator mitchellensis	Great Artesian Basin, Australia	55	Anaerobic	Ogg and Patel (2011a)
Hydrogenophilus islandicus	Graendalur, Iceland	55	H_2 oxidizer	Vésteinsdóttir et al. (2011)
Desulfosoma caldarium	Colombian Andes, Colombia	57	Sulfate reducer	Baena et al. (2011)
Jhaorihella thermophila	Green Island (Lutao), Taiwan	55	–	Rekha et al. (2011)
Fervidobacterium riparium	Kunashir Island, Russia	65	Anaerobic, cellulolytic	Podosokorskaya et al. (2011)
Schleiferia thermophila	S o Miguel Island, Azores	50	–	Albuquerque et al. (2011)
Meiothermus hypogaeus	Himekawa hot spring, Japan	50	–	Mori et al. (2012)
Laceyella sediminis	Tengchong County, China	55	–	Chen et al. (2012)
Brevibacillus aydinogluensis	Karakoc hot spring, Turkey	50	–	Iino et al. (2010)
Carboxydocella manganica	Kamchatka hot spring	58–60	Mn(IV) and Fe(III) reducer	Slobodkin et al. (2012)
Thermosyntropha tengcongensis	Chinese hot spring	60	Degrades long-chain fatty acid	Zhang et al. (2012)

Table 1.4 List of novel thermophilic archaea isolated from hot springs

Genus/species	Hot-spring location	T_{opt} (°C)	Comments	References
Sulfolobus hakonensis	Hakone, Japan	70	Acidophile	Takayanagi et al. (1996)
Sulfurisphaera ohwakuensis	Ohwaku Valley, Japan	85	Acidophile	Kurosawa et al. (1998)
Thermocladium modesties	Japan	75	–	Itoh et al. (1998)
Caldivirga maquilingensis	Philippines	85	Sulfur reducer	Itoh et al. (1999)
Sulfolobus yangmingensis	Yangming National Park, Taiwan	80	Acidophile	Jan et al. (1999)
Pyrobaculum oguniense	Kumamoto Prefecture, Japan	90–94	Facultative aerobe	Sako et al. (2001)
Caldisphaera lagunensis	Philippines hot spring	70–75	Acidophile	Itoh et al. (2003)
Caldisphaera lagunensis	Philippines	70–75	Anaerobic heterotroph	Itoh et al. (2003)
Desulfurococcus fermentans	Uzon caldera, Kamchatka	80–82	Organoheterotroph	Perevalova et al. (2005)
Ignisphaera aggregans	New Zealand	92–95	Heterotroph	Niederberger et al. (2006)
Acidobacillus saccharovorans	Kamchatka hot spring, Russia	80–85	Acidophilic anaerobe	Prokofeva et al. (2009)
Acidiplasma aeolicum	Vulcano Island, Italy	45	Chemoorganotroph	Golyshina et al. (2009)
Desulfurococcus kamchatkensis	Uzon caldera, Kamchatka Peninsula, Russia	85	Protein degrading organotroph	Kublanov et al. (2009)
Fervidicoccus fontis	Uzon caldera, Kamchatka Peninsula, Russia	65–70	Anaerobe	Perevalova et al. (2010)
Methanothermobacter thermautotrophicus	China	65	–	Ding et al. (2010)
Metallosphaera cuprina	Tengchong, Yunnan, China	65	Metal-mobilizing acidophile	Liu et al. (2011)

ancient date, in which the old centers of volcanic activity have been closed since a very long time but which still shows abundant hot-spring activity throughout its extent of nearly 60 miles north and south. Other major examples of geysers include the Valley of Geysers located in the Kamchatka Peninsula (Russia), El Tatio (Chile), Taupo Volcanic Zone (New Zealand), and Haukadalur (Iceland).

Microbial Mats

Hot springs have become quite fascinating from a microbial ecologist's point of view as large areas are created with distinct thermal gradients as the water flows over the edges of the source and gradually cools as it descends. Thermal gradients that support microbial mat communities occur in various settings: between the center of geothermal hot springs and their edges, along the effluent channels that drain hot springs, as well as along the vertical near the ocean's surface (Brock 1978). The combination of continuous supply of nutrients (including high concentrations of inorganic reduced sulfur compounds) and a high light intensity over a relatively large area results in the formation of "microbial mats" representing tightly interacting communities of microorganisms. A wide diversity of thermophilic microorganisms exists in these mats (Ward et al. 1987). Thermophilic representatives of cyanobacteria, anoxygenic phototrophic bacteria, aerobic and anaerobic heterotrophs, methanogens, and sulfate reducers are all the components of these microbial mats.

The largest and best-studied hot-spring area is Yellowstone Park, Wyoming, USA. The upper temperature limit for phototrophic microbial mats found in neutral and alkaline hot springs in North America (Yellowstone National Park) is approximately 74°C (Castenholz 1984). A significant research attention has been focused on microbial mats that occur in hot-spring waters from 50 to 75°C. In general, microbial mats in this temperature range often consist of vertically organized communities in which the photosynthetic cyanobacterium *Synechococcus lividus* lives as a top layer, while the photosynthetic green non-sulfur-like bacterium *Chloroflexus aurantiacus* lives as an undermat. *Chloroflexus* utilizes bacteriochlorophylls *a* and *c*, chromatophores with a different absorption spectrum from the chlorophyll *a* (Chl *a*), characteristic of cyanobacteria, so sunlight at wavelengths poorly absorbed by the mat's top layers can be used. It has also been evidenced that significant symbiotic interactions may occur between these two organisms (Brock 1978; Mosser et al. 1974). Other species of algae, cyanobacteria, bacteria, archaea, and fungi also inhabit microbial mats in the hot springs at different locations, which primarily depends on their temperature variations and nutrient concentration.

Cyanobacterium *Synechococcus* occurs as a monospecific surface layer on "*Synechococcus* mats" (Ward et al. 1998). The phototropic microbial mats of alkaline hot springs of Iceland are dominated by the cyanobacteria *Mastigocladus laminosus* or *Phormidium laminosum*, depending upon the temperature and sulfide concentration (Jørgensen and Nelson 1988). Furthermore, the mats contain large quantities of the green gliding phototrophic bacterium, *Chloroflexus aurantiacus*. Beneath the photosynthetic layer, anaerobic bacteria are present fermenting the

decaying mat. It has been observed that sulfate reduction is the main terminal pathway in high-sulfate (above 1 mM) hot springs, while methanogenesis is the main pathway in low-sulfate (below 1 mM) springs (Sandbeck and Ward 1981; Ward and Olson 1980). Nineteen genera and 36 species of microorganisms have been identified in various hot springs of Northern Thailand by morphometric analysis. The morphotypes, which dominated these hot springs in the temperature range of 40–80°C, are *Synechococcus lividus*, other *Synechococcus* sp., and *Phormidium boryanum* (Sompong et al. 2005). Iron-dominated microbial mats at chocolate pots hot springs (Yellowstone National Park) are composed of species of *Synechococcus*, *Oscillatoria*, *Chloroflexus*, *Pseudanabaena*, and *Mastigocladus* (Pierson and Parenteau 2000). Culture-dependent and culture-independent phylogenetic analysis of the freshwater thermal springs of Zerka Ma'in, Jordan, has been done. These hot springs are inhabited by a great diversity of thermophilic unicellular and filamentous cyanobacteria including *Thermosynechococcus*, *Chroogloeocystis*, *Fischerella* (*Mastigocladus*), and *Scytonema*. Based on 16S rRNA gene sequence analysis, it has been shown that the Zerka Ma'in strains are phylogenetically distinct from morphologically similar cyanobacteria found in hot springs worldwide (Oren et al. 2009). The isolation of a unicellular cyanobacterium with a 16S rRNA gene that showed 99% similarity with *Chroogloeocystis siderophila*, an organism originally found in iron-rich thermal environments in Yellowstone and requiring high iron concentrations for growth (Brown et al. 2005), is remarkable.

Three morphotypes of bacteria have been observed in sulfur-turf mats of Japan. Two of these have been tentatively named *Thiovibrio miyoshi* and *Thiothrix miyoshi*. Moreover, in situ ecophysiological and microscopic studies have shown that one of these bacteria, the large sausage-shaped "*Thiovibrio miyos*hi," predominates in sulfur-turf mats and oxidizes environmental sulfide to elemental sulfur and then to sulfate via thiosulfate (Maki 1991, 1993). In situ hybridization with clone-specific probes for 16S rRNA also revealed the presence of sausage-shaped bacteria in the hot-spring sulfur-turf mats in Japan (Yamamoto et al. 1998).

Diversity of Thermophilic Microbes in Hot Water Springs

Hot springs also harbor other microorganisms other than those present in the microbial mats. Various culture-dependent and culture-independent studies have been carried out to understand their diversity in hot springs. It is generally accepted that less than 1% of the extant microbial "species" have been cultured (Ward 1998) and that the majority do not grow in conventional isolation media (Vetriani et al. 1998). Investigations of microbial diversity using culture-independent methods provide a valid approach to the assessment of true microbial diversity (Jeanthon 2000).

A number of extreme thermophiles or hyperthermophiles have been isolated from terrestrial hot springs (Ghosh et al. 2003; Marteinsson et al. 2001; Takai and Sako 1999; Meyer-Dombard et al. 2005; Skirnisdottir et al. 2000; Tsubota et al. 2005; Miroshnichenko et al. 2008a, b; Wagner et al. 2008; Kublanov et al. 2009; Vésteinsdóttir et al. 2011). Phylogenetic characterization of microorganisms has been done for geothermal springs in different parts of world like Iceland (Skirnisdottir

et al. 2000; Marteinsson et al. 2001; Takacs et al. 2001), Yellowstone National Park (Hugenholtz et al. 1998; Ward et al. 1998), and Greece (Sievert et al. 2000). Phylogenetically unknown bacteria have been isolated from terrestrial hot springs (Aguiar et al. 2004; Sokolova et al. 2004; Lee et al. 2005; Nakagawa et al. 2005a; Tsubota et al. 2005; Slepova et al. 2006; Derekova et al. 2007). A great diversity of bacteria and archaea has been revealed by analysis of rRNA genes that were amplified by the polymerase chain reaction from environmental DNA (Barns et al. 1996; Burton and Norris 2000; Meyer-Dombard et al. 2005).

Hugenholtz et al. (1998) analyzed the SSU rRNA genes for studying the bacterial diversity of Obsidian Pool (OP), a Yellowstone National Park hot spring. Seventy percent of the sequence types have been found to be associated with 14 previously recognized bacterial phyla, and 30% of the sequence types were found to be unrelated with recognized bacterial divisions comprising 12 novel division-level lineages. Some of these sequences are nearly identical to those of cultivated chemolithotrophic thermophiles, including *Calderobacterium*, *Thermodesulfovibrio*, *Thermodesulfobacterium*, some Aquificales, and δ-Proteobacteria. Similarly, a 16S rRNA-based study to reveal thermophilic bacterial communities in Indonesia's thermal springs reported 22 sequences assignable to the taxa of Proteobacteria, *Bacillus*, and *Flavobacterium* (Baker et al. 2001).

Thermophilic ammonia-oxidizing bacteria capable of growth at 55°C have been enriched from geothermal springs of Kamchatka (Golovacheva 1976). A thermophilic nitrate-reducing bacterium isolated from Garga spring has been identified as *Geobacillus gargensis* sp. nov. (Nazina et al. 2004). Culture-independent 16S rRNA-based techniques have revealed the presence of *Nitrospira* relatives in subterranean hot springs of Iceland (Marteinsson et al. 2001) and in a radon-containing hot spring in South Australia (Anitori et al. 2002). The presence of nitrifying bacteria in Garga hot spring at 45–59°C has also been described. They are identified as members of *Nitrosomonas* by both conventional and molecular techniques (DGGE analysis of partial 16S rRNA sequences). Nitrite-oxidizing bacteria have been identified as the members of *Nitrospira* (Lebedeva et al. 2005). A novel aerobic, obligately mixotrophic, moderately thermophilic, thiosulfate-oxidizing bacterium *Thiomonas bhubaneswarensis* sp. nov. has been isolated from hot-spring sediment samples collected from Atri, Bhubaneswar, India (Panda et al. 2009). Members of the genus *Geobacillus* have been isolated from the volcanic hot springs. For example, various strains of *Geobacillus thermoleovorans* have been isolated from the volcanic hot springs of Waimangu Volcanic Valley, New Zealand (Malhotra et al. 2000; Noorwez et al. 2006).

Culture-independent approach has also been used to study bacterial diversity of hot spring in Bakreshwar, India. 16S ribosomal DNA clones derived from the sediment samples are found to be associated with δ-Proteobacteria (*Shewanella*), cyanobacteria (*Synechococcus elongates*), and green non-sulfur (*Thermus thermophilus*) and low-GC gram-positive bacteria (*Desulfotomaculum luciae*) (Ghosh et al. 2003). A novel species, *Anaerobranca californiensis*, has been isolated from the thermophilic alkaline hypersaline Mono Lake's sediment, California (Gorlenko et al. 2004).

A novel bacterium that transforms light into chemical energy has been discovered from Yellowstone National Park (Bryant et al. 2007). This organism has been identified by the 16S rRNA analysis and further verified by enrichment techniques and biochemical methods. This novel genus and species *Chloracidobacterium thermophilum* belongs to a new phylum, Acidobacteria. This is the third time in the past 100 years that a new bacterial phylum has been added to the list of those with chlorophyll-producing members. *Chloracidobacterium thermophilum* grows near the surface of the mats together with cyanobacteria, where there is light and oxygen, at a temperature of about 50–66°C. The organism has been found in three hot springs: Mushroom Spring, Octopus Spring, and Green Finger Pool in Yellowstone National Park. It has been found that *C. thermophilum* makes two types of chlorophyll (a and c) that allow these bacteria to thrive in microbial mats and to compete for light with cyanobacteria (Bryant et al. 2007).

Various thermophilic representatives of *Geobacillus*, *Anoxybacillus*, and *Bacillus* have been identified and characterized from diverse hot springs in Turkey by using phenotypic and genotypic methods including fatty acid methyl ester and rep-PCR profilings and 16S rRNA sequencing (Adiguzel et al. 2009). Another hot spring that has been studied is Kamchatka at Russia. Many thermophiles have been isolated from this hot spring. *Thermoproteus uzoniensis* (Bonch-Osmolovskaya et al. 1990), *Carboxydocella thermautotrophica* (Sokolova et al. 2002), *Anoxybacillus voinovskiensis* (Yumoto et al. 2004), *Carboxydocella sporoproducens* (Slepova et al. 2006), *Ammonifex thiophilus* (Miroshnichenko et al. 2008b), *Sulfurihydrogenibium rodmanii* (O'Neill et al. 2008), *Thermoanaerobacter uzonensis* (Wagner et al. 2008), and *Caldanaerobacter uzonensis* (Kozina et al. 2010) are some of the examples.

Recently whole genome of a bacterium *Acidilobus saccharovorans*, isolated from an acidic hot spring of Uzon caldera, Kamchatka (Russia), has been sequenced (Mardanov et al. 2010).

It is an obligately anaerobic acidophile with growth optimum at pH 3.5–4 and a temperature optimum of 80–85°C. It utilizes a wide range of proteinaceous and carbohydrate substrates and cannot grow lithoautotrophically on H_2 and CO_2 (Prokofeva et al. 2009). Thermophilic magnetotactic bacteria have been detected by 16S rRNA sequencing in the microbial mats of hot springs near Sierra Nevada Mountains in central California, where temperature up to 58°C has been found (Nash 2008). Population of a moderately thermophilic magnetotactic bacterium has also been discovered in Great Boiling Springs, Nevada. Amplified 16S rDNA has been obtained and sequenced, which was further authenticated by fluorescent in situ hybridization (FISH). A novel isolate *Candidatus Thermomagnetovibrio paiutensis* has been classified under the phylum Nitrospirae (Lefèvre et al. 2010).

In addition, there have been many studies on archaeal diversity in hot springs. PCR-mediated small-subunit rRNA gene (SSU rDNA) sequencing has been done to study archaeal diversity at hot-spring pools at Mt. Unzen, in Nagasaki Prefecture, Japan. This acidic hot spring which could be one of the most extreme habitats for life due to its high temperature (85–93°C) and strong acidity (pH 2.8) harbored archaeal species in majority, including thermoacidophilic *Sulfolobus* species and unidentified crenarchaeotal and korarchaeotal species (Takai and Sako 1999).

In an SSU 16S rDNA-based study of prokaryotic diversity of Bor Khlueng hot spring (Thailand), diversity of bacteria spanning 11 major lineages has been obtained. Almost 23% of the clones are classified as Acidobacteria. The other clones are grouped into the Nitrospirae, Proteobacteria, *Deinococcus–Thermus* lineage, Planctomycetes, and Verrucomicrobia. Archaeal (crenarchaeotal and korarchaeotal) clones were also obtained (Kanokratana et al. 2004).

Microbial community structure of Obsidian Pool, Sylvan Spring, and Bison Pool, Yellowstone National Park (Wyoming, USA), has been investigated by polymerase chain reaction (PCR) amplification of 16S rRNA gene sequences using environmental DNA. Diverse crenarchaea has been found to exist in all three pools, particularly affiliating with deep branching, but uncultivated organisms. Cloned DNA affiliating with the Desulfurococcales and Thermoproteales has also been identified (Meyer-Dombard et al. 2005).

A combination of both culture and culture-independent techniques revealed the presence of *Pyrobaculum* sp. and *Ignisphaera aggregans* in the near-neutral high-temperature hot spring in Rotorua, New Zealand (Niederberger et al. 2008).

Culture-independent (PCR with Crenarchaeota-specific primers and DGGE) and culture-dependent approaches have been used to study the diversity of Crenarchaeota in terrestrial hot springs of the Kamchatka Peninsula and the Lake Baikal region (Russia) and of Iceland. Both cultured (mainly hyperthermophilic) and uncultured Crenarchaeota have been obtained (Perevalova et al. 2008). Kozubal et al. (2008) isolated and characterized a thermophilic Fe-oxidizing thermophilic Cenarchaeum, *Metallosphaera*-like strain MK-1, from geothermal springs of Yellowstone National Park. This strain is shown to be a member of the order Sulfolobales, showing 94.9–96.1% sequence similarity to other known *Metallosphaera* spp. and less than 89.1% similarity to the known *Sulfolobus* spp.

Malkawi et al. (2010) investigated five major hot springs in Jordan (Ashounah, Waggas, Zara, Zarqa Ma'in, and Afra springs) using both culture-based and culture-independent approaches and obtained amplification of specific 16S rDNA sequences of bacteria, archaea, green sulfur bacteria, green non-sulfur bacteria, heliobacteria, and methanogenic archaea from metagenomic DNA extracted directly from water and mat samples from each thermal spring. Other examples of novel bacteria and archaea that have been recovered from different hot springs all over the world are presented in Tables 1.3 and 1.4.

There have been relatively few studies on isolation of fungi from hot springs. Five species of thermophilic and thermotolerant fungi have been isolated from hot springs of Northern Taiwan, which were identified as *Aspergillus fumigatus*, *Thermomyces lanuginosus* (syn. *Humicola lanuginosa*), *Humicola insolens*, *Penicillium duponti*, and *Rhizoctonia*. All these isolates can grow at temperatures ranging from 55 to 64°C (Chen et al. 2000). Several fungi have been isolated from near-neutral and alkaline thermal springs in Tengchong Rehai National Park. Internal transcribed spacer (ITS) sequencing combined with morphological analysis identified these fungi to the species level. These fungi are *Rhizomucor miehei*, *Chaetomium* sp., *Talaromyces thermophilus*, *Talaromyces byssochlamydoides*,

Thermoascus aurantiacus Miehe var. *levisporus*, *Thermomyces lanuginosus*, *Scytalidium thermophilum*, *Malbranchea flava*, *Myceliophthora* sp., and *Coprinopsis* sp. Two species, *T. lanuginosus* and *S. thermophilum*, have been found to be the dominant species (Pan et al. 2010).

1.2.1.2 Deep-Sea Hydrothermal Vents

Microbial life was earlier thought to be restricted to the thin surface layer of the planet, where the organic matter derived from photosynthesis is present and is available as an energy and nutrient source and where temperatures and chemical conditions are conducive to the known living organisms (Jørgensen and Boetius 2007), and the deep sea, which represents 88% of the Earth's area covered by seawater and 75% of the total volume of the oceans (Prieur et al. 1995), was perceived as a cold, dark, high-pressure, and nutrient-poor environment inhabited by psychrophilic, oligotrophic, and barophilic microorganisms (Prieur 1997). The discovery of deep-sea hydrothermal vents in the Galapagos Rift in 1977 made a powerful impact on the development of deep-sea biology and microbiology of hydrothermal systems (Lutz et al. 1994; Taylor et al. 1999). For the first time, an ecosystem totally based on primary production achieved by chemosynthetic bacteria was discovered.

A hydrothermal vent is a fissure in the planet's surface from which geothermally heated water emerges. Hydrothermal vents are commonly found near those areas where tectonic plates are moving apart, like volcanic areas. Hydrothermal vents are found either on land or under the deep sea. Common hydrothermal vents found on land types include hot springs, fumaroles, and geysers. Hydrothermal vents under the sea are called deep-sea hydrothermal vents (commonly known as black smokers). Hydrothermal vents occur at depths of 800–3500 m, which corresponds to hydrostatic pressures of 80–350 bars (8–35 MPa) (Miroshnichenko 2004). At deep-sea hydrothermal vents, warm and/or hot fluids are emitted. Islands of highly dense and biologically diverse prokaryotic and eukaryotic communities exist in the immediate vicinity of hydrothermal vent, as compared to the surrounding bare seafloor. These communities comprise organisms with distinct metabolism based on chemosynthesis and growth rates comparable to those from shallow-water tropical environments (Thornburg et al. 2010).

Currently, over 300 deep-sea hydrothermal vent sites are known throughout the world (Desbruyéres et al. 2006). These vent sites generally occur along a nearly continuous underwater mountain chain (midocean ridges) comprising of more than 75,000 km that remains largely unexplored for hydrothermal activity (Van Dover 2000). Figure 1.2 shows major midocean ridges and deep-sea hydrothermal vent sites throughout the world. These midocean ridges, which are located at the boundaries between the tectonic plates of Earth's crust, are the sites of incremental seafloor spreading (spreading centers) at which molten rock (magma) rises toward Earth's surface as the tectonic plates move in relation to each other.

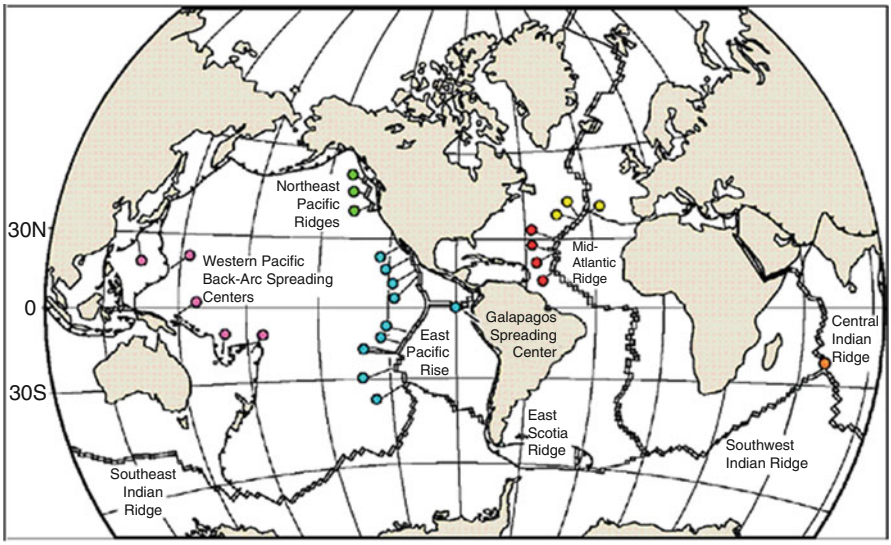

Fig. 1.2 Map showing the major midocean ridges and known deep-sea hydrothermal vent biogeographic provinces: *pink*, Western Pacific; *green*, northeast Pacific; *blue*, East Pacific Rise; *yellow*, Azores; *red*, Mid-Atlantic Ridge; *orange*, Indian Ocean (Thornburg et al. 2010)

Formation of Hydrothermal Vents

In the regions of tectonic activity, seawater interacts with the hot basaltic rocks below the ocean floor. As a result of this interaction, the water changes its chemical composition and gets overheated but still remains liquid because of the enormous hydrostatic pressure. This is called hydrothermal fluid (Miroshnichenko 2004). Hydrothermal fluids exiting vents are at the temperatures up to 400°C and are enriched with transition metals (e.g., aluminum, copper, cobalt, iron, lead, manganese, and zinc), silica, sulfides, and dissolved gases such as hydrogen and methane (Van Dover 2000; Seyfried and Mottl 1995).

The rapid mixing of these hydrothermal fluids with the surrounding cold seawater as they exit from the ocean floor causes changes in pH and temperature and the precipitation of metal sulfides and minerals (black smoke) to form columnar sulfide-rich chimney structures called black smokers. Depending on the amount of mixing with cold oceanic water before emission, hydrothermal fluids may have different physicochemical compositions (Prieur 1997). Within a year of a volcanic eruption, mature black-smoker sulfide chimneys grow 10–20 m high and may have several high-temperature orifices near the top (Tivey 1991; Haymon 1983). White smokers are the chimney structures that are formed around intermediate temperature flows (100–300°C) and formed due to precipitation of silica, anhydrite, and barite as white particles. In addition, there are several other structural variations of sulfide-rich mineral deposits, including beehives (with horizontal layering and conduits for diffuse fluid flow), flanges (where pooled hot fluids are trapped beneath a shelf-like structure), and complex sulfide mounds (Van Dover 2000).

When the hot electron-donor-rich and anoxic vent fluids meet cold electron-acceptor-rich and oxygenic ocean waters, a temperature gradient is formed, and therefore, a variety of ecological niches form in these regions characterized by different temperatures, nature of substrate, and availability of electron donors and acceptors for energy metabolism. In these regions, the chemical energy becomes available to free-living chemolithoautotrophs microorganisms and those associated with animals as symbionts. Owing to the extreme gradients and diversity in physical and chemical factors, hydrothermal vents remain incredibly fascinating to microbiologists, and most studies on deep-water microbial communities are now focused on vent habitats.

Diversity of Thermophilic Microorganisms at Deep-Sea Hydrothermal Vents

Hydrothermal vents have been one of the most fascinating environments for the search of extremophiles because of presence of high temperatures, hydrostatic pressures, reducing power, toxic chemistry, extreme pH, and fluctuations of environmental conditions (Takai et al. 2006b). In hydrothermal vents, microorganisms exist in either free-living form or remain attached to rocks, to sediment, or on/in vent animals, which in turn may feed directly on the microbes or engage in symbiotic associations to acquire fixed carbon and nitrogen (Tivey 1991; Haymon 1983; Robigou et al. 1993). Gradients of temperature and chemistry in hydrothermal vents support free-living microorganisms with a diverse physiologies and tolerances. These microorganisms can be detected hundreds of kilometers away from vent fields (Jannasch and Wirsen 1979; Juniper et al. 1988). They also form microbial mats of various colors and morphologies on the surface of basalt (Moyer et al. 1994; Santelli et al. 2008) and chimney spires (Kormas et al. 2006) and within hydrothermal sediments (Lopez-Garcia et al. 2003) where they serve as food for numerous invertebrate species (Van Dover and Fry 1994).

In the absence of light and presence of hydrothermal fluids rich in minerals, reduced compounds (including H_2S, CH_4), and CO_2, chemical energy replaces solar energy as the fuel that supports primary production by chemosynthetic bacteria and archaea (Kelley et al. 2002). Both lithotrophic and organotrophic isolates have been isolated from deep-sea vents. The majority of the recent isolates are lithotrophs, obligately or facultatively dependent on inorganic energy sources, and obligate or facultative anaerobes. This is in good agreement with the environmental conditions that exist within hydrothermal fluid. Owing to high concentration of H_2S within hydrothermal fluids (3–110 mmol per kg seawater), sulfide oxidation is a dominant microbial source of energy in vent communities (Kelley et al. 2002). The aerobes use O_2 as the electron acceptor during the energy-yielding chemosynthetic reaction, while anaerobes use CO_2, Fe^{3+}, NO_3^{2-}, or organic compounds (to oxidize H_2) (Kelley et al. 2002).

The diversity of hydrothermal vent microbial communities cannot truly be determined by artificial culture-dependent techniques, since 99% of marine microbes are unculturable (Amann et al. 1995). Thus, molecular phylogeny-based approach has been used using nucleotide-sequence analysis of the highly conserved gene for the

small-subunit (SSU) rRNA molecule (16S rRNA) (Pace 1997). New phylotypes, often representing major new lineages, have been shown consistently with each molecular analysis of microbial environments (Pace 1997; Venter et al. 2004; Sogin et al. 2006). For example, Huber et al. (2002a) identified a new archaeal phylum known as "Nanoarchaeota" by the analysis of PCR-amplified SSU rRNA genes from a defined coculture of hyperthermophilic archaea. Similar methods have indicated the emergence of a newly defined lineage distributed throughout the global deep-sea vent system called as the "deep-sea hydrothermal vent Euryarchaeota group" (DHVEG) (Takai and Horikoshi 1999).

Microbial communities of deep-sea hydrothermal vents include microorganisms of both the bacteria and archaea domains. In the last decades of the twentieth century, significant work on the diversity of thermophiles inhabiting hydrothermal environment has been done. At that time, most of known deep-sea thermophilic prokaryotes were represented by hyperthermophilic archaea (Blöchl et al. 1995; Prieur et al. 1995). The thermophilic archaeal lithotrophs included *Methanococcus* (Jones et al. 1983; Zhao et al. 1988; Jeanthon et al. 1999) and the endemic genus *Methanopyrus* (Kurr et al. 1991), characterized by temperature optimum at 98°C and maximum at 110°C, a hydrogen-oxidizing *Pyrolobus fumarii* (Blöchl et al. 1997) that could grow at even higher temperatures (optimum at 106°C and maximum at 113°C) by means of nitrate-, thiosulfate-, or microaerobic respiration and sulfate- and thiosulfate-reducing archaea *Archaeoglobus* (Burggraf et al. 1990; Huber et al. 1997). Organotrophic thermophilic archaea were represented by numerous obligately anaerobic members of Thermococcales (Zillig and Reysenbach 2001) and various species of the genera *Pyrodictium* (Pley et al. 1991) and *Staphylothermus* (Fiala et al. 1986).

At that time, thermophilic bacteria attracted much less attention of microbiologists than archaea, being represented solely by *Thermosipho melanesiensis* (Antoine et al. 1997), a member of the genus previously shown in shallow-water habitats (Huber et al. 1989), and *Desulfurobacterium thermoautotrophum* (L'Haridon et al. 1998), an obligately anaerobic hydrogen-utilizing sulfur-reducing bacterium. Many thermophilic microorganisms inhabiting deep-sea vents have been discovered since then.

Archaea generally comprise a larger fraction of microbial communities at vents compared with ocean sediments. Until recently organisms with lithotrophic sulfur respiration were not found in deep-sea thermal environments. The first thermophile of this physiological type found in a deep sea is *Ignicoccus pacificus* (Huber et al. 2000) which is an obligately anaerobic lithoautotroph utilizing only H_2, elemental sulfur, and CO_2 as the energy substrate, the electron acceptor, and the carbon source, respectively. By using specific PCR primers, another *Ignicoccus* species has been found to host symbiotic representatives of a novel archaeal kingdom Nanoarchaeota in deep-sea vents (Huber et al. 2002; Hohn et al. 2002). Kashefi et al. (2002) obtained a Euryarchaeota *Geoglobus ahangari* from the Guaymas Basin hydrothermal system in 2002. A ferric iron-reducing thermophilic archaeon has been reported from an active "black smoker" in the Juan de Fuca Ridge. This was previously called strain 121, which is now tentatively named as "*Geogemma*

barossii" (Kashefi and Lovley 2003; Lovley et al. 2004). This obligate lithoautotroph, belonging to Desulfurococcales, utilizes molecular hydrogen alone and grows at 121°C. The heat-loving vent archaea *Pyrolobus fumarii* and strain 121 (*Geogemma barossii*) hold the global record for growth at high temperatures (113 and 121°C, respectively) (Blöchl et al. 1997; Kashefi and Lovley 2003). This temperature range is currently thought to represent the upper limit for life.

Among methanogens, a few members of Methanococcaceae have been recovered from hydrothermal vents of the Central Indian Ridge, representing new species of the genera *Methanothermococcus*, *Methanocaldococcus*, and *Methanotorris* (Takai et al. 2002, 2004; L'Haridon et al. 2003). *Methanococcus infernos* (Jeanthon et al. 1998) and *Methanococcus vulcanius* (Jeanthon et al. 1999) have been isolated from Mid-Atlantic Range and East Pacific Rise, respectively.

Various organotrophic hyperthermophilic archaea have been reported from deep-sea hydrothermal chimney, Suiyo Seamount, the Izu-Bonin Arc, Japan. *Aeropyrum camini* was the first obligately aerobic hyperthermophilic abyssal archaeon found in a deep-sea environment (Nakagawa et al. 2004). Representatives of Methanococcales, Methanopyrales, Archaeoglobales, and Thermococcales have been revealed from the analysis of hydrothermal and subseafloor abyssal environment's 16S rRNA clone libraries which have also been detected by cultivation methods previously (Jeanthon 2000; Reysenbach et al. 2000a, b; Huber et al. 2002; Nercessian et al. 2003). An obligately anaerobic organotroph *Palaeococcus ferrophilus*, another new genus in the order Thermococcales (Takai et al. 2000), has also been isolated from a deep-sea chimney Myojin Knoll near Japan. Many novel representatives of the genus *Thermococcus* including *T. guaymasensis* (Canganella et al. 1997), *T. fumicolans* (Godfroy et al. 1996), *T. barophilus* (Marteinsson et al. 1999), *T. gammatolerans* (Jolivet et al. 2003), *T. coalescens* (Kuwabara et al. 2005), and *T. celericrescens* (Kuwabara et al. 2007) had been described from different hydrothermal vent sites. Representatives of some taxa like Thermoplasmatales (Reysenbach et al. 2000a, b; Huber et al. 2002) have been detected by molecular methods, which include obligate acidophiles that proliferate in inner parts of hydrothermal chimneys filled with acidic hydrothermal fluid at initial pH about 3.5. Among thermoacidophilic organisms, the representatives of Thermococcales have been detected from deep-sea vents by Prokofeva et al. (2005). A heterotrophic member of the ubiquitous, abundant, and apparently endemic deep-sea hydrothermal vent Euryarchaeota group DHVE2 had been cultivated. It shows optimum growth at a low pH and high temperature and the use of sulfur and iron as electron acceptors (Reysenbach et al. 2006).

The deep-sea hydrothermal vents are also habitat for various representatives of the domain bacteria. A species of the new genus *Persephonella*, which is related to *Aquifex* on the family level, has been isolated (Götz et al. 2002; Nakagawa et al. 2003). In 2003, Hoek et al. identified another member of Aquificaceae, *Hydrogenobacter thermolithotrophum*, by molecular techniques in a deep-sea hydrothermal environment. Two organisms growing in the temperature range from 55°C to 80°C and optimally at 70°C have been described from deep-sea thermal habitats (Jeanthon et al. 2002; Moussard et al. 2004). *Thermodesulfobacterium*

hydrogeniphilum (Jeanthon et al. 2002) utilizes hydrogen and CO_2 as sources of energy and carbon, respectively. Another isolate *Thermodesulfatator indicus* (Moussard et al. 2004) exhibits very similar phenotypic characteristics but is only distantly related to Thermodesulfobacteriaceae (<90% 16S rRNA gene sequence similarity). *Deferribacter abyssi* (Miroshnichenko et al. 2003a) has been isolated from Suiyo Seamount hydrothermal chimney (Japan) and hot vents of the East Pacific Rise. The first thermophilic member of the family Geobacteraceae, *Geothermobacter ehrlichii*, has been isolated by Kashefi et al. (2003) from hydrothermal fluid of a Juan de Fuca Ridge deep-sea vent. The family included genera capable of dissimilatory ferric iron reduction (Lovley et al. 2004), and until recently this family consisted only of mesophilic species. This organism oxidizes volatile fatty acids, organic acids, and alcohols and reduces ferric iron to ferrous and nitrate to ammonium.

Among gram-negative thermophilic bacteria, Thermaceae constitutes one of the best-studied groups. Its first representative isolated from a deep-sea habitat under aerobic conditions is *Thermus thermophilus*, a strain of a widely occurring terrestrial species (Marteinsson 1999). Recently, several members of Thermaceae have been described from different deep-sea hydrothermal habitats, one of which was *Marinithermus hydrothermalis*. It is the only obligate aerobe among new isolates. The three other Thermaceae members *Oceanithermus profundus*, *Oceanithermus desulfurans* (Miroshnichenko et al. 2003b; Mori et al. 2004), and *Vulcanithermus mediatlanticus* (Miroshnichenko et al. 2003c) are the only microaerophiles in this family.

Representatives of the order Thermotogales have also isolated from deep-sea vent environments. A thermophilic, anaerobic, piezophilic, chemoorganotrophic sulfur-reducing bacterium, *Marinitoga piezophila*, had also been reported by Alain et al. (2002b). The growth of this bacterium is enhanced by hydrostatic pressure, the optimal pressure being 40 MPa (26 MPa pressure at sampling site). Another anaerobic and organotropic member of this order *Marinitoga hydrogenitolerans* that could tolerate 100% hydrogen had been described by Postec et al. (2005). A sulfur-reducing heterotrophic species of this genus, *M. okinawensis*, has been isolated by Nunoura et al. (2007b).

Epsilonproteobacteria represent an extremely abundant and diverse group in hydrothermal vents. This has been assessed by means of culture-independent molecular phylogenetic techniques. Members of this group dominate in microbial hydrothermal mats (Moyer et al.1995; Longenecker and Reysenbach 2001) in the epibiotic microflora of deep-sea vent metazoans (Habbad et al. 1995; Campbell and Cary 2001) and diffuse flow hydrothermal fluids (Huber et al. 2003). Epsilonproteobacteria inhabiting deep-sea hot vents had been classified into six phylogenetic groups (A–F) by Corre and coworkers in 2001. The first representatives of Epsilonproteobacteria from deep-sea hydrothermal vents are *Nautilia lithotrophica* (Miroshnichenko et al. 2002) and *Caminibacter hydrogenophilus* (Alain et al. 2002a). *Caminibacter profundus* (Miroshnichenko 2004), which had been isolated from deep-sea hydrothermal system on the Mid-Atlantic Ridge, is an obligate lithoautotroph and microaerophile. Voordeckers et al. (2005) characterized another species *C. mediatlanticus*. Other

species belonging to the genus *Nautilia* (*N. nitratireducens*, *N. abyssi*, and *N. profundicola*) have also been recovered from hydrothermal vent ecosystems (Pérez-Rodríguez et al. 2010; Alain et al. 2009; Smith et al. 2008). Until the discovery of these representatives, Epsilonproteobacteria consisted of a single order Campylobacterales comprising two families: Campylobacteraceae and Helicobacteraceae (Vandamme and De Ley 1991). The representatives of the genera *Nautilia* and *Caminibacter*, together with the uncultivated Epsilonproteobacteria, form a deep phylogenetic branch within the class Epsilonproteobacteria. Hence, Miroshnichenko and coworkers (2004) proposed a separate order Nautiliales of the subclass Epsilonproteobacteria with a single family Nautiliaceae. Today, this family includes three genera: *Nautilia*, *Caminibacter*, and *Lebetimonas*, the latter one being represented by a single species *Lebetimonas acidiphila* (Takai et al. 2005). These Epsilonproteobacteria members are mostly chemolithotrophs and can utilize diverse electron acceptors. A novel thermophilic bacterium *Hydrogenimonas thermophilus* has been isolated from an in situ colonization system deployed in a superheated, deep-sea, hydrothermal vent emission at the Kairei Field on the Central Indian Ridge in the Indian Ocean. It has been classified within the previously uncultivated phylogroup {varepsilon}-Proteobacteria group A (Takai et al. 2003b). Two novel nitrifying bacteria belonging to {varepsilon}-Proteobacteria (*Nitratiruptor tergarcus* and *Nitratifractor salsuginis*) have been obtained from hydrothermal field Mid-Okinawa Trough, Japan (Nakagawa et al. 2005b). They are strict chemolithoautotrophs, which grow by respiratory nitrate reduction with H_2, forming N_2 as a metabolic product.

Another phylogenetically remarkable organism isolated from a hydrothermal sample is *Caldithrix abyssi* (Miroshnichenko et al. 2003d). This anaerobic moderately thermophilic bacterium represents a new phylogenetic phylum. The similarity value of the 16S rRNA gene of *C. abyssi* with other known microorganisms does not exceed 82.3%.

Recently, novel sulfate reducers have been isolated from deep-sea hydrothermal chimneys. Two sulfate-reducing *Desulfothermus okinawensis* (Nunoura et al. 2007a) and *Desulfovibrio hydrothermalis* (Alazard et al. 2003) are heterotrophs, while *Desulfurobacterium atlanticum* (L'Haridon et al. 2006) is a chemolithoautotroph representing a new family Desulfurobacteraceae within the order Aquificales. A sulfur-oxidizing obligate chemolithoautotroph *Hydrogenivirga okinawensis* utilizes elemental sulfur or thiosulfate as an electron donor and nitrate or oxygen as an electron acceptor (Nunoura et al. 2008). Another strictly anaerobic sulfate reducer *Thermodesulfatator atlanticus* has recently been described from a deep-sea hydrothermal vent at the Rainbow site on the Mid-Atlantic Ridge (Alain et al. 2010).

Several other novel genera have been obtained from different hydrothermal vent ecosystems. A novel genus *Caloranaerobacter azorensis*, which is anaerobic and chemoorganotrophic, had been reported from a deep-sea hydrothermal chimney sample (Wery et al. 2001). A strict anaerobe, hydrogen-oxidizing chemolithoautotroph has been isolated from a black-smoker chimney in the Suiyo Seamount hydrothermal system and named as *Balnearium lithotrophicum* (Takai et al. 2003a). A lactic acid producing facultatively anaerobic, halotolerant, and moderately

thermophilic bacterium *Exiguobacterium profundum* had been isolated from hydrothermal vent of East Pacific Rise (Crapart et al. 2007). In 2008, Imachi et al. isolated an anaerobic and novel representative of the phylum Spirochaetes, *Exilispira thermophila*. *Sulfurivirga caldicuralii*, a microaerobic chemolithoautotroph capable of using thiosulfate or tetrathionate as a sole energy source, O_2 as the sole electron acceptor, and CO_2 as the sole carbon source, has been described from a shallow marine hydrothermal system (water depth 22 m) occurring in coral reefs off Taketomi Island, Okinawa (Takai et al. 2006a). Another bacterium *Thermosipho affectus* representing a novel thermophilic anaerobic cellulolytic bacterium has been recovered from a hydrothermal vent system (Podosokorskaya et al. 2011). Some novel bacterial and archaeal thermophiles from deep-sea vents and their sites of isolation are presented in Tables 1.5 and 1.6.

The occurrence of fungi (filamentous fungi and yeasts) at deep-sea hydrothermal vents remains an underexplored topic. In contrast to surface environments, deep-sea environmental gene libraries have suggested that fungi are rare in high-pressure marine environments, and yeast forms dominate in these areas (Bass et al. 2007). This is because of the fact that fungal thermal tolerance is much lower than that of prokaryotes. Till now, no thermophilic yeasts are known from deep-sea vents (fungi are considered thermophilic if they grow at 50°C or higher temperatures and do not grow at 20°C or lower temperatures). Due to the presence of thermal gradient in hydrothermal fields (300–3°C), adequate conditions for mesophiles can also be found. Culture-dependent studies on the diversity of yeast and fungi in deep-sea environments discuss about those species that are mesophilic (Gadanho and Sampaio 2005; Burgaud et al. 2009). Due to the development of culture-independent molecular techniques, the presence of fungi has also been directly reported in deep-sea environmental DNA samples. Sequence analysis of SSU ribosomal RNA genes reveals scarce fungal diversity, but some sequences are novel (Edgcomb et al. 2002; Lopez-Garcia et al. 2003, 2007). Bass et al. (2007) reported the presence of sequences affiliated to *Debaryomyces hansenii* and novel sequences closed to *Malassezia furfur* in hydrothermal sediments. Nagano et al. (2010) investigated the diversity of fungal communities in ten different deep-sea sediment samples by PCR-mediated internal transcribed spacer (ITS) regions of rRNA gene clone analysis and reported the predominance of deep-sea phylotypes belonging to Ascomycota in these environments.

1.2.1.3 Geothermally Heated Oil and Petroleum Reserves

An oil field is a region with an abundance of oil wells extracting crude oil (petroleum) from belowground. As oil reservoirs extend over a large area, multiple wells are scattered across this area for the extraction of oil. More than 40,000 oil fields are located throughout the Earth, on land and offshore. The largest are the Ghawar field in Saudi Arabia and the Burgan field in Kuwait. The initial reservoir pressure is often enough for the recovery of oil and gas from the well that flows freely to the surface (primary recovery). When this initial pressure is depleted, water is introduced into

Table 1.5 List of novel thermophilic bacteria isolated from deep-sea hydrothermal vents

Genus/species	Site of isolation	T_{opt} (°C)	References
Thermosipho melanensis	Southwestern Pacific Ocean	70	Antoine et al. (1997)
Desulfurobacterium hydrogenophilum	Mid-Atlantic Ridge	70	L'Haridon et al. (1998)
Marinotoga camini	Mid-Atlantic Ridge	55	Wery et al. (2001)
Caloranaerobacter azorensis	Mid-Atlantic Ridge	65	Wery et al. (2001)
Caldanaerobacter subterraneus	Mid-Okinawa Trough	70	Sokolova et al. (2001)
Hydrogenobacter acidophilus	Vulcano Island	65	Stohr et al. (2001)
Carboxydobrachium pacificum	Okinawa Trough	70	Sokolova et al. (2001)
Marinotoga piezophila	East Pacific Rise	55	Alain et al. (2002b)
Caminicella sporogenes	East Pacific Rise	55–60	Alain et al. (2002c)
Persephonella marina	East Pacific Rise	73	Götz et al. (2002)
Nautilia lithotrophica	East Pacific Rise	53	Miroshnichenko et al. (2002)
Caminibacter hydrogeniphilus	East Pacific Rise	60	Alain et al. (2002a)
Thermovibrio ruber	Lihir Island, New Guinea	75	Huber et al. (2002)
Tepidibacter thalassicus	East Pacific Rise	50	Slobodkin et al. (2003)
Caldithrix abyssi	East Pacific Rise	60	Miroshnichenko et al. (2003d)
Thermodesulfatator indicus	Central Indian Ridge	70	Moussard et al. (2004)
Deferribacter abyssi	Mid-Atlantic Ridge	60–65	Miroshnichenko et al. (2003c)
Clostridium caminithermale	Atlantic Ocean Ridge	45	Brisbarre et al. (2003)
Persephonella hydrogenophila	Izu-Bonin Arc, Japan	70	Nakagawa et al. (2003)
Desulfurobacterium crinifex	Juan de Fuca Ridge	60–65	Alain et al. (2003)
Balnearium lithotrophicum	Izu-Bonin Arc, Japan	70–75	Takai et al. (2003a)
Marinithermus hydrothermalis	Izu-Bonin Arc, Japan	60–65	Sako et al. (2003)
Oceanithermus profundus	East Pacific Rise	60	Miroshnichenko et al. (2003a)
Thermovibrio ammonificans	East Pacific Rise	75	Vetriani et al. (2003)
Tepidibacter formicigenes	Mid-Atlantic Ridge	45	Urios et al. (2004)
Caminibacter mediatlanticus	Mid-Atlantic Ridge	55	Voordeckers et al. (2005)
Hydrogenimonas thermophilus	Central Indian Ridge	55	Takai et al. (2003b)
Nitratiruptor tergarcus	Mid-Okinawa Trough, Japan	55	Nakagawa et al. (2005b)
Lebetimonas acidiphila	Mariana Arc	50	Takai et al. (2005)
Vulcanibacillus modesticaldus	Mid-Atlantic Ridge	55	L'Haridon et al. (2006)
Sulfurivirga caldicuralii	Taketomi Island, Japan	50–55	Takai et al. (2006a)
Thermaerobacter litoralis	Kagoshima Prefecture, Japan	70	Tanaka et al. (2006)
Desulfothermus okinawensis	Southern Okinawa Trough	50	Nunoura et al. (2007a)
Exiguobacterium profundum	East Pacific Rise	45	Crapart et al. (2007)

(continued)

Table 1.5 (continued)

Genus/species	Site of isolation	T_{opt} (°C)	References
Marinitoga okinawensis	Southern Okinawa Trough	55–60	Nunoura et al. (2007b)
Exilispira thermophila	Okinawa Trough, Japan	50	Imachi et al. (2008)
Clostridium tepidiprofundi	East Pacific Rise	50	Slobodkina et al. (2008)
Exilispira thermophila	Okinawa Trough, Japan	50	Imachi et al. (2008)
Hydrogenivirga okinawensis	Southern Okinawa Trough	70–75	Nunoura et al. (2008)
Thermosulfidibacter takaii	Southern Okinawa Trough	70	Nunoura et al. (2008)
Nautilia abyssi	East Pacific Rise	60	Alain et al. (2009)
Rhodothermus profundus	East Pacific Rise	70	Marteinsson et al. (2010)
Thermodesulfatator atlanticus	Mid-Atlantic Ridge	65–70	Alain et al. (2010)
Nautilia nitratireducens	East Pacific Rise	55	Pérez-Rodríguez et al. (2010)
Rhodothermus profundus	East Pacific Rise	70	Marteinsson et al. (2010)
Thermosipho affectus	Mid-Atlantic Ridge	70	Podosokorskaya et al. (2011)
Thermosipho globiformans	Izu-Bonin Arc, Pacific Ocean	68	Kuwabara et al. (2011)

the injection well to pressurize the production well again. This is known as water flooding. It pushes the oil toward the production wells until oil production start again (secondary recovery).

These oil fields are one of the deep geological environments that support the existence of microorganisms. They occur at significant subsurface depth and are characterized by a high in situ temperature. Thus, these geothermally heated petroleum reservoirs represent unique biotopes that constitute ecological niches providing suitable conditions to support thermophiles (Stetter et al. 1993). They have attracted the great interest of researchers for the isolation of novel thermophilic microorganisms (Greene et al. 1997; Bonch-Osmolovskaya et al. 2003; Magot et al. 2000; Salinas et al. 2004a, b; DiPippo et al. 2009; Jayasinghearachchi and Lal 2011).

Diversity of Thermophilic Microorganisms in Oil Fields and Petroleum Reservoirs

Physiological types of microorganisms that have been isolated from these biotopes include heterotrophs (Takahata et al. 2001; DiPippo et al. 2009), sulfate reducers (Tardy-Jacquenod et al. 1996; Stetter et al. 1993; Miranda-Tello et al. 2003), sulfidogens (L'Haridon et al. 1995; Stetter et al. 1993), fermentative bacteria (Davey et al. 1993; Grassia et al. 1996; Miranda-Tello et al. 2007), manganese and iron

Table 1.6 List of novel thermophilic archaea isolated from deep-sea hydrothermal vents

Genus/species	Site of isolation	T_{opt} (°C)	References
Methanocaldococcus jannaschii	Guaymas Basin	86	Zhao et al. (1988), Jones et al. (1983)
Archaeoglobus profundus	Mid-Atlantic Ridge	92	Burggraf et al. (1990)
Methanopyrus kandleri	Guaymas Basin	110	Kurr et al. (1991)
Pyrococcus abyssi	North Fiji Basin	96	Erauso et al. (1993)
Thermococcus profundus	Mid-Okinawa Trough	90	Kobayashi et al. (1994)
Thermococcus chitonophagus	East Pacific Rise	93	Huber et al. (1995),
Thermococcus hydrothermalis		85	Godfroy et al. (1997)
Thermococcus fumicolans	North Fiji Basin	85	Godfroy et al. (1996)
Archaeoglobus veneficus	Mid-Atlantic Ridge	–	Huber et al. (1997)
Thermococcus guaymasensis	Guaymas Basin	80–90	Canganella et al. (1997)
Methanocaldococcus infernus	Mid-Atlantic Ridge	85	Jeanthon et al. (1998)
Thermococcus siculi	Mid-Okinawa Trough	90	Grote et al. (1999)
Methanocaldococcus vulcanius	East Pacific Rise	80	Jeanthon et al. (1999)
Methanocaldococcus fervens	Guaymas Basin	–	Jeanthon et al. (1999)
Thermococcus barophilus	Mid-Atlantic Ridge	95	Marteinsson et al. (1999)
Pyrococcus glycovorans	East Pacific Rise	95	Barbier et al. (1999)
Palaeococcus ferrophilus	Ogasawara-Bonin Arc., Japan	83	Takai et al. (2000)
Ignicoccus pacificus	East Pacific Rise	90	Huber et al. (2000)
Methanothermococcus okinawensis	Mid-Okinawa Trough	60–65	Takai et al. (2000)
Geoglobus ahangari	Guaymas Basin	88	Kashefi et al. (2002)
Thermococcus gammatolerans	Guaymas Basin	88	Jolivet et al. (2003)
Geogemma barossii	Juan de Fuca Ridge	105–107	Kashefi and Lovley (2003)
Methanocaldococcus indicus	Central Indian Ridge	85	L'Haridon et al. (2003)
Methanotorris formicicus	Central Indian Ridge	85	Takai et al. (2004)
Aeropyrum camini	Izu-Bonin Arc, Japan	85	Nakagawa et al. (2004)
Thermococcus coalescens	Suiyo Seamount	87	Kuwabara et al. (2005)
Thermococcus celericrescens	Suiyo Seamount, Western Pacific Ocean	80	Kuwabara et al. (2007)
Thermococcus thioreducens	Mid-Atlantic Ridge	83–85	Pikuta et al. (2007)
Pyrococcus yayanosii	Mid-Atlantic Ridge	98	Birrien et al. (2011)

reducers (Greene et al. 1997), methanogens (Rozanova et al. 1997; Ng et al. 1989; Cheng et al. 2007), acetogens (Davydova-Charakhchyan et al. 1993), and many more. Most of them are anaerobes, including a few aerobes (Nazina et al. 2001, 2005). Table 1.7 lists the novel thermophilic microorganisms isolated from various geothermal oil and petroleum reservoirs. Apart from culture-based approaches, different molecular methods, like reverse genome probing, hyb ridization with functional

Table 1.7 List of novel thermophiles isolated from geothermally heated oil and petroleum reserves

Genus/species	Location	T_{opt} (°C)	Comments	References
Thermoanaerobacter brockii subsp. *lactiethylicus*	Deep subsurface French oil well	55–60	Strict anaerobe	Cayol et al. (1995)
Thermotoga elfii	African oil-producing well	66	Strict anaerobe	Ravot et al. (1995)
Desulfotomaculum thermocisternum	North Sea oil reservoir, UK	62	Sulfate reducer	Nilsen et al. (1996)
Anaerobaculum thermoterrenum	–	55	Anaerobic citrate fermentor	Rees et al. (1995)
Deferribacter thermophilus	Beatrice oil field in the North Sea, UK	60	Anaerobe	Greene et al. (1997)
Thermosipho geolei	Oil reservoir in Siberia (Russia)	70	Anaerobic S° reducer	L'Haridon et al. (2001)
Thermotoga petrophila	Kubiki oil reservoir in Niigata, Japan	Range 47–88	Strictly anaerobic heterotroph	Takahata et al. (2001)
Thermotoga naphthophila	Kubiki oil reservoir in Niigata, Japan	Range 48–86	Strictly anaerobic heterotroph	Takahata et al. (2001)
Geobacillus subterraneus	Oil fields in Russia, Kazakhstan, and China	–	Moderately thermophile, aerobe	Nazina et al. (2001)
Geobacillus uzenensis	Oil fields in Russia, Kazakhstan, and China	–	Moderately thermophile, aerobe	Nazina et al. (2001)
Petrotoga olearia	Continental oil reservoir in Western Siberia	55	Anaerobe	L'Haridon et al. (2002)
Petrotoga sibirica	Continental oil reservoir in Western Siberia	55	Anaerobe	L'Haridon et al. (2002)
Desulfovibrio capillatus	Mexican oil field	40	Sulfate reducer	Miranda-Tello et al. (2003)
Garciella nitratireducens	Gulf of Mexico	55	Anaerobic nitrate and thiosulfate reducer	Miranda-Tello et al. (2003)

Mahella australiensis	Australian terrestrial oil reservoir	50	Anaerobe	Salinas et al. (2004b)
Petrobacter succinatimandens	Australian terrestrial oil reservoir	55	Aerobic nitrate reducer	Salinas et al. (2004a)
Petrotoga mexicana	Gulf of Mexico	55	Anaerobic S° reducer	Miranda-Tello et al. (2004)
Caldanaerobacter subterraneus	Oil fields of France	–	Anaerobe	Fardeau et al. (2004)
Thermovirga lienii	North Sea oil reservoir, UK	58	Anaerobe	Dahle and Birkeland (2006)
Methermicoccus shengliensis	Shengli oil field, China	65	Methylotroph	Cheng et al. 2007
Petrotoga halophila	Oil-producing well in Congo, West Africa	60	Fermentative	Miranda-Tello et al. (2007)
Kosmotoga olearia	Troll B oil platform, North Sea	65	Heterotroph	DiPippo et al. (2009)
Oceanotoga teriensis	Oil production well, Bombay High, India	55–58	Chemoorganotroph	Jayasinghearachchi and Lal (2011)

gene probes, 16S rDNA analysis, and immunological techniques, have been used to assess physiological and genetic diversity of thermophiles in the oil fields (Christensen et al. 1992; Leu et al. 1998; Voordouw et al. 1996, 1992).

Pyrococcus and *Thermococcus* spp. have been found in oil reservoirs (Stetter et al. 1993). Both of them derive energy by fermentation of peptides, amino acids, and sugars, forming fatty acids, CO_2, and H_2 (Fiala and Stetter 1986). *Pyrococcus furiosus* is able to ferment pyruvate, forming acetate, H_2, and CO_2 (Schäfer and Schönheit 1992). Some species of archaeal coccoid sulfate reducers occur within hot oil reservoirs and may be responsible for H_2S production or "reservoir souring" there (Stetter et al. 1993). *Archaeoglobus fulgidus* and *Archaeoglobus lithotrophicus* fulfill their energy requirements by reduction of SO_4^{2-} by H_2. *Archaeoglobus profundus* is an obligate heterotroph (Beeder et al. 1994).

The novel genera and species *Thermodesulforhabdus norvegicus* (Beeder et al. 1995) and *Desulfacinum infernum* (Rees et al. 1995) have been isolated from deep, hot oil fields. Oil fields and geothermal springs have commonly yielded moderately thermophilic members of the genus *Desulfotomaculum*, including *Desulfotomaculum geothermicum* (Daumas et al. 1988), *Desulfotomaculum australicum* (Love et al. 1993), and *Desulfotomaculum kuznetsovii* (Nazina et al. 1987). These sulfate reducers can utilize a wide substrate spectrum of low-molecular-weight alcohols and organic acids.

Species of the genera *Geotoga* and *Petrotoga* have been retrieved only from deep oil reservoirs. *Petrotoga miotherma* (Davey et al. 1993) and *Petrotoga mobilis* (Lien et al. 1998) have been isolated from petroleum reservoirs from Oklahoma and Texas and from the North Sea, respectively. Strictly anaerobic *Petrotoga olearia* and *Petrotoga sibirica* have been described from continental oil reservoir in Western Siberia by the 16S rDNA sequence analysis (L'Haridon et al. 2002). *Petrotoga mexicana* and *Petrotoga halophila* have been found in oil reservoirs of Gulf of Mexico (Miranda-Tello et al. 2004) and Congo, West Africa (Miranda-Tello et al. 2007), respectively. Recent microbiological studies performed on oil–water mixtures from different high-temperature strata of the Samotlor oil fields (Western Siberia) have identified phylogenetically diverse thermophilic organisms (Slobodkin et al. 1999; Jeanthon et al. 2000). Among them, novel species of the genera *Thermococcus* and *Thermosipho* have been isolated and characterized (L'Haridon et al. 2001; Miroshnichenko et al. 2001).

Among the aerobic, thermophilic, spore-forming bacteria, members of the genus *Geobacillus* (*G. subterraneus* and *G. uzenensis*) have been isolated (Nazina et al. 2000). *Geobacillus jurassicus* and *G. stearothermophilus* have also been described from oil reservoirs of China. These are aerobic, gram-positive, rod-shaped, moderately thermophilic chemoorganotrophs capable of growing on various sugars, carboxylic acids, and crude oil (Nazina et al. 2005).

Both molecular and culture-based methods have been used to characterize prokaryotic microorganisms associated with high-temperature, sulfur-rich oil reservoirs in California. Heterotrophic enrichments from all sites yielded sheathed rods (*Thermotogales*), pleomorphic rods resembling *Thermoanaerobacter*, and *Thermococcus*-like isolates. The major autotrophs recovered from inorganic enrichments include

isolates closely related to *Methanobacterium*, *Methanococcus*, and *Methanoculleus* species. Sequence analysis of 16S rDNA libraries generated from total community DNA yield *Thermoanaerobacter*, *Thermococcus*, *Desulfovibrio*, *Aminobacterium*, *Acidaminococcus*, *Pseudomonas*, *Halomonas*, *Acinetobacter*, *Sphingomonas*, *Methylobacterium*, and *Desulfomicrobium*, suggesting the widespread distribution of sulfur-utilizing and methane-producing thermophilic microorganisms in these oil reservoirs (Orphan et al. 2000).

Microbial biodiversity in the formation waters of the Samotlor high-temperature oil reservoir (Western Siberia, Russia) has been demonstrated by the radioisotopic, cultural, and molecular methods. Enrichment cultures indicate the presence of diverse physiological groups representing aerobic and anaerobic thermophiles and hyperthermophiles, primarily the representatives of *Thermotoga*, *Thermoanaerobacter*, *Geobacillus*, *Petrotoga*, *Thermosipho*, and *Thermococcus*. Oligonucleotide microchip analyses also revealed the presence of several groups of microorganisms that escaped cultivation, which included the representatives of *Aquificales* and members of *Desulfurococcus* and *Thermus*, which have been unknown in this habitat till now (Bonch-Osmolovskaya et al. 2003).

Thermotolerant sulfidogenic communities have been examined in the production waters of onshore oil fields in North-Eastern India. Most of the sulfur-reducing bacteria have been affiliated to the members of *Desulfovibrio*, *Desulfomicrobium*, *Desulfotomaculum*, and *Desulfobulbus* based on 16S rDNA sequence analysis. Most of the thiosulfate-reducing isolates have been affiliated with the phylum Firmicutes, including *Clostridium* and *Fusibacter*, and also with the phylum Proteobacteria, including the genera *Enterobacter* and *Citrobacter* (Agrawal et al. 2010). Anaerobic sulfate and thiosulfate reducers *Desulfovibrio capillatus* and *Garciella nitratireducens* have also been described from Mexican oil fields (Miranda-Tello et al. 2003).

1.2.1.4 Solar-Heated Soils/Sediments

Thermophilic representatives of bacteria and fungi have been isolated from solar-heated soils and sediments. The main source-producing heat in these habitats is solar heat radiation (Geiger 1965). There are over 200 identified bacterial genera, and a single soil sample may have over 2,000 genetically distinct bacteria (Torsvik et al. 1990). There has been widespread occurrence of thermophilic bacteria, filamentous fungi, and yeast in solar-heated soils at the temperatures reaching 70°C. Thermophilic spore formers belonging to *Bacillus*, *Clostridium*, and *Thermoactinomyces* have been isolated from soils. These include aerobes and facultative anaerobes (*Bacillus stearothermophilus*, *B. acidocaldarius*, *B. caldotenax*, *Thermoactinomyces vulgaris*) and anaerobes (*Clostridium thermocellum* and *C. therrnohydrosulfuricum*) (Zeikus 1979). Touzel et al. (2000) isolated a novel aerobic, thermophilic, xylanolytic, spore-forming bacterium from a farm soil in France. This gram-negative bacillus *Thermobacillus xylanilyticus* shares 91.15% sequence similarity with *Bacillus viscosus* and grows at temperatures up to 63°C.

Another novel thermoalkaliphilic obligately anaerobe has been isolated from a humid soil sample in Kenya. This gram-positive bacterium *Anaerobranca gottschalkii* grows optimally at pH 9.5 and 50–55°C (Prowe and Antranikian 2001). Novel thermophilic actinobacteria *Amycolatopsis eurytherma*, *Saccharopolyspora flava*, and *Saccharopolyspora thermophila* have been isolated from soil (Kim et al. 2002; Lu et al. 2001). Yabe et al. (2011) isolated two novel thermophilic spore-forming bacteria belonging to novel family Thermogemmatisporaceae within the class Ktedonobacteria, from fallen leaf samples in the soil. These two bacteria *Thermogemmatispora onikobensis* and *T. foliorum* grow at the temperature optima of 60–65°C and show maximum 16S rDNA homology to *Thermosporothrix hazakensis*. Zucchi et al. (2012) isolated two thermophilic actinomycetes *Amycolatopsis thermophila* sp. and *Amycolatopsis viridis* sp., thermophilic from arid soil. *Chthonomonas calidirosea* gen. nov., sp. nov., is an aerobic, pigmented, thermophilic bacterium belonging to a novel bacterial class, *Chthonomonadetes* classis nov. It was isolated from geothermally heated soil at Hell's Gate, Tikitere, New Zealand. On the basis of 16S rRNA gene analysis, this represents the first representative of this new class in the newly described phylum *Armatimonadetes*, which was formerly called as candidate division OP10 (Lee et al. 2011).

By direct solar heating, the soil surface temperature frequently rises in summer to levels allowing the development of thermophilic fungi. Here, fungi exist either as resting propagules or as active mycelia depending on the availability of nutrients and favorable environmental conditions. Widespread occurrence of thermophilic fungi in soils has been established by many studies (Taber and Pettit 1975; Awao and Mitsugi 1973; Ward and Cowley 1972; Minoura et al. 1973). Mouchacca (1995) reviewed the isolation of many thermophilic fungi [*Scytalidium thermophilum*, *Thermomyces lanuginosus* (*Humicola lanuginosa*), *Myceliophthora thermophila*, Malbranchea *cinnamomea*, *Chaetomium thermophile*, *Melanocarpus albomyces*, *Talaromyces thermophilus*, *Thermoascus aurantiacus*, *Rhizomucor pusillus*, *Myceliophthora fergusii* (*Corynascus thermophilus*), *Myriococcum thermophilum*, *Thielavia terrestris* (*Acremonium alabamense*), *Melanocarpus albomyces*, *Rhizomucor miehei*, *Rhizomucor pusillus*, *Thielavia terrestris*, *Talaromyces byssochlamydoides*, *Talaromyces emersonii*, *Talaromyces thermophilus*] from solar-heated and desert soils. Few thermophilic and thermotolerant organisms like *Chaetomium senegalense* (ascomycetes) and *Myceliophthora fergusii* (anamorphic ascomycetes) have reported for the first time from India (Salar and Aneja 2006). DNA sequence and fingerprinting approaches have also been used successfully to profile and characterize fungal communities in soils (O'Brien et al. 2005; Pérez-Piqueres et al. 2006; Anderson et al. 2008; Bates and Garcia-Pichel 2009).

Soils are the appropriate habitats for the isolation of thermophilic yeasts. *Candida thermophila* has been isolated from soil samples in Korea (Shin et al. 2001). Peter et al. (2007) observed ascospore formation in this yeast strain for the first time and therefore named its teleomorph as *Ogataea thermophila*. Two novel thermotolerant methylotrophic yeast species *Ogataea chonburiensis* and *Ogataea nakhonphanomensis* have been isolated from a tree exudate and soil collected in Thailand (Limtong et al. 2008). Three strains of a thermotolerant methylotroph

Pichia thermomethanolica have also been isolated from soils of Thailand (Limtong et al. 2005), which are later renamed as *Ogataea thermomethanolica* (Limtong et al. 2008).

1.2.2 Man-Made Thermophilic Habitats

Man-made thermophilic habitats include acid mine drainage and acidic effluents, self-heated compost piles, biological wastes, and waste treatment plants. These are comparatively lower temperature habitats, as compared to the natural habitats, and are ideal for the isolation of moderate and extreme thermophiles.

1.2.2.1 Acid Mine Drainage and Acidic Effluents

Acid mine drainage (AMD) or acid rock drainage (ARD) refers to outflow of highly acidic (pH 2.3–6.5), metal-rich fluids from metal mines or coal mines. These fluids are hot (temperature reaching up to as high as 70°C) because they are generated due to exothermic metal sulfide oxidation reactions. The predominant metal sulfide mineral in most rocks is pyrite (FeS_2) which is found in association with many commercially valued metals like Au, Ag, Cu, Zn, and Pb. These metals are present as impurities in pyrite or as sulfide minerals associated with pyrite, such as chalcopyrite ($CuFeS_2$), sphalerite (ZnS), and galena (PbS). Variable amounts of pyrites are found in the coal deposits also. Mining increases the surface area of these ores exposed to air and water. After being exposed to water and air, oxidation of metal sulfides within the surrounding rock occurs at a faster rate, leading to the formation of water enriched in sulfate, aluminum, and heavy metals (Johnson and Hallberg 2003; Ackil and Koldas 2006). This reaction serves as template for the several oxidation reactions which contributes to further increase in acidity.

Despite the extreme acidity, heat, and high concentrations of sulfate and toxic metals, a diverse range of microorganisms populate this environment. These organisms are chemoautotrophs and utilize sulfide (from ores); CO_2, O_2, and N_2 (from air); and phosphate (liberated by water–rock interaction) as electron donors. Microbial activity increases the rate of AMD formation and may be responsible for the bulk of AMD generated. DNA-based studies on the diversity of microorganisms in these environments have provided insights into many acidophilic, metal-tolerant, and thermophilic prokaryotes. Eukaryotes (protists, fungi, and yeasts) are abundant and important in some parts of acid systems like solfataras, but diversity of eukaryotes in AMD and acidic effluents still remains an underexplored topic. Some of the acid mine drainage sites include Storwartz mine, Norway; Ynysarwed, Wales; Bull House, England; Wheal Jane, England; Killingdal mine dump, Norway; King's mine stream, Norway; Parys mine, Wales; Argo Tunnel, Idaho Springs, Colorado, USA; Iron Mountain Mine, USA; Monday Creek, Ohio; Davis Pyrite Mine, Massachusetts; Hughes bore hole, Pennsylvania, Aznalcollar mine, Spain; and Rio Tinto River, Spain.

Chemistry of Acid Mine AMD

The formation of AMD from pyrite can be represented in three basic chemical reactions: pyrite oxidation, ferrous oxidation, and iron hydrolysis (Johnson 2003).

Reaction 1: Pyrite Oxidation
Pyrite oxidizes spontaneously, with either molecular oxygen or ferric iron acting as the oxidant

$$FeS_2 + 6Fe^{3+} + 3H_2O \rightarrow 7Fe^{2+} + S_2O_3{}^{2-} + 6H^+ \quad (1.1)$$

Or

$$2FeS_2 + 7O_2 + 2H_2O \rightarrow 2Fe^{2+} + 4SO_4{}^{2-} + 4H^+$$

This reaction is abiotic and may occur in anaerobic as well as aerobic environments depending on the oxidant.

Reaction 2: Ferrous Oxidation

$$4Fe^{2+} + O_2 + 4H^+ \rightarrow 4Fe^{3+} + 2H_2O \quad (1.2)$$

This reaction requires oxygen and is primarily biological in acidic (pH<4) waters. The rate of this reaction is slow at low pH; thus, it may limit the rate of AMD generation. However, microorganisms accelerate this reaction rate, thus can determine the rate of pyrite dissolution.

Some bacteria and archaea can also oxidize thiosulfate, the initial sulfur by-product of pyrite oxidation (reaction 1), and other reduced inorganic sulfur compounds (RISCs) and elemental sulfur, producing sulfuric acid as follows:

$$S_2O_3{}^{2-} + 2O_2 + H_2O \rightarrow 2H^+ + 2SO_4{}^{2-}$$

Reaction 3: Iron Hydrolysis
Further acidity results from the hydrolysis of ferric iron:

$$Fe^{3+} + 3H_2O \rightarrow Fe(OH)_3 + 3H^+ \quad (1.3)$$

Diversity of Microorganisms in AMD and Acidic Effluents

In acid mine drainage and effluents from different sites, variations occur in the temperature, ionic strength, and pH, yielding different communities characterized by different microorganisms, but all sites are restricted to a few species. This can be due to the limited number of energy-deriving reactions available in AMD environments.

Photosynthesis is an important source of energy and fixed carbon regions of AMD systems exposed to sunlight (Hiraishi et al. 2000). However, in subsurface ARD and AMD systems, the 4 major metabolic groups are detected. They are lithoautotrophs that oxidize $Fe2^+$ and S^-, organoheterotrophs that utilize carbon produced by

lithoautotrophs, lithoheterotrophic that are iron and S° oxidizers, and anaerobes that couple oxidation of sulfur or organic carbon to Fe^{3+} reduction.

Among bacteria, major representatives present in the AMD community lie within Proteobacteria, Nitrospira, Acidobacteria, and fermicutes. *Acidithiobacillus* spp. (formerly *T. ferrooxidans*, *Thiobacillus caldus*) (Kelly and Wood 2000) and *Thiobacillus* spp. are primarily responsible for acid mine drainage. They belong to γ-Proteobacteria. The moderately thermophilic *Acidithiobacillus caldus* (formerly *Thiobacillus caldus*) is a sulfur oxidizer. "*Leptospirillum ferrooxidans*" group within fermicutes division is commonly detected in AMD systems. *Leptospirillum* isolates and environmentally derived clones cluster within one of three phylogenetically distinct groups (I, II, and III) (Bond et al. 2000). Two species have been named: *L. ferrooxidans* (group I) and *L. ferriphilum* (group II) (Hippe 2000; Coram and Rawlings 2002). Group III *Leptospirillum* has only been detected via clone library analysis only (Bond et al. 2000). *Leptospirillum* isolates have been obtained from AMD environments characterized by a wide range of temperatures and pH. *L. ferrooxidans* DMZ2705 (group I) has been reported to grow in the pH range of 1.3–4.0 and an upper temperature limit of 55°C (Hippe 2000; Johnson and Roberto 1997).

Another large bacterial group named *Sulfobacillus*, which is represented by *S. thermosulfidooxidans* and *S. acidophilus*, is separate from the *Alicyclobacillus* lineage. *Sulfobacillus* spp. are moderate thermophiles and have a broad range of physical growth regimes. Some isolates that are capable of growth up to 65°C have also been isolated (Atkinson et al. 2000). They either oxidize ferrous iron or reduce ferric iron, depending on prevailing concentrations of dissolved oxygen (Bridge and Johnson 1998).

Archaeal lineages reported from AMD environments are restricted to the Thermoplasmatales and the Sulfolobales. Clones within the Thermoplasmatales (e.g., *Thermoplasma acidophilum* and *Thermoplasma volcanium*) have been detected in clone libraries created from samples collected. *T. acidophilum* and *T. volcanium* have moderately thermophilic (45–67°C) temperature and pH ranges (Darland et al. 1970; Segerer et al. 1988) typical of AMD.

One member of the Sulfolobales, *Metallosphaera prunae*, has been detected in AMD environments. Another species of this genus *Metallosphaera sedula* is a powerful oxidizer of pyrite, chalcopyrite, and sphalerite, forming sulfuric acid and solubilizing heavy metals. Two other Sulfolobales genera, *Acidianus* and *Sulfolobus*, have been isolated from AMD and also from other geothermal acidic environments. *Sulfolobus* spp. are strict aerobes growing autotrophically by oxidation of S°, S^{2-}, and H_2, forming sulfuric acid or water as end product. *Sulfolobus brierleyi* (now renamed as *Acidanus brierleyi*) and *Sulfolobus metallicus* are able to grow by leaching sulfide ores (Brierley and Brierley 1973; Huber and Stetter 1991). Several *Sulfolobus* isolates are facultative or obligate heterotrophs (Brock 1978).

They are able to reduce ferric and molybdate under microaerophilic conditions, and their growth requires low ionic strength (Brierley and Brierley 1973). *Acidanus*, like *Sulfolobus*, is able to grow by oxidation of S°, sulfides, H_2, and organic matter but is able to grow anaerobically by reduction of elemental sulfur

Table 1.8 List of thermoacidophiles isolated from acid mine drainage and acidic effluents

Thermal category	Organism	Phylogenetic affiliation	Comments
Moderate thermophile	*Sulfobacillus acidophilus*	Firmicutes	Iron oxidizer
	S. thermosulfidooxidans	Firmicutes	Iron oxidizer
	Acidimicrobium ferrooxidans	Actinobacteria	Iron oxidizer/ reducer
	Leptospirillum thermoferrooxidans	Nitrospira	Iron oxidizer
	Acidithiobacillus caldus	β/γ-Proteobacteria	Sulfur oxidizer
	Hydrogenobacter acidophilus	Aquificales	Sulfur oxidizer
	Alicyclobacillus spp.	Firmicutes	Heterotrophic
	Thermoplasma acidophilum	Thermoplasmatales	Heterotrophic
	Th. volcanicum	Thermoplasmatales	Heterotrophic
Extreme thermophile	*Acidianus brierleyi*	Sulfolobales	Iron oxidizer
	A. infernus	Sulfolobales	Iron oxidizer
	A. ambivalens	Sulfolobales	Iron oxidizer
	Metallosphaera sedula	Sulfolobales	Iron oxidizer
	Sulfolobus shibatae	Sulfolobales	Sulfur oxidizer
	Sf. solfataricus	Sulfolobales	Sulfur oxidizer
	Sf. hakonensis	Sulfolobales	Sulfur oxidizer
	Sf. metallicus	Sulfolobales	Sulfur oxidizer
	Metallosphaera prunae	Sulfolobales	Sulfur oxidizer
	Sulfurococcus mirabilis	Sulfolobales	Sulfur oxidizer
	Sulfolobus acidocaldarius	Sulfolobales	Sulfur oxidizer
	Stygiolobus azoricus	Sulfolobales	Obligate anaerobe
	Acidilobus aceticus	Sulfolobales	Obligate anaerobe

From Johnson and Hallberg (2003)

with H_2 as electron donor (Segerer et al. 1985). Environmental clones closely related to *Ferromicrobium acidophilus* have been recovered from the Richmond Mine AMD system (43°C) (Bond et al. 2000). *Acidimicrobium ferrooxidans* has been cultivated at temperatures between 34 and 57°C (Clark and Norris 1996) and has been reported from a surprisingly diverse range of environments including AMD. Table 1.8 enlists some thermoacidophiles isolated from AMD and acidic effluents.

1.2.2.2 Biological Wastes and Waste Treatment Plants

Waste materials are the unwanted or unusable materials which are disposed or are intended to be disposed. Litter is waste which has been disposed of improperly, particularly waste which has been carelessly disposed of in plain sight. Waste Management Licensing Regulations 1994 define waste as any substance or object which the producer or the person in possession of it discards or intends or is required to discard. As defined by Pongrácz and Pohjola (2004), waste is a man-made thing that has no purpose or is not able to perform with respect to its purpose. There are many waste types defined by modern systems of waste management including municipal solid waste (MSW), construction waste and demolition waste (C&D), institutional waste, commercial waste and industrial waste (IC&I), medical waste, hazardous waste, radioactive waste, and electronic waste and biodegradable waste. Waste may also be divided into solid waste or liquid waste. Solid waste includes household waste or municipal waste (food and kitchen waste, paper waste, and hazardous waste), industrial waste (factory waste, waste from mills and mine, paper and pulp effluents), agricultural waste, sewage sludge, and biomedical waste. Liquid waste includes wastewater discharged by domestic residences, commercial properties, industry, and/or agriculture and can encompass a wide range of potential contaminants and concentrations. Sewage includes domestic, municipal, or industrial liquid waste products disposed of, usually via a pipe or sewer.

Vast amounts of wastes require safe disposal. As defined by Pongrácz and Pohjola (2004), waste management is control of waste-related activities with the aim of protecting the environment and human health and resources conservation. Waste-related activities include waste-creating processes, waste handling, as well as waste utilization. The main aim of waste management besides waste evasion is turning wastes to non-wastes Pongrácz and Pohjola (2004). There are different kinds of waste management methods depending upon the kind of waste (organic or inorganic). The simplest way to handle solid waste disposal at the lowest direct cost is in landfills. In this procedure, solid wastes are deposited in low-lying and low-value land. Anaerobic and facultative microorganisms attack the organic compounds in the waste and degrade them (Senior 1995). Another method of disposal of solid domestic and agricultural waste is composting. This topic shall be dealt separately later in this chapter. Other methods include incineration, plasma gasification, anaerobic digestion, and thermophilic aerobic digestion (TAD). Liquid waste treatment methods depend on type, composition, and origin of liquid waste. Sewage waste can be treated close to where it is created (in septic tanks, biofilters, or aerobic treatment systems) or collected and transported via a network of pipes and pump stations to a municipal treatment plant. Conventional sewage treatment may involve three stages, called primary (pretreatment), secondary, and tertiary treatment. Secondary treatment is the major step of sewage treatment which relies upon use of indigenous microorganisms. It removes dissolved and suspended biological matter. Secondary treatment systems are classified as fixed-film or suspended-growth systems. Fixed-film or attached growth systems include filter beds (trickling filters) and rotating biological contactors, where the microbial biomass grows on media and the sewage passes over its surface. Suspended-growth systems include activated sludge, which involves

introduction of air or oxygen to the mixture of sludge (residual, semisolid material left from primary treated sewage), sewage, and microorganisms in an aeration tank to develop biological floc which reduces organic content of the sewage. Another secondary treatment method is anaerobic digestor, which is a slow anaerobic process that decreases organic content of the sewage.

Diversity of Thermophilic Microorganisms in Biological Wastes and Waste Treatment Plants

A vast array of thermophiles has been isolated from different anthropogenic wastes and treatment plants. Microorganisms have been described from various water sources, such as wastewaters from industrial plants, factory effluents, and wastewater treatment plants (Carreto et al. 1996, Menes et al. 2002, Kaksonen et al. 2007; Tan and Guodong 2010). A slightly halotolerant gram-positive thermophilic bacterium, *Rubrobacter xylanophilus*, has been isolated from a thermally polluted industrial runoff near Salisbury, United Kingdom. Its optimum growth temperature is approximately 60°C (Carreto et al. 1996). Cann et al. 2001 isolated two anaerobic polysaccharide-degrading thermophiles from the leachate of a waste pile from a canning factory in Hoopeston, East Central Illinois, USA. *Thermoanaerobacterium polysaccharolyticum* reduces thiosulfate to sulfide, whereas *Thermoanaerobacterium zeae* is unable to reduce thiosulfate. The temperature optimum of growth for both of these anaerobes is 65–68°C. *Anaerobaculum mobilis*, a novel anaerobic, moderately thermophilic, peptide-fermenting sulfur reducer with the optimum temperature range 55–60°C, has been isolated from an anaerobic wool-scouring wastewater treatment lagoon (Menes et al. 2002). Novel thermophilic sulfur reducers *Desulfurispora thermophila* and *Desulfotomaculum alcoholivorax* have been isolated from a sulfidogenic fluidized-bed reactor treating acidic metal- and sulfate-containing water. These fermentative bacteria grow optimally at 59–61°C and 44–46°C, respectively (Kaksonen et al. 2007, 2008). Using PCR–DGGE and real-time PCR, bacterial communities in an anaerobic reactor for treating carbazole-containing wastewater have been studied. At 70°C, dominant bacterial representatives of *Pseudomonas* sp., *Comamonas* sp., and *Diaphorobacter* sp. have been identified (Tan and Guodong 2010).

In an anaerobic digester for the treatment of wastewater, small sludge granules begin to form whose surface area is covered in aggregates of bacteria. The flow conditions create a selective environment in which only those microorganisms, which are capable of attaching to each other, survive and proliferate. Eventually the aggregates form into dense compact biofilms referred to as granules. Hence the name granular sludge is given to the sludge derived from such anaerobic digester. Microorganisms of different physiological varieties have also been isolated from sewage sludge (Shooner et al. 1996; Sekiguchi et al. 2000, 2003, 2008; Plugge et al. 2002; Manaia et al. 2003; Diaz et al. 2007). A syntrophic fatty-acid-oxidizing anaerobe, *Syntrophothermus lipocalidus*, has been isolated from granular sludge in a thermophilic upflow anaerobic sludge blanket (UASB) reactor. Crotonate is the only substrate that allows the strain to grow in pure culture. In a coculture with the

thermophilic, hydrogenotrophic *Methanothermobacter thermautotrophicus* strain DeltaH, the isolate syntrophically oxidizes saturated fatty acids (Sekiguchi et al. 2000). Another anaerobic syntrophic thermophile *Pelotomaculum thermopropionicum* has been described by Imachi et al. 2002. This propionate-oxidizing bacterium grows in coculture with *Methanothermobacter thermautotrophicus* strain DeltaH on a variety of substrates. Another thermophilic syntroph, namely, strain TPO, has been isolated from granular sludge from a laboratory-scale upflow anaerobic sludge bed reactor. This strain grows fermentatively as a pure culture in the presence of pyruvate, benzoate, fumarate, H_2CO_2, pyruvate, and lactate. Comparison of 16S rDNA sequences relates this strain to *Desulfotomaculum thermobenzoicum* (98%) and *Desulfotomaculum thermoacetoxidans* (98%) (Plugge et al. 2002). Three anaerobic, syntrophic primary alcohol- and lactate-degrading thermophilic strains of *Tepidanaerobacter syntrophicus* have also been described from municipal solid waste and sewage sludge digesters (Sekiguchi et al. 2006). A thermophilic multicellular filamentous chemoorganotrophic anaerobe, *Anaerolinea thermophila*, have been isolated from thermophilic granular sludge in an upflow anaerobic sludge blanket reactor treating fried soybean-curd manufacturing wastewater (Sekiguchi et al. 2003). From an upflow anaerobic sludge bed reactor treating brewery wastewater, a moderately thermophilic bacterium, *Aminiphilus circumscriptus*, has been reported (Diaz et al. 2007). Jiang et al. 2005 isolated a thermophilic obligately methylotrophic and methanogenic archaeon *Methanomethylovorans thermophila* that has been isolated from an upflow anaerobic sludge blanket reactor. Three obligately anaerobic, thermophilic, sulfate-reducing bacteria *Thermodesulfovibrio islandicus*, *Thermodesulfovibrio aggregans*, and *Thermodesulfovibrio thiophilus* have also been described from methanogenic sludges derived from the waste and wastewater treatment plants operating at 55°C (Sekiguchi et al. 2008).

There have been relatively few reports on the description of aerobes from wastewater and sewage sludge. On studying the microbial diversity of thermophilic aerobic suspended carrier biofilm process (SCBP) for the on-site treatment of pulp and paper mill whitewater lining by LH-PCR (length heterogeneity) amplified 16S ribosomal DNA, most prominent representatives belonging to β-Proteobacteria, Cytophaga/Flexibacter/Bacteroides group, and γ-Proteobacteria have been described (Tiirola et al. 2003). A strictly aerobic facultative autotroph *Thiobacillus thermosulfatus* has been isolated from sewage sludge samples enriched with elemental sulfur. This thermophilic organism can reduce elemental sulfur and can grow autotrophically in a temperature range of 34–65°C with optimum growth occurring at pH 5.2–5.6 and 50–52.5°C (Shooner et al. 1996). Manaia et al. (2003) described an aerobic moderately thermophilic bacterium, *Tepidiphilus margaritifer*, from water-treatment sludge aerobic digester operating at temperatures around 60°C. They described another thermophilic aerobe, *Caenibacterium thermophilum*, from digester of a municipal sludge.

There are few reports on the isolation of thermophiles from other waste and waste treatment sources. Nakamura et al. (2004) reported the characterization of a moderately thermophilic and alkaliphilic bacterium from a semicontinuous decomposing system of kitchen refuse. This novel bacterium, *Cerasibacillus quisquiliarum*, shows optimum temperature of growth at 50°C. A thermotolerant iron-oxidizing

acidophilic aerobe, *Sulfobacillus thermotolerans*, has been isolated from a gold-recovery plant, Siberia (Bogdanova et al. 2006). From a methanogenic bioreactor treating artificial solid waste, an anaerobic, moderate thermophile (temperature optima 55–58°C), *Lutispora thermophila*, has been isolated (Shiratori et al. 2008).

1.2.2.3 Self-Heated Compost Piles

Composting is a self-heating, aerobic, bio-decomposition of organic waste materials. Composting is done either on the ground (static piles or aerated piles) or can be accomplished more rapidly using a bioreactor. Under favorable conditions, self-heating static piles typically raise the temperature inside a compost pile to 55–60°C or above within few days. After a few days, there is a gradual decline in the temperature. Aerated pile process achieves faster composting rates through improved aeration by the perforated pipes. Inside the pile, temperature rises to the self-limiting levels of 70–80°C. Composting in a bioreactor is accomplished more rapidly, and the temperature of the compost piles rises to 76–78°C.

Diversity of Thermophilic Microorganisms in Compost

Microorganisms have been shown to appear in succession during the composting process. Composting is initiated by mesophilic heterotrophs. But as the temperature rises, these are replaced by the thermophilic forms. Above 60°C, thermophilic fungi become active, and further microbial heat production is due solely to bacteria and actinomycetes. Diversity of microflora in compost depends on different factors like age, substrate materials used, and temperature. Many thermophiles, which are known to be prominent in every composting process, are *Bacillus stearothermophilus*, *Thermomonospora*, *Thermoactinomyces*, and *Clostridium thermocellum*. Other bacterial representatives include both gram-negative as well as gram-positive species. Fujio and Kume 1991 isolated thermophilic strains of *Bacillus stearothermophilus* and *Thermus* sp. from a sewage sludge compost under aerobic conditions at 60°C. In a study based on cultivation as well as restriction enzyme analysis of a clone library of bacterial 16S rRNA genes, Blanc et al. (1999) revealed the presence of *Thermus thermophilus*, *Bacillus* spp., and *Hydrogenobacter* spp. in thermogenic composts. Several novel microorganisms have been isolated from the different types of composts. An aerobic thermophilic bacterium, *Geobacillus toebii*, with temperature optimum of 60°C, isolated from hay compost in Korea, has been reported by Sung et al. (2002). Other novel aerobic thermophilic bacilli, *Tuberibacillus calidus* and *Paenibacillus humicus*, have been isolated from compost pile and poultry litter compost, respectively (Hatayama et al. 2006; Vaz-Moreira et al. 2007). Several gram-negative bacteria have also been isolated from composts. *Luteimonas composti* is a yellow-pigmented gram-negative bacterium isolated from food waste compost, Kinmen County, Taiwan (Young et al. 2007). *Thermobacillus composti*, a moderately thermophilic bacterium, has been isolated from compost bioreactor (Watanabe et al. 2007). Weon et al. 2007 isolated two gram-negative thermophilic

members of *Ureibacillus*, *Ureibacillus composti*, and *Ureibacillus thermophilus* from a compost sample from a compost facility in Ichon, Korea. Two extremely thermophilic bacteria with temperature optimum of 78°C, *Calditerricola satsumensis* and *Calditerricola yamamurae*, have been described from high-temperature compost by Moriya et al. 2011. Thermophilic archaeal methanogens have also been found to dominate during composting. Derikx et al. (1989) identified strains of *Methanobacterium thermoautotrophicum*, which grow only on H_2 and CO_2 as energy and carbon source and do not require complex factors for growth. The 16S rDNA-based phylogenetic surveys of compost revealed an increase of archaeal diversity with compost maturation. 16S rDNA clones related to *Methanothermobacter* spp. and *Methanosarcina thermophila* have been found (Thummes et al. 2007).

Being traditionally studied by the culture-based methods, molecular tools like fatty acid profiling, PCR-based single-strand conformation polymorphism (PCR–SSCP) and denaturing gradient gel electrophoresis (PCR–DGGE), and 16S rRNA gene analysis are now in common use for studying the composting process. Several cloned 16S rDNA sequences from high-temperature (64–84°C) kitchen and garden waste composts have been found to be related to *Thermus* species (Blanc et al. 1999; Beffa et al. 1996). Other rDNA sequences from that study closely resembled sequences from bacteria that have not been previously associated with hot compost, including *Saccharococcus thermophilus* and *Rhodothermus marinus* (Blanc et al. 1999). Several composted materials have been found to contain ammonia oxidizer-like 16S rDNA sequences from β-Proteobacteria, using DGGE and a competitive PCR method (Kowalchuk et al. 1999). Using PCR-amplified small-subunit rRNA genes (SSU rDNA), the diversity and succession of microbial communities during composting has been studied. DNA sequencing of these molecular isolates shows similarities with gram-positive bacteria with a low and high G + C DNA content and to the SSU rDNA of γ-Proteobacteria. The amplified 18S rRNA gene sequence relates to the regions of *Candida krusei* and *Candida tropicalis* (Peters et al. 2000). Dees and Ghiorse (2001) detected high diversity of uncultivated bacteria associated with synthetic food waste compost using ARDRA. Phylogenetic analysis using sequences of 16S rDNA also revealed the presence of *Aneurinibacillus* and *Brevibacillus*, which are not commonly associated with hot compost. *Bacteroides* have been found to be the most dominant bacteria in cow manure composts using DGGE (Green et al. 2004). Xiao et al. (2011) found the presence of actinomycetes using DGGE and quantitative PCR (qPCR), in a continuous thermophilic composting process at temperatures higher than 50°C (Xiao et al. 2011).

Fungi have been cultured from all four phases of the compost cycle but appear most prevalent during the initial and middle mesophilic phases (De Bertoldi et al. 1983; Ryckeboer et al. 2003a, b). The most dominant fungi cultured from the mesophilic phases are species in the genera *Acremonium*, *Alternaria*, *Aspergillus*, *Chaetomium*, *Cladosporium*, *Emericella*, *Fusarium*, *Geotrichum*, *Mortierella*, *Mucor*, *Penicillium*, *Pseudallescheria*, *Scopulariopsis*, and *Trichoderma*. *Absidia*, *Aspergillus*, *Chaetomium*, *Coprinus*, *Mucor*, *Paecilomyces*, *Penicillium*, *Rhizomucor*, *Scytalidium*, and *Thermomyces* are thermotolerant genera that have been isolated at higher temperatures (Waksman et al. 1939; Ghazifard et al. 2001; Vijay et al. 2002; Ryckeboer et al. 2003a, b; Anastasi et al. 2005). Work on thermophilic fungi in

composts has been reviewed by Cooney and Emerson (1964), Kane and Mullins (1973), and Gray (1970). Important thermophilic fungi include *Geotrichum candidum*, *Aspergillus fumigatus*, *Mucor pusillus*, *Chaetomium thermophile*, *Thermoascus aurantiacus*, and *Torula thermophila* (Tansey and Brock 1978). In a recent culture-independent study using DGGE and analysis of rDNA clone libraries, sequences affiliated to *Candida tropicalis*, *Candida krusei*, *Cercophora*, *Neurospora*, and different Basidiomycetes have been reported from organic municipal waste compost (Bonito et al. 2010). Few fungi like *Thermomucor* (Subrahmanyam et al. 1977) and *Myceliophthora thermophila* (Sen et al. 1980) were isolated from municipal waste compost in India.

1.2.3 Other Thermophilic Habitats

Few organisms have also been isolated from the other man-made/natural thermophilic habitats apart from those that are described above. A xylanolytic enzyme-producing bacterial strain of *G. thermoleovorans* was isolated from a pulp sample from Century Paper Mills, Uttaranchal (India) (Sharma et al. 2007). A novel triangular-shaped thermophilic and sulfate-reducing archaeon *Archaeoglobus sulfaticallidus* sp. nov. was isolated from black rust formed on the steel surface of a borehole observatory (CORK 1026B) retrieved during IODP Expedition 301 on the eastern flank of Juan de Fuca Ridge, Eastern Pacific Ocean. *Archaeoglobus sulfaticallidus* sp. nov., a novel thermophilic and facultatively lithoautotrophic sulfate reducer isolated from black rust, exposed to hot ridge flank crustal fluids (Steinsbu et al. 2010). An obligately anaerobic extreme thermophilic bacterium, *Caldicoprobacter oshimai* gen. nov., sp. nov., was isolated from sheep feces. This bacterium grows optimally at 70°C and pH 7.2 (Yokoyama et al. 2010). Besides these, several thermophilic fungi have been isolated from habitats like stored peat (Kuster and Locci 1964), birds' nests (Satyanarayana et al. 1977; Tansey 1973), and stored oil palm kernels (Eggins and Coursey 1968). Subrahmanyam (1999) and Johri and Satyanaryana (1986) also reviewed the ecology of thermophilic fungi from different thermophilic habitats like hay, paddy straw, coal spoil tips, stored grains, coal mine soils, and wood chip piles.

1.3 Adaptations in Thermophiles

Cellular structure and activities are affected by various factors including temperature. For any microbe to grow at high temperature, its major components, including proteins nucleic acids and lipids, must be able to resist heat. Hence, thermophiles have accumulated various adaptations that allow them to prevail and thrive at high temperatures.

The thermostability of enzymes from thermophiles can be as high as up to 140°C (Adams and Kelly 1995). Structural studies of several thermostable proteins have

shown that some features are highly correlated with thermostability (Ladenstein and Antranikian 1998). A hydrophobic core helps exclude solvent from the internal regions of the protein, making it more resistant to unfolding. A significant increase in the proportion of NTN codons that encodes for nonpolar, hydrophobic amino acids (isoleucine, leucine, methionine, phenylalanine, and valine) has been found to be correlated with increasing optimal growth temperature for most of the bacteria and archaea. This indicates use of hydrophobicity for stabilizing proteins at high temperatures (Lieph et al. 2006). A small surface-to-volume ratio probably improves stability by conferring a compact form on the protein. A reduction in the glycine content of thermostable proteins introduces rigidity, and this, along with the extensive ionic interactions that form a network over the surface of the molecule, helps the compacted protein resist unfolding at high temperature (Ladenstein and Antranikian 1998). Thermophilic proteins show higher proportion of thermophilic amino acids (e.g., proline residues with fewer degrees of freedom). A higher content of arginine and a reduction in the total number of thermally unstable residues, such as Cys, Lys, Met, Asn, and Gln (Cicicopol et al. 1994), have been reported for thermostable proteins. Protein stability may also be assisted by accumulation of intracellular potassium and solutes such as 2,3-diphosphoglycerate.

Proteins have evolved ways to maintain protein integrity and function at high temperatures. These include increasing ion-pair content, or a change from monomeric to oligomeric structure (Cicicopol et al. 1994); additional networks of hydrogen bonds (Jaenicke and Bohm 1998); an increase in disulfide bond formation (Beeby et al. 2005), decreasing the length of surface loops that connect elements of secondary structure (Thompson and Eisenberg 1999); and exchange of amino acids to increase helix propensity of residues in α-helices. Presence of noncovalent, ionic bonds called salt bridges on a protein's surface likely has shown to play a major role in maintaining the biologically active structure of proteins (Das and Gerstein 2000).

In addition to these factors, some assisting proteins, such as molecular chaperonins, also facilitate protein thermostability. Chaperonins (heat shock proteins) function to refold partially denatured proteins. Hyperthermophilic archaea produces special classes of chaperonins that function only at the highest growth temperatures. A chaperonin known as thermosome (Andră et al. 1998) has been characterized from thermophiles *Methanopyrus kandleri*, *Pyrococcus abyssi*, and *Pyrodictium occultum*. This complex is thought to bind heat-denatured proteins, prevent their aggregation, refold them into their active form (Andră et al. 1998), and help cells survive, even at temperatures above their maximal growth temperature. For example, the cells of *Pyrococcus abyssi* can remain viable even after 1-h treatment in autoclave (121°C). Thus, the upper temperature limit at which many hyperthermophiles can survive is higher due to chaperonin activity than that the upper temperature at which they can grow.

Several factors may combine to afford heat stability to DNA in thermophiles including high levels of K^+ (Marguet and Forterre 1998), reverse DNA gyrase (van der Oost et al. 1998; Forterre et al. 1996), and histone or other DNA-binding proteins (Pereira and Reeve 1998). Cytoplasm of the hyperthermophilic methanogens *Methanopyrus* contains molar levels of potassium and cyclic 2,3-diphosphoglycerate.

This solute prevents depurination or depyrimidination of DNA, which causes mutation (Marguet and Forterre 1998) at high temperatures.

Positive supercoiling of DNA may be an important factor stabilizing DNA to high temperatures. All hyperthermophiles produce a unique protein called reverse gyrase (van der Oost et al. 1998; Forterre et al. 1996). This is a type I DNA topoisomerase. It has been shown to catalyze the positive supercoiling of closed circular DNA. For various reasons, in particular, its higher linking number, positively supercoiled DNA is more resistant to thermal denaturation than is negatively supercoiled DNA (Forterre et al. 1996). Moreover, monovalent and divalent salts enhance the stability of nucleic acids because these salts screen the negative charges of the phosphate groups and because KCl and $MgCl_2$ protect the DNA from depurination and hydrolysis (Marguet and Forterre 1998). The G–C pair of nucleic acids is more thermostable than the A–T or A–U pairs because of the additional hydrogen bond (Galtier et al. 1999). But elevated G and C ratios are not found in the genomes of thermophilic prokaryotes, although thermostability has been correlated with increase in G–C pairs of their SSU rRNA and transfer RNAs (Galtier and Lobry 1997).

In addition, other proteins in hyperthermophiles also function to maintain the integrity of the DNA duplex. Several Euryarchaeotes contain histone-like proteins, which resemble to the core histones of Eukaryotes in structure and function (Sandman et al. 1998; Pereira and Reeve 1998). They wind and compact DNA into nucleosome-like structures (Pereira et al. 1997) that maintain DNA in a double-stranded form at high temperatures (Soares et al. 2008). Small DNA-binding proteins like Sac7d in the *Sulfolobus acidocaldarius* bind to the minor groove of DNA nonspecifically and increase the melting temperature of DNA by some 40°C (Robinson et al. 1998). Archaeal histones from thermophilic methanogens like *Methanothermus fervidus* and halophiles like *Halobacterium* have been well studied.

Polyamines also play a role in DNA stability and stability of other macromolecules. These molecules (e.g., putrescine and spermidine) together with Mg^{2+} function to stabilize RNA and DNA. In thermophilic archaea such as *Sulfolobus*, polyamines help stabilize ribosomes, thereby facilitating protein synthesis at high temperatures.

High temperature also increases the fluidity of membranes. To maintain optimal membrane fluidity, the cell must adjust the membrane composition, that is, the amount and type of lipids. So, the membrane lipids of thermophiles contain more saturated and straight-chain fatty acids than mesophiles. This allows thermophiles to grow at higher temperatures by providing the right degree of fluidity needed for membrane function. Many archaeal species contain a paracrystalline surface layer (S-layer) with protein or glycoprotein that functions as an external protective barrier.

1.4 Future Perspectives and Conclusions

Natural and man-made habitats with elevated temperatures are known to exist throughout the globe. Most of the natural habitats are high-temperature zones where the source of heat is geothermal in origin. In man-made habitats, heat generation is

due to self-heating or solar heating, and temperature in these habitats can reach up to 70–80°C. An unanticipated phylogenetic and physiological diversity of thermophiles exists in the different thermophilic habitats. The representatives of all the microbial groups have been described from these habitats through cultivation-dependent as well as cultivation-independent approaches. Most of the extreme thermophiles and hyperthermophiles have been revealed from natural habitats, which include bacteria and archaea. Moderately thermophilic members include prokaryotes as well as eukaryotic fungal and algal species, which are isolated from natural as well as man-made habitats. In these habitats, thermophiles are either primary producers or consumers of organic matter. Energy conservation in primary producers occurs by anaerobic and aerobic types of respiration. Consumers gain energy either by anaerobic or aerobic types of respiration or by fermentation.

Cultivation has been a prerequisite for understanding their physiology and role in high-temperature ecosystems. Besides those isolated by culture-dependent approaches, several uncultured organisms are still to be cultivated. To cultivate these uncultivated organisms is a critical challenge in the future. Understanding their physiology through cultivation shall provide an insight into novel enzymes and metabolic products produced by them. These may further be suitable for use in novel biotechnological processes.

The understanding of genomes of the thermophilic organisms has provided valuable information on their gene sequences and high-resolution 3-D structures of their proteins. Despite the availability of such vast amount of information from thermophiles, the biophysical basis of their survival at high temperatures remains a debatable topic.

Another important aspect that needs to be considered is the upper temperature limit of life. The currently known upper limit of life is 122°C. It, however, appears improbable that this represents the endpoint of the search of hyperthermophilic organisms. The study of this aspect is important as the upper temperature limit for life is a key parameter for delimiting when and where life might have evolved on the hot Earth in the geological past, the depth to which life exists in the Earth's subsurface, and the potential for life in hot extraterrestrial environments.

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Chapter 2
Exploring the Ecology of Thermophiles from Australia's Great Artesian Basin During the Genomic Era

Christopher D. Ogg, Mark D. Spanevello, and Bharat K.C. Patel

Abstract The Great Artesian Basin (GAB) is the world's largest subsurface aquifer, underlying approximately one-fifth of subarid regions of the Australian continent and covering an area of over 1.7×106 km^2, with a water-storage capacity of 8.7×1012 m^3. The GAB provides a vital water resource for rural semiarid communities and also contains the largest onshore oil and gas reserves in Australia. The GAB is composed of alternating layers of water-bearing permeable sandstone and non-water-bearing impermeable shale. These geological formations have an immense influence on the chemical composition of GAB groundwaters, which can be bicarbonate-, chloride, sulphate or iron rich. The depth of the aquifer is estimated to be 3,000 m, and the underground water flow from the recharge areas at the edge of the basin to the discharge areas in central Australia as mound springs is estimated to be 1–5 million year^{-1}. The water is heated by the Earth's magma due to its depth, and the age of the water is calculated to be over 2 Ma. Not only do more than 5,000 free-flowing bores, with source temperatures ranging between 100 and 30°C, depending on bore depth, provide an important water resource to the outback communities, but the GAB is also a favourable environment for the growth of a wide diversity of microbial life. Distinct thriving macroscopic microbial mat communities can be seen colonising specific temperatures along the temperature gradient of runoff channels formed by the free-flowing bores. In the last two decades, a range of thermophilic and mesophilic microorganisms have been characterised from the GAB waters which include sulphate reducers, carbohydrate fermenters, strict aerobes and dissimilatory metal-reducing microorganisms (DIRM). During recent years, there has been a significant drop in the GAB groundwater pressure and volume, largely due to water leakage from corroding bores, and this is a matter of

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great concern. The isolation of metal-reducing microorganisms from the GAB environment suggests that they could be colonising the metal casing of such bores, thereby contributing to bore corrosion and subsequent complete bore failure. It is widely accepted that metal-reducing microorganisms have a large impact on the geochemistry of subsurface environments through the cycling of metals and organic matter and thereby affect water quality and taste. Furthermore, metal-reducing microorganisms have potential applications in bioremediation, mineral leaching and energy generation processes and are of evolutionary interest as metal reduction is considered to be a very ancient form of respiration. In this report, we provide an insight into the microbial diversity of this unique subsurface aquifer.

Keywords Thermophiles • Great Artesian Basin • Aquifer • Metal-reducing microorganisms • Metal leaching • Bioremediation

2.1 Introduction

Thermophiles dominate deep subsurface aquifer environments and represent the most ancient life on the planet. Furthermore, thermophiles express novel enzymes that are intrinsically stable at high temperatures that can be exploited to benefit industry and biotechnology applications, often yielding huge economic profits. In recent times, the methods used to investigate the diversity and the interactions of thermophiles within their natural environment (thermophile ecology) have been revolutionised with the onset of metagenomics and next-generation sequencing technologies. Previously, the infamous great plate count anomaly (Staley and Konopka 1985) and culture-independent 16S rRNA gene library analyses (Pace et al. 1985; Schmidt et al. 1991) revealed the immense limitations existing within the field of microbial ecology as the vast majority of microorganisms, >99% in many environments (Rolf 2004), resist cultivation using traditional approaches. Furthermore, due to culturing biases, traditional culture-dependent approaches were shown to provide inaccurate representations of microbial populations, as readily cultured microorganisms often existed in small numbers and were not dominant within their environment. This is commonly analogised as 'growing weeds' instead of the 'desired flowers'. However, the sole application of culture-independent 16S rRNA gene library analyses for microbial ecology studies provides limited benefit. Although such methods can [in theory (von Winzingerode et al. 1997)] identify the major microbial populations present, these methods provide no information regarding the physiology and function of uncultured microorganisms, which can be >99% of organisms present; i.e. culture-independent 16S rRNA gene library analyses describe 'who is there?' but not 'what they are doing?'

Enter the genomic era. Metagenomics, defined as the culture-independent genomic analysis of microbial communities (Schloss and Handelsman 2003), avoids the biases associated with culturing microorganisms by analysing DNA extracted directly from an environment. Typically, the extracted DNA is size-fractionated and ligated

into plasmids, BACs, PACs or fosmids and transformed to produce (metagenomic) libraries that represent the entire genomic complement of an environment, often with clone-insert sizes in excess of 200 kb. Metagenomic libraries are usually analysed by (1) the direct sequencing of the clone inserts; (2) using specific probes to identify inserts that contain a specific gene of interest, usually based on phylogeny or function; or (3) by performing functional screens, such as enzyme assays or microarray analyses. An alternative method to metagenomic library construction and analyses recently applied to microbial ecology studies is the direct sequencing of the extracted environmental DNA using ultra-high-throughput next-generation sequencing technologies, such as 454 pyrosequencers and solexa sequencers. These recent technologies have an advantage over metagenomic library analyses in that Mbps of sequence data can be produced in only a few days processing time, typically in the form of 400–600-nt fragments using 454 sequencing and 70–95-nt fragments using solexa sequencers. Although the assembly of short-read, mixed microbial community DNA fragments is technically challenging to say the least, tagged, paired-end libraries of 3–20 kb can be constructed and used as 'DNA scaffolds' to aid sequence assemblies. The resultant Contig sequence annotations provide an in-depth profile of the diversity and the physiological attributes of the microbial populations and hence provide an increased understanding of their evolution, activities and impacts within their environment. When such analyses are applied to high-temperature environments, the resulting sequence annotations can also be used to identify unique thermostable enzymes of potential commercial value, and microbial-evolution studies are of increased importance as thermophiles occupy the deepest branches of the phylogenetic tree of life closest to the Last Common Ancestor (LCA). When ultra-high-throughput next-generation sequencing technologies are applied to sequencing mRNA, instead of DNA, genes that are actively expressed at a specific time can be identified and quantified (transcriptomics). Thus, these technologies offer an alternative high-throughput method to microarray analyses to study gene expression. As such, these emerging technologies have revolutionised, microbial ecology investigations and are compared to the 'knife' used to unravel the 'Gordian knot' that is the uncultured microbial majority (Schloss and Handelsman 2005). As the cost of next-generation sequencing continues to decline, and the complex sequence-assembly and sequence-annotation tools continue to evolve, it is likely that the direct sequencing of mixed microbial populations using next-generation sequencers may slowly overtake metagenomic library construction and library analyses as the method of choice for microbial ecology investigations.

Compared to assembling and annotating the metagenome of a mixed microbial population, the genome sequence assembly of a single strain isolated in pure culture is vastly easier and so is likely to contain fewer errors. Genome sequence annotations of isolated novel organisms not only provides a wealth of information regarding the strains evolution and physiology, and hence likely functional roles within its natural environment, but can complement mixed microbial population sequencing projects by allowing the phylogeny of particular Contig sequences and evolutionary gene mutations between specific microbes to be identified. Furthermore, axenic cultures of a strain allows for investigations of its gene expression to be investigated

in vivo, which can be used to confirm the presence of specific sequence regions, conferring metabolic pathways predicted through genome sequence annotations. Hence, these results may be used to close existing gaps within genome sequence assemblies. It seems that isolating microbes to pure culture will therefore remain a cornerstone of microbial research, and hence there is a need to revisit and improve the more traditional culture-dependent techniques used to isolate novel strains. This chapter describes and discusses several new and modified culture-dependent approaches which have recently been used to investigate the ecology of thermophiles from Australia's Great Artesian Basin (GAB). From these recent culture-dependent enrichment studies, numerous novel thermophiles have been identified, subsequently characterised and are in various stages of having their genome sequenced and annotated. In addition to providing a more in-depth profile of these strains' phylogeny and physiology, and screening for useful thermostable enzymes, this data will be used to complement future metagenomic studies of the GAB system.

2.2 Thermophilic Microorganisms in Great Artesian Basin System

For more than the last 15 years, our laboratory has been studying the thermophilic populations existing within Australia's Great Artesian Basin (GAB). Unlike other thermal environments, the GAB is heated due to its great depth as opposed to by volcanic means, and so its physiochemical conditions differentiate it from more commonly studied thermal environments. Solfataric fields and volcanic hot springs are heated due to their close proximity to volcanic regions and therefore compared to the GAB have higher temperatures (up to 350°C) and increased levels of sulphur and sulphur-oxidised products (sulphate, thiosulphate, sulphite) which result in extremely low pH levels (as low as pH 0.5). The GAB environment is further differentiated from such environments in that its groundwaters have no exposure to sunlight, low flow rates and long recharge times (often in excess of 1,000 years). Similarly, the closed structure of thermal oil reservoirs and their high hydrocarbon concentrations differentiate them from the GAB groundwaters which are open systems (with the exception of a few isolated regions), and although the GAB does include the largest onshore oil (and gas) reserves in Australia, the majority of its groundwaters contain nominal hydrocarbon concentrations. Likewise, with few areas of exception, the GAB groundwaters contain low NaCl concentrations and thus differ greatly to thermal marine environments such as shallow and deep hydrothermal vent systems.

The GAB underlies more than 22% of the Australian continent beneath arid and subarid regions of the Northern Territory, Queensland, New South Wales and South Australia (Fig. 2.1a). Covering an area of over 1.7×10^6 km^2, with an estimated water-storage capacity of 8.7×10^{12} m^3, the GAB is considered to be the world's largest geothermal subsurface aquifer (Habermahl 1980). Since 1858, approximately

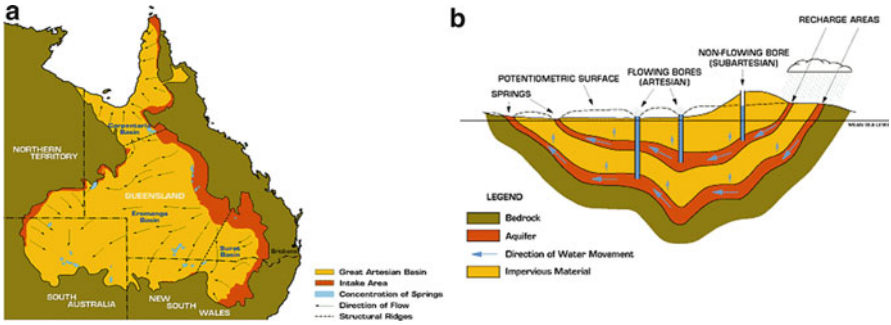

Fig. 2.1 The location, composition and groundwater flow of the Great Artesian Basin of Australia (Source: www.dnr.qld.gov.com.au). (**a**) The location of the Great Artesian Basin (GAB) of Australia, displaying areas of recharge, discharge and the direction of water flow. (**b**) The composition and flow of the Great Artesian Basin. The GAB is composed of alternating layers of permeable sandstone aquifers and impermeable siltstones and mudstones. Recharge waters, due to rainfall, occur at the edge of the basin. The confinement of the aquifer by the overlaying impermeable material pressurises the underground water (artesian). When a bore is drilled the water will rise due to this pressure

5,000 man-made artesian bore wells have been sunk into the GAB to support the domestic activities of over 180,000 Australian outback residents. In addition, the GAB groundwaters supply agriculture-, mineral- and petroleum-production industries that when combined produce approximately $4 billion (AUD) per annum. The GAB was formed between 100 and 250 Ma ago and is composed of up to four alternating layers of water-bearing permeable sandstone aquifers and non-water-bearing impermeable mudstones and siltstones (Fig. 2.1b), the sequence of which can be up to 3 km deep. The surrounding geological formations have an immense influence on the chemical composition of the GAB groundwaters, which can be bicarbonate, chloride, sulphate or iron rich. The majority of GAB groundwater recharge occurs by rainfall infiltrating the outcrops at the edge of the basin from where it flows at an estimated rate of 1–5 million year^{-1} to where the water is naturally reserved in the deep aquifer of the GAB. Such deep reserves contain groundwaters estimated to be up to 2 Ma old. Groundwater discharge occurs as natural mound springs in central Australia, in addition to groundwater expelled from the artesian bores which exhibit source temperatures ranging from ambient to boiling temperatures depending on bore depth.

Within the deep GAB subsurface waters, life is restricted to thermophiles due to the elevated temperatures and the frequently small pore spaces through which aquifer recharge occurs (Ghiorse and Wilson 1988; Lovley and Chapelle 1995). Here, thermophile communities play a major role in controlling the cycling of organic and inorganic compounds, thereby directly affecting groundwater quality and taste. Such can be spoilt by increased concentrations of dissolved iron, hydrogen sulphide and methane produced by thermophilic populations. Additionally, thermophilic populations can further negatively impact aquifer environments by increasing porosity, particularly in carbonate aquifers, and by causing damage to bores and pipelines

through microbial-influenced corrosion (MIC) processes. In recent years, a significant decrease in GAB bore pressure and outflow volume has been observed, and since 1999, the Commonwealth government has invested more than $75 million to cap and pipe the flowing bores in order to maintain and restore artesian pressure, with future plans to cap the remaining bores. Despite these efforts, water loss from leaky corroded bores still occurs and if unchecked may cause detrimental long-term effects to the future water storage, pressure and economic value of the GAB. We have previously speculated that the co-colonisation of metal- and sulphate-reducing thermophiles on the metal casing of GAB bores causes bore corrosion, leading to bore failure and significant water wastage and hardship to the outback communities who depend largely on this water resource (Ogg and Patel 2010a, b, c, 2011), and hence is of great interest.

The unique physico-chemical composition, varying temperatures and the slow rate of flow of the ancient GAB groundwaters provide a favourable environment for microbial life to thrive. This is most evident as distinct, macroscopic microbial mat communities can often be seen to colonise specific temperatures across a thermal gradient created as free-flowing bore waters cool from often near-boiling source temperatures to ambient conditions in open-ditch runoff channels used to transport water and/or hydrate livestock. Such microbial mat communities can be observed in the New Lorne bore (registered number 17263) runoff channel situated near Blackall, some 1,000 km north-west of Brisbane, QLD, Australia (24°54′48″ S and 145°08′18″ E). The New Lorne bore has a source temperature of 88°C, and within its runoff channel exists numerous distinct, filamentous thermophilic microbial mats including grey-coloured mats at 75°C, red-coloured mats at 66°C, green-coloured mats at 57°C and brown-coloured mats at 52°C (Fig. 2.2). The elevated temperatures at which these microbial mats thrive compared to the surrounding Blackall temperatures [mean annual maximum temperature of 30.2°C (http://www.bom.gov.au/climate/averages/tables/cw_036143_All.shtml)] suggest that the majority, if not all, of the constituent mat microbes originate from the GAB subsurface. Hence, investigations into the thermophile diversity present within these microbial mats provide extensive insights into the thermophile diversity present within the GAB subsurface environment.

Culture-independent 16S rRNA gene library analyses of 64 clones from the New Lorne bore outflow and a total of 96 clones from 4 distinct microbial mats existing in the bore's runoff channel indicated that *Hydrogenobacter* dominated the 88°C bore outflow, *Thermus* dominated the 75°C grey mats, *Meiothermus* dominated the 66°C red mats, cyanobacteria dominated the 57°C green mats and *Deinococci-Thermus* and α-*Proteobacteria* dominated the 52°C brown mats (Spanevello 2001). Phylogenetic diversity was found to increase as water temperatures decreased and species spanning domain *Bacteria* were identified. Such included species related to *Hydrogenobacter*; *Thermus*; *Meiothermus*; *Chloroflexus*; *Cytophaga*; *Planctomycetes*; *Rhodothermus*; *Bacillus*; *Clostridium*; *Nitrospira*; *Verrucomicrobium*; *Acidobacterium*; α-, β-, γ- and δ-*Proteobacteria*; and a diverse range of uncultured organisms (which comprised 27% of all clones).

Previous culture-dependent studies of the GAB environment have isolated and characterised a range of novel thermophilic and mesophilic microorganisms including

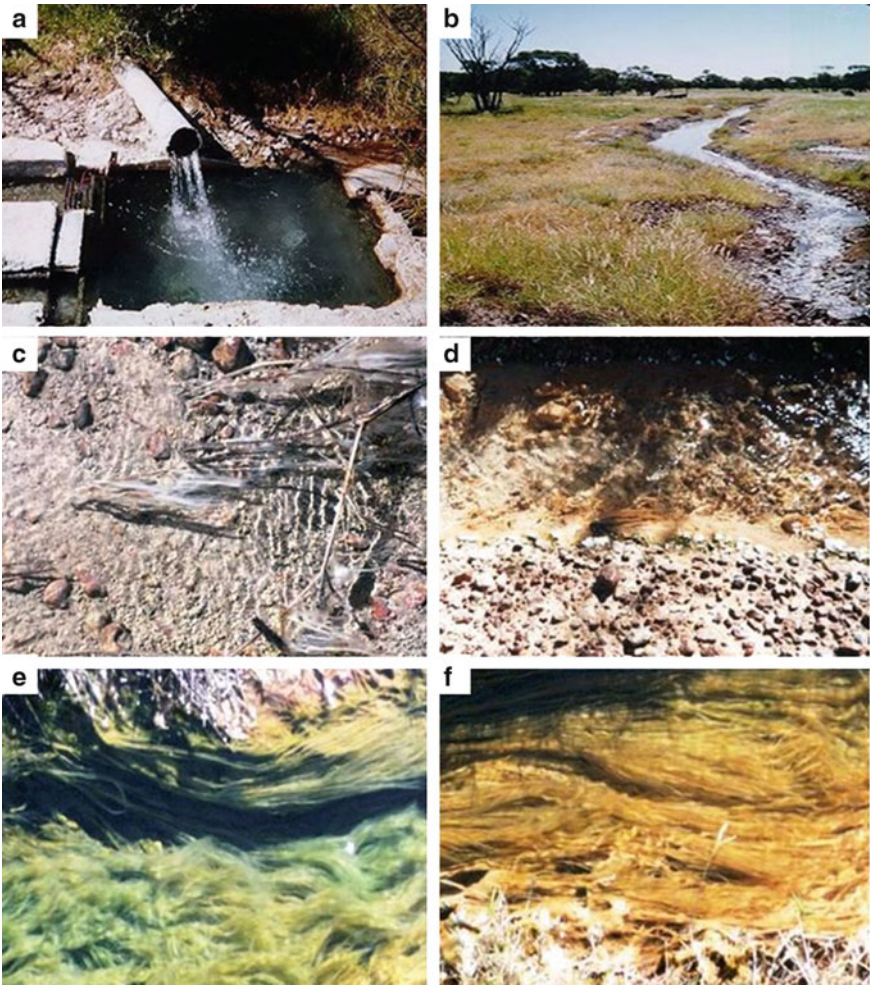

Fig. 2.2 The New Lorne bore (registered number 17263) environment. (**a**) The New Lorne bore outflow with source temperatures of 88°C flow into an open-ditch runoff channel (**b**). Within the runoff channel exists distinct filamentous thermophilic microbial mats at specific temperatures including (**c**) *grey*-coloured mats at 75°C, (**d**) *red*-coloured mats at 66°C, (**e**) *green*-coloured mats at 57°C, and (**f**) *brown*-coloured mats at 52°C

strict aerobes (Kanso and Patel 2003; Spanevello et al. 2002), anaerobic carbohydrate fermenters (Andrews and Patel 1996), sulphate reducers (Love et al. 1993; Redburn and Patel 1994) and iron(III) reducers (Kanso et al. 2002). As often, culture-dependent diversity studies fail to capture the true extent of microbial diversity within an environment by not providing the correct combinations of energy substrates and growth conditions needed to culture the majority of resident microbes, and our more recent culture-dependent studies of thermophiles from the GAB have focused on varying the

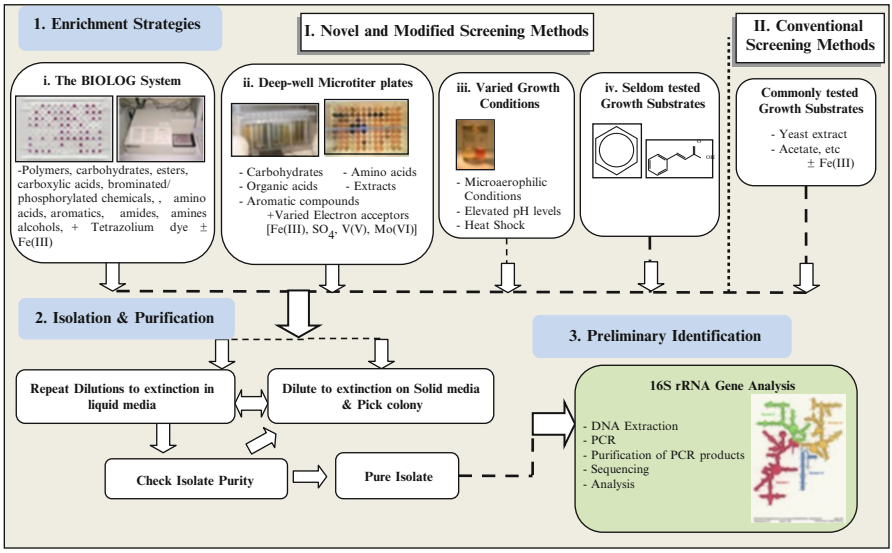

Fig. 2.3 An outline of the recent methods used to assess thermophile diversity in the GAB. Recent novel and modified enrichment screening methods included performing micro-enrichments using (i) the Biolog system (Biolog Inc.) and (ii) deep-well microtiter plates amended with varying energy sources and terminal electron acceptors. Other screens were performed in 7–10 ml of enrichment media using (iii) varied growth conditions, (iv) enrichment media supplied with seldom-used substrates such as aromatic compounds an using more commonly tested energy substrates. The enrichment cultures were subsequently purified by (mostly) performing dilutions to extinction in liquid and/or solid medium. The axenic cultures were next preliminarily identified by 16S rRNA gene analyses

combinations of terminal electron acceptors (TEAs) and energy substrates and/or growth conditions (O_2 concentrations, pH levels, etc.) provided in enrichment media to target the cultivation of previously uncultured thermophiles. These recent strategies are summarised in Fig. 2.3 and included the development of novel and modified small-scale (0.1–1 ml) high-throughput enrichments of thermophiles using (1) the Biolog system (Biolog Inc.) and (2) deep-well (1 ml) microtiter plates amended with varying energy substrates and TEAs. Other recently employed novel and modified enrichment strategies included (3) enrichments performed under varied growth conditions such as under microaerophilic conditions, at high pH levels and with incubations subsequent to heat-shock exposures (performed at 95°C for 20 min), and (4) enrichments with iron(III) and seldom-used substrates such as aromatic compounds. In addition, more conventional enrichments that used media which contained iron(III) as the TEA and commonly tested energy substrates such as yeast extract and acetate as electron donors were also performed. These recent thermophile enrichment methods, their results and potential future applications are discussed below.

There are many advantages in performing enrichments on a smaller scale (0.1–1 ml) compared to standard-size enrichments that generally use 7–10 ml of enrichment medium. Small-scale enrichments allow a decreased volume of environmental sample

Fig. 2.4 The Biolog system. (**a**) A Biolog microtiter plate with numerous purple wells resulting from the reduction of the tetrazolium dye conferring substrate utilisation. (**b**) The Biolog MicroStation and (**c**) the Biolog software which transforms the results into positive, borderline and negative scores

and growth medium to be used per enrichment, and correspondingly, a decreased incubation time is required for positive enrichment results to occur. Furthermore, as the enrichment wells within these micro-enrichments can be inoculated simultaneously using micro-well pipettes, they can be performed quickly and easily and thus in duplicate or triplicate to increase the statistical validity of the enrichment results if required.

The Biolog system was originally developed as a diagnostic tool to identify bacterial isolates based on sole-carbon-source utilisation. It consists of 95 different substrates each contained in a separate well (and a control containing no substrate), to which a minimal growth medium and inoculum are added. The wells also contain the redox dye tetrazolium violet, which turns purple in the presence of electron transfer conferring substrate utilisation (Fig. 2.4a). The absorbance of each well is determined using a Biolog MicroPlate reader, and the substrate utilisation pattern and intensity are compared on a database to identify the unknown isolate (Fig. 2.4b, c). Since 1991, Biolog MicroPlates have also been routinely used for investigating the functional diversity of whole microbial communities from environmental samples (Garland and Mills 1991). Our recent studies employed Biolog MicroPlates amended with trace amounts of yeast extract to enrich and isolate thermophiles from the red and grey New Lorne bore microbial mats. These enrichments were performed both in the presence and absence of ammonium iron(III) citrate as an added TEA. For this, the Biolog MicroPlates were inoculated as recommended by the manufacturer, with modification as described in Ogg and Patel (2009a, b, c) (and outlined in Fig. 2.5). The inoculated Biolog MicroPlates were incubated at 70°C for 4–24 h inside sealed, humified containers, after which the Biolog wells that underwent a colour change from clear to purple due to the reduction of the tetrazolium dye were scored as positive for respiration and turbid wells were scored as positive for growth. Enrichment wells that were amended with iron (III) were scored as positive for iron(III) reduction when a transformation of the reddish-brown colour of the soluble iron(III) to a dark precipitate [iron(II)] and a clearing of the media were observed. All positive wells were either streaked onto either gelrite-amended (1.5%) aerobic Media D plates (Brock and Freeze 1969; Castenholz 1969) or anaerobic iron(III) citrate-PL medium plates (Ogg and Patel 2009a, b, c), or subcultured into McCartney bottle that contained 9.5 ml of aerobic Media D and/or Hungate tubes that contained

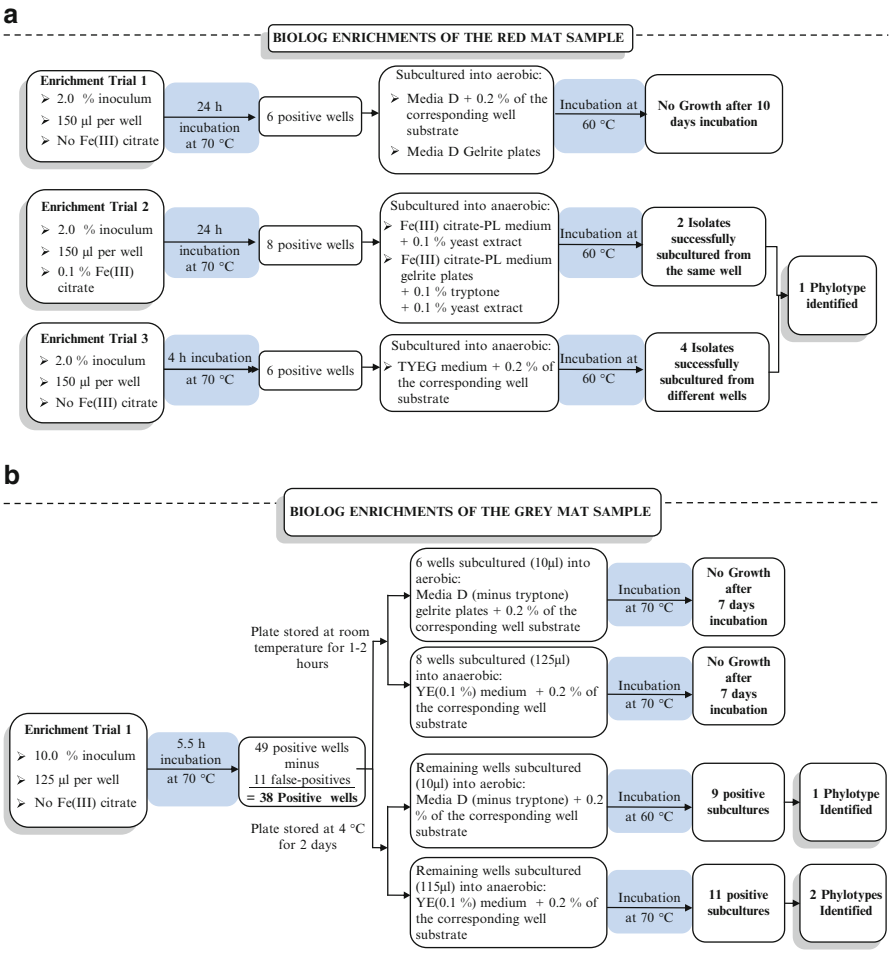

Fig. 2.5 Flowcharts outlining the Biolog enrichment methods performed with the New Lorne Bore (**a**) *red* and (**b**) *grey* microbial mat samples at 70°C. Phylogenetic analyses of the isolates obtained from the positive Biolog enrichment wells of the red and grey microbial mat samples only revealed 1 and 3 different phylotypes, respectively. Media D was prepared as described by Brock and Freeze (1969) and Castenholz (1969). Iron(III) citrate-PL medium and TYEG medium was prepared as described by Ogg and Patel (2009a)

9.5 ml of anaerobic iron(III) citrate-PL medium or YE (0.1%) medium (Ogg and Patel 2009a, b, c) as specified in Fig. 2.5. The subcultures from the Biolog MicroPlate enrichments were incubated at respective temperatures until growth occurred or for up to 7 days. A total of 6 isolates resulted from the subcultures of the various positive Biolog MicroPlate enrichments of the red mats [outlined in Fig. 2.5a, and described in Ogg and Patel (2009a, b, c)]. 16S rRNA gene analyses of more than 1,000 nt from each strain revealed that all 6 of these isolates possessed identical 16S rRNA gene sequences. One such thermophilic isolate was subsequently characterised

and described as a novel species of the genus *Caloramator*, designated *Caloramator australicus* strain RC3^T (Ogg and Patel 2009a, b, c).

Biolog MicroPlate enrichments of the grey mats were performed with an increased sample inoculum size compared to the Biolog MicroPlate enrichment trails of the red mat sample, and resultantly a total of 49 wells were initially deemed as positive for growth and/or respiration (Table 2.1). However, light microscopy of the wells A10, B2, B5, B12, C5, D9, E2, F3 and G10 did not reveal any viable cells, but instead tetrazolium precipitants were observed that are likely to have resulted from reactions between the substrate, the inoculum and the tetrazolium dye after incubations at 70°C. Notably, attempts to subculture these wells were unsuccessful (Table 2.1). Of the remaining 38 positive Biolog MicroPlate well enrichments of the grey mat sample, 20 isolates were successfully isolated and purified in aerobic or anaerobic media as specified in Table 2.1. However, 16S rRNA gene analyses of 16 of the 20 isolates revealed only 3 different phylotypes, which included *Thermaerobacter subterraneus*, *Paenibacillus timonesis* and *Fervidicola ferrireducens*, with 16S RNA gene similarities of over 99%.

These preliminary enrichment trials demonstrated that the Biolog system is not only a useful tool for investigating functional diversity, but can be modified for enrichment and isolation studies. However, the above enrichment strategies are perhaps limited by the immense impact of the media used to subculture and purify the isolates from the original Biolog MicroPlate enrichment wells. It appears that the media used for isolate purification have had the major influence in determining the dominant strain that was purified, and hence only a limited number of different phylotypes were recovered. Future enrichment studies using the Biolog system could avoid these biases by performing repeat subcultures and/or subcultures to extinction of the initial Biolog MicroPlate enrichment in Biolog MicroPlates rather than subculturing the positive wells into larger volumes of growth media, particularly those containing high levels of yeast extract. Future tests to assess the usefulness of the Biolog system for enrichment purposes could investigate and compare the uncultured microbial diversity present with the microbial diversity present in the Biolog MicroPlate enrichment subsequent to incubation and again after successive subcultures into other Biolog MicroPlates. For the latter, following a subculture, the remaining contents of the Biolog MicroPlate wells could be pooled, the total DNA extracted and the 16S rRNA genes amplified by PCR and the sequence analyses. Thermophiles of interest could then be identified and selected using specific primers to identify which Biolog MicroPlate well the organism of interest exists in. Alternatively the specific probes could be used for FISH analyses in combination with micromanipulators to isolate the strain of interest.

Due to the success of the preliminary micro-enrichment trials using the Biolog system in isolating a novel thermophile, further small-scale enrichments were performed but under improved conditions. The Biolog MicroPlates contain numerous growth substrates that are readily fermented, and so within subsequent micro-enrichment trials, these substrates were replaced with energy substrates that are seldom and/or not fermented and hence better complement TEA reduction reactions. In addition, energy substrates were employed that are not routinely used in

Table 2.1 Observations from the positive grey mat enrichment wells, their corresponding well subculture results and the phylogenies of isolated cultures

Biolog enrichment result			Well subculture result					Closest relative (nucleotides sequenced)
Well	Well substrate	Well observation	Media substrate	Anaerobic media	Aerobic media	Incubation time (days)	Designated strain name	
A3	Dextrin	Respiration	Dextrin	–	+	2	Y7A03	1 (705)
A5	Tween40	Respiration	Tween20	–	+[a]	5	Y7A05	1 (672)
A6	Tween80	Respiration	Tween80	–	–	7	–	
A10	L-Arabinose	[bc]Strong respiration & slight turbidity	Arabinose	–	ND	7	–	
B2	D-Fructose	[bc]Strong respiration	Fructose	–	ND	7	–	
B3	L-Fucose	[b]Strong respiration	Fructose	–	–	7	–	
B5	Gentiobiose	[bc]Respiration	Glucose	–	ND	7	–	
B6	α-D-glucose	Respiration	Glucose	+	–[a]	6	Y7B06	2 (1,036)
B12	D-Mannose	[b]Strong respiration	Mannose		–[a]	7	–	
C1	D-Mellibiose	Strong respiration	Glucose	–	–	7	–	
C3	D-Psicose	Strong respiration	Fructose	–	–	7	–	
C5	L-Rhamnose	[bc]Strong respiration	Rhamnose	–	ND	7	–	
D1	Acetic acid	Slight respiration	Acetate	–	–	7	–	
D6	D-Galacturonic acid	Strong respiration	Galactose	–	–	7	–	
D8	D-Glucosaminic acid	Strong respiration	Glucose	+	–[a]	5	Y7D08	ND
D9	D-Glucuronic acid	[b]Strong respiration	Glucose	–	–	7	–	
E2	Itaconic acid	[b]Respiration	Citrate	–	–	7	–	
E8	[c]Propionic acid	[c]Slight respiration	Propionate	–	ND	7	–	
E9	Quinic acid	Slight respiration	Sterilised coffee	–	–	7	–	
E12	Succinic acid	Slight respiration	Succinate	–	–	7	–	
F3	Glucuronamide	[b]Strong respiration	Glucose	–	–	7	–	

F4	L-Alaninamide	Respiration	Casamino acids	–	–	7	–	
F5	D-Alanine	[c]Respiration	Alanine	–	ND	7	–	
F6	L-Alanine	Respiration	Alanine	–	+	5	Y7F06	1 (736)
F7	L-Alanyl-glycine	Respiration	Glycine	–	–	7	–	
F8	L-Asparagine	Respiration	Asparagine	–	+	2	Y7F08	1 (758)
F9	L-Aspartic acid	Respiration	Aspartate	–	+[a]	2	Y7F09	1 (945)
F10	L-glutamic acid	Respiration	Glutamate	–	+[a]	1	Y7F10	1 (828)
F11	Glycyl-L-aspartic acid	Respiration	Aspartate	–	+	2	Y7F11	1 (928)
F12	Glycyl-L-glutamic acid	Respiration	Glutamate	–	–	7	–	
G2	Hydroxy-L-proline	Respiration	Casamino acids	–	+	2	Y7G02	1 (700)
G3	L-Leucine	Respiration	Leucine	–	–	7	–	
G4	L-Ornithine	Respiration	Casamino acids	–	–	7	–	
G5	L-Phenylalanine	Respiration	Casamino acids	–	+	1	Y7G05	ND
G6	L-Proline	Respiration	Proline	–	–	7	–	
G8	D-Serine	Respiration	Serine	–	–	7	–	
G9	L-Serine	Respiration	Serine	–	+	5	Y7G09	1 (453)
G10	L-Threonine	[bc]Strong Respiration	Threonine	–	ND	7		
G12	γ-Amino butyric acid	Respiration	Casamino acids	–	+	1	Y7G12	ND
H1	Urocanic acid	Strong Respiration	Histidine	–	–	7	–	
H2	Inosine	Respiration	Glucose	+	–	1	Y7H02	ND
H3	Uridine	Respiration	Glucose	+	–	1	Y7H03	2 (804)
H4	Thymidine	Respiration	Glucose	+	–	5	Y7H04	2 (598)
H5	Phenylethylamine	Respiration	Glucose	+	–	5	Y7H05	2 (593)
H6	Putrescine	Respiration	Casamino acids	+	–	1	Y7H06	3 (451)
H7	2-Amino ethanol	Respiration	Casamino acids	+	–	1	Y7H07	ND
H10	D,L-Glycerol phosphate	Respiration	Glycerol	–	ND	7	–	

(continued)

Table 2.1 (continued)

Biolog enrichment result			Well subculture result					Closest relative (nucleotides sequenced)
Well	Well substrate	Well observation	Media substrate	Anaerobic media	Aerobic media	Incubation time (days)	Designated strain name	
H11	α-D-Glucose-1-phosphate	Respiration	Glucose	+	–	1	Y7H11	ND
H12	D-Glucose-6-phosphate	[b]Strong Respiration	Glucose	–	–	7	–	

Key: (1)16S rRNA gene sequence similarity to *Thermaerobacter subterraneus* (AF343566) of 99–100%; (2) 16S rRNA gene sequence similarity to *Fervidicola ferrireducens* (EU443728) of 99–100%; (3) 16S rRNA gene sequence similarity to *Paenibacillus timonesis* (AY323611) of 99.1%

[a]Wells were additionally streaked (10 μl) onto aerobic Media D (minus tryptone) Gelrite (1.5%) plates amended with the corresponding substrate (0.2%), however no growth occurred after 7-day incubation at 60°C

[b]False positive results. No cells were observed via light microscopy analyses but tetrazolium precipitants were observed which likely resulted from reactions between the substrate, the inoculum and the tetrazolium dye after incubation at 70°C

[c]The entirety of the well was subcultured into 9.5 ml of anaerobic YE(0.1%) medium amended with the corresponding substrate (0.2%) and incubated at 70°C for 7 days after which no growth was observed

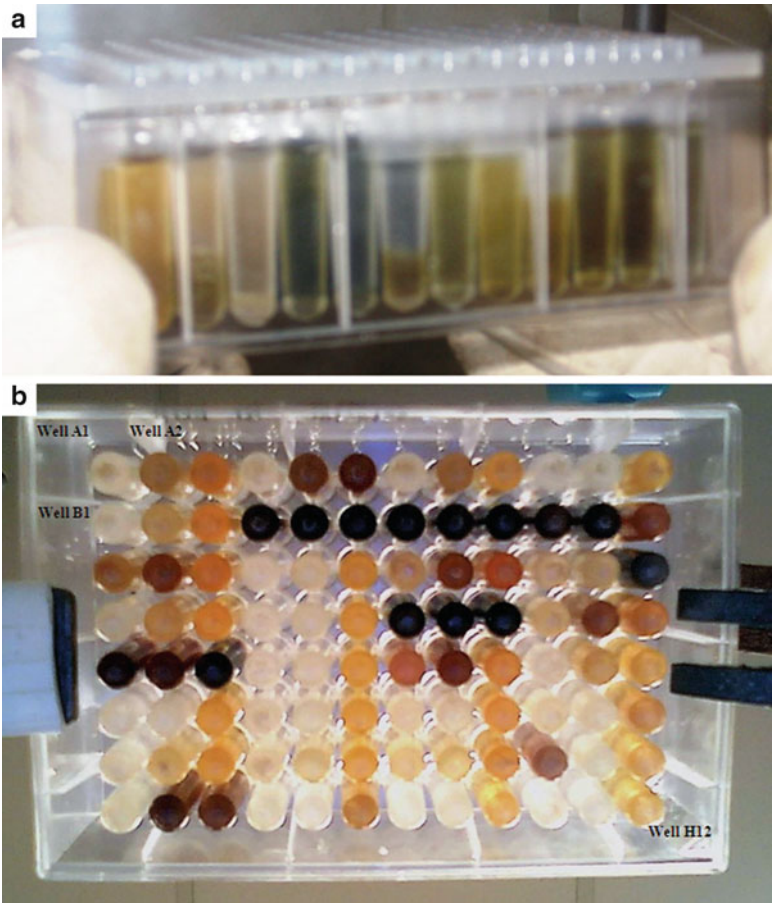

Fig. 2.6 Thermophile enrichments performed using deep-well microtiter plates. The side (**a**) and (**b**) base view of a deep-well (1 ml) microtiter plate containing an Fe(III) citrate-PL medium enrichment of the *red*, *green* and *brown* microbial mats subsequent to incubation at 50°C for 5 days is shown. Wells A1, A2, B1 and H12 are given as references. Well A1 contains a white precipitate characteristic of total Fe(III) reduction. Well B2 contains a light green colour signifying partial Fe(III) reduction. Well A2 contains a *light brown* colour signifying possible growth but no Fe(III) reduction (Note: wells B4–B11, D7–D9 and E1–E3 are invalid due to the colour produced from reactions between the growth substrates and the Fe(III))

enrichment studies and were therefore more probable to cultivate novel thermophiles. For such, deep-well microtiter plates that held a 1 ml volume in each well were selected for subsequent micro-enrichment trials (Fig. 2.6). The larger well volume of 1 ml compared with the Biolog MicroPlates wells, which hold 150 µl (maximum), allow a greater cell volume to be produced which is likely to increase the probability of a successful transfer. The Deep-well (1 ml) Microtiter plates were prepared by adding different energy substrates, which included extracts, sugar alcohols,

organic acids and aromatic compounds to each microtiter plate well from sterile anaerobic stock solutions. The substrate containing microtiter plates were subsequently dried at 50°C for 1 h and stored inside an anaerobic chamber for at least 24 h prior to enrichment to ensure that anaerobic conditions had been reached. Unlike the Biolog MicroPlates, a tetrazolium dye was not included in the deep-well microtiter plate enrichments, but instead, TEAs that produced a medium colour change when reduced were added to the enrichment medium. This enabled the easy identification of enrichment wells that were positive for TEA reduction. For this, vanadium(V) was used as a TEA for the deep-well (1 ml) microtiter plate enrichments due to its previously limited use in enrichment studies and therefore increased probability of successfully cultivating novel thermophiles. Deep-well microtiter plate enrichment media were also amended with sulphate and iron(III) to investigate the diversity of thermophilic sulphate-reducing bacteria (SRB) and dissimilatory iron(III)-reducing bacteria (DIRB) due to their considerable influence on GAB groundwater geochemistry and likely involvement in MIC processes of GAB bores. Enrichments of the deep-well microtiter plates were performed by first adding the environmental samples to Hungate tubes that contained sterile anaerobic medium fortified with either vanadium(V), sulphate or ammonium iron(III) citrate as the TEA. The inoculated medium was then dispensed into the substrate containing deep-well microtiter plates (using a multichannel pipette) inside an anaerobic chamber, and the inoculated deep-well microtiter plates were sealed and placed inside an anaerobic gas jar and incubated at either 50 or 70°C for 3 days. Following incubation, the plates were let cool inside an anaerobic chamber at room temperature for approximately 4 h to allow the cells to reach a resting growth phase prior to subculture and the enrichment wells were scored for TEA reduction. The deep-well microtiter plate enrichments amended with vanadium(V) were scored as positive for vanadium(V) reduction when a colour change from clear to a green colour was observed in an enrichment well. Sulphate-amended deep-well microtiter plate enrichments were assayed as described by Ramamoorthy et al. (2006). For this, $Fe(NH_4)_2(SO_4)_2$ was added to each well at a final concentration of 0.5% from a freshly prepared sterile anaerobic stock solution. The microtiter plate wells in which black precipitates of ferrous sulphide were produced suggestive of sulphate reduction were scored as positive. The deep-well microtiter plate wells amended with iron(III) were scored as positive for iron(III) reduction as described above. All positive wells were subcultured into Hungate tubes that contained 9.5 ml of the same medium and incubated at the respective temperature.

The microbial mat samples used for the deep-well microtiter plate enrichments amended with vanadium(V) included the grey, red, green and brown microbial mat samples from the New Lorne bore (RN 17263) runoff channel, orange/brown-coloured microbial mats (and sediment) that exist at 61–63°C in the Youranigh bore (RN 4163; source temperature 74°C) runoff channel, and the red-coloured mats that exist at 66°C in the Portland bore (RN 4995; source temperature 73°C) runoff channel. GAB bore outflow samples used for deep-well microtiter plate enrichments with vanadium(V) included those taken from the 68°C Woolscour bore (RN 1494A) and from the 46°C Mitchell bore (RN 22981). The results from

the vanadium(V) deep-well microtiter plate enrichments are shown in Table 2.2. No successful subcultures were obtained from the wells that were scored as positive for vanadium(V) reduction from the enrichments of the microbial mat samples taken from the New Lorne bore and Portland bore environments or of the Woolscour bore outflow sample. Similarly, only 9 subcultures from 15 positive wells were obtained from the Youranigh bore mat sample, and 8 subcultures from 18 positive wells were obtained from the Mitchell bore outflow enrichment.

The designated strain nomenclature of the isolates, the culture-purification method used and the results from the phylogenetic analyses of partial 16S rRNA gene sequences of these strains are given in Table 2.3. Numerous isolates including those designated strains VA09, VC01, VC08, VF08 and VG05 were successfully purified by repeat subcultures to dilution in (liquid) V(V)-PL medium (Ogg and Patel 2010a, b, c) amended with the corresponding well substrate with incubations at 50°C. Notably, the closest relatives to all of these strains are fermenters. Other vanadium(V) enrichment subcultures that required further purification were subcultured in dilution in solid (1.5% gelrite amended) V(V)-PL medium amended with the corresponding well substrate and incubated at 50°C before well-isolated colonies from cultures of VB01, VC05, VE08 and VG01 were successfully picked and transferred into the same medium and incubated at 50°C. When the specific electron donors that were used in the growth media of these strains (VB01-2, VB01-3, VC05-1, VE08-1, VG01-1 and VG01-2) and the previously isolated V(V)-reducing strains (VA09, VC01, VC08, VF08 and VG05) were excluded from the growth media, no difference in vanadium(V) reduction or growth was observed for any of these cultures. Given this and that the closest relatives of the sequenced vanadium(V)-reducing strains are fermenters, further purification of the strains VB01-3, VC05-1, VE08-1 and of the cultures VB01, VC01, VG08 and VH08 were performed by repeat dilutions to extinction in TYEG medium (Ogg and Patel 2009a, b, c). The results from the phylogenetic analyses of partial 16S rRNA gene sequences of all of the strains enriched in deep-well microtiter plates amended with vanadium(V) as the TEA are given in Table 2.4. From these deep-well enrichments with vanadium(V), three potentially novel isolates (strains VC05-2, VF08 and VG08) were successfully isolated, of which one isolate, strain VF08^T, has thus far been characterised and described as a novel species within the genus *Caloramator*, designated *Caloramator mitchellensis* (Ogg and Patel 2010a, b, c). 16S rRNA gene sequencing of 1,390 nt of the isolate designated VC05-2 revealed that the strain was most closely related to *Caloramator indicus* (GenBank ID, X75788; 16S rRNA gene similarity value of 96.6%), and 16S rRNA gene sequencing of 1,353 nt of the isolate designated VG08 revealed that the strain was most closely related to *Clostridium tunisiense* (GenBank ID, AY187622; 16S rRNA gene similarity value of 93.4%).

The results from the deep-well (1 ml) microtiter plate enrichment screens of the red, green and brown New Lorne bore microbial mats with sulphate and ammonium iron (III) citrate are shown in Table 2.4. All of the positive sulphate enrichments were successfully subcultured into Hungate tubes that contained the same media with the exception of the 2-methoxybenzoate enrichment of the green mat and the 4-methoxybenzoate enrichment of the red mat in which no growth was observed.

Table 2.2 Deep-well microtiter plate enrichments of GAB samples with vanadium(V) as the terminal electron acceptor with various growth substrates after 3-day incubation at 50°C

Energy substrates	Final concentration	New Lorne Bore microbial mat samples				Youranigh bore mats	Portland bore mats	Woolscour bore outflow	Mitchell bore outflow
		Grey	Red	Green	Brown				
		−	−	−	−	−	−	−	+
		−	−	−	−	+	−	−	++
Malate	0.2%	−	++	−	−	+	−	−	+
Formate	0.2%	−	−	−	−	+	−	−	++
Lactate	0.2%	−	−	−	−	−	−	−	−
Vanilline	4 mM	−	−	+++	−	+	−	−	++
4-Methoxybenzoate	4 mM	−	−	−	−	++	−	++	++
2-Methoxybenzoate	4 mM	−	−	−	−	++	−	−	++
Pyrogallol	4 mM	−	−	−	−	−	−	−	−
Catechol	2 mM	−	−	−	−	−	−	−	−
Cinnamate	4 mM	−	−	+	−	−	+	−	+++
4-Hydroxycinnamate	4 mM	−	−	−	−	−	−	−	−
Phenylacetate	4 mM	+	+	++	++	++	++	+	+++
4-Hydroxyphenylacetate	4 mM	−	−	−	−	++	+	−	+++
Chlorobenzoate	2 mM	−	−	−	−	−	+	−	+
Sodium benzoate	4 mM	−	+	−	−	+	−	−	+++
3-Hydroxybenzoate	4 mM	−	+	−	−	+	−	−	+++
Syringate	4 mM	−	−	−	−	+++	−	−	−
Adipate	4 mM	−	−	++	−	+++	−	−	+++
Malonate	5 mM	−	−	+	−	++	−	−	+++
o-Cresol	1 mM	−	−	++	−	++	−	−	+++
m-Cresol	1 mM	−	−	−	−	−	−	−	+++
p-Cresol	1 mM	−	−	−	−	+	−	−	+++

Key: +++ strong V(V) reduction, ++ medium level of V(V) reduction, + weak V(V) reduction, − no growth or V^{5+}-reduction

Table 2.3 The phylogenetic analyses of thermophilic vanadium-reducing isolates from the GAB bore samples. Medium compositions used for isolations are described in Ogg and Patel 2010a, b, c

Mat sample	Substrate	Isolate	Media used for purification[a]	Contig length	Nearest phylogenetic neighbour (BLASTn)	GenBank accession number	Similarity
Youranigh	Malate	VA09	Liquid V(V)-PL medium	953	*Caloramator coolhaasii*	AF104215	953/961 (99%)
Youranigh	Formate	VB01-1	TYEG medium	1,403	*Caloramator coolhaasii*	AF104215	1 360/1 379 (98%)
Youranigh	Formate	VB01-2	Solid V(V)-PL medium	1,414	*Caloramator coolhaasii*	AF104215	1 383/1 409 (98%)
Youranigh	Formate	VB01-3	Solid V(V)-PL medium + TYEG medium	1,392	*Caloramator coolhaasii*	AF104215	1 370/1 395 (98%)
Youranigh	4-Methoxybenzoate	VC01	Liquid V(V)-PL medium	955	*Thermotalea metallivorans*	EU443727	955/955 (100%)[b]
Youranigh	2-Methoxybenzoate	VC05-1	Solid V(V)-PL medium + TYEG medium	1,417	*Caloramator coolhaasii*	AF104215	1 384/1 404 (98%)
Youranigh	2-Methoxybenzoate	VC05-2	TYEG medium	1,390	*Caloramator indicus*	X75788	1 349/1 396 (96%)
Youranigh	Adipate	VG01-1	Solid V(V)-PL medium	1,336	*Thermotalea metallivorans*	EU443727	1,336/1 337 (100%)[b]
Youranigh	Adipate	VG01-2	Solid V(V)-PL medium	1,349	*Thermotalea metallivorans*	EU443727	1 349/1 351 (100%)[b]
Youranigh	Malonate	VG05	Liquid V(V)-PL medium	734	*Thermotalea metallivorans*	EU443727	734/734 (100%)[b]
Mitchell	2-Methoxybenzoate	VC08	Liquid V(V)-PL medium	797	*Caloramator coolhaasii*	AF104215	797/809 (98%)

(continued)

Table 2.3 (continued)

Mat sample	Substrate	Isolate	Media used for purification[a]	Contig length	Nearest phylogenetic neighbour (BLASTn)	GenBank accession number	Similarity
Mitchell	4-Hydroxyphenylacetate	VE08-1	Solid V(V)-PL medium + TYEG medium	1,356	*Caloramator coolhaasii*	AF104215	1 342/1 358 (98%)
Mitchell	3-Hydroxybenzoate	VF08	Liquid V(V)-PL medium	1,605	*Caloramator celere*	DQ207958	1 483/1 616(91%)
Mitchell	Malonate	VG08	Liquid V(V)-PL medium	1,353	*Clostridium tunisiense*	AY187622	1 279/1 370 (93%)
Mitchell	*p*-cresol	VH08	TYEG medium	690	*Bacillus icheniformis*	GQ375236	659/688 (95%)

[a]V(V) – PL medium was amended with the corresponding well substrate at concentrations specified in Table 2.3
[b]*Thermotalea metallivorans* strain B2-1^T was isolated as described below from the brown mats.

Table 2.4 Deep-well (1 ml) microtiter plate enrichment results of the red, green and brown New Lorne bore microbial mats with sulphate and ammonium iron(III) citrate after 5-day incubation at either 50 or 70°C

		Enrichments with sulphate at 50 C			Enrichments with iron(III) at 50°C			Enrichments with iron(III) at 70°C		
Energy substrates	Concentration	Red	Green	Brown	Red	Green	Brown	Red	Green	Brown
Control (no substrate)		–	–	–	+++	+	–	+++	–	–
Sodium benzoate	4 mM	–	–	–	+	+	++	+	–	–
3-Hydroxybenzoate	4 mM	–	–	–	++	+++	+	++	–	–
4-Hydroxybenzoate	4 mM	–	–	–	+++	++	–	++	–	–
2,5-Dihydroxybenzoate	4 mM	–	–	–	+	++	+	+	–	–
3-Chlorobenzoate	2 mM	–	–	–	+	+	–	+	–	–
Phenylacetate	4 mM	–	–	–	+	+	+	+++	–	–
4-Hydroxyphenylacetate	4 mM	–	–	–	++	+	–	+++	–	–
Cinnamate	4 mM	–	–	–	+	+++	+	+++	–	–
4-Hydroxycinnamate	4 mM	–	–	–	+	–	–	–	–	–
2-Hydroxycinnamate	4 mM	–	–	–	+++	++	–	+	–	–
2-Methoxybenzoate	4 mM	++	++	–	+	+	+	+++	–	–
4-Methoxybenzoate	4 mM	++	–	–	+	+	+	+++	–	–
Vanilline	4 mM	–	–	–	–	+	–	+	–	–
Syringate	4 mM	–	–	–	–	–	–	++	–	++
Adipate	5 mM	++	–	–	+	+	+	+++	–	–
Malonate	5 mM	–	–	–	+++	+	–	+	–	–
Starch	0.2%	–	–	–	+++	+++	+	+	–	–
Succinate	5 mM	++	–	–	+++	+	+	+++	–	–
Sodium acetate	0.2%	–	–	–	+	+	–	+++	–	–
Propionate	0.2%	–	–	–	+	++	–	+	–	–
Formate	0.2%	++	–	–	+	+	–	+++	–	–
Tryptone	0.2%	+++	–	–	++	+	+	+++	–	–

(continued)

Table 2.4 (continued)

Energy substrates	Concentration	Enrichments with sulphate at 50 C			Enrichments with iron(III) at 50°C			Enrichments with iron(III) at 70°C		
		Red	Green	Brown	Red	Green	Brown	Red	Green	Brown
Peptone	0.2%	+++	+++	−	+++	++	+++	+++	−	+
Pyruvate	0.2%	+++	−	−	+++	++	+	+++	−	−
Ethanol	0.2%	−	−	−	+++	+++	++	++	−	−
Glycerol	0.2%	+++	−	−	+++	+	−	+++	−	−
Inositol	0.2%	++	−	−	+++	+	+++	+++	−	−
Aspartate	0.2%		−	−	++	++	++	+++	−	−

Key: +++ iron(III) totally reduced producing a white precipitate/strong sulphate reduction observed, ++ partial iron(III) reduction usually producing a green colour change in the iron(III)/sulphate reduced but to a lesser extent, + growth but minimal iron(III) reduction was observed/or in the sulphate enrichments sulphate reduction was observed, − no growth or iron(III)/sulphate reduction

Notable phylotypes discovered from 16S rRNA gene sequencing of the purified cultures included the five thermophilic isolates purified from the well that contained 0.2% glycerol that were designated strains: RH04-1, RH04-2, RH04-3^T, RH04-7 and RH04-8. These strains had identical (100%) 16S rRNA gene sequences (from sequence comparisons of more than 1,000 nt of each isolate) and were most closely related to *Desulfotomaculum putei* DSM 12395^T (GenBank ID, AF053933) and *Desulfotomaculum hydrothermale* DSM 18033^T (GenBank ID, EF081293) with sequence similarity values of 95.9 and 93.7%, respectively. Strain RH04-3^T was subsequently characterised and described as *Desulfotomaculum varum* strain RH04-3^T sp. nov. (Ogg and Patel 2010a, b, c). Other notable phylotypes discovered from the deep-well microtiter plate enrichments with sulphate included 2 isolates designate RG10-1 and RG10-2 which were isolated from the well that contained 0.2% pyruvate and were purified in solid (1.5% gelrite amended) SO_4-PL medium amended with 0.2% pyruvate and TYEG medium, respectively. 16S rRNA gene sequencing of 1,217 nt of strain RG10-1 revealed that the strain was most closely related to *Caloramator australicus* (strain RC3^T, isolated above; 16S rRNA gene sequence similarity of 95.9%), and 16S rRNA gene sequencing of 1,162 nt of strain RG10-2 revealed that the strain was most closely related to *Clostridium thermocellum* ATCC 27405^T (GenBank ID, CP000568; 16S rRNA gene sequence similarity of 91.1%). Strains RG10-1 and RG10-2 have not yet been characterised.

When ammonium iron(III) citrate was provided as the TEA to the deep-well microtiter plate enrichments, a total of 60 wells were deemed as positive for iron(III) reduction and/or growth from the 87 deep-well enrichments (69.0% positive enrichment results) of the red, green and brown mat samples when incubations were performed at 50°C (Fig. 2.6b). When identical enrichments were performed but with incubations at 70°C, only 30 positive results were observed from the 87 enrichments (34.5% positive enrichment results). Notably, all but two of these positive enrichment wells at 70°C were observed from the red mat sample enrichments. The higher number of positive wells observed at 70°C found within the red mat sample enrichments corresponds to the elevated in situ temperature of the red mats (66°C) compared to the in situ temperatures of 57 and 52°C observed for the green and brown mats, respectively. The high number of positive enrichment wells observed in the ammonium iron(III) citrate-amended deep-well plate enrichments at 50°C and within the red mat enrichment at 70°C suggests that either aromatic compound oxidation linked to the reduction of iron(III) is widespread within the GAB microbial mats or that the observed iron(III) reduction is linked to the utilisation of either an alternative energy substrate transferred within the environmental sample, or utilisation of the citrate and/or the trace yeast extract present in the iron(III) citrate-PL medium. The positive results observed in the control wells (which contained no added electron donor) of the ammonium iron(III) citrate-amended deep-well plate enrichments of the red and green mat samples suggest the latter. For these reasons no subcultures or purifications of isolates from the deep-well microtiter plate enrichments amended with iron(III) were performed.

It should be noted that within the deep-well microtiter plate enrichments the aromatic compounds not only serve as potential energy substrates linked to either

microbial TEA reduction or fermentative growth, but as aromatic compounds have a toxic effect on certain microorganisms, their presence in enrichment media would selectively promote the growth of aromatic compound-tolerant microbes. Such may have contributed to the varied well observations within the vanadium(V) and ammonium iron(III) citrate enrichment plates (Tables 2.3 and 2.7) and explain the different phylotypes observed within the vanadium(V)-reducing cultures if indeed the trace yeast extract was the only energy substrate utilised in the these enrichment wells (i.e. and not the aromatic compounds). Future deep-well enrichments that employ aromatic compounds as energy sources could use gas chromatography to determine if the aromatic compounds are reduced and the corresponding end-products produced. Such studies have not yet been performed using these cultures.

The above deep-well microtiter plate enrichment strategies that employed various energy substrates and (colorimetric) TEAs resulted in the cultivation of a wider diversity of thermophiles compared to the enrichment strategies that used the Biolog system. A total of five new phylotypes were obtained from the enrichments with the deep-well plates compared to 1 new phylotype using the Biolog system. Hence, the decision to trial the enrichment strategies using substrate-amended deep-well plates instead of improving the enrichment strategies using the Biolog system is justified. Future deep-well plate enrichment studies could include other TEAs that produce a medium colour change when reduced, such as sulphite, thiosulphate, elemental sulphur, manganese(IV), cobalt(III), chromium(VI), uranium(VI), arsenate(V), technetate(VII) and selenium(III). Alternatively tetrazolium dyes can be added to the deep-well plates (as the sole TEA) to screen for thermophilic fermenters. Notably, such enrichments were trialled in this study (data not shown), but were abandoned due to complications resulting from a high number of positive wells likely resulting from the utilisation of a energy substrate transferred within the environmental sample or due to the fermentation of the trace yeast extract present in the PL medium. Other future deep-well plate enrichment studies could be performed using varied growth conditions (pH, temperature, oxygen levels, etc.) and medium constituents (electron acceptors, trace elements, vitamins, extracts, etc.) and using molecular methods to assess the culturable diversity within the deep-well plate enrichments and subsequent subcultures as described above.

Recent novel and traditional culture-dependent enrichment strategies performed in 7–10 ml of growth media that were used to investigate the ecology of thermophiles with the GAB not only supplied seldom-used energy substrates such as aromatic compounds to enrichment media but trialled the varying growth conditions (pH, temperature, oxygen concentration, etc.) in an attempt to culture novel and less readily cultured thermophiles. One such unique enrichment strategy was designed in an attempt to isolate microaerophilic iron(III)-reducers from the New Lorne bore mat samples (outlined in Fig. 2.7). These enrichments were initiated by inoculating McCartney bottles that contained 9.5 ml of aerobically prepared Fe(III) citrate-PL medium amended with either 0.2% yeast extract or 20 mM acetate as previously described (Ogg and Patel 2009a, b, c, 2010a, b, c). The enrichment cultures were incubated at 50 and 70°C until growth, determined visually by an increase in turbidity, and/or Fe(III) reduction determined as described above, was observed or for up

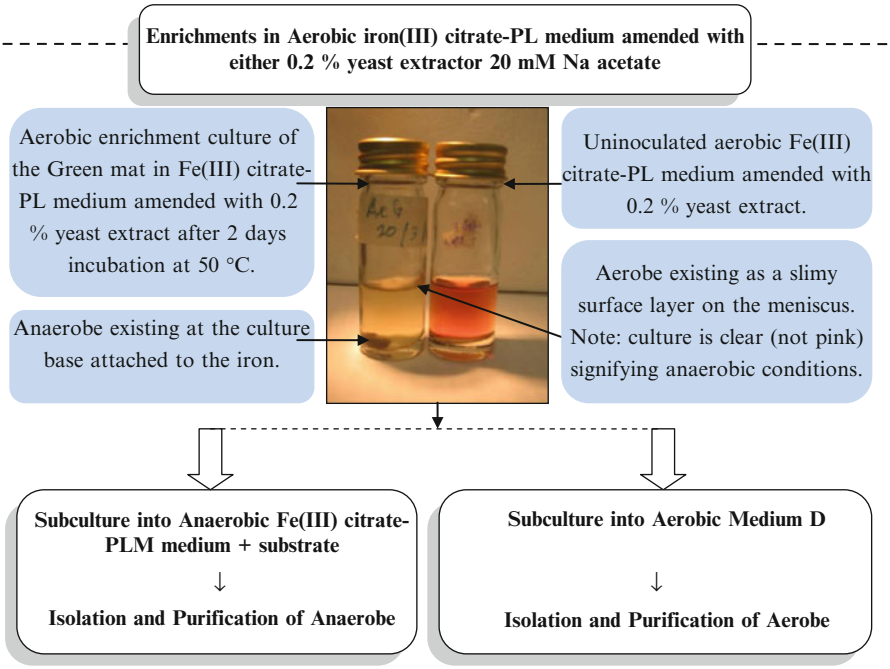

Fig. 2.7 Flowchart outlining the methods used in attempt to isolate microaerophilic Fe(III)-reducing thermophiles from the GAB

to 5 days incubation. To culture the dominant aerobes within the enrichment cultures, these cultures were streaked onto Medium D agar plates and subsequently purified. Enrichment cultures that were scored as positive for iron(III) reduction were subcultured into Hungate tubes containing 9.5 ml of (anaerobic) iron(III) citrate-PL medium amended with the corresponding growth substrate, and the resultant cultures were purified.

The results of the New Lorne Bore microbial mat enrichments that were performed in 7–10 ml of growth medium are given in Table 2.5, and resulting phylotypes from isolates of these enrichments are given in Table 2.6. These enrichment results were again reflective of the specific in situ temperatures of each mat sample, as almost all of the cultures that grew at 70°C were isolated from the grey and red mat sample enrichments. The wide diversity of DIRB observed is a somewhat expected result due to the large concentration of iron within Australian subsurface environments and the large influence that DIRB exhibit within deep aquifer environments. In Table 2.7 a summary is given of the number of novel phylotypes that were obtained from the different enrichment and isolation procedures used in the enrichments in 7–10-ml medium. Notably, the sample size is not large enough to provide statistically valid data, but nonetheless is of great interest. The enrichment and isolation strategy which was designed to provide microaerophilic conditions in attempt to culture facultative anaerobic DIRB revealed the highest proportion of

Table 2.5 Enrichment results of the 75°C grey, 66°C red, 57°C green and 52°C brown microbial mat samples from the New Lorne bore runoff channel at 50 and 70°C

Electron acceptor	Electron donor	Enrichment conditions	Microbial mat enrichment results at 50°C				Microbial mat enrichment results at 70°C				Incubation time
			Grey	Red	Green	Brown	Grey	Red	Green	Brown	
FeO(OH)	Yeast extract	Anaerobic liquid medium	+++	++	++	+++	+++	+++	–	–	3 days
Fe(III) citrate	Yeast extract	Anaerobic liquid medium	+++	+++	+++	+++	+++	+++	–	–	3 days
Fe(III) citrate	Acetate	Anaerobic liquid medium	–	+++	–	+++	+++	+++	–	–	5 days
FeO(OH)	Molecular hydrogen	Anaerobic liquid medium	–	++	++	+++	+++	+++	+++	–	3 days
Fe(III) citrate	Benzoate	Anaerobic liquid medium	–	–	++	++	–	–	–	–	10 days
Fe(III) citrate	3-Hydroxybenzoate	Anaerobic liquid medium	–	++	++	–	–	–	–	–	10 days
Fe(III) citrate	3-Chlorobbenzoate	Anaerobic liquid medium	–	–	++	–	–	–	–	–	10 days
Fe(III) citrate	4-Hydroxyphenylacetate	Anaerobic liquid medium	–	++	–	–	–	+++	–	–	10 days
Fe(III) citrate	Cinnamate	Anaerobic liquid medium	–	++	++	–	–	–	–	–	3 days
Fe(III) citrate	4-Hydroxycinnamate	Anaerobic liquid medium	–	–	++	–	–	–	–	–	3 days
Fe(III) citrate	3-Chlorobbenzoate	Solid anaerobic medium[a]	+	+	++	–	–	–	–	–	5 days
Fe(III) citrate	4-Hydroxyphenylacetate	Solid anaerobic medium[a]	–	+	+	–	–	++	–	–	5 days
Fe(III) citrate	Malonate	Solid anaerobic medium[a]	+	+	+	+	–	++	–	–	5 days

Fe(III) citrate	Cinnamate	Solid anaerobic medium[a]	+	++	++	+	−	++	−	−	5 days
Fe(III) citrate	Yeast extract	Heat shock anaerobic liquid medium	−	+++	+++	+++	+++	+++	+++	−	6 days
Fe(III) citrate	Yeast extract	Microaerophilic medium	+	++	++	++	+	+	−	−	3 days
Fe(III) citrate	Acetate	Microaerophilic medium	−	−	++	−	−	−	−	−	3 days
Fe(III) citrate	Yeast extract	High alkaline (pH 9.8) anaerobic liquid medium	−	−	−	−	−	−	−	−	(14 days)
Fe(III) citrate	Acetate	High alkaline (pH 9.8) anaerobic liquid medium	−	−	−	−	−	−	−	−	(14 days)
Fe(III) citrate	Yeast extract	High alkaline (pH 9.8) microaerophilic medium	−	+	+	−	−	−	−	−	12 days
Fe(III) citrate	Acetate	High alkaline (pH 9.8) microaerophilic medium	−	−	+	−	−	−	−	−	12 days

Key: +++ total Fe(III) reduction, ++ partial Fe(III) reduction, + growth but no Fe(III) reduction, − no growth or Fe(III) reduction
[a]Enrichments on solid anaerobic medium were performed at 50 and 65°C. The results for the 65°C enrichment are given in the 70°C column

Table 2.6 Nomenclature and phylogeny of the isolates from the enrichments of the 4 New Lorne Bore microbial mat samples performed in 7–10 ml of various growth media

Mat sample	Electron acceptor/carbon source	Enrichment method	Incubation temp (°C)	Purification method	Isolate name	Nearest phylogenetic neighbour (BLASTn)	GenBank accession number	Similarity	Taxonomy
Grey	FeO(OH)/yeast extract	Anaerobic liquid medium	70	Subcultures in dilution	Y170[a]	*Thermosediminibacter litoriperuensis*	AY703479	1,311/1,374 (95%)	*Firmicutes Thermoanaerobacteraceae*
Red	FeO(OH)/yeast extract	Anaerobic liquid medium	70	Subcultures in dilution	R170	*Thermoanaerobacter thermohydrosulfuricus*	AY701760	939/945 (99%)	*Firmicutes Thermoanaerobacteraceae*
Grey	FeO(OH)/yeast extract	Anaerobic liquid medium	50	Subcultures in dilution	Y150	*Thermincola carboxydiphila*	AY603000	960/968 (99%)	*Firmicutes Peptococcaceae*
Red	FeO(OH)/yeast extract	Anaerobic liquid medium	50	Subcultures in dilution	R150	*Geosporobacter subterraneus*	DQ643978	872/908 (96%)	*Firmicutes Clostridiaceae*
Green	FeO(OH)/yeast extract	Anaerobic liquid medium	50	Subcultures in dilution	G150	*Thermobrachium celere*	DQ207958	945/954 (99%)	*Firmicutes Clostridiaceae*
Brown	FeO(OH)/yeast extract	Anaerobic liquid medium	50	Gelrite shakes	B150	*Clostridium cylindrosporum*	Y18179	715/770 (92%)	*Firmicutes Clostridiaceae*
Grey	Fe(III) citrate/ yeast extract	Anaerobic liquid medium	70	Subcultures in dilution	Y270	*Thermosediminibacter litoriperuensis*	AY703479	836/880 (95%)	*Firmicutes Thermoanaerobacteraceae*
Red	Fe(III) citrate/ yeast extract	Anaerobic liquid medium	70	Subcultures in dilution	R270	*Thermovenabulum ferriorganovorum*	AY033493	1,380/1,431 (96%)	*Firmicutes Thermoanaerobacteraceae*
Grey	Fe(III) citrate/ yeast extract	Anaerobic liquid medium	50	Subcultures in dilution	Y250[b]	*Clostridium fervidus*	L09187	948/1,000 (94%)	*Firmicutes Clostridiaceae*
Red	Fe(III) citrate/ yeast extract	Anaerobic liquid medium	50	Subcultures in dilution	R250	*Geosporobacter subterraneus*	DQ643978	872/908 (96%)	*Firmicutes Clostridiaceae*
Green	Fe(III) citrate/ yeast extract	Anaerobic liquid medium	50	PLM gelrite shakes	G2-1	*Caloramator uzoniensis*	AF489534	1,062/1,070 (99%)	*Firmicutes Clostridiaceae*
Green	Fe(III) citrate/ yeast extract	Anaerobic liquid medium	50	PLM gelrite shakes	G2-3	*Propionispora hippei*	AJ508928	1,366/1,496 (91%)	*Firmicutes Veillonellaceae*

Brown	Fe(III) citrate/ yeast extract	Anaerobic liquid medium	50	Subcultures in dilution	B2-1	*Geosporobacter subterraneus*	DQ643978	1,342/1,400 (95%)	*Firmicutes Clostridiaceae*
Red	Fe(III) citrate/ 3-hydroxy-benzoate	Anaerobic liquid medium	50	Subcultures in dilution	Rb50-1	*Pelotomaculum thermopropionicum*	AP009389	1,344/1,537 (87%)	*Firmicutes Peptococcaceae*
Red	Fe(III) citrate/ 3-hydroxy-benzoate	Anaerobic liquid medium	1,604	Subcultures in dilution/heat shock	Rb50-2	*Pelotomaculum thermopropionicum*	AP009389	1,159/1,343 (86%)	*Firmicutes Peptococcaceae*
Red	Fe(III) citrate/3-hydroxyben-zoate	Anaerobic liquid medium	1,604	Subcultures in dilution/heat shock/ YECit	Rb50-2-Cit	*Caloramator coolhaasii*	AF104215	832/836 (99%)	*Firmicutes Clostridiaceae*
Red	Fe(III) citrate/4-hydroxyphe-nylacetate	Anaerobic liquid medium	50	Subcultures in dilution	Ri50	*Pelotomaculum thermopropionicum*	AP009389	1,292/1,450 (89%)	*Firmicutes Peptococcaceae*
Red	Fe(III) citrate/4-hydroxyphe-nylacetate	Anaerobic liquid medium	50	Subculture onto TYEG	Ri50-Gl	*Caloramator coolhaasii*	AF489534	1,040/1,056 (98%)	*Firmicutes Clostridiaceae*
Red	Fe(III) citrate/ cinnamate	Anaerobic liquid medium	50	Subculture onto TYEG	Rj50-Gl	*Caloramator uzoniensis*	AF4895234	1,364/1,381 (98%)	*Firmicutes Clostridiaceae*
Green	Fe(III) citrate/ cinnamate	Anaerobic liquid medium	50	Subculture onto TYEG	Gj50-Gl	*Caloramator uzoniensis*	AF4895234	1,383/1,403 (98%)	*Firmicutes Clostridiaceae*
Green	Fe(III) citrate/ cinnamate	Anaerobic liquid medium	50	Subculture onto TYEG	Gk50-Gl	*Caloramator coolhaasii*	AF104215	1,394/1,419 (98%)	*Firmicutes Clostridiaceae*
Green	Fe(III) citrate/ cinnamate	Gelrite shakes	50	Subcultures in dilution	G50J1	*Sporomusa ovata*	AJ279800	813/896 (90%)	*Firmicutes Veillonellaceae*
Green	Fe(III) citrate/ cinnamate	Gelrite shakes	50	Subcultures in dilution	G50J2	*Propionispora hippei*	AJ509828	1,369/1,490 (91%)	*Firmicutes Veillonellaceae*
Green	Fe(III) citrate/ malonate	Gelrite shakes	50	Subcultures in dilution	G50-malonate	*Pelotomaculum thermopropionicum*	AP009389	766/867 (88%)	*Firmicutes Peptococcaceae*

(continued)

Table 2.6 (continued)

Mat sample	Electron acceptor/carbon source	Enrichment method	Incubation temp (°C)	Purification method	Isolate name	Nearest phylogenetic neighbour (BLASTn)	GenBank accession number	Similarity	Taxonomy
Green	Fe(III) citrate/ malonate	Gelrite shakes	50	Subcultures in dilution/ TYECit	G50-malonate-Cit	*Caloramator coolhaasii*	AF489534	889/906 (98%)	*Firmicutes Clostridiaceae*
Green	Fe(III)OOH/ malate	Anaerobic liquid medium	50	PLM gelrite shakes	G3-malate	*Sporomusa sphaeroides*	AJ279801	695/745 (93%)	*Firmicutes Veillonellaceae*
Green	Fe(III)OOH/ malate	Anaerobic liquid medium	50	PLM gelrite shakes/ TYEG	G3-malate	*Caloramator coolhaasii*	AF104215	1,378/1,393 (98%)	*Firmicutes Clostridiaceae*
Red	Fe(III) citrate/ yeast extract	Microaerophilic conditions	50	Subcultures in dilution (anaerobic)	AeR	*Pelotomaculum thermopropionicum*	AP009389	808/907 (89%)	*Firmicutes Peptococcaceae*
Green	Fe(III) citrate/ yeast extract	Microaerophilic conditions	50	Subcultures in dilution (anaerobic)	AeG	*Propionispora hippei*	AJ509828	1,365/1,494 (91%)	*Firmicutes Veillonellaceae*
Brown	Fe(III) citrate/ yeast extract	Microaerophilic conditions	50	Subcultures in dilution (anaerobic)	AeB	*Clostridium cylindrosporum*	Y18179	1,310/1,421 (92%)	*Firmicutes Clostridiaceae*
Red	Fe(III) citrate/ yeast extract	Microaerophilic conditions	50	Media D agar plates	AeR-D	*Bacillus licheniformis*	EU344793	873/878 (99%)	*Firmicutes Bacillaceae*
Green	Fe(III) citrate/ yeast extract	Microaerophilic conditions	50	Media D agar plates	AeG-D	*Anoxybacillus flavithermus*	AJ586360	851/877 (98%)	*Firmicutes Bacillaceae*
Brown	Fe(III) citrate/ yeast extract	Microaerophilic conditions	50	Media D agar plates	AeB-D	*Anoxybacillus flavithermus*	AJ586360	1,288/1,322 (97%)	*Firmicutes Bacillaceae*
Red	Fe(III) citrate/ yeast extract	Microaerophilic conditions (pH 9.8)	50	Subcultures in dilution in Media D	R10YE	*Bacillus licheniformis*	AY859477	1,385/1,413 (98%)	*Firmicutes Bacillaceae*

Green	Fe(III) citrate/ yeast extract	Microaerophilic conditions (pH 9.8)	50	Subcultures in dilution in Media D	G10YE	*Anoxybacillus flavithermus*	AY643748	1,511/1,523 (99%)	*Firmicutes Bacillaceae*
Green	Fe(III) citrate/ yeast extract	Heat shock	50	Subcultures in dilution	GHS50	*Clostridium quercicolum*	AJ010962	764/814 (93%)	*Firmicutes Clostridiaceae*
Green	Fe(III) citrate/ yeast extract	Heat shock	50	Subcultured into TYEG	GHS50-Gl	*Caloramator coolhaasii*	AF104215	1,419/1,430 (99%)	*Firmicutes Clostridiaceae*

Key: *ND* Not determined

[a]Strain Y170^T was subsequently characterised and described as *Fervidicola ferrireducens* gen. nov., sp. nov. It should be note that matching phylotypes were identified in Biolog MicroPlate enrichments of the grey mat sample as described in the text

[b]It should be noted that a strain Y250 has a matching 16S rRNA gene sequence to *Caloramator australicus* sp. nov., strain RC3T isolated from Biolog microtiter plate enrichments of the red mat sample as described in the text

Table 2.7 A summary of the novel phylogenies obtained from the different enrichment and isolation strategies performed in 7–10 ml of growth medium discussed in the text

Growth substrates	Enrichment conditions	Novel phylotypes/total isolates sequenced
Fe(III)/yeast extract	Liquid anaerobic	9/13 (69%)
Fe(III)/seldom-used substrates	Liquid anaerobic	3/8 (38%)
Fe(III)/seldom-used substrates	Solid anaerobic	3/4 (75%)
Fe(III)/yeast extract	Microaerophilic enrichment/ anaerobic subculture	3/3 (100%)
Fe(III)/yeast extract	Microaerophilic enrichment/ aerobic subculture	0/3 (0%)
Fe(III)/yeast extract	Microaerophilic enrichment (pH 9.8)/aerobic subculture (pH 9.8)	0/2 (0%)
Fe(III)/Yeast Extract	Liquid Anaerobic/Heat shock	1/2 (50%)

novel phylotypes (100%). However, subsequent characterisation studies of these strains revealed that isolated strains using anaerobic Fe(III)-PL medium were strict anaerobes rather than facultative anaerobes, and so in this respect this enrichment strategy failed. From these subcultures, two novel thermophiles were isolated and subsequently described as *Fervidicella metallireducens* strain AeB^T gen. nov., sp. nov. (Ogg and Patel 2010a, b, c, 2011) and *Sporolituus thermophila* strain AeG^T gen. nov., sp. nov., (Ogg and Patel 2009a, b, c). Notably, studies of *Sporolituus thermophila* revealed that the strain did not reduce iron(III), but instead grew by fermenting the citrate supplied with the TEA [ammonium iron(III) citrate].

Enrichment and isolations that were performed in Fe(III)-PL medium amended with yeast extract (at a final concentration of 0.2%) revealed a high proportion of novel phylotypes [9/13 (69%)]. These results demonstrate the uniqueness of the GAB environment and the microbial flora that exists within GAB thermophilic mat communities. Subsequent characterisation tests of several strains isolated under these conditions resulted in the descriptions of the novel thermophiles designated: *Thermotalea metallivorans* strain B2-1^T gen. nov., sp. nov. (Ogg et al. 2010), *Fervidicola ferrireducens* strain Y170^T gen. nov., sp. nov. (Ogg and Patel 2009a, b, c) and *Thermovenabulum gondwanense* strain R270^T sp. nov. (Ogg et al. 2010).

A high proportion of novel isolates were also obtained from enrichment strategies that employed seldom-used energy substrates, namely, aromatic compounds. Most notably, BLASTn analyses of the isolates designated strain Rb50-1, strain Rb50-2, strain Ri50, strain G50-malate and strain AeR revealed they were most closely related to the type strain *Pelotomaculum thermopropionicum* strain SI (16S rRNA gene similarity of 88–89%) that was isolated from a methanogenic granular sludge in a thermophilic (55°C) upflow anaerobic sludge blanket reactor (Imachi et al. 2000). BLASTn analyses of these strains also positioned them close to the anaerobic bacterium EtOH8 (GenBank ID, AY756140), detected in a metal-reducing biofilm that exists within a Danish district heating system (Kjeldsen et al. 2007) and the iron-reducing bacterium enrichment culture clone HN-HFO19 (GenBank ID, FJ269091) detected within an arsenic contaminated paddy soil of Hunan, south China (Wang

et al. 2009) (16S rRNA gene similarity of 88–91% for both). Imach et al. (2000) report on the difficulties involved in achieving axenic conditions within the cultures of *Pelotomaculum thermopropionicum* strain SI, which was partly characterised as a synotroph, grown in coculture with *Methanobacterium thermoautotrophicum* (DSM 1053^T). This parallel difficulties encounter in this study, as cultures of strain Rb50-1, strain Rb50-2, strain Ri50, strain G50-malate and strain AeR, when subcultured onto TYEG, YECit or YE medium (Ogg and Patel 2009a, b, c), were outgrown by organisms from the genus *Caloramator* (Table 2.6). Attempts to purify these cultures by performing repeated subcultures to extinction in both solid and liquid media at differing growth phases, subsequent to exposure to heat shock (95°C for 20 min) and in growth media amended with 10 μg ml^{-1} ampicillin, streptomycin, tetracycline, penicillin and chloramphenicol, all failed to produce axenic conditions. Future studies could employ micromanipulators in combination with FISH to isolate pure cultures of this phylogenetically deep-positioned organism. Alternatively RT-PCR could be used to determine which growth medium contains the highest ratio of the novel strain, and this culture could then be used for high-throughput next-generation sequencing and in order to annotate the novel strains genome. The resulting sequence data produced from this coculture could be compared to the genomic sequencing data of strains closely related to the unwanted *Caloramator* strain existing in this culture (i.e. the sequence data could be compared to *Caloramator australicus* strain RC3^T sequence data) in order to withdraw similar sequence data, leaving only the sequence data of the novel thermophilic strain. In addition, a TYEG medium-purified subculture (containing only the undesired *Caloramator* strain) could also be sequenced (by next-generation sequencing methods) and the resulting data used to separate the matching sequence data from coculture sequence analyses. Genome sequence annotations of the novel thermophilic strain could be used for to identify metabolic pathways that would provide a means to isolate a pure culture of this strain. Phylogenetic analyses of several other strains that were isolated using Fe (III) citrate-PL medium amended with seldom-used electron donors revealed that the strains were phylogenetically placed within the genus *Caloramator*. This mimics the results observed when vanadium (V) amended to enrichment media and suggests that such strains may be utilising the trace yeast extract present in the PL medium rather than the supplied electron donors. Future studies could include gas chromatograph analyses to test the growth end products of the enrichment cultures and isolates, to determine if the aromatic compounds had been utilised.

Using the novel, modified and conventional enrichment and isolation strategies described above, numerous thermophiles from the GAB were isolated and identified. As with previous studies of the GAB microbial flora, phylogenetic analyses revealed that all of the isolates were members of phylum *Firmicutes* and included genera within the families Thermoanaerobacteraceae, Paenibacillaceae, Clostridiaceae, Peptococcaceae, Veillonellaceae and Bacillaceae. The thermophilic phylotypes detected from recent culture-dependent studies of the New Lorne Bore (RN: 17263) and the Youranigh bore (RN: 4163) microbial mat communities and from the Mitchell bore (RN:22981 A) outflow are summarised in Table 2.8. Substrate utilisation and enrichment growth temperatures of the isolates are included in this table.

Table 2.8 The phylotypes detected in the New Lorne bore (RN 17263) and the Youranigh bore (RN 4163) microbial mat communities, and within the Mitchell bore outflow

	Nearest BLASTn match	Similarity	GenBank accession number	Detection in New Lorne bore microbial mat sample				Detection in other GAB samples		Growth characteristics				
				Grey	Red	Green	Brown	Youranigh bore	Mitchell bore	Fe(III)	V(V)	SO_4	O_2	Temp. (°C)
	Family *Thermoanaerobacteraceae*													
→	*Thermosediminibacter litoriperuensis*	95%	AY703479	✓						✓				70
	[*Fervidicola Ferrireducens*]	100%	EU443728											
→	*Thermovenabulum ferriorganovorum*	96%	AY033493		✓					✓				70
	[*Thermovenabulum gondwanense*]	100%	EU443729											
	Thermoanaerobacter thermohydrosulfuricus	99%	AY701760		✓					✓				70
	Thermaerobacter subterraneus	>98%	AF343566	✓									✓	70
	Family *Paenibacillaceae*													
	Paenibacillus timonesis	99%	AY323611	✓									✓	70
	Family *Clostridiaceae*													
→	*Caloramator fervidus*	95%	L09187	✓	✓	✓				✓		–		50
	[*Caloramator australicus*]	100%	EU409943											
→	*Caloramator australicus*	95%	EU409943		✓									50
	Caloramator coolhaasii	>98%	AF104215		✓	✓		✓	✓	✓	✓			50
	Caloramator indicus	96%	X75788					✓			✓			50
	Caloramator uzoniensis	99%	AF489534		✓	✓								50
	Thermobrachium celere	99%	DQ207958			✓				✓				50
	Thermobrachium celere [*Caloramator mitchellensis*]	91%	DQ207958						✓		✓			50

Clostridium cylindrosporum	92%	Y18179			✓			✓	✓			50
[*Fervidicella metallireducens*]	100%	FJ481102										
Clostridium tunisiense	93%	AY187622						✓		✓		50
Clostridium thermocellum	91%	CP000568	✓									50
Clostridium quercicolum	93%	AJ010962		✓						✓		50
Geosporobacter subterraeneus	95%	DQ643978			✓	✓			✓	✓		50
[*Thermotalea metallivorans*]		EU443727										
Family *Peptococcaceae*												
Desulfotomaculum putei	95%	AF053933	✓								✓	50
[*Desulfotomaculum varum*]	100%	GU126374										
Pelotomaculum thermopropionicum	86–89%	AP009389	✓	✓					✓			50
Family *Veillonellaceae*												
Propionispora hippei	91%	AJ509828		✓				✓				50
[*Sporolituus thermophila*]	100%	FJ169187										
Sporomusa sphaeroides	93%	AJ279801		✓					✓			50
Sporomusa ovata	90%	AJ279800		✓								50
Family *Bacillaceae*												
Bacillus licheniformis	>98%	GQ375236	✓	✓				✓			✓	50
Anoxybacillus flavithermus	>98%	AJ586360	✓	✓	✓						✓	50

The observed substrate utilisation and enrichment temperature of each phylotype are also given. Novel isolates that have been subsequently characterised and their designated nomenclatures are given in square brackets

Key: ✓, detected; –, not observed; → ; denotes novel phylotype; Temp., enrichment temperature

2.3 Conclusion

The investigations carried out at Griffith University have demonstrated that there is a great diversity in thermophilic bacteria and archaea that thrive in the Great Artesian Basin in Australia.

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Chapter 3
Hot Environments from Antarctica: Source of Thermophiles and Hyperthermophiles, with Potential Biotechnological Applications

Patricio A. Flores, Maximiliano J. Amenábar, and Jenny M. Blamey

Abstract Antarctica, far from being an exclusively cold continent, has many geothermal sites with volcanic activity. Different islands belonging to this continent harbour different geothermal sites as thermal springs, fumaroles, hot soils and hydrothermal vents, providing ideal environments for the growth of thermophilic and hyperthermophilic microorganisms. Hyperthermophiles are an important source of enzymes and bioactive compounds with biotechnological applications.

Deception Island, a horseshoe-shaped island, is the emergent part of a young active shield volcano, which is located at the southwestern part of the Bransfield Strait and has optimal and unique geological characteristics for supporting the growth of thermophilic microbes. Several thermophilic microorganisms have been isolated from this island, and several molecular studies are being carried out for obtaining bioactive compounds and enzymes of industrial interest. This review deals with thermophilic and hyperthermophilic microorganisms isolated from Antarctica and their biotechnological potential.

Keywords Antarctica • Hyperthermophiles • Deception Island • Thermal springs • Fumarole

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3.1 Introduction

Environmental conditions in Antarctica are different from all other places around the planet. Although the Antarctic climate is mainly cold, it is far from being uniform. The Cenozoic period has seen constant volcanic activity in Antarctica, and steaming ground is actually found in a number of circumpolar islands, such as Deception Island, the South Sandwich Islands, Bouvetøya, Marion Island and Îles Kerguelen, and on continental Antarctica at Mount Erebus, Mount Melbourne, Mount Rittmann and Marie Byrd Land (Logan et al. 2000). Information derived from expeditions to Mount Erebus, Mount Melbourne and Mount Rittmann indicates that at these sites temperatures ranging from 50 to 75°C are found. Additionally, extremely low concentrations of essential nutrients (N and P) and high concentrations of metals (Cu, Zn, Cd, Pb and Hg) have been reported, suggesting that microbial life in these environments is constrained not only by high temperatures but also by low nutrient concentrations and high concentrations of toxic metals (Logan and Allan 2007). On the other hand, Lucifer Hill on Candlemas Island in the South Sandwich Islands reaches temperatures of 85°C in its active fumaroles and 0°C around inactive fumaroles at Clinker Gulch (Logan et al. 2000).

The presence of several geothermal sites in Antarctica including thermal springs, fumaroles, hot soils and hydrothermal vents provides ideal environments for the development of (hyper)thermophilic microorganisms. At present, about 90 species of hyperthermophilic archaea and bacteria have been isolated from different non-Antarctic terrestrial and marine thermal environments, including solfataric fields, hot springs and shallow or deep marine hydrothermal vents (Barry et al. 1996; Sako et al. 1996; Casamayor et al. 2001; Reysenbach and Cady 2001; Miroshinichenko and Bonch-Osmolovskaya 2006; Kuwabara et al. 2007; Osburn and Amend 2011). However, despite the presence of several geothermal sites in Antarctica, so far no studies have reported the presence of hyperthermophilic microorganisms. Moreover, only few reports have shown the presence of thermophilic bacteria on this continent (Hudson and Daniel 1988; Nicolaus et al. 1998, 2000; Logan et al. 2000; Allan et al. 2005; Poli et al. 2006; Llarch et al. 1997; Muñoz et al. 2011). This lack of information on these important extreme Antarctic sites could be due to the complexity involved in sample collection, conservation and transport; limited experimental procedures for proper handling of samples; and difficulties in isolating and growing pure cultures of new thermophiles and hyperthermophiles (Casamayor et al. 2001; Christner et al. 2001).

In this chapter, thermophilic and hyperthermophilic microorganisms isolated from Antarctica will be described in order to emphasize the importance of extreme biodiversity and its biotechnological potential.

3.2 Thermophiles from Antarctic Sites

Antarctic microorganisms are true extremophiles as they are adapted not only to low (or high) temperatures but frequently also to further environmental constraints, such as low nutrient status, low water activity, hypersalinity and UV exposure. Despite

what you may think, not only facultative and obligate psychrophiles can be found in Antarctica. To date several strains of thermophilic microorganisms belonging to different genera have been isolated from different geothermal sites of the Antarctic continent. Mainly, they can be divided into thermoacidophiles and those able to grow mainly around neutral pH. One of the first reports of thermophilic bacteria from Antarctica was derived from the analysis of Mount Erebus steam-warmed soils. The isolates obtained from these soils were all spore formers, being members of either *Clostridium* or *Bacillus* genera, and grew at 60°C (Hudson and Daniel 1988). Although there is a previous report on the isolation of bacteria from Mount Erebus fumaroles, these microorganisms were not thermophiles but presumably mesophiles (Ugolini and Starkey 1966). It is important to mention that not all the microorganisms from a hot site are thermophiles; some of them are able to resist these high temperatures but grow optimally at a mesophilic temperature. The existence of steep thermal gradients, in addition to a wide range of soil temperatures, could explain the variety of microorganisms present. It is here where some of the few Antarctic thermophiles described so far have been found.

From geothermal soil near the crater of Mount Melbourne, a novel thermophilic bacterium, *Bacillus thermoantarcticus* sp. nov., has been isolated. This microorganism grows at an optimal temperature of 63°C at pH 6.0 and has different enzymes of commercial interest such as an extracellular xylanase, an intracellular alcohol dehydrogenase and exo- and endocellular α-glucosidase(s), among others (Nicolaus et al. 1996). Besides this microorganism, Poli et al. (2006) isolated another novel thermophilic bacterium with biotechnological potential belonging to the genus *Anoxybacillus*. This spore-forming microorganism designated as *Anoxybacillus amylolyticus* sp. nov., isolated from geothermal soils on Mount Rittmann, grows optimally at 61°C and synthesizes an extracellular constitutive enzyme with amylolytic activity. Amylolytic enzymes play an important role in the biogeochemical cycle of carbon, and they are also among the most important enzymes in current biotechnological, food, detergent and pharmaceutical industries. For this reason, several efforts have been made to identify thermophilic microorganisms which produce extracellular amylases (Burhan et al. 2003; Lama et al. 1991). Logan et al. (2000) reported the isolation of aerobic, endospore-forming bacteria from soils taken from active fumaroles on Mount Rittmann and Mount Melbourne in northern Victoria Land, Antarctica, and from active and inactive fumaroles on Candlemas Island in the South Sandwich archipelago. Mount Rittmann and Mount Melbourne soils yielded a dominant, moderately thermophilic and acidophilic, aerobic endospore former growing at pH 5.5 and 50°C. Further, strains of the same organism were isolated from a cold, dead fumarole at Clinker Gulch, Candlemas Island. This microorganism was proposed as a new specie with the name *Bacillus fumarioli* sp. nov. Amplified rDNA restriction analysis, SDS–PAGE and routine phenotypic test show that the Candlemas Island isolates are not distinguishable from the Mount Rittmann strains, although the two sites are 5,600 km apart. This finding suggests that colonization of these two sites has a common source and most likely occurred from the air as free spores or spores attached to plants or from animal propagation. On the other hand, from warm soils of an active fumarole of Lucifer Hill in Candlemas Island, another aerobic

endospore-forming bacteria belonging to the genus *Bacillus* has been isolated. However, this novel specie *Bacillus luciferensis* sp. nov. grows optimally only at 30°C. These results support the fact that not only thermophiles can be detected and isolated from hot environments. Furthermore, other microorganisms belonging to the *Paenibacillus* genus have been characterized from warm soils of active fumaroles, near the northern crater of Lucifer Hill in Antarctica, and from cold soils of inactive fumaroles. It is interesting to notice that the temperature of the soil in the active fumarole ranges from 30 to 60°C and at the dead fumarole ranges from 0 to 15°C. Nevertheless, all isolates obtained from both sites showed similar growth temperature between a minimum of 0°C and a maximum of 50°C. Perhaps, the temperature ranges reflect the versatility of living organisms necessary to survive in such volcanic soils where geothermal activity at a given site is impermanent. The presence of thermoacidophilic microorganisms has also been described in the Antarctic continent. Hudson et al. (1989) reported the possible presence of *Alicyclobacillus* species from geothermal soil collected from Mount Erebus; however, detailed taxonomic description was not carried out. The authors concluded that their isolated strains could be temperature variants of *Bacillus acidocaldarius*, but a more detailed taxonomic and genetic investigation is required to substantiate this conclusion. Nicolaus et al. (1998) described the isolation of three species belonging to *Alicyclobacillus* genus from geothermal soil of Mount Rittmann. The isolates were thermoacidophilic, showing an optimum temperature of 63°C and an optimum pH of 3.5–4.0. One of the isolates was subjected to further genetic analysis, showing a 99.3% similarity with *Alicyclobacillus acidocaldarius*. However, due to its specific characteristics, the author proposed to classify this microorganism within the genus *Alicyclobacillus* as a new subspecies, *Alicyclobacillus acidocaldarius* subsp. *rittmannii*. Other two novel thermoacidophiles *Brevibacillus levickii* sp. nov. and *Aneurinibacillus terranovensis* sp. nov. were isolated from geothermal soils at Cryptogam Ridge, the northwest (NW) slope of Mount Melbourne, and from the vents and summit of Mount Rittmann, respectively. The *Brevibacillus* strains showed an optimal temperature range from 40 to 45°C and an optimum pH of 5.0–5.5; meanwhile, the *Aneurinibacillus* strains showed an optimal temperature range from 37 to 45°C and an optimum pH of 5.0–5.5 (Allan et al. 2005). A new thermoacidophilic bacterium, belonging to the *Alicyclobacillus* genus, which grows optimally at 60°C and pH 5.5 has also been isolated from Mount Melbourne (Pepi et al. 2005). This bacterium differs from other thermophiles isolated from Antarctica because it requires iron supplementation for its growth. This iron demand suggests a possible adaptation of the thermophilic strain to geochemical features of geothermal soil, in particular to that of the NW slope of Mount Melbourne, which has high bioavailable iron concentrations. It could be possible that the metabolism of this thermophilic strain from the NW slope of Mount Melbourne exchanges the chemical forms of iron, playing a role in the biogeochemical cycle of this metal. However, further studies are necessary to understand iron uptake and/or absorption mechanisms in this *Alicyclobacillus* sp. (Pepi et al. 2005). Imperio et al. (2008) described a novel thermoacidophilic bacterium, isolated from warm soils collected in the immediate vicinity of a steam vent on the NW slope of Mount Melbourne, being the second novel species recovered from this site. But

unlike the previously isolated specie (Allan et al. 2005), this bacterium belongs to the *Alicyclobacillus* genus, grows optimally at 55°C and pH 5.5 and is denominated *Alicyclobacillus pohliae* sp. nov.

3.3 Thermophiles and Hyperthermophiles from Deception Island

Of all geothermal environments in Antarctica, Deception Island (62°57′S, 60°38′W) stands out due to its singular geologic characteristics. This island is the emergent part of a young active shield volcano (Fig. 3.1) and lies in the southwestern part of the Bransfield Strait, between the Antarctic Peninsula and the South Shetland archipelago (Muñoz-Martín et al. 2005). Among its geologic characteristics, we could mention its 'horseshoe' shape, barren volcanic slopes, ash-layered glaciers, and its flooded caldera of 8–10 km diameter, which is restless and may be resurging (Cooper et al. 1998). The age of this island is less than 0.78 Ma and was probably formed by the collapse of the upper part of an ancient composite volcano that became active. As a result of this volcanic activity, the island is composed mainly of andesite effusions, a pyroclastic rock. This volcanic island has been very active during the last century: fumarolic emissions, thermal springs, hot soils and hydrothermal systems are evidence of Deception Island volcanic activity (Caselli et al. 2004). In this island, several sites of complex geology extend around the open crater. Geothermal anomalies are mainly found from Fumarole Bay to Pendulum Cove. The temperature of emissions ranges from 90 to 110°C at the fumaroles part of Fumarole Bay. The soil temperature is highly variable, reaching values between 40 and 60°C, at Whalers Bay, and more than 70°C at Pendulum Cove (Ortiz et al. 1992). Another site with high geothermal activity is Unnamed Hill where the temperatures range from 70 to 97°C.

Somoza et al. (2004) described the activity after recent eruptions, highlighting the creation of permanent fumaroles and emissions of hydrogen sulphide gas, water vapour, carbon dioxide and elemental sulphur depositions. In the Fumarole Bay area, deposition of S^o is intense along parallel tracks, and steam and boiling water (100°C) is observed at sea level. Other craters erupted andesitic tephra that formed tephra salt-rich blankets (in Telephone Bay), suggesting the involvement of sea water. The position of the mounds and volcanic cones in Deception Island coincides with the geochemical distribution patterns of the highest contents of Mn, Fe and Zn; and the values decrease progressively from the axis to the boundaries of the flooded caldera, being the lowest values detected at the Telephone Bay area (Rey et al. 1995). The highest values of As, Al, Ca, Mg and K are found between Fumarole Bay and Pendulum Cove. Currently active hydrothermal processes (fumaroles and hot springs) are associated with the area of Fumarole Bay, Telephone Bay, Pendulum Cove and Whalers Bay (Ortiz et al. 1987; Ramos et al. 1989). According to Rey et al. (1995), it has been proposed that fluids are produced by the venting of shallow aquifers heated by convective gaseous inflow from the underlying magma chamber. The main component of fumarole emissions is CO_2 (75–90%) with significant

Fig. 3.1 Map of Deception Island (South Shetland Islands, Antarctica) (Courtesy of British Antarctic Survey)

proportions of H_2S (0.3–0.9%), H_2 (0.006–0.2%), N_2 (0.77–21.6%) and O_2 (0.006–0.65%) (Somoza et al. 2004).

The presence of a large number of fumaroles and other geothermal areas makes this island specially interesting for the study of microorganisms, specifically thermophiles and hyperthermophiles. One of the most important groups of bacteria found in geothermal soils in Antarctica, as we previously described, belongs to the

aerobic endospore-forming bacteria group, which mainly includes *Bacillus* species and other related genera. These bacteria are widely distributed in natural environments, and they are able to inhabit all kinds of soil, from acid to alkaline, cold to hot and even inhabit marine waters. The thermophiles found in this group are *Alicyclobacillus*, *Aneurinibacillus*, *Anoxybacillus*, *Brevibacillus*, *Caldakalibacillus*, *Sulfobacillus*, *Thermobacillus*, *Ureibacillus* and *Vulcanibacillus*. Nearly 20% of these thermophilic endospore-forming taxa have been isolated from geothermal soils and half of them from Antarctic geothermal sites.

So far, thermophilic bacteria found in geothermal areas of Antarctica belong to the Bacillaceae family. Due to their endospore-forming capability, they can survive distribution from natural environments to a wide variety of other habitats (Logan et al. 2000). Recent taxonomic studies on the biotechnological importance of the Bacillaceae group have shown that improvements in identification and classification are needed. It is clear that knowledge of thermophilic microorganisms has increased during the last few years, but the bacteriology of geothermal soils in Antarctica, including Deception Island, needs to keep growing, helping us to understand the fascinating and versatile microbial ecology of geothermal areas (Poli et al. 2006; Logan et al. 2000).

In December 1967, cinder cones rose within Telephone Bay, Deception Island. Chilean researchers reported, as results of the eruption, the arousal of three principal craters and a satellite crater. A new island roughly oval-shaped, of about 930 m long and 200 m wide, was created. The new craters are within the northwest sector of Deception Island in Telephone Bay. The unofficial name applied by Chile to those craters is 'Isla Yelcho'. One year later, the craters were studied to determine the presence of microorganisms and cryptogams. Culture media were inoculated to study the presence of heterotrophic and chemoautotrophic, aerobic, microaerophilic and anaerobic bacteria. Although several samples contained few or no culturable microorganisms, researchers found that bacteria were generally most abundant around fumaroles, emitting moisture and CO_2. Microorganisms identified from the cinder cones included primarily soil diphtheroids and *Bacillus* spp. Most of those bacteria could grow at 2°C but also at 20°C. Although two samples were cultivated at 45 and 55°C with a high microbial count, only few thermophilic microorganisms were identified. The bacteria identified were mainly heterotrophic, non-pigmented, pale yellow or pale beige, pleomorphic or bacilloid forms, diphtheroids (*Corynebacterium*, *Mycobacterium* and *Nocardia* spp.), *Pseudomonas* sp. and *Bacillus* sp. An orange colony was identified as '*Myxococcus* sp.', which grew only at 45°C (Cameron and Benoit 1970). Although they were able to cultivate some microorganisms, the majority of them were not what they expected due to the nutrient deficiency, unbalanced ionic composition of soils and a very low organic matter content.

During 1989–1990, a scientific expedition from Spain visited Deception Island and collected water and sediment samples from four geothermal sites. Ten bacterial strains isolated from the geothermal areas of Deception Island were found to represent six distinct types of thermophilic, Gram-positive, aerobic, catalase-positive, endospore-forming rods, identified as *Bacillus* sp. They were subjected to routine phenotypic characterization and chemotaxonomic analyses. Four isolates were identified as thermophilic strains of *B. licheniformis*, *B. megaterium*, *B. lentus* and *B. firmus*. Although, those four species are all known as mesophilic microorganisms,

the Deception Island strains have an optimal growth temperature near 65°C, expanding considerably the temperature of growth for the species found in other studies. The other strains found were not identified as known species of *Bacillus* and hence may represent novel species. This is also supported by the results of G+C and DNA homology studies (Llarch et al. 1997).

3.4 Approaches for Microbiological Detection Used at Deception Island

It is now generally accepted that the vast majority of detectable microorganisms by direct methods in soil, marine and other natural samples, including those collected from much less extreme environments, cannot be assessed using only traditional methods that rely on laboratory cultivation. Consequently, the data recorded from culturable approaches are limited (Jeanthon 2000). Microbial species, including thermophiles, have traditionally been enriched and cultured in the laboratory. Identification has been mainly based on their morphology, physiology and metabolism. However, it is generally considered that up to 99.9% of microorganisms have not been cultivated or isolated in most ecosystems (Cleland et al. 2008; Aravalli et al. 1998). The identification of the culturable fraction requires isolation of pure cultures followed by testing for multiple physiological, biochemical molecular traits.

Recently, different molecular methods for studying microbial diversity have become important, which do not require the recovery of the microorganisms through the use of cultures. These methods have shown the presence of microorganisms in a wide variety of unimaginable environments. These findings mark the beginning of a new era for the investigation of such microorganisms and in particular in deepening the knowledge of their physiological properties and metabolic function within the complex microbial populations and ecosystems of which they are a part (Jeanthon 2000).

Increased knowledge on the number of 16S rRNA gene sequences of microorganisms, obtained by using culture-independent techniques, deposited at the GenBank database, has improved our understanding of the diversity of microorganisms, demonstrating that the microbial world is much larger and more diverse than reported historically based on cultivation techniques (Robertson et al. 2005). As a result, it has become clear that thermophiles are not only limited to ‘extreme’ conditions; they are a group widely distributed in the environment (Robertson et al. 2005; Aravalli et al. 1998; Rother and Metcalf 2005).

In order to broaden the scope of detection of thermophiles in Deception Island, in 2011 studies using denaturing gradient gel electrophoresis (DGGE) with the 16S rRNA gene were used to analyse bacterial diversity of thermophilic bacteria from a soil sample taken from Fumarole Bay, Deception Island. Nine bands (DI1–DI9) were obtained (Fig. 3.2), sequenced and analysed using this technique. The analysis of the information derived from these experiments indicated the presence of bacteria from the genera *Geobacillus*, *Bacillus*, *Brevibacillus* and *Thermus* and uncultured

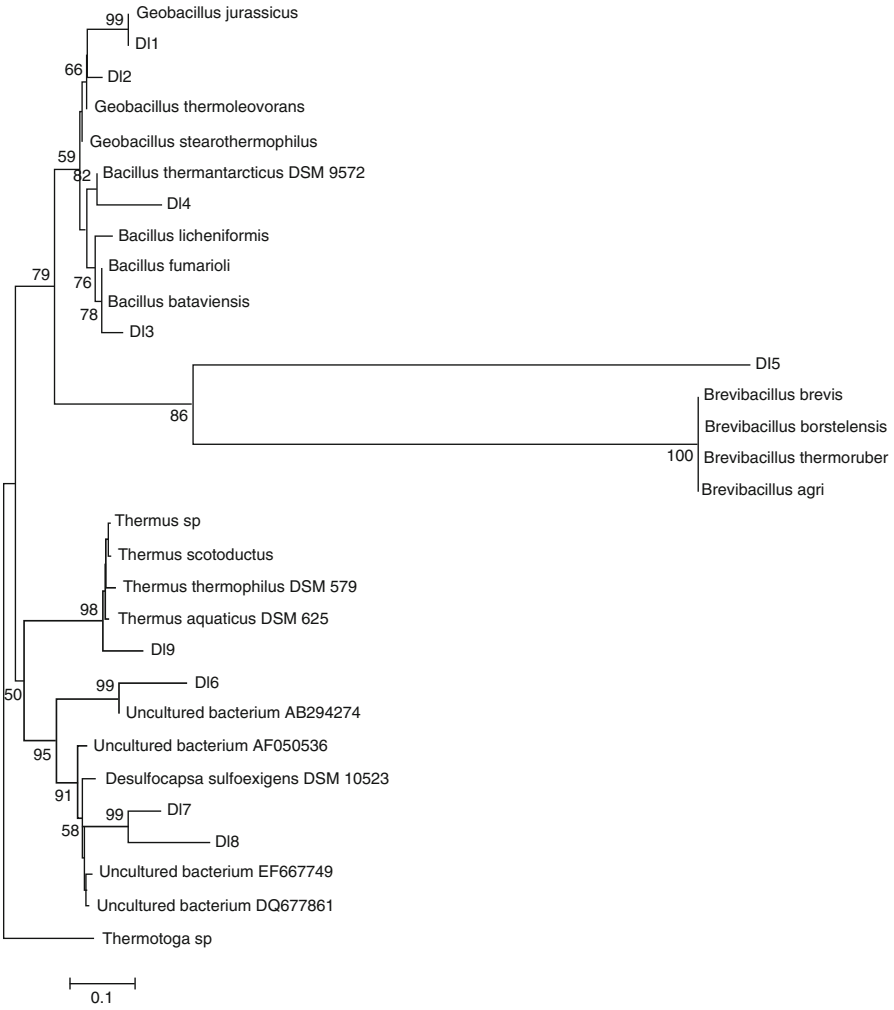

Fig. 3.2 Phylogenetic position of DGGE band sequences. The sequenced bands (DI1–DI9) were compared to 16S rDNA gene of species from the genera *Geobacillus*, *Bacillus*, *Brevibacillus* and *Thermus* and uncultured bacteria. *Thermotoga* sp. was used as the out-group. The phylogenetic tree was inferred by using the neighbour-joining method with a bootstrap test of 1,000

sulphate-reducing bacteria, some of which have been reported in other Antarctic geothermal sites (Muñoz et al. 2011).

Phylogenetic analysis using the neighbour-joining method for the partial 16S rRNA gene sequences of the thermophilic bacteria from Fumarole Bay showed three main clades (Fig. 3.2). These clades included thermophilic members from the orders *Bacillales* and *Thermus* and also uncultured sulphate-reducing bacteria. Thermophilic members of the order *Bacillales clade* can be divided into three

groups composed by the genera *Geobacillus*, *Bacillus* and *Brevibacillus*. Although members of this clade are closely related, they show a variety of phenotypically heterogeneous species and exhibit a wide range of nutritional requirements, physiology, metabolic diversity and DNA base composition (Xu and Coté 2003).

Three different bands obtained from the DGGE analysis corresponded with the uncultured sulphate-reducing bacteria. It has been previously reported that thermophilic sulphate-reducing bacteria belong to the genera *Desulfotomaculum*. For example, *Desulfotomaculum geothermicum* and *Desulfotomaculum hydrothermale* have been isolated from other geothermal sites (Daumas et al. 1988; Haouari et al. 2008). These bacteria are probably implicated in the mineralization of organic carbon derived from primary production by the benthic community. Using DGGE information, our group at Biociencia was able to cultivate *Geobacillus jurassicus*, *Brevibacillus thermoruber* and *Bacillus fumarioli* from Deception Island samples. The last two microorganisms have a maximum growth temperature under 60°C. The characterization of *G. jurassicus* indicated that this strain has a temperature range from 50 to 72°C. Due to its resistance to high temperatures, *G. jurassicus* (Fig. 3.3a) could be a good model for future studies of thermal adaptations and also an important source of different compounds for biotechnological applications. (Muñoz et al. 2011). Additionally, another thermophilic microorganism isolated from Unnamed Hill, Deception Island, during Antarctic Scientific Expedition ECA47 (Unpublished data, Fundación Biociencia, Chile) was characterized biochemically and identified by 16S rRNA gene analysis as *Bacillus gelatini* (Fig. 3.3b). This is the first report of *B. gelatini* isolated from Antarctica. This microorganism showed an optimum temperature and pH of 50°C and 8.0, respectively.

Microorganisms capable of growth not only around neutral pH but also at other pH could be detected in Deception Island. At present, only two members of *Alicyclobacillus* genus have been isolated from geothermal sites in Antarctica: *A. pohliae* (Imperio et al. 2008) and *A. acidocaldarius*, subsp. *rittmannii* (Nicolaus et al. 1998). The presence of members of this genus has never been reported in Deception Island. Correa-Llanten reported a thermoacidophilic bacterium isolated from geothermal soils collected on Unnamed Hill, Deception Island. This bacterium, identified as strain CC2, grows aerobically at temperatures between 30 and 70°C (optimum 55°C) and pH range from 2.0 to 7.0 (optimum pH 4.0). It was identified as a member of the genus *Alicyclobacillus* and showed high identity with *Alicyclobacillus acidocaldarius* subsp. *acidocaldarius*. However, unlike other members of *Alicyclobacillus* genus, this microorganism has an important antioxidant defense system that makes it tolerant to high levels of UV radiation.

3.5 Biotechnology of Antarctic Thermophiles

Microorganisms growing at high temperatures need special adaptations such as thermostable enzymes and proteins with increased molecular stability, structural flexibility and a different composition of membrane phospholipids to give them the ability to maintain a nearly constant fluid membrane at different growing temperatures.

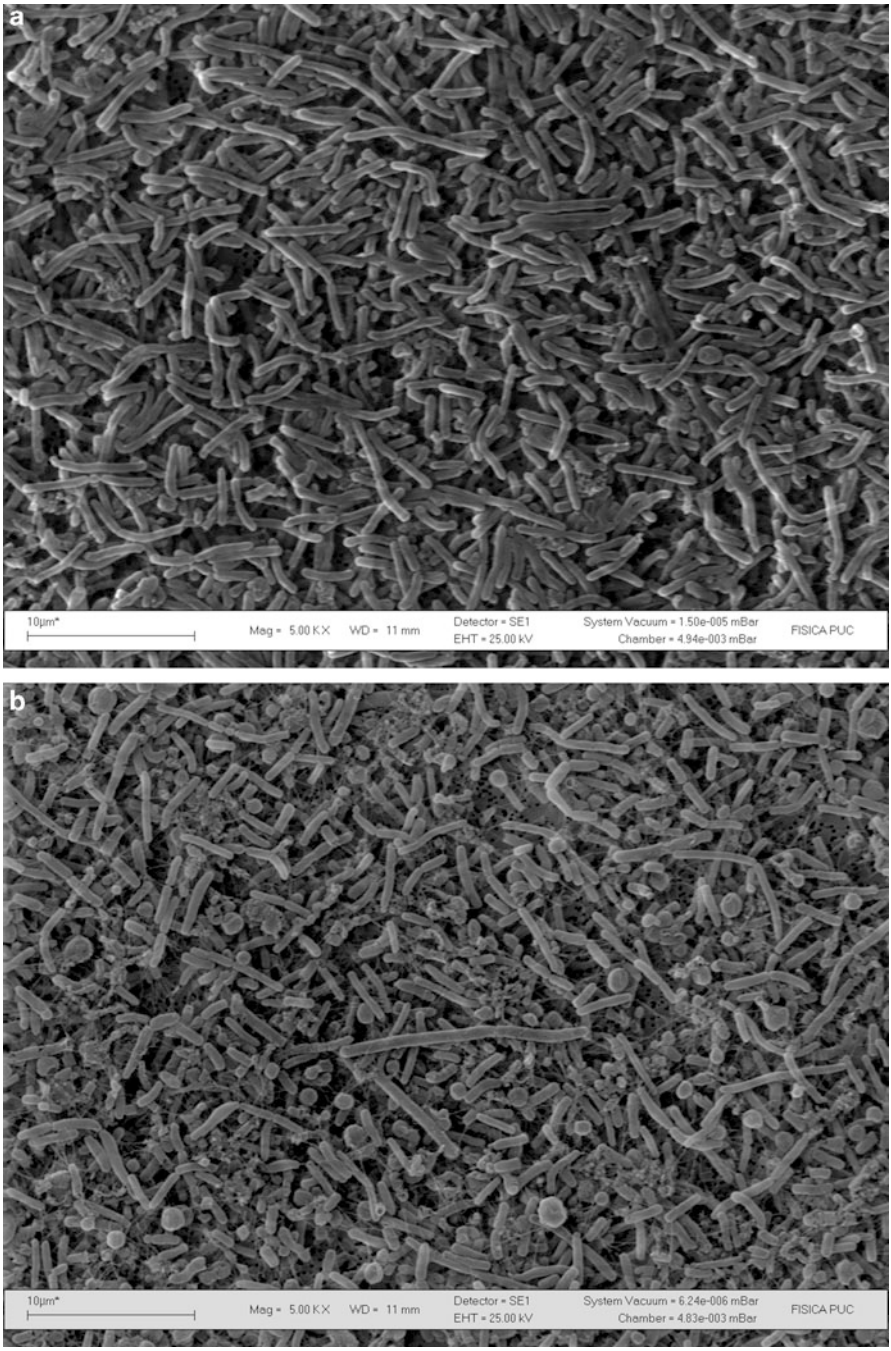

Fig. 3.3 Bacterial morphology by scanning electron microscopy of *G. jurassicus* (**a**) and *B. gelatini* (**b**) isolated from Deception Island. The photograph is magnified 5,000 times

In particular, enzymes produced by thermophiles and hyperthermophiles, also called thermozymes, are the focus of interest in both academic and industrial research, mainly because of their high thermal stability (resistance to inactivation at high temperatures) and thermofilia (optimal activity at high temperatures). Generally, these enzymes are adapted to function optimally at the microorganism growth conditions. Therefore, the extreme temperature range, pH and salinity, of which the microorganism is isolated, in many cases define the conditions for optimal activity of the enzyme to be detected (Li et al. 2005; Schiraldi and De Rosa 2002; Kujo and Ohshima 1998). Thermozymes play an important role in the growing biotech market with applications ranging from agriculture to biomedicine and industry.

To solve industrial demands for thermostable enzymes, the number of studies investigating hyperthermophiles and thermophilic microorganisms has increased in the recent years, attempting to solve the problem of protein instability, which causes great economic loss. One of the enzymes of great scientific and applied interest is glutamate dehydrogenase (GDH). This enzyme plays a major role in the metabolism of carbon and nitrogen (Diruggiero and Robb 1995). It is widely distributed in eukaryotes, bacteria and archaea. Its physiological function is to reversibly catalyze oxidative deamination of glutamate to alpha-ketoglutarate and ammonium (Miñambres et al. 2000; Kujo et al. 1998).

Glutamate dehydrogenase and other oxidoreductases have been highly regarded for use as biosensors, particularly for their specificity and ability to operate in photochemical and electrochemical systems. The use of mesophilic enzymes as biosensors has been relatively problematic due to their instability at high temperatures, organic solvents and denaturing agents (Helianti et al. 2001). Many commercial kits for the quantification of ammonium and glutamate in biological fluids or food products are based on redox reaction of $NAD(P)^+$ (Humbert et al. 2007; Yamaguchi et al. 2005). In food and wine industry, GDH has been widely used to determine the degree of food decomposition due to the bacterial degradation of proteins, peptides and amino acids (Cheuk and Finne 1984).

From *B. gelatini*, isolated from Deception Island, a thermostable GDH has been purified. The measurement of the specific activity of this GDH at high temperatures has been standardized, for the purification of this enzyme. The purified enzyme showed a higher specific activity and thermostability in comparison to the most important commercial GDH, which is currently obtained from bovine liver.

Another microorganism isolated and characterized from Unnamed Hill belongs to the *Geobacillus* genus (Muñoz et al. 2013). This bacterium has a very active and thermostable extracellular lipase, showing a wide range of temperature for its activity between 20 and 85°C. This property makes the enzyme a very useful tool for different biotechnological applications where high temperatures are required, including detergent and leather industries. Moreover this thermostable lipolytic enzyme could be used in the synthesis of biopolymers and biodiesel but also for the production of pharmaceuticals, agrochemicals, cosmetics and flavours.

From *Alicyclobacillus* strain CC2, the group at Biociencia purified a very active and thermostable superoxide dismutase that has tolerance for UV radiation. This enzyme showed an optimal activity at pH 7.4 (at 21°C) and maintains

more than 80% of activity when incubated for 6 h at 50°C (Correa et al.). From a biotechnological point of view, SOD enzymes have several commercial applications, including protection of high-value biomolecules such as lipids and proteins, in the packaging and storage of food, pharmaceuticals and cosmetic products.

3.6 Hyperthermophiles from Antarctic Hydrothermal Vents

Despite the presence of several geothermal sites in Antarctica, so far no information about the presence of hyperthermophilic microorganisms on this continent is available. Due to the difficulties in isolating and growing pure cultures of new hyperthermophiles and to the fact that only a small fraction of the microorganisms found in natural habitats are revealed by culture-dependent techniques, we have studied the archaeal diversity present in a hydrothermal site of Deception Island based on a combined strategy using culture-dependent and culture-independent methodologies (Amenábar et al. 2012). Blastn results of the retrieved sequences from DGGE of 16S rRNA gene showed that most of the archaeal sequences did not share close similarity with any cultured archaea described. However, they were similar to environmental clones obtained from deep-sea hydrothermal vents. One of these sequences was of high interest because it shows 88% of identity with an uncultured archaeon from hydrothermal sediments from the Bransfield Strait, Antarctica. Other retrieved sequences matched with cultured species of the hyperthermophilic order Thermococcales, members of which are known as common inhabitants of deep-sea hydrothermal vents (Huber et al. 2002; Nercessian et al. 2003, 2004). Our group at Biociencia was able to isolate an anaerobic hyperthermophilic archaeon, called BX13 strain, from Deception Island. The purity of this strain was tested microscopically (Fig. 3.4) and confirmed by DGGE (single band was observed). Despite the wide distribution

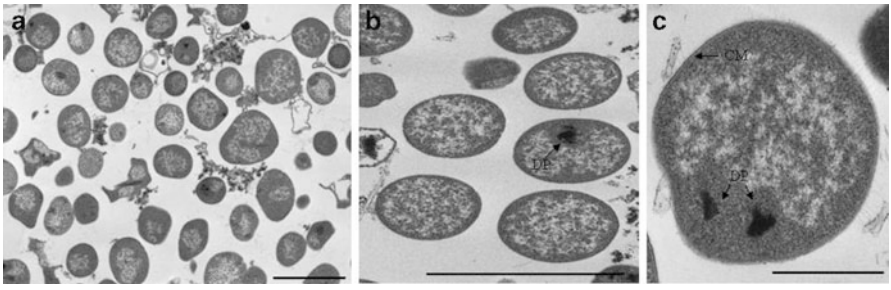

Fig. 3.4 Transmission electron micrographs of strain BX13 (**a**–**c**). Thin sections revealed that cells are irregular cocci with variable size (**a**, **b**). A cell with clustered dense particles (**c**). *CM* cytoplasmatic membrane, *DP* dense particle. Bars, 3.0 μm (**a**, **b**) and 0.8 μm (**c**)

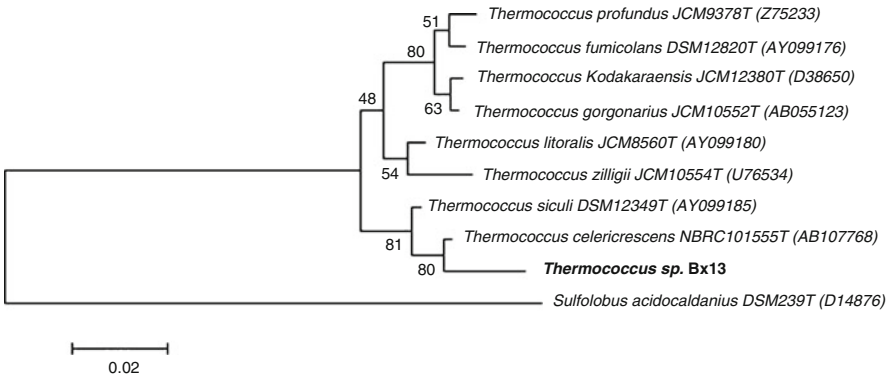

Fig. 3.5 Phylogenetic position of BX13 16S rRNA gene sequence, among closely related species. The tree was constructed using the neighbour-joining method. Numbers at nodes represent bootstrap percentages (1,000 replicates). Bar, 2 nucleotide substitutions per 100 nucleotides

of *Thermococcus* genus in geothermal areas, including deep-sea and shallow marine hydrothermal vents and terrestrial thermal springs (Zillig and Reysenbach 2001), to date, this genus has never been reported in Antarctica. The sequence of strain BX13 was highly similar to the sequence of *Thermococcus celericrescens* (96% of identity), a fast-growing and cell-fusing hyperthermophilic archaeon from a deep-sea hydrothermal vent (Kuwabara et al. 2007). Phylogenetic analysis of the 16S rRNA gene revealed that *T. celericrescens* and *T. siculi* were the nearest neighbours of the BX13 strain (Fig. 3.5).

Cells of strain BX13 are irregular cocci, with a diameter generally between 0.6 and 2 μm (Fig. 3.4 a, b). Cells as large as 3 μm in diameter with a similar appearance were also observed, suggesting that they are likely to fuse during growth, as occurs in *T. coalescens* and *T. celericrescens* (Kuwabara et al. 2005, 2007). Strain BX13 grows in a temperature range from 50 to 90°C. No growth was observed at 45°C or below, nor at 95°C or above, when the strain was incubated up to 1 week. Salinity range for growth of strain BX13 was 1–5% NaCl. No growth was observed at 0.5% NaCl or below or at 5.5% NaCl or above when the strain was incubated up to 1 week. As described previously by Kuwabara et al. (2007), *T. celericrescens* has a maximum temperature for growth under 85°C; meanwhile, BX13 strain can grow at higher temperatures (90°C). The salinity range was also similar among these two microorganisms. Morphologically, BX13 cocci showed intracellular spots that appeared dark on electronic microscopy forming a cluster of dense particles (Fig. 3.4c), a shared characteristic with the cell-fusing hyperthermophile *T. coalescens* (Kuwabara et al. 2005). It is probable that these dense particles contain Fe and S. The presence of these dense particles could be associated to later stages of growth, as occurs in *T. coalescens* (Kuwabara et al. 2005). Despite the similarities of strain BX13 to the cell-fusing hyperthermophiles *T. celericrescens* and *T. coalescens*, which were isolated from a hydrothermal site at a depth of 1,380 m, this new isolated

archaeon was found in a hydrothermal site at a depth of 4 m. Further characterization is required in order to establish the novelty of this new *Thermococcus* strain BX13 (Amenábar et al. 2012).

3.7 Bransfield Strait

Hydrothermal vents revealed a vast and previously unknown habitat on Earth which harbours a wide range of microbial communities (Martin et al. 2008). Its fluctuating conditions, including temperature gradients, oxygen levels and multiple forms of chemical energy, such as methane, hydrogen and hydrogen sulphide, promote the growth of metabolically diverse organisms, including hyperthermophiles (Nercessian et al. 2005; Miroshinichenko and Bonch-Osmolovskaya 2006; Moussard et al. 2006).

Bransfield Strait is a narrow marginal basin that separates the South Shetland Islands from the Antarctic Peninsula. It consists of three subbasins: the eastern, central and western Bransfield Basins, which rifting zone is tectonically and geologically unique (Lawyer et al. 1996). The rift process (back-arc oceanic spreading) is influenced by a slow subduction mechanism (Barker and Austin 1994; Barker et al. 2003), but during the Mesozoic and most of the Cenozoic, it was an active margin (Barker et al. 1991), characterized by the subduction of the former Phoenix oceanic plate under the Antarctic plate (Barker and Lawver 1988). Oblique subduction ceased progressively from southwest to northeast during the Cenozoic, as a result of successive ridge crest–trench collisions (Herron and Tucholke 1976; Barker 1982). Island-arc volcanism associated with the subduction at the South Shetland Trench ceased 20–25 Ma, but in the last million years, renewed volcanism occurred at least three South Shetland Islands such as Livingston, Greenwich and King George, and volcanism occurred in conjunction with rifting that produced the Bransfield Basin (Fisk 1990).

This allowed the generation of a hybrid basalt in the Bransfield Basin between the two types of plate interactions (Barker et al. 2003). Volcanic edifices were created in the central rift composed by MORB (mid-ocean ridge basalt) and the younger arc type (100,000 years) (Gracia et al. 1996). Although it is an area with back-arc hydrothermal activity, it has characteristics that indicate it is a recently active rift (Gracia et al. 1996), with seismic and volcanic activity of 0.3 Ma (Pelayo and Wiens 1989) where the crust is relatively thin (Klinkhammer et al. 2001). Therefore, due to this tectonic situation, active hot zones that were created are represented by the existence of three volcanic islands: Bridgeman, Penguin and Deception, accompanied of several submarine structures (Gracia et al. 1996; Prieto et al. 1998). The presence of high temperatures in different hydrothermal sites of the Bransfield Strait has also been described (Klinkhammer et al. 2001). A recent microbial prospection of hydrothermal vents of the Bransfield Strait by our group at Biociencia has revealed the presence of thermophilic microorganisms belonging to the Bacteria and Archaea domain (unpublished data).

Full characterization of these new microorganisms is still ongoing work. Biological and biotechnological implications of this research are of great interest.

3.8 Unexpected Biodiversity: Permafrost as a Fingerprint of the Past

As mentioned before, thermophiles and hyperthermophiles are widely distributed in hot environments and have been isolated from terrestrial and marine thermal environments such as solfataric fields, hot spring and shallow or deep marine hydrothermal vents (Barry et al. 1996; Sako et al. 1996; Casamayor et al. 2001; Reysenbach and Cady 2001). However, these microorganisms have also been described in other regions with lower temperatures such as hydrothermal fluids between 3 and 30°C (Holden et al. 1998). Another low-temperature site where thermophiles could be found is permafrost.

Permafrost represents a stable and unique physicochemical complex, which maintains life for incomparably longer time than any other known habitat. Moreover, if we take into account the depth of the permafrost layers, it is easy to conclude that they contain a total microbial biomass many times higher than that of the soil cover. Thawing of the permafrost renews the physiological activity and exposes ancient life to modern ecosystems. This great mass of viable matter is peculiar to permafrost only.

Permafrost microorganisms represent a unique material for research on microbial evolution and low-temperature adaptation, because they may possess unique mechanisms that allow them to maintain viability for very long periods. Therefore, permafrost is of great interest for research in microbiology, biotechnology, ecology, molecular biology, palaeontology and the newly emerging field of Astrobiology (Gilichinsky et al. 2007).

The presence of permafrost in Antarctica supposes the possibility to find those microorganisms that lived million years ago in these sites. In fact, the oldest cells are dated back to five million of years ago in Antarctica. Despite of these characteristics, Antarctic permafrost has not received as much biological study as has been devoted to the ice sheet, though permafrost is more stable, older and inhabited by more microbes. This makes it a potentially more significant microbial reservoir than ice sheets. Due to the stability of the subsurface physicochemical regime, Antarctic permafrost is a balanced natural environment and contains numerous microbes as well as the end products of their metabolism. Various ancient microorganisms, which includes prokaryotes and eukaryotes, anaerobes and aerobes and whose age corresponds to the longevity of the permanently frozen state of the soils, survive under permafrost conditions and have been isolated from Antarctic permafrost (Gilichinsky et al. 2007). Notably, the microbial community within the permafrost has the optimal growth at room temperature. According to the growth temperature classification of Morita (1975), are not psychrophilic, or restricted to permanently cold habitats, but are predominantly ‘psychrotrophic’ (Gilichinsky 2002).

Culture-dependent and culture-independent methods were used for the study of microbial ecology in samples of Antarctic permafrost. Both methods identified important groups of microorganisms. The distribution of the major groups found were Gram positives (up to 45%) and Proteobacteria (up to 25%). These groups include microorganisms of the genus *Arthrobacter*, *Micrococcus*, *Rhodococcus*,

Bacillus, *Pseudomonas* and Enterobacteriaceae, among others (Gilichinsky et al. 2007). Both culture-dependent and culture-independent methods revealed that these microorganisms were closely related to strains isolated from other ecosystems with very different geological characteristics.

Different permafrost samples from Deception Island were incubated by the group of Biociencia, using minimum mineral media supplemented with a carbon and nitrogen source. After 24 h of incubation at 85°C, thermophilic archaea were preliminary identified on the cultures, placing permafrost as an important source of thermophiles.

This confirms the fact that permafrost is a good reservoir of microorganisms, which allow the finding of unexpected microorganisms.

3.9 Conclusions

The knowledge of microbial life forms from Antarctica has steadily increased in recent years. The pace of progress and interest in answering questions regarding microbial diversity from this extreme continent is continuously growing.

Million of years of isolation have produced unique adaptive responses to these extreme environments of Antarctica, so the study of biodiversity and the mechanisms of adaptation to extreme conditions has generated not only fundamental but also applied interest.

Antarctica not only is the source of psychrophilic microorganisms, but it possesses several geothermal areas of interest for the study and isolation of thermophiles and hyperthermophiles. As reported in this work, several strains of thermophiles have been described from Antarctica, confirming that thermophilic microorganisms have a worldwide distribution and not just restricted to areas of high temperatures as we historically used to think.

The importance of studying thermophilic microorganisms and hyperthermophiles not only lies in the investigation of the mechanisms of adaptation to high temperatures but are key in biotech industry, where thermozymes and other biocompounds play a lead role due to its high specific activity at elevated temperatures, significantly higher thermostability and resistance against organic solvent denaturation. The examples of thermophilic enzymes presented on this work, with higher thermostability and robustness than enzymes currently used in industry, confirm the great potential of these microorganisms and their specific environmental adaptations.

It is clear that our knowledge of Antarctic microorganisms has increased in recent years. However, it is a fact that by using culture-dependent techniques, it is possible to recover no more than 1% of the existing microorganisms. Measuring the genetic diversity based on analysis of 16S rRNA and functional genes has been an additional avenue for the discovery of many enzymes of biotechnological application. The fast growth and use of culture-independent techniques, in particular DGGE, have enabled a leap in knowledge and understanding of microbial ecology, becoming

a key tool for the identification of new species and signalling an auspicious way for both microbiological and biotechnological investigations.

In general, analysis of multiple approaches applied to single environments has enhanced the robustness of our understanding of the various high-temperature environments and the biodiversity they harbour.

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Chapter 4
Bacterial and Biochemical Properties of Newly Invented Aerobic, High-Temperature Compost

Takahiro Yoshii, Toshiyuki Moriya, and Tairo Oshima

Abstract Compost is the environmentally safe and the most economical way to treat organic waste. Due to heat emitted by fermentation processes, the compost inside is hot, and therefore, a variety of thermophilic bacteria thrive in compost. The thermophiles play an important role in the degradation of biopolymers in compost. This chapter deals with microbial community in a newly developed aerobic, high-temperature compost, where temperature reaches 100°C or even higher. Physical, chemical, biochemical, and microbial analyses of the high-temperature compost are presented. The technical problems associated with the biochemical and microbial analyses are also discussed.

Keywords Extreme thermophiles • *Calditerricola satsumensis* • *Calditerricola yamamurae* • DGGE • Real-time PCR • Zymogram • *Thermus thermophilus*

4.1 Introduction

Composting is a classical way to treat organic wastes produced from house kitchens, restaurants, agriculture fields, cattle farming, and food industries. Composting has many advantages over burning. Composting is economical, safe, and environmentally favorable. To burn organic wastes, a large amount of oil is needed. Thus, we can reduce emissions of CO_2 if organic waste is decomposed by composting instead of burning. Composting does not require sophisticated equipment, whereas burning requires expensive combustion furnaces.

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Composting is environmentally safe since composting does not produce harmful substances such as nitrogen oxides (NOx), sulfur oxides (SOx), and dioxins. The final products can be used as fertilizers and soil conditioners and thus contribute to improvements of production of food resources. Composting kills pathogenic microbes and viruses. Composting processes also kill seeds of weeds. Since burning is now generally prohibited in many countries, composting becomes more important for decomposing organic wastes than before.

Heat emitted from fermentation processes of microorganisms causes the inside of compost piles to become hot, and compost is one of the important environments for isolating thermophiles (Finstein and Morris 1975). According to the literature, the inside temperature reaches up to 75–80°C (Golueke 1972; Saiki et al. 1978). The isolation of many moderate thermophiles belonging to the genera *Geobacillus, Bacillus*, and *Clostridium* and related species has been reported. Methanogens, including thermophilic methanogens, which belong to Archaea have also been isolated from the traditional compost.

Recently the Sanyu Company in the city of Kagoshima, Japan, invented an aerobic, high-temperature compost process in which the internal temperature often exceeds 95°C or even 100°C. This high-temperature composting rapidly degrades wastes from cattle farming including bones, skins, or even dead bodies of animals (Oshima and Moriya 2008). In this chapter, the bacterial and biochemical nature of the high-temperature composting will be described.

4.2 Compost and Thermophiles

Composting can be considered to be microbial degradation of organic materials by aerobic respiration. Due to heat emitted from microbial respiration, the compost pile is hot inside; usually the temperature of the central portion of the compost pile reaches up to 80°C. It is well known that thermophiles play important roles in thermogenic composting. In the past, compost was the main resource for isolation of thermophilic bacteria (Finstein and Morris 1975).

The best known thermophile in compost is *Geobacillus stearothermophilus* (formally *Bacillus stearothermophilus*) which is a moderate thermopile and can grow up to 75°C. *G. stearothermophilus* is an aerobic, gram-positive, spore-forming rod. This thermophile had been the most extensively studied species until extreme thermophiles such as *Thermus* species were isolated from hydrothermal environments such as hot springs in 1960s.

Microbial flora changes during the composting processes, and the change has been investigated for a long time. When compost fermentation starts by mixing the organic waste with matured compost soil, temperature raises with time, and within a few days the temperature inside reaches the maximum. Along with the temperature change, population of mesophiles decreases and that of thermophiles increases.

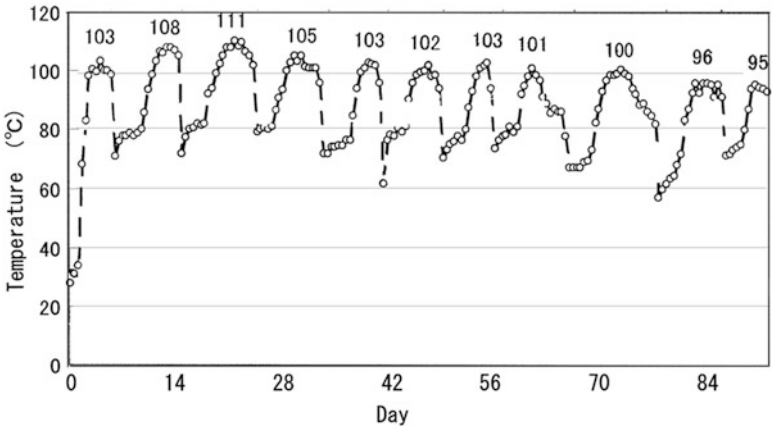

Fig. 4.1 An example of temperature change of an aerobic, high-temperature compost. Temperature at 50 cm below the center of the top surface was measured

4.3 High-Temperature Compost

Since burning was restricted due to environmental issues, organic waste from city life has been treated by composting in many cities. Thus, the size of a compost pile was enlarged. The inside temperatures of these large-scale compost are higher than those of the traditional compost. We have analyzed the aerobic high-temperature compost developed by Sanyu Company in Kagoshima city in Japan (Oshima and Moriya 2008).

A typical example of temperature changes of an aerobic high-temperature compost is shown in Fig. 4.1. In this experiment, temperature was measured at 50 cm below the top surface of a composting pile with an alcohol thermometer. The inside temperature reaches up to 90–110°C within a few days after the raw materials had been mixed with the matured compost soil as shown in Fig. 4.1. The temperature starts to decrease after 5–6 days to about 80–90°C. The compost is thoroughly stirred using a shovel car every week. By this procedure, a large amount of water vapor escapes and compost cools down.

After the turning, the temperature rises again within a day or so. Often 100°C or higher temperature is recorded in the first 3–4 weeks. The temperature at about 5cm below the surface or 5cm above the floor is around 65–70°C, and thus, most part of the compost pile is hotter than 70°C.

The aerobic, high-temperature composting has three unique advantages over the traditional compost: firstly, since inside temperature is higher than 70°C, unfavorable pathogenic microorganisms can be killed quickly; secondly, degradation rates of organic wastes is faster than those of the traditional compost, for instance, an animal body, pig or even beef including their bones, disappears within a few weeks; and thirdly, auxiliary materials such as wood tips or husk are not necessary to add

Fig. 4.2 An example of high-temperature compost pile

to the high-temperature compost, which are needed to adjust water content of compost piles in the traditional composting. The water content of the aerobic, high-temperature compost is controlled by changing the ratio of organic waste to be decomposed and the matured compost soil used as inoculum.

The water content is one of the most crucial factors to operate the aerobic, high-temperature compost. Usually organic waste or active sludge of which water content is around 80% is mixed well with the roughly equal amount of the matured compost soil of which water content is around 15%; the water content of the resulted mixture is around 50%. A typical procedure is as follows: matured compost (91.3 t) and sewage sludge cake (76.1 t) which is dewatered by adding calcium hydroxide are mixed thoroughly, and the mixture is stacked to make a pile of 5×3×8 m (width×height×length). Figure 4.2 illustrates a compost pile at a factory of Sanyu Company.

The fermentation processes start immediately after mixing, and the temperature starts to elevate as already shown in Fig. 4.1. Water content decreases gradually during the composting, especially at a time of stirring every week. The water content of the final product is around 15%, and the final product can be used as inoculum to decompose new organic waste. It takes 6–10 weeks to reduce the water content to 15%. Aeration is another crucial factor. Air is continuously supplied from the bottom of a compost pile through pipes with small holes which are buried on the ground floor. Air compressors are used, and the flow rate should be adjusted to keep the possible highest temperature inside of the pile.

Carbon and nitrogen content decreases rapidly in the first half of the composting process. Total carbon content of the starting mixture is about 20–25 mg/g wet weight of compost and drops to 15–20 mg/g in the final stage. Total nitrogen content also

gets reduced during the composting process. The ratio of total C to total N is almost constant throughout the fermentation and is around 8.5–10. Most of nitrogen in the original waste can be outgassed in the form of NH_3. Nitrate is not detected, but nitrate is formed rapidly when the matured compost is mixed with garden soil or farming soil. The pH is around 8 and is almost constant during the composting process.

4.4 Bacterial Community of the High-Temperature Compost

At first, microbial flora of the final products is analyzed by using viable count techniques. Some typical results of bacterial populations of the final product are shown in Table 4.1. In traditional compost, usually the final product contains around 10^8 culturable aerobic mesophiles plus 10^4–10^5 culturable moderate thermophiles per g wet weight of compost. Mesophiles are defined as microorganisms which form colonies below 55°C, and thermophiles are those which form colonies at 55°C.

As shown in Table 4.1, the aerobic high-temperature compost contains about 1×10^7 culturable mesophiles and about 3×10^6 spores per g wet weight of the compost. Thermophiles detected are about 3×10^6 per gram. The ratio of thermophiles to mesophiles is about 1:3, and this value is about 100–1,000 times larger than that of the traditional compost.

Taking into account that the inside of the compost pile is hot, we expected that the detected mesophiles would be mostly spore formers and vegetative cells would not be present in the course of active degradation of waste. Thus, these mesophiles can scarcely contribute to the decomposition of organic substances. Assuming that culturable thermophiles are 1% of the whole thermophiles in compost, it can be assumed that the aerobic high-temperature compost contains around 10^8–10^9 thermophile cells per gram.

We investigated some of the thermophiles isolated from the compost. It was found that the majority of the isolates belong to genera *Geobacillus*, *Bacillus*, *Saccharococcus*, and *Thermaerobacter* and the related genera as expected.

Table 4.1 Bacterial colony-forming units (CFU) per g wet weight of the final products of different compost factories

Sample taken from	Mesophiles	Spore-forming mesophiles	Thermophiles
Factory in Osaka	3.0×10^7	1.6×10^6	2.9×10^6
Factory in Kohchi	7.7×10^6	7.2×10^6	3.9×10^6
Factory in Saga	3.3×10^9	1.7×10^7	5.5×10^6
Factory in Hokuto	6.3×10^6	5.1×10^6	4.8×10^6

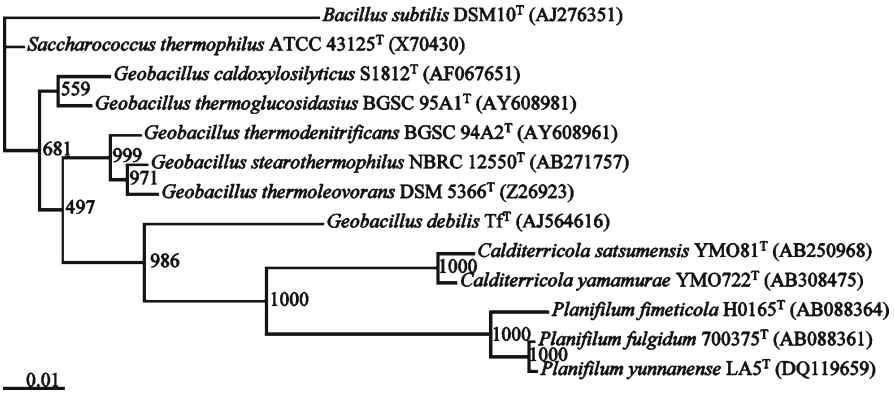

Fig. 4.3 Phylogenetic tree constructed with 16S rDNA gene sequences (1,409 nt) using Phylip neighbor-joining analysis and the Kimura two-parameter mode. *Bar*, 1 substitution in 100 nt. *Numbers* given at the nodes represent bootstrap percentage values (1,000 analysis). The dendrogram was rooted using the sequence of *Bacillus subtilis* as an out-group

4.5 *Calditerricola satsumensis* YMO81 and *C. yamamurae* YMO722

In the course of the analyses of bacteria in the aerobic, high-temperature compost, we could isolate new extreme thermophiles. We named the new strains to be *Calditerricola satsumensis* YMO81^T and *C. yamamurae* YMO722^T (Moriya et al. 2011). The highest growth temperature of *C. satsumensis* is 82°C. They are the first extreme thermophiles isolated from compost. The new isolates are long rod (0.2×3 μm), gram stain negative, nonspore forming, and nonmotile. The G+C content of the chromosomal DNA of *C. satsumensis* YMO81 was 70%. Although BLAST search of 16S rRNA gene sequences shows that the closest species to the isolates is *Planifilum yunnanense* strain LA5^T (Yan et al. 2007), but the homology was only 91%, suggesting that the isolates belong to a new genus. We proposed a new genus *Calditerricola* under the family Bacillaceae. DNA-DNA hybridization homology between *C. satsumensis* YMO81^T and *C. yamamurae* YMO722^T was only 51%, indicating that they are different species to each other. An example of the phylogenetic analyses on these new isolates is illustrated in Fig. 4.3.

Both strains were deposited at the Japan Collection of Microorganisms, RIKEN Bioresource Center (JCM); the German Collection of Microorganisms and Cell Cultures (DSMZ); and the American Type Culture Collection (ATCC). *C. satsumensis* is weakly halophilic and 2% NaCl is required for the best growth. The grow rate of the new isolates is fast; doubling time of the isolates is about 30 min under optimum conditions. In the stationary growth phase, the isolates die gradually at 80°C or even at 4°C. These isolates could not grow in rich media such as Lysogeny broth or Soybean-casein digest medium.

Colony formation can be observed after overnight incubation at 75°C on a gellan gum plate. However, the colony-forming efficiency of the isolates is quite low. The rate was much improved by the addition of $FeSO_4$ and $VOSO_4$ (100 μg/l and 2 mg/l, respectively). Even in the presence of $FeSO_4$ and $VOSO_4$, one colony can be formed from 100 cells. The reasons why the colony formation rate is so low, and why some metal ions improved the viability, are not yet understood. The low viability rate probably suggests that *Calditerricola* species belong to "unculturable bacteria." Since *C. satsumensis* and *C. yamamurae* die rapidly at high temperature and also at ambient temperature, it is problematic to store them. The authors tested many different methods to store them in a laboratory. Storage at −80°C with glycerol is one of the simplest ways to store most of bacteria. *Calditerricola* species die under the conditions, and therefore, this method is not applicable. Second method is freeze-drying. In this method, the cells are suspended in a selected dispersion medium. We tested many dispersion media. The best dispersion medium for *C. satsumensis* and *C. yamamurae* consists of (g/100ml of 0.1 mM phosphate buffer, pH 7.0) sodium glutamate, 3; ribitol (=adonitol), 1.5; and cysteine hydrochloride, 0.05. Though usually the cells suspended in the dispersion solution are freeze-dried, it is advisable to disperse a drop of the cell suspension on a piece of small filter paper strip in an ampoule and then dried under vacuum without prior freezing. This modified method is called liquid-drying (or simply L-dry) method (Malik 1990) and improved the rate of viability of *Calditerricola* species. A slightly different way we adopted successfully is to dry a small drop of the cell suspension (without the filter paper) directly under vacuum without freezing. These microorganisms seem to be highly sensitive to freezing. To our experience, L-dry method on a paper strip is the best for long-term storage of *C. satsumensis* and *C. yamamurae*.

Recently we found that Microbank™ Bacterial and Fungal Preservation System (Pro-Lab Diagnostics Inc., Round Rock, TX) is the best for short-time storage of the thermophile cells. This system consists of beads and a special cryopreservation solution in a small plastic tube. To make a storage ampoule, pour a small amount of the cell suspension or culture medium into the plastic tube and mix well with the "cryopreservation solution" and then remove and discard the liquid by using a sterilized micro-tip. The remaining beads in the plastic tube are stored at −80°C. The cells bind to the beads. To revive the cells, pick up a bead with a set of sterilized tweezers and drop it into a fresh culture medium at 75–80°C in advance. It is the easiest and quickest way, and the survival rate is high. By these procedures, *Calditerricola* species can be stored for at least 6 months. This method can be applied for many thermophiles other than *C. satsumensis* as well as for mesophiles.

C. satsumensis YMO81 required the presence of ten essential amino acids for growth (Moriya et al. 2011); the essential amino acids are glutamine, methionine, histidine, isoleucine, leucine, lysine, phenylalanine, serine, tryptophan, and valine. In the compost, many species produce and secrete proteolytic enzymes as described in the latter part of this chapter, and these amino acids are easily available.

Polyamines are noncyclic (viz., nucleic acid bases are excluded), aliphatic organic compounds which contain two or more amino nitrogen atoms in a molecule.

Polyamines are essential for cell proliferation and play important roles in a variety of biological reactions such as DNA, RNA, and protein biosyntheses. Especially polyamines are important for life at high temperature, since they interact with acidic cell components such as nucleic acids and membrane lipids (polyamines interact with phospholipids) (Terui et al. 2005; Grosjean and Oshima 2007).

It is known that most of extremely thermophilic organisms produce unusual polyamines in addition to the standard polyamines, especially long-chain polyamines such as caldohexamine and caldopentamine and branched polyamines such as tetrakis (3-aminopropyl)ammonium (Oshima 2007, 2010; Morimoto et al. 2010). *C. satsumensis* YMO81^T and *C. yamamurae* YMO722^T produce spermidine, spermine, and a unique branched polyamine, N^4-aminopropylspermine $(NH_2(CH_2)_3)_2N(CH_2)_4NH(CH_2)_3NH_2$ as the major polyamine components. Small amounts of 1,3-diaminopropane, putrescine, and agmatine are also found. The intracellular concentration of the branched polyamine, N^4-aminopropylspermine, increases when the growth temperature is raised. In contrast, this unique polyamine is not detected from the cells grown at lower temperatures such as 60°C. The finding suggests that N^4-aminopropylspermine is one of the key substances for life of *Calditerricola* species at high temperatures.

4.6 Analyses on Microbial Flora Using DGGE Technique

Culture-dependent investigations on microbial community of a compost have a great disadvantage. Unfortunately the culturable bacteria in natural environment such as soil and compost are thought to be only 0.1–10% (Rozak and Colwell 1978; Torsvik et al. 1990; Van Elsas et al. 1997). Majority of bacteria in soil and compost have been neglected by classical studies based on isolation of bacteria and characterization of the isolates. However, introduction of sophisticated DNA technology in microbial ecology has opened a new era for the studies on microbial structure of environment. Now we can analyze the microbial population of compost without isolation and cultivation.

DGGE technique (Muyzer et al. 1993; Amann et al. 1995; Head et al. 1998; Kowalchuk et al. 1997; Heuer et al. 1997; van Wintzingerode et al. 1997; Felske et al. 1998; Nakatsu et al. 2000; Duineveld et al. 2001) is now widely used for investigation of microbial flora in environment. This consists of isolation of DNA from microbial habitats, use of PCR for amplification of 16S rDNA with suitable primers, and use of denaturing gradient gel electrophoresis for separation of the PCR products. Though the method is powerful for tracking microbial populations in space and time and it has been used by many investigators, there are many problems to be solved in such studies in future (van Wintzingerode et al. 1997).

DNA isolation from compost is one example of the DGGE-associated problems (Griffiths et al. 2000; Pan et al. 2010; Inceoglu et al. 2010). We temporarily selected ISOIL kit (Nippon Gene Co., LTD., Tokyo, Japan) for the isolation of DNA.

We perform the isolation according to the manufacturer's instructions. However, contamination of small amount of humic acid is unavoidable. In future, we have to compare the procedures with and without using a bead beater. Also purification procedures to remove the contaminated humic acid are another subject to be studied in future.

The techniques that we have used for analyzing the structure of bacterial community in the compost are briefly described. RNA was extracted from each sample using PowerSoil RNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, USA), and reverse-transcribed into complementary DNA (cDNA) using QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). PCR for denatured gradient gel electrophoresis (DGGE) was carried out using TaKaRa Ex Taq Hot Start Version (Takara Bio, Kyoto, Japan) and primers, 357F-GC (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3′) and 907R (5′-CCC GTC AAT TCM TTT GAG TTT-3′), that were designed to amplify bacterial 16S rRNA genes. Thermal cycler conditions consisted of the following 30 cycles: denaturation at 93°C for 30 s, annealing at 65°C (reducing 5°C every 10 cycles) for 30 s, and extension at 72°C for 30 s. PCR products were detected with ethidium bromide after 1.5% agarose gel electrophoresis. DGGE was performed according to Muyzer et al. using the DCode universal mutation detection system (Bio-Rad, Hercules, CA). Denaturing gradient gel, 1-mm thickness and 160 × 160 mm, was prepared in 0.5 × Tris acetate-EDTA (TAE) buffer. The concentration gradient used was 6–12% polyacrylamide and 2.1–4.9 M urea with 12–28% formamide. Electrophoresis was carried out at 100 V for 780 min in 0.5 × TAE buffer at 60°C. SYBR Green I (Invitrogen Japan, Tokyo, Japan) was used to stain the gel after electrophoresis. Separated DGGE bands were excised using a plastic tip and washed with sterile water. The PCR re-amplification was performed using the same primer set without GC-clump. The sequences of each DGGE band were determined by FASMAC Co., Ltd. (Kanagawa, Japan). The sequence similarities were phylogenetically analyzed using BLAST program provided by the National Center for Biotechnology Information (NCBI) and the DNA Data Bank of Japan (DDBJ).

DGGE band patterns of 16S rRNA gene based on DNA had continued to change during composting process (Fig. 4.4a, Table 4.2). As the temperature of compost exceeded 80°C from 40°C, the most remarkable difference was observed between band patterns of the fermentation start (1st day) and the 5th day. The sequences of two of the predominant bands in 5 days can be assigned to *C. satsumensis* and were detected in each sample till 25 days (Fig. 4.4a; bands 5 and 6). The sequence of the major band in days 15 and that of 20 were almost identical to the partial 16S rRNA sequence of *Thermus thermophilus* (Fig. 4.4a, band 11). The bands found in late period (days 25–38) could be attributed to *Sphaerobacter thermophilus* (Fig. 4.4a, band 17), *Saccharomonospora viridis* (Fig. 4.4a, band 18), and a species belonging to the genus Planifilum (Fig. 4.4a; bands 15 and 16). Members of the genus *Bacillus* were dominant in the latter period of composting (Fig. 4.4a; bands 20 and 21). The bands related to the genus *Thermaerobacter* (the closest species is *T. composti* or *T. marianensis*) were presented in every sample throughout the composting (Fig. 4.4a, band 9).

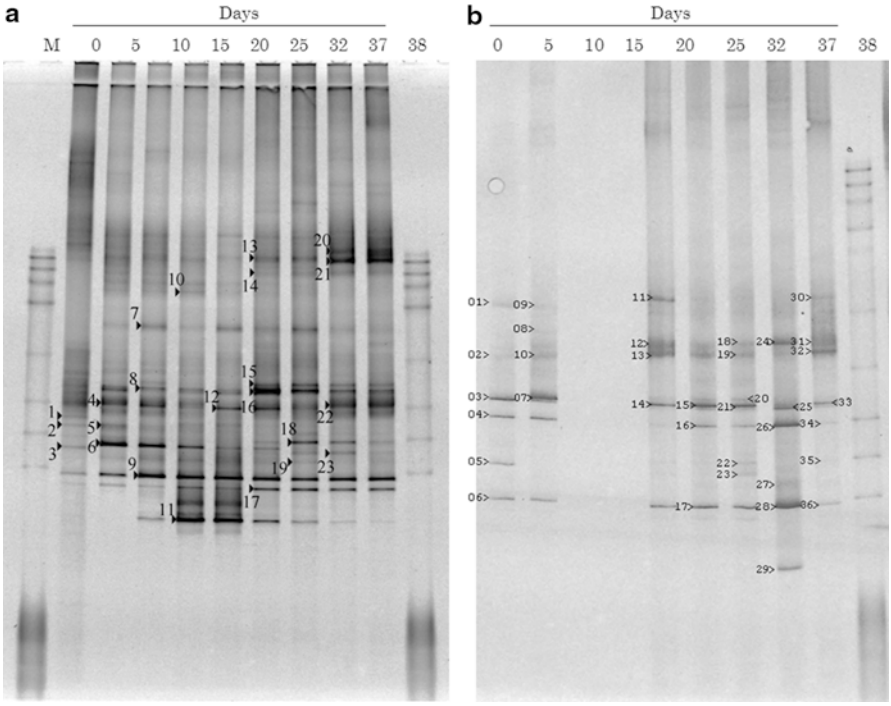

Fig. 4.4 DGGE band profiles of 16S rRNA genes extracted from an aerobic, high-temperature compost. The *arrows* on DGGE gel indicate the excised bands in order to determine the base sequences (see Tables 4.2 and 4.3). *Gel A* DNA-based profiles, *Gel B* RNA-based profiles, *lane M* DGGE marker II (Nippon Gene Co., LTD., Tokyo, Japan)

One of the serious problems associated with DGGE analyses of microbial community is viability of the microorganisms. DNAs in dead cells can be extracted and are undistinguishable from those of living cells. One possible way to solve this problem is to extract and analyze RNAs instead of DNAs. We carried out the RNA analyses of the aerobic, high-temperature compost. In our preliminary experiments, *E. coli* could not survive for 5–10 min after they had poured into the actively fermented compost, which was, however, detected after 24 h in the compost, suggesting that DNAs in dead cells can be stable up to 24 h at 70–90°C. RNA disappeared much faster than DNA.

The results of DGGE fingerprints based on RNA were greatly different from those of DNAs (Fig. 4.4b, Table 4.3). The band was not detected from compost samples in days 10 and 15. A DNA band assigned to *C. satsumensis* was found only in the early stages of composting (Fig. 4.4b, band 05). The dominant bands in almost all compost samples had high similarity to the genus *Planifilum* (Fig. 4.4b; bands 02, 03, 04, 07, 08, 14, 15, 16, 21, 24, 26, 33, and 34) and *Thermaerobacter* (Fig. 4.4b; bands 06, 17, 27, 28, and 36).

Table 4.2 Species identification based on base sequences of bands detected on DGGE shown in Fig. 4.4a

Band	Name	Similarity (%)
1	*Barnesiella intestinihominis* JCM 15079	87
2	*Barnesiella viscericola*	87
3	*Methylocaldum szegediense* OR2	88
4	*Planifilum yunnanense* LA5	96
5	*Calditerricola satsumensis*	100
6	*Calditerricola satsumensis*	100
7	*Bacillus* sp. R-7336	99
8	*Planifilum fimeticola* H0165	95
9	*Thermaerobacter marianensis* DSM 12885	99
10	*Anoxybacillus toebii* NS1-1	99
11	*Thermus thermophilus* HB8	100
12	*Planifilum yunnanense* LA5	99
13	*Bacillus* sp. R-7336	96
14	*Bacillus* sp. R-6491	99
15	*Planifilum fimeticola* H0165	95
16	*Planifilum fimeticola* H0165	96
17	*Sphaerobacter thermophilus* DSM 20745	100
18	*Saccharomonospora viridis* DSM 43017	100
19	*Thermanaeromonas toyohensis* ToBE	87
20	*Bacillus bataviensis* CCGE2104	98
21	*Bacillus* sp. R-6491	99
22	*Planifilum yunnanense* LA5	100
23	*Actinomadura* sp. IMMIB L-889	100

4.7 Abundance Ratio of *C. satsumensis* in the Composting Process

For the determination of relative abundance of *C. satsumensis* in the compost microbial community, we conducted a quantitative PCR using SYBR premix Ex Taq GC (Takara Bio) and Thermal Cycler Dice Real-Time System (TP800, Takara Bio). Universal primers, EU350F (5′-TAC GGG AGG CAG CAG-3′) and EU500R (5′-CCG CGG CTG CTG GCA C-3′), were used in order to estimate amounts of 16S rDNA of eubacteria. The specific forward primer for *C. satsumensis* was designed on the base sequences of 16S rRNA genes, YMO16F (5′-TTT TCG CGT GAA GCC TTC G-3′), and EU500R primer was used as the reverse primer. PCR program was run as follows: the first denaturation at 98°C for 2 min, followed by 40 cycles of 10 s at 98°C and 1 min at 68°C. The quantitative analyses of DNAs were carried out using the recorded melting curves of DNAs according to the instructions of the manufacturer. Abundance ratio of *C. satsumensis* per whole eubacterial cells is shown in Fig. 4.5. The abundance ratio was below 0.1% at the start of compost. It increased

Table 4.3 Species identifications based on base sequences of bands detected on DGGE shown in Fig. 4.4b

Band	Name	Similarity (%)
01	*Anoxybacillus toebii*	99
02	*Planifilum yunnanense*	95
03	*Planifilum yunnanense*	99
04	*Planifilum yunnanense*	99
05	*Calditerricola satsumensis*	99
06	*Thermaerobacter subterraneus*	98
07	*Planifilum fimeticola*	96
08	*Planifilum fimeticola*	95
09	*Anoxybacillus toebii*	94
10	*Planifilum fimeticola*	91
11	*Bacillus thermoamylovorans*	92
12	*Bacillus thermoamylovorans*	97
13	*Bacillus thermoamylovorans*	97
14	*Planifilum fimeticola*	95
15	*Planifilum fimeticola*	95
16	*Planifilum yunnanense*	99
17	*Aerothermobacter marianas*	97
18	*Planifilum fimeticola*	90
19	*Planifilum fimeticola*	90
20	*Planifilum fimeticola*	90
21	*Planifilum fimeticola*	95
22	*Thermaerobacter subterraneus*	85
23	*Thermaerobacter composti*	91
24	*Planifilum yunnanense*	95
25	*Planifilum yunnanense*	91
26	*Planifilum yunnanense*	95
27	*Thermaerobacter subterraneus*	95
28	*Aerothermobacter marianas*	98
29	*Thermus thermophilus*	99
30	*Bacillus subtilis*	96
31	*Bacillus subtilis*	98
32	*Bacillus subtilis*	97
33	*Planifilum fimeticola*	96
34	*Planifilum yunnanense*	99
35	*Saccharomonospora viridis*	95
36	*Aerothermobacter marianas*	98

to 23% rapidly within the first 5 days. The predominance of *C. satsumensis* continued until the day 15, the period in which the temperature of the compost is highest. Then the abundance ratio decreased till the end of composting process and returned to the low level (<0.1%) which is identical to the figure at the start. There is some inconsistency between the analyses based on RNAs (cDNA) and DNAs. RNA analyses indicated that the abundance ratio at the start is the highest (>7%), and then it fell down to less than 1% immediately by 5th day.

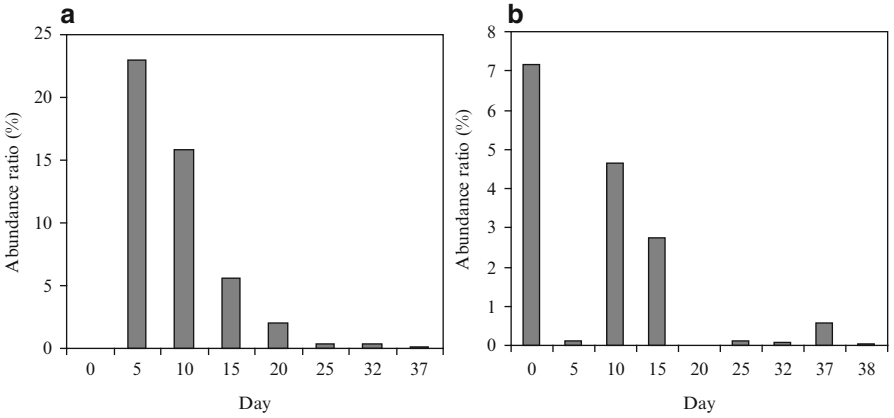

Fig. 4.5 Changes of the relative abundance *C. satsumensis* during the composting process. The relative abundance of *C. satsumensis* in each sample is represented as the ratio of *C. satsumensis* 16S rDNA/total amount of eubacteria 16S rDNAs. Each 16S rDNA amount was determined using DNA (**a**) or RNA (**b**) extracted from compost

As mentioned, DGGE is a powerful method to analyze the microbial community in environment. However, the method is able to detect only 20–40 major species at a time. Often *C. satsumensis* could not be detected in the final stages of high-temperature composting, though the bacterium flourishes instantly and becomes the most abundant species in the compost when the final compost is mixed with new organic waste. In contrast, often DGGE detects DNA from dead or dormant cells, and it is difficult to distinguish actively growing cells from dead cells. Other problems to be solved in future include that often a band seen on DGGE plates does not represent a single species. Improvement on the sensitivity as well as the accuracy of band separation would be desirable (Sekiguchi et al. 2001).

4.8 Population Changes of *Thermus thermophilus* and *Thermaerobacter composti* in the Composting Process

T. thermophilus (Oshima and Imahori 1974) and *T. composti* are extremely thermophilic bacteria growing at temperatures above 75°C and have been isolated from compost soils (Beffa et al. 1996; Yabe et al. 2009). The 16S rDNA genes which are highly homologous to those of *T. thermophilus* or *T. composti* were detected on the DNA-based DGGE (Fig. 4.4a, band 11 or 9). We analyzed the abundance of these bacteria using a quantitative real-time PCR. Specific primers for *T. thermophilus* were designed TTH-F (5′-GCC TAA GAC ATG CAA GTC GT-3′) and TTH-R (5′-CAA AGC CCT TTG GAC AC-3′). To perform the quantitative PCR of *T. composti* 16S rDNA, TN457R (5′-TCC TCA CCC CCG ACC TTC-3′) were designed as a reverse primer and was used with EU350F. The cells of *T. thermophilus* accounted for more than 40% of the total cells in the compost between days 15 and 20, and it is the most dominant organism at this stage. However, the bacterial

population decreased greatly during the late phase of composting process to less than 5%. The abundance ratio of *T. composti* drifted about in a range of 1–4% except the early phase. The highest value of the abundance ratio of *T. composti* was 4% at around the day 20. The findings suggest that these two thermophilic eubacteria as well as *Calditerricola* species play important roles in the aerobic, high-temperature compost.

4.9 Analyses on Hydrolytic Enzyme Activities

To analyze the enzymatic activities of the compost, we performed the activity staining method (zymogram analyses). The enzymes were extracted from compost directly into a buffer solution. Then, the enzyme proteins were collected by ammonium sulfate precipitation (70% saturation) and concentrated in a small volume. The zymogram for gelatinases (collagenases) was carried out according to Nakamura et al. (2004) with slight modifications. Each enzyme sample was loaded on a 10% polyacrylamide gel plate containing 0.8% gelatin. After the electrophoresis, the gel plate was placed in Tris-HCl buffer and incubated at 70°C for 3 h. After the incubation, the gel was stained by Coomassie Brilliant Blue and was destained using a mixture consisting of 10% acetate, 15% methanol, and 75% distilled water. The hydrolysis of gelatin results in the formation of the clear zones on the gel. It seems that the change of gelatinase activity is related to the change of the microbial community structure during composting. Two major gelatinases were detected; one with larger molecular weight was detected only in the first half of the compost processes, whereas the smaller enzyme was constantly produced and secreted into compost soil throughout the entire composting process (Fig. 4.6a).

The carboxymethyl cellulase (CMCase) and xylanase activities were also measured in similar manner using carboxymethyl cellulose (CMC) and xylan instead of gelatin in the zymography gel plates. Congo red was used for staining residual CMC and xylan. The amylase activity in the compost was determined using soluble starch instead of gelatin. The undegraded starch was stained by iodine solution.

The determination of esterase activity in the compost samples was carried out using a polyacrylamide gel without gelatin (Miller and Karn 1980). After the electrophoresis, the gel was incubated in Tris-HCl buffer containing α-naphthyl acetate and fast blue RR at 70°C for 30 min. The esterases were visible as colored bands resulting from the formation of diazo dye complex.

The amylase activities were detected in the early and late stages of the composting process, and low-molecular-weight amylases were found only in the late phase (Fig. 4.6b). CMCase activities were more abundant only in the late (days 25–37) than early stages (days 0–20) (Fig. 4.6c). Thermostable xylanases of different molecular weight were found in all samples except day 15 (Fig. 4.6d). The high esterase activities were found in middle phase of composting process as opposed to other enzymes (Fig. 4.6e).

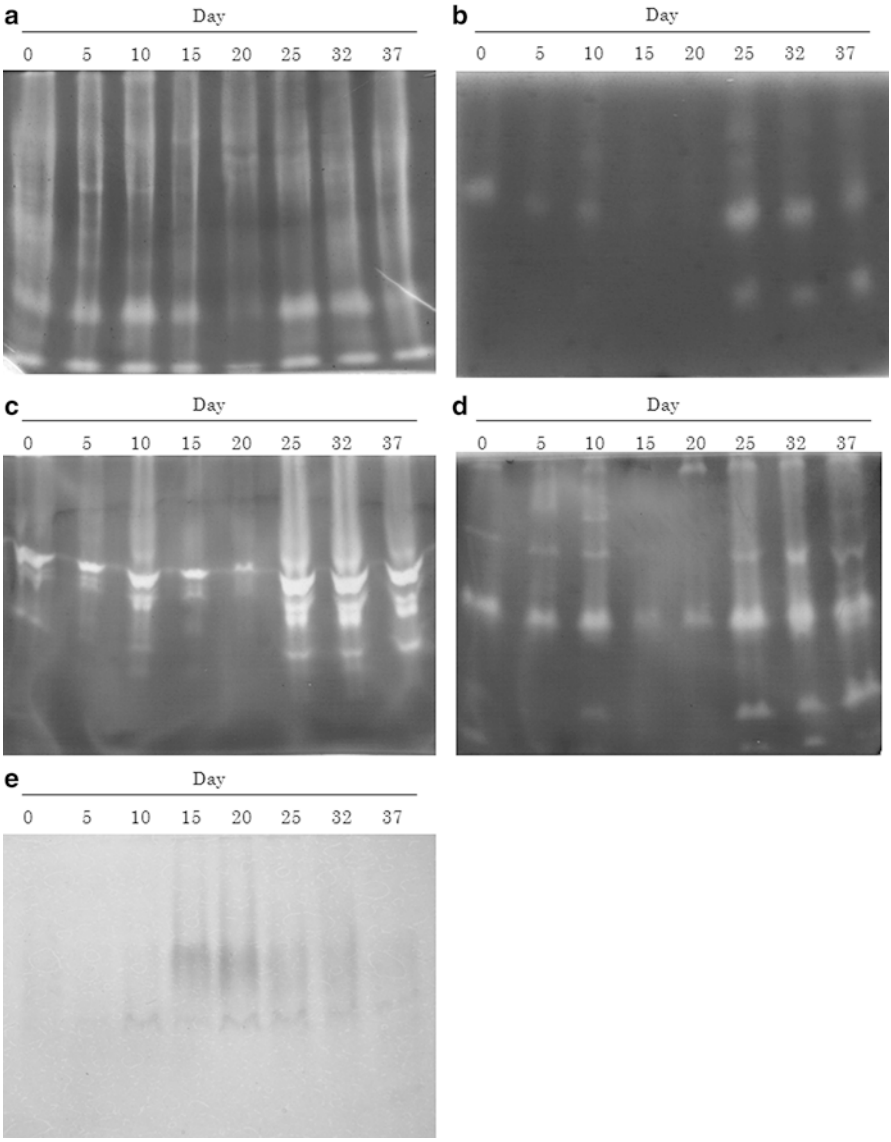

Fig. 4.6 The zymogram of gelatinases (**a**), amylases (**b**), carboxymethyl cellulases (**c**), xylanases (**d**), or esterases (**e**) extracted directly from the aerobic, high-temperature compost

As mentioned above, *T. thermophilus* is one of the most dominant species in the middle phase of the high-temperature composting. In this context, it is noteworthy that two papers reported the production of lipolytic enzymes by *T. thermophilus* isolated from compost (Beffa et al. 1996; Fuciños et al. 2005).

4.10 Conclusions

Newly developed aerobic, high-temperature compost seems to be an interesting process from viewpoint of pure science as well as applied research. Often temperature exceeds 100°C inside. Organic wastes including so-to-speak "hard to digest waste" such as skins, bones, and straw are digested within relatively short period. Temperature changes during the compost process suggest that both mesophiles and thermophiles equally play crucial roles in the compost.

New extremely thermophilic bacteria (eubacteria), *Calditerricola satsumensis* and *C. yamamurae*, were isolated from an aerobic, high-temperature compost. They are different from the representative thermophiles of traditional composts such as *Geobacillus stearothermophilus* in that they are gram stain negative and nonspore formers. Their cellular polyamine compositions entirely differ from those of *Bacillus* and *Geobacillus* species. Though they can be easily isolated from high-temperature composts which were stored for long time such as several months and can grow actively under suitable conditions, these new isolates cannot survive at high temperature as well as ambient temperature, suggesting that the isolates are deeply adapted to the environment of the compost.

DGGE is a powerful tool to analyze structure of bacterial community in compost, though there are many technical problems to be solved in future. Our DGGE analyses implied that the bacterial community of the aerobic, high-temperature compost varies dynamically during the fermentation process. The whole bacterial community of the compost can be regarded as a "super organism" (Wilson and Sober 1989).

The aerobic, high-temperature compost seems to be a promising resource for hunting enzymes of industrial interest. Hydrolytic enzymes for collagen, keratin, cellulose, starch, and so on were detected, and they are heat resistant without exception.

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Chapter 5
Role of Thermophilic Microflora in Composting

Seema Rawat and Bhavdish Narain Johri

Abstract Composting is a process that converts organic waste to a humus-like end product and is not only a waste treatment technique but also a recycling method as the end product can be used in agriculture as fertiliser, in gardening or in landscaping. Microbes play a key role as degraders during the composting process; mesophilic microflora constitutes the pioneer component, while thermophilic microflora, the climax and also the dominant component contributes significantly to the quality of compost. The dynamics of microbial community in different compost ecosystems change in context with qualitative and quantitative changes in physico-chemical conditions of compost. Optimisation of compost quality is directly linked to composition and succession of microbial communities in compost ecosystem. This necessitates the monitoring and characterisation of the microbial community composition, patterns and dynamics of species diversity at spatial scales. The biasedness and time-consuming nature of cultivation-based approaches severely limits spatial and temporal intensity of sampling, but with the advent of culture-independent molecular techniques, new insights into the composition of uncultivable communities have been gained. It has also been now possible to define the causes of time-dependent changes in the health of microbial community on the basis of observed 16S and 18S rDNA diversity. The genomic information represented by such a 'community genome' offers a tremendous resource for examining the extent and patterns of microbial genetic diversity and metabolic capabilities in the natural ecosystem. These studies also provide basic knowledge on mechanisms that control species coexistence, which have fundamental applications since they offer the framework

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that serves in maintaining, restoring and manipulating the diversity in natural compost ecosystem and hastening the process of composting besides improving the quality of compost.

Keywords Composting • Compost ecosystem • Microbial diversity • Mesophilic microflora • Thermophilic microflora • Pioneer community • Climax community • Microbial community succession • Community genome

5.1 Introduction

Composting is an age-old practice of decomposition of plant and animal matter, resulting into nutritionally rich compost, which dates to the early Roman era since Pliny the Elder (AD 23-79) who refers to compost in his writings. It is, however, different from the natural process of decomposition of large amounts of organic material on a regular basis by microorganisms which results into humus and is a mean of nutrient turnover in the natural ecosystem. The latter takes place at a slow pace which can be accelerated by creating conditions ideal for growth and activity of the decomposers. This accelerated process of decomposition of organic matter by a mixed population of microorganisms in a warm, moist, aerobic environment has been termed as 'composting' (Rawat et al. 2005). It can be either 'passive' or 'active', involving complex physico-chemical interactions between the organic matter and decomposer, resulting into the end products: compost (soil-like material composed of more resistant residues of the organic matter, breakdown products and dead and living microorganisms along with products formed from further chemical reaction between these materials), carbon dioxide, water and heat. The compost acts as a soil conditioner (as it improves soil structure, texture, aeration and water retention), a fertiliser, a substitute for peat in horticulture, a natural pesticide and a microbial additive to increase enzyme activity and also adds humus (Perucci 1990; Jakobsen 1995; Eklind et al. 1998; Hoitink and Boehm 1999; Li and Jang 1999; Odlare 2005). The high organic matter content and biological activity makes it effective in a wide range of applications including use in gardening, landscaping, horticulture, agriculture, production of mushroom and biogas (methane), control of erosion, balancing soil pH, land and stream reclamation, wetland construction and as landfill cover. Composting has become an increasingly important strategy for the treatment of municipal organic waste. It is a far better and an ideal eco-friendly approach for disposal and decomposition of wastes compared to landfilling and burning as the latter poses environmental problems. Regardless of the products formed, the active component mediating biodegradation and conversion process during composting is the resident microbial community. The knowledge about the microorganisms present in composts, their coexistence and the ways they replace each other during the different stages of the biological degradation process should help to ensure a high quality of the final compost product (Steger et al. 2007b).

The microbial community changes with the change in physico-chemical conditions of compost. Thermophilic microflora constitutes the predominant component which provides selectivity to compost. Thus, the optimisation of compost quality is directly linked to composition and succession of microbial communities in the composting process. This means that tools are required to monitor and characterise the composition, patterns and dynamics of species diversity during composting and to relate them to compost quality. While analysis of the presence and distribution of different operational taxonomic units (OTU) within a population provides insights into the ecological functioning of communities, the study of taxonomic structure alone limits insight into the ecological relevance of the community structure. The exclusion or addition of different microflora does not necessarily change the resultant functions of the community (White and Findlay 1988). The change in abundance of an individual group may not necessarily equate to meaningful shifts in the community function.

The biasedness of cultivation approaches, employed in the past to study microbial populations, inevitably favoured growth of some community members due to selective nature of media, and thus, it could have excluded majority of the indigenous microbes (Troussellier and Legendre 1981). Moreover, the reported culturable microbes comprise of only 1% of the total diversity and the cultivation methods are time-consuming and henceforth severely limit the spatial and temporal intensity of sampling and the associated description of the population structure (Garland and Mills 1991). This has led to the development of cultivation-independent approaches which have given new insights into the composition of unculturable microbial communities and also in microbial community succession. It is now becoming possible to define the causes of time-dependent changes in the health of microbial community on the basis of observed genetic diversity (Purohit et al. 2005). These include assessment of microbial diversity based on *in situ* extracted phospholipids (Herrmann and Shann 1997; Boggs et al. 1998; Klamer and Baath 1998), measurement of carbon source utilisation by substrate-extracted microbial cell consortia (Inssam et al. 1996; Boggs et al. 1998) and nucleic acid-based techniques (Kowalchuk et al. 1999; Peters et al. 2000). The combination of signature lipid and nucleic acid-based analyses has greatly expanded the specificity and the scope for assessing the microbial community composition in composts (Steger et al. 2007b). These approaches also provided basic knowledge of mechanisms that control species coexistence, which have fundamental applications since they offer the framework that serves biologists interested in maintaining and restoring diversity in natural ecosystems.

This chapter covers various types of composting, physico-chemical aspects of composting and structure of thermophilic microbial community and their functionality in various compost ecosystems. Readers may find that available information on several aspects of compost ecosystem is scanty which is due to horizontal advancements in some areas and also because compost represents a complex ecological system from the viewpoint of microbial distribution and activity. A special emphasis has been given to mushroom compost ecology since this complex man-made ecosystem harbours a complete spectrum of microbial diversity and is also unique with respect to conditions under which the mushroom crop is grown and relatively short time required to complete the successional cycles which are not matched elsewhere.

5.2 Composting

Composting has been a part of our global culture since ancient times. Traditionally, it was done for piling organic materials and allowing it to stand in the agricultural field until the next planting season, at which time the dark-brown-coloured material would be ready for soil application. It was modernised beginning in the 1920s in Europe as a tool for organic farming. The practice started on large scale in 1921 with the setting up of the first industrial station in Austria for the transformation of urban organic materials into compost. The advent of effective microorganism technology has brought tremendous change in composting. The noted personages cited for propounding composting within farming are Rudolf Steiner, (founder of biodynamic farming), Annie France-Harrae, Sir Albert Howard, Lady Eve Balfour, J. I. Rodale (founder of Rodale Organic Gardening), E. E. Pfeiffer (who developed scientific practices in biodynamic farming), Paul Keene (founder of Walnut Acres in Pennsylvania) and Scott and Helen Nearing (who inspired the back-to-land movement of the 1960s).

Composting represents an astonishing example of solid-state fermentation (SSF) wherein a crude variety of wastes such as sewage sludge, refuse, animal manure, industrial wastes, food wastes, leaves, tree bark, agriculture residues and abattoir residues can be treated through microbial route irrespective of their suitability as feedstock for compost production (Rawat et al. 2005). Refuse (municipal solid waste) is partly compostable but poses problems due to its extreme heterogeneity as it consists of food scrapes (garbage), paper, glass, plastic, metal, sweepings, yard waste, ash, etc. The organic rich fraction after separation can however be composted; the whole municipal solid waste can also be passed through the composting stage (mass composting), possibly with subsequent segregation (Satyanarayana and Grajek 1999).

The basic aims of composting according to Miller (1994) are as follows: (1) achievement of a suitable bulk density (compost makes a more physically stable landfill and can be easily stored, transported and disposed off than the original material as bulk density of former is higher), (2) modification of complex polysaccharides and plant materials, (3) biological removal of readily available nutrients to avoid overheating, (4) building up of an appropriate biomass and a variety of microbial products, (5) establishment of selectivity, (6) conversion of nitrogen into stable organic form and (7) sanitation, i.e. killing of pathogenic microbes, larvae and weeds.

The various methods of composting are as follows: (a) sheet composting (it is also a quite long method which is carried out by spreading organic material on the surface of the soil or untilled ground and allowing it to decompose naturally. It does not destroy pathogens and composting materials should be limited to plant residue and manure), (b) trench composting (it is a relatively simple and a slow method involving filling of organic materials into trenches (usually 6–8 in. deep) which are then covered with soil. It also does not destroy pathogens), (c) in-vessel composting (it is the large-scale composting which involves loading of composts in closed reactors like metal tanks, steel drums or concrete containers of very large size. The vessel

is designed to rotate at 3–5 revolutions per hour. It can produce stabilised compost in 3–4 days and destroys pathogens, thus providing selectivity to compost), (d) bin composting (it involves pellets fastened together to form a box and lined with wire mesh. To limit the degree of turning and to permit air to flow through the pile, the structure can be elevated, or perforated pipes can be incorporated into the structure), (e) windrow composting (it is a means of indoor composting in which windrows are large enough to retain heat and small enough to facilitate air to its centre) and (f) aerated static pile composting (it involves air for the operation by placing the heap on holed piping that allows circulation) (Hultman 2009).

Composting is done at small scales like decomposition of domestic wastes as well as large scales like decomposition of industrial wastes. It is carried out *via* either batch or a continuous mode. Batch mode is a long process comprising of four sequential phases: the mesophilic phase (or moderate-temperature phase), thermophilic phase (or high-temperature phase), cooling phase and curing phase. The initial stage of decomposition of mass of organic matter, initiated by mixing and wetting the substrates, the mesophilic stage, which lasts for a couple of days, is governed by the mesophilic microflora, which uses up the readily available nutrients. The aerobic fermentation (composting) commences as a result of growth and activity of microorganisms resulting in release of heat, ammonia and CO_2 as by-products along with other unpleasant-smelling compounds. The metabolic activity of the mesophilic microorganisms results in rise in temperature which paves way for the development of thermophilic microflora which initiates the second phase of composting. This phase of composting starts very rapidly and may last days or weeks or even months. It is the thermophilic stage that results in maximum decomposition of organic matter besides sanitation. During the cooling stage, mesophilic microflora recolonises the compost and degrades more resistant organic matter (Tiquia et al. 2002; Hiraishi et al. 2003). The final phase of composting is called curing, ageing or maturing stage, which is long (may last up to several months) and an important one since it provides a safety net for destruction of the pathogens. Uncured compost can produce phytotoxins, besides depriving soil of oxygen and nitrogen, and can contain high levels of organic acids (Mathur et al. 1993).

In the continuous mode of composting, similar phases occur but they are not as apparent as in the batch mode, and these could occur concurrently rather than sequentially. A well-designed continuous system can eliminate the need for a mesophilic stage and operate continuously at thermophilic temperatures. This mode offers a means of decomposition of putrescible materials quickly under close process control.

Municipal compost is prepared by batch mode, while garden composting is performed in a continuous mode. Mushroom compost and vermicompost, though prepared by batch mode, are significantly different from general municipal compost.

According to temperature, composting can be of two types: (a) hot composting, the most efficient method for producing quality compost, carried out using windrow or bin method or in-vessel method, in a relatively short time, and (b) cold composting, a long method of composting which may last up to years.

5.3 Types of Compost

All composts, namely, mushroom compost, vermicompost, industrial compost, municipal compost and garden compost, are unique with respect to the raw material used, their way of preparation, physico-chemical conditions and the end product generated.

Mushroom compost is prepared very rapidly (18–24 day) and does not involve curing stage. It is prepared, either by long method (LMC) or short method (SMC), from various agro-residues, namely, wheat straw/paddy straw/sugarcane bagasse, as a base material along with other additives, namely, chicken manure, calcium ammonium nitrate, urea, superphosphate, muriate of potash, wheat bran and gypsum, as additives. LMC is the primitive, cheap method involving only one phase (without pasteurisation) (Mantel et al. 1972). SMC is a quick method constituting of a general advance in controlled composting (Sinden and Hauser 1950) and involves two sequential phases: phase I (an uncontrolled self-heating process initiated by mixing and wetting the ingredients as they are stacked in windrows which are periodically turned and watered at approximately 2 days interval) and phase II which is the indoor process of pasteurisation, carried out in tunnels.

Vermicompost (also called vermicast or worm castings), obtained by the decomposition of organic materials, namely, straw, shredded newspaper, saw dust and horse manure using surface feeding worms, usually red wigglers, white worms and earthworms, especially *Eisenia foetida* (red wiggler) and other microorganisms, is rich in microbial activity and plant growth regulators, and fortified with pest repellence attributes as well. It differs from other composts as it is prepared by a mesophilic process and contains a higher content of humic acid (50–70%). It is carried out both indoor in specially designed worm boxes and large-scale outdoor, involving decomposition in pits, heaping above ground on polythene sheets, in tanks, in commercial biodigester and in cement rings. The organic material is sprinkled with phosphate powder and cow dung slurry and is allowed to decompose for 15–20 days. The earthworms are released once heat generated has been cooled down. The vermicompost is ready in about 2 months if agricultural waste is used and about 4 weeks if sericulture waste is used as substrate (Tognetti et al. 2005; Vivas et al. 2009).

Industrial compost is prepared *via* a large-scale composting system involving various techniques, namely, in-vessel method, aerated static pile composting, sheet composting, anaerobic digestion and high-fibre method. Municipal compost is prepared from the decomposition of yard waste, food scraps, leaves and other domestic wastes in large bins. It differs from backyard compost in the size of containers and also the factors such as flow of air and temperature are controlled more effectively.

5.4 Physico-Chemical Aspects

Composting is a very dynamic process involving quick changes in physico-chemical conditions due to microbial activity which in turn also poses a selection pressure on the succession of microbial communities and thus has a profound effect on the entire

process. The essential parameters which affect composting are temperature, ammonia, carbon dioxide, moisture and C:N ratio of the substrate. The initial phase of composting is the most dynamic part of the process and is characterised by rapid increases in temperature, large swings in pH and the degradation of simple organic compounds (Schloss et al. 2003). The mass of decomposing organic materials is an exception to most ecosystems as it not only results into intensive heat production due to the metabolic activity of microorganisms but also acts as an effective retention system, resulting in a significant rise in temperature. Generally, self-heating occurs when organic materials are assembled provided there is sufficient mass, at least one ton, for insulation, and that moisture, aeration and nutrition level are adequate (Satyanarayana and Grajek 1999).

Temperature is one of the key indicators for microbiological reactions of composting processes. It is an important parameter to monitor composting efficiency, because it affects not only the biological reaction rates and the dynamic population of microbes but also the physico-chemical characteristics of composts (Namkoong et al. 2002; Antizar-Ladislao et al. 2005). Temperature is directly proportional to the biological activity within the composting system. As the metabolic rate of the microbes accelerates, the temperature within the system increases, while with the decrease in the metabolic rate, the system temperature decreases. The temperature in the composting stack usually reaches as high as 70°C within 2 or more days (Rawat and Johri 2002). A peak temperature of 70°C can be attained within 4 days in metrowaste composting system meant for processing 150 t of general municipal refuse daily (Rawat 2004). A maximum temperature of 67°C was observed in wheat straw composting after 8 days and remained above 50°C for about 3 weeks (Chang and Hudson 1967); on the other hand, composting of grass cuttings can attain 66°C within 16 h, reaching a maximum of 76°C after 48 h before a decline. The difference in temperature recorded is attributed to the type of material and other factors, such as age of the plant material (Satyanarayana and Grajek 1999). In pig manure and chicken manure compost too, maximum temperatures can reach up to 75°C (Kowalchuk et al. 1999). The composting of garden wastes occurs at 60–70°C during the initial phase by self-heating, while the later phases operate at 45°C (Pedro et al. 2001). Partanen et al. (2010) observed that the temperature in the pilot-scale compost rose quickly to the thermophilic stage. Within 2 days after feeding waste into the feeding end of the drum, the average temperature exceeded 50°C, while in the full-scale composting unit, the thermophilic phase was reached only temporarily in the unloading end of the drum, 3–4 days after feeding (average 45°C), and more consistently in the tunnel compartment (50–70°C), 4–7 days after feeding.

The size of the compost pile is crucial not only for temperature build-up but also for maintenance of appropriate microbial equilibrium and successional pattern. In phase I of mushroom compost, a gradient of 20°C (outer region of stack) to 70°C (centre) exists, while phase II runs at lower temperature of 50–60°C (Johri and Rajni 1999; Peters et al. 2000). The high temperature (75–80°C) of compost pile is necessary to induce Millard reactions, i.e. fixing of free ammonia through reactions with carbohydrates and lignatious polymers. Millard reaction transforms carbohydrates into chemical form which is accessible to the mushroom crop but not to

competitors and, hence, provides for compost selectivity. High temperature is also necessary for the chemical incorporation of nitrogen into stable form within the compost (Johri and Rajni 1999).

The initial pH of the compost varies between 6 and 7 (Chang and Hudson 1967). During the early stages of composting, a rapid growth of microbes reduces the pH value due to the formation of short-chain organic acids, mainly lactic acid and acetic acid, and thereafter, the pH value of the composting materials rises gradually due to the increment in the amount of ammonia generated by the biochemical reactions of nitrogen-containing materials (Khiyami et al. 2008). During vermicomposting the pH changes from acidic to neutral, while in municipal waste, poultry manure–saw dust mixtures and grass composts, initial pH of 4.5–6.0, stabilise within 7–9, possibly due to the loss of organic acids through volatilisation and microbial decomposition and the release of ammonia through mineralisation of organic nitrogen (Rawat 2004). Peters et al. (2000) have reported the decrease in pH from 5.6 to 4.5 within the first 2 days of mushroom composting followed by an increase to 7.0, while Rawat (2004) reported variation in pH from 6.7 to 8.6 (end of phase I) with phase II composting pH stabilising at 7.8. In a field-scale composter, the pH varied between 7.5 and 8.10 during the composting of garden wastes (Pedro et al. 2001). Sundberg et al. (2004) reported low pH as an inhibiting factor in the transition from mesophilic phase to thermophilic phase in composting of household waste. The respiration rate was strongly reduced at 46°C and pH below 6.0, compared to composts with a higher pH or lower temperature. The pH in the composting of a mixture of date palm wastes, date palm pits and shrimp and crab shell wastes in vessel system ranged between 7.4 and 8.4 (Khiyami et al. 2008).

Moisture content has significant effects on enzyme activities and microbial respiration of the composting process (Horng 2003; Margesin et al. 2006). A moisture content of 50–60% is ideal for composting and microbial activity (Horng 2003). Water content depends on the properties of the organic components within the composting mixtures (Li and Jang 1999). In general, 50% moisture is the minimum requirement for maintaining high microbial activity (Liang et al. 2003). Very high moisture can cause the compost pile to go anaerobic and emit obnoxious odours, while low moisture will not favour the growth of microorganisms. The ratio between dry matter, water and air is an important factor in composting because raw materials have different moisture contents. Straw compost has high organic matter and can therefore retain more water. In mushroom compost, the optimum moisture is generally 75% at the time of filling, 69% at the time of spawning and 66% after spawn run (Johri and Rajni 1999; Rawat 2004). During the composting of garden wastes, the moisture content is reduced from initial 49–25% (Pedro et al. 2001). The change in moisture content affects change in temperature during composting.

An increase in electrical conductivity (8.28–33.86 mmhos cm^{-1}) occurs from zero-day to end of phase I of mushroom compost with a sharp decline to 23.11 at peak-heat stage and slight increase (25.60) at the end of phase II (Rawat 2004). The changes in oxygen concentration in the compost atmosphere between turnings represent net result of O_2 utilised by microorganisms and that replenished by convection and diffusion through compost. During the composting process oxygen is used

up quickly by the microbes as they metabolise the organic matter. The change in O_2 concentration is related with temperature. In the centre of compost pile, where the temperature is highest, anaerobic conditions exist. When the stack is turned, heat loss occurs and the resulting temperature of about 50°C stimulates microbial activity and depletes oxygen. Turning of the stack also results in a change in physical environment whereby the existing temperature gradients are disturbed. The loss and recovery of oxygen in the compost atmosphere is thus largely due to combined influence of temperature, which affects microbial activity and changes the ventilation pattern (Johri and Rajni 1999). As O_2 becomes depleted, the composting process slows down and temperature is declined. Steger et al. (2005) observed a rapid increase in microbial activity and biomass in the early thermophilic phase in 200 litre laboratory compost reactor at 16, 2.5 and 1% O_2, although this period was delayed at the lower O_2 concentrations. Starch and fat were the main substrates utilised at all three O_2 levels during this period. The depletion of the starch fraction coincided with the beginning of microbial biomass decrease, suggesting starch to be an important carbon substrate for the growth of thermophilic microorganisms during composting. At 16% O_2, 10Me fatty acids from actinomycetes and cyclopropyl fatty acids (from gram-negative bacteria) became more important with time, whereas 18:157t was characteristic at 2.5 and 1% O_2, indicating a more stressed bacterial community at the lower O_2 concentrations. Although adequate composting was achieved at O_2 levels as low as 2.5 and 1%, it is not recommended to compost at such low levels in large-scale systems, because the heterogeneous gas transport through the material in these systems might lead to anaerobic conditions and inefficient composting.

The CO_2 evolution on the surface of compost pile shows good correlation with microbial activity compared to the number of propagules. For example, it has been shown that whereas the total number of propagules is low during peak heating, CO_2 evolution is high due to higher rate of respiration of the abundant thermophilic microflora (Johri and Rajni 1999). Respiratory CO_2 of *Scytalidium thermophilum* was documented to be the likely reason for the growth-promotory effect of this fungus on mushroom yield (Weigant 1992).

Johri and Rajni (1999) reported that decomposition during composting was dependent upon carbon, nitrogen, lignin and carbohydrate composition of the organic material and expressed this relationship as:

$$CO_2 \text{ evolved} = \frac{\sqrt{\%\text{ carbohydrate}}}{\text{C/N residues} \times \%\text{lignin}}$$

The rate of decomposition in compost pile is maximum during the first few days followed by a decline and ceases after sometime although large quantities of cellulose and hemicellulose are still present. In mushroom compost, about 60–70% polysaccharides are consumed by compost microorganisms; however, only 15–25% of total polysaccharides are used during mushroom production. The absolute amount of lignin remains unaltered though changes occur in the degree and condensation of lignins during composting because general microflora is devoid of Basidiomycetes.

During spawn running and fruiting, about 15% of wall polysaccharides are utilised from compost, and thus, considerable amounts (17–31%) remain at the end of mushroom production (Iiyama et al. 1996).

Ishii et al. (2000) observed that, in a laboratory-scale composting process of garbage, the total dissolved organic carbon (TDOC) decreased from 30 to around 15 mg-C g (wet weight)$^{-1}$ in the first 4 days, rose once on the ninth day and then decreased gradually. The organic and amino acids (except for acetate) declined in the first 9 days and were exhausted after 9 days. In contrast, acetate increased in the first 9 days from 0.1 to 0.6 mg-C g (wet weight)$^{-1}$ and was then exhausted after 13 days. The sum of the organic acids measured decreased from 5.6 to 0.3 mg-C g (wet weight)$^{-1}$ for the first 4 days and were exhausted after the curing phase. The water-soluble humic substance began to increase in the curing phase and maintained its maximum value of 11 mg-C g (wet weight)$^{-1}$ until the end of the experiment.

The particle size also influences composting. The ideal particle size is around 2–3 in.. In some cases like composting of grass clippings, the raw material may be too dense to permit adequate airflow or may be too moist, and therefore, a bulking agent (straw, dry leaves, paper, cardboard, etc.) should be added to allow for proper ventilation. Mixing materials of different sizes and texture also helps in aerating the compost.

The rate of decomposition is markedly influenced by size as well as C:N ratio of the composting material. The variation in composition (% by dry weight basis) of municipal compost to garden composts ranges from 25.0 to 80.0 (organic matter), 8.0- to 50.0 (carbon) and 0.4 to 3.5 (nitrogen); a C:N ratio of 30:1 is considered ideal for the activity of most microbes. The composting period is governed by a number of factors including temperature, moisture, oxygen, particle size, the carbon-to-nitrogen ratio and the degree of turning involved. Generally, effective management of these factors will accelerate the composting process and allows compost to be prepared in 2–3 weeks (Johri and Rajni 1999).

5.5 Microbial Spectrum of Compost

Compost is a unique man-made ecosystem which harbours a complete spectrum of microbial diversity. The microbial abundance, composition and activity change substantially during the composting process and are correlated with high microbial diversity and low activity in matured compost.

Microbial community succession during composting is a classical example of how the growth and activity of one group of organisms can create conditions necessary for the growth of others. Several generations of microorganisms succeed each other during composting wherein each crop of microbial form utilises the available material in the substrate as also the cellular components of its predecessors for growth, spread and sustenance. Compost stability is strongly related to microbial activities during the composting process. Microbiological parameters can serve as indicators of compost maturity (Eiland et al. 2001; Benito et al. 2003). The study of community structure and diversity by various workers (Straatsma et al. 1994a; Beffa et al. 1996; Peters

et al. 2000; Rawat et al. 2005) has been instrumental in manipulating the compost environment in order to quicken the composting process and to improve the compost quality.

5.5.1 Structural Diversity

The compost is a rich reservoir of microbial types, comprising of mesophilic and thermophilic bacteria, fungi and actinomycetes. Bacteria are the most common of all the microorganisms found in the compost, but the predominant component is fungi. Protozoa help consume bacteria and fungi, balancing out the composting cycle.

The mesophilic microflora forms the pioneer community which rapidly breaks down soluble, readily degradable compounds, resulting in production of heat which raises the temperature of compost and thus paves the way for thermophilic microflora at or above 45°C; the latter forms the climax community of compost. During the thermophilic phase, high temperature accelerates the breakdown of proteins, fats and complex carbohydrates like cellulose and hemicellulose, the major structural molecules in plants. As the supply of these high-energy compounds becomes exhausted, the compost temperature gradually decreases and mesophilic microorganisms once again take over for the final phase of 'curing' or maturation of the remaining organic matter. The structural divergence and species distribution are probably most significantly affected by temperature distribution. Thermophilic forms exhibit less structural divergence compared to mesophilic counterparts (Takaku et al. 2006).

Bacillus has been documented to be the predominant bacterial component of majority of compost ecosystems (Strom 1985; Libmond et al. 1995; Rawat 2004). The diversity of bacilli is fairly high at temperatures from 50 to 55°C but decreases dramatically at 60°C or above. In composting of table scraps, 87% of isolates, recovered between 49 and 67°C, were *Bacillus,* namely, *B. circulans*, *B. coagulans*, *B. licheniformis, B. stearothermophilus* and *B. subtilis* (Strom 1985). The predominance of *Bacillus,* namely, *B. pallidus*, *B. stearothermophilus* and *B. thermodenitrificans,* in hot composts was also characterised using amplified ribosomal DNA restriction analysis (ARDRA) by Blanc et al. (1997).

In garden and domestic waste composts, 76.1% of the thermophilic bacterial flora isolated from thermogenic, post-thermogenic and maturation phase of compost was assigned by their RAPD (randomly amplified polymorphic DNA) profiles to just two species, *B. licheniformis* and *B. thermodenitrificans.* The other species assigned by RAPD profiles were *B. sporothermodurans* (4.9%), *B. thermosphaericus* (7.4%) and *Terrabacter tumescens* (5.0%). The phylogenetic analysis revealed that the two most abundant isolates belonged to the genera *Aneurinibacillus* and *Brevibacillus*, which are not commonly associated with the hot composts. The clone library contained sequences that clustered within the genus *Bacillus*, an exception was a *Lactobacillus*-type sequence, but none of the sequences could be assigned to

obligate thermophilic *B. stearothermophilus* which is commonly believed to dominate the hot compost (Dees and Ghorse 2001). In municipal solid waste compost, staphylococci dominate during the mesophilic phase and at the beginning of the thermophilic phase, whereas bacilli predominate during the remainder of the compost cycle (Hassen et al. 2001).

Schloss et al. (2003) observed two significant shifts in the composition of the microbial community, one between 12 and 24 h and the other between 60 and 72 h of composting using automated 16S–23S rRNA intergenic spacer amplification (ARISA). They found that sequences related to lactic acid bacteria were most common during the first 60 h and *Bacillus*-type sequences were most common between 72 and 96 h. *Bacillus* and *Paenibacillus* spp. and members of Enterobacteriaceae were observed to be predominant bacteria during composting of sugar cane bagasse and coast-cross straw compost prepared for the production of *Agaricus brasiliensis* (Silva et al. 2009).

In mushroom compost bacteria comprise of much lower proportion of total biomass although it is difficult to define the specifics of the domineering species (Johri and Rajni 1999). Straatsma et al. (1994a) have however reported a biomass ratio of 1:1.8 of bacteria to fungi after phase II of composting, while according to Weigant (1991) the ratio is 1:0.9 in conventional compost and 1.0:2.3 in the experimental compost. In mushroom compost maximum bacterial diversity at 50°C was found among morphotypes of third turning of phase I (H'=0.83), least among those of peak-heat stage of phase II (H'=0.02), while maximum species richness was at first turning of phase I compost (R1=5.54 and R2=4.45) and least during third turning of phase I (R1=2.36 and R2=2.08). Thermophilic bacterial morphotypes isolated at 65°C were less structurally diverse as compared to those isolated at 50°C. Maximum diversity at 65°C was found among morphotypes of third turning of phase I (H'=0.63), while second turning of phase I was least diverse (H'=0.00) but rich in species (R1=4.18 and R2=3.14); peak-heat stage was least rich in species make-up (R1=0.58 and R2=1.17) (Rawat 2004).

Bacillus megaterium, *B. subtilis* and *B. licheniformis* (AY 871062) had been sequenced from zero-day mushroom compost; *B. licheniformis* (AY 871063) from end of phase I compost; *B. subtilis* (AY 871054) and *B. cereus* (AY 871057) from pasteurisation; and *B. subtilis* (AY 871059) from end of phase II compost. Other bacterial species sequenced were *Ochrobactrum* sp. from conditioning and *Arthrobacter arilatiti* (AY 871058) from end of phase II compost (Agarwal 2006).

In a monitored composting bin system, bacilli were present only in fresh wastes, and during the peak-heating phase of biowaste (vegetable, fruit and garden waste) composting, the bacterial community structure based on denaturing gradient gel electrophoresis (DGGE) pattern during the steady state of semicontinuous treatment of organic solid waste showed DNA sequence, closest to *Bacillus licheniformis*. Fluorescence *in situ* hybridisation (FISH) analysis showed that the bacterium corresponding to the major band accounted for 30% of total eubacterial cell count at the steady state, indicating it to be the key microorganism in the biodegradation process. Addition of the compost to soil caused a substantial shift in community

composition in soil, mainly within the members of the *Cytophaga–Flavobacterium–Bacteroides* group (Rawat and Johri 2002).

Beffa et al. (1996) reported for the first time the presence of genus *Thermus* in thermogenic (65–82°C) composts taken from 2- to 5-week-old organic waste samples. Majority of the isolates were probably *Thermus* strains, which had adapted to the conditions prevalent in hot compost ecosystem. The nutritional characteristics, total protein profiles, DNA–DNA hybridisation and restriction fragment length polymorphisms (RFLP) profiles of 16S rDNA showed that *Thermus* strains isolated from hot composts were closely related to *T. thermophilus* HB8 except strain JT4. All isolates except JT4 showed 65–71% DNA–DNA homology with *T. thermophilus* and 50–62% with *T. aquaticus* and poor DNA–DNA homology with *T. filiformis* (39–48%) and *T. ruber* (30–40%). Isolate JT4 showed 72% homology with *T. aquaticus*. The isolates were found to have similar RFLP profiles as *Thermus thermophilus* HB 8, while strains of *T. thermophilus*, *T. aquaticus*, *T. filiformis* and *T. ruber* showed different RFLP profiles. Miyatake and Iwabuchi (2005) observed *Thermus* spp. and *Bacillus* spp. at 66°C in cattle manure compost.

The raw and cured compost samples from a large-scale urban composter contained metal- and antibiotic-tolerant bacteria (<log 3.0 to log 8.5 CFU g^{-1} compost) as well as high numbers (as high as log 7.4 CFU g^{-1} dry weight compost) of thermophilic bacteria. Biolog profiles indicated a wide diversity of gram-negative bacteria, namely, *Klebsiella*, *Pseudomonas*, *Serratia* and *Xanthomonas.* A gram-positive bacterium, *Corynebacterium jeikeium*, was also isolated from cured compost (Andrews et al. 1994). A moderately high number of obligately autotrophic *Hydrogenobacter* spp. and facultatively autotrophic *Bacillus schlegelii* growing at temperatures above 70°C were isolated from hot composts (Beffa et al. 1996). Low G+C gram-positive bacteria constituted a dominant fraction of the bacterial community during hot composting (55–70°C) phase (Alfreider et al. 2002).

In spent mushroom compost, sequencing of the 16S rDNA amplicon assigned 12 of the 14 OTUs to gram-positive bacteria, associated with the genera *Arthrobacter*, *Bacillus*, *Brevibacterium*, *Carnobacterium*, *Desemzia*, *Exiguobacterium*, *Microbacterium*, *Paenibacillus* and *Staphylococcus* of the bacterial divisions Firmicutes and Actinobacteria. Two bacterial groups had phylogenetic links with the genera *Comamonas* and *Sphingobacterium*, belonging to β-Proteobacteria and Bacteroidetes, respectively. Two potentially novel bacteria associated with the genera *Bacillus* and *Microbacterium* were reported (Ntougias et al. 2004). Sequences similar to Acetobacteria and *Lactobacillus* were frequently detected in the early stages of drum composting. In tunnel stages of composting, the bacterial community comprised of Actinobacteria, *Bacillus*, *Lactobacillus* and *Thermoactinomyces* (Hultman 2009).

Partanen et al. (2010) observed that over 500 sequences, out of 1,500 full-length 16S rRNA gene sequences, were present only as singletons in both pilot- and full-scale composting plants. Most of the similar sequences observed can be divided into six main groups: *Acetobacter*, Actinobacteria, *Bacillus*, *Clostridium*, *Lactobacillus* and *Thermoactinomyces*. The abundance of *Acetobacter* and *Lactobacillus* groups was found to be related to low pH, whereas the presence of Actinobacteria was related to the age, i.e. time elapsed after the feeding of composting material.

Actinomycetes, though not a predominant component, yet constitute an important microbial component of the composting process and contribute extensively by their ability to produce antibiotics and enzymes and the ability to degrade complex and recalcitrant molecules especially lignocellulosics. Some species appear during the thermophilic phase, while others become important during the cooler curing phase, when only the most resistant compounds remain in the last stages of the formation of humus.

Thermophilic actinomycetes grow extensively during phase II of mushroom compost as evident from the white wefts of mycelium ('fire fang') in the compost. *Nocardia brasiliensis*, *Pseudonocardia thermophila*, *Saccharomonospora viridis*, *Saccharopolyspora rectivirgula*, *Streptomyces diastaticus*, *Streptomyces griseus*, *Streptomyces rectus*, *Streptomyces thermoviolaceus*, *Streptomyces thermovulgaris*, *Streptomyces violaceoruber, Thermoactinomyces chromogena*, *Thermoactinomyces curvata*, *Thermoactinomyces fusca*, *Thermoactinomyces thalophilus* and *Thermoactinomyces vulgaris* have been reported from phase II compost (Fergus 1964; Kleyn and Wetzler 1981).

Actinomycete population in composts is often dominated by the species of *Thermomonospora* (McCarthy and Broad 1984). *T. alba, T. curvata*, *T. lineata* and *T. vulgaris* constitute the dominant component of domestic waste compost (Resz et al. 1977). Lacey (1973) observed the predominance of *Saccharomonospora*, *Saccharopolyspora rectivirgula*, *Streptomyces* spp., *Thermoactinomyces* spp. and unidentified white thermophiles in experimental composts indoors, while during turning of compost outdoors, *Saccharomonospora* sp., *Streptomyces* spp., *Thermoactinomyces* spp. and *Thermomonospora* spp. were the dominant components; population sizes ranged from 1.8 to 4.9×10^5 cfu m^{-3} air.

Streptomyces spp. was dominant in composts made from lucerne, oat straw and maize stalks, while *Thermoactinomyces vulgaris* was dominant in grass composts (Lacey 1973). During urban waste composting, streptomycetes represented the most abundant taxa. *Amycolata, Microtetraspora*, *Thermomonospora chromogena* colonies and a new form *Thermocrispum* have also been reported (Korn-Wendisch et al. 1995). *Thermomonospora* spp., including *Thermomonospora chromogena* and *Microtetraspora,* were less numerous than is sometimes found in mushroom compost. The populations at shredding stage were less thermotolerant than those dispersed from compost piles. Rawat (2004) reported that in mushroom compost the population count ($\log_{10}$CFU) of thermophilic actinomycetes varies from 3.42 to 6.48 till the end of phase I of composting and from 6.34 to 6.15 during phase II composting. Actinomycetes, and especially members of the genus *Streptomyces*, are well represented with a population density of $2–3 \times 10^8$ CFU g^{-1} during composting of sugar cane bagasse and coast-cross straw compost prepared for the production of *A. brasiliensis* (Silva et al. 2009).

Fungi are the most predominant component of compost. *Aspergillus*, *Chaetomium*, *Humicola*, *Mucor*, *Penicillium* and *Thermomyces* are the dominant fungi of compost ecosystems. Species of *Aspergillus* and *Mucor* are predominant in composting of biowaste (Ryckeboer et al. 2003). *Aspergillus fumigatus* and *Humicola grisea* var. *thermoidea* have been reported to be the dominant members of the spent mushroom compost. Other fungi reported from spent mushroom compost are *Aspergillus flavus,*

Aspergillus nidulans, Aspergillus terreus, Aspergillus versicolor, Chrysosporium luteum, Mucor spp., *Nigrospora* spp., *Oidiodendron* spp., *Paecilomyces* spp., *Penicillium chrysogenum, Penicillium expansum, Trichoderma viride* and *Trichuris* spp. (Kleyn and Wetzler 1981).

In paddy straw compost, *Chaetomium thermophile*, *Humicola* spp. and *Sporotrichum thermophile* have been reported to be abundantly present (Satyanarayana 1978). Antagonism appears to play a significant role in determining the population structure. The volatiles of *Chaetomium thermophile* and *Sporotrichum thermophile* can inhibit conidial germination of *Humicola lanuginosa* by impairing essential metabolic processes, whereas *Chaetomium thermophile* suppresses mycelial growth of *Humicola lanuginosa* and *Torula thermophila.* However, effect of the fungistatic volatile factors in compost ecosystem is only marginal in view of high temperature at which they grow (Johri and Rajni 1999).

Satyanarayana and Grajek (1999) observed that the colonising ability of thermophilic fungi on paddy straw was directly proportional to the inoculum concentration. For example, colonisation by *Humicola lanuginosa, S. thermophile* and *T. thermophila* increased with higher inoculum dose. During peak-heating period, only a few thermophilic fungal propagules were present exhibiting high rate of respiration. However, Johri and Rajni (1999) reported that thermophilic fungi were not present at peak high temperature in wheat and broad bean straw composts. When it cooled down to 51.5°C, *Myriococcum albomyces, Penicillium dupontii* and *S. thermophile* were found in abundance.

Thermophilic fungi grow extensively during the last phase of composting in mushroom compost from the spores that survive the pasteurisation temperature (Straatsma et al. 1989). Thus, they contribute significantly towards the quality of compost. However, their presence throughout the course of composting is largely responsible for the maintenance of biological equilibrium that ultimately leads to unique selectivity wherein *A. bisporus* multiplies without competition. These fungi influence growth of *A. bisporus* at three distinct levels (Weigant 1992): First, they decrease concentration of ammonia in compost which otherwise would counteract the growth of the mycelium. Second, they immobilise nutrients in a form, which improves apparent availability to the mushroom mycelium. Third, they exert direct growth-promotory influence on the mushroom mycelium, namely, *S. thermophilum*. The course of fungal succession is partially dependent on the ecophysiological conditions in compost (Satyanarayana et al. 1992).

The pioneer thermophilic mycoflora of mushroom compost comprises of fast-growing and rapidly sporulating fungi such as *Aspergillus fumigatus* and *Rhizomucor* spp. with a pH optima below 7.0 and temperature optima of about 40°C. When self-heating and ammonification starts and pH reaches 9.0, the pioneer flora disappears and paves way for *Talaromyces thermophilus* and *Thermomyces lanuginosus*; during massive heat production these fungi possess moderate growth rate, as they exhibit high thermal death point and pH tolerance, but do not degrade cellulose. At the end of the composting process, about 50–70% of the compost biomass is constituted by thermophilic fungi (Sparling et al. 1982; Weigant 1992). While most of the species are eliminated, *S. thermophilum* appears as near-exclusive species after

phase II composting and constitutes a climax species in the mushroom compost along with thermophilic actinomycetes (Straatsma et al. 1994b). The number of CFU of *S. thermophilum* in fresh matter of phase II is about 10^6 g^{-1} compost (Bilai 1984); however, actinomycetes and bacteria appear to play a decisive role in successful colonisation by this thermophile. The presence of *S. thermophilum* throughout the composting period, i.e. from zero day, dominance during phase II and at the end of phase II is supported by its relative abundance (0.68) as observed by Rawat (2004) and earlier observations of Straatsma et al. (1994a) on the subject.

In mushroom compost maximal diversity among thermophilic fungal morphotypes is observed with fourth turning of phase I compost (H'=2.14, E1=0.89 and D=8.88) and least by peak-heat stage morphotypes (H'=0.75 and D=1.80). End of phase I is most rich in species make-up (R1=4.07 and R2=3.37). Fourth turning of phase I compost is maximally diverse for thermophilic community. The least fungal diversity is observed at peak-heat stage (Rawat 2004). This is not unusual since only limited fungal species spectrum has been reported from this stage of composting (Straatsma et al. 1994a).

In the beginning of phase II of mushroom compost, thermophilic fungi and actinomycetes extensively colonise the plant matter until temperature reaches 60°C, as an outcome of slow peak heating for about 2 days (Straatsma et al. 1994a). The high temperature of the first indoor period of phase II kills most of the pathogenic and nonpathogenic microorganisms, except the spores of actinomycetes and thermophilic fungi such as *Scytalidium thermophilum* (Straatsma et al. 1991); the latter was most abundant at the end of phase II compost (abundance=0.68) (Rawat 2004). Klamer et al. (1998) reported *A. fumigatus* and *Rhizomucor pusillus* as predominant species before peak heating and *P. variotii*, *S. thermophilum* and *T. lanuginosus* as dominant forms after peak heating. Tewari (2000) reported the presence of *H. lanuginosa* and *S. thermophilum* during peak-heat stage of phase II composting.

Thermophilic fungi of the *Torula–Humicola* complex are a necessary and dominant component of the community in mushroom compost during phase II. *S. thermophilum* is a natural inhabitant of compost ingredients, including drainage from compost, and has been documented to be present throughout composting. Dominance of *S. thermophilum* has been reported by several workers (Straatsma et al. 1991; Vijay 1996; Klamer et al. 1998; Rajni et al. 1998), while *H. grisea* var. *thermoidea* and *H. insolens* have been described by others (Fergus 1964). They are inherently close partners in the degradation processes in compost and provide selectivity to compost (Straatsma et al. 1989; Opden Camp et al. 1990). Rajni et al. (1998) and Rawat (2004) observed nearly similar microbial distribution pattern in compost as reported by Straatsma et al. (1991) with predominance of *S. thermophilum* although inputs in the European and Indian composts are substantially different. In 20-day schedule of compost preparation, *S. thermophilum* was detected from the first turning (i.e. after fifth day of composting) till the fifth turning (i.e. 17th day), whereas species of *Paecilomyces* were present only during the last turning, i.e. 20th day. Two isolates of *Malbranchea cinnamomea* were recorded between the second and fifth turning stage (8th to 17th day). The presence of *Paecilomyces* sp. and *M. cinnamomea*, which are normally slow growers, provided an opportunity to evaluate their

influence on *in situ* mycelial extension of *A. bisporus*. At 24th day of composting, the population of *S. thermophilum* was 10^8 propagules g^{-1} of compost.

The genetic variation exhibited by *Torula–Humicola* complex has drawn wide attention. Azevedo et al. (1999) distinguished homokaryotic strains of *Humicola grisea* var. *thermoidea* in two groups displaying uniform DNA profile reflecting heterogeneity in the wild genome using RAPD analysis. Straatsma and Samson (1993) studied the genetic diversity among *Scytalidium thermophilum* isolates using RAPD analysis. These strains exhibited a distinct pattern of amplified DNA bands. Rajni (1999) studied the genetic diversity between the black and white strains of *S. thermophilum* by RAPD analysis. A distinct band pattern was exhibited by these isolates. The protein profiling of *Torula–Humicola* complex exhibited that *S. thermophilum* was more closely related to *H. grisea* var. *thermoidea,* while the latter was similar to *H. insolens* than *H. lanuginosa*; *H. insolens* and *H. lanuginosa* were more distantly related. The RAPD analysis and sequence analysis of ITS region of rDNA exhibited wide genetic variation in *Torula–Humicola* complex (Lyons et al. 2000). RAPD analysis of 34 geographically diverse isolates revealed two distinct groups showing differences in the banding pattern. An examination of the genetic distance matrix indicated differences between isolates belonging to *S. thermophilum* cultural types 1 and 2. The sequence analysis of ITS 1, 5.8 S and ITS 2 region of rDNA suggested high homology between the isolates with minor sequence variation. Genetic distance values, among type 1 and 2, varied by a value of 0.005%. The RAPD groupings mirror closely the morphological and thermogravimetric data for *S. thermophilum* isolates and provide further evidence of the variation, which exists between the species complex (Straatsma and Samson 1993; Lyons and Sharma 1998). Isolates of *S. thermophilum* bear close similarity to those of *H. grisea* var. *thermoidea* and *H. insolens*. RAPD analysis showed that a majority of structurally or functionally dominant fungal species recovered from different stages of composting belonged to *Torula–Humicola* complex (Rawat 2004). During composting of sugar cane bagasse and coast-cross straw compost, prepared for the production of *A. brasiliensis*, the filamentous fungi exhibited much lower population densities and were less diverse than other microorganisms, although *A. fumigatus* was present during the whole composting process and after pasteurisation (Silva et al. 2009).

The fungal diversity was found to be high and phylotypes similar to yeasts were abundantly found in the full-scale drum and tunnel processes. In addition to phylotypes similar to *Candida*, *Geotrichum* and *Pichia*, moulds from genus *Penicillium* and *Thermomyces* were also observed in tunnel stages of composting. Zygomycetes were detected in the pilot-scale composting processes and in the compost piles. Basidiomycota became abundant in the cooling and maturation phase of the compost (Hultman 2009).

5.5.2 Microbial Community Succession

The genetic profiling techniques which are cultivation independent have great potential in identifying the population structure and community succession. These techniques utilise DNA or RNA extracted directly from environmental samples and

amplification of signature genes by PCR or reverse transcription-PCR (RT-PCR) with primers, bind to conserved regions and produce homologous gene fragments. The products can be subsequently analysed to determine their nucleotide differences by techniques such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), terminal restriction fragment length polymorphism (t-RFLP) and single-stranded conformation polymorphism (SSCP) (Muyzer et al. 1993; Lee et al. 1996; Liu et al. 1997; Schwieger and Tebbe 1998). SSCP has the potential to be more easily applied in contrast to DGGE and TGGE, as no GC clamps or construction of gradient gel is required (Lee et al. 1996). However, it is quite essential to compare diversity results obtained by both cultivation-dependent and cultivation-independent methods for better understanding and for eliminating the biases associated with genetic profiling.

Kowalchuk et al. (1999) detected β-subgroup proteobacterial ammonia oxidiser-like sequences in commercial mushroom compost by separating the products of PCR and RT-PCR by DGGE and identifying them by hybridisation with a hierarchical set of oligonucleotide probes designed to detect ammonia oxidiser-like sequence clusters. However, there are known examples of thermophilic ammonia-oxidising bacteria; cell activity is probably impaired or destroyed by high temperatures (Focht and Verstraete 1977); thus, cell survival at high temperatures may be facilitated by the formation of microniches, (Derikx et al. 1990) and the moisture content may contribute towards cell survival (Gromov and Pavlenko 1989).

Microbial succession analysed in a laboratory-scale composting process of garbage by DGGE profiles revealed bacteria closely related to *Leuconostoc paramesenteroides*, *Pediococcus acidilactici* and *Staphylococcus pisciferm entans* in the mesophilic phase till 4th day; *Bacillus* especially *B. badius* or *B. coagulans* were predominant in thermophilic phase. *Corynebacterium urealyticum*, *Gracilibacillus halotolerans* and *Virgibacillus proomii* were present. In the curing phase, sequences were related to *Alcaligenes* sp., *Alloiococcus otitis*, *Clostridium fervidus*, *Clostridium limosum* and *Sphingobacterium multivorum*. In maturation phase DNA sequence related to *Arthrobacter* sp. was present (Ishii et al. 2000).

DGGE and 16S rDNA analyses of microbial community of garden wastes in a field-scale composter revealed that *Bradyrhizobium* sp., *Propionibacterium* sp., *Pseudomonas* sp. and *Methylobacterium* sp. were present throughout the process. Different *Bacillus* spp. thrived at the thermophilic or the mesophilic stage, while *Clostridium* sp. was only found at the initial phase of the process. *Caulobacter* sp. and *Staphylococcus* sp. existed during the later phase of the composting period (Pedro et al. 2001).

Poulsen et al. (2005) observed that the genetic and functional diversity of the indigenous microbial communities in compost samples could be linked using DGGE and simultaneously measuring the enzymatic activity of chitinase. Two types of compost, a garden compost and a household waste compost, showed different genetic diversity as measured by PCR-DGGE of total DNA extracted from the composts and also had different chitinase activity, 0.46 and 3.97 μmol 4MU $h^{-1} \times g$ dry matter, respectively. The addition of chitin in the composts induced a change in both the bacterial and fungal genetic diversity when compared to the non-amended

compost samples. The N-mineralisation in the household waste compost was apparently increased by the addition of chitin, while such an effect was not observed in the garden compost.

In garbage composting, the DGGE profiles and clone library analysis revealed that the microbial community drastically changed during the composting process from the thermophilic to the maturing stages. The dominant bacterial group changed from the phylum Firmicutes in the thermophilic stage to the phylum Bacteroidetes in the maturing stage (Takaku et al. 2006). Time-dependent changes, in the structure of the actinobacterial community in composting of organic household wastes, were revealed by the DGGE analysis of 16S rRNA genes after nested PCR with Actinobacteria-specific primers (Steger et al. 2007a). As composting proceeded, actinobacterial communities changed mainly between the 6th and 13th weeks and from the 29th week onwards. A band that only occurred at the sixth week had a sequence closely affiliated to the genus *Corynebacterium*. Two major bands at the beginning became weaker during the process and contained sequences that were closely related to *Thermobifida fusca*. Other bands had sequences that were affiliated to the genera *Saccharomonospora*, *Saccharopolyspora* and *Streptosporangium*. In the later stages of composting (37th–57th weeks), members of the genera *Actinomadura*, *Microbacterium*, *Streptomyces* and *Thermocrispum* were also found in the material. A rather weak band at the 47th week appeared as the dominant band at the 57th week and was identified as *Arthrobacter*.

Actinobacteria are believed to play a major role in organic matter degradation and humification processes in composts. Temperature is an important selective factor for the development of Actinobacteria populations in composts, and they constitute a substantial part of the community in the later compost stages. Based on DGGE and sequencing of 16S rRNA genes with Actinobacteria-specific primers, a shift from members of *Corynebacterium*, *Rhodococcus* and *Streptomyces* at the onset of composting to species of thermotolerant Actinobacteria like *Saccharomonospora viridis*, *Thermobifida fusca* and *Thermobispora bispora* in the cooling phase occurs (Steger et al. 2007b).

The three methods based on 16S rRNA gene sequence, namely, DGGE of PCR-generated rRNA gene fragments; partial rRNA gene clone libraries; and a microarray of oligonucleotide probes targeting rRNA gene sequences, applied to monitor changes in the microbial communities in the compost prepared from sewage sludge and yard waste, indicated distinctive community shifts during curing of compost. Proteobacteria were the most abundant phylum in all cases; Bacteroidetes and Gammaproteobacteria were ubiquitous. During the midcuring stage, Actinobacteria were dominant. Different members of nitrifying bacteria and cellulose and macromolecule-degrading bacteria were found throughout the curing process. In the cured compost, bacterial population shifts were observed after the composted organic matter and other biochemical properties had stabilised (Danon et al. 2008).

In a large-scale completely mixed composting reactor using plastic bottle flakes as bulking agent, DGGE analysis and physico-chemical parameters revealed that bacterial community succession occurred in four phases: (1) at the start of operation and pH decreasing period (day 0–3); (2) pH decreasing and increasing period (day

4–11); (3) middle term, moisture content decreasing and maximum temperature increasing period (day 12–16); and (4) latter term, temperature decreasing period (day 17–24). *Lactobacillus* spp. and *Bacillus coagulans* were detected from the initial phase and middle term, respectively. 16S rDNA clone analysis showed that the dominant bacteria shifted from the order Lactobacillales to Bacillales and Actinomycetales (Watanabe et al. 2009). DGGE analysis in a composting reactor operated with woodchips as bulking agent revealed that the dominant bacteria were members of the order Bacillales. However, these aerobic bacteria decreased to 14%, and anaerobes or facultative anaerobes arose when the decomposition rate of organic compounds dropped following aggregation of the contents (Watanabe et al. 2010).

A study of community succession during 18-day-long mushroom compost using SSCP along with sequencing of subcloned products revealed the presence of lactobacilli at the early stage of composting (Peters et al. 2000). Of 12 molecular isolates detected during heating phase through primers targeting V4–V5 region of eubacterial 16S r RNA genes, 5 were closely related to the genus *Bacillus* and 1 to genus *Clostridium*. Two *Bacillus*-related isolates could be detected throughout the composting process. One of these isolates showed perfect homology to a known 16S rRNA gene sequence (*B. badius*), but the other had only 92.3% identity to a known sequence. This dominant isolate was possibly related to a group of gram-positive bacteria with a low G + C DNA content, not characterised yet. Two sequences were related to γ-Proteobacteria. Four of the five molecular isolates, obtained using primers targeting V3 region of 16S rRNA genes of actinomycetes, were closely related to *Detolaasinbacter* sp. (99.0%), *Streptomyces nodosus* (98.3%), *S. thermodiastaticus* (100.0%) and *Streptosporangium vulgare* (98.2%). These four sequences were detected throughout composting, but their intensities, especially those related to *S. nodosus* and *S. thermodiastaticus,* increased continuously during the process. The two dominant products detected in SSCP profiles of 18S rRNA gene-targeted PCR were related to two yeast sequences, both members of the genus *Candida*.

Alfreider et al. (2002) observed a number of Enterobacteriaceae and members of the genus *Lactobacillus* in the initial composting stage at 41°C; low G + C gram-positive bacteria constituted a dominant fraction of the bacterial community during hot composting phase (55–70°C). The community succession of actinomycetes in mushroom compost was investigated by Peters et al. (2000) employing SSCP wherein four of the five molecular isolates were closely related to *S. nodosus*, *S. thermodiastaticus*, *S. vulgare* and *Detolaasinbacter* sp.

SSCP profiles of 18S community of different stages of mushroom compost revealed a total of 95 distinct bands (Rawat 2004). The banding pattern of community underwent a rapid change with the onset of composting; this corroborated well with the reports of Peters et al. (2000). Zero-day compost samples consisted of many faint bands which disappeared with the onset of composting; during peak-heat stage only prominent bands were present. Principal component analysis (PCA) of SSCP profiles revealed that the two samples of zero-day compost (PWI and PWII) were quite similar, while different stages of phase I compost shared nearly identical profile pattern; patterns of cropping sequences were quite similar to each

Fig. 5.1 PCA of 18S community of different stages of mushroom compost

other (Fig. 5.1). The community profiles of phase I compost were found to be identical to each other; maximum diversity was observed during end of phase I compost, casing soil and cropping stage (H'=0.37) and least at peak-heat stage (H'=0.22).

The dendrogram (Fig. 5.2), based on Jaccard's similarity coefficient, exhibited a 15–66% similarity among the 18S community of different stages of mushroom compost and revealed that the community of all the samples could be divided into three main groups (Rawat 2004). Group I comprised of zero-day (PWI and PWII) community exhibiting 26% similarity. Group II could be further subdivided into two groups: The first subgroup comprised of phase I community. Second (T2) and third (T3) turning community shared maximum similarity at 66% level, while first turning (T1) community shared a similarity of 32% with that of second and third community. Fourth turning (T4) and end of phase I (filling) community showed 51% similarity with each other. The second subgroup comprised of peak-heat, conditioning and end of phase II compost (spawning) community. Peak-heat and conditioning communities shared 38% similarity with each other and 30% similarity with end of phase II compost community.

The overall similarity between the two subgroups was 30%. The second group had casing soil, cropping, airing and drenching communities. PWI, PWII, T2, T4, spawning, cropping, airing and drenching community shared a maximum similarity of 66% with each other and 44% similarity with spawn-run community. Drenching community shared 42% similarity with that of spawn-run, cropping and airing community. Casing community showed 22% similarity with cropping, airing and drenching community. The similarity among these three subgroups was quite low, 16% between group I and group II, while they both shared 15% similarity with group III.

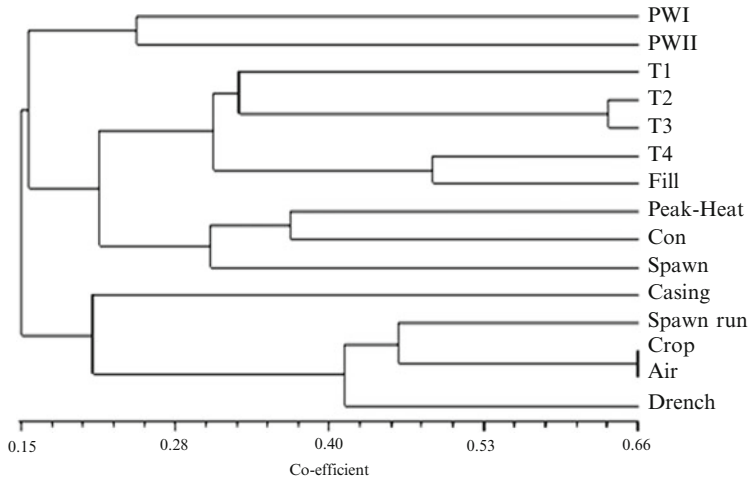

Fig. 5.2 Cluster analysis of 18S community of different stages of mushroom compost based on Jaccard's coefficient

Klamer et al. (1998) studied the succession of mycoflora during 8 months of composting of *Miscanthus* straw and pig slurry in well-insulated containers. Before peak heating, *A. fumigatus* and *R. pusillus* were dominant. Forms developing after peak heating could be divided into two groups: those appearing from day 15 to 27 and others developing from day 50 to 225. The first group was dominated by *Paecilomyces variotii, Scytalidium thermophilum* and *Thermomyces lanuginosus,* and the second by *Acremonuim* spp. and *Thermomyces lanuginosus.* The Brillouin diversity index changed with temperature; diversity was high before peak heating, low during elevated temperature and increased again during the third phase of composting. Temperature was the main controlling parameter that changed fungal community during the first month of composting. Based on principal component analysis (PCA), it could be concluded that when the temperature reached an ambient level, only minor change in fungal community was detected although some changes in certain species were still discernible.

The study of physiological diversity of compost by techniques like phospholipid fatty acid analysis (PLFA) and metabolic fingerprinting has been instrumental in tracking changes in microbial communities and thus understanding the *in situ* community structure (Garland and Mills 1991; Petersen et al. 1991; Kennedy and Busacca 1995; Inssam et al. 1996; Boggs et al. 1998; Campbell and Cooper 1999; Cahayani et al. 2002). The PLFA markers of bacteria (15:0 and 17:0) were observed to decline over time along with an indicator of aerobic bacteria, i.e. 16:1 ω7C. Neither of the markers of fungi, i.e. 18:2 ω6C and 18:3 ω6C, changed significantly over time, indicating that the proportion of fungi in the community varies little with compost maturation although the type of fungi present or active at any stage could vary significantly (Boggs et al. 1998). Cahayani et al. (2002) observed that biomarkers of gram-positive bacteria and actinomycetes were predominant at the thermophilic stage (i.e. first 2 weeks) and those of gram-negative bacteria at the curing stage.

The time-dependent change in the PLFA composition in composting of organic household wastes was due to a shift from fatty acids typical for eucaryotic cells towards iso- and anteiso-branched bacterial fatty acids and 10-methyl-branched fatty acids, which are typical for gram-positive bacteria and/or thermophiles and Actinobacteria, respectively. Monounsaturated fatty acids were most dominant in the later compost stages and indicated the establishment of a new community of gram-negative bacteria. A similar shift in fatty acid composition was seen; the higher proportion of fatty acids derived from microorganisms at the end of composting was accompanied by a shift in their composition towards dominance of bacterial fatty acids. The concentrations of the 10-methyl-branched fatty acids typical for Actinobacteria followed approximately the same pattern of change as the total PLFA concentrations (Steger et al. 2007a).

Community analysis of composting of dairy manure and pine-shave beddings based on carbon source utilisation as tool revealed that microbial utilisation of γ-aminobutyric acid was increased over time, while histidine was utilised at similar level at all sampling times. The ability to utilise sucrose, galactose and fructose increased, while trehalose utilisation decreased during composting (Boggs et al. 1998).

Rawat (2004) studied physiological diversity of different stages of mushroom compost by 'metabolic fingerprinting' employing Biolog. Average well colour development (AWCD) was higher for mesophilic community than the thermophiles. Principal component analysis (PCA) revealed maximum similarity between first sample of pre-wetting compost (PWI) and second turning of phase I and second sample of pre-wetting compost (PWII) and airing community among mesophiles (Fig. 5.3a). PCA biplot divided mesophilic community under three clusters: Cluster I comprised of third turning, end of phase I composting, spawn-run, fourth turning, end of phase II composting, second turning, PWI and drenching community; cluster II comprised of peak-heat, casing, conditioning and cropping community; and cluster III comprised of PWII, airing and first turning community.

Thermophilic community were also divided into three clusters by PCA biplot (Fig. 5.3b): Cluster I comprised of drenching, spawn-run and PWI community; cluster II comprised of casing, second turning, end of phase I composting, first turning, third turning, peak-heat, conditioning and end of phase II (spawning) community; and cluster III comprised of PWII, cropping, airing and fourth turning community. The physiological distribution of community, however, did not correlate well with the structural distribution. Maximum physiological diversity was exhibited within the mesophiles representing zero-day community and least among peak-heat community. Among thermophiles, zero-day community was maximally diverse and conditioning community of phase II the least.

Quinone profile method has been used to track community changes in composting (Tang et al. 2004; Yu et al. 2007). Yu et al. (2007) observed that mesophilic bacteria containing MK-7 and mesophilic fungi containing Q-9 as major quinone were predominant. Actinobacteria indicated by a series of partially saturated and long-chain menaquinones were preponderant during the thermophilic period.

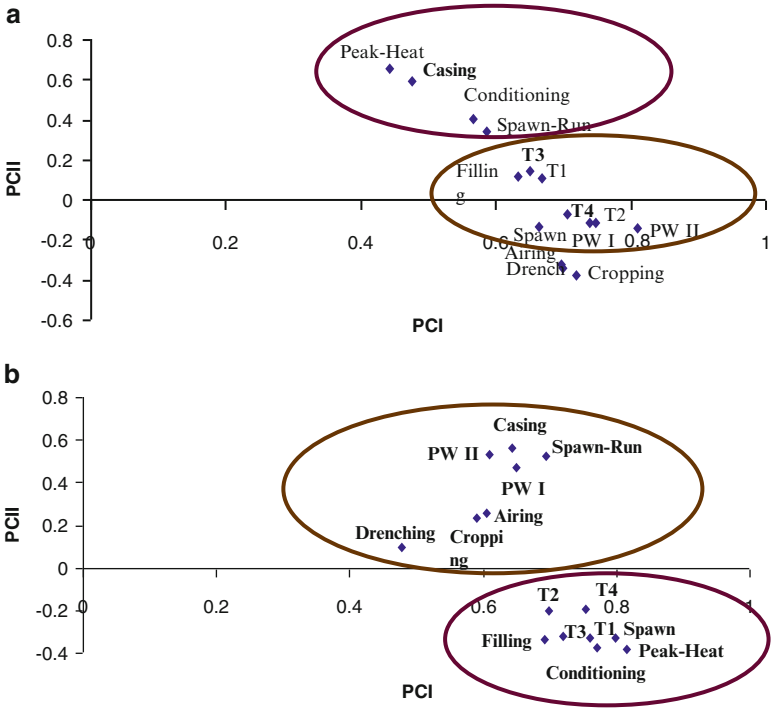

Fig. 5.3 (**a**) PCA of physiological diversity of mesophilic microflora in mushroom compost based on metabolic fingerprinting. (**b**) PCA of physiological diversity of thermophilic microflora in mushroom compost based on metabolic fingerprinting

The COMPOCHIP microarray, applied to three different compost types (green compost, manure mix compost and anaerobic digested compost) of different maturity (2nd, 8th and 16th weeks), revealed that the bacterial composition of the three composts was different at the beginning of the composting process and became more similar upon maturation. Certain probes (targeting *Sphingobacterium*, *Actinomyces*, *Xylella/Xanthomonas/Stenotrophomonas*, *Microbacterium*, *Verrucomicrobia*, Planctomycetes, low G+C and α-Proteobacteria) were more influential in discriminating between different composts (Franke-Whittle et al. 2009).

The fungal phylotypes, revealed by cloning and sequencing of fungal internal transcribed spacer (ITS) region of samples of different stages of composting in a full-scale and a pilot-scale composting reactors, could be grouped into those that dominated the mesophilic low pH initial phases (sequences similar to genera *Candida, Dipodascaceae* and *Pichia*) and those found mostly or exclusively in the thermophilic phase (sequences clustering to *Candida*, *Rhizomucor* and *Thermomyces*), but a few were also present throughout the whole process (Hultman et al. 2010).

5.6 Thermophilic Microflora in Compost Management

Compost harbours a number of guilds as it harbours not only structurally diverse microflora but also functionally active communities. The *in situ* functionality of each microbial component especially the extracellular enzymatic machinery, namely, polysaccharases, proteases and lipases, plays an important decisive role in successful colonisation and succession in mushroom compost (Rajni et al. 1998; Johri et al. 1999; Rawat and Johri 2002; Rawat et al. 2005).

The wide exploration of enzymatic machinery of the individual microbial component and their *in situ* enzymatic action in their own niche would help in greater understanding of the role played by them in compost management. However, the available information appears largely biased towards the functional role of thermophilic mycoflora of compost probably because they are a dominant component of the functional niche occupied by compost microbiota. The wide enzymatic potential exhibited by these fungi along with enzyme multiplicity with different physicochemical characteristics helps in functioning of each component under different biophysical conditions. The increase in cellulolytic and amylolytic activity during composting is a reflection of change in the population and community structure of the resident microflora. The enzymatic diversity of compost microbiota is known to result in specificity and successional change besides providing a niche to various species to survive in the absence of simple sugars (Rawat and Johri 2002).

In compost the pioneer microflora, in general, can utilise simple sugars, but such biota disappear soon and only those organisms with wide polysaccharolytic ability persist. The cellulolytic and hemicellulolytic ability of thermotolerant *A. fumigatus* allows it to persist in the wheat straw compost, whereas thermophilic *Mucor pusillus* does not recur even after temperature became suitable for growth due to lack of polysaccharolytic ability. *Humicola lanuginosa* persists throughout composting due to its ability to lead commensal life with others along with its cellulolytic and hemicellulolytic ability. *Chaetomium thermophile, Humicola insolens, Humicola lanuginosa* and *Talaromyces dupontii* develop abundantly in the 'plateau' period and rapidly utilise cellulose and hemicellulose. When compost temperature drops, thermophilic *Sporotrichum thermophile* and mesophilic *Coprinus cinereus* and *Clitopilus pinsitus* appear which can utilise cellulose and hemicellulose in wheat straw at a slower rate (Chang and Hudson 1967).

The success of microflora in competitive saprophytic colonisation (CSC) such as that operative in plant residues and soil depends upon its intrinsic ability to decompose that substrate and the ability to succeed in the competition. The strongly cellulolytic and hemicellulolytic *A. fumigatus, S. thermophile* and *T. thermophila* exhibit greater colonisation ability than the weakly cellulolytic *Humicola lanuginosa* (Johri and Satyanarayana 1984). The dominance of *S. thermophilum* has been attributed to the presence of complete complement of polysaccharolytic enzyme machinery (Rawat 1998; Tewari 2000; Rawat et al. 2005). The hydrolytic potential of such thermophilic fungi is responsible for solubilisation of complex ingredients of compost and making the nutrient available to *A. bisporus*. The stimulation of

growth of mushroom mycelium by cellulose-decomposing mycoflora has been well documented (Stanek 1969; Straatsma et al. 1994a; Johri and Rajni 1999).

Mushroom compost that harbours high population of thermophilic flora yields more mushroom produce (Shandilya 1982; Vijay 1996). The selectivity of compost is brought about by the static population of thermophilic flora, which becomes inactive at the time of spawning. The thermophilic microbial biomass is a concentrated source of nutrients required for the growth of *A. bisporus*. Thermophilic fungi appear to use up all the readily available nutrients during the process of composting, and thus, a major portion of the available nutrients is locked up inside their cells (Betterley 1993). *Agaricus bisporus* possesses the complement of enzymes, namely, β-*N*-acetylgalactosaminidase, laminarinase and protease, by which it can degrade thermophilic bacterial, fungal and actinomycete mycelium for its own growth (Fermor and Grant 1985; Rawat et al. 2005). Sparling et al. (1982) reported that microbial biomass contributed less than 10% to mushroom biomass, and therefore, *A. bisporus* probably obtained bulk of its carbon nutrition from straw. However, the microbial biomass can act as a concentrated source of nitrogen and minerals. The selectivity of compost is lost if dormant thermophilic biomass is destroyed by heat or chemicals (Ross and Harris 1983), and growth rate of *A. bisporus* mycelium is reduced on sterilised compost (Wood and Matchman 1980). Rawat (2004) found that dominant functional forms in mushroom compost were representatives of T3 stage.

The role of *Scytalidium thermophilum*, predominant component of compost, in compost management has been well documented by various workers (Ross and Harris 1983; Straatsma et al. 1989; Johri and Rajni 1999; Rawat 2004; Rawat et al. 2005). Among various groups actively engaged in studying the ecology of mushroom compost, Straatsma's group in Holland had laid considerable emphasis on population dynamics of *S. thermophilum* for improved compost management. The disappearance of ammonia and selectivity of compost for the growth of *A. bisporus* mycelium that occurs in phase II at temperature 45–55°C is linked to the presence of *S. thermophilum* (Ross and Harris 1983). The density of *S. thermophilum* was found to be positively correlated with mushroom yield (Straatsma et al. 1989). The causal relationship between the presence of *S. thermophilum* and the crop yield of mushroom remains still obscure. Straatsma et al. (1991) observed that this fungal species merely affects the radial extension rate rather than having a positive influence on the surface growth rate of *A. bisporus* mycelium. It reduces the growth of pathogenic microorganisms by virtue of inhibitory influence. Respiratory CO_2 of this species may play a stimulatory role (Weigant et al. (1992), but under different experimental conditions, neither volatiles nor CO_2 were stimulatory (Straatsma et al. 1994a). The mere presence of *S. thermophilum* is however quite essential. Ross and Harris (1983) suggested that visible but dormant biomass of this species in composts fills an otherwise biological vacuum, which in turn allows growth of *A. bisporus* mycelium. The disappearance of ammonia and selectivity of compost for the growth of *A. bisporus* mycelium that occurs in phase II at temperature 45–55°C are linked to the presence of *S. thermophilum* (Ross and Harris 1983). Other thermophilic fungal species such as *Chaetomium thermophilum*, *Malbranchea sulfurea*, *Myriococcum thermophilum*, *Stilbella thermophila*, *Thielavia terrestris*

and two unidentified Basidiomycetes were also found to be promotory for mycelial growth of *A. bisporus* on sterilised compost along with *S. thermophilum* (Straatsma et al. 1994a).

Considering the fact that culturable microbial populations are limited on account of our poor understanding of their nutritional requirements, detailed, *in situ* enzymatic investigations are likely to provide a better understanding of the relationship between structural and functional diversity of thermophilic fungal community. Iiyama et al. (1996) observed that the loss of cellulose and lignocellulose and increase in protein content during the composting period was a result of increased polysaccharolytic activity of the fungal biomass; this resulted in increased level of reducing sugars. In mushroom compost the level of enzymes was found to increase from zero-day to the end of phase I compost; thereafter, it decreased continuously and the results were well corroborated with the population structure (Rawat 2004).

It has been observed that *in situ* changes in lignocellulose and cellulose and loss in weight during composting are corroborated with the activity of thermophilic fungi. The analysis of plant residues after decomposition by pure thermophilic fungal cultures resulted in biochemical changes that were similar to those observed during composting of organic materials by natural mixed microflora (Satyanarayana 1978). During the 24-day composting sequence of button mushroom, Rajni (1999) reported that the level of organic carbon decreased from 18.12 to 10.57%, while that of cellulose and lignocellulose from 32.3 to 23.0% and 52.4 to 43.1%, respectively. An increase of 77.5 and 86% in protein and reducing sugar level was observed. The level of amylase, endo-cellulase and exo-cellulase also changed. Based on the regression analysis between the population of *Scytalidium thermophilum* and chemical parameters, a 92.5% change in lignocellulose was found as a result of rise in population of *S. thermophilum* in compost. The levels of dehydrogenase activity decreased by 72%, protease by 32% and xylanase and cellulase by 50% during peak heat, while during phase II and post peak-heat stage of mushroom compost, levels again increased by 71, 23 and 33.3%, respectively. These changes were found to be well corroborated with the change in population structure of compost microflora (Tewari 2000).

Yu et al. (2007) reported that hemicellulose and cellulose were partially degraded during initial stage of composting of agricultural wastes, and thereafter, the degrading ratio was almost unaltered due to high temperature followed by the large decomposition during the temperature-falling phase (12–20 day) and initial stage of the second fermentation (21–40 day of composting). Lignin was slightly decomposed during the initial stage of composting. When the temperature was lower than the maximum value during thermophilic phase, lignin was greatly degraded until the temperature began to fall. The microbes containing Q-9 or Q-10(H2) as major quinone were found to be the most important hemicellulose- and cellulose-degrading microorganisms during composting, while the microorganisms containing Q-9(H2) as major quinone and many thermophilic Actinobacteria were believed to be responsible for lignin degradation during agricultural waste composting.

Miyatake and Iwabuchi (2005) observed that the highest level of enzyme activity of thermophilic bacteria was observed at 54°C. The highest levels of superoxide

dismutase (SOD) and catalase activity in thermophilic bacteria were observed at 54°C and decreased sharply after 60°C. The decline in activity did not coincide with microbial extinction but with a decrease in metabolic activity. Extracellular lactate dehydrogenase (LDH) activity and the species diversity index value at 60°C were almost the same as those at 54°C. At 63°C, extracellular LDH activity reached the highest level, and the species diversity index value was the lowest, indicating that bacterial diversity was reduced and certain bacteria died at 63°C. An increase in SOD activity was observed at 70°C without a corresponding increase in catalase activity. Dehydrogenase activity is the most suitable indicator of compost stability and maturity (Tiquia 2005).

Stanek (1969) observed stimulation of growth of mushroom mycelium by cellulase-decomposing microflora, mainly actinomycetes and fungi. It is, however, difficult to draw a conclusive relationship between restricted cellulolysis and growth promotion of *A. bisporus* since species such as *A. fumigatus* and *Corynascus thermophilus* are cellulolytic but not growth promotory, whereas the reverse is true for *Chaetomium thermophilum* and *Sporotrichum thermophile*. Thus, growth-promotory species can be cellulolytic but not necessary pioneer colonisers of the compost biota. Such an influence is exerted by the climax species of mushroom compost, *Scytalidium thermophilum*, perhaps due to production of a complete complement of enzyme machinery (Rawat 1998, 2004; Tewari 2000).

An increase in cellulase activity and decrease in laccase activity was observed after the addition of casing soil to the surface of compost colonised by *Agaricus bisporus* (Gillman et al. 1994). The increase in cellulolytic and amylolytic activity during composting is a reflection of change in the population and community structure of the resident microflora. The enzymatic diversity of compost microbiota is known to result in specificity and successional change besides providing a niche to various species to survive in the absence of simple sugars.

The importance of polysaccharolytic enzymes in mushroom compost has led some to stimulate composting by supplementing the substrate with commercial enzymes. Savoie and Libmond (1994) observed that microbial enzyme activities, number of bacteria and solubilisation of carbon and nitrogen were greater in compost treated with polysaccharidases. However, this had no positive effect on mushroom yield. Libmond et al. (1995) observed that supplementation of wheat straw with Express (trade name of polysaccharidase complex) reduced the time of mushroom composting besides releasing low quantities of readily available sugars, increased enzyme activities and number of microorganisms, particularly aerobic bacterial population in the substrate.

Some workers have exploited the enzymatic potential of thermophilic fungi by pre-inoculation of compost with thermophilic fungi. Salar and Aneja (2007) observed the growth of *Agaricus bisporus* on sterile compost pre-colonised with four thermophilic fungi, namely, *Chaetomium thermophile, Malbranchea sulfurea, Thermomyces lanuginosus* and *Torula thermophila*, either singly or in different combinations. A mixed inoculum of *Malbranchea sulfurea* and *Torula thermophila* was found to be the best among the various treatments that promoted the growth of *A. bisporus* to the plateau of 7.7 mm day^{-1}, and the yield of the mushroom was

almost twice compared to the pasteurised control. The effect of *T. lanuginosus* when inoculated singly or in combination with other thermophilic fungus/fungi in compost was insignificant resulting in lower growth rates. The study revealed that thermophilic fungi provide for compost selectivity and protection against negative effects of compost bacteria on mycelial growth of *A. bisporus.* Improved growth of *A. bisporus* mycelium in composts treated with *S. thermophilum* has been extensively reported in literature (Ross and Harris 1983; Weigant 1992; Straatsma and Samson 1993; Straatsma et al. 1994a, b; Rawat 2004; Salar and Aneja 2007). Straatsma et al. (1994a) reported that nine thermophilic fungi, namely, *Chaetomium thermophilum*, an unidentified *Chaetomium* sp., *Malbranchea sulfurea*, *Myriococcum thermophilum*, *S. thermophilum*, *Stilbella thermophila*, *Thielavia terrestris* and two unidentified Basidiomycetes, promoted mycelial growth of *Agaricus bisporus* on sterilised compost.

The wide enzymatic potential exhibited by thermophilic fungi with different physico-chemical characteristics helps in the functioning of each component under different biophysical conditions. They also exhibit enzyme multiplicity which helps them function efficiently under different ecophysiological conditions. Multiplicity of hemicellulolytic enzymes has been widely reported in *Chaetomium thermophile* var. *coprophile*, *Humicola grisea* var. *thermoidea*, *Melanocarpus albomyces*, *Talaromyces emersonii*, *Thermoascus aurantiacus* and *Scytalidium thermophilum* (Thakur et al. 1992; Tuohy et al. 1993; Johri and Rajni 1999; Rawat 2004; Rawat et al. 2005).

5.7 Conclusions

Compost represents an interesting example of thermogenic, solid-state fermentation process that results from a succession of microbial communities. While it is a complex ecosystem, study of structural diversity complemented with functional diversity provides a fair picture of the species spectrum and their associated interactions within the substrate. Application of phenotypic and genetic tools with culturable and non-culturable components of the microbial diversity, coupled to utilisation of diversity indices and PCA analysis, has permitted deeper insights at subtle changes that result in physico-chemical and biological conditioning of compost as also to suggest how artificial inoculation could hasten the composting process. The survival strategies and competitive behaviour of microflora in this interesting niche requires to be understood. The *in situ* functionality of bacteria, actinomycetes and mesophilic fungi is still poorly known, whereas tapping the biopotentiality of microflora in this niche would help in hastening the composting process besides improving the quality of compost by pre-inoculation of microflora. This approach while being widely practised in mushroom compost has currently become popular in other composting systems as Effective Microorganism Technology but is at its nascent stage. New vertical dimensions will add to our existing knowledge, which would throw light into the adaptive behaviour of the microflora in compost ecosystem besides helping unravel

new and novel forms. The present moment of organic agriculture augurs well for use of microbial consortia in ecotechnological endeavours of which composting is an essential component. Use of modern molecular tools will permit better and more defined picture of community-level changes as also the role of dominant forms in deriving strategies for more effective degradative processes and conservation tools.

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Chapter 6
Metal Bioremediation by Thermophilic Microorganisms

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Abstract Environmental pollution with toxic metals is a severe threat to biota and human health. Microbe-mediated bioremediation of such contaminants has emerged as a potential alternative to conventional treatment methods. Thermophilic microorganisms, owing to their natural ability to survive and flourish under elevated temperatures along with other stressful environmental conditions including high concentrations of heavy metals, have developed various adaptation strategies to cope with harsh environments, which may offer enormous opportunities for bioremediation of heavy metals at higher temperatures. Thermophilic microorganisms, being common in geological and anthropogenic thermal environments with high concentrations of dissolved metal, possess unique cell wall structures and metabolic and enzymatic properties that may contribute in metals–thermophiles interactions. Biosorption/bioaccumulation of metals is most effective and widely used approach for the bioremediation. The nature and extent of metal biosorption onto thermophilic bacteria may differ greatly from the mesophilic organisms. Microbial transformation of metal through oxidation/reduction reactions plays a critical role in metal speciation, distribution, and thus altered toxicity in the ecosystems, which may be implemented in metal recovery and remediation. Both sulfate- and metal-reducing bacteria have profound application in metal bioremediation. Thermophilic bacteria with higher metal tolerance and metabolic characteristics at high temperature may exhibit enhanced metal solubilization through sulfur- or iron-oxidizing processes. Thermophilic microbial community can perform both degradative and productive functions through coupling of metal reduction with oxidation of a variety

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of organic and inorganic substrates. Thermophilic bacteria are also able to reduce a wide spectrum of metals including Mn (IV), Cr (VI), U (VI), Tc (VII), Co (III), Mo (VI), Au (I, III), and Hg (II) which can be used for the immobilization of toxic metals/radionuclides during bioremediation of hot wastewater of disposal sites of radioactive wastes having temperature range favorable for thermophiles for a long period of time. This chapter discusses various modes of metal-microbe interactions in thermophilic bacteria for their promising application in bioremediation.

Keywords Thermophiles • Bioremediation • Heavy metals • Environment • Metal resistance • Microbe–metal interaction • Metal oxidation • Metal reduction • Bioaccumulation • Biosorption

6.1 Introduction

Bioremediation can be defined as a pollution treatment technology that exploits microorganisms to reduce, eliminate, contain, and transform environmental contaminants to benign products (Tabak et al. 2005). This technology is considered as an effective and eco-friendly alternative to conventional remediation strategies with significant disadvantages including expensive process economics, incomplete remediation, high reagents/energy requirements, and generation of large sludge volume and other waste products for disposal (Kazy et al. 2006). Metals, being ubiquitous in the biosphere, constitute about 75% of the known elements and are essential for human civilization. Various natural (mineral rock weathering, volcanic eruptions, etc.) and anthropogenic (mining, smelting, industrial, agricultural, energy production, waste disposal, etc.) activities have redistributed and concentrated metals in terrestrial and aquatic habitats, thus adversely affecting ecosystem structure and function posing serious threat to biota and human health (Gadd 2010). Particularly, disposal of wastes from metal excavation and processing facilities have created tremendous pollution in some soils and sediments, which have motivated attention to the detoxification of metal-contaminated environments (Tabak et al. 2005). Microorganisms are exposed to metals long before anthropogenic activities have created metal pollution. Many metals (Na, K, Ca, Cu, Co, Mg, Mn, Mo, Ni, Fe, and Zn) are essential at trace concentrations for microbial growth and survival, yet all can exert toxicity when present at elevated levels. Some metals (Cd, Hg, Pb, Cs, etc.) are highly toxic and have no known biological function (Gadd 2010). Metal toxicity results from the displacement of essential ions from their active binding sites, interaction with ligands, or generation of free radicals leading to protein inactivation and DNA damage (Bruins et al. 2000; Nies 1999). Microorganisms inhabiting contaminated environments, therefore, have evolved various survival strategies to restrict intracellular metals within permissible limits. Owing to their small size, high surface to volume ratio and metabolic versatility, microbes can frequently interact with these contaminants that strongly regulate environmental fate of metals by altering their physical and chemical state and therefore their solubility, mobility, bioavailability,

and toxicity (Barkay and Schaefer 2001; Gadd 2004). Such metal-microbe interactions are of various types including (1) bioaccumulation and biosorption, (2) biotransformation, (3) bioprecipitation, and (4) biomineralization. Microorganisms are also able to execute an array of metal resistance mechanisms to cope with metal toxicity by regulating intracellular metal concentrations in sublethal levels. These are (1) efflux of metals that enter cells by either specific or nonspecific transporters, (2) intracellular compartmentalization within safe sectors of cell reducing cytoplasmic availability of metals, (3) intra- or extracellular entrapment of metals by complexation with microbially generated ligands, and (4) enzymatic transformations reducing metal toxicity, etc. (Nies 1999; Chatziefthimiou et al. 2007). All such interaction/resistance processes have immense importance in determining metal mobility, bioavailability, and thus toxicity in the environment; some of which may be harnessed as the basis of metal bioremediation strategies (Tabak et al. 2005).

During last three decades, extensive research has been carried out to decipher almost all modes of microbial metal resistance/interaction in mesophilic microorganisms for their potential application in bioremediation, while the potency of thermophilic organisms has largely been unexplored. Thermophilic microorganisms are capable of growing above 45°C (moderately thermophilic), while some grow best at further elevated temperatures and are grouped as extreme thermophiles having optimum growth temperature between 70 and 80°C and hyperthermophiles exhibiting maximum growth above 80°C (Charlier and Droogmans 2005). Thermophiles can be found within phototrophic bacteria (cyanobacteria, purple and green bacteria), bacteria (*Bacillus*, *Clostridium*, *Thiobacillus*, *Desulfotomaculum*, *Thermus*, lactic acid bacteria, *Actinomycetes*, *Spirochetes*, and numerous other genera) and also within the domain archaea having the most thermophilic ones (*Pyrococcus*, *Pyrolobus*, *Thermococcus*, *Thermoplasma*, *Sulfolobus*, and Methanogens) (Rothschild and Mancinelli 2001). It seems more likely that thermophiles in specific environments might have evolved metal resistance and other adaptation mechanisms to adjust with naturally harsh conditions since the evolution of life on earth. Such microorganisms are naturally exposed to elevated concentrations of metals resulting from geological activities (Miroshnichenko 2004). The organisms capable of growing at the highest metal concentrations are the iron- and sulfur-oxidizing microbes including thermophilic archaea. Metal resistance in these organisms could be an adaptation mechanism to cope with very acidic environments with high metal loads (Valls and de Lorenzo 2002). However, compared to the mesophilic organisms, metal resistance mechanisms conferring the ability of thermophiles to adapt with high metal concentrations are poorly investigated (Llanos et al. 2000; Miroshnichenko 2004). As a thermoadaptation mechanism, thermophiles have adjusted the composition of cell membrane including increase in acyl chain length and degree of saturation of fatty acids, branching, and cyclization, thus maintaining optimal membrane structure and function in high temperature that could increase the fluidity and permeability of membranes (Rothschild and Mancinelli 2001; Charlier and Droogmans 2005). Thermostability of proteins depends on various strategies such as increased amounts of bulky hydrophobic and charged residues and less nonpolar residues to stabilize the core and periphery of

the protein, increased ion-pair content, and formation of higher order oligomers decreasing flexibility at room temperature, multiple amino acid substitution, introduction of negatively charged amino acid residues and metal-binding sites, etc. (Daniel and Cowan 2000; Rothschild and Mancinelli 2001; Kumar and Nussinov 2001; Santos and da Costa 2002). Thermostability of nucleic acids can be achieved by high intracellular concentrations of salts, polyamines, and low-molecular weight DNA-binding proteins. DNA stabilization by reverse gyrase or histones is also known in majority of thermophilic archaea or in Euryarchaeota. High G + C content is correlated with the thermostability of ribosomal and transfer RNAs of thermophiles (Galtier et al. 1999; Rothschild and Mancinelli 2001; Miroshnichenko 2004). Several enzymes of DNA metabolism having unusual properties might be involved in DNA repair at high temperature. A specific DNA repair system for thermophilic organisms was also predicted through genome context analysis (Koulis et al. 1996; Wood et al. 1997; Makarova et al. 2002). The unique cell wall structures and other thermoadaptation mechanisms may contribute significantly in their interactions with metals, which may differ qualitatively and quantitatively from the mesophilic organisms (Hetzer et al. 2006).

Ever since the discovery of thermophilic microorganisms, scientists have been interested in the diversity of such extremophiles for various reasons including fundamental interests, biotechnological applications, and economic benefit (Charlier and Droogmans 2005; Spain and Krumholz 2011; Hasyima et al. 2011; Gugliandolo et al. 2012). Much attention has been paid on the molecular basis of thermoadaptation strategies involving cellular macromolecules. In recent years, it has been realized that thermophiles might have developed diverse metal resistance mechanisms to adapt with high concentrations of dissolved metals associated with their extreme environmental living conditions (Llanos et al. 2000; Miroshnichenko 2004; Watkin et al. 2009). Availability of complete genome sequences of a number of thermophilic bacteria retrieved from metal-rich environments provides insights into biologically catalyzed metal–sulfide oxidation and reduction of iron and other metals (Table 6.1). Comparative genomics was used to identify pathways and proteins involved (directly or indirectly) with microbial interactions with metals (Maaty et al. 2009). Thus, intensive investigations on thermophilic microbial metal resistance and their various modes of metal interactions can enrich our present knowledge for the improvement of metal bioremediation strategies (Hetzer et al. 2006; Poli et al. 2009; Chatterjee et al. 2010; Ozdemir et al. 2012; Sheoran et al. 2010). However, the success of bioremediation depends on complex interactions between biological and physicochemical processes such as availability of appropriate microorganisms with relevant attributes and favorable environmental factors, all that greatly influence remedial action. Bioremediation goal can be achieved through biologically mediated (a) concentration, and thus reduction in volume of metal-contaminated matrices, and (b) transformations in oxidation state leading either to precipitation that immobilizes metals in place or to solubilization that increases metal mobility accelerating their removal/recovery (Barkay and Schaefer 2001; Tabak et al. 2005). Thermophilic microbial interactions with metallic contaminants offer enormous opportunities to develop remedial strategies for cleaning up of contaminated environments associated

Table 6.1 List of thermophilic microorganisms whose complete genome sequences are available

Organism	Source	Genome size (kb)	Possible role in metal transformation	GenBank accession no.
Metallosphaera sedula DSM 5348	*M. sedula* DSM 5348	2,200	Sulfide oxidation	NC_009440.1
Hydrogenobacter thermophilus TK-6, DSM 6534	Soil near hot spring in Izu Peninsula, Japan	1,743	Ferredoxin reduction	CP002221.1
H. thermophilus TK-6, DSM 06534	Soil near hot spring in Izu Peninsula, Japan	1,742	ND	CP002221.1
Symbiobacterium thermophilum IAM 14863	Compost in Hiroshima, Japan	3,566	Arsenate efflux	NC_006177.1
Thermanaeromonas toyohensis ToBE, DSM 14490	Geothermal aquifer at a depth of 550 m, Japan	3,400	ND	NR_024777.1
Thermanaerovibrio acidaminovorans Su883, DSM 6589	Upflow anaerobic sludge bed reactor of a sugar refinery, Breda, Netherlands	1,848	ND	CP001818.1
Thermoanaerobacter brockii Ako-1, DSM 3389	Lake sediment; Lake Kivu, Africa	2,234	ND	NZ_ACQZ00000000.1
Thermoanaerobacter ethanolicus X514	Anaerobic enrichment culture from a deep subsurface, USA	2,457	Iron, cobalt, chromium, manganese, and uranium reduction	NC_010320.1
Thermoanaerobacter italicus Ab9, DSM 9252	Mud, thermal spa; Italy	2,451	Production of thermostable pectate-lyases	CP001936.1
Thermoanaerobacter pseudethanolicus 39E	Thermal springs in Yellowstone National Park, USA	2,362	Thermostable L-lactate dehydrogenase production	NC_010321.1
Thermoanaerobacter wiegelii Rt8.B1	Fresh water hot spring in New Zealand	2,746	Reduction of iron, manganese, and other metals	NZ_ADXD01000169.1

(continued)

Table 6.1 (continued)

Organism	Source	Genome size (kb)	Possible role in metal transformation	GenBank accession no.
Thermobaculum terrenum YNP1, ATCC BAA-798	Geothermal heated soil, located near the confluence of Rabbit Creek and Firehole River, Yellowstone National Park, USA	3,101	ND	CP001825.1
Thermobispora bispora R51, DSM 43833	Decaying manure	4,189	ND	NC_014165.1
Thermococcus kodakarensis KOD1	Solfatara on Kodakara Island Kagoshima, Japan	2,088	ND	NC_006624.1
Thermococcus sp. AM4	2,600-m depth in the East Pacific Rise	2,084	Chromium reduction	NZ_DS999064.1
Thermocrinis albus HI 11/12, DSM 14484	Grayish filaments collected from a hot streamlet in Hveragerdi, Iceland	1,500	Arsenic oxidation	CP001931.1
Thermodesulfovibrio yellowstonii DSM 11347	Thermal vent in Yellowstone Lake in Wyoming	2,003	Sulfur oxidation	NC_011296.1
Thermus thermophilus HB8	Thermal vent in Japan	1,849	Arsenite oxidation	NR_037066.1
Thermotoga neapolitana DSM 4359	Black smoker in the bay near Naples, Italy	1,884	Thiosulfate reduction	NC_011978.1
Thermotoga petrophila RKU-1	Production waters of the Kubiki oil reservoir in Niigata Japan	1,823	Iron oxidation	NC_009486.1
Dictyoglomus thermophilum H-6-12, ATCC 35947	Slightly alkaline Tsuetae Hot Spring in Kumamoto Prefecture, Japan	1,959	Thiosulfate reduction	NC_011297.1
Thermomicrobium roseum DSM 5159	Toadstool Spring in Yellowstone National Park	2,006	Mercury reduction	NC_011959.1
Thermomonospora curvata DSM 43183	Municipal refuse compost sample	5,639	Sulfur oxidation	NC_013510.1

Thermosediminibacter oceani JW/IW-1228P, DSM 16646	Deep-sea sediments of Peru margin	2,280	Hydroxymethylbutenyl pyrophosphate reduction	CP002131.1
Thermosipho melanesiensis BI429	Gills of the deep-sea vent hydrothermal mussel *Bathymodiolus brevior* from Southwestern Pacific Ocean	1,915	Heavy metal transportation	NC_009616.1
Pyrolobus fumarii	Wall of a black smoker hydrothermal vent on the Mid-Atlantic Ridge	1,850	Sulfur oxidation	AB245540.2
Fervidobacterium pennivorans	Hot spring, mud, sediment	2,166	ND	NC_017095
Marinitoga piezophila KA3	Deep-sea, hydrothermal vent, marine, Pacific Ocean	2,244	Sulfur-reducing bacteria	NC_016751
Leptospirillum ferrooxidans C2-3	Fresh volcanic deposit	2,559	Iron-oxidizing bacterium	NC_017094
Caldisericum exile AZM16c01	Caldisericum exile AZM16c01	1,558	Thiosulfate-reducing bacteria	NC_017096

with high temperature and other stressful factors. Fundamental understanding of such interactions at all possible levels including characterization of (a) inhabitant thermophilic microbial communities in contaminated geothermal and other habitats, (b) their modes of interactions with metals, and (c) genes that code for metal resistance/interaction processes is critical for designing bioremediation strategies. In this chapter, thermophilic microbial metal resistance and their interactions with various metals and metalloids have been discussed highlighting their vast potentials in metal bioremediation.

6.2 Critical Review and Analysis

6.2.1 Heavy Metal Resistance by Thermophiles

Thermophilic microorganisms occupy natural habitats often enriched with heavy metals and develop strategies to resist metal toxicity. Metal resistance among the thermophiles may develop either as part of their metabolic activities (coupled with electron transfer reactions to and/or from iron and sulfur mainly during growth) or as specialized mechanism to reduce metal toxicity (by efflux pump-mediated extrusion of accumulated metal or by enzymatic conversion), thereby providing competitive advantage to survive and flourish under such extreme conditions (Dopson et al. 2003; Chatziefthimiou et al. 2007; Watkin et al. 2009). Recent studies on diversity of metal-resistant microbes across diverse environments including the most extreme ones clearly imply that metal resistance systems were evolved long before anthropogenic release of metal contaminants (Llanos et al. 2000). Prevalence of high metal tolerance among chemolithoautotrophic acidophilic bacteria and archaea and the presence of metal resistance genes in their genomes are the testimony for such inferences (Dopson et al. 2003). In recent time, heavy metal-resistant thermophilic bacilli (*Bacillus sphaericus*, *Bacillus pumilus*, *Paenibacillus larvae*, and *Geobacillus stearothermophilus*) were isolated from Ma'een hot springs in Jordan and used as immobilized, dead, and live biomass for bioremediation of heavy metals present in the wastewater effluent of Jordan Rotogravure Establishment (JRE) (Al-Daghistani 2012). The investigator showed that although the overall sorption efficiency does not vary significantly within the temperature range 37–70°C, thermophilic bacilli exhibited a maximum metal-binding capacity at temperatures 60 and 70°C. The affinity of *Bacillus* for heavy metals adsorption was found to be Cu > Cr > Ni for *B. sphaericus* and Cr > Cu > Ni for other *Bacillus* species. Apart from the isolation of various thermophilic metal-resistant microorganisms (both bacteria and archaea) followed by molecular characterization of metal-resistant determinants, several studies have identified genes encoding metal resistance in thermophiles. Several metal-resistant genes (viz., *chrA*, *merA*, *cadA*, *cadC*, *arsB*, *arsC*, *arsR*, *arr*, *acr*(*3*)*p*, and *czc*) that mostly confer resistance by encoding efflux pumps and reductases (for arsenate, chromate, and mercury) have been detected in thermophilic bacteria and archea (Fig. 6.1, Table 6.2).

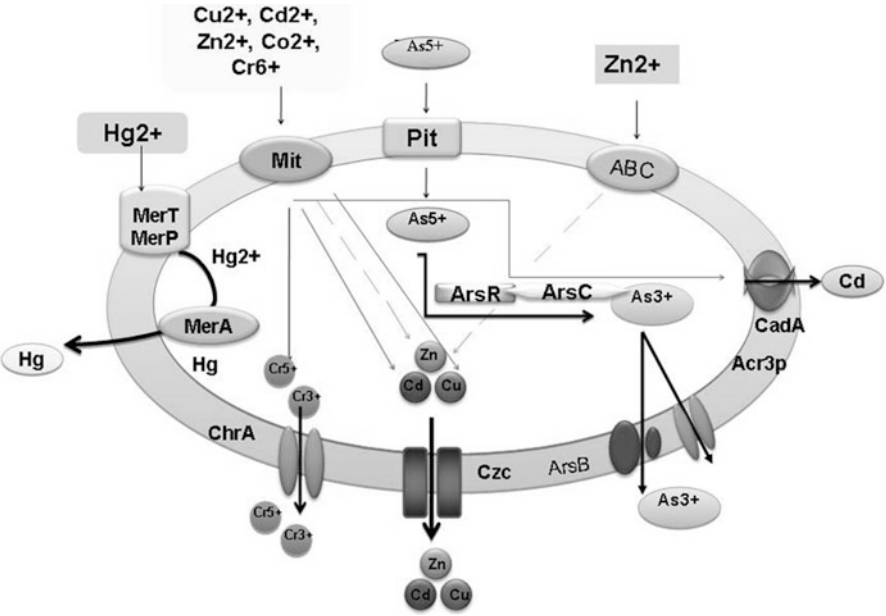

Fig. 6.1 Heavy metal resistance systems present in thermophilic microorganisms. Heavy metal ions may be accumulated by the inorganic transport systems MIT/PIT or uptake transporters (*ABC*). Metal ions may be effluxed out by specific inducible systems (*Czc*/*CadA*). Reduction of metals [As(V), Cr(VI), Hg(II)] to their less toxic form may be accomplished by specific reductase (*ArsC*/*ChrA*/*MerA*)

Heavy metal (Cd, Cu, and Ni) toxicity on pure cultures of thermophilic methanogenic *Methanobacterium thermoautotrophicum* (MT) and TAM, a thermophilic, acetate-decarboxylating, methanogenic bacterium, was studied by Ahring and Westermann (1985). It was observed that Cd and Cu toxicity depends upon the presence of Ni in the medium. Llanos et al. (2000) tested Cd, Zn, Co, and Ni sensitivity of 30 thermophilic strains (*Bacillus* spp., members of *Thermotogales* and *Thermococcales*) isolated from heavy metal-rich hydrothermal vent sites at Lau Basin (southwestern Pacific) together with their Cd accumulation potential. Compared to the members of *Thermotogales* that showed resistance to only Co, members of the *Thermococcales* were highly resistant to both Cd and Zn. Watkin et al. (2009) studied Cu, Zn, Ni, and Co tolerance by a number of moderately thermophilic bacteria closely related to *Acidithiobacillus caldus*, *Acidimicrobium ferrooxidans*, and *Sulfobacillus thermosulfidooxidans*. In another report, Poli et al. (2009) investigated heavy metal resistance by six thermophilic bacteria, namely, *Anoxybacillus amylolyticus*, *Geobacillus thermoleovorans*, *G. thermoleovorans* subspecies *stromboliensis*, *G. toebii* subspecies *decanicus*, *Bacillus thermantarcticus*, and *Thermus oshimai*, isolated from different environmental sites. For the test isolates (except *G. toebii* subspecies *decanicus*), Hg was the most toxic metal followed by Cu (or Cd in case of *A. amylolyticus*, *B. thermantarcticus*, and *T. oshimai*), and Fe was the least toxic. These investigators also observed a correlation between

Table 6.2 Metal resistance genes detected in thermophilic bacteria and archaea

Gene/ protein	Bacteria	Source	GenBank accession no.
ChrA	*Geobacillus kaustophilus*	Deepest ocean	BAD75953
ChrA	*G. kaustophilus*	Deepest ocean	YP_147521
ChrA	*Geobacillus* sp.	*Geobacillus* sp. C56-T3	YP_003671410
ChrA	*G. thermoglucosidasius*	*G. thermoglucosidasius*	ZP_06811590
ChrA	*Thermosipho melanesiensis* BI429	*T. melanesiensis* BI429	ABR31746
ChrA	*Fervidobacterium nodosum* Rt17-B1	F. nodosum Rt17-B1	ABS60223
ChrA	*Geobacillus thermoleovorans CCB_US3*	G. thermoleovorans CCB_US3	YP_004984151
merA	*Brevibacillus* sp.	Mercury-rich geothermal springs	DQ835531
merA	*Anoxybacillus* sp.	Mercury-rich geothermal springs	DQ835532
merA	*Anoxybacillus* sp.	Mercury-rich geothermal springs	DQ835533
merA	*Anoxybacillus* sp.	Mercury-rich geothermal springs	DQ835534
merA	*Anoxybacillus* sp.	Mercury-rich geothermal springs	DQ835536
merA	*Anoxybacillus* sp.	Mercury-rich geothermal springs	DQ835535
merA	*Anoxybacillus* sp.	Mercury-rich geothermal springs	DQ460032
MerA	*A. flavithermus*	*A. flavithermus*	YP_002315777
MerA	*A. flavithermus*	*A. flavithermus*	ACJ33792
MerA	*Anoxybacillus* sp.	*Anoxybacillus* sp. HT14	ABF29526
MerA	*Anoxybacillus* sp.	*Anoxybacillus* sp. FB9	ABJ53183
MerA	*Anoxybacillus* sp.	*Anoxybacillus* sp. HT8	ABJ53182
MerA	*Anoxybacillus* sp.	*Anoxybacillus* sp. FB7	ABJ53181
MerA	*Anoxybacillus sp.*	*Anoxybacillus* sp. FB6	ABJ53180
MerA	*Anoxybacillus* sp.	*Anoxybacillus* sp. FB5	ABJ53179
MerA	*G. kaustophilus*	Deepest ocean	YP_148949
MerA	*Hydrogenobacter thermophilus* TK-6	*H. thermophilus* TK-6	YP_003432979
CadA	*Streptococcus thermophilus*	*S. thermophilus*	AJ315964
CadA	*G. stearothermophilus*	*G. stearothermophilus*	AF098974
CadA	*S. thermophilus*	*S. thermophilus*	CAE52410
CadA	*S. thermophilus*	*S. thermophilus*	CAC87152
CadA	*S. thermophilus*	*S. thermophilus*	CAE52398
CadA	*S. thermophilus*	*S. thermophilus*	CAC87153
CadC	*S. thermophilus*	*S. thermophilus*	AJ315964
CadC	*G. stearothermophilus*	*G. stearothermophilus*	AF098974
CadC	*S. thermophilus*	*S. thermophilus*	CAE52412
CadC	*S. thermophilus*	*S thermophilus*	CAE52397
CadC	*G. stearothermophilus*	*G. stearothermophilus*	AAL27800
ArsB	*Hydrogenobacter thermophilus*	*H. thermophilus* TK-6	YP_003433426
ArsB	*Pelotomaculum thermopropionicum*	*P. thermopropionicum* SI	YP_001211004
ArsB	*A. flavithermus*	Geothermal hot springs and manure	YP_002315766

(continued)

Table 6.2 (continued)

Gene/ protein	Bacteria	Source	GenBank accession no.
ArsB	*Syntrophothermus lipocalidus*	*S. lipocalidus* DSM 12680	ADI02931.1
ArsB	*Spirochaeta thermophila*	*S. thermophila* DSM 6192	YP_003875301
ArsB	*Fervidobacterium nodosum*	*F. nodosum* Rt17-B1	YP_001409998
ArsB	*S. thermophila*	*S. thermophila* DSM 6192	YP_003875301
ArsB	*S. thermosulfidooxidans*	*S. thermosulfidooxidans*	P30329
ArsB	*Deferribacter desulfuricans* SSM1	*D. desulfuricans* SSM1	YP_003497365
ArsC	*A. flavithermus*	*A. flavithermus* WK1	YP_002316821
ArsC	*G. kaustophilus*	*G. kaustophilus* HTA426	YP_148858
ArsC	*A. flavithermus*	*A. flavithermus* WK1	ACJ34836
ArsC	*G. kaustophilus*	*G. kaustophilus* HTA426	BAD77290
ArsC	*S. thermophila*	*S. thermophila* DSM 6192	YP_003873915
ArsC	*S. thermophila*	*S. thermophila* DSM 6192	ADN01642
ArsC	*A. flavithermus*	*A. flavithermus* WK1	YP_002316471
ArsC	*G. kaustophilus*	*G. kaustophilus* HTA426	BAD75102
ArsC	*Campylobacter lari*	*C. lari*	BAK55635
ArsC	*Synechococcus sp.* JA-2-3B'a(2–13)	*S. sp.* JA-2-3B'a	YP_478568
ArsC	*Exiguobacterium sp.* AT1b	*E. sp.* AT1b	ACQ69743
ArsR	*P. thermopropionicum*	*P. thermopropionicum* SI	YP_001211592
ArsR	*S. lipocalidus*	*S. lipocalidus* DSM 12680	ADI02934
ArsR	*Caldicellulosiruptor bescii*	*C. bescii* DSM 6725	YP_002574477
ArsR	*Thermotoga lettingae*	*T. lettingae* TMO	YP_001470954
ArsR	*F. nodosum*	*F. nodosum* Rt17-B1	YP_001411110
ArsR	*C. saccharolyticus*	*C. saccharolyticus* DSM 8903	ABP66847
ArsR	*T. lettingae*	*T. lettingae* TMO	YP_001470879
ArsR	*T. petrophila*	*T. petrophila* RKU-1	YP_001245324
ArsR	*S. thermophila*	*S. thermophila* DSM 6192	YP_003874799
ArsR	*H. thermophilus*	*H. thermophilus* TK-6	YP_003433422
ArsR	*A. flavithermus*	*Anoxybacillus flavithermus* WK1	YP_002316707
ArsR	*G. kaustophilus*	*Geobacillus kaustophilus* HTA426	YP_149077
ArsR	*D. desulfuricans*	*D. desulfuricans* SSM1	YP_003495320
ArsR	*Thermosynechococcus elongatus*	*T. elongatus* BP-1	BAC08043
ArsR	*S. thermosulfidooxidans*	*S. thermosulfidooxidans*	CAB12340
Acr3	*P. thermopropionicum*	*P. thermopropionicum* SI	YP_001211202
Acr3	*P. thermopropionicum*	*P. thermopropionicum* SI	BAF58833
Acr3	*D. desulfuricans*	*Deferribacter desulfuricans* SSM1	YP_003496669
Acr3	*D. desulfuricans*	*Deferribacter desulfuricans* SSM1	BAI80913

(continued)

Table 6.2 (continued)

Gene/ protein	Bacteria	Source	GenBank accession no.
Acr3	*S. lipocalidus*	*S. lipocalidus* DSM 12680	ADI02931
Acr3	*S. lipocalidus*	*S. lipocalidus* DSM 12680	YP_003703496
Arr	*D. desulfuricans*	*Deferribacter desulfuricans* SSM1	YP_003496662
Arr	*D. desulfuricans*	*Deferribacter desulfuricans* SSM1	BAI80906
CzcD	*A. flavithermus*	*Anoxybacillus flavithermus* WK1	YP_002314741
CzcD	*A. flavithermus*	*Anoxybacillus flavithermus* WK1	ACJ32756

decrease in α-amylase biosynthesis and activity in response to heavy metals in *A. amylolyticus*, indicating that this enzyme could be useful in developing potential sensitive bioassay for heavy metal detection.

Among other metals, isolation and characterization of mercury-resistant microbes from several geothermal springs got special attention (Schelert et al. 2004, 2006; Chatziefthimiou et al. 2007). Along with Pb and Cd, Hg belongs to the class B heavy metals (soft metals) and is extremely poisonous at low concentration (Schelert et al. 2006). Among bacteria, mercury resistance is well studied and is usually plasmid borne (Barkay et al. 2003). Active resistance to Hg (II) is regulated by *merR*, a unique representative of the winged helix-turn-helix (WHTH) family of bacterial transcription factors (Aravind et al. 2005). MerR controls production of mercuric reductase (MerA) along with other related components (Barkay et al. 2003). Schelert et al. (2004, 2006) have studied interaction between Hg and hyperthermophilic *Sulfolobus solfataricus* and revealed the occurrence of mercury resistance in members of the archaeal domain. Members of the phylum *Crenarchaeota*, including the *S. solfataricus*, inhabit metal-rich geothermal environments. Proliferation under these conditions is accompanied by induction of active metal detoxification pathways encoded by homologs of mercuric reductases, MerA, that operates under the control of MerR. MerR represents a unique class of transcription factors that exert both positive and negative regulation on gene expression (Schelert et al. 2004, 2006). In *S. solfataricus*, the level of resistance afforded by these proteins was found to be considerably lower than that of other mercury-resistant bacteria (Simbahan et al. 2005). It has been found that *S. solfataricus* though sensitive to mercuric chloride, a low-level adaptive response, could be induced as a result of upregulation of *merA* transcription by mercury challenge. Mercuric ion (Hg^{2+}) inactivates generalized transcription but simultaneously derepresses transcription of mercuric reductase (MerA) by interacting with the archaeal transcription factor MerR (Schelert et al. 2006). On either side of *merA*, two additional Hg(II)-inducible genes, *merH* and *merI*, were also detected. Further experiments by creating mutant strains that lack both *merA* and *merRA* Hg^{2+}-mediated transient derepression of *mer* expression "implicated the existence of a secondary system or alternative cellular reductases for metal detoxification" (Schelert et al. 2006). Chatziefthimiou et al. (2007) have investigated mercury resistance in several facultative thermophilic and chemolithoautotrophic, thiosulfate-oxidizing

bacteria (with close lineage to *Anoxybacillus* spp., *Geobacillus* spp., *Bacillus thermoalkalophilus*, and *Brevibacillus thermoruber*) isolated from mercury-rich hot sulfidic springs and indicated that Mer-mediated Hg(II) reduction was the dominant resistance mechanism among these strains. Based on several similar studies, it was suggested that bacterial genera affiliated to the order *Bacillales* are common in mercury-rich high temperatures and geothermal sources of mercury in acidic to slightly alkaline environments (Simbahan et al. 2005; Glendinning et al. 2005; Chatziefthimiou et al. 2007). Phylogenetic analysis of *merA* genes by the later investigators further showed genetic incongruence, suggesting evolution by horizontal gene transfer (HGT) of the *merA* gene.

Protein phylogenetic analysis of mercury reductase (MerA) in bacteria and thermophilic archaea revealed some interesting findings. In bacterial origin, the catalytic site of MerA is comprised of a conserved pyridine nucleotide–disulfide oxidoreductase domain (PFAM 0007) with two active cysteines (C207 and C212) (Schiering et al. 1991). Unlike other members of this large family, MerA has two additional unique regions: (1) a short C-terminal extension having two additional active cysteines (C628 and C629) and (2) an extended N-terminus that promotes metal recruitment critical for metal reduction. Additionally, two conserved tyrosines (Y48 and Y605) that facilitate catalysis are also present in bacteria (Simbahan et al. 2005). In contrast to these, MerA among the archaea lacked the N-terminal metal recruitment domain and a C-terminal active tyrosine. Recently, Simbahan et al. (2005) have investigated the community structure and distribution of the forms of MerA in an interacting community comprising members of both prokaryotic domains. Both culture-based and culture-independent analyses indicated the presence of thermophilic crenarchaeal (*S. solfataricus*) and Gram-positive bacterial (*Sulfobacillus* and *Alicyclobacillus*) members within the naturally occurring mercury-rich geothermal environment. Analysis of archaeal and bacterial MerA sequences supported the hypothesis that both forms of MerA were present. The study indicated that the archaeal MerA is a new form of the enzyme that provides strong advantage to *S. solfataricus* in mercury-contaminated environment. The authors clearly showed that despite strong "environmental selection for mercury resistance and the proximity of community members, MerA retains the two distinct prokaryotic forms and avoids genetic homogenization" (Simbahan et al. 2005).

Phylogenetic analysis of amino acid sequences of thermophilic *mer*A gene retrieved from NCBI GenBank revealed presence of discrete clades well separated from those of mesophilic origin (Fig. 6.2). Three distinct clades represented the MerA sequences from thermophilic bacterial, mesophilic bacterial, and archaeal domains were observed. Interestingly, MerA from *Anoxybacillus* spp., *G. kaustophilus*, showed a close relationship forming a single monophyletic group. MerA from archeal (*S. solfataricus*) origin also formed a separate clade leaving the MerA from mesophiles as distinct members. Apart from these studies, several other investigations have yielded sequence of metal-resistant determinants (e.g., *cad*, *czc chr*, *ars*, *arr*, and *acr*) form thermophilic microorganisms and deposited in NCBI database. Sequence of such genes/proteins was retrieved and analyzed to obtain a preliminary idea about their phylogenetic lineage.

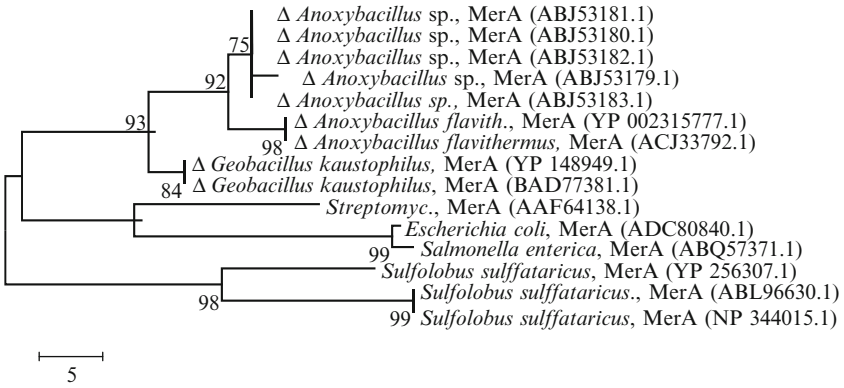

Fig. 6.2 Neighbor-joining tree depicting phylogenetic relation between the mercuric reductase (*MerA*) of thermophilic bacteria, thermophilic archaea, and mesophilic bacteria. The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of MerA. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. Phylogenetic analyses were conducted in MEGA4. Δ indicates genes from thermophilic bacteria

6.2.1.1 Cadmium-Resistant Genes (*cad* and *czc*)

Cadmium resistance in bacteria is provided by either ATP-dependent cadmium efflux transporters or metallothionein-based metal binding and detoxification system (Silver and Phung 1996). Cadmium resistance system found in Gram-positive bacteria encodes Cd exporting P-type ATPase (CadA pump) along with CadC (regulator protein). Function of these two genes was originally studied in *Staphylococcus aureus* (Endo and Silver 1995). Although, homologs of these genes have been identified later in many mesophilic bacteria (e.g., *Bacillus firmus*, *Listeria monocytogenes*, *Lactococcus lactis*, *Listeria innocua*, and *Stenotrophomonas maltophilia*), their detection in thermophilic microorganisms remains scanty. So far, both *cadA* and *cadC* genes have been reported only from two genera, namely, *Streptococcus thermophilus* and *Geobacillus stearothermophilus* (Schirawski et al. 2002). Phylogenetic analysis of thermophilic CadA showed its lineage with that of mesophilic *E. coli* CadA, while the CadC from thermophilic *S. thermophilus* and *G. thermophilus* represented its distinctness from *E. coli* CadC (Fig. 6.3). In addition to *cad* system, *czc* system that encodes proteins responsible for transporting cobalt, zinc, and cadmium to or from cell interior is also detected in thermophilic bacteria *Anoxybacillus flavithermus* (Saw et al. 2008). This protein is a member of RND protein family which is involved in bacterial transport of heavy metals (*C. metallidurans*), nodulation (*Mesorhizobium loti*), and cell division (*E. coli*) (Saier et al. 1994). In bacteria and archaea, members of this superfamily are involved in transport of heavy metals, hydrophobic compounds, amphiphiles, and nodulation factors.

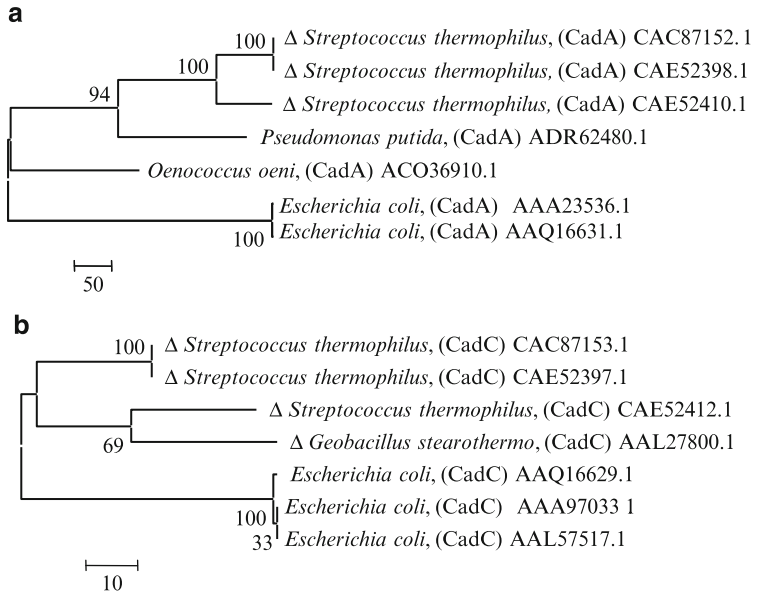

Fig. 6.3 Neighbor-joining tree depicting phylogenetic relation between the CadA (**a**) and CadC (**b**) of thermophilic bacteria. The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. Phylogenetic analyses conducted in MEGA4. Δ indicates genes from thermophilic bacteria

6.2.1.2 Chromium Resistance Gene (*chr* A)

Several bacterial mechanisms of resistance to chromate have been reported. The best characterized mechanisms comprise reduction of Cr (VI) to Cr (III) and efflux of chromate ions from the cell cytoplasm (Nies 1999). Chromate efflux by the ChrA transporter has been established. ChrA functions as a chemiosmotic pump that effluxes chromate from the cytoplasm using the proton-motive force (Alvarez et al. 1999). In thermophilic bacteria, the protein for chromate reductase (ChrA) was detected only in *G. kaustophilus* and *G. thermoglucosidasius* (Takami et al. 2004). Phylogenetic analysis revealed that although ChrA sequences from thermophilic origin are closely related, they share a common evolutionary node with that of mesophilic bacteria (*E. coli*) (Fig. 6.4).

6.2.1.3 Arsenite Resistance (*ars*) Operon

One of the best characterized, and probably the most widespread, arsenic resistance system in microorganisms is the *ars* gene system (Cervantes et al. 1994; Oremland

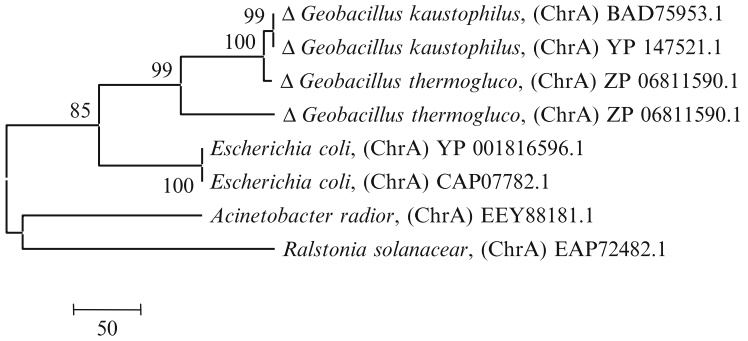

Fig. 6.4 Neighbor-joining tree depicting phylogenetic relation between chromate reductase (*ChrA*) proteins and thermophilic bacteria. The evolutionary history was inferred using the neighbor-joining method. The bootstrap consensus tree inferred from 500 replicates. Phylogenetic analyses were conducted in MEGA4. Δ indicates genes from thermophilic bacteria

and Stoltz. 2003). This system consists of a series genes coding for transmembrane pump system (*ars*B), arsenate reductase (*arsC*), and a regulatory gene (*arsR*) (Silver and Phung 1996). In this mechanism, ArsC catalyzes the first step in arsenic detoxification reduction, reduction of arsenate [As (V)] to arsenite [As (III)] using reducing equivalents derived from glutathione (GSH) via glutaredoxin (GRX). This protein was well characterized in many mesophilic bacteria including *Pseudomonas* sp., *Bacillus* sp., *Shewanella* sp., *Mycobacterium* sp., *Klebsiella* sp., *Clostridium* sp., and *Staphylococcus* sp. (Mukhopadhyay and Rosen 2002) along with few thermophilic bacteria like *A. flavithermus*, *G. kaustophilus*, and *Spirochaeta thermophila* (Saw et al. 2008; Takami et al. 2004; Angelov et al. 2010). Analysis of ArsC sequences from several mesophiles and thermophiles revealed a distinct evolutionary lineage (Fig. 6.5). All the ArsC sequences from thermophilic origin were highly related, forming a separate clade from that of mesophiles.

The other important members of *ars* operon are *arsB* and *arsR*. The gene *arsB* encodes the efflux pump membrane protein, while the *arsR* is a trans-acting inducer responsive repressor codes for a repressor protein that regulates *ars* gene expression (Francisco et al. 1990). Following reduction of arsenate, the reduced arsenite is pumped out from the cell through this pump. The ArsB protein is well characterized in most of the arsenate-reducing and arsenate-resistant mesophilic bacteria like *Pseudomonas* sp., *Shewanella* sp., *Bacillus* sp., and *Staphylococcus* sp. (Rosen 1999; Wu et al. 1992), while in case of thermophilic bacteria, this protein was found only within limited number of spices including *Hydrogenobacter thermophilus*, *Pelotomaculum thermopropionicum*, *S. thermophila*, *A. flavithermus*, and *Syntrophothermus lipocalidus* (Saw et al. 2008; Angelov et al. 2010; Arai et al. 2010; Kosaka et al. 2008). In few thermophilic bacteria including *P. thermopropionicum*, *Deferribacter desulfuricans*, and *S. lipocalidus*, another arsenite transporter Acr3 was detected (Takami et al. 2004; Takai et al. 2003). Acr3p transporters perform a similar function like ArsB, but the two proteins have no significant sequence similarity. The phylogenetic relation among the Acr3p sequences is presented in Fig. 6.6.

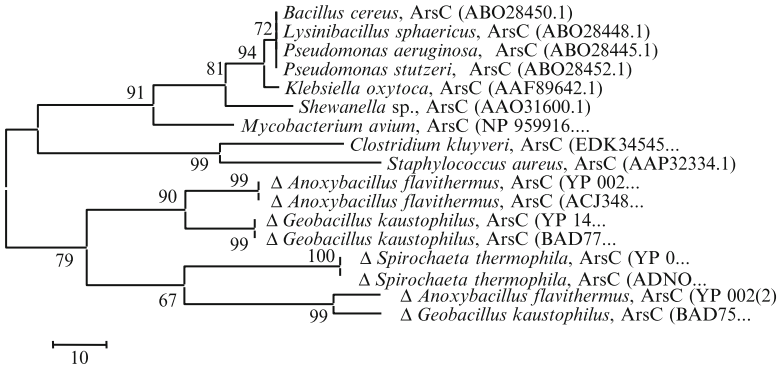

Fig. 6.5 Neighbor-joining tree depicting phylogenetic relation between arsenate reductase (*ArsC*) proteins and thermophilic bacteria. The evolutionary history was inferred using the neighbor-joining method. The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. Phylogenetic analyses were conducted in MEGA4. Δ indicates genes from thermophilic bacteria

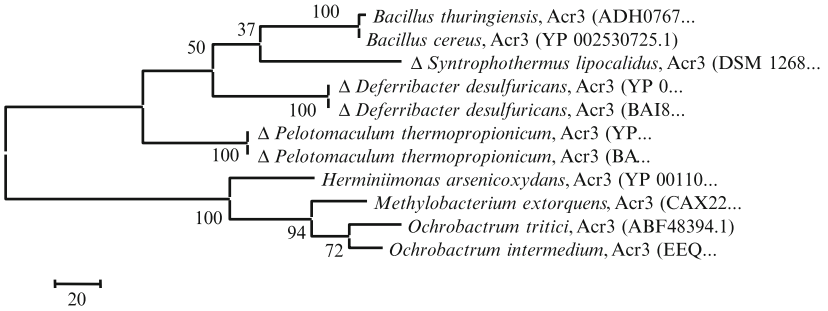

Fig. 6.6 Neighbor-joining tree depicting phylogenetic relation between arsenite transporter (*Acr3p*) proteins and thermophilic bacteria. The evolutionary history was inferred using the neighbor-joining method. The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Poisson correction method and are in units of the number of amino acid substitutions per site. Phylogenetic analyses were conducted in MEGA4. Δ indicates genes from thermophilic bacteria

The ArsR protein was characterized in both mesophilic (*Klebsiella* sp., *Halobacterium* sp., *Bacillus* sp., *Microcystis* sp., *Pantoea* sp., *Pseudomonas* sp., *Staphylococcus* sp.) as well as thermophilic (*P. thermopropionicum*, *S. lipocalidus*, *Caldicellulosiruptor bescii*, *Thermotoga lettingae*, *H. thermophilus*, *S. thermophila*, *G. kaustophilus*, *Deferribacter desulfuricans*, etc.) bacteria (Ong et al. 2010; Saw et al. 2008; Angelov et al. 2010; Arai et al. 2010; Kosaka et al. 2008; Takami et al. 2004; Takai et al. 2003).

6.2.1.4 Arsenite Respiratory Reductase (*arr*)

Arsenite respiratory reductase (Arr) system is well distributed within diverse dissimilatory arsenate-reducing prokaryotes capable of using arsenate as a respiratory electron acceptor while oxidizing various aromatic compounds. The Arr that has been characterized from *Shewanella* sp. strain ANA-3 (Saltikov and Newman 2003) is a periplasmic dimethyl sulfoxide (DMSO)-type reductase (2,596 bp, 854 amino acids, or 95.2 kDa) that reduces arsenate to arsenite and considered as reliable biomarker for arsenate respiration (Richey et al. 2009). Arsenite reductase gene (*arrA*), encoding the Arr protein, has been studied from a number of mesophilic bacteria (*Bacillus selenitireducens*, *Desulfitobacterium hafniense*, *Wolinella succinogenes*, *Bacillus arseniciselenatis*, *Chrysiogenes arsenatis*, and *Sulfurospirillum barnesii*) while its presence in thermophiles is much less (Afkar et al. 2003). The only thermophilic bacteria reported to have Arr protein is *Deferribacter desulfuricans* (Takai et al. 2003).

6.2.2 Metal Biosorption and Bioaccumulation by Thermophiles

Microorganisms can physically remove metals from aqueous solution by direct cellular interaction leading to intracellular bioaccumulation or biosorption on cell surface. Sequestration of toxic metals by biosorption/bioaccumulation is considered to be one of the most practical and widely used approaches for bioremediation (Barkay and Schaefer 2001; Tabak et al. 2005; Gadd 2010). Biosorption is a metabolism-independent passive sequestration of metals, while bioaccumulation refers metabolism-dependent intracellular metal accumulation by live cells. Living cells accumulate metal usually in two phases: a first rapid binding onto cell surface ligands followed by relatively slower intracellular transport via either metal specific or nonspecific broad ion channels/pumps. Biosorption, on the other hand, results mainly due to complex interaction (ion exchange, adsorption, microprecipitation, and electrostatic interaction) with cellular metal-binding groups (carboxylic, phosphate, amino, hydroxyl, etc.) facilitating metal sequestration within various cell sectors (Schiewer et al. 2000). In last three decades, metal biosorption and bioaccumulation by mesophilic bacteria is studied extensively with developments of several metal removal systems of industrial use. Thermophiles, although occupy several natural with elevated metals habitats (hot springs, deep-sea hydrothermal vents, volcanic areas, etc.), their uptake by thermophilic bacteria, however, is relatively less studied. Few investigations have focused on metal uptake by thermophilic bacteria with optimal growth temperature between 45 and 80°C (Wightman et al. 2001; Burnett et al. 2006a, b, 2007; Hetzer et al. 2006). As mentioned by Burnett et al. (2007), due to their elevated growth temperature optima, thermophiles have different cell wall configurations than mesophiles, and their reactivity toward aqueous metal ions (in terms of rate, selectivity, and extent of adsorption) may also be different. Daughney et al. (2010) have also indicated that the distinct cellular properties of thermophiles that confer thermostability and solute impermeability may influence their reactivity

toward dissolved metals and protons. It has been further indicated that specifically, both the relative amounts and the dissociation constants for thermophilic functional groups (especially for the most acidic COOH site) may be different from previously studied mesophiles (Burnett et al. 2007 and references therein). Capasso et al. (1996) identified a high-molecular weight cadmium-binding protein in Cu-resistant bacterium *Bacillus acidocaldarius* present as an essential component of the mechanism mediating recovery from heavy metal toxicity. Burnett et al. (2007) have indicated that metals bind to the thermophilic bacterium *A. flavithermus* in the preferential order Mn ≈ Ni < Zn < Cd < Pb ≈ Cu, and the metal binding is facilitated by complexation with carboxyl, phosphoryl, and hydroxyl moieties. Hetzer et al. (2006) tested Cd sensitivity and adsorption in a number of thermophilic bacteria affiliated to the genera *Aneurinibacillus*, *Anoxybacillus*, *Bacillus*, *Brevibacillus*, and *Geobacillus*. Following initial screening, selected genera like *Geobacillus*. *G. thermocatenulatus*, and *G. stearothermophilus* used for detail investigation. This study revealed that a functional group with a pK value of approximately 3.8 accounts for 66 and 80% of all titratable sites for *G. thermocatenulatus* and *G. stearothermophilus*, respectively, is dominant in Cd^{2+} adsorption reactions. Biosorption of Cd, Cu, Ni, Zn, and Mn on *G. toebii* ssp. *decanicus* and *G. thermoleovorans* ssp. *stromboliensis* was investigated in a batch-stirred system by Ozdemir et al. (2009). The equilibrium adsorptive quantity was determined to be a function of several parameters (solution pH, contact time, biomass concentration, initial metal concentrations, and temperature). Daughney et al. (2010) investigated proton and metal adsorption properties of *Thermococcus zilligii*. The data indicated that the archaeon displays significantly lower overall sorption site density over the previously studied thermophilic bacteria *A. flavithermus*, *G. stearothermophilus*, *G. thermocatenulatus*, and *Thermus thermophilus*. These investigators reported that thermophilic bacteria and archaea display lower sorption site densities than the mesophilic microorganisms, thus indicating to a general pattern of total concentration of cell wall adsorption sites per unit biomass being inversely correlated to growth temperature.

Biosorption of heavy metals from industrial wastewater by *Geobacillus thermodenitrificans* was studied by Chatterjee et al. (2010). Metal-binding capacity of the *G. thermodenitrificans* isolated from Damodar River, India, was assessed using synthetic metal solutions and industrial wastewater. Biosorption preference of dead bacterial biomass for the synthetic metal solutions was found to be in the following order: $Fe^{+3} > Cr^{+3} > Co^{+2} > Cu^{+2} > Zn^{+2} > Cd^{+2} > Ag^{+} > Pb^{+2}$. Ozdemir et al. (2012) have reported bioaccumulation and heavy metal resistance of Cd^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, and Mn^{2+} ions by thermophilic *Geobacillus toebii* ssp. *decanicus* and *G. thermoleovorans* ssp. *stromboliensis*.

6.2.3 Metal Transformation by Thermophiles

Microbe-mediated oxidative and/or reductive transformations of toxic metals greatly influence metal solubility, mobility, and toxicity and have found potential application

in bioremediation (Nealson and Cox 2002; Umrania 2006). These redox-mediated processes include autotrophic and heterotrophic leaching, reductive precipitation, sulfate reduction, and metal sulfide precipitation (Gadd 2004). Microbial transformation of metals serves multiple functions ranging from energy generation during anaerobic growth (dissimilatory metal reduction), biosynthesis (assimilatory metal reduction), and reduction related to metabolic process supporting growth but provides only detoxification function and finally oxidation during chemolithotrophic metabolism (Barkay and Schaefer 2001; Slobodkin 2005; Rawlings and Johnson 2007). From bioremediation perspective, all three processes are of great importance as most of the metals in their reduced form are less soluble and less toxic over their oxidized counterparts (except, arsenic, selenium, etc.). Microorganisms can reduce a wide variety of toxic metals and radionuclides considered as environmental contaminants either by direct enzymatic reduction that involves use of oxidized forms of these metals as terminal electron acceptor or alternatively as secondary consequence of metabolic processes unrelated to the transformed metals eventually leading to their precipitation (Valls and de Lorenzo 2002; Tabak et al. 2005). Microbial reduction of metal is a topic of great scientific and technological interest as these transformations play crucial roles in biogeochemical cycling of elements and offer the basis for wide range of biotechnological applications (including but not limited to bioremediation, bioenergy, astrobiology, and our efforts to explore life beyond this planet) (Nealson and Cox 2002; Lloyd 2003; Cockell 2010).

6.2.3.1 Reductive Transformation Mediated Metal and Radionuclide Precipitation

Mechanism of metal and radionuclide reduction by several bacteria is well studied (Lloyd 2003; Wall and Krumholz 2006; Hau and Gralnick 2007). Biogeochemical cycles of both modern as well as ancient biosphere are incredibly influenced by microbial reduction of metals. For all the contemporary environments, microbial processes that reduce Fe, Mn, Cr, and U are not only interrelated with the cycles of C, O, and S that have considerable ecological effect, but more importantly, the reduction of Fe(III) and Mn(IV) by microorganisms is the main process providing for the oxidation of organic matter (Lovley et al. 1991; Nealson and Saffarini 1994; Straub et al. 2001). In the ancient biosphere, such reduction processes possibly played greater role as Fe(III) was probably evolutionarily the first and, for a certain period, major oxidant of organic carbon (Walker 1987). In recent time, metal reduction by microbes, particularly by thermophilic bacteria and archaea, provokes a great deal of interest for environmental restoration. As the oxidized forms of many toxic metallic contaminants (viz., U, Tc, Cr, etc.) are highly soluble in aqueous milieu with increased mobility and toxicity over their reduced forms that are insoluble and often precipitate from solution, microbial reduction of these elements becomes a subject of immense interest to clean up metal-contaminated environments. Metal reduction by thermophilic microorganisms is particularly more relevant for remediation of metal and radionuclides within nuclear wastes and waste treatment

facility that use anaerobic sludge reactor with temperature range exceeding that of mesophiles. In this context, activities of dissimilatory metal-reducing microorganisms are of particular importance as they can alter the solubility of radionuclides by either direct enzymatic reduction or by indirect mechanisms catalyzed via a range of electron-shuttling compounds (Lloyd et al. 2002; Anderson et al. 2003; Slobodkin et al. 2006).

Dissimilatory metal-reducing bacteria (DMRB) can couple the reduction of a variety of metals to cellular respiration and growth. As narrated by Nealson and Cox (2002), the excitement of this metabolic group lies not only in the elucidation of a new type of metabolism but also in the potential use of these abilities for the removal of toxic organics and in their ability to reduce (and thus, detoxify) other toxic metals. Dissimilatory reduction of iron, uranium, selenium, chromium, technetium, gold, cobalt, and molybdenum by a number of thermophilic microorganisms is reported. Reduction of these metals and even radionuclides by thermophilic bacteria and archaea is considered only useful for developing bioremediation strategies at elevated temperatures but also in recovering useful metals from low-grade ores (Rawlings et al. 2003; Khijniak et al. 2005; Slobodkin et al. 2006; Slobodkina et al. 2007). Thermophilic microorganisms capable of Fe^{3+} reduction are well studied since the first report on *Sulfolobus acidocaldarius* by Brock and Gustafson in 1976 and subsequently, by Slobodkin et al. (1995) followed by the report on thermophilic Fe(III)- and Mn(IV)-reducing bacterium *Bacillus infernus* by Boone et al. (1995). Since then, many thermophilic bacteria and archaea capable of performing organotrophic with fermentable substrates or chemolithoautotrophic growth with molecular hydrogen reducing Fe(III) to Fe(II) are reported (Zavarzina et al. 2007). Microorganisms capable of dissimilatory iron reduction have been isolated and characterized from diverse thermal ecosystems including hydrothermal vents, geothermally heated soils and subsurface water/sediment, high-temperature oil fields, and in sulfide chimneys (black smokers) (Slobodkin 2005). Representatives of thermophilic microorganisms capable of dissimilatory reduction of Fe^{3+} are present in both the domains of prokaryote. In bacterial domain, the members are distributed across phylogenetically diverse groups like low G+C Gram-positive bacteria (*B. infernus*, *Thermoterrabacterium ferrireducens*, *Thermovenabulum ferriorganovorum*, *Thermosinus carboxydovorans*, and species of the genera *Thermoanaerobacter*, *Anaerobranca*, and *Sulfobacillus*), *Actinobacteria*, *Deferribacteres*, *Thermus-Deinococcus* group, *Aquificae* group, *Thermotogae*, and *Thermodesulfobacteria* (Slobodkin 2005). Mechanism of Fe^{3+} reduction by a limited number of thermophilic bacteria is studied. Vadas et al. (1999) have done extensive characterization of the iron reductase of the archaeon *Archaeoglobus fulgidus*, belonging to NAD(P)H:FMN oxidoreductases (Vadas et al. 1999). Dissimilatory iron reductase activity of another archaeon *Pyrobaculum islandicum* was found to be not related to *c*-type cytochromes (Childers and Lovley 2001). Recently, Gavrilov et al. (2007) have characterized membrane-bound Fe^{3+}–EDTA reductase activities of the thermophilic Gram-positive dissimilatory iron-reducing bacterium *T. ferrireducens*. These investigators have observed that similar to the mesophilic Gram-negative dissimilatory iron reducers (*Shewanella* and *Geobacter*),

T. ferrireducens also employs *c*-type cytochromes to transfer electrons for Fe(III) reduction. However, in contrast to the membrane-bound iron reductase activities of *G. sulfurreducens*, *T. ferrireducens* reductase showed preferential utilization of Fe (III)–EDTA as the electron acceptor.

Metal reduction by thermophilic microbes poses great advantage for precipitation/immobilization of toxic metals and radionuclides (U, Cr, Tc, Co, etc.) in the biotechnological processes of hot wastewater treatment or heated nuclear waste streams and thermally insulated contaminated environments where temperature is elevated by the decay of long-lived radionuclides or in the subsurface depositories of nuclear waste due to geothermal heating (Chernyh et al. 2007; Brim et al. 2003). Since most of the hydrothermal fluids are found to be enriched with heavy metals (Co, Mo, Cr, and U) at elevated concentration, it is not surprising that thermophiles will reduce these metals as well, thus providing exciting opportunities for their application in metal and radionuclide bioremediation. Enzymatic uranium reduction has been shown in *Thermus scotoductus*, *P. islandicum*, *Thermoanaerobacter* sp., and *T. ferrireducens* (Kashefi and Lovley 2000; Kieft et al. 1999; Roh et al. 2002). In comparison to the earlier reports that fail to demonstrate conservation of energy for growth during U(VI) reduction, *T. ferrireducens* could couple organotrophic growth to the reduction of sparingly soluble U(VI) phosphate. In all cases, reduced uranium is found to be precipitated as either uraninite (UO_2) or U(IV)–calcium phosphate (ningyoite). In contrast to the preexisting impression that at uranium-contaminated sites, solid-phase U(VI) present in sediments is resistant to microbial reduction (Ortiz-Bernad et al. 2004), results with *T. ferrireducens* showed that in its U(VI) form, uranium could be potentially bioavailable. Results of this study extend the limited number of known uranium-reducing microorganisms and demonstrated that there are biogenic transformations of U-phosphate minerals that may take place in uranium ore deposits and uranium-contaminated environments (Khijniak et al. 2005). In recent time, formation of such biogenic UO_2 is considered to be a fascinating and important nanoscale biogeological material owing to its long-term structural stability, crucial to the viability of microbial bioremediation strategies (Lee et al. 2010).

Thermophilic bacteria like *P. islandicum*, *T. ferrireducens*, and *Tepidibacter thalassicus* and archaea like *Thermococcus pacificus* and *Thermoproteus uzoniensis* have been studied for technetium [^{99}Tc (VII)] reduction (Gavrilov et al. 2004; Chernyh et al. 2007). Technetium (Tc^{99}), a β-emitting fission product of ^{235}U is long-lived radioactive pollutant of significant environmental concern. The pertechnetate anion (TcO_4^-) of technetium is highly soluble and mobile, acts as sulfate analogue, and despite its artificial nature, gets assimilated by plants and thus bioaccumulated in food chain (Lloyd and Macaskie 2000). Enzymatic reduction of highly soluble pertechnetate anion (Tc(VII)) to insoluble low-valence form is considered to an effective strategy for its immobilization. Technetium reduction under anaerobic condition has been demonstrated by a number of mesophilic bacteria, mostly iron-/sulfur-reducing, fermentative, and aerobic bacteria have been well documented (Chernyh et al. 2007 and references therein). Kashefi and Lovley (2000) reported anaerobic pertechnetate reduction at 100°C by washed cell suspension of the archaeon *P. islandicum*. Chernyh et al. (2007) have done a thorough

investigation on Tc(VII) by isolated thermophilic bacteria and archaea. The Euryarchaeota *T. pacificus* and the Gram-positive bacterium *T. thalassicus* isolated from shallow- and deep-sea hydrothermal vents having high concentrations of heavy metals and elevated level of radiation were used. Washed cell suspension of these organisms showed Tc(VII) reduction with molecular hydrogen as an electron donor forming insoluble Tc(IV) precipitates. The results demonstrated the potential of thermophilic bacteria and archaea for Tc immobilization during bioremediation of contaminated environments and in biotechnological processes (Chernyh et al. 2007).

Thermophilic bacteria capable of reduction of chromium (VI), selenite [Se(IV)], and tellurite [Te(IV)] are also reported. Members of bacterial genera including *Thermoanaerobacter*, *Thermus* sp., *Deinococcus geothermalis*, and the hyperthermophilic *archaeon* and *P. islandicum* are known to be capable of reducing chromate (Kieft et al. 1999; Brim et al. 2003; Kashefi and Lovley 2000). *T. ferriphilum* and *Thermosinus carboxydivorans* have a selenite-reducing capacity, while representatives of the genus *Thermus* have shown their ability to reduce both selenite and tellurite (Slobodkin et al. 2006; Sokolova et al. 2004; Chiong et al. 1988). Recently, Slobodkina et al. (2007) have reported Cr(VI), Se(IV), and Te(IV) reduction by a moderately thermophilic, facultatively anaerobic bacterium *Bacillus thermoamylovorans*. It was shown that although these metals exert bacteriostatic effect on the bacterium, their reduction provides a detoxification mechanism (Slobodkina et al. 2007).

Apart from these direct enzymatic reductions of heavy metals and radionuclides, indirect reduction of soluble contaminants within the various environments may facilitate bioremediation (Tabak et al. 2005). Indirect metal reduction, leading to precipitation/immobilization, could be accomplished by various metal-reducing or sulfate-reducing bacteria and archaea. Thermophilic microorganisms capable of coupling oxidation of organic compounds or hydrogen to the reduction of iron [Fe(III)], manganese [Mn(IV)], or sulfur [S(IV)] (in the form of SO_4) are reported (Kashefi and Lovley. 2000; Sheoran et al. 2010). Microbiologically produced Fe(II), Mn(III), and S(II) (as H_2S) can in turn chemically reduce metals or radionuclides to yield separate or multicomponent insoluble species (Tabak et al. 2005, and references therein). Sulfate-reducing prokaryotes (SRP) (including both bacterial and archaeal domains) inhabit a variety of sulfate-rich reducing environments, and among these, geothermal vents are the major habitat. Castro et al. (2000) have made a detailed phylogenetic analysis of thermophiles wherein bacterial members represented by the genera *Thermodesulfobacterium* and *Thermodesulfovibrio* inhabiting high-temperature environments and archaeal thermophilic SRP (genus *Archaeoglobus*) thriving in marine regions at temperatures above 80°C have formed two distinct groups. Activity of thermophilic SRP is considered highly relevant for the treatment of various acid mine drainage and acid rock drainage enriched with several toxic metals and sulfate along with their application in immobilization of radionuclides and metals from nuclear wastes (Sheoran et al. 2010). Role of sulfate-reducing bacteria in bioremediation of multiple heavy metals containing acid rock drainage has recently been reviewed by Sheoran et al. 2010.

6.2.3.2 Oxidative Solubilization of Metals

Chemolithotrophic microorganisms catalyze direct or indirect oxidation of reduced metals as they obtain their energy by either oxidizing ferrous iron or reducing sulfur compounds as electron donor, thereby converting the insoluble metal to a soluble form. Microbe-mediated metal oxidation leading to solubilization and extraction (biomining) has become one of the most successful and expanding areas of biotechnology for recovering metals from low-grade ores to concentrates (Rawlings and Johnson 2007). Biomining exploits acidophilic microorganisms to recover valuable metals (i.e., Cu and Au) from ores in biohydrometallurgical processes conducted at temperatures ranging from ambient to 80°C (Auernik et al. 2008). The most important microorganisms in mineral biooxidation processes that operate at ≤40°C are believed to be a consortium of Gram-negative bacteria including iron- and sulfur-oxidizing *Acidithiobacillus ferrooxidans*, sulfur-oxidizing *At. thiooxidans*, *At. caldus*, and iron-oxidizing *Leptospirillum ferrooxidans* and *L. ferriphilum* (Rawlings et al. 2003). At higher temperatures (~50°C), *A. caldus*, *Leptospirillum* spp., Gram-positive genera *Sulfobacillus* and *Acidimicrobium*, and members of the archaeal genus *Ferroplasma* are generally predominant. Extremely thermoacidophilic archaea from the genera *Sulfolobus*, *Acidianus*, and *Metallosphaera* are reported to be involved in mobilization of metals from metal sulfides at higher temperature (Auernik et al. 2008). In recent time, thermophilic microorganisms have gained considerable interest not only in biomineralization of metal-containing ores and concentrates but at the same time, for the treatment of metal-containing wastes generated from various metal industries and mines. Metal-containing solid wastes such as slag, ash, sludge, and residues generated from primary and secondary nonferrous processing industries contain large quantities of heavy metals including As, Cd, Cr, Ni, Pb, Cu, Hg, and Zn considered harmful to the environment (Agrawal et al. 2004; Liu et al. 2008; Cheng et al. 2009). Mine overburden and mine tailings generated from mining activities contain sulfide ores (including the pyrite FeS_2). Microbial oxidation and dissolution of sulfide minerals produce environmentally detrimental acid mine drainage (AMD) and more commonly acid rock drainage (ARD) comprised of various heavy metals and sulfate at highly acidic pH, posing considerable adverse effect to groundwater and surface water through heavy metal contamination and acidification (Sheoran et al. 2010). Bioleaching/biosolubilization of metal sulfide ores is considered to be an ideal alternative for the mitigation of pollution even at mining sites. It has been found that maximum rates and yields of metal extraction can be enhanced at elevated temperatures (Norris 1990). It has also been emphasized that there is a need to search metal tolerant, metal absorbent, as well as moderate thermophilic acidophilic organisms for biogeotechnological applications (Umrania 2006). Recovering metal values and removing the hazardous elements from the wastes are important not only for saving metal resources but also for protecting the environment (Agrawal et al. 2004; Cheng et al. 2009). Microbe-mediated metal solubilization and recovery have been rapidly developed in recent decades for its advantages, which include mild reaction condition, low-energy consumption, simple process, low environmental impact, and being suitable for low-grade

mine tailings and residues. In contrast to such applications, there are only few studies on the application of this innovative technique for recovering metals and reducing the toxicity of metal-containing residues (Guo et al. 2008). Metal oxidation leading to its solubilization by thermophilic microorganisms has recently been studied for smelting slag with low concentrations of metals and electronic scraps. Thermophiles are thought to make bioleaching more efficient for the process to run at higher temperatures which result in a faster reaction rates (Cancho et al. 2007). Guo et al. (2010) have studied solubilization of Pb/Zn from smelting slag using moderate thermophilic *Bacillus* spp., *Sporosarcina* spp., and *Pseudomonas* spp. These investigators demonstrated that metal oxidation by indigenous thermophilic bacteria can be a promising process for solubilization and removal of toxic metals (As, Cu, Mn, and Zn) from the Pb/Zn smelting slag that aids its bioremediation. Owing to the higher rates of metal bioleaching by thermophilic microorganism, recent attention has been paid toward their application in treatment of metal-containing electric and electronic wastes for the recovery and recycling of industrially important and precious metals (Cu, Pb, Ni, Ag, Au, Pt, etc.) (Ilyas et al. 2007; Cui and Zhang 2008). Ilyas et al. (2007) demonstrated that metals can be recovered from electronic scrap by microbial leaching using metal preadapted moderately thermophilic bacterium *S. thermosulfidooxidans*. Recently, Umrania (2006) has examined the potential use of acidothermophilic bacteria for bioremediation of toxic heavy metals from metal-rich industrial wastes. Seventy-two thermophilic bacteria were initially isolated, screened for metal tolerance and metal biosorption, and finally, one strain designated as *ATh*-14 was studied for metal sulfide oxidation-based bioremediation. The selected strain showed maximum adsorption of Ag > Pb > Zn > As > Ni > Cr in chalcopyrite and thus appeared to be a suitable candidate in bioleaching and bioremediation (Umrania 2006). Gihring and Banfield (2001) investigated arsenic oxidation by a newly isolated *Thermus* strain. Along with various anthropogenic sources, geothermal fluids, capable of leaching rock, are also considered to be a significant source of arsenic that contributes to arsenic mobilization into ground and surface waters (Umrania 2006). Gihring and Banfield (2001) reported that *Thermus* strain HR13 is capable of rapidly oxidizing inorganic As(III) to As(V) without any gain of metabolic energy, while under anaerobic condition, the bacterium can grow by coupling oxidation of lactate to As(V) reduction. *Thermus* HR13 therefore may contribute both toward arsenic mobilization as well as arsenic removal from geothermal waters.

6.3 Future Perspectives

Bioremediation of metal-contaminated soils, sediments, and waters is a multifaceted business that exploits diverse biological activities ranging from single molecules to entire microorganisms (Tabak et al. 2005). To make it more attractive, this technology must be adapted with specific contamination problems in specific environment. Developments of *in situ* treatment methods, treatment of mixed wastes or radioactive

wastes, particularly in high temperature environments, are some of the main targets. Knowledge of microbial processes that are operative at high temperature can be employed in metal decontamination under such conditions. Recent observations on multiple metal and radionuclide [Cr(VI), Co(III), U(VI), and Tc(VII)] reduction abilities of hyperthermophilic bacterium *P. islandicum* resulting in the formation of insoluble uranium deposits at temperature nearly 100°C in hydrothermal vents provide evidence about the extraordinary capability of thermophiles, which could be relevant for metal bioremediation. Further study in this direction may result in novel strategies for *in situ* treatment of metal-contaminated environments (Lovley and Coates 2000; Coates and Anderson 2000; Valls and de Lorenzo 2002). The distribution and diversity of thermophilic microorganisms inhabiting contaminated sites and their genes that code for the attributes required to cope with harsh environments having elevated concentrations of metals should be taken into account for future research as these insights are invaluable in metal bioremediation. Metagenomic investigations can provide key information of phylogenic and functional diversity of thermophiles in specific environments. Also, such studies will provide direct insights on the metabolic potentials of these indigenous microorganisms and on their genetic determinants relevant to their functions in natural habitat. Metagenome sequencing from high-temperature geothermal environments provides excellent tool for accessing and characterizing the predominant members of such microbial communities as well as their functional attributes relevant for survival and growth of these extremophiles (Inskeep et al. 2010).

Intensive research should be focused on the various modes of metal resistance and interaction in thermophilic microorganism, which may contribute to improvement in bioremediation strategies. For instance, little is currently known about enzymatic metal transformations by such microorganisms, which may find potential application in metal immobilization in contaminated sites. Studies on some chemolithotrophic, thermophilic, and anaerobic bacteria, or on thermophilic bacterial heavy metal resistance/accumulation/transformation characteristics, or on the metal efflux/transformation proteins/enzymes of such microorganisms may contribute to the progress in bioremediation (Spada et al. 2002; Kashefi et al. 2003; Umrania 2006; Sokolova et al. 2007; Chatterjee et al. 2010; Onyenwoke et al. 2009; Barkay et al. 2010). Development of recombinant thermophilic strain(s) incorporating desirable genes for metal bioremediation from suitable mesophilic or other extremophilic microorganism may open up new possibilities. Engineering extremely radiation-resistant thermophilic bacterium *Deinococcus geothermalis* has generated a Hg(II)-resistant strain capable of reducing Hg(II), Fe(III)-nitrilotriacetic acid, U(VI), and Cr(VI) at elevated temperatures and in presence of ionizing radiation (Brim et al. 2003). These characteristics support the prospective development of this thermophilic radiophile for bioremediation of radioactive mixed waste environments with high temperatures (Brim et al. 2003). Improvement in our understanding of metal resistance and transport mechanisms in thermophilic bacteria and archaea is imperative to explore the potential of such organisms in metal and radionuclides bioremediation at elevated temperature.

Biosurfactants or other extracellular polymers from thermophilic microorganisms can also be used in solubilization and mobilization of metals from polluted soils and sediments. These molecules are competitive in soil treatments as they have excellent surface properties and are environment friendly than synthetic compounds (Tabak et al. 2005). Polymer engineering through the introduction of required genes of the biosynthetic pathways may further enhance the promising possibilities of these polymers in metal bioremediation (Gutnick and Bach 2000). The metal-microbe interaction processes taking place in the cell microenvironment need to be better elucidated so that such well-characterized systems can be used efficiently. Similar to mesophilic organisms, genetic engineering of thermophilic bacterial biosorbents and exploration of novel-binding molecules, such as designed phytochelatin analogues (Bae et al. 2000) and selected polypeptides with new affinities, may further enhance metal bioaccumulation/biosorption (Valls and de Lorenzo 2002). Genomic and proteomics approaches will provide us access to new and enticing activities of such extremophiles. The availability of genome sequences of some of the thermophilic microorganisms inhabiting metal polluted environments will be very advantageous for further research (Valls and de Lorenzo 2002; Auernik et al. 2008).

6.4 Conclusions

Thermophilic microbial metal resistance and interactions offer enormous opportunities to develop bioremediation strategies for cleaning up of contaminated environments associated with high temperature and other stressful factors. Such microorganisms are abundant in geological/anthropological thermal environments with high concentrations of dissolved metals and possess unique cell wall/membrane structures as well as enzymatic and metabolic properties, which may contribute in their metal resistance and interaction processes. The nature and extent of metal accumulation onto thermophilic microorganisms may differ qualitatively and quantitatively from that of mesophilic organisms. Thermophilic microorganisms with elevated metal resistance can exhibit enhanced metal solubilization through sulfur- and iron-oxidizing activities, which may be implemented in metal recovery and remediation. Thermophilic microbial community can perform both degradative and productive functions as evident from the ability of the iron-reducing thermophiles to couple metal reduction with oxidation of a variety of organic and inorganic substrates. Thermophiles are capable of reducing a wide spectrum of metals/radionuclides, which can be exploited for the immobilization of toxic metals/radionuclides in the environment. Thermophilic microbial metal transformations may have potential applications in *in situ* and *ex situ* bioremediation of contaminated soils, sediments, and waters. Development of recombinant thermophilic strain(s) incorporating desirable genes for metal bioremediation from suitable mesophilic or other extremophilic microorganism may open up new possibilities. Engineered extremely radiation-resistant thermophilic bacterium is capable of reducing several metals and radionuclides at elevated temperatures and in the presence of radiation, which

indicates the prospective development of thermophilic radiophiles for bioremediation of radioactive mixed waste environments with high temperature. Improvement in our understanding of metal resistance and transport mechanisms in thermophilic bacteria and archaea is imperative to explore the potential of such organisms in metal and radionuclides bioremediation at elevated temperature. Genomic/proteomic approaches and availability of genome sequences may provide us new informations on diverse activities of such extremophiles.

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Chapter 7
CO-Oxidizing Anaerobic Thermophilic Prokaryotes

T. Sokolova and A. Lebedinsky

Abstract Being a potent electron donor ($E^{0'}_{CO/CO2} = -520$ mV), CO may serve as an energy source for anaerobic prokaryotes. The main sources of CO in hot environments inhabited by anaerobic thermophiles are volcanic exhalations and thermal degradation of organic matter. A number of phylogenetically diverse anaerobic prokaryotes, both Bacteria and Archaea, are known to metabolize CO. CO transformation may be coupled to methanogenesis, acetogenesis, hydrogenogenesis, and sulfate or ferric iron reduction. This review will mainly focus on the diversity, ecology, physiology, and certain genomic features of the hydrogenogenic species, which are most numerous among the currently recognized thermophilic anaerobic carboxydotrophs and many of which were isolated and described in recent years. Among them are diverse Firmicutes, Dictyoglomi, and Eury- and Crenarchaeota. Despite their phylogenetic diversity, they employ similar enzymatic mechanisms of the $CO + H_2O \rightarrow CO_2 + H_2$ process. The key enzyme of anaerobic CO utilization, the Ni-containing CO dehydrogenase, forms in hydrogenogens an enzymatic complex with the energy-converting hydrogenase, and genomic analysis shows this enzymatic complex to be encoded by a single-gene cluster.

Keywords CO • Carboxydotrophs • CO dehydrogenase • CO transformation • Hydrothermal environments • Volcanic gases

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7.1 Introduction

CO is a possible substrate for growth of thermophilic prokaryotes in their natural habitats – hot volcanic environments. The reported concentration of CO in volcanic gases varies from 0.6 to 5540 ppm (for references, see Sokolova et al. 2009). The main sources of CO in hydrothermal environments are eruptive (Menyailov and Nikitina 1980; Greenland 1986; Symonds et al. 1994; Allard and Barton 2004) or fumarolic volcanic gases (Garofalo et al. 2007; Tassi et al. 2003, 2005). A minor source of CO in hot volcanic environments may be its production in the course of thermochemical or photochemical degradation of organic matter (Conrad and Seiler 1985; Schade et al. 1999; Hellebrand and Schade 2008). One more minor source of CO is its formation as a side product by some thermophilic anaerobes (Conrad and Thauer 1983; Diekert et al. 1984). However, sources of CO mentioned as minor in hot volcanic environments, i.e., thermochemical degradation of organic matter and CO production by some thermophilic anaerobes as a side product, probably play a more important role in other thermophilic habitats, such as bioreactors or composts.

7.2 CO Transformation in Hot Volcanic Environments

CO is a potent electron donor ($E^{0\prime}_{CO/CO2} = -520$ mV); thus, it is an energy-rich substrate that can serve as an electron donor for the growth of lithotrophic microorganisms; however, its utilization by microorganisms is restricted because of its high toxicity to metal-containing enzymes (Thauer et al. 1974; Fauque et al. 1988; Adams 1990). However, a number of anaerobic thermophilic prokaryotes, among which there are carboxydotrophic hydrogenogens, methanogens, acetogens, and sulfate and ferric iron reducers, can use CO as a source of energy for growth; some organisms use CO as a cell carbon source as well. The concentration of CO dissolved in hot spring water is usually rather low (Shock et al. 2005; Kochetkova et al. 2011). An active process of CO transformation by thermophilic anaerobic community was demonstrated in several hot springs of Uzon Caldera (Kamchatka Peninsula) (Kochetkova et al. 2011). The actual rate of anaerobic CO transformation by the microbial community was determined for one of studied springs to be 120 μmol l^{-1} of sediment day^{-1}. For all the hot springs studied, the results of radioisotopic tracing experiments showed that active anaerobic transformation of CO took place and that the major part of CO (89–99%) was converted to CO_2. Production of ^{14}C-labeled methane was not detected, and the production of ^{14}C-labeled volatile fatty acids, presumably acetate, was comparatively low: it did not exceed 4.8%. These data indicate that either hydrogenogenic carboxydotrophy or CO oxidation in the course of anaerobic respiration are dominant processes of CO transformation in the habitats studied. However, methanogenesis from CO could not be completely excluded, as it proceeds through formation of CO_2 as an intermediate (Stupperich and Fuchs 1984), and thus, significant dilution of isotope with the HCO_3^- ions present in hydrothermal water should reduce significantly the sensitivity of the method. Anaerobic

respiration processes, such as sulfate reduction (Parshina et al. 2005a, b) or Fe(III) reduction (Slobodkin et al. 2006), could also be responsible for anaerobic CO oxidation in these thermophilic environments.

Thus, it was demonstrated that CO is a substrate that is present and is utilized in natural hot environments under conditions suitable for moderately, extremely, and hyperthermophilic prokaryotes (Kochetkova et al. 2011).

7.3 Hydrogenogenic Carboxydotrophic Thermophilic Anaerobes

The main feature of hydrogenogenic carboxydotrophic prokaryotes is their ability to grow producing hydrogen as the only reduced product of the oxidation of carbon monoxide in the reaction with water:

$$CO + H_2O = CO_2 + H_2\,\Delta G^{0'} = -20\ \text{kJ/mol CO}.$$

The representatives of thermophilic hydrogenogenic carboxydotrophic bacteria were first found by V. A. Svetlichny in hot springs of Kunashir Island (Kurils) (Svelichny et al. 1991b). Currently, there are 17 hydrogenogenic carboxydotrophic species belonging to Firmicutes, Dictyoglomi, Euryarchaeota, and Crenarchaeota (Table 7.1).

7.3.1 Genus Carboxydothermus

This genus belongs to the family *Thermoanaerobacteraceae* in the class *Clostridia* (Ludwig et al. 2009) and is the taxon containing first described thermophilic hydrogenogenic carboxydotrophic species (Svetlichny et al. 1991a, b; Slobodkin et al. 2006; Wiegel 2009). Cells of *Carboxydothermus* species are straight to slightly curved rods, 0.3–0.5 × 1.3–2.7 μm; they have cell wall of the Gram-positive structure. All *Carboxydothermus* species are obligate anaerobes, extreme thermophiles, and neutrophiles. The optimal temperature for growth is in the range of 65–70°C. All *Carboxydothermus* species can utilize CO, but they are facultative carboxydotrophs and can grow chemolithotrophically on CO or on other substrates. Genus *Carboxydothermus* currently contains four species (Table 7.1): the three hydrogenogenic species, *C. hydrogenoformans* (Svetlichny et al. 1991a, b), *C. siderophilus* (Slepova et al. 2009), and *C. islandicus* (Novikov et al. 2011), and one nonhydrogenogenic carboxydotrophic species, *C. ferrireducens* (Slobodkin et al. 1997; Gavrilov et al. 2003; Slobodkin et al. 2006), which grows on CO only in the presence of ferric iron hydromorphic oxide, without production of hydrogen. The species of *Carboxydothermus* were isolated from

Table 7.1 Hydrogenogenic CO-oxidizing prokaryotes

Organism	CO-trophy	Autotrophy/ organotrophy	Acceptors	Acceptors during growth on CO	Optimal temperature (°C)/optimal pH of growth	Reference
Domain *Bacteria*						
Phylum *Firmicutes*, Class *Clostridia*, Order *Thermoanaerobacterales*, Family *Thermoanaerobacteraceae*						
Carboxydothermus hydrogenoformans	Facultative	+/+	AQDS, S^0, SO_3^{2-}, $S_2O_3^{2-}$, fumarate, nitrate	AQDS, fumarate	70–72/7.0	Svetlichny et al. (1991b), Henstra and Stams (2004)
Carboxydothermus siderophilus	Facultative	–/–	Fe(III), AQDS	Fe(III), AQDS	70/7.0	Slepova et al. (2009)
Caldanaerobacter subterraneus subsp. *pacificus*	Facultative	–/+	$S_2O_3^{2-}$	–	70/6.8–7.1	Sokolova et al. (2001), Fardeau et al. (2004)
Caldanaerobacter strain 2707	Facultative	–/+	$S_2O_3^{2-}$	–	75/7.0	
T. hydrosulfuricus subsp. *carboxydivorans*	Facultative	–/+	AQDS, Fe(III), S^0, SO_3^{2-}, $S_2O_3^{2-}$, MnO_2	ND	70/6.3–6.8	Balk et al. (2009)
Phylum *Firmicutes*, Class *Clostridia*, Order *Clostridiales*, Family *Peptococcaceae*						
Thermincola carboxydiphila	Obligate	–/–	–	–	55/8.0	Sokolova et al. (2005)
Thermincola ferriacetica	Facultative	–/+	Fe(III) oxide, AQDS, MnO_2, $S_2O_3^{2}$	ND	57–60/7.0–7.1	Zavarzina et al. (2007)
Thermincola potens	Facultative	ND/+	Fe(III) oxide, MFC anodes	ND	55/6.8	Byrne-Bailey et al. (2010), Wrighton et al. (2008)
Desulfotomaculum carboxydivorans	Facultative	+/–	SO_4^{2-}, SO_3^{2-}, $S_2O_3^{2-}$	SO_4^{2-a}	55/6.8–7.2	Parshina et al. (2005b)

Phylum *Firmicutes*, Class *Clostridia*, Order *Clostridiales*, Unclassified *Clostridiales*						
Carboxydocella thermautotrophica	Obligate	+/–	–	–	58/7.0	Sokolova et al. (2002)
Carboxydocella sporoproducens	Facultative	+/+	–	–	60/6.8	Slepova et al. (2006)
Carboxydocella ferrireducens	Facultative	–/+	Fe(III), AQDS	Fe(III)	60/6.8	
Phylum *Firmicutes*, Class *Negativicutes*, Order *Selenomonadales*, Family *Veillonellaceae*						
Thermosinus carboxydivorans	Facultative	–/+	Fe(III), SeO_3^{2-}, $S_2O_3^{2-}$	Fe(III), SeO_3^{2}	60/6.8–7.0	Sokolova et al. (2004a)
Phylum *Firmicutes*, Class *Thermolithobacteria*, Order *Thermolithobacterales*, Family *Thermolithobacteraceae*						
Thermolithobacter carboxydivorans	Facultative	+/+	–	–	70/6.8–7.0	Sokolova et al. (2007)
Phylum *Dictyoglomi*, Class *Dictyoglomia*, Order *Dictyoglomales*, Family *Dictyoglomaceae* *Dictyoglomales*; *Dictyoglomaceae* Family *Dictyoglomaceae*						
Dictyoglomus carboxydivorans	ND	–/ND	ND	ND	75/ND	Slepova et al. (2007)
Domain *Archaea*						
Phylum *Euryarchaeota*, Class *Thermococci* or *Protoarchaea*, Order *Thermococcales*, Family *Thermococcaceae*						
Thermococcus AM4	Facultative	–/–	S^0	S^0	85/7.0	Sokolova et al. (2004b)
Thermococcus onnurineus	Facultative	–/+	S^0	ND	80/8.5	Lee et al. (2008)
Thermococcus barophilus	Facultative	–/+	S^0	S^0	85/6.0	
Phylum *Crenarchaeota*, Class *Thermoprotei* or *Crenarchaeota*, Order *Thermoproteales*, Family *Thermofilaceae*						
Thermofilum carboxydotrophus	Facultative	–/ND	ND	ND	92/ND	Kochetkova et al. (2011)

The hierarchical classification is according to the J. P. Euzéby's site http://www.bacterio.cict.fr/classification.html

geographically distant hot springs characterized by pH values close to neutral and temperatures in the range of 60–80°C. *C. hydrogenoformans, C. siderophilus,* and *C. islandicus* were isolated from hot springs of Kunashir Island (Kurils), Geyser Valley (Kamchatka), and Iceland, respectively. *C. ferrireducens* was isolated from a hot spring in Yellowstone National Park, USA (Slobodkin et al. 1997). All *Carboxydothermus* species grow well on high CO concentrations (100% v/v in the gas phase). The type species is *Carboxydothermus hydrogenoformans* (Svetlichny et al. 1991a). Currently, *C. hydrogenoformans* is the best-studied thermophilic hydrogenogen. *C. hydrogenoformans* enzymes involved in hydrogenogenic CO oxidation were thoroughly studied (Svetlitchnyi et al. 2001, 2004a, b; Soboh et al. 2002; Dobbek et al. 2001, 2004). The complete genome of *C. hydrogenoformans* was sequenced (Wu et al. 2005). *C. islandicus,* as well as *C. hydrogenoformans,* grows on CO chemolithoautotrophically. Two other species require yeast extract for growth (0.2 g l^{-1}). *C. hydrogenoformans* performs fermentative growth on pyruvate accompanied by hydrogen, acetate and CO_2 formation, or nonhydrogenogenic growth with various electron donors and electron acceptors: it reduces AQDS (9,10-anthraquinone-2,6-disulfonate) with CO, H_2, glycerol, lactate, or formate or it reduces sulfite, thiosulfate, sulfur, nitrate, and fumarate with lactate (Henstra and Stams 2004). *C. siderophilus*, in contrast to other *Carboxydothermus* species, can grow only in the presence of hydromorphic ferric iron oxide or AQDS in the medium during the chemolithotrophic growth on CO or chemoheterotrophic growth on other substrates, and it does not use other electron acceptors like sulfite, thiosulfate, sulfur, nitrate, and fumarate (Slepova et al. 2009). *C. islandicus* does not reduce any of acceptors tested, i.e., hydromorphic ferric iron oxide, AQDS, sulfate, sulfite, thiosulfate, sulfur, nitrate, or fumarate. In addition to chemolithotrophic growth on CO, it performs fermentative growth on pyruvate (Novikov et al. 2011).

7.3.2 Genus Thermincola (Sokolova et al. 2005)

Genus *Thermincola* (Table 7.1) is currently classified as a member of family *Peptococcaceae*, although rRNA analysis suggests only a weak relationship to other *Peptococcaceae* members (Ludwig et al. 2009). The genus contains two validly described species – *Thermincola carboxydiphila* (Sokolova et al. 2005) and *T. ferriacetica* (Zavarzina et al. 2007) – and one not validated species *Thermincola potens,* the complete genome of which has been sequenced (Byrne-Bailey et al. 2010). Cells of all species are straight rods with cell wall of the Gram-positive-type structure. *T. ferriacetica* cells are sporeforming. Representatives of *Thermincola* species are strict anaerobes and moderate thermophiles. *T. carboxydiphila* was isolated from the sample of cyanobacterial mat, mud, and hot water from slightly alkaline hot spring at Baikal Lake rift zone. *T. ferriacetica* was isolated from ochre deposits in a hot spring at Kunashir Island. *T. carboxydiphila* is alkalitolerant, the pH optimum for growth is 8.0, and *T. ferriacetica* is neutrophilic.

Both species grow on 100% CO hydrogenogenically; during chemolithotrophic growth on CO, both species require additional sources of cell carbon in the form of acetate or yeast extract. They do not grow on fermentable substrates. *T. carboxydiphila* is an obligate carboxydotroph. In addition to growth on CO, *T. ferriacetica* can grow at the expense of ferric iron reduction with hydrogen or acetate or some other organic substrates.

Thermincola potens strain JR (Byrne-Bailey et al. 2010) has been isolated from a biofilm that originated from an anoxic marine marsh sediment and developing at the anode of a thermophilic microbial fuel cell. It was the dominant member of the current-producing bacterial community (Wrighton et al. 2008; Mathis et al. 2008). The isolate shares 99% 16S rRNA gene sequence identity with *T. carboxydiphila* and *T. ferriacetica*. This strain couples acetate oxidation to the reduction of insoluble electron acceptors: microbial fuel cell (MFC) anodes and hydrous ferric oxide (Wrighton et al. 2008). It is also capable of growth with CO as the sole electron donor and carbon source (Byrne-Bailey et al. 2010).

7.3.3 Genus *Carboxydocella* (Sokolova et al. 2002)

Genus *Carboxydocella* is classified as a member of Family XVI *Incertae sedis* within *Thermoanaerobacterales* (Ludwig et al. 2009). The genus contains four species: the three thermophilic hydrogenogenic carboxydotrophic species, *Carboxydocella thermautotrophica* (Sokolova et al. 2002), *C. sporoproducens* (Slepova et al. 2006), and *C. ferrireducens* (our unpublished data), and one non-carboxydotrophic species, *C. manganica* (Slobodkina et al. 2012). Cells of *Carboxydocella* species are rods of various lengths with cell wall of Gram-positive-type structure. *C. thermautotrophica* and *C. ferrireducens* cells are motile due to peritrichous flagella. *C. sporoproducens* cells are nonmotile and spore-forming. Representatives of the genus *Carboxydocella* are strict anaerobes, moderate thermophiles, and neutrophiles. All *Carboxydocella* species were isolated from hot springs at Kamchatka Peninsula: *C. thermautotrophica,* from hot spring at Geyser Valley; *C. sporoproducens,* from the bottom hot spring in Karymsky Lake; and *C. ferrireducens,* from a hot ground layer at a thermal field of Uzon Caldera. All *Carboxydocella* species except *C. manganica* grow on 100% CO hydrogenogenically. *C. manganica* does not grow not only at 100% CO in the gas phase but also at lower concentrations (Slobodkina et al. 2012). *C. thermautotrophica* and *C. sporoproducens* grow on CO chemolithoautotrophically; *C. ferrireducens* requires an additional source of cell carbon in the form of acetate or lactate or pyruvate or yeast extract. *C. ferrireducens* can reduce Fe(III) during the growth on CO in the presence of hydromorphic ferric iron oxide, although hydrogen remains the main reduced metabolic product. *C. thermautotrophica* cannot reduce Fe(III) or Mn(IV) with CO, lactate, or molecular hydrogen. In contrast, *C. sporoproducens* can grow and reduce Fe(III) and Mn(IV) with lactate as electron donor at least in three culture transfers. Among *Carboxydocella* species,

C. thermautotrophica is the only obligate CO-troph. Two other hydrogenogenic species can grow organotrophically on several substrates.

7.3.4 Genus Thermosinus

The genus belongs to the family Veillonellaceae within the phylum Firmicutes, class *Negativicutes*, and order Selenomonadales (Ludwig et al. 2009). The genus is currently represented by a single species *T. carboxydivorans* (Sokolova et al. 2004b). Cells are curved rods motile by means of lateral flagella. Cell wall structure is of the Gram-negative type. *T. carboxydivorans* grows on CO both in the presence and absence of sodium sulfide in the medium (E_h − 250 mV or + 50 mV, respectively). It grows chemolithotrophically on CO forming equimolar amounts of H_2 and CO_2. *T. carboxydivorans* grows organotrophically on some carbohydrates or on pyruvate. During growth on CO, sucrose, or lactose, it reduces ferric iron. Selenite is reduced to elemental selenium during *T. carboxydivorans* growth on CO. Neither ferric iron nor selenite causes a significant shift in the ratio of H_2 and CO_2 produced. During the growth on CO, elemental sulfur, thiosulfate, sulfate, and nitrate do not stimulate growth and are not reduced. However, thiosulfate enhances growth rate and cell yield during growth on glucose, sucrose, or lactose; in this case, the fermentation products are acetate, H_2S, and CO_2. During glucose fermentation, acetate, H_2, and CO_2 are produced. Lactate, acetate, formate, and H_2 are not utilized, either in the absence or presence of ferric iron, thiosulfate, sulfate, sulfite, elemental sulfur, or nitrate. Growth is completely inhibited by penicillin, ampicillin, streptomycin, kanamycin, and neomycin. Draft genome sequence is available from NCBI (NZ_AAWL00000000).

7.3.5 Genus Caldanaerobacter

The genus belongs to the family *Thermoanaerobacteraceae* within the order *Thermoanaerobacterales*. It contains a few CO-trophic hydrogenogenic strains. *C. subterraneus* subsp. *pacificus* JM (Fardeau et al. 2004), formerly *Carboxydobrachium pacificum* (Sokolova et al. 2001), was the first hydrogenogenic CO-trophic thermophile isolated from deep-sea hot environment (Okinawa Trough). The species *Caldanaerobacter subterraneus* also contains another hydrogenogenic CO-trophic strain 2707 isolated from freshwater hot spring at Kunashir Island. Cells of both strains are very thin long rods. Cells of *C. subterraneus* subsp. *pacificus* JM sometimes are branching, and strain 2707 cells are sporeforming. Both strains are strict anaerobes, extreme thermophiles, and neutrophiles. In addition to lithotrophic hydrogenogenic growth on CO, they grow organotrophically on some fermentable substrates. Both strains reduce thiosulfate during organotrophic growth. Strain 2707 reduces hydromorphic ferric oxide during the growth on peptone. Draft genome sequence is available from NCBI for JM (NZ_ABXP00000000).

7.3.6 Genus Thermoanaerobacter

Representatives of this genus are widely distributed in various thermal environments. *Thermoanaerobacter* species are strictly anaerobic, thermophilic, rod-shaped bacteria, growing between 55 and 75°C, and most of them form round to oval terminal spores. Although the end products are mainly acetate, lactate, ethanol, H_2, and CO_2, the most abundant end product depends on the species and the growth conditions. Generally, thiosulfate can be used as electron acceptor in anaerobic respiration. There are 13 validly described species belonging to the genus (Onyenwoke and Wiegel 2009). The only hydrogenogenic carboxydotrophic representative of the genus, *T. hydrosulfuricus* subsp. *carboxydivorans,* has been isolated from a geothermal spring in Ayas, Turkey (Balk et al. 2009). The isolate cells are straight to curved rods which stain Gram-positively. They are sporeforming with terminal, round, heat-resistant endospores. Cells are 0.3–0.4 μm in diameter and 3.5–10 μm in length, occurred singly, in pairs, or in long chains. *T. hydrosulfuricus* subsp. c*arboxydivorans* grows both in the presence and absence of thiosulfate on a number of fermentable substrates including peptone, various sugars, xylan, starch, pectin, inulin, and cellobiose. End products of sugar fermentation and thiosulfate reduction are dependent on growth conditions, particularly on pH or thiosulfate presence. The main products of sugar fermentation are lactate, acetate, ethanol, alanine, H_2, and CO_2. In addition to thiosulfate, elemental sulfur, sodium sulfite, ferric iron, MnO_2, anthraquinone-2,6-disulfonate (AQDS), and arsenate can serve as electron acceptors. Sulfate, nitrate, and selenite cannot be utilized. *T. hydrosulfuricus* subsp. c*arboxydivorans* grows in an atmosphere containing up to 25% of CO. CO oxidation is coupled to equimolar H_2 and CO_2 formation. Cell-free extracts exhibit CO dehydrogenase activity (Balk et al. 2009).

7.3.7 Thermolithobacter carboxydivorans

Hydrogenogenic carboxydotrophic species *Thermolithobacter carboxydivorans* is a representative of the recently established class *Thermolithobacteria*, affiliated to Firmicutes (Sokolova et al. 2007). The single hydrogenogenic carboxydotrophic strain of this species was isolated from a freshwater hot spring at Raoul Island, Kermadec Archipelago. The initial description assigned this strain to *Carboxydothermus restrictus* on the basis of phenotypic similarity to *Carboxydothermus hydrogenoformans* (Svetlichnyi et al. 1994). Later, 99% similarity was revealed of the 16S rRNA gene sequences of this isolate and three strains of ferric iron-reducing chemolithoautotrophic bacteria *Ferribacter thermautotrophicus* isolated by J. Wiegel from hot springs of Yellowstone National Park and Fiji Island. DNA–DNA hybridization level between the carboxydotrophic and ferric iron-reducing strains was 35%. Surprisingly, strains of *Carboxydothermus restrictus* and *Ferribacter thermautotrophicus* are hardly similar physiologically. Strain R1 grows on CO hydrogenogenically, and it does not reduce Fe(III), does not grow on hydrogen at the excess of AQDS and does not reduce it, and does not reduce AQDS during the growth on CO. Strain R1 does not grow on CO in

the presence of NO_3^-, Fe(III) oxide/hydroxide, Fe(III) citrate, or SO_3^{2-}. Strain R1 grows on CO in the presence of SO_4^{2-}, $S_2O_3^{2-}$, or fumarate, but the presence of electron acceptors does not stimulate growth. R1 does not reduce SO_4^{2-}, $S_2O_3^{2-}$, or elemental sulfur in media supplemented with yeast extract, formate, acetate, pyruvate, citrate, succinate, lactate, or H_2:CO_2. Strain R1 also does not grow on the H_2:CO_2 mixture with NO_3^-, SO_3^{2-}, or fumarate. Physiological characteristics and the 16S rDNA sequence analysis indicated that *Carboxydothermus restrictus* and *Ferribacter thermautotrophicus* strains represent two novel species of the novel genus *Thermolithobacter* within the *Firmicutes* branch. Levels of 16S rRNA gene sequence similarity between the lineage containing *Thermolithobacter* and members of the other classes of the *Firmicutes* were less than 85%, and *Thermolithobacter* representatives appeared to form their own lineage. It was therefore proposed that the *Thermolithobacter* represent a new class within phylum BXIII *Firmicutes* (Gibbons and Murray 1978; Garrity et al. 2002), Thermolithobacteria. Class Thermolithobacteria was described to contain the order Thermolithobacterales ord. nov., the family Thermolithobacteraceae fam. nov., and the genus *Thermolithobacter* gen. nov.

7.3.8 Genus Dictyoglomus

The hydrogenogenic representative of phylum "Dictyoglomi," class Dictyoglomia, order *Dictyoglomales*, and family *Dictyoglomaceae, Dictyoglomus* sp. 1512 (Kochetkova et al. 2011), is currently the single thermophilic hydrogenogenic bacterium not affiliated to "Firmicutes." It was isolated from a hot spring at Uzon Caldera, Kamchatka, in which active process of CO transformation was demonstrated using radioisotopic tracing. The isolate is neutrophilic extremely thermophilic anaerobic bacterium, which grows on CO if its concentration in the gas phase does not exceed 15%. The isolate oxidizes CO to CO_2, producing equimolar quantities of H_2 from water. The optimum CO concentration in the gas phase is 5%. The isolate grows on CO significantly slower than other thermophilic hydrogenogens. The generation time of growth on 5% of CO is about 60 h. It grows on CO chemolithoheterotrophically but requires 0.2 g l^{-1} of yeast extract for growth. Cells of the isolate are long thin filaments. Analysis of 16S rDNA sequence revealed that the novel isolate belongs to Dictyoglomi. The highest similarity of 16S rDNA sequence (98.6%) was found with 16S rRNA of *Dictyoglomus thermophilum* (Saiki et al. 1985).

7.3.9 Hydrogenogenic Carboxydotrophic Representatives of Thermococcus

Thermococcus sp. AM4 was isolated from 1 of 13 enrichments of coccoid cells growing on CO with H_2 and CO_2 production at 80°C obtained from 24 samples of hydrothermal venting structures collected at East Pacific Rise 13 °N (Sokolova et al. 2004b). It was described as the first hydrogenogenic carboxydotrophic representative

of Archaea. Cells of strain AM4 are cocci 1–1.5 μm in diameter. Cells are motile by means of tuft of flagella. The isolate grows at 60–90°C and has a broad optimum temperature interval of 70–80°C. The pH range for growth is 5.5–8.5, with an optimum pH 6.5–7.0. Strain AM4 grows on 100% of CO in the gas phase producing equimolar quantities of H_2 and CO_2, similarly to all other hydrogenogenic carboxydotrophs. In the absence of CO, strain AM4 grows on some peptide substrates with elemental sulfur as electron acceptor under a nitrogen atmosphere. In the presence of elemental sulfur, *Thermococcus* sp. AM4 grows on CO with the production of hydrogen sulfide but not the hydrogen (our unpublished data). 16S rRNA gene analysis revealed high similarity of strain AM4 with *T. gammatolerans* (99.5%), T. kodakarensis (99.2%), *T. peptonophilus* (99.2%), *T. guaymasensis* (99.2%), and *T. fumicolans* (99.1%).

In silico hybridization of the genomes of strain AM4 and *T. gammatolerans* EJ3 yielded an ANI value (average nucleotide identity of shared protein-coding genes) of 87% (about 80% of genes shared). This ANI value is lower than the 95–96% value shown to correspond to the 70% DNA–DNA hybridization level accepted to delimit microbial species (Goris et al. 2007; Richter and Rosselló-Móra 2009; Tindall et al. 2010). Thus, AM4 phylogenetically represents a close to *T. gammatolerans* but distinct species.

Another hydrogenogenic species of the genus, *Thermococcus onnurineus* NA1 (Lee et al. 2008), was isolated from PACMANUS thermal field at the East Manus Basin in heterotrophic conditions on peptone with elemental sulfur. The analysis of whole genome sequence revealed the presence of a gene cluster containing genes for carbon monoxide dehydrogenase and energy-converting hydrogenase (Lee et al. 2008). Indeed, it was demonstrated that the strain NA1 can grow hydrogenogenically in the atmosphere of 100% CO (Lee et al. 2008).

From the deep-sea hydrothermal vents of Mid-Atlantic Ridge and Lau Spreading Center, we isolated six strains of CO-trophic hydrogenogenic hyperthermophilic Archaea. 16S rRNA gene sequence analysis revealed that new isolates are close to *Thermococcus barophilus* MP^T (nearly 100% identity). We also demonstrated hydrogenogenic growth on CO for the type strain *Thermococcus barophilus* MP^T.

The capacity for hydrogenogenic carboxydotrophy was tested for some other representatives of *Thermococcales*: *Pyrococcus furiosus, Thermococcus peptonophilus, T. profundus, T. chitonophagus, T. stetteri, T. gorgonarius, T. litoralis* and *T. pacificus.* The tested strains did not oxidize CO (100% in the gas phase) (Sokolova et al. 2004a).

Thus, it may be inferred that hydrogenogenic carboxydotrophic hyperthermophiles, particularly phylogenetically diverse thermococci capable of this process, may be abundant members of the microbial communities inhabiting deep-sea hot vents.

7.3.10 Hydrogenogenic Representative of Thermofilum

Strain 1505 was isolated from a hot spring at the slope of Mutnovsky volcano (Kamchatka Peninsula) (Kochetkova et al. 2011). Cells of the isolate are very thin long rods 15–50 μm in length. The isolate grows chemolithotrophically on

45% CO in the gas phase at 92°C producing equimolar quantities of H_2 and CO_2. Yeast extract (0.2 g l^{-1}) is required for growth. Strain 1505 can also grow fermentatively on yeast extract. 16S rRNA gene sequence analysis revealed that the closest relative of strain 1505 is *Thermofilum pendens* Hrk-5T (Zillig et al. 1983) with sequence similarity 97.8%. Based on 16S rRNA gene sequence analysis and physiological features, it was suggested to assign strain 1505 to a new species of the genus *Thermofilum, T. carboxydotrophus,* sp. nov. 16S rRNA gene sequence analysis revealed 100% sequence identity of strain 1505 to environmental clones obtained from samples taken from different hot springs of Yellowstone National Park (USA) (Barns et al. 1994; Reysenbach et al. 2000). Thus, it is highly possible that representatives of this species are widespread in terrestrial hot springs.

7.4 CO-Oxidizing Dissimilatory Fe(III)-Reducing Thermophiles

Few strains are known to be capable of Fe(III) reduction during the growth on CO. Among them are the hydrogenogenic carboxydotrophs *Carboxydothermus siderophilus, Carboxydothermus islandicus, Thermincola ferriacetica, Thermosinus carboxydivorans, and Carboxydocella ferrireducens.* These species grow on CO reducing ferric iron but still producing hydrogen as a main product. The only true dissimilatory Fe(III)-reducing CO-oxidizing thermophile is *Carboxydothermus ferrireducens* (Slobodkin et al. 2006), formerly *Thermoterrabacterium ferrireducens,* which was isolated from a hot spring in Yellowstone National Park (Slobodkin et al. 1997). This organism can grow by utilizing organic substrates or H_2 as electron donors, and, apart from Fe(III) or AQDS, it can also reduce sulfite, thiosulfate, elemental sulfur, nitrate, and fumarate (Slobodkin et al. 1997; Henstra and Stams 2004) during organotrophic growth. *Carboxydothermus ferrireducens* grows on CO with ferrihydrite as electron acceptor, forming magnetite precipitate and not producing hydrogen or acetate (Slobodkin et al. 2006), or with AQDS or fumarate as electron acceptors (Henstra and Stams 2004). Unlike *C. hydrogenoformans*, this bacterium cannot grow on CO without electron acceptors.

7.5 CO-Oxidizing Hydrogen Sulfide Producing Bacteria and Archaea

7.5.1 CO-Utilizing Sulfate-Reducing Bacteria and Archaea

Few thermophilic sulfate-reducing bacteria, *Desulfotomaculum nigrificans*, *D. kuznetsovii* and *D. thermobenzoicum* subsp. *thermosyntrophicum,* and *Desulfotomaculum carboxydivorans,* were found to be capable of anaerobic CO oxidation (Parshina et al.

2005a, b). These bacteria have been isolated from bioreactors. The capacity for anaerobic CO oxidation was not demonstrated for sulfate-reducing bacterial strains isolated from hot volcanic environments.

It was shown that the anaerobic extremely thermophilic euryarchaeote *Archaeoglobus fulgidus* VC-16 is capable of autotrophic growth with CO. *A. fulgidus* VC-16 was isolated from submarine solfataric fields near Vulcano Island, Italy (Stetter et al. 1987). *Archaeoglobus* species occur in both shallow and abyssal marine hydrothermal systems (Burggraf et al. 1990). *A. fulgidus* strains were also isolated from hot oil field waters (Beeder et al. 1994; Stetter 1988, 1993). *Archaeoglobus fulgidus* VC-16 can oxidize CO both in the presence and absence of sulfate. In the first case, the oxidation of CO to CO_2 is coupled to sulfate reduction; acetate and formate are formed as minor products. In the absence of sulfate, the only products of CO metabolism are acetate, formed via the reductive acetyl-CoA pathway with formyl-methanofuran as intermediate, and formate (Henstra et al. 2007). *A. fulgidus* can also completely oxidize various organic compounds in the presence of sulfate or grow chemolithoautotrophically on H_2 with thiosulfate but not with sulfate as electron acceptor (Zellner et al. 1989).

7.5.2 *Thermococci Growing at the Expense of Sulfidogenic Oxidation of CO*

Recently, we have demonstrated the ability of some *Thermococcus* species to grow at the expense of CO oxidation with elemental sulfur. In this case, hydrogen production is very low or negligible. This ability was shown for *T. barophilus* strains MP^T and Ch5 and for the type strains of *T. chitonophagus*, *T. gammatolerans,* and *T. profundus.*

7.6 CO-Oxidizing Thermophilic Methanogenic Archaea

The only thermophilic methanogen demonstrated to be able to grow on CO is *Methanothermobacter thermautotrophicus* ΔH (Zeikus and Wolfe 1972). The strain was isolated from sewage sludge. *M. thermautotrophicus* has been found in various hot environments, including hot springs in Yellowstone National Park and Iceland (Sandbeck and Ward 1982) and biogas reactors. It converts CO according to the equation $4CO + 2H_2O = CH_4 + 3CO_2$. The growth rate on CO was only 1% of that on CO_2/H_2 (Daniels et al. 1977). The ability to grow on CO was more studied for mesophilic methanogens. Among them, six representatives of the genera *Methanosarcina, Methanobacterium,* and *Methanobrevibacter* were reported to grow on CO (Daniels et al. 1977; O'Brien et al. 1984; Rother and Metcalf 2004). It may be expected that the capacity for growth on CO can also be found in other thermophilic methanogens.

7.7 CO-Oxidizing Thermophilic Homoacetogenic Bacteria

Many homoacetogens can grow on CO. CO conversion has been documented for ten homoacetogens, four moderate thermophiles among them: *Moorella thermoacetica, Moorella thermoautotrophica, Thermoanaerobacter kivui,* and *Moorella perchloratireducens,* recently described by Balk et al. (2008). *M. thermoautotrophica* and *M. thermoacetica* can grow on CO at high partial pressures as sole energy source (Savage et al. 1987; Diekert and Thauer 1978; Daniel et al. 1990). *M. thermoacetica* and *T. kivui* can oxidize CO during growth on other substrates (Diekert and Thauer 1978; Yang and Drake 1990). Most *M. thermoacetica* strains (11 of 13 tested) possess this ability (Daniel et al. 1990). Homoacetogens grow on CO according to the equation (Drake and Daniel 2004)

$$4CO + 2H_2O \rightarrow CH_3COOH + 2CO_2 \left(\Delta G = -41.4 \text{ kJ / mol CO}\right).$$

M. thermoautotrophica, originally isolated from a hot spring (Wiegel et al. 1981), was found in temporarily heated soils (see Drake and Daniel 2004) and cyanobacterial mats in hot springs (Bateson et al. 1989). As shown by blastn at the NCBI site (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), 16S rRNA genes exhibiting 99% similarity with *Moorella thermoacetica* have been obtained from bioreactors, hot springs, and oil reservoirs. The diverse metabolic capacities of this organism make it highly competitive. It grows both autotrophically and heterotrophically, fermentatively, or by respiration using various electron donors and acceptors (Drake and Daniel 2004). In the presence of nitrate, *M. thermoacetica* and *M. thermoautotrophica* can grow on CO if O-methyl groups (of vanillate or syringate) are provided. In these organisms, CO_2 reduction both to THF-CH_3 and to CO is blocked by nitrate (Drake and Daniel 2004).

7.8 Occurrence of Genetic Determinants of Anaerobic Carboxydotrophy in the Genomes of Thermophiles

The diversity of anaerobic carboxydotrophs can be estimated not only from isolation and cultivation data but also from data of genomics.

7.8.1 Distribution of Genetic Determinants of Carboxydotrophy

Utilization of CO by anaerobes is catalyzed by Ni-containing CO dehydrogenases (Ni-CODHs) and acetyl-CoA synthases (ACSs) (Ragsdale 2004). Ni-CODHs and Ni-CODH–ACS complexes are widespread among anaerobes and are found in methanogens, acetogens, sulfate reducers, and iron reducers. Ni-CODH–ACS

complexes catalyze catabolic and anabolic acetyl-CoA synthesis and catabolic acetyl-CoA cleavage. In these reactions, CO is an intermediate that travels along a hydrophobic channel between the Ni-CODH and ACS active sites (Ragsdale 2004). The capacity to use exogenous CO by Ni-CODH–ACS complexes is a debatable topic. Many relevant papers infer direct and preferential incorporation of CO into the acetate carboxyl group (Stupperich and Fuchs 1984; Martin et al. 1985; Henstra et al. 2007), but preferential incorporation of CO_2-derived CO has also been reported (Ragsdale 2004), and most probably this is the case at ecologically relevant CO concentrations.

It may be expected that the main enzymes responsible for the utilization of exogenous CO by anaerobes are the Ni-CODHs that are not part of the Ni-CODH–ACS complex. Our analysis at the "BLAST with microbial genomes" site (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) of 1590 microbial species with available genomes (about 150 thermophiles) revealed 131 species (Table 7.2), including 35 thermophiles (Table 7.3) that contained Ni-CODH genes (Techtmann et al. 2012). Note that these values are considerably higher than King and Weber (2007) estimated the frequency of occurrence of the aerobic CO-oxidizing capacity (8 of 330 genomes). Of the 131 species whose genomes contained one or more Ni-CODH genes, 20, including six thermophiles (Table 7.2), encoded only Ni-CODHs as part of Ni-CODH–ACS gene clusters. It may be speculated that these organisms are mainly capable of dealing with endogenous CO. The remaining genomes contained Ni-CODH genes additional to those in Ni-CODH–ACS gene clusters or lacked ACS genes (Tables 7.2 and 7.3). It may be speculated that these organisms are adapted to deal with exogenous CO. Several organisms, including the thermophile *Thermodesulfovibrio yellowstonii*, harbor only ACS genes but not Ni-CODH genes, which is a somewhat puzzling occurrence pattern. It has been shown by Svetlitchnyi et al. (2004a, b) for the autotroph *Carboxydothermus hydrogenoformans* that, at high CO concentrations, acetate synthesis involves ACS but not Ni-CODH. However, *Thermodesulfovibrio yellowstonii* has been shown by Parshina et al. (2005) to be inhibited by CO concentrations as low as 2% during growth on pyruvate.

Ni-CODH genes occurred in the genomes of *Archaea* and *Bacteria* belonging to ten phyla. As far as thermophiles are concerned, Ni-CODH genes occurred in the genomes of *Archaea* and *Bacteria* from five phyla (Table 7.3).

Among the species whose genomes encode Ni-CODHs, there are obligate and facultative anaerobes and an obligate aerobe (*Azotobacter vinelandii*). Among thermophiles, facultatively anaerobic are *Geobacillus thermoglucosidasius, Geobacillus* sp. Y4.1MC1, and *Persephonella marina;* others are obligate anaerobes.

Whereas the presence of a Ni-CODH–ACS complex appears to be a genus-level character (not to mention autotrophic methanogens, among which this trait is ubiquitous), the presence of additional or sole Ni-CODH(s) is most often a species-level character. However, in a few genera (e.g., *Carboxydothermus, Thermincola*), all known representatives are so far carboxydotrophic (judging from cultivation and/or genomic data). On the other hand, in a few species, the

Table 7.2 Numbers of microbial species whose genomes exhibit a particular occurrence pattern of Ni-CODH and ACS genes

Total		With Ni-CODH and/or ACS genes	With only ACS gene(s)	With more ACS genes	With Ni-CODH and ACS gene(s) in equal proportion	With more Ni-CODH genes	With only Ni-CODH genes(s)
Entire NCBI database for blast with prokaryotic genomes							
Species	1,590	138	7	3	20	33	75
Thermophiles and hyperthermophiles							
Species	ca. 150	36	1	2	8	8	15

Note: The table is based on our analysis performed in January 2011 at the NCBI "BLAST with microbial genomes" site (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi)

Table 7.3 Numbers of genes encoding Ni-CODHs and ACSs and numbers of Ni-CODH–ACS, ECH, and Ni-CODH–ECH gene clusters in the genomes of thermophiles that contain at least one Ni-CODH or ACS gene

Species	Ni-CODH genes	ACS genes	Ni-CODH–ACS gene clusters	ECH gene clusters	Ni-CODH–ECH gene clusters		Growth on CO	Production of H_2 from CO
					"Classic" *coo* type	Other		
Domain *Bacteria*								
Phylum *Aquificae* Class *Aquificae* Order *Aquificales* Family *Hydrogenothermaceae*								
Persephonella marina	1	0	0	0	0	0	ND	ND
Phylum *Nitrospirae* Class "*Nitrospira*" Order "*Nitrospirales*" Family "*Nitrospiraceae*"								
Thermodesulfovibrio yellowstonii	0	1	0	1	0	0	–	–
Phylum *Firmicutes* Class *Bacilli* Order *Bacillales* Family *Bacillaceae*								
Geobacillus thermoglucosidasius	1	0	0	1	0	1	+[a]	+[a]
Geobacillus sp. Y4.1MC1	1	0	0	1	0	1	ND	ND
Phylum *Firmicutes* Class *Clostridia* Order *Clostridiales* Family *Clostridiaceae*								
Clostridium thermocellum	1	0	0	1	0	0	ND	ND
Phylum *Firmicutes* Class *Clostridia* Order *Clostridiales* Family *Peptococcaceae*								
Thermincola potens	4	1	1	1	1	0	+	ND
Phylum *Firmicutes* Class *Clostridia* Order *Natranaerobiales* Family *Natranaerobiaceae*								
Natranaerobius thermophilus	1	0	0	0	0	0	ND	ND
Phylum *Firmicutes* Class *Clostridia* Order *Thermoanaerobacterales* Family *Thermoanaerobacteraceae*								
Ammonifex degensii	1	1	1	1	0	0	ND	ND

(continued)

Table 7.3 (continued)

Species	Ni-CODH genes	ACS genes	Ni-CODH–ACS gene clusters	ECH gene clusters	Ni-CODH–ECH gene clusters		Growth on CO	Production of H_2 from CO
					"Classic" *coo* type	Other		
Caldanaerobacter subterraneus subsp. *pacificus*	1	0	0	2	0	1	+	+
Caldanaerobacter subterraneus ssp. *tengcongensis*	1	0	0	2	0	1	ND	ND
Carboxydothermus hydrogenoformans	5	1	1	1	1	0	+	+
Moorella thermoacetica	2	1	1	1	0	0	+	–
Phylum *Firmicutes* Class *Clostridia* Order *Thermoanaerobacterales* Unclassified *Thermoanaerobacterales*								
Caldicellulosiruptor hydrothermalis	1	0	0	1	0	0	ND	ND
Caldicellulosiruptor kristjanssonii	1	0	0	1	0	0	ND	ND
Caldicellulosiruptor lactoaceticus	1	0	0	1	0	0	ND	ND
Caldicellulosiruptor saccharolyticus	1	0	0	1	0	0	ND	ND
Thermosediminibacter oceani	3	1	1	0	0	0	ND	ND
Phylum *Firmicutes* Class *Negativicutes* Order *Selenomonadales* Family *Veillonellaceae*								
Thermosinus carboxydivorans	3	0	0	1	1	0	+	+

Domain *Archaea*								
Phylum *Crenarchaeota* Class *Thermoprotei* Order *Thermoproteales* Family *Thermofilaceae*								
Thermofilum carboxydotrophus[b]	1	0	0	2	0	1	+	+
Phylum *Euryarchaeota* Class *Archaeoglobi* Order *Archaeoglobales* Family *Archaeoglobaceae*								
Archaeoglobus fulgidus	3	1	0	0	0	0	+	–
Ferroglobus placidus	3	1	1	0	0	0	ND	ND
Phylum *Euryarchaeota* Class *Methanobacteria* Order *Methanobacteriales* Family *Methanobacteriaceae*								
Methanothermobacter marburgensis	1	1	1	2	0	0	ND	ND
Methanothermobacter thermautotrophicus	1	1	1	2	0	0	+	–
Phylum *Euryarchaeota* Class *Methanobacteria* Order *Methanobacteriales* Family *Methanothermaceae*								
Methanothermus fervidus	1	1	1	1	0	0	ND	ND
Phylum *Euryarchaeota* Class *Methanococci* Order *Methanococcales* Family *Methanocaldococcaceae*								
Methanocaldococcus fervens	1	1	1	2	0	0	ND	ND
Methanocaldococcus infernus	1	2	0	2	0	0	ND	ND
Methanocaldococcus jannaschii	2	2	1	2	0	0	ND	ND
Methanocaldococcus vulcanius	2	2	1	2	0	0	ND	ND
Methanocaldococcus sp. FS406-22	2	2	1	2	0	0	ND	ND
Phylum *Euryarchaeota* Class *Methanococci* Order *Methanococcales* Family *Methanococcaceae*								
Methanothermococcus okinawensis	1	2	1	2	0	0	ND	ND

(continued)

Table 7.3 (continued)

Species	Ni-CODH genes	ACS genes	Ni-CODH–ACS gene clusters	ECH gene clusters	Ni-CODH–ECH gene clusters		Growth on CO	Production of H_2 from CO
					"Classic" *coo* type	Other		
Phylum *Euryarchaeota* Class "*Methanomicrobia*" Order *Methanosarcinales* Family *Methanosaetaceae*								
Methanosaeta thermophila	3	1	1	0	0	0	ND	ND
Phylum *Euryarchaeota* Class *Methanopyri* Order *Methanopyrales* Family *Methanopyraceae*								
Methanopyrus kandleri	2	1	1	1	0	0	ND	ND
Phylum *Euryarchaeota* Class *Thermococci* Order *Thermococcales* Family *Thermococcaceae*								
Thermococcus barophilus	1	0	0	3	0	1	+[a]	+[a]
Thermococcus gammatolerans	1	0	0	3	0	0	+[a]	–[a]
Thermococcus onnurineus	1	0	0	4	0	1	+	+
Thermococcus sp. AM4	2	0	0	3	0	1	+	+
Phylum *Korarchaeota*								
"*Candidatus* Korarchaeum cryptofilum"[c]	2	0	0	0	0	0	ND	ND

Notes: The table is based on our analysis performed in January 2011 at the NCBI "BLAST with microbial genomes" site (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). The hierarchical classification is according to the J. P. Euzéby's site (http://www.bacterio.cict.fr/classifphyla.html) as of August 28, 2011
ND no data

[a]According to our unpublished data

[b]The genome was sequenced at the Centre "Bioengineering," Russian Academy of Sciences and analyzed using the AutoFACT annotation tool (Koski et al. 2005), followed by manual curation

[c]The genome was analyzed at the NCBI BLAST site (http://blast.ncbi.nlm.nih.gov/Blast.cgi)

presence of Ni-CODH gene(s) is a strain-level character (among thermophiles, currently no examples are known).

In no case did our analysis reveal plasmid localization of Ni-CODH (in case of the molybdenum-containing CODHs of aerobes, plasmid localization has been shown for the genes encoding the enzyme of *Oligotropha carboxidovorans* (Fuhrmann et al. 2003)).

In the metabolism of many microorganisms possessing Ni-CODHs, the so-called energy-converting hydrogenases (ECHs, Table 7.2) play an important role. ECHs form a subclass within the class of [NiFe]-hydrogenases (Hedderich 2004; Vignais and Billoud 2007). ECHs are multisubunit membrane-bound enzyme complexes able to pump ions out of cells at the expense of proton reduction with low-potential electrons, including those accepted by Ni-CODH from CO ($E^{0'}$ is −520 mV for the CO/CO_2 couple and −414 mV for the H_2/H^+ couple). ECHs can also mediate the reverse transfer of electrons from hydrogen to low-potential electron carriers at the expense of the transmembrane ion gradient; these electrons can then be used for the reduction of CO_2 to CO, used further in acetate synthesis (Meuer et al. 2002; Hedderich 2004). The role of Ni-CODH–ECH gene clusters is discussed in the subsection devoted to hydrogenogenic carboxydotrophs.

7.8.2 Genetic Determinants of Carboxydotrophy in Thermophilic Methanogens

Methanothermobacter thermautotrophicus DH, which, as discussed above, is capable of weak growth on CO, contains in its genome a single Ni-CODH gene, and, judging from its genomic environment, this Ni-CODH is part of a Ni-CODH–ACS complex (Table 7.3). *Methanopyrus kandleri* and *Methanosaeta thermophila* contain in their genomes additional Ni-CODH genes beyond Ni-CODH–ACS gene clusters and thus seem to be better able to use exogenous CO than *M. thermautotrophicus* (Table 7.3). However, their ability to grow on CO has not been tested.

7.8.3 Genetic Determinants of Carboxydotrophy in Thermophilic Acetogens

The genome of *Moorella thermoacetica* contains two Ni-CODH genes (Table 7.3). Only one of them is part of a Ni-CODH–ACS gene cluster. Thus, *M. thermoacetica* is among those rather numerous organisms in which CO oxidation is not just a side effect of the capacity for acetate synthesis or cleavage. *Archaeoglobus fulgidus* VC-16, which can oxidize CO both in the presence of sulfate sulfidogenically and in the absence of sulfate acetogenically, contains in its genome three Ni-CODH genes and one ACS gene (Table 7.3).

7.8.4 Genetic Determinants of Thermophilic CO Oxidation with Yet-Unknown Acceptors

Ferroglobus placidus and *Thermosediminibacter oceani,* the ability of which to grow on CO has not been tested, contain in their genomes three Ni-CODH genes and one ACS gene (Table 7.3). Thus, these organisms are likely to grow at the expense of CO oxidation with some (or probably all) of the electron acceptors that they can utilize: for *F. placidus,* these are nitrate, thiosulfate (Hafenbradl et al. 1996), and Fe(III) (Tor et al. 2001) and for *Thermosediminibacter oceani,* thiosulfate, elemental sulfur, and Mn(IV) (Lee et al. 2005).

A single Ni-CODH gene is present in the genomes of the following organisms with untested ability to grow on CO: the facultative anaerobe *Persephonella marina*, able to use as acceptors O_2, nitrate, thiosulfate, and Fe(III) (Götz et al. 2002); the anaerobic alkalithermophile *Natranaerobius thermophilus*, using fumarate, thiosulfate, nitrate, and Fe(III) (Mesbah et al. 2007); and, paradoxically, the fermenters *Clostridium thermocellum* and *Caldicellulosiruptor* spp., for which no electron acceptors have been reported (Rainey et al. 2009; Rainey 2009).

7.8.5 Genetic Determinants of Carboxydotrophy in Thermophilic Hydrogenogens

The key enzymes of the process $CO + H_2O \rightarrow CO_2 + H_2$ are the enzymes already considered above: the Ni-containing CO dehydrogenase (Ni-CODH) (Svetlitchnyi et al. 2001) and the energy-converting hydrogenase (ECH), which form the CO-oxidizing H_2-producing enzymatic complex, studied in the thermophile *Carboxydothermus hydrogenoformans* (Soboh et al. 2002). The gene cluster encoding this complex was also studied in the mesophile *Rhodospirillum rubrum* (Fox et al. 1996; Kerby et al. 1997). The remarkable similarity of the two gene clusters, called *coo* gene clusters, was noted (Soboh et al. 2002). Comparison of the *coo* gene clusters of *C. hydrogenoformans* and *R. rubrum* is presented in Fig. 7.1a. A fact deserving attention is the close disposition in both gene clusters of the Ni-CODH gene *cooS* and the gene *cooH* of the ECH catalytic subunit.

With the aim to search for the *coo* gene cluster in phylogenetically diverse thermophilic hydrogenogenic carboxydotrophs, we designed primers proceeding from the consensus sequence of the Ni-CODH genes of *R. rubrum* (*cooS*) and *C. hydrogenoformans* (*cooS-I*) and from the consensus sequence of the ECH gene *cooH* of these two organisms (Fig. 7.1b). With the use of these primers, the presence of rather closely related genes (72–89% identity of nucleotide sequences with the corresponding genes of *C. hydrogenoformans*) and their localization within a single-gene cluster was demonstrated in *Thermosinus carboxydivorans, Carboxydocella thermautotrophica, Thermincola carboxydiphila*, *Thermolithobacter carboxydivorans,* and *Desulfotomaculum carboxydivorans* (Fig. 7.1c; Lebedinsky et al. 2005; Techtmann et al. 2012).

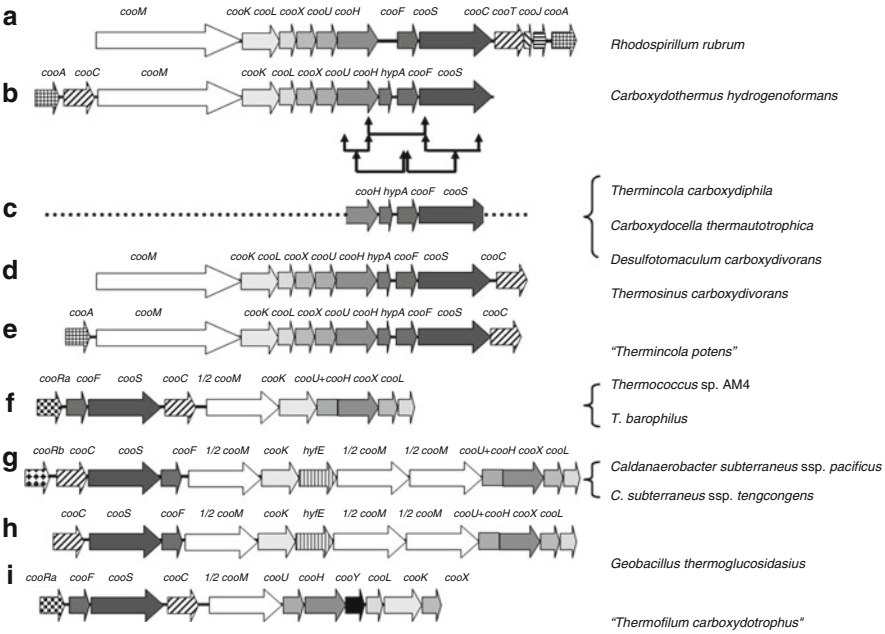

Fig. 7.1 (**a**) The *coo* gene clusters of *Rhodospirillum rubrum* and *Carboxydothermus hydrogenoformans* include the Ni-CODH gene (*cooS*), a ferredoxin-like protein gene (*cooF*), genes of a six-subunit ECH (*cooM–cooH*), and genes of Ni-CODH and hydrogenase maturation proteins. (**b**) Primer systems designed by us proceeding from the consensus sequences of the Ni-CODH genes *cooS* of *R. rubrum* and *cooS1* of *C. hydrogenoformans,* genes of the ECH catalytic subunits (*cooH)* of these organisms, and genes of ferredoxin-like proteins (*cooF*). (**c**) The fragment of the *coo* gene cluster of diverse thermophilic hydrogenogenic carboxydotrophs, amplified with the primers designed. (**d**) The *coo* gene cluster of *Thermosinus carboxydivorans* (based on our analysis of the genome shotgun sequence available from NCBI (NZ_AAWL00000000)). The transcriptional regulator gene *cooA* is located in the *Thermosinus* genome apart from the *coo* gene cluster. (**e**) The *coo* gene cluster of *Thermincola potens* (based on our analysis of the genome (Byrne-Bailey et al. 2010) available from NCBI (NC_014152)). (**f**) The Ni-CODH–ECH gene cluster in carboxydotrophic thermococci. *cooRa* is our name for the gene of a transcriptional regulator absent from the "classical" *coo* cluster, where the regulator gene is *cooA*. The adjoining Na+/H+ antiporter genes located downstream (see Lim et al. 2010) are not shown. (**g**) The Ni-CODH–ECH gene cluster in *C. subterraneus* ssp. *pacificus* and *C. subterraneus* ssp. *tengcongensis. cooRb* is our designation for a transcriptional regulator gene unrelated to *cooRa* or *cooA*. (**h**) The Ni-CODH–ECH gene cluster in *Geobacillus thermoglucosidasius*. (**i**) The Ni-CODH–ECH gene cluster in *Thermofilum carboxydotrophus. cooY* is our designation for a gene absent in other *coo* clusters

Thus, it was demonstrated that the coo enzyme complex, encoded by the *coo* gene cluster, plays a key role in hydrogenogenic carboxydotrophy in phylogenetically diverse bacteria. However, the primers that we designed failed to produce positive PCR result with the hydrogenogenic carboxydotrophs *Thermococcus* sp. AM4 and *Caldanaerobacter subterraneus* subsp. *pacificus.* Analysis of the genomes of these organisms, shotgun sequenced at J. Craig Venter Institute, showed that both these genomes contain a Ni-CODH gene clustered with ECH genes (Lebedinsky et al. 2008; Techtmann et al. 2012), but these gene clusters considerably differ from the

"classical" *coo* gene cluster in terms of the gene primary structure and order (Fig. 7.1f, g). A Ni-CODH–ECH gene cluster very similar to that present in the genome of *Thermococcus* sp. AM4 was found in the genome of *Thermococcus onnurineus* (Lee et al. 2008; Lim et al. 2010; Fig. 7.1f); this thermococcus was tested for the ability to grow on CO and proved to be capable of hydrogenogenic carboxydotrophy (Lee et al. 2008). Our analysis of the genome of the crenarchaeote *Thermofilum carboxydotrophus,* capable of hydrogenogenic carboxydotrophy, showed that it contains a Ni-CODH–ECH gene cluster (Fig. 7.1i) whose dehydrogenase module (the transcriptional regulator gene *cooRa*, the electron transfer protein gene *cooF*, the Ni-CODH gene *cooS*, and the nickel-insertion protein gene *cooC*) is similar to that of the thermococcal gene cluster, while the hydrogenase module (*cooM–cooL* genes) differs from those of other known Ni-CODH–ECH gene clusters in terms of the gene primary structure and order.

Thus, all hydrogenogenic carboxydotrophs studied in this respect contain a gene cluster encoding Ni-CODH–ECH enzymatic complexes. On the other hand, we found that the occurrence of Ni-CODH–ECH gene clusters is most probably restricted to hydrogenogenic carboxydotrophs. Our analysis at the "BLAST with microbial genomes" site (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) of 1590 microbial species with available genomes (about 150 thermophiles, Table 7.2) revealed five cases of the occurrence of a Ni-CODH–ECH gene cluster in organisms whose ability to grow on CO has not been tested: these are the mesophile *Rhodopseudomonas palustris* BisB18 and the thermophiles *Caldanaerobacter subterraneus* subsp. *tengcongensis, Geobacillus thermoglucosidasius, Geobacillus* sp. Y4.1MC1, and *Thermococcus barophilus* (Fig. 7.1f, g, and h). Most probably, these are actually not exceptions: for *Thermococcus barophilus* and *G. thermoglucosidasius*, our tests already demonstrated the capacity for hydrogenogenic growth on CO (Techtmann et al. 2012). In organisms in which the Ni-CODH–ECH interplay results in the reverse reaction, i.e., in CO_2 reduction by H_2 to CO at the expense of transmembrane ion gradient (this interplay of Ni-CODH and ECH, important for autotrophic CO_2 fixation via Wood–Ljungdahl pathway (Ragsdale 2004) and proven for at least *Methanosarcina barkeri* (Meuer et al. 2002), is probably widespread), the Ni-CODH and ECH genes do not cluster. Thus, the presence of a Ni-CODH–ECH gene cluster can be used as a marker of hydrogenogenic carboxydotrophy when interpreting genomics and metagenomics data. Our primers specific for the *coo* gene cluster may also be an informative tool for molecular ecological studies, since they reveal many (although not all) hydrogenogenic carboxydotrophs and do not produce false-positive results concerning their presence.

Taking into account that the Ni-CODH–ECH complex is an efficient, compact, and "self-sufficient" enzymatic machine, is encoded by a common gene cluster, and is scattered over the phylogenetic tree, it could be expected that, in its evolution, interspecies horizontal transfer has played an important role. However, the topology of the relatedness dendrograms constructed for the proteins of the Ni-CODH–ECH enzymatic complexes shows that the main way of evolutionary inheritance of the discussed gene clusters is vertical inheritance. Figure 7.2 presents comparison of 16S-rRNA-based dendrograms of relatedness of hydrogenogenic carboxydotrophs

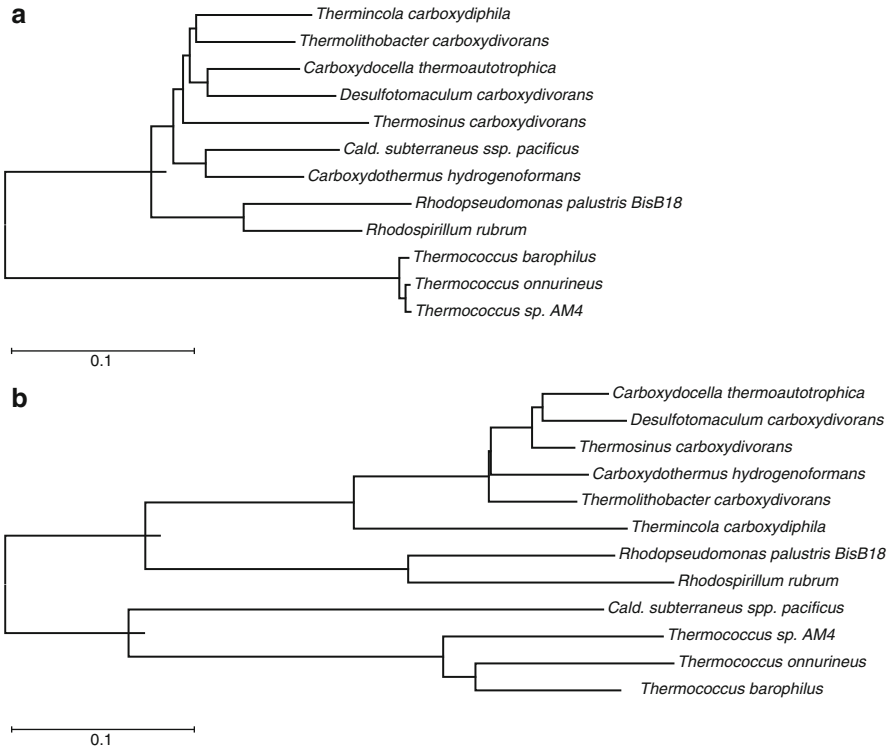

Fig. 7.2 (**a**) 16S-rRNA-based dendrogram of relatedness of hydrogenogenic carboxydotrophs and (**b**) dendrograms of relatedness of the Ni-CODHs encoded by the Ni-CODH–ECH gene clusters. The sequences were aligned by MAFFT v. 6 (Katoh and Toh (2008); http://align.bmr.kyushu-u.ac.jp/mafft/online/server/). Dendrograms were constructed by the neighbor-joining method after calculation of evolutionary distances with Jukes and Cantor and Poisson corrections, respectively (the methods are implemented in the TREECONW v. 1.3b software package (Van de Peer and De Wachter (1994); http://bioinformatics.psb.ugent.be/software/details/TREECON))

and dendrograms of similarity of the Ni-CODHs encoded by the Ni-CODH–ECH gene clusters. The similarity dendrograms that we constructed for the ECH subunits encoded by the discussed cluster are not presented; they are on the whole congruent to the Ni-CODH dendrogram shown in Fig. 7.2b. The few exceptions (*C. subterraneus* subsp. *tengcongensis,* Geobacilli, and *Thermofilum carboxydotrophus*) were deliberately omitted when constructing the trees in Fig. 7.2 to allow it to demonstrate the general tendency. Comparison of the dendrograms in Fig. 7.2a, b leads to a conclusion that interspecies horizontal transfer of the discussed gene cluster is, in any case, not frequent. No recent events of horizontal transfer can be deduced from comparison of Figs. 7.2a, b. However, the gene cluster of *Caldanaerobacter subterraneus* ssp. *tengcongensis* has experienced a recent event of xenologous replacement of *cooS* and *cooF* genes, which is evident from their distinct positions in the relatedness trees (not shown in Fig. 7.2) and from their G+C content 20 points

higher than that of the genome; the donor of the genes is unknown. More ancient occurrence of horizontal transfer events requires special analysis. The understanding of the evolution of hydrogenogenic carboxydotrophy needs further studies, including those that will involve sequence data from new microorganisms.

7.9 Conclusions

Being a potent electron donor ($E^{0'}_{CO/CO2} = -520$ mV), CO serves as an energy source for various anaerobic prokaryotes. The main sources of CO in hot environments inhabited by anaerobic thermophiles are volcanic exhalations and thermal degradation of organic matter. Anaerobic transformation of CO by microbial communities of hot volcanic environments has been demonstrated. A number of phylogenetically diverse anaerobic prokaryotes, both *Bacteria* and *Archaea*, are known to metabolize CO. CO transformation can be coupled to methanogenesis, acetogenesis, hydrogenogenesis, sulfur, sulfate, or ferric iron reduction. Genomic analysis gives indications that anaerobic carboxydotrophy may be inherent in a wider range of organisms than it is currently recognized. Most numerous among the currently known thermophilic anaerobic carboxydotrophs are hydrogenogens. Among them are diverse *Firmicutes*, *Dictyoglomi*, and *Eury-* and *Crenarchaeota*. Despite their phylogenetic diversity, they employ similar enzymatic mechanisms of the $CO + H_2O \rightarrow CO_2 + H_2$ process. The key enzyme of anaerobic CO utilization, the Ni-CODH, forms in hydrogenogens an enzymatic complex with the energy-converting hydrogenase (ECH), and genomic analysis shows this enzymatic complex to be encoded by a single-gene cluster, whose presence can be used as a marker of hydrogenogenic carboxydotrophy when interpreting genomics and metagenomics data. Notably, most of the currently known species of hydrogenogens were isolated under 100% CO, but some species grow only at low CO concentrations in the gas phase. This calls for studies aimed to explain mechanisms of high resistance to CO and the evolutionary significance of their conservation and opens prospects for expanding the diversity of anaerobic carboxydotrophs by using low CO concentration in cultivation tests and isolation procedures.

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Chapter 8
Biomineralization in Geothermal Environments

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Abstract Biomineralization is the process in which various organisms internally or externally produce inorganic minerals as biominerals such as bones, teeth, shells, and invertebrate exoskeletons. The magnetites, iron deposits, gold deposits, calcium carbonates, calcium phosphates, and silicates are well-known examples of the biominerals. Although silica is the most abundant compound in the earth's crust and its precipitation is an important geological process in many geothermal environments, it is not useful for microorganisms. Recent research efforts revealed that both inorganic chemical reactions and microbial activity can be implicated in the formation of siliceous deposits. The extremely thermophilic bacteria within the genus *Thermus* are predominant component in the indigenous microbial community in siliceous deposits formed in pipes and equipment of geothermal power plants, which contributes to the rapid formation of huge siliceous deposits. In vitro examination suggested that *Thermus* cells induced precipitation of supersaturated amorphous silica during the exponential growth phase. A silica-induced protein (Sip) was isolated from the cell envelope fraction. The amino acid sequence of Sip was similar to that of the solute-binding protein of the Fe^{3+}-binding ABC transporter. Furthermore, Sip promotes silica deposition on the surfaces of cells, after which the silicified outer membrane may serve as a "suit of armor" that confers resistance to peptide

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antibiotics. Dissolved silica in geothermal hot water may be a significant component in the maintenance and survival of microorganisms in nutrient-limited niches. And thus, thermophilic bacteria may use biosilicification for their own survival. This chapter reviews the formation of siliceous deposits by thermophilic bacteria in geothermal environments.

Keywords Biomineralization • Silica • Biosilicification • *Thermus* • Silica-induced protein • Ferric iron-binding ABC transporter • Geothermal environment • Biofilm

8.1 Introduction

Biomineralization is the process by which living organisms internally or externally produce inorganic minerals known as biominerals. Familiar examples of biomineral products include bones, teeth, shells, and invertebrate exoskeletons. As reviewed in detail elsewhere (Lowenstam and Weiner 1989; Baeuerlein 2000), these minerals include the magnetites and other iron-containing deposits (Pardoe and Dobson 1999), gold deposits within unicellular organisms (Reith et al. 2006), silicates in diatoms and sponges (Shimizu et al. 1998; Sumper and Brunner 2008), and both calcium carbonates and calcium phosphates in nonvertebrate shells (Walters et al. 1997). It also includes the magnetites, calcium carbonates, and, more commonly, the calcium phosphate phases found in vertebrate tissues. In all cases of biomineralization, the cell directs the mineral formation process: by expression of proteins that act as nucleators in the cell or in the extracellular matrix or which prevent mineral formation in unwanted sites, by production of enzymes that modify the functions of these proteins, and by the regulation of ion transport. The proteins' properties determine their abilities to affect biomineralization and to interact with other proteins, with cells, and with the biomineral.

In the process of biomineralization, biominerals are formed at mild conditions (physiological temperature, pressure, and pH) by biological macromolecules. Fundamental studies of biomineralization process have led to the development of strategies for the synthesis of hybrid organic/inorganic structures. Nowadays, biomineralization contains the fields where the researchers use information gained from the bioextracts, and it has inspired materials chemists to design biomimetic analogues and develop bio-inspired synthetic schemes for mineral formation. The aim of biomimetics is to mimic the natural way of producing minerals. Many man-made crystals require elevated temperatures and strong chemical solutions, whereas the organisms have long been able to lay down elaborate mineral structures at ambient temperatures. Thus, biomineralization is by definition a multidisciplinary field that draws on researchers from biology, chemistry, geology, material science, and beyond (Estroff 2008).

Biominerals differ from pure inorganic minerals in that they are composed of organic macromolecules and inorganic hybrids, with a variety of biological macromolecules involved in their morphogenesis and maintenance. On the basis

of the formation process, Lowenstam (1981) classified biomineralization into two types:

The first type includes processes in which organisms accumulate mineral precursor ions to form mineral phases in specific shapes and at specific locations. Such processes form minerals for specific purposes or functions, for example, skeletal structure formation. These processes are referred to as biologically controlled mineralization (BCM), and the most commonly known biominerals are formed in this manner. A good example of BCM is the formation of a frustule primarily composed of silica (SiO_2) in diatoms, a type of unicellular algae. Diatoms selectively take up the silica precursor, silicic acid ($Si(OH)_4$), from water in their environment using silicon transporter (SIT) (Hildebrand et al. 1998; Hildebrand 2003). Silicic acid is then transported by organelles known as silica deposition vesicles (SDV), following which it is deposited as silica in a specific pattern and acted upon by peptides known as silaffins to form the frustule (Kröger et al. 2001). Frustule formation is essential for the development of diatoms; therefore, they cannot grow without a supply of silicic acid. Organisms such as diatoms conduct biologically controlled mineralization, with strict control over the mineralization process for their survival.

In contrast, the second type of biomineralization occurs without biological control. This is known as biologically induced mineralization (BIM) and results in the formation of biominerals as mere by-products of metabolic activity or as a result of interactions between the cell surface and surrounding water. Specifically, it involves processes in which substances such as the waste products of metabolism (e.g., O_2, OH^-, HCO_3^-, Fe_2^+, NH_4^+, H_2S) and ions with oxidation states that have been altered by enzyme action (e.g., oxidation of Fe (II) and Mn (II)) react with mineral precursor ions to form mineral phases without a defined shape or crystal structure, depending on conditions such as charge states and chemical composition of the cell surface. It is well known that iron (III) hydroxide, magnetite, calcium carbonate, silica, and other mineral phases are formed on bacterial cell surfaces; however, the majority of these compounds are formed by BIM (Fortin et al. 1997). The earliest known examples of products formed by this process are fossils of silicified organisms found in rock strata from 1.9 Ga (Brasier et al. 2011). BIM processes result from a variety of causes; however, bacteria seem to lack the means to control them.

Bacterial biominerals do not form skeletal structures with specific shapes or crystalline architectures such as those observed in diatoms. In addition, the minerals produced are not essential for bacterial development. These two factors would imply that no clear goal exists for bacterial mineral precipitation. However, we know that large amounts of various mineral precursors were dissolved in the environment since 3.8 Ga, the period in which life is commonly thought to have originated. On the basis of this knowledge, it is possible to conclude that life on earth and biomineral formation originated at the same time. Life on earth has evolved through adaptation to the environment and has sometimes survived by altering the environment itself. Despite having evolved over such a vast period of time, would bacteria continue to use incongruous mineralization processes? In this chapter, we have explored the clever survival strategies hidden behind the seemingly meaningless mineralization processes in bacteria that use biominerals.

8.2 Silica Chemistry in Geothermal Environments

As described above, biomineralization is observed in a wide variety of species, and various minerals are formed in the cells of these species. In this review, we exclusively deal with silica precipitation in bacteria. Silica is the most abundant compound in the earth's crust, accounting for approximately 60% of its mass. Silica precipitation is an important geological process in many geothermal environments. A siliceous deposit called "sinter" has been widely observed in geothermal environments such as hot spring systems, and many microbial-like structures are found attached to amorphous silica within the sinter (Fig. 8.1) (Inagaki et al. 1997, 2001; Handley et al. 2005; Kyle et al. 2007). From this, we can infer that some type of interaction occurs between silica and bacteria; however, we first explain the chemical properties of silica in geothermal environments.

Hot water in geothermal regions originates from hydrothermal reservoirs deep underground, where geothermal water reaches solution equilibrium with subterranean rock and becomes saturated with silica (SiO_2), the main component of the rock. Water temperatures in hydrothermal reservoirs reach 200–300°C as a result of high pressure and heat from magma. Therefore, the concentration of silica in geothermal water must be greater than the solubility of amorphous silica at 100°C (approximately 400 ppm). When this water is discharged at the earth's surface, degassing, rapid cooling, and evaporation cause sudden supersaturation with silica. Supersaturation with most other inorganic substances results in solute precipitation above the solubility limit and maintenance of the saturating concentration at the water temperature; however, these events are not observed with silica.

In the environment, a dissolve form of silicon below pH 8 and at low concentrations is monomeric silicic acid ($Si(OH)_4$). The solubility of amorphous silica is the concentration of silicic acid that dissolves after sufficient contact between water and amorphous silica under a given temperature, pressure, and pH. A supersaturated silica solution occurs when the total concentration of silicic acid in an aqueous solution exceeds the solubility of amorphous silica. In this supersaturated state, the dehydration of hydroxyl groups (silanol groups: Si-OH) occurs between silicic acid molecules, leading to the formation of oligomers (dimers, trimers, and tetramers) with siloxane bonds (Si-O-Si). Oligomer formation is followed by repeated polymerization of oligomers and dissolved silicic acid to form spherically shaped silica particles with diameters of 1–5 nm. Ostwald ripening of these particles gives rise to a solution of colloidal silica particles with diameters greater than 5 nm. Because of the unequal charge that results from the silanol group and the siloxane bond, the colloidal silica surface has a residual negative charge (Fig. 8.2). Consequently, the colloids repel each other, thereby maintaining their dissolved states and preventing silica precipitation, resulting in supersaturated silica solutions. Both the soluble content of silicic acid monomers and supersaturated content of colloidal silica are present in a supersaturated silica solution.

The formation of siliceous deposits on the surface of equipment and in pipelines of geothermal power plants presents serious economic problems, related to energy

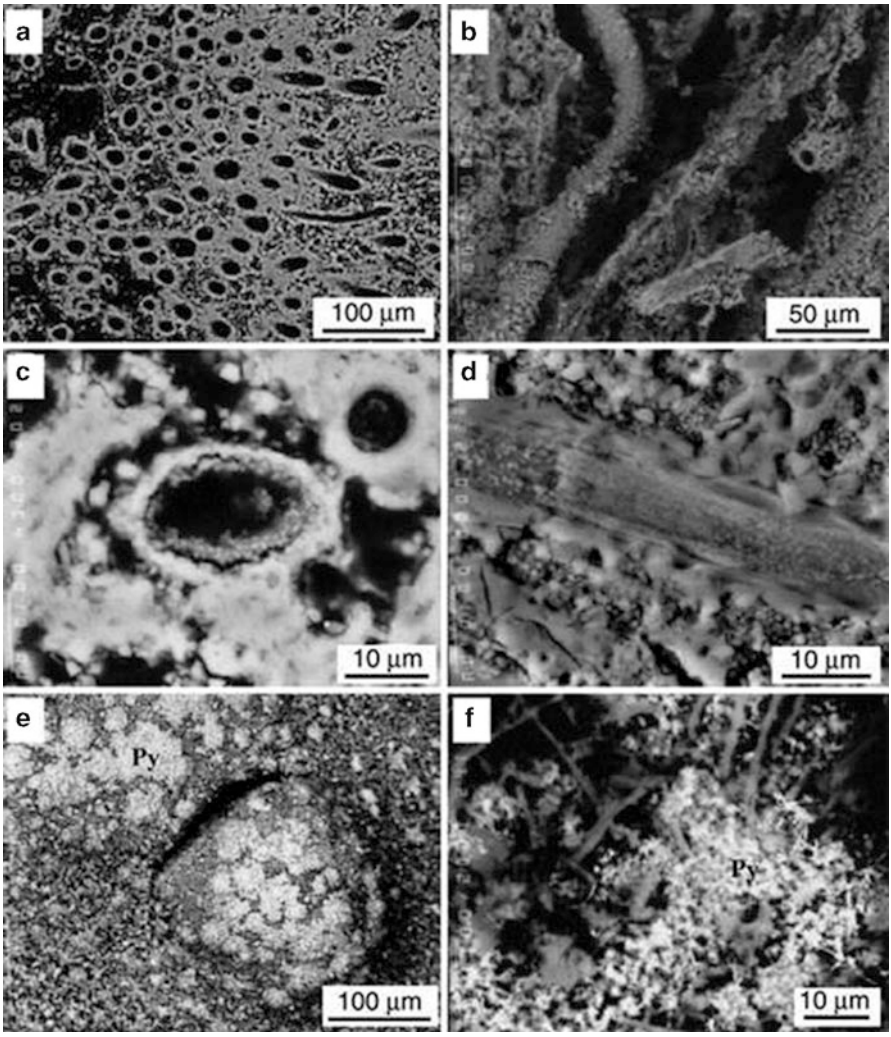

Fig. 8.1 EPMA micrograph of microbial textures preserved in the sinter sediment at the Steep Cone hot spring, Yellowstone National Park. The *gray* part reveals amorphous silica. Organic material is *dark* to *black* and indistinct. (**a**) Section of filamentous microbes embedded in amorphous silica precipitates. (**b**) Silicified filamentous microbes. (**c**, **d**) Amorphous silica spherules precipitated on sheathes of filamentous microbes. (**e**) Framboidal aggregates of pyrite (*Py*) precipitated on the surface of amorphous silica layer. (**f**) Close association of silicified filamentous microbes and spinous pyrite (*Py*) formed in a boiling pool (96°C) (Reprint from Inagaki et al. (2003) with permission from Springer)

losses, increased costs of cleaning and maintenance, loss of production, or even abandoning a production and reinjection wells (Fig. 8.3). We examined microbial participation in formation of siliceous deposits. The result suggested the presence of bacteria-like structures and DNA in the siliceous deposits. The existence of

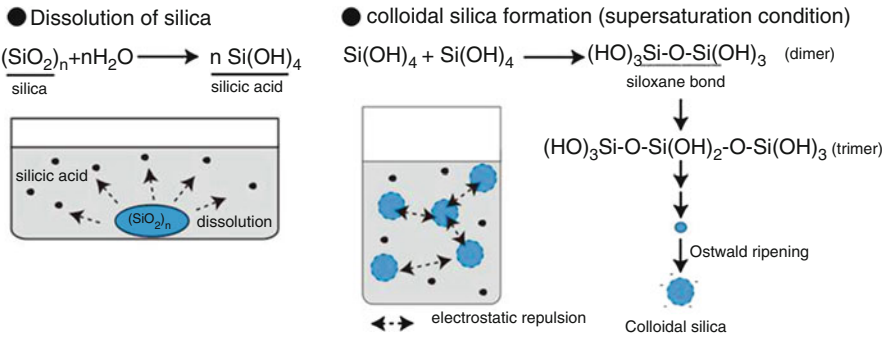

Fig. 8.2 Schematic diagram of silica dissolution and silicic acid polymerization

microorganisms and the diversity of the bacterial community in the siliceous deposits formed in a geothermal environment were investigated (Fig. 8.3). Then we performed a series of experiments using the extremely thermophilic bacteria *Thermus thermophilus* TMY from a sample of silica scale taken from the geothermal power plant in Japan (Fujino et al. 2008). The formation of biosilica (silica precipitate formed by biomineralization) was observed in culture at 75°C with silica concentrations greater than the saturation concentration (300 ppm) of silicic acid (Fig. 8.4) (Inagaki et al. 1998). Approximately 50~60% of total silicic acid was deposited during growth of the bacteria. Under these conditions, the difference between the original and dissolved silica concentrations after culture suggests that only silica corresponding to the supersaturated content is precipitated as biosilica. On the basis of this result, we assumed that colloidal silica is associated with biosilica formation. The same phenomena were observed with other *Thermus* strains, *T. thermophilus* HB8, HB27, AT-62, and *T. aquaticus*. Another study has shown little affinity between bacteria and silicic acid monomers (Fein et al. 2002). On the basis of these findings, we suggest that supersaturated concentrations of silicic acid are essential for biosilica formation by bacteria and that colloidal silica is a precursor of biosilica.

As described earlier, the colloidal silica surface has a residual negative charge; however, the bacterial surface also has a negative charge, except under extremely low pH conditions. The interaction between the bacterial surface and colloidal silica requires a driving force that can overcome this electrostatic repulsion. The following theories for this driving force have been suggested: (1) hydrogen bond formation between the hydroxyl group on the bacterial surface (exopolysaccharide, EPS) and silanol group on the colloidal surface, (2) cross-linking between the bacterial surface and colloidal silica caused by metallic cations, and (3) direct electrostatic interaction between the negative charge of the colloidal silica surface and protonated amino group of the protein-rich biofilm secreted by the bacterial surface (Phoenix et al. 2002, 2003; Lalonde et al. 2005). However, none of these interactions have been clearly demonstrated. Apart from these physicochemical interactions, it is possible that the driving force is a protein that interacts exclusively with silica, similar to the

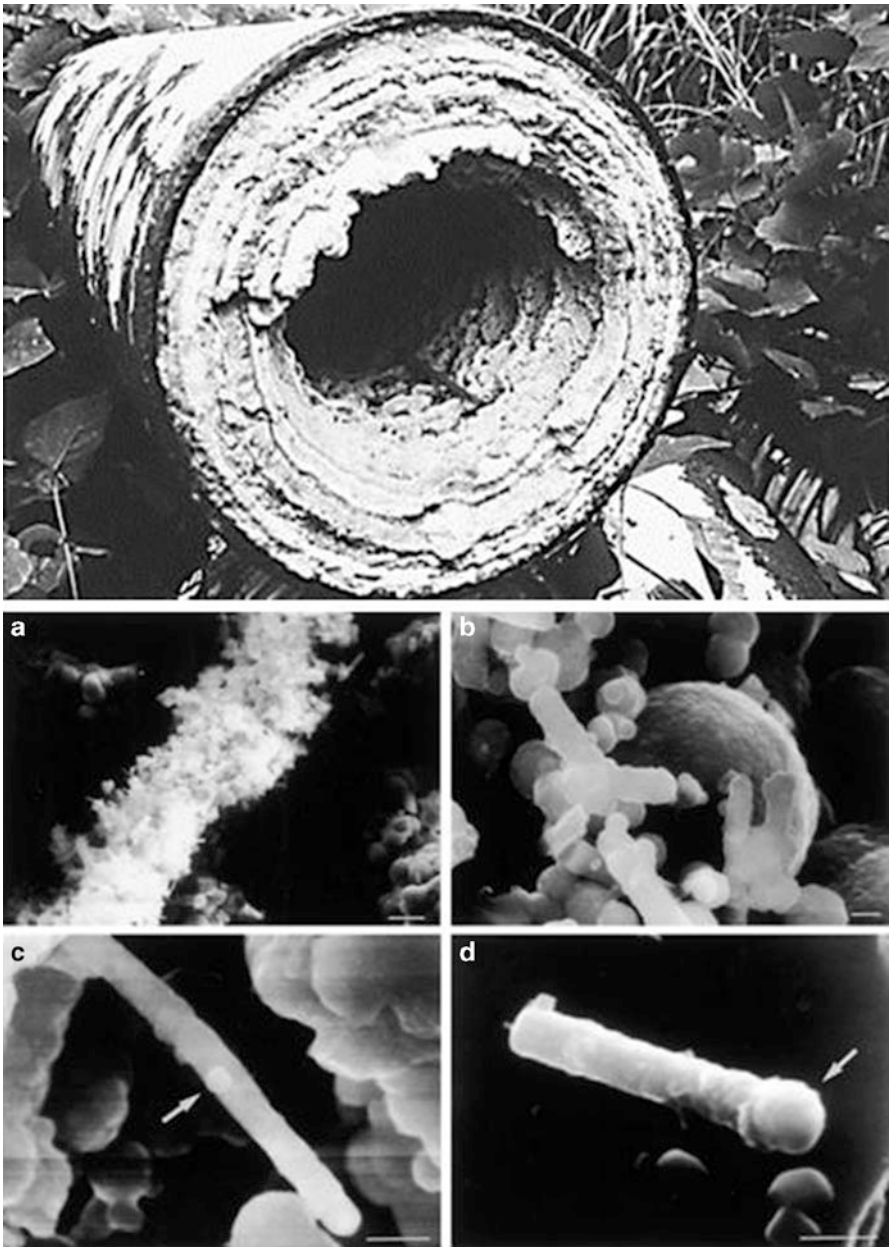

Fig. 8.3 (*Upper*) Photograph of silica scale formed in a pipeline at the Otake geothermal power plant. Rapid formation of silica scale is considered to be associated with the population and effects of microorganisms, and serious economic and industrial problems ensue. (*Bottom*) EPMA micrograph of silica scale formed in the Otake geothermal power plant in Oita Prefecture, Japan. (**a**) Aggregation of silica particles. Bar, 10 μm. (**b**) Rod-shaped bacteria-like structures and spherical silica particles. Bar, 1 μm. (**c** and **d**) Rod-shaped bacteria-like structures attached to the colloidal silica particles (*arrow*) on the surface. Bar, 1 μm (Reprint from Inagaki et al. (1997) with permission from John Wiley & Sons, Inc.)

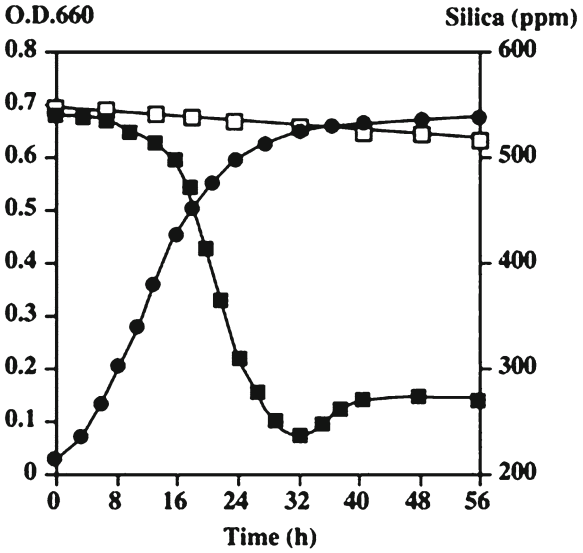

Fig. 8.4 Biosilicification with an extreme thermophilic bacterium *T. thermophilus* TMY isolated from silica scale. A relationship between cell growth (*closed circles*) and silica concentration in culture medium supernatant (*closed squares*). Silica concentrations in the absence of cell growth are indicated by *open squares*. Cultivation was performed at 75°C. Supersaturated amorphous silica was precipitated at the exponential growth phase of *Thermus* cells. The final concentration of silica, at approximately 280 ppm, corresponds to the solubility of amorphous silica in hot water at 75°C (Kitahara 1960) (Reprint from Inagaki et al. (1998) with permission from Japan Society for Bioscience, Biotechnology, and Agrochemistry)

transporters seen in biologically controlled mineralization (Aramaki et al. 2004; Yokoyama et al. 2004). However, until date, there have been no reports of proteins involved in bacterial silica precipitation.

8.3 Thermophilic Bacterial Response to Silica Stimulation

We propose that the surface layer of bacterial cells has a profound connection with biosilica formation in *Thermus* species and that silica concentration plays an important role as an external factor (Inagaki et al. 1998). *Thermus* strains were cultured in media containing various silica concentrations (0–600 ppm). By extracting the surface layer proteins, we found that the production of an approximately 35-kDa protein is only induced under supersaturated silica conditions (greater than 300 ppm silica at 70°C) (Doi et al. 2009). This protein was called a silica-induced protein (Sip), and it became clear that the expression of this protein is initiated within 1 h of the addition of supersaturated silica concentrations. This protein was treated as an iron-binding ATP-binding cassette (ABC) transporter on the basis of its internal amino acid sequence. Iron-binding ABC transporters have primarily been studied in

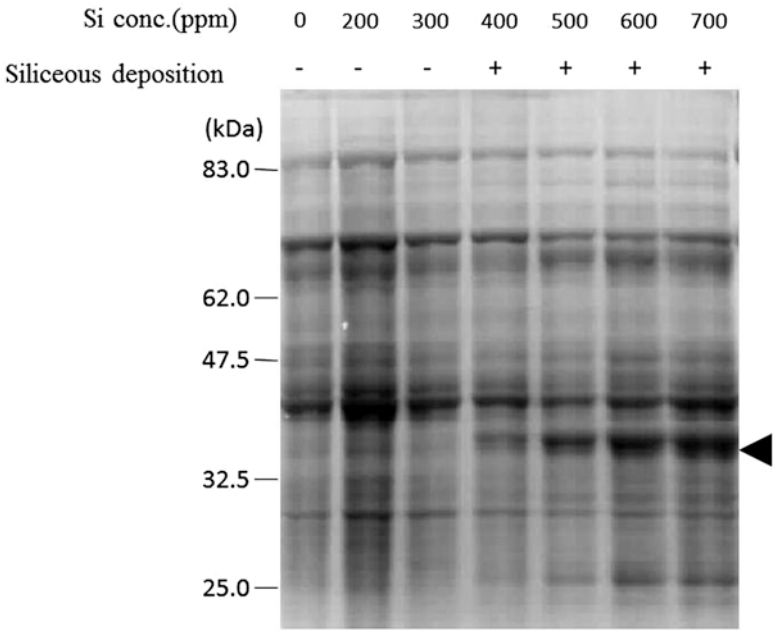

Fig. 8.5 Relationship between silica concentration and the production of the Sip protein in *T. thermophilus* TMY. Cells were cultivated in TM medium containing various concentrations of silicic acid. An *arrowhead* indicates the Sip (Reprint from Doi et al. (2008) with permission from American Society for Microbiology)

pathogenic bacteria such as *Serratia marcescens* (Angerer et al. 1992), *Neisseria gonorrhoeae* (Adhikari et al. 1996), and *Actinobacillus actinomycetemcomitans* (Rhodes et al. 2007). Their function is uptake of iron ions from their environment at the time of infection and during pathogen manifestation; however, how they interact with silica is not known.

To better understand the function of this protein, *T. thermophilus* HB8 was cultured in iron-deficient media and media supersaturated with silicic acid, and microarray analysis was performed. Transcription levels of genes related to iron transport, including the iron-binding ABC transporter, significantly increased under both the conditions, suggesting that iron deficiency results on the addition of silica. This deficiency may be caused by a decrease in available iron ions caused by binding to the negatively charged colloidal silica surface. In other words, the cell interprets the presence of colloidal silica as a signal that indicates a decrease in the concentration of available iron ions. We predicted that a deficiency of iron, which is essential for growth, would inhibit bacterial propagation. However, the growth curve observed in cultures with 600 ppm silica was the same as that in cultures without silica (Fig. 8.5). This finding indicates that iron deficiencies caused by silica are not serious enough to inhibit growth. Interestingly, transcription levels of efflux transporters for heavy metals also increased significantly in the presence of colloidal silica (Spada et al. 2002). The use of nearly pure sodium metasilicate (Na_2SiO_3) in this study did not

eliminate the possibility of heavy metal contamination of the culture. Therefore, the fact that heavy metal efflux transporters were found despite the absence of heavy metals likely means that the cell detected the presence of colloidal silica, intentionally accumulated heavy metal ions on its surface, and used their cross-linked structures to promote silica binding.

In recent years, silica-induced protein expression has been reported in the cyanobacterium *Anabaena* sp. PCC 7120 (Konhauser et al. 2008). Similar to our findings with *T. thermophilus*, induced expression was not observed under low silica concentrations but was observed under high silica concentrations (300 ppm). In addition, a protein with a molecular weight of 116 kDa was detected. This protein is also reported to have some homology with membrane transporters; however, this has yet to be investigated in detail. Furthermore, the secretion of large quantities of EPS and precipitation of biosilica restricted to the biofilm under supersaturated conditions have been observed in *Sulfurihydrogenibium azorense*, a species of the autotrophic order Aquificales (Lalonde et al. 2005). Such findings indicate for the first time that specific proteins and biological macromolecules are involved in biosilica formation and suggest that bacteria might precipitate silica intentionally.

8.4 Merits of Biosilicification

Until recently, silica formation in bacteria was merely thought to be the formation of a site for inorganic deposition of high concentrations of silica from solution. However, bacteria actually seem to respond to silica in their environment and actively cause its precipitation. A major question in biosilica formation is whether or not bacteria can maintain growth in a silicified state. In experiments on biosilica formation using *Thermus* species, we found that growth rates under supersaturated silica conditions and those without silica were approximately equal. This finding indicates that supersaturated silica does not have an effect on growth in *Thermus* species. Phoenix et al. (2006) reported that silicification of the cyanobacterium *Calothrix* sp. occurred after culture for 12 days with 300 ppm silica, and they observed the formation of a silica layer several microns in thickness around the cell circumference. However, even these mineralized cells exhibited the same photosynthetic performance as nonmineralized cells, leading to the conclusion that silicification does not lead to cell death. Therefore, rather than saying that the cells are embedded in silica, it might be more appropriate to say that they are clad in a suit of "silica armor." Phoenix and Konhauser (2008) proposed the following benefits that bacteria gain from their armor:

1. A shield from harmful ultraviolet rays
   Protection from harmful ultraviolet (UV) rays has been suggested as one of the benefits of biosilica formation. UV rays are deleterious to life, and those with wavelengths at 240 and 270 nm are particularly harmful as they are absorbed by DNA and cause damage. Silica does not absorb the light necessary for photosynthesis (400–700 nm) but absorbs UV rays and dampens their strength by an

order of magnitude. Phoenix and his coworkers had also reported that silicified cells exhibit considerable UV resistance than unsilicified cells (Phoenix et al. 2001). This finding is also consistent with the physicochemical hypothesis that photosynthetic bacteria can grow 1–10 mm below sinter surfaces in high-altitude, low-latitude hot spring systems. These areas have more powerful UV rays than those at the ground level, and it is thought that photosynthetic bacteria can survive as a result of the intensity of UVB rays being attenuated within 1 mm of the sinter surface. The finding that the light necessary for photosynthesis reaches depths of 5–10 mm is consistent with the wavelength range at which bacteria grow (Phoenix et al. 2006).

2. A safety barrier against attack from other organisms
   Another function of biosilica that has been proposed is protecting against consumption by protists such as amoebas. Although such organisms do not exist in geothermal habitats, if silicified bacteria were to be ingested by phagocytosis, the silica armor would probably be consumed with the rest of the cell. However, if many bacteria accumulate using silica and achieve a certain size, it may become impossible to digest them by phagocytosis. This hypothesis has not been proven, and experiments to verify it have not been performed. However, it is likely that the silica armor is a safety barrier against attack by other organisms.

   Instead of the protists mentioned above, our research group focused on antibiotics produced by bacteria and conducted a study on the susceptibility of *T. thermophilus* to various antibiotics such as chloramphenicol, kanamycin, ampicillin, tetracycline, bacitracin, colistin, and polymyxin B by measuring broth turbidity in silica-present and silica-absent cultures (Iwai et al. 2010). Acquired drug resistance to chloramphenicol, kanamycin, ampicillin, and tetracycline was not observed in either silica-present or silica-absent cultures; however, improved growth was observed in silica-present cultures with bacitracin, colistin, and polymyxin B (Fig. 8.6). This finding suggests that the presence of silica contributes to acquired drug resistance. Seven multidrug efflux transporter genes located in the *T. thermophilus* HB8 genome may be associated with acquired drug resistance. Using quantitative real-time PCR, we tracked transcription levels of each of these genes under the conditions of this experiment; however, we did not find significant increases in their transcription levels. We concluded that acquired drug resistance in *T. thermophilus* results from biosilica formation. The antibiotics to which silica-present *T. thermophilus* cultures were resistant (bacitracin, colistin, and polymyxin B) are all drugs that use the cellular membrane and are produced by members of the genus *Bacillus* and other related species that grow in geothermal environments. We are of the opinion that *T. thermophilus* uses large amounts of dissolved silica in its environment, precipitates silica on its surface, and prevents attack from antibiotics with its silica armor.

3. Biosilica as a “growth locus”
   Silica deposited by microorganisms is hydrated amorphous silica ($SiO_2 \cdot nH_2O$) and not SiO_2. If it were to be dehydrated over a long period of time, it would change into a highly crystalline substance. However, when it is first formed, biosilica resembles a hydrated gel, presumably because of the fact that water is

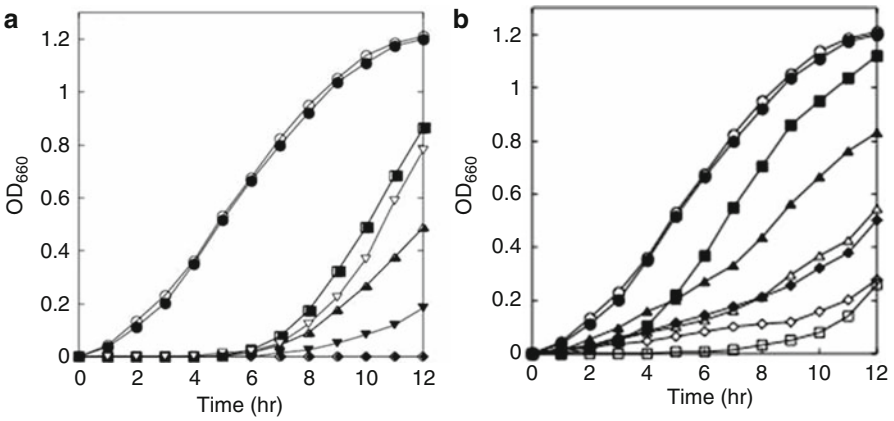

Fig. 8.6 Effect of antibiotics on the growth of *T. thermophilus* TMY in the presence and absence of supersaturated silica. (**a**) Effect of antibiotics which inhibit protein or cell wall synthesis. *Open symbols* show the growth curve in the absence of supersaturated silicic acid. *Filled symbols* show the growth curve in the presence of supersaturated silicic acid (600 ppm). ○ ● no antibiotics, □ ■ chloramphenicol (1 μg/ml), △ ▲ kanamycin (5 μg/ml), ◇ ◆ ampicillin (0.1 μg/ml), ▽ ▼ tetracycline (0.5 μg/ml). (**b**) Effect of peptide antibiotics on the growth. *Open symbols* show growth curves in the absence of supersaturated silicic acid. *Closed symbols* show growth curves in the presence of supersaturated silicic acid (600 ppm). ○ ● no antibiotics, ◇ ◆ bacitracin (0.5 μg/ml), □ ■ colistin (5 μg/ml), △ ▲ polymyxin B (5 μg/ml) (Reprint from Iwai et al. (2010) with permission from Nature Publishing Group)

retained in the gaps present in the porous structure of amorphous silica. These biosilica particles are like sponges and are convenient for temporary moisture retention.

Many geysers exist in geothermal environments; however, a regular constant outflow of hot water is not guaranteed. In other words, bacteria have to survive in an environment where the water supply may cease at any moment. By using moisture stored in this biosilica sponge, the cells attached to biosilica can survive in such an environment even if the water supply temporarily stops.

Another possible benefit of biosilica is the increase in robustness that occurs with the inclusion of biosilica in biofilms. In electron microscopy of samples from geothermal environments, a structure composed of many cells embedded in a biomineral matrix is observed rather than individual biomineral-clad cells. It seems that cells in this state have greater structural integrity than individually mineralized cells. The strength of such a structure is also dependent on the biofilm density (Beyenal and Lewandowski 2002). In addition, it is possible that by forming a biofilm with a complex structure, the surface area of the biofilm in contact with the surrounding water facilitates more efficient nutrient transport and accumulation. We speculate that biominerals are involved in the morphogenesis and structural maintenance of this type of biofilm (Fig. 8.7). Whether or not substance transport is possible in cells that are completely embedded in biominerals is an important question. However, as demonstrated in the experiment using

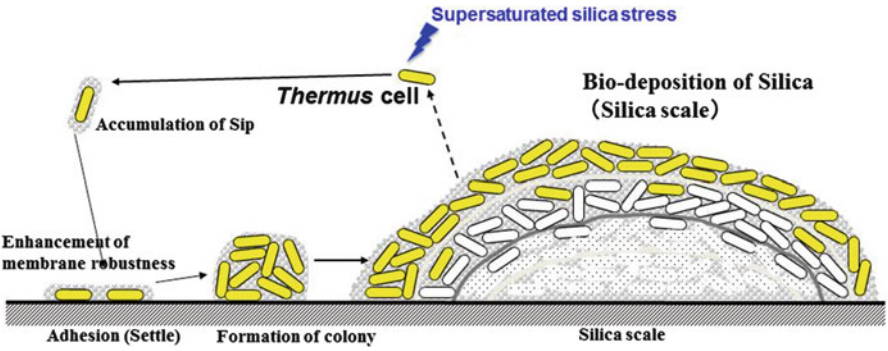

Fig. 8.7 Schematic diagram of biosilicification with *T. thermophilus* in a geothermal environment

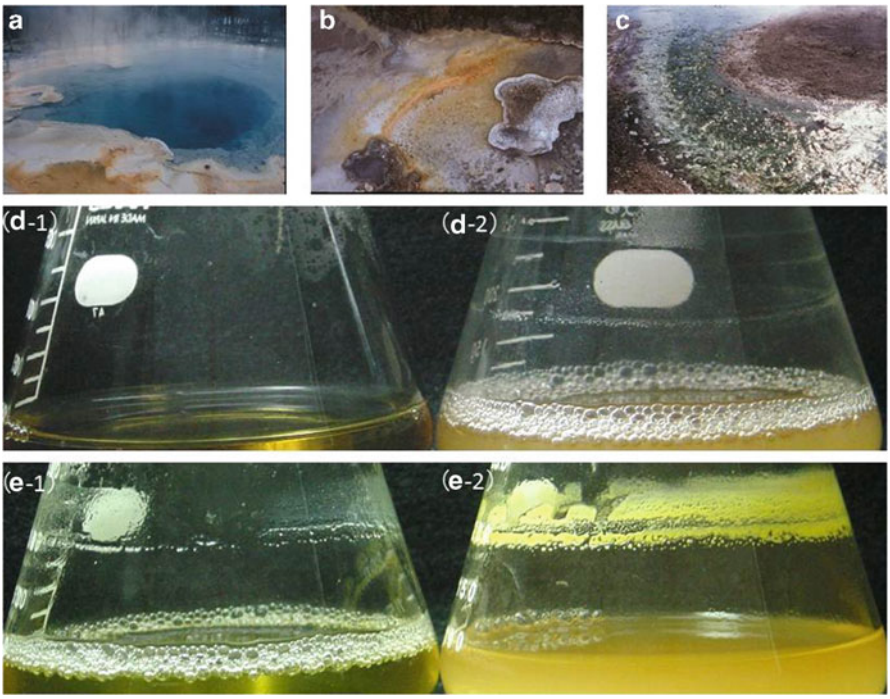

Fig. 8.8 Silica deposition in geothermal area and in vitro biosilicification with *T. thermophilus*. (**a**) Deposited sinter formations along the edge of boiling water pool. A huge number of hyperthermophilic archaea make their habitat in the edge (>90°C). (**b**) Flow channel of scalding hot waters overflowed from the boiling water pool (70~80°C). Many *yellow*- or *orange*-colored colonies or biofilms of *Thermus* species with silica deposition are observed. (**c**) *Blue* or *green* mattes of cyanobacteria or algae thrive in the scalding waters (40°C). (**d-1**) TM broth. (**d-2**) Culture of *T. thermophilus* in TM broth. (**e-1**) TM broth containing 600 ppm silica. (**e-2**) Culture of *T. thermophilus* in TM broth containing 600 ppm silica

Calothrix sp., it was clear that sufficient mineral-mediated carbon dioxide transport occurs to enable normal photosynthesis. This mineral transport may occur via the pores present in the hydrated amorphous silica gel. This phenomenon is not seen with any other minerals and may be one of the reasons why bacteria use silica in their biofilms.

In geothermal regions, hot water gushes from the underground, and mound-shaped sinter terraces are formed by microbial action and the cooling effect of the outside air. Water ejected from the top of the sinter flows along the surface, and a mat of microorganisms that can cope with the high temperature can be observed along its route. This microbial mat displays different colors depending on the temperature zone; hyperthermophiles appear closest to the vent where the water temperature is the highest, followed by extreme thermophiles and then by moderate thermophiles. Hyperthermophilic bacteria such as *Thermus* species produce a biosilica-containing biofilm to remain in their ideal growth temperature range in geothermal environments; therefore, biosilicification can help to ensure growth sites. In fact, the biosilica which was observed in the sinter terrace adhered at the upper edge of the Erlenmeyer flask containing *Thermus* culture with supersaturated silica (Fig. 8.8). The formation of siliceous deposits on the surface of equipment and in pipelines of geothermal power plants might be resulted in a similar bacterial response.

8.5 Conclusions

The inorganic chemical reactions as well as microbial activity appear to be involved in the formation of siliceous deposits. The dissolved silica in geothermal hot water could be a significant component in the maintenance and survival of microbes in the nutrient-limited niches.

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Chapter 9
Phylogeny and Biological Features of Thermophiles

Takashi Itoh and Takao Iino

Abstract Thermophilic prokaryotes constitute a quite large group among the currently known prokaryotic species. At present, more than 220 genera and 580 species of the thermophiles have been isolated from various thermal and non-thermal environments and characterized. They are classified in more than 16 phyla of the two domains, Archaea and Bacteria. This chapter provides an overview of their phylogenetic relationships and their phenotypic features in the light of the current prokaryote systematics. Possible reasons for the phylogenetic diversity of the thermophiles in geothermal habitats are also discussed.

Keywords Thermophile • Archaea • Bacteria • 16S rRNA • Genome • Systematics • Phylogeny

9.1 Introduction

Thermophilic organisms distribute widely within the two prokaryotic domains, *Archaea* and *Bacteria*, and there are also some examples of the "thermophilic Eukaryotes" that can grow up to 50–60 °C (Brock 1986). Although there is no clear definition of the term "thermophile," here, it is used for prokaryotes growing optimally at 50 °C and higher for convenience. Where necessary, the thermophiles are further subdivided into three categories: moderate thermophiles (growing optimally 50–70 °C), extreme thermophiles (growing optimally 70–80 °C), and hyperthermophiles (growing optimally more than 80 °C: Stetter et al. 1990).

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At early attempt to draw the universal phylogenetic tree by small subunit (SSU) rRNA sequences, the presence of hyperthermophiles with deep short lineages on both bacterial and archaeal domains had led the idea that the last universal common ancestor (LUCA) might have been a hyperthermophile (Stetter 1995). Soon after, it was challenged by different studies and observations: some claimed that the hyperthermophilic LUCA model was derived by artifacts due to the long-branch attraction or compositional bias in the G+C (guanine+cytosine)-rich rRNA genes of the hyperthermophiles (Gribaldo and Philippe 2002). Forterre et al. (2000) observed that reverse gyrase in hyperthermophilic bacteria represented by *Aquifex* and *Thermotoga*, the protein which is thought to stabilize DNA at high temperature and distribute among hyperthermophiles, were more likely to be transferred from hyperthermophilic archaea, implying that the hyperthermophily in bacteria was derived by the secondary adaptation. Even so, the phylogenetic analysis of the archaeal reverse gyrases implied that the archaeal ancestor was hyperthermophilic (Forterre et al. 2005). Furthermore, as reviewed by Gaucher et al. (2010), laboratory experiments on the thermostability of several putative ancestral proteins, which were reconstructed from the sequences of extant modern organisms, fit the hypothesis that LUCA was thermophilic or hyperthermophilic. Therefore, the ancient thermophiles, not necessary to be hyperthermophiles, could have had important roles in the evolution to modern prokaryotes.

This chapter aims at providing an overview of the phylogenetic relationships and biological (taxonomic) features of the thermophiles in the light of current prokaryotic systematics that is mainly based on the 16S rRNA gene phylogeny. In addition, probable phylogenetic history of some thermophilic taxa would be elucidated from comparative genomic analyses.

9.2 Phylogeny and Systematics of Prokaryotes

At present, the most commonly accepted taxonomic scheme of prokaryotes is primarily based on the 16S rRNA gene phylogeny, and it arranges prokaryotes in the hierarchical taxonomic structure from domain to species, as revealed in the latest edition of the *Bergey's Manual of Systematic Bacteriology* (2nd edition: Garrity and Holt 2001; Ludwig and Klenk 2001). Primacy of the small subunit (SSU) rRNA gene as a phylogenetic marker in the prokaryotic systematics has been approved theoretically and practically (Ludwig and Schleifer 1999; Ludwig and Klenk 2001). The molecules distribute ubiquitously with constant function and are less subjected to the horizontal gene transfers (HGTs). The length, approximately 1.5 K bases, is large enough in terms of the information content, and presence of the conserved and variable regions in the molecule allows to resolve the prokaryotic phylogeny broadly from domain to species levels. In addition, the relative ease in the current analytical procedure (i.e., presence of universal primers for the gene amplification and sequencing, adequate or permissible length to complete the whole/almost whole gene sequence) has allowed to accumulate the sequence data. Consequently a huge database of the 16S rRNA gene sequence data has been constructed in the public domain.

On the other hand, several drawbacks have been pointed out at the same time, e.g., presence of heterogeneous copies in a single strain of some taxa and low phylogenetic resolving power among closely related strains (Ludwig and Klenk 2001; Stackebrandt and Goebel 1994). Furthermore, SSU rRNA gene represents only a single gene, and the inference of the phylogenetic relationships of organisms by the single gene can be biased.

According to the Genomes OnLine Database (GOLD v. 4.0: http://www.genomesonline.org/cgi-bin/GOLD/index.cgi), more than 3,170 completed genome sequences are published until now (as of June, 2012), and this number is certainly surging by the advancement of the gene sequencing technologies and computational developments as well as the promotion of several microbial genome projects such as "A Genomic Encyclopedia of Bacteria and Archaea" (GEBA: Wu et al. 2009a). However, because of the evolutionary events that would hamper the phylogenomic inferences, such as HGT, gene loss, and gene duplication, the analytic methods to compare gene sequences cannot be applied simply to complete the genome-based phylogenies (Snel et al. 2005). Furthermore, a variety of genomic information leads to develop different types of methods to cluster the genomes by different researchers. According to Snel et al. (2005), the approaches can be divided into five categories: (a) alignment-free genome tree based on statistics properties of the complete genome, (b) gene content tree based on the presence and absence of genes, (c) genome trees based on chromosomal gene order, (d) genome trees based on average sequence similarities, and (e) phylogenomic trees based either on the collection of phylogenetic trees derived from shared gene families or on a concatenated alignment of those gene families. The algorithms to reconstruct trees are further categorized to distance, maximal parsimony, maximum likelihood, and Bayesian methods. Nevertheless, the genome-based phylogenies have been giving new insights into the evolutionary relationships of the prokaryotes and sometimes offer more reasonable interpretations than those derived from the SSU rRNA gene information only.

9.3 Phylogenetic Relationships and Biological Features of the Thermophiles

In the following subsections, the phylogenetic relationships and some phenotypic features of the thermophiles in the two prokaryotic domains, *Archaea* and *Bacteria*, are overviewed in the light of the current 16S rRNA gene-based systematic scheme. Where possible, the phylogenetic views from genome or certain gene-set comparison are included.

9.3.1 Archaea

At present, the domain *Archaea* comprises two phyla: *Crenarchaeota* and *Euryarchaeota*. In addition to these two phyla, *Korarchaeota, Nanoarchaeota, Thaumarchaeota*, and *Aigarchaeota* are regarded as provisional phyla, as described

below. Certain members of these provisional phyla are maintained as a pure culture, coculture, or highly enriched culture; nevertheless, they do not represent any taxa with nomenclatural standings at this moment. Among the phyla *Crenarchaeota* and *Euryarchaeota*, thermophilic members with nomenclatural standings are listed in Table 9.1.

The phylum *Crenarchaeota* is composed of a single class, *Thermoprotei*, including five orders: *Thermoproteales, Desulfurococcales, Sulfolobales*, and recently proposed *Acidilobales* (Prokofeva et al. 2009) and *Fervidicoccales* (Perevalova et al. 2010). All of the known members of the phylum *Crenarchaeota* are thermophilic (mostly hyperthermophilic). The order *Thermoproteales* accommodates rod-shaped members of the family *Thermoproteaceae* and a filamentous member of the family *Thermofilaceae*. In the 16S rRNA phylogenetic tree of the family *Thermoproteaceae* (Itoh 2003), two genera, *Thermoproteus* and *Pyrobaculum*, form a rather coherent cluster, while the other three genera are distantly related to the cluster of *Thermoproteus*/*Pyrobaculum* and to each other. *Thermocladium* forms an independent lineage, while the phylogenetic positions of *Caldivirga* and *Vulcanisaeta* are rather ambiguous. In a phylogenetic tree by means of the CBF5 and RadA proteins, *Thermocladium, Caldivirga*, and *Vulcanisaeta* were clustered together, excluding the *Thermoproteus*/*Pyrobaculum* (Yokobori et al. 2009). The lineage of the family *Thermofilaceae* is more deeply branched. Members of the order *Desulfurococcales* are coccoid or disk-shaped in cell morphology, anaerobic except for the genus *Aeropyrum*, and neutrophilic or weakly acidophilic growing optimally at pH 5.0–8.0, isolated from both terrestrial and marine habitats. Two families, *Desulfurococcaceae* and *Pyrodictiaceae*, are known in this order. On the 16S rRNA gene phylogenetic tree, the latter forms a coherent cluster, while the former is rather heterogeneous and may be subdivided into the following three clusters (Itoh 2003) represented by the genera *Desulfurococcus*, *Ignicoccus*, and *Aeropyrum* from the respective clusters. The genus *Ignicoccus* grows chemolithotrophically, while other genera in this family grow heterotrophically or mixotrophically. Genomic comparison of *Ignicoccus hospitalis*, the host archaeon of the obligately symbiotic *Nanoarchaeum equitans* (see below), suggested that this lineage has lost most of the genes associated with a heterogeneous metabolism that is characteristic of most of the *Crenarchaeota* strains (Podar et al. 2008). The order *Acidilobales* was created to accommodate acidophilic, heterotrophic, coccoid, extremely thermophilic, or hyperthermophilic members: *Acidilobus* and *Caldisphaera* (Prokofeva et al. 2009). All of the known species were isolated from terrestrial acidic hot springs and grew optimally around pH 4. Based on the 16S rRNA gene sequence phylogenetic analyses, the members are closely related to *Desulfurococcales*; however, separateness of *Acidilobales* from *Desulfurococcales* is still debatable. Depending on the alignments or phylogenetic analyses, the order can be positioned as if it represents a clade of *Desulfurococcales* (e.g., Itoh 2003; Boyd et al. 2007). The order *Fervidicoccales* was proposed more recently to accommodate slightly acidophilic, moderately thermophilic archaea isolated from terrestrial hot springs (Perevalova et al. 2010). The order *Sulfolobales* is represented by thermoacidophilic, regularly or irregularly coccoid archaea found in terrestrial

Table 9.1 List of thermophilic archaeal genera

Genus	Species number	Temp. range (°C)	Temp. optimum (°C)	pH optimum
Phylum *Crenarchaeota* – Class *Thermoprotei* – Order *Thermoproteales* – Family *Thermoproteaceae*				
Thermoproteus	3	74–102	85–90	5.0–6.8
Caldivirga	1	60–92	85	3.7–4.2
Pyrobaculum	6	68–104	90–102	6.0–7.0
Thermocladium	1	45–82	75	40
Vulcanisaeta	2	65–99	85–90	4.0–4.5
Phylum *Crenarchaeota* – Class *Thermoprotei* – Order *Thermoproteales* – Family *Thermofilaceae*				
Thermofilum	1	<95	85–90	5.0–6.0
Phylum *Crenarchaeota* – Class *Thermoprotei* – Order *Acidilobales* – Family *Acidilobaceae*				
Acidilobus	2	60–92	80–85	3.5–4.0
Phylum *Crenarchaeota* – Class *Thermoprotei* – Order *Acidilobales* – Family *Caldisphaeraceae*				
Caldisphaera	1	45–80	70–78	3.5–4.5
Phylum *Crenarchaeota* – Class *Thermoprotei* – Order *Desulfurococcales* – Family *Desulfurococcaceae*				
Desulfurococcus	5	63–97	80–92	6.0–6.5
Aeropyrum	2	70–100	95–95	7.0–8.0
Ignicoccus	3	70–98	90	5.5–6.0
Ignisphaera	1	85–98	92–95	6.4
Staphylothermus	2	70–98	85–98	6.0–6.5
Stetteria	1	70–102	95	6.0
Sulphobococcus	1	70–95	85	7.5
Thermodiscus	1	75–98	90	5.0–7.0
Thermosphaera	1	65–90	85	6.5
Phylum *Crenarchaeota* – Class *Thermoprotei* – Order *Desulfurococcales* – Family *Pyrodictiaceae*				
Pyrodictium	3	80–110	97–105	5.5
Hyperthermus	1	75–108	95–107	7.0
Pyrolobus	1	90–113	106	5.5
Phylum *Crenarchaeota* – Class *Thermoprotei* – Order *Fervidococcales* – Family *Fervidococcaceae*				
Fervidococcus	1	55–85	65–70	5.5–6.0
Phylum *Crenarchaeota* – Class *Thermoprotei* – Order *Sulfolobales* – Family *Sulfolobaceae*				
Sulfolobus	6	50–95	65–80	2.0–4.0
Acidianus	4	45–96	70–90	1.5–2.5
Metallosphaera	4	50–80	65–75	3.0–3.5
Stygiolobus	1	57–89	80	2.5–3.0
Sulfurisphaera	1	63–92	84	2.0
Sulfurococcus	2	40–85	60–75	2.0–2.6
Phylum *Euryarchaeota* – Class *Methanobacteia* – Order *Methanobacteriales* – Family *Methanobacteriaceae*				
Methanothermobacter	6	37–75	55–70	7.0–8.0
Phylum *Euryarchaeota* – Class *Methanobacteia* – Order *Methanobacteriales* – Family *Methanothermaceae*				
Methanothermus	2	<97	83–88	6.5
Phylum *Euryarchaeota* – Class *Methanococci* – Order *Methanococcales* – Family *Methanococcaceae*				
Methanothermococcus	2	17–75	60–65	5.1–7.0
Phylum *Euryarchaeota* – Class *Methanococci* – Order *Methanococcales* – Family *Methanocaldococcaceae*				
Methanocaldococcus	6	48–92	80–85	6.0–6.5
Methanotorris	2	45–91	75–88	5.7–6.7

(continued)

Table 9.1 (continued)

Genus	Species number	Temp. range (°C)	Temp. optimum (°C)	pH optimum
Phylum *Euryarchaeota* – Class *Methanomicrobia* – Order *Methanomicrobiales* – Family *Methanomicrobiaceae*				
Methanoculleus	1 (7)	37–65	55	7.0
Phylum *Euryarchaeota* – Class *Methanomicrobia* – Order *Methanomicrobiales* – Incertae sedis				
Methanolinea	1	35–55	50	7.0
Phylum *Euryarchaeota* – Class *Methanomicrobia* – Order *Methanosarcinales* – Family *Methanosarcinaceae*				
Methanosarcina	1 (10)		50	6.0–7.0
Methanomethylovorans	1 (2)	42–58	50	6.5
Phylum *Euryarchaeota* – Class *Methanomicrobia* – Order *Methanosarcinales* – Family *Methanosaetaceae*				
Methanosaeta	1 (3)	≤70	55–65	6.7
Phylum *Euryarchaeota* – Class *Methanomicrobia* – Order *Methanosarcinales* – Family *Methermicoccaceae*				
Methermicoccus	1	<70	65	6.0–6.5
Phylum *Euryarchaeota* – Class *Halobacteria* – Order *Halobacteriales* – Family *Halobacteriaceae*				
Haloarcula	1 (9)	>55	53	6.5–7.0
Haloferax	3 (11)	≤55	42–53	7.3–7.4
Halorhabdus	1 (2)	17–55	50	6.7–7.1
Halorubrum	2 (25)	≤56	50	7.0–7.5
Haloterrigena	2 (9)	37–60	50	7.0–7.5
Natrialba	1 (6)	20–55	50	9.0
Phylum *Euryarchaeota* – Class *Thermoplasmata* – Order *Thermoplasmatales* – Family *Thermoplasmataceae*				
Thermoplasma	2	33–67	59–60	1.0–2.0
Phylum *Euryarchaeota* – Class *Thermoplasmata* – Order *Thermoplasmatales* – Family *Ferroplasmaceae*				
Acidiplasma	2	15–65	45–54	1.0–1.6
Phylum *Euryarchaeota* – Class *Thermoplasmata* – Order *Thermoplasmatales* – Family *Picrophilaceae*				
Picrophilus	2	45–65	60	0.7
Phylum *Euryarchaeota* – Class *Thermoplasmata* – Order *Thermoplasmatales* – Incertae sedis				
Thermogymnomonas	1	38–68	60	3.0
Phylum *Euryarchaeota* – Class *Thermococci* – Order *Thermococcales* – Family *Thermococcaceae*				
Thermococcus	27	40–103	75–93	5.8–9.0
Palaeococcus	2	45–88	80–83	6.0–6.5
Pyrococcus	5	70–108	95–103	6.0–8.0
Phylum *Euryarchaeota* – Class *Archaeoglobi* – Order *Archaeoglobales* – Family *Archaeoglobaceae*				
Archaeoglobus	5	60–95	70–83	6.0–7.0
Ferroglobus	1	65–95	85	7.0
Geoglobus	2	50–90	81–88	6.8–7.0
Phylum *Euryarchaeota* – Class *Methanopyri* – Order *Methanopyrales* – Family *Methanopyraceae*				
Methanopyrus	1	84–122[a]	98–105	6.3–6.6

Only genera and species having standing in the prokaryote nomenclature as of June 2012 are shown. The number of total species is shown in parenthesis in case that the genera comprise both thermophilic and non-thermophilic species. For more information of the genus or species, see Bergey's Manual of Systematic Bacteriology, 2nd ed., vol. 1 (2001), vol. 3 (2009) and the following website: List of Prokaryotic names with Standing in Nomenclature (http://www.bacterio.cict.fr/index.html: maintained by J. P. Euzéby); Taxonomy Browser (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Root: maintained by NCBI); Bergey's Taxonomic Outlines (http://www.bergeys.org/outlines.html: maintained by Bergey's Manual Trust)

[a]Pressurized at 0.4 MPa

geothermal habitats. They are aerobic, facultative aerobic or strictly anaerobic depending on the genera, although such differentiation is not supported by the phylogenetic analyses (Fuchs et al. 1996). On the comparison of the protein content for the genome-sequenced archaea, *Pyrobaculum aerophilum*, *Aeropyrum pernix*, and *Sulfolobus* spp. (representing *Thermoproteales*, *Desulfurococcales*, and *Sulfolobales*, respectively), the lineage of *Pyrobaculum* branched earlier than the other two lineages on the phylogenetic tree; besides, *Aeropyrum* and *Sulfolobus* share larger number of proteins in common (Gao and Gupta 2007). Therefore, the orders *Sulfolobales* and *Desulfurococcales* may share common ancestor excluding the order *Thermoproteales*.

The phylum *Euryarchaeota* includes phenotypically diverse group of archaea, such as hyperthermophiles, thermoacidophiles, methanogens, and halophiles. The classes *Thermococci*, *Archaeoglobi*, and *Methanopyri* comprise hyperthermophiles exclusively. The classes *Methanococci* and *Methanobacteria* contain both hyperthermophiles and mesophiles, while the class *Methanomicrobia* does a few moderate thermophiles. Moderate thermophiles and mesophiles exist in the class *Thermoplasmata*. The order *Thermoplasmatales* contains four moderately thermophilic genera as shown in Table 9.1. They thrive in moderately thermal and strongly acidic habitats including self-heating coal refuse piles, acidic hot springs, solfataric fields, and acid mine drainage. All species are aerobic or facultatively anaerobic, and extremely acidophilic. Cells of the genera *Thermoplasma*, *Thermogymnomonas*, and *Acidiplasma* lack an S-layer cell wall, while *Picrophilus* has it. On the 16S rRNA gene-based phylogenetic analysis, it formed a clearly separated lineage which is diverged later from *Methanomicrobia*/*Halobacteria* lineages (Ludwig and Klenk 2001). Several genome-based approaches, however, have it emerged quite early near the base of *Archaea* or *Euryarchaeota* (see Snel et al. 2005). Presumably, this phenomenon is due to a bias by HGT, particularly from members of *Sulfolobales* of the phylum *Crenarchaeota* which share similar thermoacidophilic habitats with the order *Thermoplasmatales* (Ruepp et al. 2000). The genome analyses based on the ribosomal proteins, which are relatively immune to HGT, support the late emergence of the order *Thermoplasmatales* (Matte-Tailliez et al. 2002). It should be mentioned that another thermoacidophilic archaeon "*Aciduliprofundum boonei*," representing an independent cluster (known as DHVE2) but relatively close to the class *Thermoplasmata*, has been isolated from deep-sea hydrothermal vents (Reysenbach et al. 2006). It was a strict anaerobe using elemental sulfur or ferric iron as an electron acceptor and growing optimally at 70 °C and pH 4.5. The class *Thermococci* comprises a single order and family as shown in Table 9.1. They are heterotrophic cocci utilizing proteinaceous substances as carbon and energy sources. The *Pyrococcus* species have higher growth temperature optima (≥95 °C) than the other two genera. Most species were isolated from marine geothermal habitats, while a few *Thermococcus* species from terrestrial hot springs or a deep oil reservoir. In the 16S rRNA gene phylogenetic tree, members of the genera *Thermococcus* and *Pyrococcus* are rather closely related to each other, while the genus *Palaeococcus* is separated from the two genera. At present, the genus *Thermococcus* comprises 27 species which are separated into at least three clades, represented by *T. litoralis*, *T. barophilus*, and *T. celer* (Itoh 2003). The class *Thermococci* diverges from the

deep position of the euryarchaeal lineage by the small subunit (SSU) rRNA gene phylogeny or most of the genome phylogenies. The class *Archaeoglobi* houses three genera in a single family *Archaeoglobaceae* (Table 9.1). All species in this order are hyperthermophilic, regular or irregular cocci isolated from marine hydrothermal vents or subterrestrial oil reservoirs. For the *Archaeoglobi* species, sulfur is not utilized as an electron acceptor or even inhibitory, unlike most of the hyperthermophiles of the phylum *Crenarchaeota* or the class *Thermococci*. *Archaeoglobus* uses sulfate, sulfite, or thiosulfate as electron acceptor. *Ferroglobus* oxidizes Fe(II), while *Geoglobus* strain reduces Fe(III). Some *Archaeoglobus* strains produce methane weakly. *Archaeoglobus* had been regarded as a deeply branching lineage between *Thermococcus* and *Methanococcus* by the 16S rRNA sequence analysis; however, this topology turned out to be an artifact derived from the GC bias of the sequences (Woese et al. 1991). Based on the transversion analysis of the 16S and 23S rRNA sequences, it was replaced in the *Methanomicrobiales-Methanosarcinales-Halobacteriales* cluster. Such placement of *Archaeoglobus* is supported by several phylogenetic analyses based on the genome sequences (e.g., Matte-Tailliez et al. 2002; Gao and Gupta 2007) Thermophilic methanogens distribute each of methanoarchaeal orders, while hyperthermophilic methanogens are restricted to the orders of *Methanopyrales*, *Methanobacteriales*, and *Methaonococcales*. *Methanopyrus kandleri* is the sole member of the class *Methanopyri*; it is a rod-shaped hyperthermophilic methanogen isolated from a hydrothermal vent system of deep seas. Members of *Methanobacteriales* are rod-shaped methanogens, except for *Methanosphaera*, and extant cultivable species are isolated from terrestrial habitats. The SSU rRNA phylogeny supports monophyly of the respective three orders; however, a phylogenetic approach based on ribosomal proteins cluster them together, terming Class I methanogens (Bapteste et al. 2005). According to the 16S rRNA gene phylogenetic tree, the lineage of *Methanopyrus kandleri* is branched at the deepest position of the euryarchaeotic clade, while the gene content analysis of *Methanopyrus kandleri* revealed that it was rather clustered with the other genome-sequenced *Methanothermobacter thermautotrophicus* and *Methanocaldococcus jannaschii* (Slesarev et al. 2002). Brochier et al. (2004) point out that the evolutionary rate of transcription protein dataset (containing RNA polymerase subunits and transcriptional factors) is much higher than that of translational datasets based on ribosomal proteins, yielding the phylogenetic tree in which *Methanopyrus kandleri* is diverged at the base of the phylum *Euryarchaeota*. With the latter dataset, *Methanopyrus* is grouped together with *Methanococcales* and *Methanobacteriales*. The phylogenetic analysis on the fusion of 53 ribosomal proteins (Bapteste et al. 2005), as well as the gene content analysis based on arCOGs (Makarova et al. 2007), also favor the *Methanopyrus kandleri* lineage clustered with those of *Methanococcales* and *Methanobacteriales*. The comparison of the genomes between hyperthermophilic *Methanocaldococcus jannaschii* and mesophilic *Methanococcus maripaludis* revealed that approximately two-thirds of ORFs were conserved in both genomes and many pathways and functions were held in common (Hendrickson et al. 2004). In contrast, reverse gyrase was observed in *Methanocaldococcus jannaschii* only. Besides, tungsten-containing formylmethanofuran dehydrogenase is found in

Methanocaldococcus jannaschii, while molybdenum-containing formylmethanofuran dehydrogenase in *Methanococcus maripaludis*; the dependency of molybdenum and tungsten at different growth temperatures is in agreement with the cases observed in the other methanogenic archaea (Hochheimer et al. 1998).

The existence of *Korarchaeota* was predicted in 1994 by means of the environmental 16S rRNA gene survey from hot springs in Yellowstone National Park (YNP; Barns et al. 1994, 1996). The 16S rRNA sequences of the *Korarchaeota* clones predicted that they were placed in a deep-branching lineage other than the *Crenarchaeota* and *Euryarchaeota*. More recently, the genome sequence of "*Candidatus* Korarchaeota cryptofilum" which was maintained in a mixed community of hyperthermophiles from an YNP hot spring was determined (Elkins et al. 2008). The phylogenetic analyses based on combined small and large rRNA subunits, conserved single-gene, and conserved concatenated proteins illustrate that "*Candidatus* Korarchaeota cryptofilum" sit in a deep archaeal lineage, yet had an affinity to *Crenarchaeota*. Nevertheless, the gene content analysis revealed that several cellular systems, such as cell division, DNA replication, tRNA maturation, are more like those of *Euryarchaeota*. Thus, the *Korarchaeota* lineage might have retained a set of archaeal cellular features existed before the bifurcation to the *Crenarchaeota* and *Euryarchaeota* lineages.

The provisional phylum *Nanoarchaeota* was found as the first obligately parasitic, hyperthermophilic archaea attaching to cell surface of the specific host archaeon *Ignicoccus* thriving in the deep-sea hydrothermal vent (Huber et al. 2002). Cells of the sole species described, "*Nanoarchaeum equitans*," was spherical ranging only about 400 nm in diameter, and its genome size was only 490,855 bp, which was at least three times smaller than any other archaeal genomes. This genome encodes the machinery for information processing and repair but lacks many of metabolic genes, like ones for biosynthesis of lipids, cofactors, amino acids, and nucleotides (Waters et al. 2003). The phylogenetic analyses derived from the 16S rRNA genes or ribosomal proteins revealed that it diverged early in the archaeal lineage (Hohn et al. 2002; Waters et al. 2003). However, Brochier et al. (2005) tested the placement of *Nanoarchaeota equitans* using a dataset of concatenated ribosomal proteins and claimed that the deep origin of *Nanoarchaeota* in the archaeal lineage may be biased by the coupled effect of its fast evolutionary rate and lateral gene transfers. They suggested that *Nanoarchaeota* possibly related to *Thermococcales* in the phylum *Euryarchaeota*. The gene content analysis based on the arCOG was also compatible with this hypothesis (Makarova et al. 2007). However, another phylogenetic analysis based on ribosomal proteins and unique conserved proteins, chosen from arCOG by the phylogenetic profiles, favored the early divergence of *Nanoarchaeota* (Csúrös and Miklós 2009). Thus, the phylogenetic position of the phylum *Nanoarchaeota* remains an open question.

Based on the initial 16S rRNA gene sequence analyses, it had been demonstrated that hyperthermophilic member of the phylum *Crenarchaeota* and certain non-thermophilic archaeal lineages, known as Group I archaea, detected from temperate and cold environments such as open oceans and forest soils shared the base on the phylogenetic trees (DeLong 1992; Schleper 2005). However, the genomic analyses of

"*Candidatus* Cenarchaeum symbiosum" as well as two other marine ammonia-oxidizing archaea representing the non-thermophilic *Crenarchaeota* revealed that they are different from the hyperthermophilic *Crenarchaeota* members, being included in the provisional phylum called "*Thaumarchaeota*" (Brochier-Armanet et al. 2008; Spang et al. 2010). The provisional phylum may include certain thermophiles such as ammonia oxidizing "*Candidatus* Nitrosocaldus yellowstonii" which is maintained in a thermophilic enrichment culture at 72 °C (de la Torre et al. 2008). On the other hand, another provisional phylum, *"Aigarchaeota,"* is proposed for "*Candidatus* Caldiarchaeum subterraneum" that thrives in hot water in a subsurface gold mine (Nunoura et al. 2011). This archaeon is unique in that it has a eukaryotes-like ubiquitin protein modification system; however, it may be regarded as a deeply branching "*Thaumarchaeota*" order by the ribosomal-protein phylogeny and the characteristic gene content (Brochier-Armanet et al. 2011).

9.3.2 Bacteria

Within domain Bacteria, the thermophiles also distribute widely in most of the phyla; however, abundance of the thermophiles varies from phylum to phylum. Hyperthermophilic or extremely thermophilic species occur predominantly in the phyla *Aquificae* and *Thermotogae* representing the deep-branching groups on the basis of the 16S rRNA gene phylogeny. Still the thermophiles occupy portions of major clusters in several phyla, such as *Firmicutes* and *Chloroflexi*, whereas only a handful of moderate thermophiles occur sporadically or exceptionally in other phyla, like *Actinobacteria* and *Bacteroidetes*. Distribution of the thermophiles with nomenclatural standings in the domain *Bacteria* is listed in Table 9.2.

Members of the phylum *Aquificae* grow chemolithotrophically oxidizing H_2 under microaerophilic growth conditions. Species of the genus *Aquifex*, including the genome-sequenced strain "*Aquifex aeolicus*," are hyperthermophilic, while the other members are extremely thermophilic or moderately thermophilic. Molecular signatures as revealed by "indel" (inserts and deletions) in four widely distributed proteins are congruent with the phylogeny based on the 16S rRNA gene (Griffiths and Gupta 2006). By the phylogenetic analysis on the 16S rRNA gene sequence, it revealed the deep phylogeny being separated from the other hyperthermophilic bacterium *Thermotoga maritima*, in the bacterial domain; however, such placement is not supported by the phylogenetic analyses of the other genes (Deckert et al. 1998). A recent concatenated informational protein reveals that the phylum *Aquificae* represented by "*Aquifex aeolicus*" could be placed as a neighbor to the phylum *Thermotogae* or the class *Epsilonproteobacteria* as an alternative possibility (Boussau et al. 2008). The phylum *Thermotogae* comprises hyperthermophilic and thermophilic bacteria, growing optimally from 45 to 80 °C, represented by eight genera as shown in Table 9.2. They are fermentative strictly anaerobic bacteria, and cells are encapsulated in an outer sheath-like envelope that may balloon at the cell ends ("toga"). They distribute widely in volcanically or geothermally heated environments, such as marine hydrothermal

Table 9.2 List of thermophilic bacterial genera

Genus	Species number	Temp. range (°C)	Temp. optimum (°C)	pH optimum
Phylum *Aquificae* – Class *Aquificae* – Order *Aquificales* – Family *Aquificaceae*				
Aquifex	1	67–95	85	6.8
Hydrogenivirga	2	55–85	70–75	6.5–7.5
Hydrogenobaculum	1		65	3.0–4.0
Hydrogenobacter	3	60–85	70–78	7.5
Thermocrinis	3	44–89	75–85	5.9–7.0
Phylum *Aquificae* – Class *Aquificae* – Order *Aquificales* – Family *Desulfobacteraceae*				
Desulfurobacterium	3	40–85	70–75	6.0–6.2
Balnearium	1	45–80	70–75	5.4
Thermovibrio	3	50–88	75–80	5.5–6.2
Phylum *Aquificae* – Class *Aquificae* – Order *Aquificales* – Family *Hydrogenothermaceae*				
Hydrogenothermus	1	45–80	65	7.0
Persephonella	3	50–80	70–73	6.0–7.2
Sulfurihydrogenibium	5	40–80	60–75	6.0–7.5
Venenivibrio	1	45–75	70	5.4
Phylum *Aquificae* – Class *Aquificae* – Order *Aquificales* – Incertae sedis				
Thermosulfidibacter	1	55–78	70	5.5–6.0
Phylum *Thermotogae* – Class *Thermotogae* – Order *Thermotogales* – Family *Thermotogaceae*				
Thermotoga	9	47–90	65–80	7.0–7.5
Defluviitoga	1	37–65	55	6.9
Fervidobacterium	6	41–90	65–80	6.5–7.8
Geotoga	2	30–60	45–50	6.5
Kosmotoga	2	20–80	60–65	6.8–7.1
Marinitoga	5	25–70	55–65	5.5–7.0
Oceanotoga	1	25–70	55–58	7.3–7.8
Petrotoga	6	25–65	55–60	6.0–7.5
Thermococcoides	1	45–75	65	7.0
Thermosipho	7	35–80	65–75	6.0–7.6
Phylum *Thermodesulfobacteria* – Class *Thermodesulfobacteria* – Order *Thermodesulfobacteriales* – Family *Thermodesulfobacteriaceae*				
Thermodesulfobacterium	5	45–85	65–75	6.5–7.0
Caldimicrobium	1	52–82	75	7.0–7.2
Thermodesulfatator	2	55–80	65–70	6.3–7.5
Phylum *Deinococcus- Thermus* – Class *Deinococci* – Order *Thermales* – Family *Thermaceae*				
Thermus	10	37–85	65–72	6.0–8.5
Marinithermus	1	50–73	68	7.0
Meiothermus	10	30–70	50–60	7.5–8.5
Oceanithermus	2	30–68	60	6.5–7.5
Rhabdothermus	1	37–75	65	7.3
Vulcanithermus	1	37–80	70	6.7
Phylum *Chloroflexi* – Class *Chloroflexi* – Order *Chloroflexales* – Family *Chloroflexaceae*				
Chloroflexus	2	40–66	52–60	7.6–8.4
Roseiflexus	1		50	7.5–8.0

(continued)

Table 9.2 (continued)

Genus	Species number	Temp. range (°C)	Temp. optimum (°C)	pH optimum
Phylum *Chloroflexi* – Class *Anaerolineae* – Order *Anaerolineales* – Family *Anaerolineaceae*				
Anaerolinea	2	42–60	50–55	7.0
Belilinea	1	45–65	55	7.0
Phylum *Chloroflexi* – Class *Caldilineae* – Order *Caldilineales* – Family *Caldilineaceae*				
Caldilinea	2	37–65	55	7.0–8.5
Phylum *Chloroflexi* – Class *Ktedonobacteria* – Order *Ktedonobacterales* – Family *Thermosporotrichaceae*				
Thermosporothrix	1	31–58	50	7.0
Phylum *Chloroflexi* – Class *Ktedonobacteria* – Order *Thermogemmatisporales* – Family *Thermogemmatisporaceae*				
Thermogemmatispora	2	45–74	60–65	7.0
Phylum *Chloroflexi* – Subclass *Sphaerobacteridae* – Order *Sphaerobacterales* – Suborder *Sphaerobacterineae* – Family *Sphaerobacteraceae*				
Sphaerobacter	1		50–60	8.5
Phylum *Chloroflexi* – Class *Thermomicrobia* – Order *Thermomicrobiales* – Family *Thermomicrobiaceae*				
Thermomicrobium	1	≤85	70–75	8.2–8.5
Phylum *Nitrospirae* – Class *Nitrospira* – Order *Nitrospirales* – Family *Nitrospiraceae*				
Thermodesulfovibrio	5	40–70	55–65	6.5–7.5
Phylum *Deferribacteres* – Class *Deferribacteres* – Order *Deferribacterales* – Family *Deferribacteraceae*				
Deferribacter	4	25–75	60–65	6.5–6.7
Calditerrivibrio	1	30–65	55	7.0–7.5
Phylum *Proteobacteria* – Class *Alphaproteobacteria* – Order *Rhizobiales* – Family *Hyphomicrobiaceae*				
Dichotomicrobium	1	20–65	44–50	8.0–8.5
Phylum *Proteobacteria* – Class *Alphaproteobacteria* – Order *Rhizobiales* – Family *Rhodobiaceae*				
Tepidamorphus	1		45–50	7.5–8.5
Phylum *Proteobacteria* – Class *Betaproteobacteria* – Order *Burkholderiales* – Family *Burkholderiaceae*				
Thermothrix	2	60–87	73–78	7.0–7.5
Phylum *Proteobacteria* – Class *Betaproteobacteria* – Order *Burkholderiales* – Family *Comamonadaceae*				
Caldimonas	2		50–55	7.0–8.0
Phylum *Proteobacteria* – Class *Betaproteobacteria* – Order *Burkholderiales* – Incertae sedis				
Tepidimonas	4	<65	50–55	7.0–8.5
Thiomonas	1 (8)	34–65	50–53	5.2–5.6
Thiobacter	1	35–62	50–55	6.5–7.0
Phylum *Proteobacteria* – Class *Betaproteobacteria* – Order *Hydrogenophilales* – Family *Hydrogenophilaceae*				
Hydrogenophilus	3	35–67	50–63	6.5–7.0
Tepidiphilus	1	<61	50	

(continued)

Table 9.2 (continued)

Genus	Species number	Temp. range (°C)	Temp. optimum (°C)	pH optimum
Phylum *Proteobacteria* – Class *Gammaproteobacteria* – Order *Chromatiales* – Family *Chromatiaceae*				
Thermochromatium	1	<57	48–50	7.0
Phylum *Proteobacteria* – Class *Gammaproteobacteria* – Order *Chromatiales* – Family *Thioalkalipiraceae*				
Thioprofundum	1 (2)	30–55	50	7.0
Phylum *Proteobacteria* – Class *Gammaproteobacteria* – Order *Xanthomonadales* – Family *Xanthomonadaceae*				
Thermomonas	2 (5)	18–60	37–50	
Phylum *Proteobacteria* – Class *Gammaproteobacteria* – Order *Methylococcales* – Family *Methylococcaceae*				
Methylococcus	1 (4)		45–50	6.5–7.0
Methylocaldum	1 (3)	37–62	55	
Methylothermus	2	36–67	55–60	5.8–6.8
Phylum *Proteobacteria* – Class *Deltaproteobacteria* – Order *Desulfovibrionales* – Family *Desulfohalobiaceae*				
Desulfothermus	2	35–65	50–65	5.9–6.8
Desulfonauticus	2	30–64	45–58	7.0–7.8
Phylum *Proteobacteria* – Class *Deltaproteobacteria* – Order *Desulfuromonadales* – Family *Geobacteraceae*				
Geothermobacter	1	35–65	55	
Phylum *Proteobacteria* – Class *Deltaproteobacteria* – Order *Desulfurellales* – Family *Desulfurellaceae*				
Desulfurella	4	33–77	52–60	6.4–7.2
Hippea	3	40–75	53–65	4.5–6.5
Phylum *Proteobacteria* – Class *Deltaproteobacteria* – Order *Syntrophobacterales* – Family *Syntrophobacteraceae*				
Desulfacinum	2	37–65	60	7.1–7.5
Desulfosoma	1	50–62	57	6.8
Thermodesulforhabdus	1	44–74	60	6.9
Phylum *Proteobacteria* – Class *Epsilonproteobacteria* – Order *Campylobacterales* – Family *Hydrogenimonaceae*				
Hydrogenimonas	1	35–65	55	5.9
Phylum *Proteobacteria* – Class *Epsilonproteobacteria* – Order *Nautiliales* – Family *Nautiliaceae*				
Nautilia	4	25–68	40–60	6.0–7.0
Caminibacter	3	45–70	55–60	5.5–7.1
Lebetimonas	1	30–68	50	5.2
Phylum *Firmicutes* – Class *Clostridia* – Order *Clostridiales* – Family *Clostridiaceae*				
Clostridium	14 (177)	22–70	49–65	6.0–10.3
Caloramator	7	37–80	50–68	6.0–8.2
Caloranaerobacter	1	45–65	65	7.0
Caminicella	1	45–65	55–60	7.5–8.0
Fervidicella	1	37–55	50	7.0
Tepidibacter	2 (3)	33–60	45–50	6.0–6.8

(continued)

Table 9.2 (continued)

Genus	Species number	Temp. range (°C)	Temp. optimum (°C)	pH optimum
Tepidimicrobium	2	25–67	50–60	7.5–8.5
Thermobrachium	1	43–75	66	8.2
Thermohalobacter	1	45–70	65	7.0
Phylum *Firmicutes* – Class *Clostridia* – Order *Clostridiales* – Family *Caldicoprobacteraceae*				
Caldicoprobacter	1	44–77	70	7.2
Phylum *Firmicutes* – Class *Clostridia* – Order *Clostridiales* – Family *Defluviitaleaceae*				
Defluviitalea	1	45–60	50	7.0–7.5
Phylum *Firmicutes* – Class *Clostridia* – Order *Clostridiales* – Family *Eubacteriaceae*				
Garciella	1	25–60	55	7.5
Phylum *Firmicutes* – Class *Clostridia* – Order *Clostridiales* – Family *Heliobacteriaceae*				
Heliobacterium	1 (5)	25–56	52	6.0–7.0
Phylum *Firmicutes* – Class *Clostridia* – Order *Clostridiales* – Family *Peptococcaceae*				
Desulfotomaculum	15 (27)		50–66	
Pelotomaculum	1 (5)	45–65	55	7.0
Thermincola	2	37–70	55–60	7.0–8.0
Phylum *Firmicutes* – Class *Clostridia* – Order *Clostridiales* – Family *Syntrophomonadaceae*				
Syntrophothermus	1	45–60	55	6.5–7.0
Thermohydrogenium	1	45–75	65	7.0–7.4
Thermosyntropha	2	50–70	60–66	8.1–8.9
Thermovirga	1	37–68	58	6.5–7.0
Phylum *Firmicutes* – Class *Clostridia* – Order *Clostridiales* – Incertae sedis				
Anaerobaculum	3	28–65	55–60	7.0–7.6
Anaerobranca	4	30–67	50–60	8.5–9.5
Thermanaerovibrio	2	40–70	55–65	6.5–8.1
Sulfobacillus	3 (5)	17–60	45–60	1.7–2.0
Symbiobacterium	1	45–65	60	7.5
Thermaerobacter	5	50–80	70–75	7.0–8.5
Phylum *Firmicutes* – Class *Clostridia* – Order *Halanaerobiales* – Family *Halanaerobiaceae*				
Halothermothrix	1	45–68	60	6.5–7.0
Phylum *Firmicutes* – Class *Clostridia* – Order *Natranaerobiales* – Family *Natranaerobiaceae*				
Natranaerobius	2	26–56	52–53	9.5
Natronovirga	1	<58	51	9.9
Phylum *Firmicutes* – Class *Clostridia* – Order *Thermoanaerobacterales* – Family *Thermoanaerobacteraceae*				
Thermoanaerobacter	14	30–85	55–75	5.8–8.5
Ammonifex	2	57–82	70–75	6.8–7.5
Caldanaerobacter	2	40–85	65–75	6.5–7.5
Caldanaerobius	3	40–72	60–68	6.8
Carboxydothermus	4	48–78	65–72	5.5–7.2
Fervicola	1	55–80	70	7.0
Gelria	1	37–60	50–55	7.0
Moorella	5	42–70	56–65	5.5–8.5
Tepidanaerobacter	1	25–60	45–50	6.0–7.0

(continued)

Table 9.2 (continued)

Genus	Species number	Temp. range (°C)	Temp. optimum (°C)	pH optimum
Thermacetogenium	1	40–65	58	6.8
Thermanaeromonas	1	55–73	70	6.5
Phylum *Firmicutes* – Class *Clostridia* – Order *Thermoanaerobacterales* – Family *Thermodesulfobiaceae*				
Thermodesulfobium	1	37–65	50–55	5.5–6.0
Caldicellulosiruptor	8	45–83	65–82	7.0–7.5
Coprothermobacter	2	35–70	55–70	6.8–7.5
Phylum *Firmicutes* – Class *Clostridia* – Order *Thermoanaerobacterales* – Incertae sedis				
Caldanaerovirga	1	44–74	62–66	8.4
Carboxydocella	3	26–78	58–60	6.5–7.0
Mahella	1	30–60	50–65	7.5
Thermoanaerobacterium	7	35–72	55–70	5.2–7.8
Thermosediminibacter	2	43–76	64–68	7.5–8.4
Thermovenabulbum	2	45–76	63–65	6.7–7.0
Thermovorax	1	50–81	71	7.0–7.5
Phylum *Firmicutes* – Class *Bacilli* – Order *Bacillales* – Family *Bacillaceae*				
Bacillus	11 (190)	<80	50–70	4.2–8.0
Aeribacillus	1		50–70	60
Anoxybacillus	16	30–75	50–66	6.0–9.7
Caldalkalibacillus	2	42–65	50–60	8.2–8.5
Calditerricola	2	56–83	72–78	7.0–7.5
Cerasibacillus	1	30–55	50	8.0–9.0
Geobacillus	16	35–75	55–70	6.2–8.0
Microaerobacter	1	37–70	55	6.5–7.0
Saccharococcus	1	≥78	68–70	
Vulcanibacillus	1	37–60	55	7.0
Phylum *Firmicutes* – Class *Bacilli* – Order *Bacillales* – Family *Alicyclobacillaceae*				
Alicyclobacillus	15 (20)	20–70	42–65	1.5–5.5
Phylum *Firmicutes* – Class *Bacilli* – Order *Bacillales* – Family *Paenibacillaceae*				
Thermobacillus	2	32–63	50–55	7.8–9.0
Brevibacillus	1 (17)	35–65	55	7.0
Phylum *Firmicutes* – Class *Bacilli* – Order *Bacillales* – Family *Planococcaceae*				
Ureibacillus	5		50–55	7.0–8.0
Phylum *Firmicutes* – Class *Bacilli* – Order *Bacillales* – Family *Thermoactinomycetaceae*				
Thermoactinomyces	2		50–55	
Laceyella	4	28–70	48–55	
Planifilum	3	50–75	55–70	
Thermoflavimicrobium	1		55	
Phylum *Firmicutes* – Class *Bacilli* – Order *Bacillales* – Incertae sedis				
Thermicanus	1		55–60	6.5–7.0
Phylum *Firmicutes* – Class *Negaivicutes* – Order *Selenomonadales* – Family *Veillonellaceae*				
Thermosinus	1	40–68	60	6.8–7.0

(continued)

Table 9.2 (continued)

Genus	Species number	Temp. range (°C)	Temp. optimum (°C)	pH optimum
Phylum *Firmicutes* – Class *Thermolithobacteria* – Order *Thermolithobacterales* – Family *Thermolithobacteraceae*				
Thermolithobacter	2	40–78	70–73	6.8–7.3
Phylum *Actinobacteria* – Class *Actinobacteria* – Subclass *Acidimicrobidae* – Order *Acidimicrobiales* – Suborder *Acidimicrobineae* – Family *Acidimicrobiaceae*				
Acidimicrobium	1		45–50	2.0
Phylum *Actinobacteria* – Class *Actinobacteria* – Subclass *Acidimicrobidae* – Order *Acidimicrobiales* – Suborder *Acidimicrobineae* – Incertae sedis				
Aciditerrimonas	1	35–58	50	3.0
Phylum *Actinobacteria* – Class *Actinobacteria* – Subclass *Rubrobacteridae* – Order *Rubrobacterales* – Suborder *Rubrobacterineae* – Family *Rubrobacteraceae*				
Rubrobacter	3	30–70	45–60	7.5–8.0
Phylum *Actinobacteria* – Class *Actinobacteria* – Subclass *Rubrobacteridae* – Order *Thermoleophilales* – Family *Thermoleophilaceae*				
Thermoleophilum	2	45–70	60	6.5–7.5
Phylum *Actinobacteria* – Class *Actinobacteria* – Subclass *Actinobacteridae* – Order *Actinomycetales* Suborder *Streptosporangineae* – Family *Thermomonosporaceae*				
Thermomonospora	2		40–50	7.5–11.0
Phylum *Actinobacteria* – Class *Actinobacteria* – Subclass *Actinobacteridae* – Order *Actinomycetales* Suborder *Streptosporangineae* – Family *Streptosporangiaceae*				
Thermopolyspora	1	40–60	45–55	
Phylum *Actinobacteria* – Class *Actinobacteria* – Subclass *Actinobacteridae* – Order *Actinomycetales* Suborder *Propionibacterineae* – Family *Nocardioidaceae*				
Thermasporomyces	1	35–62	50–55	7.0
Phylum *Actinobacteria* – Class *Actinobacteria* – Subclass *Actinobacteridae* – Order *Actinomycetales* Suborder *Pseudonocardineae* – Family *Pseudonocardiaceae*				
Amycolatopsis	2 (51)	10–50	50	
Thermobispora	1	<65	55	
Thermocrispum	2	20–62.5	45–50	
Phylum *Actinobacteria* – Class *Actinobacteria* – Subclass *Actinobacteridae* – Order *Actinomycetales* Suborder *Streptomycineae* – Family *Streptomycetaceae*				
Streptomyces	8 (528)	20–55	50	
Phylum *Actinobacteria* – Class *Actinobacteria* – Subclass *Actinobacteridae* – Order *Actinomycetales* – Suborder *Frankineae* – Family *Acidothermaceae*				
Acidothermus	1	37–65	55	5.0
Phylum *Spirochaetes* – Class *Spirochaetes* – Order *Spirochaetales* – Family *Spirochaetaceae*				
Spirochaeta	2 (18)		48–68	
Phylum *Spirochaetes* – Class *Spirochaetes* – Order *Spirochaetales* – Incertae sedis				
Exilispira	1	37–60	50	7.0
Phylum *Bacteroidetes* – Class *Bacteroidetes* – Order *Bacteroidetales* – Family *Bacteroidaceae*				
Acetothermus	1	<60	58	7.0–7.5
Acetomicrobium	1 (2)		58	
Phylum *Bacteroidetes* – Class *Bacteroidetes* – Order *Bacteroidetales* – Family *Marinilabiaceae*				
Anaerophaga	1	37–55	50	6.7–6.8

(continued)

Table 9.2 (continued)

Genus	Species number	Temp. range (°C)	Temp. optimum (°C)	pH optimum
Phylum *Bacteroidetes* – Class *Bacteroidetes* – Family *Rhodothermaceae*				
Rhodothermus	2	50–85	65–80	7.0
Phylum *Bacteroidetes* – Class *Bacteroidetes* – Incertae sedis				
Thermonema	2	35–70	60	6.5–7.5
Phylum *Bacteroidetes* – Family *Schleiferiaceae*				
Schleiferia	1	30–60	50	7.5–8.5
Phylum *Caldiserica* – Class *Caldisericia* – Order *Caldisericales* – Family *Caldisericaceae*				
Caldisericum	1	55–70	65	6.5
Phylum *Armatimonadetes* – Class *Chthonomonadetes* – Order *Chthonomonadales* – Family *Chthonomonadaceae*				
Chthonomonas	1	55–73	68	5.3
Phylum *Dictyoglomi* – Class *Dictyoglomia* – Order *Dictyoglomiales* – Family *Dictyoglomaceae*				
Dictyoglomus	2	50–86	72–78	7.0–7.2
Unknown affiliation				
Caldithrix[a]	2	40–70	60	6.8–7.0

See the footnote of Table 9.1

[a]According to the 16S rRNA gene phylogeny, the representatives of the genus *Caldithrix* are most closely related to those of the genus *Deferribacter*, although the similarities are very low (Miroshnichenko et al. 2010)

systems, terrestrial hot springs, and oil wells. Owing to the 16S rRNA gene-based phylogenetic analysis, it was thought to position in the deepest phylogenetic branch, along with another hyperthermophilic bacterium *Aquifex*, within the bacterial domain (Stetter 1995). However, the comparative analysis of the *Thermotoga maritima* genome sequence suggested that the highest percentage (24 %) of the genes were most similar to archaeal genes, indicating HGT may have occurred between the two domains (Nealson et al. 1999). Besides, the placement of the deep-branching lineage predicted by the 16S rRNA gene phylogeny is not supported by the genome-based phylogeny. For instance, phylogenetic analysis of concatenated 31 universal protein families placed *Thermotoga maritima* clustered with *Aquifex aeolicus* (Ciccarelli et al. 2006), while the gene content analysis put it next to *Caldanaerobacter tengcongensis* (formerly *Thermoanaerobacter tengcongensis*) in the phylum Firmicutes (Yang et al. 2005). By analyzing four additional *Thermotogales* members (i.e., *Thermotoga petrophila*, *Thermotoga lettingae*, *Thermosipho melanesiensis*, and *Fervidobacterium nodosum*), Zhaxybayeva et al. (2009) showed that the ribosomal proteins placed this order as a sister group to *Aquificae*, whereas majority of genes were derived from *Archaea* and *Firmicutes*. The phylum *Thermodesulfobacteria* constitutes only three genera encompassing anaerobic thermophiles reducing sulfate or thiosulfate from marine or terrestrial geothermal habitats. *Thermodesulfobacterium commune* is known to have non-isoprene branched glycerol diethers and monoethers (Langworthy et al. 1983), which are partly analogous to the archaeal glycerolipid. In addition to these validated three genera, "*Geothermobacterium ferrireducens,*" which reduces ferric iron as electron acceptor and has the highest growth temperature range up to 100 °C

(optimally 85–90 °C) within the bacterial domain, is described (Kashefi et al. 2002). Thermophilic species of the tentative phylum *Deinococci-Thermus* are only encountered in the order *Thermales*, represented by six genera as shown in Table 9.2. Species of the genera *Thermus* and *Meiothermus* are aerobic or facultatively anaerobic rods and mostly thrive in terrestrial hot springs. On the other hand, members of the other four genera are aerobic, microaerophilic, or facultatively anaerobic and isolated from marine hydrothermal systems. The phylum *Chloroflexi* is a relatively small group of bacteria, but there are six classes at present. Typical members of the phylum *Chloroflexi* are filamentous Gram-negative bacteria showing gliding mobility and grow phototrophically or heterotrophically. Several members of this phylum show moderate thermophily, and they are generally isolated from hot springs with or without microbial mats. Thermophilic members of the classes *Anaerolineae* and *Cladilineae* are non-photosynthetic, chemoorganotrophic, filamentous anaerobes. The extant strains of the former class were isolated from thermophilic activated sludge. Rod-shaped, extremely thermophilic *Thermomicrobium roseum*, which had been assigned in an independent phylum *Thermomicrobia*, and rod-shaped moderately thermophilic *Sphaerobacter thermophilus*, formerly classified as the deepest branching member of the phylum *Actinobacteria*, were transferred to this phylum as a result of the 16S rRNA gene sequence analysis (Hugenholtz and Stackebrandt 2004). Interestingly, *Thermomicrobium roseum* has a megaplasmid encoding a complete system for the chemotaxis, suggesting its flagellum-based motility has been acquired by HGT (Wu et al. 2009b). In addition, non-validated species "*Thermobaculum terrenum*," which grows optimally at 67 °C, pH 7.0, is revealed to have a close affinity to *Sphaerobacter thermophilus* and *Thermomicrobium roseum* (Kunisawa 2011). The phylum *Firmicutes* is one of the largest groups in the bacterial domain, and all members possess a rigid cell wall. There are a number of thermophilic species distributed, particularly among the families *Bacillaceae*, *Clostridiacea*, and the order *Thermoanaerobacteriales*. On the phylogenetic position of the latest order *Thermoanaerobacteriales*, some uncertainty is pointed out whether it should stay in this phylum or constitute separate novel phyla (Ludwig et al. 2009). The phylum *Actinobacteria* houses a large number of Gram-positive bacteria, but fewer numbers of thermophiles are known. Species of *Rubrobacter* and *Thermoleophilum* grow optimally at 60 °C. The phylum *Proteobacteria* constitutes at present the largest and phenotypically most diverse group of bacteria. Moderately thermophilic species occur sporadically. Among this phylum, two *Thermothrix* species correspond to extreme thermophiles, but their type strains seem to be lost (see List of Prokaryotic names with Standing in Nomenclature, by J. P. Euzéby: http://www.bacterio.cict.fr/). The phylum *Bacteroidetes* covers the so-called *Cytophaga-Flavobacterium-Bacteroides* (CFB) group which contains limited numbers of thermophilic species. Thermophilic genera *Rhodothermus* and *Thermonema* represent the deepest branches in this phylum. Several members of the phyla *Nitrosospira* and *Deferribacteres* are also moderate thermophiles. Within the phylum *Acidobacteria*, no thermophilic species have been isolated and described so far, but establishment of a highly enrichment culture of an aerobic phototrophic "*Candidatus* Chloracidobacterium thermophilum" belonging to this phylum was demonstrated (Bryant et al. 2007). The phylum *Dictyoglomi* is currently represented by a single genus, *Dictyoglomus*, comprising two species isolated from hot springs.

The two phyla *Caldiserica* and *Armatimonadetes* accommodate respective single species, which formerly represented candidate divisions OP5 and OP10, respectively (Hugenholtz et al. 1998). On the other hand, no bacterial phyla have been proposed to accommodate an extremely thermophilic genus *Caldithrix* represented by two species. It is most closely related to the phylum *Deferribacteres*, but the phylogenetic analysis does not support the inclusion of the genus *Caldithrix* in this phylum. There are also several thermophilic species belonging to the phylum *Cyanobacteria*. Although it forms a major lineage in the domain *Bacteria*, nomenclature of the taxa is governed by the *Botanical Code of Nomenclature* rather than the *Bacteriological Code*, and its taxonomic scheme is structured according to phenotypic characteristics rather than the 16S rRNA gene phylogeny (Garrity and Holt 2001). *Mastigocladus laminosus, Synechococcus lividus, Synechococcus vulcanus*, and *Thermosynechococcus elongatus* are known to be thermophilic.

9.3.3 Diversification of the Thermophiles in Geothermal Habitats

As overviewed above, overwhelmingly diverse thermophiles flourish on the Earth, particularly in geothermal habitats. Besides, as predicted by many of culture-independent molecular ecological studies, there are diverse phylogenetic groups of as-yet-cultivated microorganisms in the geothermal environments (e.g., Barns et al. 1994; Hugenholtz et al. 1998; Takai and Horikoshi 1999). Here possible reasons of the wide phylogenetic diversity of the thermophiles are considered below:

Firstly, wide diversity of the geothermal environments exists on Earth (Brugess et al. 2007). In addition to the temperature range, the pH range of the geothermal environments is also broad, from strongly acidic solfataric surface to alkaline carbonate-rich hot spring. Redox potentials are also variable from highly reduced subterrestrial water holes passing volcanic gases to ground surfaces exposed by air. Various geochemical reactions in the geothermal environments provide a variety of electron acceptor/donor substances. Thus, the thermal habitats harbor various physiologically different prokaryotes.

Secondly, the geothermal environments must have prevailed throughout geological history on Earth; thus, at a certain point in history, they may have functioned as one of the oldest habitats for life on Earth (Reysenbach and Cady 2001). Thus, the ancient thermophiles might have evolved generating a variety of descendant lineages during the geological history, and many of them might have survived. This scenario does not imply that LUCA, or ancestral *Archaea* or *Bacteria*, was represented by the thermophiles or hyperthermophiles. One spot of the geothermal habitats may not last so long in the geological time, but they have existed always anywhere on Earth. Thus, the lineages of the thermophiles might have been relieved from extinction due to change of environments. Besides, the descendant lineages might have some capacities to coexist with each other. In addition, island-like nature of the thermal habitats might have promoted the diversification. Verification of the hypothesis could be challenging, but further ecological and genomic analyses of the diverse thermophiles will enhance our understanding of the phylogenetic histories of the thermophiles.

9.4 Future Perspective

At present, the 16S rRNA gene-based systematic scheme seems to be justified as a starting point for pursing the ideal classification based on the natural and evolutional relationships. Some of the phyla or orders defined by the 16S rRNA gene sequence similarity are phenotypically homogenous. Most of comparative genomic analyses supported the classes, phyla, and orders defined by the 16S rRNA gene-based phylogeny. On the other hand, certain phylogenetic issues such as the location of LUCA or the chronological divergence order of the major phyla on the "tree of life" have not reached a scientific consensus. Because the thermophiles are thought to share characteristics with the early life forms, further analyses of the thermophiles would also play pivotal role in the inference of these phylogenetic issues.

In addition, a variety of thermophiles have been serving as potential model organisms that may help understand possible mechanism of genomic diversification or environmental adaptation, which could be driving forces for the microbial evolution. For example, genomic comparison of various "*Sulfolobus islandicus*" isolates from different geographic locations is providing a new insight on the dispersal and diversification of these thermoacidophilic archaea thriving terrestrial hot springs (Reno et al. 2009). Hyperthermophilic bacterium *Thermotoga maritima* and related strains are now considered as a model organism to investigate gene transfer and genomic plasticity (Nesbø et al. 2006). Genomic and proteomic comparison of the hyperthermophilic, moderately or extremely thermophilic, and mesophilic methanogens in the same order (i.e., *Methanobacteriales* and *Methanococcales*) may elucidate the relationships of the temperature adaptation and microbial evolution. Further phylogenetic comparison of a parasitic hyperthermophile *Nanoarchaeum equitans* and the host hyperthermophilic archaeon *Ignicoccus hospitalis* may help understand the effect of parasitism/symbiosis on the microbial evolution.

9.5 Conclusions

The number of the thermophiles isolated has rapidly increased during the last three to four decades, reaching more than 220 genera and 580 species. Even so, we have resolved only a fraction of their diversity and ecological roles in the thermal environments. Therefore, we have to explore and study more diverse thermophiles in thermal environments. Entering the twenty-first century, the genomic analyses become common tools, and computational sciences have been advancing; therefore, our knowledge of phylogenetic history of the thermophiles will be greatly enhanced in the near future.

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Chapter 10
Biology, Biodiversity and Application of Thermophilic Viruses

Kristine Uldahl and Xu Peng

Abstract Where there is life, there are viruses. Thermophilic viruses are essential in regulating the structure and composition of microbial communities in the terrestrial and marine hot environments. Moreover, they constitute an important source of novel enzymes of high biotechnological and industrial potential. This chapter focuses on the application potential of thermophilic viruses. Central to this topic are the many novel genetic and morphological features discovered in thermophilic viruses. In order to fully appreciate the huge potential these viruses have in basic research and biotechnology, we will introduce recent results derived from studies of structural adaptations, functional genomics, metagenomics, virus–host interactions and virus life cycle, all areas with remarkable characteristics. In the end, a section will cover more specific application areas including thermophilic viruses as nano-building blocks.

Keywords Thermophilic viruses • Genomics • Life cycle • Biotechnology • Application

10.1 Introduction

Since the first discovery of microorganisms in the boiling hot springs of Yellowstone National Park by Thomas Brock in 1966, many thermophilic organisms including bacteria, Archaea and fungi have been isolated from different hot environments.

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Where there is life, there are viruses. Viruses infecting thermophilic microorganisms are abundant in terrestrial and marine hot environments and have been isolated from a range of hot environments all over the world, including hydrothermal vents, acidic and neutral hot springs and self-heating organic material such as compost.

Viruses constitute a major component of the biosphere and play a significant role in nutrient and energy cycling of carbon, nitrogen and phosphorus (Krupovic et al. 2011). Moreover, viruses have important impacts on the evolution of their hosts. For example, viruses can influence the genetic diversity of prokaryotes by horizontal gene transfer among different species. As killers of bacteria and Archaea, viruses play an essential role in regulating the structure and composition of microbial communities (Weinbauer and Rassoulzadegan 2004). Importantly, thermophilic viruses can serve as model systems for studying life at high temperatures, and they constitute a valuable source of novel enzymes of high biotechnological and industrial potential.

Viruses are genetic elements that require host resources for their replication. They have single- or double-stranded RNA or DNA genomes ranging in size from a few thousand to a million bases. In order to thrive and multiply, viruses have to transmit from one host to another. When they exit the cell, they have to survive in the natural environment of the host (Madigan and Brock 2009). Thus, viruses have an extracellular form that enables them to exist outside the host for extended periods of time. Thermophilic viruses have adapted to hot environments same as their hosts. Thermophiles refer to organisms that grow at temperatures above 45°C, and if the temperatures are above 80°C, they are called hyperthermophiles. Above 65°C only prokaryotic life forms are found, and Archaea are the more successful domain (Lengeler et al. 1999). Archaea are adapted to conditions of chronic energy stress and have adaptations in membrane composition and metabolic pathways that provide a competitive advantage in a range of extreme environments (Valentine 2007). Although both Archaea and bacteria exist in hot environments, there is a trend that more Archaea and fewer bacteria are found when the temperature increases. This is also reflected by the temperature range of host cells for thermophilic bacteriophages and archaeal viruses. As illustrated by Fig. 10.1, the optimal growth temperatures for hosts of bacteriophages range from 45 to 75°C, while the hosts of archaeal viruses have optimal growth temperatures from 65 to 100°C. This implies that thermophilic bacteriophages and especially thermophilic archaeal viruses must employ some special strategies to cope with the detrimental high temperatures when they are outside of their host. It is worth noting that the number of thermophilic viruses isolated so far is still limited, and the distribution illustrated by Fig. 10.1 may change when more viruses are obtained. Moreover, as seen in Fig. 10.2, the locations where thermophilic viruses have been sampled are few due to the natural obstacles of hot environments. Thus, it is only with the development of advanced equipment that isolation and ecological studies of thermophilic and especially hyperthermophilic viruses have been possible.

This chapter focuses on the application potential of thermophilic viruses. Central to this topic are the many novel genetic and morphological features discovered in

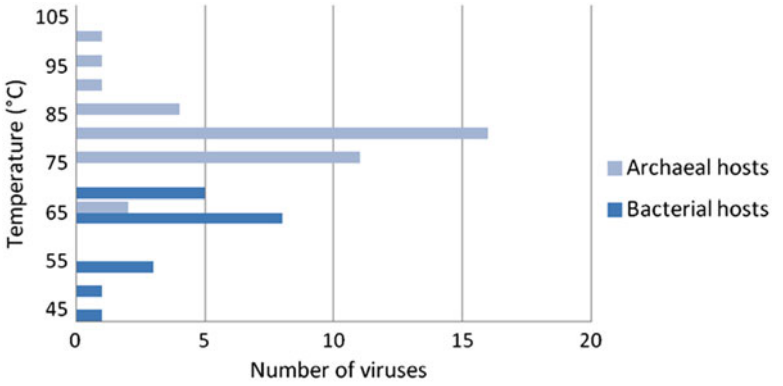

Fig. 10.1 The optimal growth temperature of thermophilic viral hosts. The graph shows an overview of the number of archaeal viruses and bacteriophages that infect hosts which have an optimal growth temperature exceeding 45°C

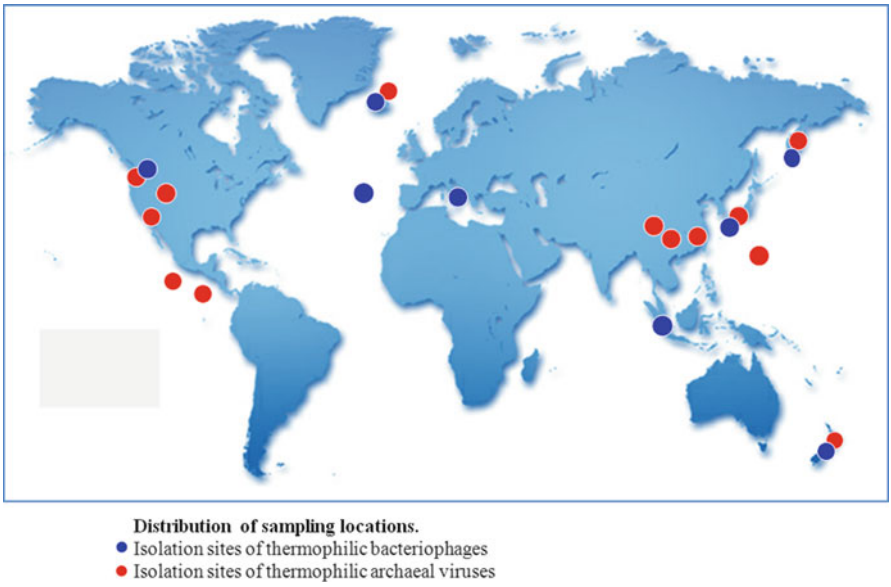

Fig. 10.2 Distribution of sampling locations

thermophilic viruses. In order to fully appreciate the huge potential these viruses have in basic research and biotechnology, we will introduce recent results derived from studies of structural adaptations, functional genomics, metagenomics, virus–host interactions and virus life cycle, all areas with remarkable characteristics. At the end, a section will cover more specific application areas including thermophilic viruses as nanobuilding blocks.

10.2 Virion Morphotypes of Thermophilic Viruses

Thermophilic viruses are much less studied compared to the mesophilic viruses. In approximately 5,600 known viruses, only a few have been isolated from thermophiles (Lin et al. 2011). Tables 10.1 and 10.2 present 57 isolated thermophilic viruses, and the majority belong to archaeal genera. Although the number of known thermophilic viruses is limited, the morphological diversity is exceptional and includes unique morphotypes (Prangishvili and Garrett 2005), providing us with unique forms and complex features for possible future applications.

The thermophilic archaeal viruses are extremely diverse and can be categorised into nine distinct families including *Fuselloviridae, Bicaudaviridae, Ampullaviridae, Clavaviridae, Lipothrixviridae, Rudiviridae, Globulaviridae, Myoviridae* and *Siphoviridae* and four taxa of uncertain affiliation, represented by five novel viruses: STIV1, STIV2, PAV1, STSV1 (Pina et al. 2011) and TPV1 (Gorlas et al. 2012). Enrichment cultures from hot environments exhibit a range of morphotypes including exceptional forms not previously observed for any dsDNA virus. The newly described morphotypes of archaeal viruses include spindle, droplet and bottle shapes (Arnold et al. 2000a; Mochizuki et al. 2010). These morphotypes are not rare in hot environments, and electron microscopy studies from terrestrial hot springs suggest that spindles, filaments, rods and spheres predominate (Garrett et al. 2010). The thermophilic archaeal viruses have been isolated from several archaeal genera including *Sulfolobus, Acidianus, Thermoproteus, Aeropyrum, Stygiolobus, Pyrobaculum, Methanobacterium, Pyrococcus* and *Thermococcus* (Pina et al. 2011). *Pyrococcus* and *Pyrobaculum* have optimal growth temperatures of 100°C demonstrating the extreme temperatures the thermophilic archaeal viruses have to adapt to.

Most of the isolated thermophilic bacteriophages exhibit a head–tail morphology similar to that of a T bacteriophage and belong to the families of *Myoviridae* and *Siphoviridae*. Interestingly, in an extensive survey done by the Promega Corporation in which 115 *Thermus* bacteriophages were isolated, only 45% of the bacteriophages were tailed. This was surprising because tailless bacteriophages of all types comprise only 4% of about 5,150 bacteriophages observed in the electron microscope. Of tailless thermophilic bacteriophages, three have been classified, two belong to the family of *Tectiviridae* (Yu et al. 2006) and one belongs to the family of *Inoviridae*. The thermophilic bacteriophages have been isolated from six bacterial genera including *Bacillus, Geobacillus, Thermus, Meiothermus, Rhodothermus* and *Thermomonospora* (Lin et al. 2011).

It is noteworthy that out of the seven described virion morphotypes, the bacterial viruses are represented by three, and the archaeal viruses are represented in all seven morphotype categories.

10.2.1 Spindle-Shaped Viruses

Viruses with a spindle- or lemon-shaped morphotype have been observed in both terrestrial and marine hot environments. Viruses with spindle-shape morphology, single or two tailed, are common in and exclusive to the domain of Archaea

Table 10.1 Morphology, taxonomical classification and characteristics of thermophilic archaeal viruses

Morphology	Family/genus	Virus	Host	Temp. host (°C)	Genome size	Lytic	Isolation site	References
Bacilliform	*Clavaviridae*[a]	APBV1	*Aeropyrum*	90	5.2	–	Japan, coastal hot spring	Mochizuki et al. (2010)
Bottle	*Ampullaviridae*	ABV	*Acidianus*	75	24	–	Italy, acidic hot spring	Haring et al. (2005)
Droplet	*Guttaviridae*	SNDV	*Sulfolobus*	80	20		New Zealand	Arnold et al. (2000a)
Head-tail	*Siphoviridae*	ψM1	*Methanobacterium*	65	30.4	–	From culture	Meile et al. (1989)
		ψM2		65	26.1	–		Pfister et al. (1998)
Linear	*Lipothrixviridae*	AFV1	*Acidianus*	75	21	–	Yellowstone hot spring	Bettstetter et al. (2003)
		AFV2		75	31.7	–	Italy, acidic hot spring	Prangishvili et al. (2006)
		AFV3		75	40.4	–		Vestergaard et al. (2008a)
		AFV6		75	39.5	–	Italy	
		AFV7		75	36.8	–		
		AFV8		75	38.1			
		AFV9		75	41.1		Russia, acidic hot spring	Bize et al. (2008)
		SIFV	*Sulfolobus*	80	40.8	–	Iceland, solfataric field	Arnold et al. (2000b)
		TTV1	*Thermoproteus*	85	15.9	+	Iceland, hot mud hole	Janekovic et al. (1983)
		TTV2		85	–		Iceland	
		TTV3		85	–			
	Rudiviridae	SIRV1	*Sulfolobus*	80	32.3	+	Iceland, solfataric field	Zillig et al. (1994)
		SIRV2		80	35.4	+		Prangishvili et al. (1999)
		SRV	*Stygiolobus*	80–90	28	–	Azores, hot spring	Vestergaard et al. (2008b)
		ARV1	*Acidianus*	75	24.6	–	Italy, acidic hot spring	Vestergaard et al. (2005)
Spherical	*Globulaviridae*	PSV1	*Pyrobaculum*	100	28.3	–	Yellowstone hot spring	Haring et al. (2004)
		TTSV1	*Thermoproteus*	85	21.6	–	Indonesia, hot spring	Ahn et al. (2006)
	Unclassified	STIV	*Sulfolobus*	80	16.6	+	Yellowstone hot spring	Rice et al. (2004)
		STIV2		80	17.6	+	Iceland, hot spring	Happonen et al. (2010)

(continued)

Table 10.1 (continued)

Morphology	Family/genus	Virus	Host	Temp. host (°C)	Genome size	Lytic	Isolation site	References
Spindle	*Fuselloviridae*	SSV1	*Sulfolobus*	80	15.4	–	Japan	Palm et al. (1991)
		SSV2		80	14.7	–	Iceland, hot spring	Stedman et al. (2003)
		SSV4		80	15.1	–	Iceland, hot spring	Peng (2008)
		SSV5		80	15.3	–	Iceland, hot spring	Redder et al. (2009)
		SSV6		80	15.6	–	Iceland, hot spring	
		SSV7		80	17.6	–	Iceland, hot spring	
		SSVk1		80	17.3		Russia, acidic hot spring	Wiedenheft et al. (2004)
		SSVrh		80	16.4		Yellowstone hot spring	
		ASV1	*Acidianus*	75	24.1	–	USA	Redder et al. (2009)
	Bicaudaviridae	ATV		75	62.7	+	Italy, acidic hot spring	Prangishvili et al. (2006)
	Unclassified	STSV1	*Sulfolobus*	80	75.2	–	China, acidic hot spring	Xiang et al. (2005)
		PAV1	*Pyrococcus*	95–100	18	–	Deep sea hot vent	Geslin et al. (2003)
		TPV1	*Thermococcus*	85	21.5	–	Deep sea hot vent	Gorlas et al. (2012)

The temperature of the host refers to the optimum growth temperature of the Bacterium from which the virus was isolated from
Genome size shows the viral genome size in kb. A lytic life cycle state is identified with a (+) and a lysogenic life cycle without apparent lysis of host cells during any stage is identified with a (–)

[a]The approval of the family *'Clavaviridae'* is pending at the ITV

Table 10.2 Morphology, taxonomical classification and characteristics of thermophilic bacteriophages

Morphology	Family/genus	Virus	Host	Temp. host (°C)	Genome size	Lytic	Isolation site	References
Linear	*Inoviridae*	PH75	*Thermus*	70–75	6.5	–	New Zealand, hot spring	Pederson et al. (2001)
Head-tail	*Myoviridae*	φTMA	*Thermus*	70–75	151.5	+	Japan, hot spring	Tamakoshi et al. (2011)
		φYS40		65	152.4	+	Japan, hot spring soil	Matsushita and Yanase (2009)
		TS2126		70–75	90		Iceland, hot tap water	Blondal et al. (2005b)
		RM378	*Rhodothermus*	65	130		Iceland	Blondal et al. (2003)
		MMP17	*Meiothermus*	50–65	33.5–39.5	+	China, hot spring	Lin et al. (2010)
		D6E	*Geobacillus*	65	34.7	+	Deep sea hot vent	Wang and Zhang (2010)
		GBSV1		65			China, offshore hot spring	Liu et al. (2009)
		BV1		65	35	+[a]	China, inshore hot spring	Liu et al. (2010)
	Siphoviridae	GVE2		65–70	40.9	+	Deep sea hot vent	Liu and Zhang (2008)
		TSP4	*Thermus*	70–75	80	+	China, hot spring	Lin et al. (2010)
		P23-45		70–75	84.2	+	Russia, acidic hot spring	Minakhin et al. (2008)
		P74-26		70–75	83.3	+		
	Unclassified	BVW1	*Bacillus*	65–70	18	+	Deep sea hot vents	Liu et al. (2006)
		Tb1	*Thermomonospora*	45	43	+	Compost, self-heated organic material	Lawrence et al. (1986)
		Tf2		55	35	+		
		Tf3		55	45	+		
		Tf4		55	36	+		
Head, no tail	*Tectiviridae*	φIN93	*Thermus*	65	19.6	+	Japan, hot spring soil	Matsushita and Yanase (2009)
		P23-77		70–75	17	+	New Zealand, hot spring	Jaatinen et al. (2008)

The temperature of the host refers to the optimal growth temperature of the bacterium from which the virus was isolated from
Genome size shows the viral genome size in kb. A lytic life cycle state is identified with a (+) and a lysogenic life cycle without apparent lysis of host cells during any stage is identified with a (–)
[a]Evidence suggests the bacteriophage is lytic

(Garrett et al. 2010). Thermophilic archaeal viruses with spindle shape have been divided into two families *Fuselloviridae* and *Bicaudaviridae*, and three viruses await classification.

The Fuselloviridae Family. All the known fuselloviruses infect and propagate in the hyperthermophilic genera *Sulfolobus* and *Acidianus,* including eight *Sulfolobus* spindle-shaped viruses (SSV1, 2, 4, 5, 6, 7, k1 and rh) and one *Acidianus* spindle-shaped virus (ASV1) (Table 10.1). The spindle-shaped virions are approximately 55–60 nm wide and 80–100 nm long (Fig. 10.3a). The exceptions are the viruses SSV6 and ASV1, whose virions tend to be pleomorphic (Redder et al. 2009). At one of the pointed ends of the spindle shape, a short tail of thin fibres is attached, which appear to be extremely sticky and readily attach to cellular fragments. The thin terminal fibres can also attach to the same type of fibres in other virions, which can produce rosette-like aggregates (Redder et al. 2009).

The Bicaudaviridae Family. The *Bicaudaviridae* family has only one member, the *Acidianus* two-tailed virus (ATV). The virions are exceptional in that they are extruded from host cells as tailless spindle-shaped particles, which then develop long tails at each pointed end. The tailless and the two-tailed virions have different virion dimensions. The tailless virions exhibit an average length of 243 nm and a maximum width of 119 nm, while the two-tailed virions show an average length of 744 nm and a maximum width of 85 nm (Fig. 10.3b) (Prangishvili et al. 2006). The tail development happens at temperatures above 75°C and independently of host cells or any energy sources (Haring et al. 2005). The only other known examples of extracellular viral morphogenesis comprise initial steps of infection or final steps in particle assembly and budding, and these are triggered on the cell surface of the host. The tails consist of tubes that terminate in an anchor-like structure. One function of the elongated, flexible tails might be to enhance the probability of virion adsorption to a new host cell (Prangishvili et al. 2006).

Three spindle-shaped viruses remain to be classified, *Pyrococcus abyssi* virus 1 (PAV1), *Thermococcus prieurii virus* 1 (TPV1) and *Sulfolobus tengchongensis* virus 1 (STSV1). PAV1 virions are approximately 120 nm long and 80 nm wide with a 15 nm long tail terminating in fibres. It contains a dsDNA of 18 kb (Geslin et al. 2003). STSV1 produces spindle-shaped virions (230 nm × 107 nm) with a single tail of variable length (Xiang et al. 2005). Similar to ATV, the spindle size of STSV1 seems inversely proportional to the length of the tail, that is, the longer the tail of a specific virion, the smaller the spindle is. This might reflect a reorganisation of structural components on the virions. The recently characterised TPV1 has a lemon-shaped virion approximately 140 nm long and 80 nm wide with a 15 nm long tail terminating in fibres, similar to the structure observed for PAV1. TPV1 contains a dsDNA of 21.5 kb (Gorlas et al. 2012).

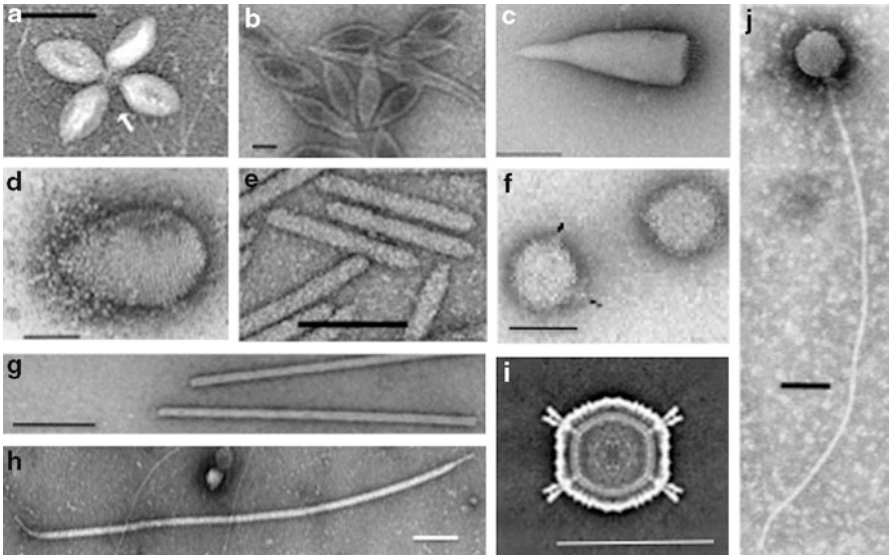

Fig. 10.3 Electron micrographs of (**a**) SSV6 (Redder et al. 2009); (**b**) ATV (Prangishvili et al. 2006); (**c**) ABV (Haring et al. 2005); (**d**) SNDV (Arnold et al. 2000a); (**e**) APBV1 (Mochizuki et al. 2010); (**f**) PSV (Haring et al. 2004); (**g**) SIRV2 (Prangishivili et al. 1999); (**h**) AFV3 (Vestergaard et al. 2008a); (**i**) STIV2 (Happonen et al. 2010) and (**j**) P23-45 (Minakhin et al. 2008). The *white arrow* in (**a**) indicates the interaction between tail fibers of four virions. I shows the central section of a cryo-EM picture of STIV2. Scale bars, 50 nm in D; 100 nm in (**a**), (**b**), (**c**), (**f**), (**i**) and (**j**); 200 nm in (**e**), (**g**) and (**h**)

10.2.2 Bottle-Shaped, Droplet-Shaped and Bacilliform Viruses

The virions of three archaeal viruses, ABV, APBV1 and SNDV, were all isolated from extremely hot terrestrial environments, and all of them display morphological features that are so unique that each of the viruses has been assigned to a new family.

The Ampullaviridae Family. The virion of *Acidianus* bottle-shaped virus (ABV) is structurally one of the most complex virions in the viral world and infects the hyperthermophilic *Acidianus* genus. The complex form resembles a bottle approximately 230 nm long and 4–75 nm wide (Fig. 10.3c). The lipid-containing envelope encases a cone-shaped core formed by a torroidally supercoiled nucleoprotein filament. At the broad end of the bottle shape, a disc is present to which 20 (±2) short, thick filaments are attached (Haring et al. 2005). Their function is still unknown. The virion seems to absorb to the host cell through its narrow end, suggesting that the tip of the virion could be involved in viral adsorption and channelling viral DNA into host cells.

The Guttaviridae Family. The *Sulfolobus neozealandicus* droplet-shaped virus (SNDV) exhibits a complex droplet morphology. The droplet is approximately 90 nm wide

and 180 nm long and carries multiple long, thin fibres that are attached at its pointed end (Fig. 10.3d). The surface appears to be helically ribbed and has been compared to resemble a beehive (Arnold et al. 2000a). The circular dsDNA genome has not been sequenced, and the virus does not presently exist in culture collections.

The Clavaviridae Family. The *Aeropyrum pernix* bacilliform virus 1 (APBV1) has a short stiff bacillus form. The virions are 140 nm long and 20 nm wide (Fig. 10.3e). One end is pointed; the other is rounded. The circular dsDNA genome of 5.2 kb is the smallest genome of known prokaryotic dsDNA viruses (Mochizuki et al. 2010). The approval of the family *Clavaviridae* is pending at the ITV.

10.2.3 Linear Viruses

Linear viruses represent the main virion morphotype in extreme geothermal environments. Linear viruses of bacteria and Eukarya carry either ssDNA or ssRNA. Interestingly, linear archaeal viruses isolated from hot environments carry dsDNA. The linear archaeal viruses have been classified into two new families: the stiff, rodlike *Rudiviridae* and the flexible, filamentous *Lipothrixviridae*. One thermophilic filamentous bacteriophage is known, which belongs to the *Inoviridae* family.

The Lipothrixviridae Family. Eleven members belonging to this family have been isolated, including seven *Acidianus* filamentous viruses (AFV1, 2, 3, 6, 7, 8 and 9), one *Sulfolobus islandicus* filamentous virus (SIFV) and three *Thermoproteus tenax* viruses (TTV1-3) (Table 10.1). Lipothrixvirus filaments are surrounded by envelopes containing lipids obtained from the host. The filament is approximately 24 nm wide, ranging in length from 900 nm (AFV1) to 2,000 nm (AFV3 and SIFV) (Fig. 10.3h). Lipothrixviruses carry identical terminal structures at both ends, indicating both ends are able to bind to cell receptors. Members of this family show considerable diversity in their terminal structures. These structures can represent claws (AFV1), T-bars (AFV9), mop-like structures (SIFV), three (AFV1) or six (SIFV) short fibres or tips resembling bottle brushes (AFV2) (Pina et al. 2011). The lipothrixvirus SIFV has a termini structure that ends in a mop-like structure to which six tail fibres are attached. This structure unfolds like a spider's legs, before attaching to receptors on the host cell membrane (Arnold et al. 2000b). Apparently, members of the *Lipothrixviridae* family evolved different structures for cellular attachment, providing great flexibility in host recognition.

The Rudiviridae Family. This family contains two *S. islandicus* rudiviruses (SIRV1 and 2), one *Acidianus* rod-shaped virus (ARV1) and one *Stygiolobus* rod-shaped virus (SRV) (Table 10.1). The rod-shaped virions are nonenveloped, approximately 23 nm wide and 610–900 nm long (Fig. 10.3g). The length of the linear virion matches the length of the genomic dsDNA, a phenomenon also characteristic of bacterial inoviruses, which carry a ssDNA genome. The virion body is a tubelike superhelix formed by linear dsDNA and multiple copies of a highly glycosylated, DNA-binding capsid

protein (Vestergaard et al. 2008b). A plug is located at each end of the virion. It is approximately 50 nm wide and carries three short terminal fibres.

The Inoviridae Family. The only thermophilic member of this family, PH75, infects the bacterium *Thermus thermophilus* (Pederson et al. 2001). The filamentous virions are 6 nm wide and 1,000–2,000 nm long. The terminal structures of inoviruses are not identical. One end functions as attachment site to host cells. The virion attaches to the target cell via protein binding to a pilus. Pilus retraction then pulls the virion to the host's internal membrane. The virions comprise a helical capsid surrounding a core of circular ssDNA. Unlike other bacterial viruses, PH75 does not lyse host cells but exit by extrusion.

10.2.4 Spherical Viruses

Two spherical virus families have been isolated from geothermal environments, *Globulaviridae* that infects Archaea and *Tectiviridae* that infects bacteria. Two spherical hyperthermophilic archaeal viruses, STIV1 and STIV2, still need to be assigned to a family.

The Globulaviridae Family. The family currently comprises two viral species, *Pyrobaculum* spherical virus (PSV) and *T. tenax* spherical virus 1 (TTSV1) (Fig. 10.3f; Table 10.1). PSV infects the anaerobic hyperthermophilic archaea, *Pyrobaculum*, which has a temperature optimum of a 100°C. The two viruses are highly similar in morphological and genomic properties. The spherical virions consist of a lipid envelope, which encases a helical nucleocapsid containing a linear dsDNA genome with inverted terminal repeats. The sphere of PSV has a diameter of approximately 100 nm (Pina et al. 2011).

STIV Viruses. The hyperthermophilic *Sulfolobus* turreted icosahedral viruses, STIV1 and STIV2 (Fig. 10.3i), remain taxonomically unclassified. The two viruses share a similar icosahedral structure and appear to be closely related. The virions have non-tailed icosahedral structures with an internal lipid monolayer, which encases the circular dsDNA (Fulton et al. 2009). They share an architectural similarity with viruses of the bacteriophage family, *Tectiviridae*. Image reconstruction of the STIV virion revealed a unique virus architecture including complex, turret-like projections extending from each of the vertices, which may be involved in host recognition.

The Tectiviridae Family. Two thermophilic bacteriophages have been assigned to this family, P23-77 and φIN93 (Table 10.2). The virions of tectiviruses consist of an icosahedral protein capsid with an internal membrane vesicle that encloses the linear dsDNA genome. The internal membrane contains lipids organised as a bilayer underneath the protein capsid, which can give a layered shell appearance of the spherical virion (Jaatinen et al. 2008). There is no indication of any tail structure. Both thermophilic tectiviruses have small genomes of approximately 17–20 kb, possessing only the basic functions needed for survival in hot environments.

10.2.5 Head–Tail Viruses

Several thermophilic viruses with head–tail morphotypes have been isolated from hot environments, most of which infect bacteria. Only two thermophilic archaeal viruses with head–tail morphology have been isolated so far; they infect the thermophilic *Methanobacterium*. The isolated head–tail viruses fall into the two families *Myoviridae* and *Siphoviridae*, and five still remain to be classified. The head has icosahedral symmetry and the tail structure differs in length and construct.

The Myoviridae Family. Eight thermophilic viruses belong to this family, with three infecting *Thermus*, one infecting *Rhodothermus* and *Meiothermus* each, and three infecting *Geobacillus* (Table 10.2). The hexagonal heads are approximately 60 nm wide, and the contractile tails vary in length between 80 and 150 nm. In T4, a mesophilic myovirus, the contractile tail ends with a complex baseplate with six long fibres radiating from it. These fibres are used for cell attachment (Comeau et al. 2007). A similar structure has been observed in the thermophilic myovirus φTMA. Like a typical myovirus, such as T4, the tail tube of φTMA protrudes from the bottom of the baseplate when the tail sheath contracts. Moreover, electron microscopy study showed lipid vesicles that appeared to be bound to the bottom of the baseplate of the φTMA page particles (Tamakoshi et al. 2011). Bacteriophage T4 is a very complex virus, more than 40 different proteins form the mature virion and the dsDNA genome comprises approximately 172 kb. Among the isolated thermophilic myoviruses, φTMA, φYS40 and RM378 have genome sizes ranging from 130 to 152 kb; the rest have genomes smaller than 100 kb. It has been hypothesised that the large genomes of φTMA, φYS40 and RM378 may encode a complexity similar to that of T4.

The Siphoviridae Family. Four thermophilic bacteriophages and two thermophilic archaeal viruses belong to this family (Table 10.2). The hexagonal heads are approximately 60 nm wide, and the tails have a length of approximately 150 nm and a width of about 8 nm. The tails are noncontractile with a helical structure; at the end of the tail, short terminal fibres are attached. The virions have nonenveloped dsDNA genomes, which have undergone extensive genetic exchange (Hendrix et al. 1999). The thermophilic siphovirus, TSP4, infecting *Thermus* sp. isolated from Southwest of China, has an extremely long and flexible tail of 785 nm in length and 10 nm in width. It has been found that the end of the tail frequently absorbs to cell debris, indicating attachment structures. Despite their exceptional habitats being separated by thousands of kilometres, the morphological characteristics of TSP4 showed high similarity with the thermophilic siphoviruses P23-45 (Fig. 10.3j) and P47-26, which have been isolated from hot springs in the Far East of Russia (Lin et al. 2010).

Five viruses with head–tail morphology remain to be classified, BVW1, Tb1 and Tf2-4. *Bacillus* virus W1 (BVW1) has a long tail of 300 nm in length and 15 nm in width, and the hexagonal head has a diameter of 70 nm. It contains a dsDNA of 18 kb (Liu et al. 2006). Tb1 has been isolated from *Thermonospora alba* and Tf2-4 have been isolated from *Thermonospora fusca*. All four *Thermonospora* bacteriophages possess polyhedral heads and long tails. The tail lengths for Tb1 and

Tf3 are approximately 260–280 nm and for Tf2 and Tf4 approximately 122–125 nm. Tail flexibility has been observed for the longer-tailed bacteriophages; however, flexibility is not apparent for the shorter-tailed types. The four bacteriophages contain dsDNA of 35–45 kb (Lawrence et al. 1986).

10.3 The -Omics Era and Gene Regulation of Thermophilic Viruses

Genomics and functional genomics have been instrumental in the past two decades in the field of biology. Although the number of thermophilic virus isolates is limited, the genomic and functional genomic data originated from them have provided insights into the diversity, ecology and gene regulation of these exceptional organisms.

10.3.1 Genomic Properties

All thermophilic viruses isolated so far package circular or linear dsDNA genomes, with the exception of bacteriophage PH75 which packages circular ssDNA. The genomes of thermophilic viruses vary considerably in size. The smallest genomes belong to the archaeal virus APBV1 (5.2 kb) and the bacteriophage PH75 (6.5 kb). The largest genomes are found among thermophilic bacteriophages φYS40 and φTMA, which range from 151 to 153 kb. The largest archaeal viral genome is found in STSV1 (75.2 kb). Genome size in prokaryotes is often a good predictor of metabolic complexity; it has been hypothesised that the large genomes of φTMA and φYS40 may encode a complexity similar to that of the bacteriophage T4.

The genomes of most isolated thermophilic viruses have been sequenced, providing a wealth of information about the genetic diversity of these viruses. However, the genes of thermophilic viruses generally yield few significant matches to sequences in public sequence databases, and most predicted gene products lack recognisable functions and homologs in extant databases. Identified functions in archaeal viruses are confined to a few proteins. For example, in STIV1 the structure of A197 reveals a GT-A fold that is common to many members of the glycosyltransferase superfamily, suggesting a glycosyltransferase activity for A197. Viruses commonly decorate their proteins with sugars as means of regulating interactions with their hosts. While viruses can utilise their host's glycosylation machinery, it is clear that some also encode their own proteins for specialised glycosylation needs, for example, some lytic bacteriophages glycosylate their DNA to protect it from host restriction enzymes (Larson et al. 2006). In SIRV1 and SIRV2, Holliday junction cleaving enzymes have been characterised. Holliday junction resolving enzymes are ubiquitous and found in all living cells. They are essential for DNA recombination, recombination-related DNA repair and recombination-dependent DNA replication (Birkenbihl et al. 2001). Moreover, SIRV1 and SIRV2 both encode a dUTPase

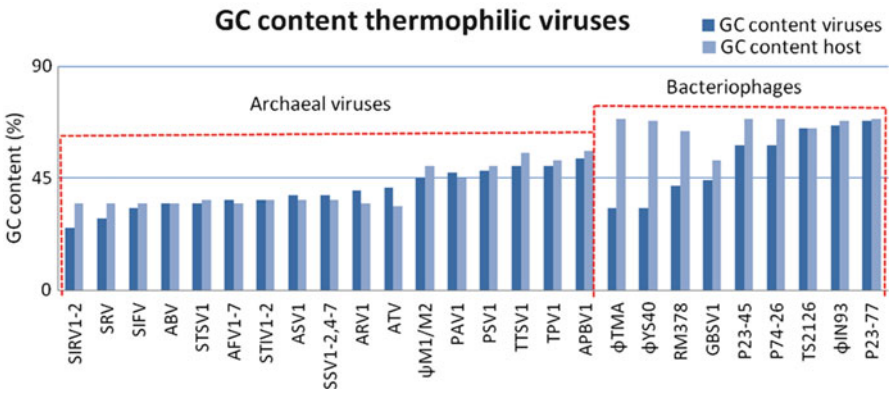

Fig. 10.4 Graph comparing the GC content of thermophilic archaeal viruses and bacteriophages with their respective hosts. More than half of the bacteriophages have a GC content of 45% or higher. Only one third of the archaeal viruses have a GC content of 45% or higher

which is involved in nucleotide metabolism (Prangishvili et al. 1998). Similarly, another thermophilic rudivirus, ARV1, encodes a thymidylate synthase (Vestergaard et al. 2005). These enzymes function in adjacent steps of the de novo synthesis pathway of thymidine nucleotides, and either enzyme can help to maintain a low dUTP–dTTP ratio, thereby, minimising misincorporation of uracil into DNA (Chen et al. 2002). This is important at high temperatures when dCTP deaminates more rapidly to yield dUTP. Thus both enzymes are most likely important for efficient replication and for the stability of the hyperthermophilic rudiviruses. In SSV1 an integrase has been identified, encoding a site-specific recombination system, suitable for further investigations into the regulatory mechanisms in the SSV1 genome (Muskhelishvili et al. 1992). Other functional categories characterised in archaeal viruses including polymerases, ligases and nucleases will be discussed in the application section. Furthermore, structural genomics has produced insights into the functions of some previously unknown viral genes. This was reviewed recently (Krupovic et al. 2012) and will not be discussed in this chapter.

One surprise when studying thermophilic viral genomes has been the variance in their genomic GC content. The GC content of nucleic acids is known to be correlated with the stability of their double helix. GC pairs are more stable than AT pairs because they have an additional hydrogen bond. Thus higher GC base pairing leads to higher thermal stability of the DNA (Galtier and Lobry 1997). This can be exploited by organisms living in thermal environments. It has been suggested that high GC content may be a selective response to high temperature. However, a study comparing GC content and optimal growth temperature for numerous prokaryotes failed to demonstrate the predicted correlation (Hurst and Merchant 2001). Looking at GC content of thermophilic viruses emphasises this finding. Approximately 35% of the thermophilic archaeal viruses and 55% of the thermophilic bacteriophages have a GC content of 45% or higher (Fig. 10.4). This was surprising given the relatively higher optimal growth temperatures of archaeal hosts than bacterial hosts (Fig. 10.2).

One extreme example is the archaeal rudiviruses SIRV1 and SIRV2 which have a GC content of 25% while the hyperthermophilic host, *Sulfolobus*, has an average GC content of about 37%. Apparently, factors other than GC base pairing, such as DNA-binding protein(s) in virions, play major roles in protecting viruses against heat.

10.3.2 Metagenomics

The classification of viruses has traditionally been based on morphological characteristics as demonstrated by the previous section. The system can only be used for viruses that are abundant enough for both microscopic study and genomic sequencing, which significantly biases our view of diversity towards the culturable fraction of the virus community (Pride and Schoenfeld 2008). One factor limiting the discovery of thermophilic viruses has been the reliance on culture-dependent methods for virus isolation. Hot environments are characterised by extreme conditions. For example, deep sea hydrothermal vents have strong physicochemical gradients such as temperatures from 2°C to more than 350°C and high hydrostatic pressure, lack of solar energy and prevalence of chemosynthesis (Gorlas et al. 2012). No viral-cultivation study can fully mimic the temperature and pressure extremes that characterise deep sea hydrothermal vents; this imposes a selective pressure preventing the propagation of viruses unable to adapt to laboratory conditions. Studies have shown that only 1–15% of microbial organisms are culturable under laboratory conditions, demonstrating the amount of genomes still to be discovered (Singh et al. 2009). The introduction of viral metagenomics has revolutionised the field of environmental virology by allowing the exploration of viral communities in a variety of environments, including extreme environments. Viral metagenomics involves viral particle purification followed by library construction and sequencing (Rosario and Breitbart 2011). Viral metagenomic studies of hot environments have provided information concerning viral biogeography, diversity and community structure and new viral types and contributed to the discovery of a potential archaeal RNA virus.

There are approximately 26 published metagenomic studies investigating viral communities, among which, five investigate hot environments. From these studies, it is clear that environmental viral communities are different from those observed in culture. Analysis of viral metagenomes has shown that about 30% of these sequences have detectable similarity to those in GenBank and about half of these are most similar to other known viruses (Rosario and Breitbart 2011). The high percentage of unknown sequences demonstrates the vast novelty of genetic information to be obtained from viruses. Despite the lack of sequence identification from viral metagenomes, studies have used this technique to catalogue viruses in environmental samples based on the identifiable sequences and to investigate community composition through statistical analyses.

A metagenomic analysis of samples from Yellowstone hot springs investigated viral biogeography at a local and global scale (Schoenfeld et al. 2008). Two pools

were investigated, Bear Paw (74°C) and Octopus (93°C). The two metagenomic libraries, one from each pool, showed a relative close similarity (nearly 25%). This was surprising, given that microbial populations are temperature-dependent and the surface temperature of the two hot springs differ by 19°C. Alignment of the metagenomes to whole-genome sequences of six cultivated thermophilic viruses revealed striking conservation of certain sequences. Comparison with the genome of PSV showed median identities of 60 and 51% to Bear Paw and Octopus, respectively. PSV was isolated from a hot spring with notably different geochemistry and more than 30 km away from both hot springs. The high median identities between PSV and the two metagenome libraries illustrate the global conservation of sequences in hot springs with different physical and biogeochemical properties (Schoenfeld et al. 2008). This correlates with the findings that groups of highly similar *Sulfolobus* viruses and *Thermus* bacteriophages have been isolated from hot springs on different continents (Wiedenheft et al. 2004; Yu et al. 2006).

In general, viral metagenomics has revealed an enormous diversity and abundance of viruses in all environments. Estimates of viral diversity suggest the existence of several thousand unidentified virus types (Weinbauer and Rassoulzadegan 2004). With metagenomics, it is possible to assemble complete genomes of unidentified virus-like particles obtained from environmental viromes, thus identifying novel viruses. A metagenomic study investigating an enriched environmental sample from a hot spring in Yellowstone national park yielded two novel viral genomes, HAV1 and HAV2. Neither viral genome shows any clear similarity to other known archaeal viruses; only HAV2 shows morphological similarities with the two-tailed spindle-shaped ATV virus and limited sequence similarity between two genes. HAV1 has a linear 23 kb genome and HAV2 has a circular 18 kb genome; both have dsDNA. They yielded few significant matches in public sequence databases, reinforcing the notion of wide genetic diversity in archaeal viruses. The study attempted to identify the hosts of the two viruses by isolating archaeal strains from the environmental sample. However, none of the isolated strains were infected (Garrett et al. 2010). Based on the morphological and limited sequence, similarities to ATV, HAV1 and HAV2 were inferred as archaeal viruses. It can be difficult to identify potential archaeal hosts, especially because no reliable protocol procedures for transfection of viral DNA into potential hosts have been developed. Metagenomic analyses can be a helpful tool for identifying new viruses, although information concerning hosts and virus life cycle can be difficult to confirm.

Identification of viral hosts is crucial in understanding the role of viruses in an ecosystem. It would aid in determining how viruses impact the microbial diversity of a given ecosystem, as they are the only predators in hot environments above 60°C. A study tried to identify specific hosts for a viral assemblage obtained from a hydrothermal vent in the Northeast Pacific. The viral metagenome was compared with a comprehensive database of spacers derived from the clustered regularly interspaced short palindromic repeat (CRISPR) adaptive immune system (Anderson et al. 2011). The CRISPR system is an antiviral defence mechanism found in both Archaea and bacteria (Marraffini and Sontheimer 2010). Cells incorporate genomic sequences as CRISPR spacers from invading viruses and plasmids into their CRISPR

loci. When invading viruses or plasmids have a match to a pre-existing CRISPR spacer sequence in the host genome, these elements are recognised as pathogenic invaders. Thus, CRISPR spacer sequences provide an adaptive, heritable record of past infections and express small CRISPR RNAs that guide targeting of invasive nucleic acids, rendering the cell immune to these viruses (Marraffini and Sontheimer 2010). The CRISPR loci act effectively as libraries of previous viral infections which can be compared with environmental viromes, thus potentially identifying virus–host relationships. A study by Anderson et al. (2011) showed that CRISPR spacers from vent isolates and from thermophiles in general have a higher percentage of matches to the vent virome than to other marine or terrestrial hot spring viromes. Spacers derived from strains belonging to 23 different taxonomic groups matched sequences from the hot vent virome; most notably, a wide range of both archaeal and bacterial taxonomic groups has CRISPR spacers matching the marine vent viromes. Interestingly, a high percentage of hits to spacers from mesophilic hosts suggested that the marine vent virome was comprised of viruses that have the potential to infect diverse taxonomic groups of multiple thermal regimes in both the bacterial and the archaeal domains (Anderson et al. 2011).

Metagenomics has also resulted in findings that suggest the existence of novel positive-strand RNA viruses that probably replicate in hyperthermophilic archaeal hosts and are highly divergent from RNA viruses that infect eukaryotes and bacteria. To date, all archaeal viruses possess dsDNA genomes, except one that possess ssDNA. Finding a RNA virus infecting an archaeal host could enhance our knowledge of RNA viruses and shed light on the origin of the enormous diversity of RNA viruses infecting eukaryotes (Bolduc et al. 2012). Further studies need to identify the potential hosts of the RNA viruses, possibly through sequence comparison between the RNA viral genomes and CRISPR spacers of cellular genomes from the same environment.

Advances in the field of metagenomics will provide more information concerning important aspects of viral ecology. Moreover, functional viral metagenomics are being developed to discover novel viral enzymes that can be used for diagnostic and biotechnological purposes (Schoenfeld et al. 2010).

10.3.3 Functional Genomics and Gene Regulation

A virus is totally dependent on its host cell for viral reproduction. In order to multiply, a virus must take control of its host cell's molecular machineries. Perhaps the most important mechanism for achieving this control is a viral code for strong positive signals to promote viral gene expression and other signals to repress expression of some cellular genes. When a cell is infected, much is going on inside the cell at the molecular level, such as transcription of the incoming viral genes to form viral mRNAs, and their translation to produce early viral proteins, including the enzymes necessary to replicate viral DNA (Collier and Oxford 2006). Transcriptomics is a useful tool for investigating the subtle but definite changes in

gene expression of both the host and the virus in the period of a virus infection. The transcriptome reflects the genes that are expressed at any given time. Therefore, it can provide insights into which genes are being up- or downregulated during an infection cycle. Moreover, viral transcriptomics can be used to compare transcription patterns of viruses under a wide range of conditions (Walther et al. 2011).

Microarray analysis of an infected culture of *Sulfolobus solfataricus* with STIV1 has revealed insights into the timing and extent of virus transcription, as well as differential regulation of host genes. The infection cycle of STIV1 is lytic with almost all cells in a culture being killed by the virus. Transcription of viral genes was first detected at 8 h post infection (hpi), and at 16 hpi, most viral genes were expressed. The majority of the viral genes are transcribed between 16 and 24 hpi. Around 24 hpi, a shift takes place from virus replication to preparation for lysis, with general cell lysis detected at 32 hpi. Although the expression starts at different timepoints for different genes, little temporal control was observed. During the infection, 177 host genes were determined to be differentially expressed, with 124 genes upregulated and 53 genes downregulated. The upregulated genes were dominated by genes associated with DNA replication and repair and those of unknown function, suggesting that STIV1 uses host proteins to aid the replication of its own DNA (Ortmann et al. 2008). An important upregulated gene concerns an ESCRT (endosomal-sorting complex required for transport) III homolog. The ESCRT III complex serves in eukaryotes as a protein-sorting machinery and functions as a pair of molecular scissors that cuts cell endosomale membranes (Wollert et al. 2009). The ESCRT III homolog has recently been reported essential for the cell division in *Sulfolobales* (Ettema and Bernander 2009); the upregulation may suggest its involvement in the release of STIV1 virions. The downregulated genes, mostly detected at 32 hpi, were associated with energy production and metabolism (Ortmann et al. 2008). Interestingly, the majority (94%) of the host genes showed no differential regulation between infected and uninfected cells, suggesting that the virus life cycle is tailored to avoid host stress response.

An infection study of SSV1 with *S. solfataricus* as a host investigated the transcriptome fluctuations of a virus with a lysogenic life cycle. Upon infection, the SSV1 genome is rapidly and site specifically integrated into a tRNA gene of the host paralleled by a short slowdown of growth. Even after infection with an excess of virus particles, the host recovers well and often grows even better than before, as if the presence of the viral genome gives some advantage (Schleper et al. 1992). Upon UV irradiation of the host cells, a strong replication of the viral DNA is induced and large amounts of particles (up to 100 per cell) are released into the culture medium. This occurs without apparent lysis of the host cells. However, at this stage, the cell growth is significantly retarded. The study found that the first viral transcripts already can be found after 1 hpi, while most viral genes are active at 8.5 hpi. The viral genes are clustered at 9 operons expressed into 10 transcripts, comprising both regulatory and structural genes. The regulatory genes are the first to be transcribed, and the genes coding for the coat protein of the virus are produced at a later stage. Similar to those of eukaryotic viruses and bacteriophages, the transcripts of SSV1 can be categorised according to their time of appearance

and putative functional roles, showing that SSV1 exhibits a tight chronological transcriptional regulation (Frols et al. 2007). With respect to host gene regulation, transcriptomes were compared between UV irradiated and nonirradiated cells for both infected and uninfected cultures, and the response to UV irradiation was then compared between infected and uninfected cells. Only small differences in genome-wide analysis were detected between infected and uninfected cells, possibly due to the indirect comparison.

Very few studies have investigated the change in gene expression profiles when a bacteriophage infects a hyperthermophilic bacterium. A study analysing the interaction between GVE2, a deep sea thermophilic bacteriophage, and its host *Geobacillus* sp. E263 showed differential regulation of host genes. A comparison of protein/gene expression profiles of GVE2-infected and noninfected *Geobacillus* sp. E263 bacteria revealed that among the 20 differentially expressed host genes, 13 were upregulated and 7 were downregulated in response to GVE2 infection. Based on homology searches in GenBank, 19 of the 20 proteins involved in GVE2 infection shared homology with proteins of known function. The 19 proteins have diverse metabolic functions and can be grouped into three different categories based on cellular function, suggesting a coordinated response to virus infection (Wei and Zhang 2010).

One of the current limitations of transcriptomic methods is that they require millions of cells as a starting material. Therefore, a future challenge will be the determination of transcriptome maps for single cells, which will open an avenue for investigating the regulatory heterogeneity in a microbial population upon infection (Sorek and Cossart 2010). This may lead to the discovery of advantageous genes/defence mechanisms in some cells of a given population. Furthermore, single cell transcriptomics will also allow studies of the transcriptome of individual cells from unculturable species, a major advantage with the high amount of unculturable thermophilic viruses and their hosts. Another field that could advance our understanding of thermophilic viruses' impact on their environments is metatranscriptomics. The analyses of the ecology in hyperthermophilic environments are generally less complex than that of aquatic or soil systems, making it easier to deal with big dataset covering many organisms (Walther et al. 2011). Comparing metatranscriptomes from different hot environments could elucidate the impact of viruses, helping us understand the roles viruses play in ecological and geochemical processes in hot environments.

10.4 Virus–Host Interactions

The number of studies characterising virus life cycles in hot environments is limited, and the interactions have rarely been characterised in depth, beyond the basic level. According to studies on isolated archaeal virus–host systems, the dominant type of the viral life cycle differs from that of bacterial systems (Abedon 2009). The majority of known thermophilic archaeal viruses establish a chronic infection in which virions are continuously produced, and the host cells remain alive although the

growth rate is often retarded. Adaptation to energy stress conditions is hypothesised to be the crucial factor that distinguishes Archaea from Bacteria (Valentine 2007) and may be what has driven and even favoured such virus–host relationships. The only hyperthermophilic bacteriophage that exhibits a chronic mode of infection is the filamentous bacteriophage of the *Inoviridae* family, PH75, which does not lyse host cells even when virions are actively produced (Pederson et al. 2001). This infection mode is rare for bacteriophages but appears to be common among thermophilic archaeal viruses and is often referred to as the carrier state. However, the carrier state can be interrupted and transformed into a lytic state by stress factors such as UV irradiation. The hallmark of the lytic life cycle is the lysis of the host cells when virions are released, thus killing the host. The lytic cycle is the mode of reproduction for all hyperthermophilic bacteriophages with the exception of PH75. A few archaeal viruses are reported to be purely lytic (TTV1, STIV and SIRV). Determining the nature of the infection cycle is not always easy. For example, the rudivirus SIRV2 was thought to establish a carrier state infection, mainly based on the lack of a decrease in OD in infected cultures. However, the virus lyses the cells. The exceptional egress mechanism involved in the release of virions from the cell results in lysed cells in form of empty spheres. The cells appear intact and OD measurements lack the sophistication to identify that the measured cells are no longer 'living'. Thus, other thermophilic archaeal viruses could turn out to be lytic upon further investigations.

10.4.1 Carrier State Infection

AFV1 is similar to most thermophilic archaeal viruses. Thus, it does not kill its host during reproductive infection. A study investigating the virus–host interactions between AFV1 and its host showed that the growth rate of host cells, *Acidianus hospitalis*, is nearly completely blocked in the initial stage of infection. The cell growth recovers slowly after initial infection, and from 2 hpi, a generation time of 20 h is observed, in contrast to a generation time of 11 h for uninfected cells. Mature virus particles start to be released from infected cells 4–5 hpi. After several successive dilutions of virus-carrying cultures and prolonged incubation, the virus was still present in host cells, indicating a stable carrier state of host–virus relationship. Under certain growth conditions, a balance between production of virions and multiplication of host cells can be observed. DNA bands originating from the viral AFV1 genome could not be distinguished in the restriction digestion pattern of total cellular DNA at any stage of infection. This indicates a low intracellular copy number of virus DNA, probably less than 10 copies per cell (Bettstetter et al. 2003). Another thermophilic virus, which reproduces by combining a lysogenic life cycle with various levels of viral particle production in a carrier state mode, is SSV2. A comparative study of SSV2 physiology in the natural host *S. islandicus* versus the foreign host *S. solfataricus* provided evidence of differently regulated SSV2 life cycles in the two hosts. An initial infection of the foreign host *S. solfataricus* retards

cell growth. However, when SSV2 settles in the foreign host *S. solfataricus*, host growth is no longer inhibited. This is in strict contrast to what is observed in the initial infection stage. Neither does the SSV2 genome exceed over a few copies per cell throughout extended cultivation. This suggests that a series of interactions between SSV2 and its foreign host leads to a coexistence harmony between them. Further studies may reveal whether this conversion of host response is a general scheme for the host defence of *S. solfataricus* to a viral infection. In the natural host *S. islandicus*, SSV2 replication is characterised by a physiological induction. Viral genome copy number increases up to 50-fold within 4 hpi after the sudden halt of cell growth at OD_{600} of about 1.3 which corresponds to a late exponential growth phase. Growth inhibition of the host correlates with the virus replication induction. Interestingly, the inhibition effect is reversible. When released into a fresh medium, the SSV2-induced *S. islandicus* culture resumes their exponential growth and hosts regain control over SSV2 replication (Contursi et al. 2006).

10.4.2 Lytic Life Cycle

Virus–host interactions in hyperthermophilic bacteriophages are not well studied. Therefore, the best examples of lytic thermophilic virus–host interactions are from the domain of Archaea. As mentioned earlier, only few thermophilic archaeal viruses are lytic. However, the life cycles of the lytic viruses, STIV1 and SIRV2, have revealed a unique mechanism of virion release (Prangishvili and Quax 2011). The viruses differ significantly in their morphological and genomic properties but exploit the same mechanism for the release of mature virions from the host cell. A study done by Bize et al. (2009) investigated the life cycle of SIRV2. SIRV2 infects *S. islandicus* and virions are assembled in the cystoplasm of the host cell; approximately 8–10 hpi, they start to be released through well-defined apertures in the cell envelope. Formation of these openings is preceded and facilitated by the generation of virus-induced cellular structures of pyramidal shape, virus-associated pyramids (VAPs), located at the cell envelope and pointing outwards. The VAPs perforate the membrane and S-layer and opens up to release the preassembled virions in the cytoplasm to the surrounding environment. After virion release, the cell envelope remains as a stable empty shell. The timing of VAP disruption and virus release must be strictly controlled by virus-encoded functions, such that cell lysis does not occur until the virions have been assembled, as for any lytic virus (Bize et al. 2009). The extent of modifications caused by SIRV2 on the host cells results in a radically transformed cell, which functions as a complex viral factory. It has been suggested that the lysogenic life cycle provides an intracellular refuge for virus populations in hot environments. However, viruses with a purely lytic life cycle such as SIRV2 demonstrate that virus particles can survive in extreme ecosystems long enough to encounter new host cells. The SIRV2 virions are well adapted to hot environments, being almost as stable at 80°C as bacteriophages infecting mesophilic bacteria are at 37°C (De Paepe and Taddei 2006).

10.5 Applications

The previous sections have covered thermophilic viral characteristics, including novel features not previously observed. Many of these features may have application potential in basic research or biotechnology. However, application development in thermophilic viruses has so far concentrated on a few identified enzymes and an emerging interest to use them as nanobuilding blocks. Virus-derived vectors and enzymes have been essential research tools since the first days of molecular biology, and compared to mesophilic viruses, an obvious advantage of thermophilic viruses is their thermostability. The next section will summarise thermophilic viral enzymes which are already trademarked or have the potential for biotechnological/industrial exploitation. Moreover, the potential of thermophilic viruses as nanobuilding blocks will be addressed.

10.5.1 Application of Thermophilic Viral Enzymes in Biotechnology and Basic Research

Many research applications of enzymes are centred on nucleic acid metabolism. Especially DNA polymerases have been used in molecular biology techniques such as whole-genome amplification, PCR and DNA sequencing (Tabor and Richardson 1987; Saiki et al. 1988; Zhang et al. 1992). Viral polymerases are highly diverse in terms of primary amino acid sequence and biochemical activities (Schoenfeld et al. 2010) and are functionally distinct from their cellular counterparts. Many applications of DNA polymerases depend on thermostability up to 95°C, making hyperthermophilic viruses ideal subjects for discovering viral DNA polymerases with biotechnological potential. Recently, a thermostable DNA polymerase from a viral metagenome was found to be a potent RT-PCR enzyme (Moser et al. 2012). Out of 21,198 Sanger sequence reads derived from a viral metagenome library constructed from Octopus hot spring (93°C) in Yellowstone National Park, hundreds of potential *pol* genes were identified and 59 complete *pol* genes were tested for polymerase activity. Among these, 3173 Pol demonstrated both high thermostability and innate reverse trancriptase (RT) activity (Table 10.3). As the first reported virus-derived thermostable RT Pol, 3173 Pol also exhibited high sensitivity and high specificity comparable to two-enzyme RT-PCR systems. Obviously, 3173 Pol fulfils the requirements for a facile single-enzyme RT-PCR reagent (Moser et al. 2012). The 3173 Pol-based PyroScript RT-PCR master mix (Lucigen) provides demonstrated advantages over the commonly used two-enzymes RT-PCR systems. Among other documented performance problems, the two-enzyme RT-PCR systems require an initial low temperature reverse transcription that reduces specificity, increases reaction time and impairs synthesis through complex secondary structures. In contrast, the high thermostability of 3173 Pol should allow a ‘hot start’ (Moser et al. 2012).

Table 10.3 Current and future uses of thermophilic viruses

Enzyme/nanoparticles	Viral source	Current and emerging applications	References
ssDNA/RNA ligase 1	TS2126	Production of ssDNA templates for rolling-circle replication or rolling-circle transcription experiment	EpiCentre (2012)
		Production of ssDNA templates for RNA polymerase and RNA polymerase inhibitor assays	Blondal et al. (2005b)
Polynucleotide ligase 1	RM378	Phosphorylation of nucleic acids with modified phosphates for subsequent modifications and/or labeling	Blondal et al. (2005a) and Prokazyme (2012)
Nonspecific nuclease	GBSV1	RNA sequencing Removal of nucleic acids during protein purification Antiviral agents	Song and Zhang (2008)
Protein-primed DNA polymerase	ABV	PCR Genome amplification	Peng et al. (2007)
3173 DNA polymerase	Metagenome	RT-PCR Genome amplification DNA sequencing	Moser et al. (2012)
Nanoparticles	SSV1 SIRV2	Nanocompartments Imaging Drug delivery	Reiter et al. (1987) and Steinmetz et al. (2008a)

PCR has been used for about two decades as a powerful tool in molecular biology. However, the size of DNA product that can be amplified by PCR is still limited. With the best PCR enzyme to date, Phusion, a maximum of 20 kb can be reached. Although the bacteriophage phi29 DNA polymerase presents the highest processivity described so far for a DNA polymerase, and up to 70 kb fragments can be generated (Blanco et al. 1989; Rodriguez et al. 2005), the enzyme is not thermostable and therefore cannot be used in PCR. Biochemical and structural studies have shown that the high processivity of Phi29 DNA Pol is dependent on one of the two insertions in the protein sequence which are a characteristic of protein-primed DNA Pol families (Rodriguez et al. 2005). Recently, a putative protein-primed DNA polymerase gene was identified from the genome of the hyperthermophilic archaeal virus ABV which also contains the two insertions in the protein sequence (Table 10.3) (Peng et al. 2007). This feature may render the ABV Pol a high processivity, which, in combination with the high thermostability, makes the enzyme a promising candidate for PCR amplification of large DNA fragments.

DNA polymerases constitute just one group of useful enzymes; other viral enzymes with distinct and useful properties have recently been discovered in thermophilic viruses. Thermostable RNA ligases 1 from bacteriophages TS2126 and RM378 were recently isolated and characterised (Table 10.3) (Blondal et al. 2003, 2005a, b). RNA ligases 1 have the ability to ligate single-stranded nucleic acids by catalysing the ATP-dependent formation of phosphodiester bonds between 5′-phosphate and 3′-hydroxyl termini of single-stranded RNA or DNA. The biological role of bacteriophage RNA ligases has been primarily studied in T4, where the T4 RNA ligase 1, together with the bacteriophage encoded polynucleotide kinase, repairs cleaved tRNA molecules, thereby counteracting the defence mechanism of the bacterial host. The actual role of RNA ligases in TS2126 and RM378 bacteriophages has not been determined, although it is likely that these enzymes are a part of a repair machinery that responds to RNA degradation by the host. The T4 RNA ligase is a very important tool in molecular biology and is used in numerous protocols. Applications include RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) which can be used in mapping the 5′ and 3′ends of RNA molecules (Liu and Gorovsky 1993), ligation of oligonucleotide adaptors to cDNA or single-stranded primer extension products for PCR (Zhang et al. 1992) and various 5′ nucleotide modifications of nucleic acids. For example, a rapid 5′-labelling method of single-stranded DNA/RNA was developed based on the utilisation of an adenylated intermediate in the reaction of T4 RNA ligase. This method is useful for fluorescence-, isotope- or biotin-labelling of the 5′ ends of both oligo- and polynucleotides (Kinoshita et al. 1997).

Investigations into the overall identity of the RNA ligase 1 sequences from TS2126 and RM378 showed low similarity to each other. The amino acid sequence of RNA ligase 1 from the TS2126 bacteriophage showed more similarity to T4 (18%) than RM378 ligase 1 (15%), indicating that the two proteins of thermophilic origin have evolved and adapted independently to the elevated thermal conditions (Blondal et al. 2005a). The characterisation of TS2126 RNA ligase 1 revealed that it is stable at 60–65°C for an extended time period but loses activity at higher temperatures, similar to the thermostability of RM378 RNA ligase 1. When comparing the activity of TS2126 and RM378 and a commercial T4 RNA ligase 1, the TS2126 ligase showed ~30 and 10 times higher specific activity, respectively. This striking difference in ligation efficiency was also observed in the ssDNA ligation experiments where TS2126 RNA ligase 1 was much more effective than RNA ligases from RM378 and T4. The TS2126 RNA ligase 1 exhibits extremely high activity, high ligation efficiency and moderate thermostability; thus, it can be used in the RLM-RACE assay at elevated temperatures (65°C). This may produce better results if the 5′ donor end of mRNA molecules has a secondary structure that inhibits efficient ligations with T4 RNA ligase at 37°C (Blondal et al. 2005b). The ability to ligate ssDNA at elevated temperatures may also lead to a major advancement in specific applications in molecular biology. In fact, patents covering RNA ligases 1 from both TS2126 and RM378 have been sold to Epicentre® (an Illuminia® company). The RNA ligase 1 from TS2126 is already commercially available under the registered trademark CircLigase™. The thermostable ATP-dependent ligase that

catalyses intramolecular ligation (i.e. circularisation) of ssDNA templates having a 5′-phosphate and a 3′-hydroxyl group can be used for very specific applications. In contrast to T4 DNA ligase and Ampligase® DNA ligase, which ligate dsDNA ends, CircLigase™ ligates ends of ssDNA. The enzyme is therefore useful for making circular ssDNA molecules from linear ssDNA. Circular ssDNA molecules can be used as substrates for rolling-circle replication or rolling-circle transcription. Linear ssDNA of >30 bases is circularised by the CircLigase™ enzyme. Under standard reaction conditions, virtually no linear concatamers or circular concatamers are produced. In addition to its activity on ssDNA, CircLigase™ also has activity in ligating a single-stranded nucleic acid having a 3′-hydroxyl ribonucleotide and a 5′-phosphorylated ribonucleotide or deoxyribonucleotide (EpiCentre 2012). This can be used to map transcription termination sites of prokaryotic RNAs.

Following the characterisation of RNA ligase 1, a polynucleotide kinase (PNK) was identified and characterised in RM378 (Table 10.3), elucidating a defence mechanism against the host similar to that observed in T4. RNA ligase 1 functions together with PNK 1; together, they repair cleaved tRNA molecules. Whereas RNA ligase 1 is essential for ligation of the cleaved tRNA molecules, PNK 1 modifies the tRNA fragments to make appropriate substrates for the ligation step (Silber et al. 1972; Lillehaug and Kleppe 1977). PNK 1 is a bifunctional nucleic acid processing enzyme with 5′-kinase and 3′-phosphatase activities, catalysing the restoration of 5′-phosphate and 3′-hydroxyl termini in cleaved ssRNA and ssDNA. It functions in two major ways: (1) to remove the 2′:3′-cyclic phosphate from the 5′ tRNA fragment and (2) to add a phosphate group to the 5′-hydroxyl group of the 3′-tRNA fragment using ATP as the phosphate donor (Wang et al. 2002). The RNA ligase 1 and the PNK 1 are thus part of the same system, acting in concert to repair RNA and DNA. A study showed that RM378 PNK 1 carries out the same or very similar processes as those of the T4 PNK 1 (Blondal et al. 2005a). Characterisation of the RM378 PNK 1 protein showed thermostability up to 60–65°C; this is consistent with the optimum temperature of the natural environment of its host, *Rhodothermus marinus*. The RM378 PNK 1 shares only its 5′-kinase domain with the PNK family but no apparent homology to the 3′-phosphatase domain in that family. Its similarity with the T4 5′-kinase is low, but they share the P-loop motif, which is characteristic of many phosphotransferase families. Because of its unique phosphatase domain, RM378 PNK 1 resembles the mammalian PNKs rather than other phage PNKs in terms of domain arrangement (Zhu et al. 2004; Blondal et al. 2005a). This is a very interesting observation, because eukaryotic PNKs function as repair enzymes on double-stranded DNA. The T4 PNK 1 is used for labelling the 5′-termini of nucleic acids, and the labelled products can be used as markers for gel electrophoresis, primers for DNA sequencing, primers for PCR and probes for hybridisation (Stahl et al. 1991; Hilario 2004). The RM378 PNK 1 may have advantages in some protocols due to its stability at higher temperatures. The company Prokazyme is selling the RM378 PNK 1 under the registered trademark ThermoPhage™ polynucleotide kinase, and their protocol mentions several application areas including labelling of nucleic acids using 32P-γ-ATP for probes and DNA sequencing, phosphorylation of nucleic acids for subsequent ligation for cloning, phosphorylation of

oligonucleotides for ligase reaction like the ligase chain reaction and similar procedures and phosphorylation of nucleic acids with modified phosphates (i.e. thiol-phosphates) for subsequent modifications and/or labelling. ThermoPhage™ polynucleotide kinase is the only available thermostable enzyme of its kind (Prokazyme 2012) opening up for exploration of high-temperature applications with a PNK.

Another study characterised a novel nonspecific nuclease from the thermophilic bacteriophage GBSV1 (Table 10.3) (Song and Zhang 2008). Nucleases are defined as a group of enzymes which are capable of hydrolyzing the phosphodiester linkages of nucleic acids. According to the substrates they hydrolyze, nucleases are divided into two groups: sugar specific nucleases (deoxyribonucleases and ribonucleases) and sugar nonspecific nucleases. Sugar nonspecific nucleases are characterised by their ability to hydrolyze both DNA and RNA without exhibiting pronounced base preferences. Sugar nonspecific nucleases play very important roles in different aspects of basic genetic mechanisms, including DNA salvage, repair, recombination and degradation. Moreover, they are involved in nutrition, scavenging for nucleotides and phosphates for growth and metabolism (Hsia et al. 2005). They have been isolated from a wide variety of sources including fungi, bacteria and viruses. The majority of these enzymes are intracellular, but some have been reported to be extracellular in nature (Legerski et al. 1978; Rangarajan and Shankar 1999). The ability of sugar nonspecific endonucleases to recognise a wide variety of nucleic acid structures has led to considerable efforts to evaluate their role in different cellular processes as well as application as analytical tools to study nucleic acid structure. Applications also include rapid sequencing of RNA, the removal of nucleic acids during protein purification and the use as antiviral agents (Rangarajan and Shankar 1999; Song and Zhang 2008). Compared with mesophilic enzymes, thermostable nucleases may possess novel properties in structures and biological functions. The novel GBSV1 nonspecific nuclease purified from GBSV1 is the first nuclease isolated from a thermophilic virus. GBSV1 nonspecific nuclease is able to degrade a variety of nucleic acids, including RNA, ssDNA and dsDNA that is either circular or linear. It is active at temperatures ranging from 20 to 80°C with an optimal temperature of 60°C, which is higher than those of most reported nonspecific nucleases. GBSV1 nonspecific nuclease could be obtained in large quantity by expression in *E. coli*. This would facilitate its biotechnological applications (Song and Zhang 2008).

10.5.2 Industrial Potential of Thermophilic Viruses

Thermophilic viruses have adapted to extreme hot environments. Besides high temperature, hot environments are often accompanied by a set of harsh conditions, consisting of physical extremes (e.g. pressure or radiation) and geochemical extremes such as salinity and pH. All biomolecules within the virus particles, including proteins, nucleic acids and possibly lipids, must be adapted to these conditions. Therefore, thermophilic viruses offer a source of thermostable enzymes that display outstanding stability against high temperatures and can often cope with a set of harsh conditions. Driven by increasing industrial demands for biocatalysts that

can cope with industrial process conditions, considerable efforts have been devoted to the search for such enzymes. Biocatalysis uses natural catalysts, such as enzymes, to perform chemical transformations on organic compounds compared with organic synthesis; biocatalysts often have far better chemical precision, which can lead to more efficient production of single stereoisomers, fewer side reactions and a lower environmental burden. Approximately 3,000 different enzymes have been identified, and many of these have found their way into biotechnological and industrial applications (van den Burg 2003). However, there is a demand for enzymes which can withstand industrial reaction conditions. The reasons to exploit enzymes that are stable and active at elevated temperatures are obvious. At elevated temperatures, the solubility of many reaction components, in particular polymeric substrates, is significantly improved. Moreover, the risk of contamination, leading to undesired complications, is reduced at higher temperatures (van den Burg 2003). As a result, increasing attention is given to microorganisms that are able to thrive in hot environments. The exceptional diversity and novelty of thermophilic archaeal viral genomes indicates that novel enzymes may be encoded. One example is the putative provirus XQ2 entrapped in the genome of *S. solfataricus* P2 (She et al. 2001). The genome of the XQ2 is about 60 kb which encodes many hypothetical proteins as well as recognisable metabolic enzymes. The sizes of thermophilic viral genomes range from 5 to 153 kb, showing the coding capacity for metabolic genes. Recently, an EU funded collaborative project, HotZyme, was initiated aiming at a global investigation of biodiversity of the hot environments on Earth. Most importantly, novel hydrolases with improved performance in different industrial fields are expected to be discovered from thermophilic microorganisms and their viruses (HotZyme 2012).

10.5.3 Nanotechnological Potential of Thermophilic Viruses

A virus is a nanoscaled biomolecular unit composed of genes, protecting capsid proteins, and eventually envelopes. In recent years, naturally occurring bionanoparticles, including virus capsids and protein cages, have been studied and utilised as templates and building blocks for applications in biotechnology. Virus capsids and protein cages have received special attention because they are naturally self-assembled with atomic precision. Viral nanoparticles (VNPs) have several advantages such as their nanometre range size, their propensity to self-assemble into monodisperse nanoparticles of discrete size and shape, their high degree of symmetry and polyvalency, their relative ease of producing large quantities of material, and their exceptional stability, robustness and biocompatibility (Steinmetz et al. 2008b). The particles are composed of programmable units, which can be modified by either genetic modification or chemical bioconjugation methods. Due to these properties, viruses hold promise for development as amenable platforms for diverse applications in biotechnology, electronics and medicine (Wiedenheft et al. 2007). Naturally occurring protein cage architectures, such as the spherical cagelike architectures of ferritins, can mediate the deposition of ‘hard’ inorganic materials within the spatial confines of a ‘soft’ protein container. For example, Cowpea chlorotic mottle

virus (CCMV) is composed of 180 identical coat proteins that self-assemble to form a protein cage where RNA is encapsulated (Speir et al. 1995). CCMV undergoes reversible swelling when pH varies. Capsid swelling of CCMV at pH levels greater than 6.5 results in pore opening of the capsid protein assembly (Tama and Brooks 2002). Capsid protein swelling allows for the exchange of molecules, which in turn enables the release or entrapment of target molecules (Douglas and Young 1998). The development of VNPs for nanotechnological applications often requires that they withstand harsh conditions during either fabrication or use. Bioconjugation technique often requires the organic solvent dimethyl sulfoxide (DMSO) in concentrations of at least 20% by volume, and many applications rely on temperature stability. This has prompted efforts aimed at exploiting the thermostability and unique features found in hyperthermophiles. A recent study investigated *S. islandicus* rod-shaped virus 2 (SIRV2) as a candidate VNP (Table 10.3) (Steinmetz et al. 2008a). SIRV2 particles were found to be stable in two different solvent/water mixtures that are of relevance for bioconjugation and mineralisation reactions. Moreover, SIRV2 particles offer attachment sites allowing selective chemical modification. It was found that the major coat protein (CP) forms the virus body while the minor CP is located in the tail fibres at the end of the particles. Interestingly, amine reactivity showed that the minor CP could be selectively labelled, and this labelling reaction targeted the ends of the particles only. This suggests that various functional molecules can be installed at different positions in the VNP, offering multiple and various chemical attachment sites. SIRV2 remained intact and infectious in DMSO in concentrations up to 50% by volume for at least 6 days and is naturally stable at 80°C and pH 3. Overall, SIRV2 represents an extremely stable and structurally interesting VNP with the potential for novel biotechnological applications (Steinmetz et al. 2008a).

10.5.4 Perspectives

Enzymes from thermophilic viruses offer the opportunity to greatly expand the reaction conditions of biocatalysis. Furthermore, unique features in morphology and virus–host interactions may be used in tailored applications in biomedicine and nanotechnology. However, the current repertoire of viral enzymes only hints at their overall potential. The most commonly used enzymes are derived from a surprisingly small number of cultivated viruses, which is remarkable considering the enormous morphological and genomic diversity of viruses revealed over the past decade (Schoenfeld et al. 2010). Developments in the cultivation and production of thermophilic viruses, and developments related to the cloning and expression of their genes in heterologous hosts, will help in the search of novel enzymes with biotechnological/industrial potential. In addition, thermophilic viruses are likely to be useful tools for studying host evolution and host biochemical pathways, providing us with information concerning life in hot environments. Thermophilic viruses have many unique features not observed in other studied viruses; there may be a potential to exploit these features in biomedicine.

Viral genomes are relatively simple compared with those of their hosts and contain a comparatively high proportion of genes coding for structural proteins (e.g. coat and tail) together with proteins involved in nucleic acid metabolism and lysis (Schoenfeld et al. 2010). The density of certain genes in viral genomes offers a great advantage when looking for novel enzymes. For example, a typical bacterial genome of about 2 Mb contains only a single *pol I* gene (coding for DNA polymerase I). By contrast, between 20 and 40 *pol* genes per 2 Mb were found in viral metagenomic sequences. Metagenomic approaches have been used in ecological investigations of hot environments. However, viral metagenomics can also be used to uncover novel biological features of viruses, ultimately, producing useful enzymes. Many challenges are associated with metagenomic-based enzyme discovery. An inherent difficulty in viral metagenomics is isolating adequate amounts of genomic material for large-insert library construction (Edwards and Rohwer 2005). Another challenge arises during sequence assembly. Very large scale sequencing of viral metagenomics should permit the assembly of large contiguous stretches of DNA and potentially entire genomes. However, the high degree of sequence polymorphism within viral populations has largely confounded attempts to assemble large contigs with high confidence (Schoenfeld et al. 2008). Assembly at high stringency tends to prevent misassembling noncontiguous parts of the genome, but such stringent assembly can lead to overestimation of unique viral types and can prevent discovery of genes, enzymes and genomes. The identification of viral genes from sequence assemblies is difficult owing to the high diversity of viral genes and the relatively low numbers of viral genomes in public sequence databases. Moreover, most viral coding sequences (even for a well-studied bacteriophage such as T4) have no similarity to any known genes. This is especially distinct for thermophilic viruses of the Archaea (Prangishvili and Garrett 2005), lowering the likelihood of finding genes by similarity. These problems are being addressed, and viral metagenomics offers a mean of exploring genetic diversity within the vast uncultivated portion of thermophilic viruses.

10.6 Conclusions

Thermophilic viruses show a huge potential as useful tools in a broad field of biotechnology. Novel genetic and morphological features, in combination with a huge unexplored diversity, promise new discoveries which are essential to the development of new applications both in industry and basic research.

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Part II
Genomics, Metagenomics and Biotechnology

Chapter 11
Genomics of Thermophilic Bacteria and Archaea

Takaaki Sato and Haruyuki Atomi

Abstract An overview of the genome analyses of (hyper)thermophilic archaea and bacteria has been provided in this chapter. Basic information such as the species with published genome sequences, their genome size, and predicted gene number are presented. Genes that are specifically present on the genomes of (hyper)thermophiles are described. Various strategies to utilize the genome sequences for novel enzyme discovery are discussed with several examples. A brief overview of the wide range of omics research that has been performed with (hyper)thermophiles is also briefly dealt with.

Keywords Genomics • Thermophiles • Archaea • Bacteria • Enzyme discovery • Omics

11.1 Introduction

It is now a little more than three decades since the complete sequence of bacteriophage ϕX174 DNA was reported (Sanger et al. 1977a). The length of the DNA was 5,375 bp, containing nine open reading frames. At present, complete genomes from a wide range of organisms, from microbes to plants and animals, which consist of millions or even billions of base pairs, have been or are being sequenced. An overview of the current status of published genome sequences and ongoing genome

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sequencing projects can be found at the Genomes OnLine Database (GOLD) at http://www.genomesonline.org/. There are now over 3,000 genome sequences that have been published, with approximately 150 from the Archaea, 2,700 from the Bacteria, and 170 from the Eucarya. In terms of ongoing projects, there are roughly 200 projects on archaeal genomes, more than 6,000 for bacteria, and 2,000 for eukaryotes.

This dramatic increase in sequence data reflects the constant improvements in DNA sequence technology. During the past few decades, the dideoxy method, developed by Sanger (Sanger et al. 1977b), and its many modified and automated methods have played a predominant role in DNA and genome sequencing technology. In recent years, however, several methods that rely on completely different chemistry have been developed. These next-generation sequencing (NGS) technologies (Mardis 2008; Metzker 2010) allow the sequencing of giga base (Gb) nucleotides or more per run and include the Genome Analyzer$_{II}$ (GA$_{II}$) from Illumina/Solexa, the Genome Sequencer (GS) FLX+ from Roche/454 Life Sciences, and the SOLiD4 from Life Technologies/Applied Biosystems. The GA$_{II}$ system utilizes reversible terminator-based sequencing, the GS system utilizes pyrosequencing, and the SOLiD4 system relies on di-base probe recognition mediated by ligation. For template preparation, the GA$_{II}$ system uses solid-phase amplification, whereas the latter two systems utilize emulsion PCR.

Owing to the progress in PCR and sequencing techniques, it is now also possible to sequence nucleic acids that are obtained directly from environmental samples in mass. This metagenome approach is frequently used to examine the population or diversity of microbes in a given environment (Perevalova et al. 2008; Reigstad et al. 2010). As the methodology does not require cultivation, biases brought about by differences in growth characteristics can be avoided and thus allow us to recognize microbes that cannot be grown under conventional culture conditions. In addition, by assembling these sequences, entire genome sequences of non-cultured and/or non-isolated microorganisms can be determined (Barns et al. 1996; Nunoura et al. 2011; Schleper et al. 1998). The sequence data provide a huge library for sequence-based gene screening, and the assembled metagenomes should give us valuable clues as to what is necessary for these organisms to grow in the laboratory.

After determining the raw nucleotide sequence, there comes the annotation stage, where we must identify regions that correspond to open reading frames and predict their functions. This is still a difficult task, and in some cases subjective decisions are unavoidable. How long should an open reading frame be to consider it as a functional gene? How similar should a deduced protein sequence be to allow us to predict functional analogy? Playing it on the safe side may prevent us from overlooking genes or provide more accurate function predictions, but it also has its drawbacks. When we shorten the threshold for the regions we allow to be considered as genes, the number of genes escalates dramatically. If we set a high standard for function prediction, we end up with a huge number of genes with no functional prediction or general functional prediction only. Significant advances have been and are being made in annotation programs, leading to higher accuracy in gene annotation.

In many published bacterial/archaeal genome analyses, we find that the functions of more or less half of the genes are not predictable from their primary structures. In addition, experimental studies have revealed that the functional predictions are, in numerous cases, not accurate. It is therefore most likely that we do not understand the roles of more than half of the genes that are present on bacterial and archaeal genomes.

11.2 Critical Review and Analysis: Genomics

11.2.1 The Genomes of Thermophilic Bacteria and Archaea

There are 2,967 complete published genome projects listed in the Genomes OnLine Database (GOLD). Among them, 133 genomes are of thermophilic and hyperthermophilic organisms. Genome sequences from 47 hyperthermophilic archaea, 12 hyperthermophilic bacteria, 13 thermophilic archaea, and 61 thermophilic bacteria have been determined (Tables 11.1 and 11.2).

Among the (hyper)thermophilic archaea, 28 sequences are from the Crenarchaeota, 29 from the Euryarchaeota, 1 from the Korarchaeota, and 1 from the Nanoarchaeota. There are several metagenome sequences from the Archaea, and *Caldiarchaeum subterraneum* has been suggested to be a thermophile and classified to Aigarchaeota. There are a number of orders within which there are four or more genome sequences available from (hyper)thermophiles: Desulfurococcales (11), Thermoproteales (10), Thermococcales (9), Sulfolobales (7), Methanococcales (7), and Archaeoglobales (4). On the other hand, Methanopyrales (*Methanopyrus kandleri*) and Methanosarcinales (*Methanosaeta thermophila*) have only one (hyper)thermophilic member whose genome has been determined.

In the case of Bacteria, Firmicutes (32), Thermotogae (10), Aquificae (6), Chloroflexi (4), and Actinobacteria (3) are relatively well-analyzed phyla in terms of genome analysis, with three or more genome sequences available. On the other hand, the phyla Nitrospirae, Bacteroidetes, Deferribacteres, Proteobacteria, and Verrucomicrobia include only one thermophile member with a genome sequence.

11.2.2 Signatures in the Genomes of (Hyper)thermophiles

It is considered that proteins from (hyper)thermophiles tend to contain fewer Cys, Met, Asn, and Gln residues than other amino acid residues, and they are usually buried inside the proteins, as these amino acids are labile under high temperature (Taylor and Vaisman 2010). Taylor reported that the proportions of Cys, Asp, Asn, Gln, and Ser residues are relatively low in proteins from extremely thermophilic and hyperthermophilic organisms, while those of Glu, Arg, and Val are relatively high

Table 11.1 Genome sequences from the thermophilic and hyperthermophilic archaea

Phylum	Order	Species[a]	Strain	Size(kb)	ORFs	Reverse gyrase	FBPase V	Growth temperature (T/H)[b]		Publication
Crenarchaeota										
	Desulfurococcales	*Aeropyrum pernix*	K1	1,669	2820	+	+	70–100	H	DNA Res 6:83–101
		Desulfurococcus fermentans	Z-1312, DSM 16532	1,389		+	+	80–82	H	Unpublished
		Desulfurococcus kamchatkensis	1221n	1,365	1471	+	+	85	H	J Bacteriol 191:2371–2379
		Desulfurococcus mucosus	07/1, DSM 2162	1,314	1421	+	+	85	H	SIGS 4(2):173–182
		Hyperthermus butylicus	DSM 5456	1,667	1671	+	+	95–108	H	Archaea 2:127–135
		Ignicoccus hospitalis	Kin4/I, DSM 18386	1,297	1544	+	+	70–98(90)	H	Genome Biol 9(11):R158
		Ignisphaera aggregans	AQ1, S1,DSM 17230	1,875	2061	+	+	92	H	SIGS 3(1):66–75
		Pyrolobus fumari	1A,DSM 11204	1,843	2038	+	+	106	H	SIGS 4(3):381–392
		Staphylothermus hellenicus	P8, DSM 12710	1,580	1716	+	+	85	H	Unpublished
		Staphylothermus marinus	F1, DSM 3639	1,570	1661	+	+	92	H	BMC Genomics 10:145
		Thermosphaera aggregans	M11 TL, DSM 11486	1,316	1457	+	+	85	H	SIGS 2(3):245–259
	Sulfolobales	*Acidianus hospitalis*	W1	2,137	2424	+	+	50–80	T	Extremophiles 15:487–497
		Metallosphaera cuprina	Ar-4	1,840	2077	+	+	65	T	J Bacteriol 193:3387–3388

	Metallosphaera sedula	DSM 5348	2,191	2388	+	+	70	T	Appl Environ Microbiol 74:682–692
	Sulfolobus acidocaldarius	DSM 639	2,225	2344	+	+	70–75	T	J Bacteriol 187:4992–4999
	Sulfolobus islandicus	L.D.8.5	2,722	3127	+	+	75–85	H	Proc Nat Acad Sci 106(21):8605–8610
	Sulfolobus solfataricus	P2	2,992	3169	+	+	50–87	H	Proc Nat Acad Sci 98:7835–7840
	Sulfolobus tokodaii	7, JCM 10545	2,694	2982	+	+	80	H	DNA Res 8:123–140
Thermoproteales	*Caldivirga maquilingensis*	IC-167	2,077	2055	+	+	85	H	Unpublished
	Pyrobaculum aerophilum	IM2	2,222	2659	+	+	74–104	H	Proc Nat Acad Sci 99:984–989
	Pyrobaculum arsenaticum	PZ6	2,121	2455	+	+	95	H	Unpublished
	Pyrobaculum calidifontis	JCM 11548	2,009	2237	+	+	90–95	H	Unpublished
	Pyrobaculum islandicum	DSM 4184	1,826	2110	+	+	100	H	Unpublished
	Thermofilum pendens	Hrk 5	1,781	1964	+	+	88	H	J Bacteriol 190:2957–2965
	Thermoproteus neutrophilus	V24Sta	1,769	2094	+	+	85	H	Unpublished
	Thermoproteus tenax	Kra1	1,841	2100	+	+	86	H*	PLoS ONE 6(10):e24222
	Thermoproteus uzoniensis	768-20	1,936	2229	+	+	86	H*	J Bacteriol 193:3156–3157

(continued)

Table 11.1 (continued)

Phylum	Order	Species[a]	Strain	Size(kb)	ORFs	Reverse gyrase	FBPase V	Growth temperature (T/H)[b]		Publication
		Vulcanisaeta distributa	IC-017, DSM 14429	2,374	2592	+	+	90	H	SIGS 3(2):117–125
Euryarchaeota										
	Archaeoglobales	*Archaeoglobus fulgidus*	VC-16	2,178	2519	+	+	60–95	H	Nature 390:364–370
		Archaeoglobus profundus	Av18, DSM 5631	1,563	1907	+	+	82	H	SIGS 2(3):327–346
		Archaeoglobus veneficus	SNP6	1,901	2194	+	+	75	H	Unpublished
		Ferroglobus placidus	AE DII12DO, DSM 10642	2,196	2622	+	+	85	H	Unpublished
	Methanobacteriales	*Methanothermobacter marburgensis*	Marburg	1,634	1806	–	+	65–70	T	J Bacteriol 192:5850–5851
		Methanothermobacter thermautotrophicus	Delta H	1,751	1894	–	+	65–70	T	J Bacteriol 179:7135–7155
		Methanothermus fervidus	V24S, DSM 2088	1,243	1361	+	+	83	H	SIGS 3(3):315–324
	Methanococcales	*Methanocaldococcus fervens*	AG86	1,485	1630	+	+	49–89	H	Int J Syst Bacteriol 49:583–589
		Methanocaldococcus infernus	ME	1,328	1513	+	+	55–91	H	Int J Syst Evol Microbiol 53:1931–195
		Methanocaldococcus jannaschii	DSM 2661	1,664	1878	+	+	85	H	Science 273:1058–1073
		Methanocaldococcus sp.	FS406-22	1,760	1879	+	+	90	H	Unpublished
		Methanocaldococcus vulcanius	M7, DSM 12094	1,746	1792	+	+	49–89	H	Int J Syst Bacteriol 49:583–589

	Methanothermococcus okinawensis	IH1	1,662	1662	+	+	40–75	T	Unpublished
	Methanotorris igneus	Ko15	1,854	1843	+	+	85	H	Unpublished
Methanopyrales	*Methanopyrus kandleri*	AV19	1,694	1768	+	+	80–101	H	Proc Nat Acad Sci 99:4644–4649
Methanosarcinales	*Methanosaeta thermophila*	PT	1,879	1833	–	+	55–60	T	Unpublished
Thermococcales	*Pyrococcus abyssi*	GE5	1,765	2059	+	+	67–103	H	Genome Res 11:981–993
	Pyrococcus furiosus	JCM 8422	1,908	2180	+	+	70–103	H	Meth Enzymol 330:134–157
	Pyrococcus horikoshii	OT3	1,738	2366	+	+	80–102	H	DNA Res 5:55–76
	Pyrococcus sp.	NA2	1,861		+	+	70–103	H	J Bacteriol 193:3666–3667
	Pyrococcus yayanosii	CH1	1,716	1952	+	+	98	H	J Bacteriol 193:4297–4298
	Thermococcus gammatolerans	EJ3	2,045	2210	+	+	88	H	Genome Biol 10:R70
	Thermococcus kodakarensis	KOD1	2,088	2411	+	+	85	H	Genome Res 15:352–363
	Thermococcus onnurineus	NA1	1,847	1976	+	+	80	H	J Bacteriol 190:7491–7499
	Thermococcus sibiricus	MM739	1,845	2107	+	+	78	H	Appl Environ Mircobiol 75:4580–4588
Thermoplasmatales	*Picrophilus torridus*	DSM 9790	1,549	1608	–	+	60	T	Proc Nat Acad Sci 101:9091–9096
	Thermoplasman acidophilum	DSM 1728	1,564	1580	–	+	45–63	T	Nature 407:508–513

(continued)

Table 11.1 (continued)

Phylum	Order	Species[a]	Strain	Size(kb)	ORFs	Reverse gyrase	FBPase V	Growth temperature (T/H)[b]		Publication
		Thermoplasma volcanium	GSS1	1,584	1660	–	+	33–67	T	Proc Nat Acad Sci 97:14257–14262
	Unclassified eury	*Aciduliprofundum boonei*	T469	1,486	1544	+	+	70	T	Unpublished
Nanoarchaeota		*Nanoarchaeum equitans*	Kin4-M	490	643	+	–	70–90	H	Proc Nat Acad Sci 100:12984–12988
Korarchaeota		Candidatus *Korarchaeum cyrptofilum*	OPF8	1,590	1707	+	+	74–93	H	Proc Nat Acad Sci 105:8102–8107
Aigarchaeota		Candidatus *Caldiarchaeum subterraneum**		1,681	1730	+	+	Metagenome	T	Nucleic Acids Res 39:3204–3223, 2011

The information shown in this Table are based on data provided by Genomes OnLine Database (GOLD)

[a]Organisms added to the Table are indicated with asterisks

[b]*H* hyperthermophile, *T* thermophile. Data modified here are indicated with asterisks

Table 11.2 Genome sequences from the thermophilic and hyperthermophilic bacteria

Phylum	Order	Species	Strain	Size(kb)	ORFs	Reverse gyrase	FBPase V	Growth temperature	(T/H)[a]	Publication
Actinobacteria	Actinomycetales	*Thermobispora bispora*	R51, DSM 43833	4,189	3662	–	–	55–65	T	SIGS 2(3): 327–346
		Thermomonospora curvata	DSM 43183	5,639	5061	–	–	65	T	SIGS 4(1):13–22
	Rubrobacterales	*Rubrobacter xylanophilus*	DSM 9941	3,225	3330	–	–	60	T	Unpublished
Aquificae	Aquificales	*Aquifex aeolicus*	VF5	1,551	1663	+	+	96	H	Nature 392:353–358
		Desulfurobacterium thermolithotrophum	BSA, DSM 11699	1,541	1594	+	–	70	T	Unpublished
		Hydrogenobacter thermophilus	TK-6, DSM6534	1,743	1963	+	+	70	T	J Bacteriol, Epub
		Persephonella marina	EX-H1	1,930	1981	+	–	73	T	J Bacteriol 191:1992–1993
		Thermocrinis albus	HI 11/12, DSM 14484	1,500	1652	+	+	80	H	SIGS 2(2):194–202
		Thermovibrio ammonificans	HB-1, DSM 15698	1,682		+	–	60–80	T	Unpublished
Bacteroidetes	Sphingobacteriales	*Rhodothermus marinus*	R-10, DSM 4252	3,386	2962	–	–	65	T	SIGS 1(3):283–291
Chloroflexi	Anaerolineales	*Anaerolinea thermophila*	UNI-1	3,532	3235	–	+	50–60	T	Unpublihsed
	Chloroflexales	*Chloroflexus aurantiacus*	J-10-fl	5,258	3966	–	–	52–60	T	BMC Genomics 12:334
	Thermomicrobiales	*Thermomicrobium roseum*	DSM5159	2,006	1925	–	–	70	T	PLoS ONE 4:e4207
	Unclassified	*Thermobaculum terrenum*	YNP1, ATCC BAA-798	3,101	2930	–	–	65–92	T	SIGS 3(2):153–162

(continued)

Table 11.2 (continued)

Phylum	Order	Species	Strain	Size(kb)	ORFs	Reverse gyrase	FBPase V	Growth temperature	(T/H)[a]	Publication
Deferribacteres	Deferribacterales	*Deferribacter desulfuricans*	SSM1, DSM 14783	2,234	2184	–	–	40–70	T	DNA Res, Epub
Deinococcus-thermus	Thermales	*Marinithermus hydrothermalis*	T1, DSM 14884	2,269		–	+	67.5	T	Unpublished
		Meiothermus ruber	DSM 1279	3,097	3105	–	–	50–60	T	SIGS 3(1):26–36
		Oceanithermus profundus	506, DSM 14977	2,303	2301	–	–	60	T	SIGS 4(2):210–220
		Thermus aquaticus	Y51MC23	2,338	2593	–	+	70	T	Unpublished
		Thermus scotoductus	SA-01, ATCC 700910	2,346	2506	–	+	65	T	BMC Genomics 12(1):577
		Thermus thermophilus	HB27	1,894	2278	–	+	68	T	Nat Biotechnol 22:547–553
		Thermus thermophilus	HBB	1,849	2307	+	+	70–75*	T	Unpublished
Dictyoglomi	Dictyoglomales	*Dictyoglomus thermophilum*	H-6-12, ATCC 35947	1,959	1912	+	–	78	T	Unpublished
		Dictyoglomus turgidum	DSM6724	1,855	1744	+	–	75	T	Unpublished
Firmicutes	Aquificales	*Sulfurihydrogenibium yellowstonense*	SS-5	1,543	1713	+	–	70	T	J Bacteriol 191:1992–3
	Bacillales	*Alicyclobacillus acidocaldarius*	104-IA, DSM 446	3,018	3032	–	–	60	T	SIGS 2(1):9–18
		Anoxybacillus flavithermus	WK1, DSM 2641	2,846	2863	–	–	60–65	T	Genome Biol 9:R161
		Caldalkalibacillus thermarum	TA2.A1	2,986	3105	–	–	70	T*	J Bacteriol 193:4290–4291
		Geobacillus kaustophilus	HTA426	3,544	3733	–	–	40–75	T	Nucleic Acids Res 32:6292–6303
		Geobacillus thermodenitrificans	NG80-2	3,607	3705	–	–	65	T	Proc Nat Acad Sci 104:5602–5607

	Geobacillus thermoglucosidasius	C56-YS93	3,893	4040	–	–	55–65	T*	Unpublished
Clostridiales	*Symbiobacterium thermophilum*	IAM 14863	3,566	3529	–	–	60	T	Nucleic Acids Res 32:4937–4944
	Thermaerobacter marianensis	7p75a, DSM 12885	2,844	2435	–	+	75	H	SIGS 3(3):337–345
	Thermaerobacter subterraneus	C21, DSM 13965			–	+	70	T	Unpublished
Halanaerobiales	*Halothermothrix orenii*	H 168	2,578	2366	–	–	60	T	PLoS ONE 4:e4192
Selenomonadales	*Thermosinus carboxydivorans*	Nor1	3,000	2862	–	–	60	T	Unpublished
Thermoanaerobacterales	*Ammonifex degensii*	KC4	2,129	2210	+	+	70	T	Unpublished
	Caldicellulosiruptor bescii	Z-1320, DSM 6725	2,919	2832	+	–	75	T	Unpublished
	Caldicellulosiruptor hydrothermalis	108	2,770	2686	+	–	79	H	J Bacteriol 193:1483–1484
	Caldicellulosiruptor kristjanssonii	177R1B	2,786	2691	+	–	78	H	J Bacteriol 193:1483–1484
	Caldicellulosiruptor kronotskyensis	2002	2,843	2643	+	–	70	T	J Bacteriol 193:1483–1484
	Caldicellulosiruptor lactoaceticus	6A, DSM 9545	2,674	2545	+	–	68–75	T	J Bacteriol 193:1483–1484
	Caldicellulosiruptor obsidiansis	OB47	2,532	2389	+	–	79	H	J Bacteriol 192:6099–6100
	Caldicellulosiruptor saccharolyticus	DSM 8903	2,970	2873	+	–	79	T	Appl Environ Microbiol 74:6720–6729
	Carboxydibrachium pacificum	JM, DSM 12653	2,294	1737	–	+	70	T	Unpublished

(continued)

Table 11.2 (continued)

Phylum	Order	Species	Strain	Size(kb)	ORFs	Reverse gyrase	FBPase V	Growth temperature	(T/H)[a]	Publication
		Carboxydothermus hydrogenoformans	Z-2901	2,401	2765	–	+	78	T	PLoS Genet 25:e65
		Coprothemobacter proteolyticus	DSM5265	1,424	1541	–	–	63	T	Unpublished
		Thermoanaerobacter brockii	Ako-1, DSM 3389	2,344	2408	–	+	65	T	J Bacteriol 192:6494–6496
		Thermoanaerobacter italicus	Ab9, DSM 9252	2,451	2478	–	–	70	T	Unpublished
		Thermoanaerobacter mathranii	A3, DSM11426	2,306	2362	–	–	70–75	T	Unpublished
		Thermoanaerobacter pseudethanolicus	39E	2,362	2427	–	+	65	T	Appl Environ Microbol 77:7998–8008
		Thermoanaerobacter sp.	X513	2,456	2534	–	–	60	T	Unpublished
		Thermoanaerobacter sp.	X514	2,457	2467	–	+	60	T	Appl Environ Microbiol 77:7998–8008
		Thermoanaerobacter tengcongensis	MB4T/ JCM11007	2,689	2726	+	+	75	T	Genome Res 5:689–700
		Thermoanaerobacterium thermosaccharolyti-cum	DSM 571	2,785	2830	–	–	55–60	T	Unpublished
		Thermosediminibacter oceani	JW/IW-1228P, DSM 16646	2,280	2348	–	+	52–76	T	SIGS 3(2):108–116
Nitrospirae	Nitrospirales	*Thermodesulfovibrio yellowstonii*	DSM11347	2,003	2033	–	–	65	T	Unpublished

Proteobacteria	Unclassified	*Nitratiruptor* sp.	SB155-2	1,877	1959	+	–	37–65(55)	T	Proc Nat Acad Sci 104:12146–12150
Sprochaetes	Spirochaetales	*Spirochaeta* sp.	Buddy	3,316		–	–	65	T*	Unpublished
		Spirochaeta thermophila	DSM 6192	2,472	2255	–	–	65	T*	J Bacteriol 192:6492–6493
Thermodesulfobacteria	Thermodesulfobacteriales	*Thermodesulfatator indicus*	CIR 29812, DSM 15286	2,322	2291	+	+	70	T	Unpublished
		Thermodesulfobacterium sp.	OPB45	1634	1687	+	+	70	T	Unpublished
Thermotogae	Thermotogales	*Fervidobacterium nodosum*	Rt17-B1	1,948	1895	+	–	70	T	Unpublished
		Thermosipho africanus	TCF52B	2,016	1911	+	–	75	T	J Bacteriol 191:1974–1978
		Thermosipho melanesiensis	B1429	1,915	2002	–	–	70	T	Unpublished
		Thermotoga lettingae	TMOT	2,135	2141	–	–	65	T	Proc Nat Acad Sci 106(14)
		Thermotoga maritima	MSB8	1,860	1961	+	–	80	H	Nature 399:322–329
		Thermotoga naphthophila	RKU-10	1,809	1768	+	–	80	H*	Unpublished
		Thermotoga neapolitana	DSM4359	1,884	1988	+	–	70–75	H	Unpublished
		Thermotoga petrophila	RKU-1	1,823	1911	+	–	80	H	Unpublished
		Thermotoga sp. RQ2	RQ2	1,877	1917	+	–	76–82	H	J Bacteriol 193:5869–5870
		Thermotoga thermarum	LA3, DSM5069	2,039	2066	+	–	80	H	Unpublished
Verrucomicrobia	Methylacidiphilales	*Methylacidiphilum infernorum*	V4	2,287	2521	–	–	60	T	Biol Direct 3:26

The information shown in this Table are based on data provided by Genomes OnLine Database (GOLD)

[a] *H* hyperthermophile, *T* thermophile. Data modified here are indicated with asterisks

(Taylor and Vaisman 2010). The abundant number of Glu residues may be a result of the necessity of a large number of ionic interactions in the proteins for maintaining proper conformation at high temperatures (Taylor and Vaisman 2010).

When we focus on genes that are present only in hyperthermophiles, there are some clear signature genes. All hyperthermophiles possess a reverse gyrase gene without any exception, regardless of whether the organism is a member of the Bacteria or Archaea (Table 11.1). It had been believed that reverse gyrase was the sole gene entirely specific to hyperthermophiles (Forterre 2002). However, as the number of genome sequences accumulated, it became apparent that the gene is also present in several extremely thermophilic organisms (Table 11.1). The presence of the gene has still not been observed on any mesophile genome. Reverse gyrase displays positive supercoiling activity and this had been suggested to play a role in genome thermostability, but further studies have suggested that it exhibits a number of diverse functions (Hsieh and Plank 2006; Kampmann and Stock 2004). A genetic analysis of the gene has indicated that the presence of the gene does provide an advantage for life at high temperatures (Atomi et al. 2004), but the physiological role(s) of the enzyme in hyperthermophiles is still not clearly established.

Another gene that is relatively specific to hyperthermophiles is the class V fructose-1,6-bisphosphatase gene. The gene is widely spread among the hyperthermophiles but can also be found in several extreme thermophiles and mesophiles (Tables 11.1 and 11.2). Five hyperthermophilic bacteria, *Thermotoga maritima*, *Thermotoga neapolitana*, *Thermotoga petrophila*, *Thermotoga* sp. RQ2, and *Thermocrinis albus*, and two archaeal hyperthermophiles, *Ignisphaera aggregans* and *Nanoarchaeum equitans*, are the exceptions at present. The properties of this protein will be described below.

11.3 Critical Review and Analysis: Functional Genomics

11.3.1 Genome-Based Strategies for Novel Enzyme Discovery

Genome sequences provide a huge amount of information that allows us to obtain an overview of the presence or absence of various biological systems in a given organism. In terms of metabolism, the presence of orthologous genes corresponding to the individual enzymes that constitute a given pathway suggests that the pathway is present in the organism, whereas their absence indicates otherwise. In many cases, however, there are situations where although most of the genes of a given metabolic pathway are present, several genes seem to be lacking. These genes are often referred to as "missing" genes and have been a focus of metabolic research in a wide range of organisms. Enzymes that fulfill the functions of these "missing" genes are at least structurally novel, as they cannot be identified by primary structure. They may also utilize chemistry that is distinct from conventional enzymes or even catalyze different reactions that represent new metabolic routes. In any case, the presence of

"missing" genes can be regarded as an opportunity for novel gene and enzyme discovery.

On the genomes of many (hyper)thermophilic archaeal species, genes homologous to previously known fructose-1,6-bisphosphatases (FBPase) and fructose-1,6-bisphosphate aldolases (FBPald), which are key enzymes of sugar metabolism, had not been identified.

Another case was the fact that all (hyper)thermophilic archaea did not harbor a complete set of genes that constitute the pentose phosphate pathway (PPP), necessary for the synthesis of nucleic acids (Fig. 11.1). In addition, several genes necessary for the biosynthesis of cofactors, coenzyme A and flavin adenine dinucleotide, were also not found on many of the archaeal genomes. These problems have been solved during the past decade utilizing various strategies. The most straightforward approach is to identify the missing enzyme/protein by activity measurements and purification. Another alternative is a comparative genomics approach. Searching the genome databases for genes that display a distribution that correlates with its necessity can provide valuable hints in identifying or at least narrowing down candidate genes. Furthermore, one can take into account the location of genes; there are chances that genes that are located in close vicinity to one another on the genome, especially if they form a gene cluster or operon, are functionally linked. In the following section, several examples of the search for "missing" genes that involve hyperthermophiles and/or archaea will be described.

11.3.2 Examples of Novel Enzyme Discovery Initiating from Genome Sequence Data

FBPase is the key enzyme of gluconeogenesis that is responsible for the dephosphorylation of fructose 1,6-bisphosphate (FBP) to fructose 6-phosphate (F6P). As it functions in the reverse direction to that of the glycolytic enzyme phosphofructokinase (PFK), which utilizes ATP/ADP hydrolysis for phosphorylation of F6P, FBPase and PFK must be strictly regulated in order to avoid a futile cycle in which energy is lost. A candidate for the "missing" FBPase was initially proposed through three-dimensional structural examination of a *myo*-inositol monophophatase from the hyperthermophilic archaeon *Methanocaldococcus jannaschii* (FBPase IV: Stec et al. 2000), which indicated that the enzyme could accommodate FBP. A second candidate was identified by purifying the protein exhibiting FBPase activity in another hyperthermophilic archaeon *Thermococcus kodakarensis* grown under gluconeogenic conditions (FBPase V: Rashid et al. 2002). Gene disruption experiments have indicated that the latter enzyme is the physiologically relevant FBPase, at least in *T. kodakarensis* (Sato et al. 2004). As mentioned above, FBPase V homologs display a relatively wide distribution among the hyperthermophilic archaea and bacteria and most likely are responsible for the FBPase reaction in these organisms. Similar strategies have been pursued to identify a number of important "missing" genes. By directly examining enzyme activity in cell extracts,

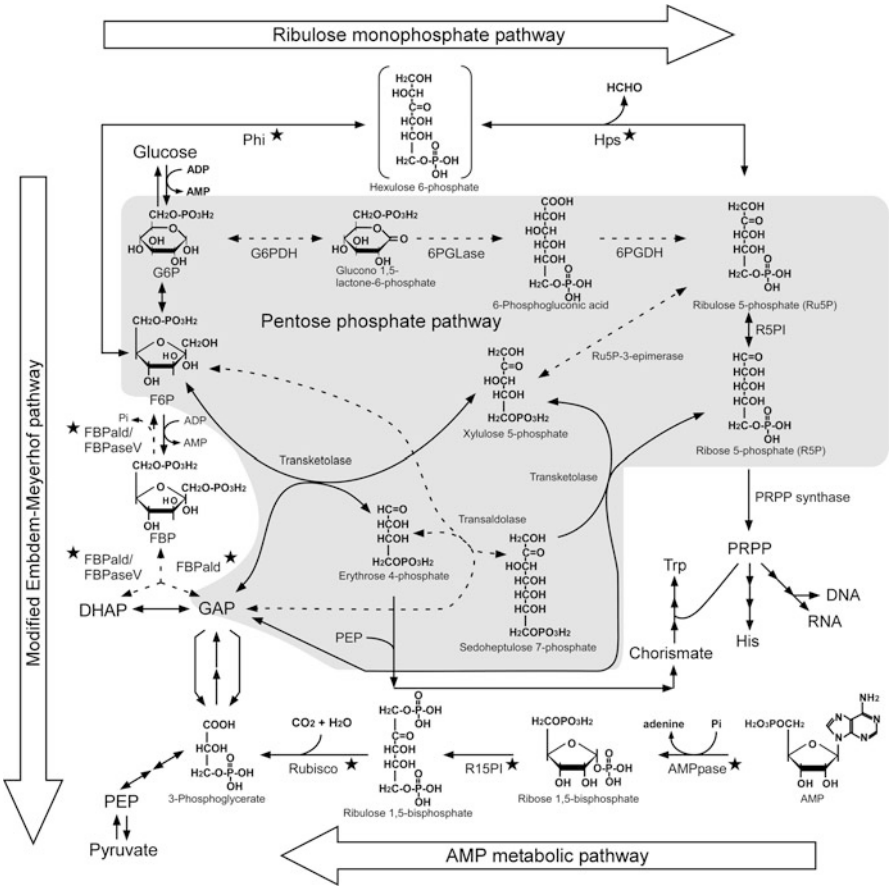

Fig. 11.1 An illustration of the metabolic pathways in *T. kodakarensis* involved in sugar/nucleotide metabolism. The modified Embdem-Meyerhof pathway, the ribulose monophosphate pathway, and the AMP metabolic pathway described in the text are indicated with *arrows*. The pentose phosphate pathway is *shaded*. *Dotted arrows* indicate reactions whose corresponding genes are not found on the genomes of *T. kodakarensis* and other (hyper)thermophilic archaea. Novel proteins described in the text are indicated with *stars*. Enzyme abbreviations: *FBPald/FBPase V* bifunctional fructose-1,6-bisphosphate aldolase/fructose-1,6-bisphosphatase V, *Phi* 6-Phospho-3-hexuloisomerase, *Hps* 3-Hexulose-6-phosphate synthase, *G6PDH* glucose-6-phosphate dehydrogenase, *6PGLase* 6-phosphoglucono-δ-lactonase, *6PGDH* 6-phosphogluconate dehydrogenase, *AMPpase* AMP phosphorylase, *R15PI* ribose-1,5-bisphosphate isomerase, *Rubisco* ribulose-1,5-bisphosphate carboxylase/oxygenase. Compound abbreviations: *G6P* glucose 6-phosphate, *F6P* fructose 6-phosphate, *FBP* fructose 1,6-bisphosphate, *DHAP* dihydroxyacetone phosphate, *GAP* glyceraldehyde 3-phosphate, *PEP* phosphoenolpyruvate, *PRPP* phosphoribosylpyrophosphate

a novel 2-keto-3-deoxygluconate aldolase has been identified in the thermophilic acidophile *Picrophilus torridus* (Reher et al. 2010).

By taking note of similarity in primary structure towards yeast phosphatidylinositol synthase, a novel archaetidyl inositol phosphate synthase was discovered

in *Methanothermobacter thermautotrophicus* (Daiyasu et al. 2005; Morii et al. 2009). Additionally, a comparison of three-dimensional crystal structures, as in the case of the identification of FBPase IV, has led to the identification of a novel, archaeal riboflavin kinase (Mashhadi et al. 2008).

FBPald catalyzes the reversible cleavage of FBP to glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP), a key step in both glycolysis and gluconeogenesis. The first candidate for hyperthermophilic/archaeal FBPald was proposed based on the fact that an atypical *dhnA* gene in *Escherichia coli* exhibits FBPald activity (Galperin et al. 2000; Thomson et al. 1998) and that archaeal species including most of the hyperthermophiles harbor this *dhnA* homolog. The protein products encoded by this gene from *Thermoproteus tenax* and *Pyrococcus furiosus* were confirmed to display metal-independent FBPald activity and thus designated as class IA FBP aldolase (Fig. 11.1, FBPald) (Siebers et al. 2001). Judging from its kinetic properties, class IA FBP aldolases were considered to mainly participate in glycolysis, although classical FBP aldolases are known to also catalyze the reverse reaction. In the search for identifying a novel FBPald that functions in gluconeogenesis, Say et al. discovered that the FBPase V protein also exhibits FBPald activity (Say and Fuchs 2010). This result indicated that this protein is a bifunctional enzyme responsible for the conversion from GAP/DHAP to F6P, exclusively functioning in gluconeogenesis (Fig. 11.1, FBPald/FBPase V).

Most of the archaeal (hyper)thermophiles do not harbor complete sets of genes encoding the components of PPP. A comparative genomics study led to solve this problem. Soderberg pointed out that among the many archaea that lacked a complete set of the PPP genes, many harbored genes that encoded the two enzymes of the ribulose monophosphate (RuMP) pathway (Soderberg 2005). Although the RuMP pathway had been considered to function as a pathway to detoxify formaldehyde by fixing it to ribulose 5-phosphate (Kato et al. 2006; Sakai et al. 1999; Yanase et al. 1996; Yurimoto et al. 2002), the pathway in archaea was proposed to function in the reverse direction, thereby generating pentose phosphates from F6P. This hypothesis was confirmed by gene disruption experiments in *T. kodakarensis* (Orita et al. 2006). Furthermore, although *M. jannaschii* harbors a complete set of PPP genes, metabolite analysis indicated that this organism also provides pentoses via the RuMP pathway and not the PPP (Fig. 11.1, Phi and Hps) (Grochowski et al. 2005). Other studies based on comparative genomics that led to the discovery of novel enzymes/pathways in the archaea include those on the biosynthesis of CoA (pantothenate kinase and phosphopantothenate synthase) (Yokooji et al. 2009) and heme biosynthesis (*S*-adenosyl-L-methionine-dependent uroporphyrinogen III methyltransferase and precorrin-2 dehydrogenase) (Storbeck et al. 2010). It should be noted that one of the novel carbon fixation pathways in Archaea, the 3-hydroxypropionate/4-hydroxybutyrate cycle, was also discovered by this strategy (Berg et al. 2007).

There are also cases in which genes encode an enzyme that is apparently isolated from a metabolic point of view. These genes are here designated as “lonely” genes. Due to their metabolic isolation, the metabolic functions of these “lonely” genes are of course unknown. One example is the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) genes present in a number of members of the Euryarchaeota.

Rubisco is well known as the key enzyme of the Calvin-Benson-Bassham (CBB) cycle for carbon dioxide fixation (Bassham et al. 1953; Hartman and Harpel 1994). Although many of the archaeal Rubiscos have actually been shown to display carboxylase/oxygenase activity (Ezaki et al. 1999; Watson et al. 1999), the presence of the gene is not limited to autotrophic archaea, and a number of signature genes of the CBB cycle, especially the gene encoding phosphoribulokinase which generates the Rubisco substrate, are not found on the archaeal genomes. The function of this "lonely" gene was elucidated by a comparative genomics approach. Genes that co-occurred with archaeal Rubiscos were focused upon and examined taking catalytic promiscuity (O'Brien and Herschlag 1999) into consideration. Archaeal Rubiscos are now considered to participate in a novel AMP metabolic pathway consisting of AMP phosphorylase, ribose-1,5-bisphosphate isomerase, and Rubisco (Sato et al. 2007).

The pathways in thermophiles responsible for amino acid biosynthesis are also a focus of attention. In terms of lysine biosynthesis, two classical pathways had been known; the diaminopimelic acid (DAP) pathway which is utilized by the majority of bacteria (Bukhari and Taylor 1971) and plants (Hudson et al. 2005) and the α-aminoadipic acid (AAA) pathway, the predominant route in eukaryotes (Bhattacharjee 1985; Xu et al. 2006) (Fig. 11.2). Although *Thermus thermophilus* HB27 is a member of thermophilic bacteria, its lysine biosynthetic pathway had been obscure. In this organism, a third lysine biosynthetic pathway was discovered by genetic and enzymatic analyses of a gene cluster that complemented lysine auxotrophy of a mutant strain (Kobashi et al. 1999; Kosuge and Hoshino 1997, 1998; Nishida et al. 1999). Aspartate kinase catalyzes the first step of the DAP pathway in which aspartate is converted to 4-phosphoaspartate, a precursor of threonine, methionine, and lysine. The disruption of aspartate kinase in *T. thermophilus* resulted in auxotrophy toward only threonine and methionine, suggesting the presence of a lysine biosynthetic pathway independent of aspartate kinase. In order to investigate lysine biosynthesis in this organism, four lysine auxotrophic mutants were prepared with chemical mutagenesis. Addition of α-aminoadipic acid restored lysine autotrophy in two of these mutants, whereas DAP did not complement the lysine auxotrophy in any of the mutants (Kobashi et al. 1999). Analysis of a gene cluster that rescued the lysine auxotrophy in *T. thermophilus* indicated that lysine is synthesized via a chimeric pathway consisting of a portion of the AAA pathway from 2-oxoglutarate to α-aminoadipic acid and a pathway from α-aminoadipic acid to lysine that resembles the initial portion of arginine biosynthesis from glutamate (Nishida et al. 1999). The pathways clearly illustrate how organisms utilize similar chemistry for different biosynthetic purposes. As shown in Fig. 11.2, pathways responsible for the conversion from oxaloacetate to glutamate, 2-oxoglutarate to α-aminoadipic acid, and 2-oxoisovalerate to leucine follow the same chemistry. Similarities are also obvious between the pathway in *T. thermophilus* from α-aminoadipic acid to lysine and the initial portion of arginine biosynthesis from glutamate.

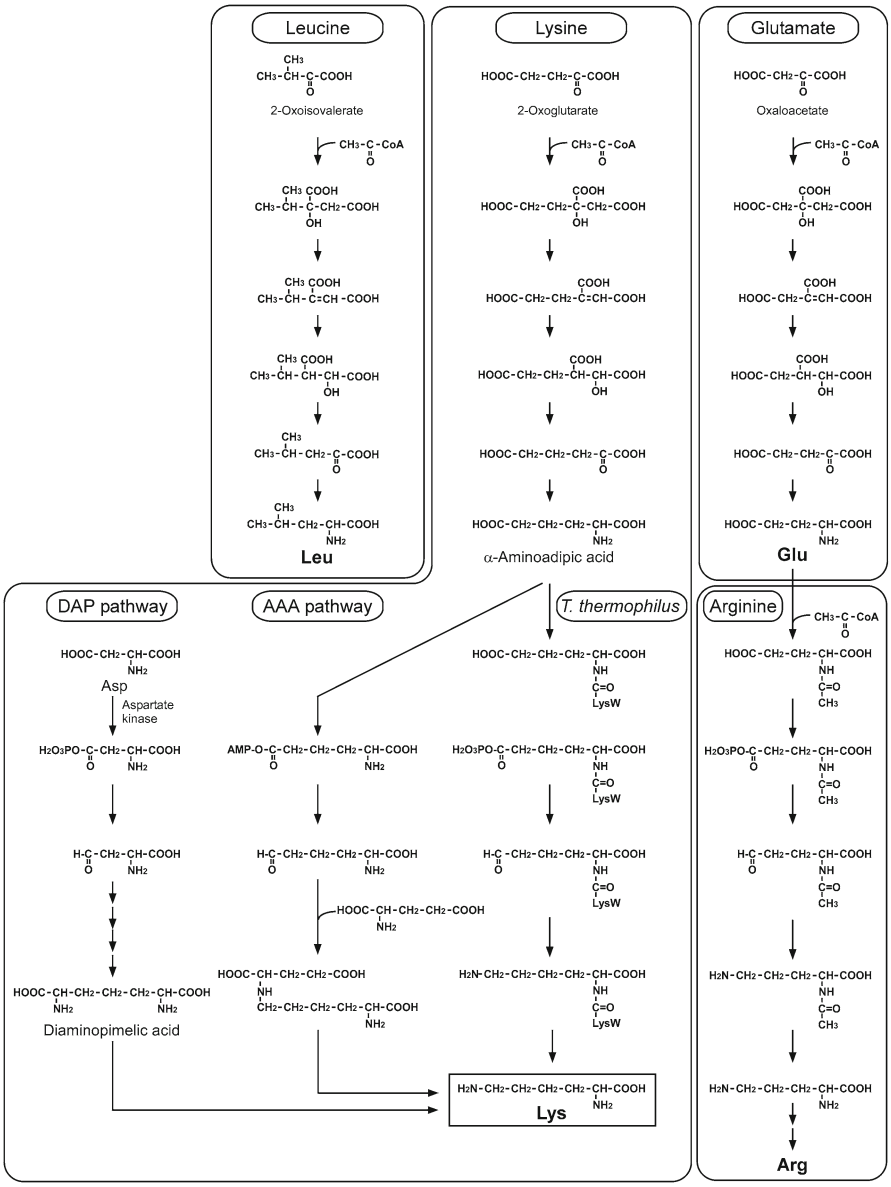

Fig. 11.2 An illustration of the lysine biosynthesis pathway in *T. thermophilus* and a comparison with other biosynthesis pathways involved in the synthesis of glutamate, leucine, and arginine. The classical pathways for lysine biosynthesis, the DAP pathway and AAA pathway, are also shown. LysW is a protein utilized to mask the α-amino group of α-aminoadipic acid

11.3.3 Research on CRISPRs in Hyperthermophilic Archaea

CRISPRs (Clustered Regularly Interspaced Short Palindromic Repeats) are regions which contain multiple, short-sequence repeats separated by spacer sequences. They were first identified in *Escherichia coli* (Ishino et al. 1987; Jansen et al. 2002; Mojica et al. 2005) and have been found in a large number of bacterial and archaeal genomes, including many (hyper)thermophiles. Research on CRISPRs can be regarded as an example of a field that initiated and developed with gene/genome sequences. Although much still needs to be understood, CRISPRs, along with the Cas (CRISPR-associated) proteins, are considered to function as a prokaryotic immune system against exogenous genetic elements such as plasmids and viruses. In *P. furiosus*, it was found that the CRISPR-Cas effector complex prepared from cell extracts recognizes RNA as a substrate. It cleaves complementary target RNAs at a specific distance from the 3′ end of the integral RNAs in vitro (Hale et al. 2009). Furthermore, the nucleotide binding site recognized by Cas6 has been determined (Carte et al. 2010), and a crystal structure of the Cas6 protein bound with a repeat RNA sequence has also been solved (Wang et al. 2011). On the other hand, in *Sulfolobus solfataricus*, along with confirming in vitro endonuclease activity of Cas proteins (SSO1404 (Beloglazova et al. 2008) and SSO2201 (Han and Krauss 2009)), immune activity against viral infection dependent on the CRISPR-Cas system was also demonstrated in vivo (Gudbergsdottir et al. 2011; Manica et al. 2011). It has also been suggested that partial deletions of the CRISPR loci and random dynamic recombination including entire deletions of CRISPR-Cas effectors can occur spontaneously in *Sulfolobus* (Gudbergsdottir et al. 2011).

11.3.4 Genome-Based Omics Research on (Hyper)thermophiles

Complete genome sequences provide the basis for a wide range of omics research. Transcriptome research based on DNA microarrays has been performed on a number of hyperthermophiles (Walther et al. 2011). For example, with *P. furiosus*, transcriptomics have been utilized to identify genes that are involved in carbon (Lee et al. 2006; Schut et al. 2003) and sulfur (Schut et al. 2001) metabolism, the heat (Shockley et al. 2003) and cold (Weinberg et al. 2005) shock responses, and oxidative stress (Strand et al. 2010). *S. solfataricus* and *Sulfolobus acidocaldarius* have also been well studied in terms of carbon sources (Brouns et al. 2006; Snijders et al. 2006) and against a variety of stress conditions (Frols et al. 2007; Simon et al. 2009; Tachdjian and Kelly 2006). In combination with the disruption of transcriptional regulator genes, the technique has been used to identify regulons in *T. kodakarensis* (Kanai et al. 2010). Among the hyperthermophilic bacteria, studies on *T. maritima* are abundant, examining responses toward various sugars (Conners et al. 2005), co-culture with *M. jannaschii* (Johnson et al. 2006), and antibiotic challenge (Montero et al. 2007). Proteomic studies have been performed on *M. jannaschii* in relation to flagellin modification (Mukhopadhyay et al. 2000) and methanogenesis (Zhu et al. 2004). *Thermococcus onnurineus* has been studied with a focus on energy

metabolism and hydrogen production (Yun et al. 2011). Proteome analyses have been utilized to study the relationship between the two hyperthermophilic archaea *Ignicoccus hospitalis* and *N. equitans* (Giannone et al. 2011). In *P. furiosus*, proteome analysis was utilized to identify membrane proteins (Holden et al. 2001), multiprotein complexes (Menon et al. 2009), and metalloproteins (Sevcenco et al. 2009). Recently, the iTRAQ (isobaric tags for relative and absolute quantitation) method has been demonstrated in *S. solfataricus*. In this method, proteins are tagged with iTRAQ reagents and separated and detected with liquid chromatography and MS/MS, respectively. As isobaric tag in iTRAQ reagents contains reporter groups, the ratio of signal intensity in the MS analysis represents the quantitative ratio of proteins in the cells. This method has been applied to identify the membrane proteome in this organism (Pham et al. 2010), as well as to identify genes/proteins involved in ethanol (Chong et al. (2007b)) and *n*-propanol metabolism (Chong et al. (2007a)). A system biology approach (SulfoSYS-project) that integrates a complete set of genomic, transcriptomic, proteomic, metabolomic, and enzymatic information for production of a silicon cell model is actively being carried out, and results on central carbon metabolism have been published (Zaparty et al. 2010).

11.4 Conclusions

As in the case of other organisms, the genomes of a large number of (hyper)thermophiles have been and are being examined. These sequences reveal that there are not many genes that are specific to (hyper)thermophily, with only a few examples including reverse gyrase and the bifunctional FBPald/FBPase V. Although not limited to (hyper)thermophiles, the genome sequences have provided the basis to study the mechanisms of various biological systems, including metabolism. Strategies such as identification of "missing" genes or enzymes metabolically linked to "lonely" genes have led to the discovery of a number of novel enzymes and pathways. The genome sequences have also helped us to utilize a wide range of omics approaches on the hyperthermophiles. These strategies are being applied to biological functions other than metabolism and will surely lead to further exciting discoveries in biology.

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Chapter 12
Comparative Genomics of Thermophilic Bacteria and Archaea

Satoshi Akanuma, Shin-ichi Yokobori, and Akihiko Yamagishi

Abstract Elucidation of the origin and the early evolution of life is fundamental to our understanding of ancient living systems and of the ancient global environment where early life evolved. A number of molecular phylogenetic trees have been constructed by comparing the homologous gene sequences.

In this chapter, we have reviewed the universal trees constructed based on different types of genetic information. The tree topology was different depending on the type of the gene analyzed as well as the method used. The root of the universal tree is most likely placed between the bacterial branch and the common ancestor of Archaea and Eucarya. However, there are possibilities that the root may be within the bacterial branches.

Monophyly of Archaea is rather controversial. Though the rRNA tree suggested the monophyly, other types of the tree are also reported. The conclusive result where the Eucarya originated within/outside of the branch of Archaea is yet to come.

The growth temperature of the ancient organism has long been a topic that has interested many scientists. Theoretical works suggested mesophilic, thermophilic, and hyperthermophilic origin of life, depending on the report. Experimental test analyzing the effect of each or combination of ancestral amino acid residues suggested the hyperthermophilic origin of life. However, we cannot totally deny the possible artifact based on the method used for the estimation of ancestral sequences possessed by the ancestral organisms.

Keywords Commonote • LUCA • Ancient protein • Archaea • Bacteria • Eucarya

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12.1 Introduction

Elucidation of the origin and the early evolution of life is fundamental to our understanding of ancient living systems and of the ancient global environment where early life evolved. Extant genes are evolutionary descendants of ancient genes. Consequently, information on the traits of ancient genes is embedded in the sequences of extant genes. A number of molecular phylogenetic trees have been constructed by comparing the homologous gene sequences. However, the information used in constructing the phylogenetic trees has been limited. The topologies of the trees largely depend on the genes analyzed. In this chapter, we first review the phylogenetic trees built by several different ways and their possible interpretation. We then discuss the nature of the last universal common ancestor predicted from such phylogenetic analyses. Finally, we introduce several studies where ancient proteins were reconstructed by the combination of computational prediction and experimental resurrection of ancient genes. Although the last common ancestor has been often referred to as LUCA, LCA, and senancestor, we use herein "Commonote" (Yamagishi et al. 1998).

12.2 Topology of Universal Trees

Universal trees have been constructed using information carried by sequences of genes. In this section, we will review the point to be considered to obtain the true universal trees, as well as the genes to be used for the construction of the universal tree.

12.2.1 Ribosomal RNA Gene Trees

Small subunit ribosomal RNA (SSU rRNA) gene sequences have been most widely used for phylogenetic analyses. All living organisms contain rRNAs, which are the main components in ribosome involved in protein synthesis. Although the copy numbers of ribosomal RNA genes are often multiple, they are almost identical in an organism (see Hillis and Dixon 1991). SSU rRNA sequences of uncultured organism as well as isolated ones have been extensively analyzed (Barns et al. 1994; Ward et al. 1990). Phylogenetic trees for all of life were constructed by comparing SSU rRNA (gene) sequences and consequently suggested monophyletic status of Bacteria, Archaea, and Eucarya (Woese et al. 1990).

In the SSU rRNA trees, hyperthermophilic or thermophilic organisms are found at the basal position of Archaea and Bacteria (i.e., Woese et al. 1990; Stetter 2006), suggesting the (hyper)thermophilic ancestry of Archaea and Bacteria (Woese et al. 1990; Stetter 2006; Yamagishi et al. 1998). However, hyperthermophilic and thermophilic SSU rRNA genes often show relatively high G+C contents (e.g., Wang and Hickey 2002). Because varied nucleotide compositions among operational taxonomic units (OTUs) may have resulted in the unreliable phylogenetic trees (e.g., Loomis and Smith

1990; Hasegawa and Hashimoto 1993), it could be that placing hyperthermophilic and thermophilic organisms at the basal position of Archaea and of Bacteria is the artifact caused by the high G+C content of their SSU rRNA genes. In addition, evolutionary rates of hyperthermophilic and thermophilic SSU rRNA genes are often slower than those of other organisms (see Woese's tree: Woese et al. 1990). Large variation of evolutionary rate among taxa can also cause the unreliable phylogenetic tree (e.g., Philippe and Laurent 1998). Under the situation, fast-evolving taxa tend to form a group and slow-evolving taxa form another group in a phylogenetic tree. Thus, fast- (and moderately) evolving SSU rRNA genes form a cluster different from clusters consisting of slowly evolving hyperthermophilic and thermophilic SSU rRNA genes.

Fast-evolving taxa tend to be placed near the basal position of the phylogenetic tree. This observation is referred to as "long branch attraction (LBA)." Eukaryotic genes generally show faster evolutionary rate than do bacterial and archaeal genes (e.g., the tree reported in Woese et al. (1990)). Therefore, it is difficult to examine the precise phylogenetic position of Eucarya in the universal tree.

Organisms with parasitic life often show accelerated evolutionary rate. If an evolutionary model where evolutionary pattern and rate are invariable among sites and across time was used, the substitution rate may be underestimated for first-evolving sites and branches, and overestimated for invariant/slow-evolving sites and branches. Accordingly, fast-evolving taxa tend to be placed near the basal position of the tree (LBA). *Nanoarchaeum equitans*, a parasite of another archaeon, is the only member of *Nanoarchaeota* and often represented to be the basal group of Archaea (Huber et al. 2002; Waters et al. 2003). Because *N. equitans* has a long branch in the archaeal tree, the basal position of *N. equitans* may be an artifact due to LBA.

12.2.2 Protein Gene Trees

Many genes encoding proteins have been also used for building universal trees. Elongation factors (EFs) trees (see below for more details) suggested the monophyly of each Archaea, Bacteria, and Eucarya (Iwabe et al. 1989; Baldauf et al. 1996). The monophylies of the three groups were also suggested by the analysis of RNA polymerase sequences (Iwabe et al. 1991) and of ribosomal protein sequence analysis (Fournier and Gogarten 2010). Puigbò et al. (2010) suggested that Archaea and Bacteria tend to show similar phylogenetic trend based on about 100 universal trees.

12.2.3 Genome Trees

Increasing number of complete genome sequences (see public databases such as genbank, EMBL, and DDBJ) enables the phylogenetic analyses based on the genome information. To obtain the reliable phylogenetic tree using genome level information, all of the genes to be analyzed must be orthologous. However, it is not easy to judge

if the protein genes are orthologous or not. For example, the archaeal elongation factor 1α (aEF-1α) is generally regarded as the ortholog of eukaryotic EF-1α (eEF-1α). However, aEF-1α is functionally similar to eukaryotic release factor 3 and HBS1, which are paralogs of eEF1α (Saito et al. 2010). Can we think that aEF-1α is "true" ortholog of eEF-1α? It is not easy to answer this question. We, however, need to remember that the aEF-1α might be under certain selection pressure different from eEF-1α. Similar situation is found for EF-G that is responsible for the translocation of peptidyl tRNA during elongation process of translation. In certain bacteria, EF-G contributes to the ribosomal recycling in the termination process of translation (Zavialov et al. 2005). Some bacterial lineages and mitochondria contain two types of EF-Gs that show different characteristics (Suematsu et al. 2010). Even though the two EF-Gs share the same origin, evolutionary constraints (e.g., rate of substitution, invariable residues) are expected to be different if the roles of these proteins are different.

To obtain a reliable phylogenetic tree, horizontal gene transfer (HGT) should be absent from the analysis. Certain bacterial species have natural transformation ability (e.g., *Thermus* spp., Claverys et al. 2009; Koyama et al. 1986; Hidaka et al. 1994). Horizontal gene transfer event occurred frequently during the early evolution of Bacteria and Archaea. For instance, 24% of protein genes within the *Thermotoga maritima* genome are likely to be the descendants of archaeal genes (Nelson et al. 1999). Horizontal gene transfer between Eucarya and Bacteria may have occurred during the early stage of eukaryote evolution, for example, *Tunicata* (or *Urochordata*), one of three subphyla belonging to *Chordata*. Tunicates are the only multicellular animals producing cellulose. Recent studies suggested that the common ancestor of tunicates might have acquired bacterial cellulose synthetase genes (Sagane et al. 2010).

The numbers of the genes suitable for phylogenetic analysis are limited. Only 31 protein gene families were used for the analysis by Ciccarelli et al. (2006). Most of them are members of the protein families related to translation and transcription. They have reported an unrooted tree including Archaea, Bacteria, and Eucarya. In the tree, Bacteria can be divided into three groups: the basal group is represented by Firmicutes including *Bacilli, Clostridia*, and *Mycoplasmatales*; the second group includes *Actinobacteria* and *Bacteroidetes*; and the third group consists of *Proteobacteria, Cyanobacteria, Deinococcus-Thermus* group, and thermophilic *Thermotogales* and *Aquificales*. Given a monophyletic group of Bacteria, the tree suggests that the common ancestor of Bacteria was not (hyper)thermophilic although Firmicutes include thermophilic species (it should be noted that the root cannot be placed on this tree). The tree is not consistent with the SSU rRNA tree. Moreover, Harris et al. (2003) proposed a different type of the tree obtained from genome data. They analyzed 80 conserved clusters of genes throughout the three domains and found that 50 of them show similar topology to the SSU rRNA gene tree.

A problem for the genome-based phylogenetic analyses with primary sequences is how to select the genes (regions) for analyses. Although more than 1,000 complete genomes have been opened to public (see public database such as GenBank), the function of several 10% of predicted protein genes are not known. As mentioned above, only tens of the protein genes were used for phylogenetic analyses by Ciccarelli et al. (2006) and Harris et al. (2003).

12.2.4 Other Approaches

Other approaches for reconstruction of universal tree also have been reported. Wang et al. (2007) used protein structures to infer relationship among life. They compared presence/absence of each protein structure (or protein structure family) among species of which complete genome sequences are known. By counting the number of protein structures conserved among species, they estimated the relationship among species. In their conclusion, Eucarya and Archaea appear as monophyletic groups and do not seem to form a group in their (unrooted) tree.

Another method to determine the direction of evolution is to utilize the evolutionary event that can indicate direction of evolution. Existence and absence of retrotransposon in certain loci has been used for phylogenetic analyses (e.g., Shimamura et al. 1997) because retrotransposon is first transcribed and then inserted into other positions of genome from the original position by reverse transcription of the RNA followed by insertion event. Sharing the same retrotransposon sequence at the same locus within their genomes suggests that these two species diverged after the period when the retrotransposon was inserted to the position. However, this kind of phylogenetic marker is rare.

12.3 Placing the Root on the Universal Tree

To identify the root of extant organisms, a multiple gene (protein) tree of paralogous genes which might be duplicated into two or more prior to the age of Commonote has been constructed. Commonote has been positioned at the different branches, depending on the type of the gene and the analytical method used.

12.3.1 The Root on the Bacterial Branch

The root is most often placed between Bacteria and common ancestor of Archaea and Eucarya. Iwabe et al. (1989) reconstructed the multiple gene tree of EF-Tu/EF-1α and EF-G/EF2. Since these proteins are essential for the elongation process of translation, the date of diversification to EF-Tu/EF-1α and EF-G/EF-2 was assumed to be before the age of Commonote. Therefore, the Commonote is expected to be located on the branch connecting the EF-Tu/EF-1α clade and the EF-G/EF-2 clade. Accordingly, the EF-G/EF-2 clade can be used as the out-group of EF-Tu/EF-1α clade. In turn, the EF-Tu/EF-1α clade can be used as the out-group of EF-G/EF-2. In the trees of Iwabe et al. (1989), Archaea is the sister group of Eucarya, and Bacteria is their sister group. The similar result was reported by using larger dataset of these proteins (Baldauf et al. 1996). Gogarten et al. (1989) also reported the close relationship between Archaea and Eucarya based on the H^+ ATPase sequences. However, in the case of the H^+ ATPase and its homologs, complicated evolutionary

history has been pointed out (Hilario and Gogarten 1993; Lapierre et al. 2006), since HGT is known for this protein gene. Woese et al. (1990) adopted the position of Commonote in the Bacteria branch to the small subunit ribosomal RNA tree and proposed the three domains of life, Archaea, Bacteria and Eucarya.

Brown and Doolittle (1995) also reconstructed a composite tree for three closely related aminoacyl-tRNA synthetases: that is, valyl tRNA synthetase, leucyl tRNA synthetase, and isoleucyl tRNA synthetase. From the isoleucyl tRNA synthetase part of the tree, Brown and Doolittle (1995) suggested the Archaea/Eucarya clade. However, after the report, Brown et al. (2003) have noticed the two types of bacterial isoleucyl tRNA synthetase present. One of them shows mupirocin resistance, and this type is found in limited lineage of bacteria. Brown et al. (2003) suggested that the bacterial isoleucyl tRNA synthetase resistant to mupirocin appeared independently to the mupirocin-sensitive bacterial isoleucyl tRNA synthetase, and the eukaryotic isoleucyl tRNA synthetase may originate from the mupirocin-resistant isoleucyl tRNA synthetase. In the earlier work of Brown and Doolittle (1995), mupirocin-resistant isoleucyl tRNA synthetases were not included. Therefore, at least from the isoleucyl tRNA synthetase data, we cannot conclude the monophyly of the Archaea/Eucarya clade. On the other hand, another analyses of aminoacyl-tRNA synthetases (seryl, tyrosyl, and tryptophanyl tRNA synthetases) had suggested similar relationship among Archaea, Bacteria, and Eucarya to the tree of Iwabe et al. (1989) (Kollman and Doolittle 2000), although threonyl tRNA synthetase did not.

When we use base or amino acid sequences for reconstruction of molecular phylogenetic trees, we have to distinguish orthologs from paralogs. Orthologs have common ancestry and share the same function in the biological processes. On the other hand, paralogs have common ancestry, but have different functions in the biological processes. For example, human elongation factor 1α (EF-1α) and chimpanzee EF-1α are orthologs since they share their ancestry and the same biological function in the translation. On the other hand, EF-1α and mitochondrial EF-Tu are paralogs. Though both proteins are responsible for elongation process of translation to bring aminoacylated tRNAs to the A site of ribosome, eukaryotic EF-1α works in cytoplasm, while mitochondrial EF-Tu works in mitochondria. When we want to reconstruct species tree, accidental inclusion of paralogous genes (proteins) may mislead to wrong trees. However, the discrimination of the orthologs from the paralogs is not obvious (see discussion above on the aEF-1α).

12.3.2 The Commonote as a Member of Bacteria

12.3.2.1 Cavalier-Smith's Hypothesis

Several recent studies have suggested that the Commonote was the member of Bacteria. In other words, the root was placed within the Bacterial branches. Cavalier-Smith (2002, 2006a, b, 2010), for example, has suggested that Commonote is in Eobacteria. In his hypothesis, the oldest extant lineage is

Eobacteria. Eobacteria in his term include *Chloroflexi*. *Negibacteria* (overlapping with gram-negative bacteria) includes *Eobacteria* and *Glycobacteria* (consists of *Cyanobacteria, Proteobacteria*, and so on) with two-layered surface membrane. *Eobacteria* are older groups in the course of evolution in the Cavalier-Smith's hypothesis. *Posibacteria* (overlapping with gram-positive bacteria) with single-layered membrane was originated from *Negibacteria* (*Glycobacteria*) in his view. From *Posibacteria,* common ancestor of Eukaryotes and Archaebacteria might be appeared.

His hypothesis on the evolution of life depends on several observations, one being the structure of membranes surrounding cells. Most of others are also presence/absence of certain structures (proteins and other high-weight molecules) in the cell. Cavalier-Smith divided life into two classes, one is the class of two-membrane organisms and another is the class of single-membrane organisms (Cavalier-Smith 2002, 2006a, b, 2010). In addition, he predicted evolutionary direction from two-membrane organisms to single-membrane organisms. This direction is derived from his "obcell" hypothesis for the origin of cell (Cavalier-Smith 2001).

The hypothesis of Cavalier-Smith on the early evolution of life and the evolution of Bacteria, Archaea, and Eucarya depend on the various topics he gathered. However, in general, it is very difficult to tell the evolutionary directions of traits. Although the discussion of Cavalier-Smith (2001, 2002, 2006a, b, 2010) is fruitful for the research on early evolution of life, the standing position is very different from others: most of them find bases in the molecular phylogenetic analyses. Only when we accept his obcell hypothesis, the direction of evolution of traits, and then the direction of evolution of life, can be accepted.

12.3.2.2 Lake's Hypothesis

Recent studies of Lake's group also suggested the bacterial origin of all extant living organisms based on the indel analyses of various pairs of protein genes which diversified prior to the era of Commonote, such as EF-Tu/EF-G (Skophammer et al. 2007; Lake et al. 2008, 2009). They also delineated the early evolution of life using a ring instead of a tree (Rivera and Lake 2004). They suggested the possible close relationship between Archaea and *Firmicutes* (in particular *Bacilli*), based on the indel analyses (absence/presence of residues at the well-conserved region). Then, they placed the root of all of life between *Actinobacteria* and *Clostridia* (Lake et al. 2008, 2009). In other words, *Actinobacteria* and *Clostridia* show most primitive characteristics in their analyses. Lake's group suggested that Commonote was the gram-positive bacteria similar to *Actinobacteria* and/or *Clostridia*. This conclusion is opposite to the conclusion on the early evolution of life presented by Cavalier-Smith (2002, 2006a, b, 2010), although both conclusions suggest that Commonote was not Archaea, but Bacteria. We need to note that some alignment comparisons used by Lake's group have been questioned (e.g., Valas and Bourne 2009).

12.4 Are Archaea Monophyletic?

Another issue remained to be answered is whether Eucarya is a subgroup of Archaea or a distinct group from Archaea. The SSU rRNA tree (Woese et al. 1990) suggested that Eucarya and Archaea are distinct monophyletic groups.

12.4.1 On the Origin of Eucarya

Determination of the origin of eukaryotic cell is challenging. Substantial evidence has been accumulated on the bacterial origin of mitochondria and plastids (chloroplasts). Molecular phylogenetic analyses have suggested that the mitochondrion is derived from *Alphaproteobacteria* (e.g., Andersson et al. 1998) and the plastid from *Cyanobacteria* (e.g., Rodríguez-Ezpeleta et al. 2005). Therefore, the early eukaryotic cells incorporated bacterial genes through mitochondria and plastid symbiosis. Because mitochondria are the organelle responsible for respiration and related metabolism, and because plastid is responsible for photosynthesis, many eukaryotic metabolic genes are the descendants of early bacterial genes.

Very few evidence is present regarding the origin of cytoplasm and nucleus. The transcription and translation systems of Eucarya are similar to those of Archaea rather than of Bacteria (e.g., Werner 2007; Benelli and Londei 2009). Therefore, Eucarya are thought to be relatives of Archaea rather than Bacteria. However, there are tens of hypothesis on the origin of nucleus (see the review by Martin 2005). Some of them are going to be reviewed in the following sections.

12.4.2 Eocyte Hypothesis

Lake and his colleagues have proposed the “Eocyte” hypothesis (Lake et al. 1984; Rivera and Lake 1992). Eocytes are one of the subgroups of Archaea, which include some groups in *Crenarchaeota*. They have analyzed the phylogeny using the indel trait. Since nucleotide insertion/deletion event is thought to occur much less frequently than base substitution during evolution, parallel evolution does not seriously affect the phylogenetic analyses. Accordingly, shared insertion/deletion between orthologous sequences can be a good phylogenetic signal. By comparing the existence of indels in the EF-Tu/EF-1α/EF-G/EF-2 alignment, Lake and his coworkers concluded that the Eocytes (*Crenarchaeota*) are the sister group of Eucarya.

Recent phylogenetic analyses of combined data of large and small ribosomal RNA genes, as well as concatenated protein genes, supported the Eocyte hypothesis (Cox et al. 2008; Foster et al. 2009). In these analyses, a method allowing heterogeneity of nucleotide composition through time was adopted. The evolutionary rates

of ribosomal RNA genes are accelerated in Eukaryotic lineage (Cox et al. 2008). Therefore, we cannot rule out the possibility that Eucarya being the distinguished group from Archaea because of the long branch attraction in the Woese's tree (Woese 1987; Woese et al. 1990). In the case of Woese's molecular phylogenetic analyses of SSU rRNAs, the Jukes-Cantor model (1969) was used for the estimation of evolutionary distances. This is the simplest substitution model for nucleotide sequences, and the effects of transversions on the sequence evolution might be overestimated. In addition, there was no consideration on the rate heterogeneity among sites and branches, in Woese's original tree, because of the limitation of analytical technique in those days.

12.4.3 Other Hypothesis on the Archaeal Origin of Eucarya

Euryarchaeota is also a candidate of the closest relative to Eucarya. Martin and his collaborators proposed the "hydrogen" hypothesis to explain the origin of mitochondria and hydrogenosomes (Martin and Müller 1998). According to Martin and Müller (1998), the methanogens are thought to be archaeal "host" for bacterial symbiosis and the origin of eukaryotic cells. In agreement to the hypothesis, several molecular studies proposed that Eucarya are the closest relatives to methanogens (e.g., Sandman et al. 1990).

It has been argued that *Thermoplasmatales* or their close relatives were hosts of eukaryotic cells (e.g., Searcy et al. 1978; Margulis 1996). *Thermoplasmatales* lack cell wall (Darland et al. 1970) and therefore can be good hosts for the intracellular symbiosis. Currently, there are few molecular evidences that directly support the close relationship between Eucarya and *Thermoplasmatales* (Pisani et al. 2007; see also Shimizu et al. 2007). In addition, *Thermoplasma* (and *Archaeoglobus*) MreB, a bacterial/archaeal homolog of actin, is closely related to eukaryotic actin rather than to those of methanogens (Hara et al. 2007), although the direct ancestor of eukaryotic actin may be different.

The universal tree has been used to obtain the information regarding the origin of Eucarya. Yutin et al. (2008) analyzed eukaryotic protein genes that were the descendants of archaeal genes and found that most of them were the sister group of all archaeal orthologs. The result suggests that the Archaea and Eucarya form different monophyletic groups; they are separated before the dispersion of each group. Pisani et al. (2007) reported that eukaryotic genes show high affinity to *Alphaproteobacteria, Cyanobacteria,* and *Thermoplasmatales*. If the affinity to *Alphaproteobacteria* is caused by the mitochondrial origin of the genes and if affinity to *Cyanobacteria* is caused by the plastid origin of the genes, nuclear genes of Eucarya may most closely related to those of *Thermoplasmatales*.

Recently, Kelly et al. (2011) suggested the *Thaumarchaeal* origin of Eucarya based on the presence/absence of protein gene families. They also suggested the ancestral characteristics in methanogens in Archaea.

12.5 Was the Commonote Thermophile?

12.5.1 Theoretical Analyses on the Growth Temperature of Commonote

The growth temperature of the ancient organism has long been a topic that has interested many scientists (Gaucher et al. 2010). In a well-accepted phylogenetic tree constructed by SSU rRNA sequences, hyperthermophilic Archaea and Bacteria are represented in the deepest and shortest branches (Woese 1987; Achenbach-Richter et al. 1988). Based on this observation, Stetter described that the common ancestors of Archaea and of Bacteria were likely hyperthermophilic (Stetter 2006). Given hyperthermophilic ancestry both for archaeal and bacterial lineages, the Commonote is also parsimoniously thought to have been thermophilic. However, it cannot be ruled out the hypothesis that the most ancestral organism lived in a colder environment and subsequently adapted to higher temperatures (Greaves and Warwicker 2007).

Reverse gyrase is an ATP-dependent type I DNA topoisomerase that introduce positive supercoils into circular DNAs in vitro (Kikuchi and Asai 1984). Although the precise role of reverse gyrase in vivo is still unknown, the facts that the protein is found only in thermophiles and that all known hyperthermophiles contain this protein suggest an essential role of reverse gyrase in the adaptation of life to very high temperatures (Forterre 2002). Indeed, a reverse gyrase knockout *Thermococcus kodakaraensis* mutant can grow at 90 °C but not at 93 °C at which the growth of wild-type *T. kodakaraensis* can be observed (Atomi et al. 2004). Therefore, although reverse gyrase is not the absolute requirement, the emergence of this enzyme was crucial in the origin of hyperthermophiles. An important structural feature of reverse gyrase is that the protein is composed of two non-related domains, a topoisomerase domain and a helicase domain (Declais et al. 2000). It is apparent that these two domains could not have been fused to produce a single-chained reverse gyrase molecule before topoisomerase and helicase families were diverged. Therefore, assuming that reverse gyrase is an essential protein for hyperthermophilic organisms (Heine and Chandra 2009), the primitive microorganisms could not be hyperthermophilic. In addition, eukaryotic type I DNA topoisomerase interacts with helicases *in vivo*, suggesting that type I topoisomerases and helicases originated and evolved independently in mesophiles or thermophiles and later recruited to hyperthermophiles (Forterre 1996). This argument suggests that hyperthermophiles descended from less thermophilic organisms, but does not preclude the idea that reverse gyrase had evolved prior to the appearance of the last universal common ancestor.

Galtier et al. (1999) established a model of sequence evolution and estimated the ancestral G+C content in rRNA sequence. The underlying idea of this approach is that a greater G+C content may reflect a higher growth temperature. The inferred G+C content was not compatible with high temperature-living organisms. They therefore concluded that the Commonote was likely a mesophile. However, a different

phylogenetic algorithm led to a contradicted conclusion. Di Giulio reanalyzed the same genome data set by using maximum parsimony and then claimed that the last universal common ancestor is a thermophile or hyperthermophile (Di Giulio 2001). In the following two papers, Di Giulio provided further evidences supporting the hypothesis that the last universal common ancestor was a hyperthermophile (Di Giulio 2003a, b).

Ancestral amino acid compositions were also computed. Brooks et al. estimated amino acid compositions of a set of proteins postulated to have existed in the last universal common ancestor using an expectation-maximization method (Brooks et al. 2004). The calculated amino acid composition of this protein set was more similar to the observed composition of the same set in extant thermophilic species than in extant mesophilic species. They therefore concluded that the Commonote lived in a thermophilic environment.

Becerra et al. focused on the evolution of protein disulfide oxidoreductases and then implicated the thermostabilities of proteins in the Commonote. The results imply that disulfide oxidoreductase sequence was missing in genome of the last universal common ancestor, suggesting non-thermophilic ancestry (Becerra et al. 2007). However, it should be noted that disulfide bond formation is not necessarily required for the high thermostability of thermophilic proteins. Indeed, a number of thermophilic and hyperthermophilic proteins lack or contain few cysteine residues (Cambillau and Claverie 2000).

Recently Boussau et al. conducted computational analyses of both rRNA and protein sequences (Boussau et al. 2008). The results suggested that the Commonote was mesophilic and, subsequently, the common ancestor evolved divergently to thermophilic ancestors of Bacteria and of Archaea-Eucarya that were adapted to high temperature, possibly in response to a climate change of early earth.

Thus, a number of theoretical studies have argued the growth temperature of the last universal common ancestor, but these studies remained inferential due to the lack of empirical testing. In the next section, we will describe some experimental studies to assess if the Commonote was thermophilic, performed in authors' laboratory.

12.5.2 Experimental Testing if the Commonote Was (Hyper) thermophilic

Ancestral sequences of a particular protein can be inferred by comparison with extant homologous protein sequences (Messier and Stewart 1997; Bielawski and Yang 2003; Thornton 2004). We have developed an experimental way to assess the antiquity of hyperthermophilic organisms using an inferred amino acid sequence of a protein postulated to exist in the Commonote. In this experimental method, inferred ancestral residues are introduced into an extant protein and then the thermal stabilities of the resulting mutant proteins are examined. If the Commonote was thermophilic, the mutant proteins, each of which contains one or a few inferred

ancestral residues, are expected to show the trend toward enhanced thermal stability when compared to the wild-type protein.

Miyazaki et al. inferred an ancestral amino acid sequence of 3-isopropylmalate dehydrogenase (IPMDH), which might be hosted by the Commonote. The inferred ancestral residues were then introduced into the IPMDH originated from the hyperthermophilic archaeon, *Sulfolobus tokodaii* (Miyazaki et al. 2001). When the thermal stabilities of the resulting mutant proteins were investigated by measuring the remaining activity after heat treatment and the change in 222-nm ellipticity upon thermal unfolding, at least five of the seven ancestral mutants tested showed thermal stability higher than that of the wild-type IPMDH. A homologous protein from the extremely thermophilic bacterium, *Thermus thermophilus*, was also used for the experimental testing as another model protein. Watanabe et al. designed 12 ancestral mutants each containing an ancestral amino acid residue that was postulated to be present in the common ancestor of Bacteria and Archaea (Watanabe et al. 2006). When the thermal stabilities of the designed mutants were compared to the wild-type IPMDH from *T. thermophilus*, at least 6 of the 12 ancestral mutants designed exhibited enhanced thermal stability. A similar trend was also observed when we constructed ancestral mutant proteins of isocitrate dehydrogenase (ICDH) from the extremely thermophilic archaeon, *Caldococcus noboribetus* (Iwabata et al. 2005). At least four of the five ancestral mutants, each containing an ancestral amino acid residue, showed thermal stability higher than that of the wild-type ICDH. Thus, the ancestral amino acid residues tend to increase the thermostability of metabolic proteins originating from thermophilic and even hyperthermophilic organisms. The results provide experimental evidences for existence of extremely thermostable proteins in the last universal common ancestor, supporting the hypothesis that the Commonote was a hyperthermophile.

A similar experiment was also performed using a protein involved in the translation system of *T. thermophilus* (Shimizu et al. 2007). Phylogenetic trees of the proteins involved in translation system often show the same topology as the rRNA tree, which is frequently used in phylogenetic analysis. The function of the translation system is universal because all organisms on the earth have a translation system. Furthermore, aminoacyl-tRNA synthetases must be primordial proteins that emerged early in evolution. Therefore, the evolution of an aminoacyl-tRNA synthetase is likely coincided to the evolution of host organisms. In addition, probably because mutations occurred in aminoacyl-tRNA synthetases would affect survival of the organisms, the sequences of the proteins are well conserved. Therefore, it is unlikely that modification of the functions and horizontal transfer of the genes have been frequent during evolution. Thus, it is advantageous to use an aminoacyl-tRNA synthetase for a phylogenetic analysis. Shimizu et al. deduced a possible ancestral amino acid sequence of glycyl-tRNA synthetase (GlyRS) from a maximum-likelihood tree of α_2-type GlyRS (Shimizu et al. 2007). An individual or pairs of the inferred ancestral amino acids were introduced into GlyRS from *T. thermophilus*, and the thermal stabilities of the resulting eight mutant proteins were evaluated by monitoring the change in 222-nm ellipticity upon thermal unfolding. As a result, six mutants showed higher thermostability than wild-type GlyRS, suggesting that

the Commonote possessed extremely thermophilic translation enzymes. The result is again compatible with the hyperthermophile common ancestry. However, as discussed below, it cannot be fully precluded that the observed trend for enhanced thermostability of mutant proteins is an artifact of the ancestral design method (Williams et al. 2006).

As described above, introduction of ancestral residues further enhanced the thermostabilities of the proteins involved in a metabolic pathway or a translation system of the (hyper)thermophiles with the probability between 50 and 80%. Therefore, the ancestral design method is a useful technique of designing mutant enzymes with higher thermostability that only relies on the primary amino acid sequences of homologous proteins. We also found that the extent to which thermostability of the mutants with an introduced ancestral residue enhances is directly correlated with the degree to which residues surrounding the mutation site are conserved (Yamashiro et al. 2010).

Consensus approach is a very similar way to improve thermal stability of a protein using a multiple amino acid sequence alignment of homologous proteins. This method is based on the hypothesis that, at a given position of a multiple sequence alignment of homologous proteins, most frequently occurring amino acids contribute to the thermostability of the protein more than other less frequently occurring amino acids. In 1994, Steipe et al. first rationalized the feasibility of this approach using statistical thermodynamics (Steipe et al. 1994). They analyzed the amino acid sequences of the immunoglobulin variable VL and VH domains. The theoretical basis of their design is that randomly occurring mutations are often destabilizing and, therefore, mutations tend to destabilize proteins if selection pressure is absent. However, during the actual evolution, mutations that caused reduced stability insufficient to maintain protein's specific tertiary structure have been hardly selected. Consequently, the frequency of a given residue in the multiple sequence alignment of a protein correlates with the contribution of the amino acid to protein stability. Hence, the most frequent amino acid at any position among homologous immunoglobulin variable VL or VH domains should contribute to the stability of the domain greater than that with an amino acid rarely seen in the homologous sequences. They calculated a statistical free energy from the frequencies of occurrence of a particular amino acid at a given site and designed proteins with specific amino acid residue substitutions, producing mutant proteins with improved stability at a high probability. Later, the consensus approach method was used to enhance other proteins: for example, phytase (Lehmann et al. 2000, 2002), the SH3 domain from the yeast actin-binding protein 1 (Rath and Davidson 2000), the DNA-binding domain of the tumor suppressor p53 protein (Nikolova et al. 1998), and GroEL minichaperones (Wang et al. 1999). The consensus design concept was also applied to improve thermal stability of chorismate mutase from *Escherichia coli* by using artificially generated functional protein sequences selected from binary-patterned libraries (Jackel et al. 2010).

The consensus design approach and the ancestral design approach frequently resulted in the same residue substitutions because consensus residues often originated from the ancestral residues. Therefore, it remain unclear if the enhanced thermostability of the proteins with an ancestral amino acid could be ascribed to the antiquity of the residue or if the enhanced thermostability is attributable to the

statistic free energy. To clarify the reason why ancestral mutations tended to improve protein stability, Watanabe et al. analyzed all of the ancestral mutants of IPMDHs and ICDHs designed to date (Watanabe et al. 2006). In authors' laboratory, the thermal stabilities of IPMDHs from *Bacillus subtilis* and *Saccharomyces cerevisiae* had been improved by an evolutionary molecular engineering technique that consisted of random mutagenesis and selection (Akanuma et al. 1998, 1999; Tamakoshi et al. 2001). Some of the mutants isolated by evolutionary engineering have an ancestral residue at the mutated site. Therefore, the thermostabilizing ancestral amino acids found in the experimental evolution were also incorporated into the analysis. Watanabe et al. classified the ancestral mutations into two groups from the viewpoint of the consensus approach, that is, dominant ancestral residues and minor ancestral residues (Watanabe et al. 2006). The dominant residues are the residues that occupied a given site most frequently in the amino acid sequence alignment of 75 IPMDHs (or of 79 ICDHs in the case of the ICDH mutants). When the dominant residue is not coincident to the ancestral residue at a site, the ancestral residue was designated as a minor ancestral residue. Among the 15 mutants with a dominant ancestral residue, ten led to improved thermal stability. Similarly, out of six mutants with a minor ancestral residue, four improved the thermal stability of the proteins. Because the rate of improving thermal stability by introducing the ancestral residue was not related to whether the ancestral residue was dominant, the stabilization effect of the ancestral residues cannot be attributed to the consensus residue: that is, statistical free energy. However, the analyzed data are limited and therefore not sufficient to justify that the increased stability of the mutant proteins into which an ancestral amino acid is introduced is only related to the inherent nature of ancestral sequences. Very recently, we predicted the sequence for the deepest nodal position of a phylogenic tree composed of 16 gyrase B subunit sequences, which was then synthesized and characterized (Akanuma et al. 2011). Notably, the ATPase domain of the reconstructed gyrase B is more thermally stable than is a corresponding sequence containing the most frequently occurring amino acids among the 16 gyrases. The thermal stability of the designed protein is likely due in part to the antiquity of some of the inferred residues. However, it would be also possible that the ancestral design algorithm simply corrected for the potential inclusion of erroneous residues in the reconstructed sequence that would have been caused by the use of limited number of homologous amino acid sequences (Akanuma et al. 2011). Further evidences are, therefore, required to conclude that the results of our experimental testing really support the (hyper)thermophilic ancestor hypothesis.

12.6 Computer Prediction and Experimental Reconstruction of Ancient Proteins

Information about the ancient environment of Earth is often obtained from fossil records. In contrast, no tangible remnants of the primitive protein forms hosted by ancient organisms that lived more than 3,500 million years ago are preserved

(Schopf 1993). However, in addition to the currently available genome information that has provided growing database of homologous protein sequences, recent advance in phylogenetic analysis and whole-gene-synthesis technique have made it possible to reconstruct the genes encoding ancient proteins in laboratories. Therefore, predicting ancestral protein sequences and characterizing the properties of the reconstructed proteins are one of the most powerful means available for studying the traits of ancient proteins. Procedures to reconstruct ancestral genes and several examples of resurrection experiments are discussed in greater detail in an excellent review by Thornton (2004).

The empirical reconstruction of ancient proteins was used as a novel tool for improving our knowledge of environmental temperatures experienced by ancient bacteria. Gaucher et al. have reproduced the ancestral elongation factor Tu (Gaucher et al. 2003, 2008). They estimated the growth temperature of the common ancestor of Bacteria according to the concept that the denaturation temperature of a protein reflects the living temperature of the host organism. Based on the resurrection experiment, they reported that the common ancestor of bacteria was thermophilic, rather than hyperthermophilic or mesophilic. However, it is well known that a single random mutation, insertion, deletion, or substitution can drastically decrease the thermal stability of a protein. Therefore, it cannot be ruled out that the common ancestor of Bacteria was a hyperthermophilic organism. Conversely, Williams et al. have pointed out that an inaccurate estimation of ancestral amino acids has a risk to overestimate the thermostability and other properties of ancestral proteins (Williams et al. 2006). To assess the reliability of the properties of ancestral proteins reconstructed by various methods, they performed an evolution simulation of computationally generated population. Using the resulting data, they compared the thermodynamic properties of the true ancestral sequences with those of ancestral sequences inferred by maximum parsimony, maximum likelihood, and Bayesian inference. As the result, they found that reconstruction by maximum parsimony or maximum likelihood tends to overestimate thermodynamic stabilities although the two methods can effectively predict accurate ancestral amino acids. In contrast, Bayesian inference sometimes predicts less probable ancestral amino acids, but the method is more reliable guide to ancestral thermodynamic properties. Nevertheless, there may still be anxiety to use incorrect models, even when Bayesian inference is used. It is therefore important to keep in mind that none of the reconstruction methods provide a perfect success for predicting ancestral amino acid residues. Thus, although phylogenetic reconstruction of ancestral protein sequences is a powerful way for studying early evolution of life, any conclusion obtained from such studies relies largely on the accuracy of the reconstructed sequences.

Similar resurrection experiments have been also applied to eukaryotic proteins; ancestral reconstruction has been used to understand the evolution of ethanol production/consumption in yeast (Thomson et al. 2005) and the evolutionary trajectory of changes in substrate specificity of hormone receptors (Bridgham et al. 2006; Ortlund et al. 2007). Thus, the reconstruction method is currently a common technique to study the molecular evolutions of genes, proteins, and life.

12.7 Conclusions

In this chapter, we have reviewed the universal trees constructed based on different types of genetic information. The tree topology was different depending on the type of the gene analyzed as well as the method used. The root of the universal tree is most likely placed between the bacterial branch and the common ancestor of Archaea and Eucarya. However, there are possibilities that the root may be within the Bacterial branches.

Monophyly of Archaea is rather controversial. Though the rRNA tree suggested the monophyly, other types of the trees have been also reported. The conclusive result where the Eucarya originated within/outside of the branch of Archaea is yet to come.

The growth temperature of the ancient organism has long been a topic that has interested many scientists. Theoretical works suggested mesophilic, thermophilic, and hyperthermophilic origins of life, depending on the report. Experimental test analyzing the effect of each or combination of ancestral amino acid residues suggested the hyperthermophilic origin of life. However, we cannot totally deny the possible artifact originated from the method used for the estimation of ancestral sequences possessed by the ancestral organisms.

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Chapter 13
Host-Vector Systems in Thermophiles

Takahiro Inoue and Yoshihiko Sako

Abstract The potential application of thermophiles and their enzymes for industry is enormous where the development of host-vector systems in thermophiles is essential. Compared to mesophilic organisms, the host-vector systems in thermophiles are lagging behind. However, in recent years a number of host-vector systems were developed for thermophilic bacteria and also for some archaea. Host-vector systems in thermophiles are more advanced than is commonly believed.

In this chapter, firstly, we introduce basic genetic methodology for host-vector systems in thermophiles including transformation, selectable markers, vectors, and hosts (recipient cells). We show transformation methods used for thermophiles, selectable markers that are effective for thermophiles, some *Escherichia coli*-host shuttle vectors, and important property of the host.

Secondly, we show the actual application systems for the host-vector systems in thermophiles, expression vectors, reporter gene systems, and targeted gene disruption (replacement) method.

Finally, we introduce some commercial and potential application of thermophile host-vector systems. Homologous and heterologous expression of the thermophilic proteins which were difficult to produce in full active form from mesophilic hosts was successfully developed in thermophiles, especially in *T. thermophilus*, and several hyperthermophilic archaea using their host-vector systems, "genetic and metabolic engineering," were developed for biofuel production in thermophiles, especially in thermophilic ethanologens. And, directed evolution methods were developed for thermo-adaptation of mesophilic proteins in thermophiles such as *T. thermophilus*.

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13.1 Introduction

Since the discovery of thermophiles, these microorganisms have been useful with special attention paid to their thermostable enzymes. Stability of enzymes and activity at high temperature are important properties for industrial applications, e.g., performing processes at high temperature can reduce risk of contamination and improve solubility of substrates and transfer rates.

Nearly all thermostable enzymes are produced using heterologous expression in mesophilic host-vector systems, typically *Escherichia coli*. However, this approach has several limitations because several proteins require cofactors, the appropriate posttranslational processing, active chaperones, or high temperatures to fold correctly. Differences in GC content and codon bias have also been shown to hamper gene expression in mesophiles (Bjornsdottir et al. 2007; Hidalgo et al. 2004). Therefore, the enzymes produced from thermophiles are difficult to produce in full active form from mesophilic hosts (Jenney and Adams 2008). To overcome these problems, recently alternative methods for overexpression of these proteins were successfully developed using host-vector systems in thermophiles.

Thermophilic fermentative culture may have particular application for biofuel production (bioethanol, biodiesel, methan, hydrogen, etc.) (Barnard et al. 2010). Thermophiles are often able to tolerate changes in environmental conditions such as pH and temperature change, and a number of thermophiles ferment structurally complex carbohydrates important in biofuel production (Taylor et al. 2009). Further, thermophilic industrial fermentations reduce risk of microbial contamination. However, there are a number of disadvantages. One is many thermophiles are inhibited by their products. To overcome these disadvantages, we have improved the desirable cell properties using "genetic and metabolic engineering" of the host-vector system.

The potential application of thermophiles and their enzymes for industry is enormous where the development of host-vector systems in thermophiles is essential. Compared to mesophilic organisms, the host-vector system technology in thermophiles is lagging behind due to several constrains and differences. However, in recent years a number of host-vector systems were developed for thermophilic bacteria and also for other archaea. Host-vector systems in thermophiles is more advanced than is commonly believed and requires further discussion.

The two principal requirements of a host-vector system are (1) the availability of a cloning vehicle encoding a readily selectable genetic trait and (2) a means of introducing the trait into the host organism. In the first part of this review, we will present the basic methods for thermophilic host-vector systems. In the second part, we present several commercial and potential applications of thermophile host-vector systems.

13.2 Critical Review and Analysis: Methods for the Host-Vector System

The primary requirement to develop host-vector systems in thermophiles is to establish an efficient plating method for host organisms at high incubation temperatures. Plate culture of thermophiles requires preventing gel dehydration at high temperatures since evaporation of water changes the medium composition which is critical in achieving plating efficiencies. Therefore, to improve the plating methods at high temperatures, some thermophiles can be cultivated on solid media (Allers and Mevarech 2005; Erauso et al. 1995; Jiang et al. 2006; Tyurin et al. 2006).

In the following sections, we will review the basic genetic methodology for host-vector systems in various thermophiles including transformation, selecting markers, vectors, and hosts.

13.2.1 Transformation

The development of selectable markers and transformation protocols are closely related to each other. Without selectable markers, it is impossible to quantify transformation efficiency. Conversely, the development of selectable markers requires efficient transformation protocols (Mevarech and Allers 2007).

Generally, transformation protocols follow three common steps. The first step is the preparation step where the recipient host bacteria are made receptive to uptake of exogenous DNA. The second is the shock step where the host cells in the presence of the exogenous DNA are subjected to a nonlethal shock. Finally, in the recovery step, the cells are incubated to restore the cell membrane after acquiring the exogenous DNA. The methods of transformation include $CaCl_2$ treatment and heat shock-dependent transformation, electro-transformation, protoplast and spheroplast methods and others, e.g., conjugation and transduction. Although naturally competent organisms are found in various phyla, the number in each is known to be relatively small (Shaw et al. 2010). We summarized in Table 13.1 the thermophiles previously reported showing transformations and the methods used.

13.2.1.1 Natural Transformations

In thermophilic bacteria, *Thermus thermophilus* HB27 (and other strains) and *Thermus aquaticus* YT1 exhibit high natural transformation competence (Hidaka et al. 1994; Koyama et al. 1986). Based on a genetic analysis, a model for the natural competence of *T. thermophilus* has also been proposed (Averhoff 2004). Recently, Shaw et al. reported natural competence in several *Thermoanaerobacter* and *Thermoanaerobacterium* strains previously believed to be difficult to transform (Shaw et al. 2010). In the thermophilic archaea, natural transformation events in

Table 13.1 Transformation methods used for thermophilic bacteria and thermophilic archaea

Method	Host	References
Thermophilic bacteria		
Natural transformation	*Th. thermophilus*	Koyama et al. (1986)
	Th. aquaticus	Koyama et al. (1986)
	Tab. saccharolyticum	Shaw et al. (2010)
	Tab. thermosaccharolyticum	Shaw et al. (2010)
	Tab. aotearoense	Shaw et al. (2010)
	Tab. xylanolyticum	Shaw et al. (2010)
	Tabr. ethanolicus	Shaw et al. (2010)
	Tabr. pseudethanolicus	Shaw et al. (2010)
	Tabr. brockii	Shaw et al. (2010)
Electroporation	*Th. thermophilus*	de Grade et al. (1998)
	R. marinus	Bjornsdottir et al. (2005)
	C. thermocellum	Tyurin et al. (2004)
		Tyurin et al. (2005)
	Tab. saccharolyticum	Mai et al. (1997)
		Tyurin et al. (2005)
	Tabr. thermosaccharolyticum	Klapatch et al. (1996) and Tyurin et al. (2005)
	Tabr. ethanolicus	Peng et al. (2006)
	Tabr. mathranii	Yao and Mikkelsen (2010)
	G. thermoglucosidasius	Taylor et al. (2008)
	B. coagulans	Rhee et al. (2007)
		van Kovács et al. (2010)
Ploto-Sphelo-plast	*Tab. thermohydrosulfuricum*	Soutscheck-Bauer et al. (1986)
	C. thermocellum	Zyprian and Matzura (1986)
	G. stearothermophilus	Wu and Welker (1989)
	T. maritime	Yu et al. (2001)
	T. neapolitana	Yu et al. (2001)
Conjugation	*Th. thermophilus*	Ramírez-Acros et al. (1998)
Thermophilic archaea		
Natural transformation	*Tc. kodakaraensis*	Sato et al. (2005)
	M. thermoautotrophicum	Worrell et al. (1988)
	P. furiosus CoM1	Lipscomb et al. (2011)
$CaCl_2$ and heat shock	*Tc. kodakaraensis,*	Sato et al. (2003)
	P. furiosus,	Aagaard et al. (1996) and Waege et al. (2010)
	S. acidocaldarius	Aagaard et al. (1996)
Electroporation	*S. solfataricus*	Albers and Driessen (2007)
	S. acidocaldarius	Kurosawa and Grogan (2005)
	S. islandicus	Deng et al. (2009)
	A. pernix	Sako et al. (unpublished)
Proto-Shero plast	*P. abyssi*	Lucas et al. (2002)
Conjugation	*S. solfataricus*	Grogan (1996)
		Schleper et al. (1995)
Transduction	*M. thermoautotrophicum*	Meile et al. (1990)

Th: *Thermus*; R: *Rhodothermus*; Tab: *Thermoanaerobacterium*; Tabr: *Thermoanaerobacter*; C: *Clostridium*; B: *Bacillus*; G: *Geobacillus*; T: *Thermotoga*; Tc: *Thermococcus*; M: *Methanobacterium*; P: *Pyrococcus*; S: *Sulfolobus*; and A: *Aeropyrum*

Thermococcus kodakaraensis, *Pyrococcus furiosus* COM1, and *Methanobacterium thermoautotrophicum* were reported (Sato et al. 2005; Lipscomb et al. 2011; Worrell et al. 1988).

13.2.1.2 $CaCl_2$ Treatment and Heat Shock-Dependent Transformation

Heat shock after treatment with a cation ($CaCl_2$ is most widely used in thermophiles), a method that is commonly used for *E. coli*, can be used with a few thermophilic archaea; however, this method is not generally efficient. In *Pyrococcus furious* and *Sulfolobus solfataricus*, $CaCl_2$ and heat shock-dependent transformation was observed (Waege et al. 2010; Aagaard et al. 1996).

13.2.1.3 Electro-Transformation (Electroporation)

Transformation by electroporation is usually more efficient than the $CaCl_2$ method and applicable to more species. Electroporation is efficient for a number of thermophilic bacteria and archaea. However, it is not universally applicable and electroporation usually needs to be optimized for a number of parameters. Various electroporation conditions have been studied for low-G+C thermophilic gram-positive bacteria, e.g., *Thermoanaerobacterium* and *Thermoanaerobacter* (Peng et al. 2006; Tyurin et al. 2004, 2005). Kurosawa and Grogan studied various electroporation conditions for *Sulfolobus acidocaldarius* (Kurosawa and Grogan 2005).

13.2.1.4 Transformation Using Protoplasts and Spheroplasts

This method is only effective in species where proto- and spheroplasts can be generated readily accomplished, usually by removing the cell wall. The common transformation reagent is 15–40% polyethylene glycol (PEG). For most bacterial cells, this method has been replaced by facile methods. However, this is still an important method for some archaea including *Pyrococcus abyssi* that cannot use the $CaCl_2$ or electroporation methods (Lucas et al. 2002). Liposome-mediated transformation was observed in *Thermotoga maritima* and *Thermotoga neapolitana* (Yu et al. 2001).

13.2.1.5 Other Transformation Methods

Phage-mediated transduction is reported in *M. thermoautotrophicum* Marburg (Meile et al. 1990). Genetic exchange was observed during cell mating in *S. solfataricus* and *S. acidocaldarius*, and this characteristic has been applied to transform these organisms (Schleper et al. 1995; Grogan 1996). By permeabilizing the cell wall with 50-mM Tris-HCl (pH8.3), *Thermoanaerobacter thermohydrosulfuricum* was transformed (Soutscheck-Bauer et al. 1986).

13.2.2 Selectable Markers

13.2.2.1 Auxotrophic Selectable Markers

Since many bacterial antibiotics are ineffective against thermophiles, especially in thermophilic archaea, host-vector systems in thermophiles have developed auxotrophic selectable markers. They are genes involved in several metabolic pathways, and the markers are used to complement deletion mutations in these genes. Thymidine, tryptophan, and uracil auxotrophic markers are particularly useful since thymidine is absent in some commercial yeast extracts and tryptophan and uracil are absent in some commercial casamino acids. This methodology is used to higher advantage in thermophiles than cell growth inhibitors as we will discuss below. However, this method requires preparing auxotrophic host cells and in many cases, a minimal medium (Wartrin et al. 1995). Further, gellan gum used as plate solidifier for (hyper)thermophiles contains trace nucleic acids where false background selection for a uracil auxotroph can be high (Martusewitsch et al. 2000; Sato et al. 2003). Recently, an auxotrophic selectable marker which allows genetic manipulation in nutrient-rich medium including yeast extracts and tryptone was developed in *T. kodakaraensis* (Santangelo et al. 2010). The mutant lacking the arginine decarboxylase gene, which converts arginine to agmatine, has an agmatine requirement even in the nutrient-rich medium.

13.2.2.2 Cell Growth Inhibitors

Cell growth inhibitors and their corresponding resistance genes are widely used as selectable markers in bacteria and eukaryotic cells. However, at high temperatures most cell growth inhibitors are unstable and therefore not effective. Further, most resistance genes in mesophilic microbes are not effective when expressed in a thermophilic host. Additionally, most bacterial cell growth inhibitors are harmless to archaea. Searching for effective inhibitors that are thermostable and efficient for archaea and improving stability of resistance genes at high temperatures, selectable markers for thermophiles have been developed. Although cell growth inhibitors for thermophiles are limited as mentioned above, these compounds allow selection of transformants in nutrient-rich mediums.

Resistance to cell growth inhibitors include (1) decomposition or modification of the inhibitor, (2) modification of the target molecule to reduce binding of the inhibitor, (3) overproduction of the target molecule, (4) use of an alternative pathway that is insensitive to the inhibitors, and (5) active efflux pumping of the inhibitor (Mevarech and Allers 2007).

13.2.2.3 Cell Growth Inhibitors for Bacteria

Kanamycin is the most widely used selectable marker in thermophilic bacteria using the kanamycin nucleotidyltransferase gene (*kat*) from *Geobacillus stearothermophilus* whose thermostability has been increased up to 70°C using in vitro mutagenesis

(Liao et al. 1986; Matsumura and Aiba 1985). Recently, bleomycin and hygromycin B resistance were used for *T. thermophilus* (Brouns et al. 2005; Nakamura et al. 2005). The two articles describe thermostabilization established resistance to bleomycin and hygromycin B by directed evolution of the corresponding resistance genes using the *T. thermophilus* host-vector system. In low-G+C thermophilic gram-positive bacteria, kanamycin, chloramphenicol, erythromycin, and lincomycin have been used where these antibiotics have stability at high temperatures (Tyurin et al. 2006).

13.2.2.4 Cell Growth Inhibitors for Archaea

Although most bacterial antibiotics and growth inhibitors are ineffective in archaea, several exceptions are used as selectable markers in archaea (Aagaard et al. 1994, 1996). Hygromycin B has been shown to inhibit the growth of *S. solfataricus*. The resistance marker of a thermostable *E. coli* gene encoding for hygromycin B phosphotransferase was adopted into *S. solfataricus* using error-prone PCR (Cannio et al. 2001). Butanol or benzyl alcohol also inhibits the growth of some thermophilic archaea. In *P. furiosus* and *S. acidocaldarius*, the *adh* gene encoding alcohol dehydrogenase from *G. stearothermophilus* and *S. solfataricus* was used as a selectable marker (Contursi et al. 2003; Aravalli and Garret 1997). Regardly, simvastatin was reported to inhibit the growth of *T. kodakaraensis* where overexpression of the 3-hydroxy-3methylgutaryl coenzyme A (HMG-CoA) reductase gene gives resistance against simvastatin (Matsumi et al. 2007). As almost all thermophilic archaea are presumed to require HMG-CoA reductase for their membrane lipid biosynthesis, this marker (simvastatin, mevinolin, and other statin compounds) system has the potential for application in many other thermophilic archaea. This marker system has been recently applied to several hyperthermophilic archaea (Deng et al. 2009; Lipscomb et al. 2011; Zheng et al. 2012; Zhang and Whitaker 2012).

13.2.3 Vectors

A lot of vectors for thermophiles have been developed. Here, we described only a few of the important ones (Table 13.2). All of these vectors are shuttle vectors, based on *E. coli* plasmids. Using shuttle vectors, the cloning can be performed in *E. coli* before the vector is introduced into the host. The form, e.g., modification and topology (Charbonnier and Forterre 1994; Schreier et al. 1999; Vee Aune and Aachmann 2010), of the plasmids influences the transformation efficiency (see Sect. 13.2.4).

13.2.3.1 Shuttle Vectors

Generally, the vector itself consists of at least three parts: (1) a replicon in the host (recipient cells), (2) a selectable marker for the host, and (3) an *E. coli* vector portion that has an origin in replication and a selectable marker in *E. coli* (Berkner and

Table 13.2 Shuttle vector for thermophilic bacteria and thermophilic archaea

Vector							
				E. coli	Host		
Name	Selectable marker	Size (kb)	Copynumber	Vector Part	Replicon	Host	References
Thermophilic bacteria							
pMY	*kat*[a]	8.4	3–40	pUC19	pTSP1	*Th. thermophihus*	Lasa et al. (1992)
pMK18	*kat*	5	n. d.	pUC18	EM2	*Th. thermophihus*	de Grade et al. (1999)
pRM100	*trpB* [b]	7	6–8	pUC18	pRM21	*R. marimus*	Bjornsdottir et al. (2005)
pJY1, 2	*cat* [c], *kat*	4.8	n. d.	pBluescript	pRQ7	*T. maritime* *T. neapolitana*	Yu et al. (2001)
pIKM1	*kat*	6.3	n. d.	pUC9	pIM13	*C. thermocelham* *Tab. saccharolyticum*	Tyurin et al. (2004) Mai and Wiegel (2000)
pCTC1	*ermE*[d]	7.2	n. d. (high)	pUC19	pAMβ1, RP4	*Tab, thermosaccharolyticum*	Klapatch et al. (1996)
pTE16	*cat, erm*E	7	n. d. (low)	ColEH1	oriCP	*Tabr. ethanolicus*	Peng et al. (2006)
p3CHPT	*kat*	–	–	pUC19	–	*Tabr. Mathranii*	Yao and Mikkelsen (2010) Mikkelsen (unpublished)
pUCG18	*kat*	6.3	n. d.	pUC18	pBST1	*G. thermoghucosidasius*	Taylor et al. (2008)
pRP9	*Cat*	2.9	n. d.	pUC19	pLM6	*G. stearothermophihus*	de Rossi et al. (1994)
pMSR10	*Cat*	6	n. d.	pUC19	pMSR0	*B. coagulas*	Rhee et al. (2007)
Thermophilic archaea							
pAG21	*adh*[e]	6.5	n. d.	pUC19+rom/rop	pGT5	*S. acidcaldarius* *P. furiosus*	Aravalli and Garret (1997)
pA-pN	*pyrE, F*	9	2–8	pBluescript deletion derivative	pRN1	S. acidcaldarius	Berkner et al. (2007)
pHZ2	*pyrE, F*	11.2	3–5	pGEM3Z	pRN2	*S. islandicus*	Deng et al. (2009)
pSSR	*hmg*[h]	8.1	n.d.	pGEM3Z	pRN2	*S. islandicus*	

pSeSD	*pyrE, F*	8.4	n.d.	pGEM3Z	pRN2	*S. islandicus*	
pEX	*hph*[f]	6.4	1–2	pGEM5Zf	SSV1	*S. solfataricus*	Cannio et al. (1998)
pMJ03	*pyrE, F, lacS*[g]	21.8	Integrated(1)	pUC18	SSV1	*S. solfataricus*	Jonuscheit et al. (2003)
pMSSVlacS	*lacS*	9.6	1–130	pUC18 deletion derivative	pSSVx/ SSV2	*S. solfataricus*	Aucelli et al. (2006)
pYS2	*pyrE*	6.4	20–30	pLitmus38	pGT5	*P. abyssi*	Lucas et al. (2002)
pYS3	*hmg*[h]	6.7	1–2	pUC19	pGT5	*P. furiosus*	Waege et al. (2010)
pJFW027	*pyrF*	6.75	1	pJFW017	Cdc6/Orcl	*P. furiosus*	Farkas et al. (2011)
pTK01	*trpE*[i]	7.5	3	pCR2.1-TOPO	pTN1	*Tc. kodakaraensis*	Santangelo et al. (2008)
pTS535	*trpE*, 6-metyhl Purine (TK0664)	5.4	n.d.	pUC118	pTN1	*Tc. kodakaraensis*	Santangelo et al. (2010)

n.d. not determined
[a]Codes for kanamycin nucleotidyltransferase
[b]Codes for tryptophan synthetase
[c]Codes for chloramphenicol acetyltransferase
[d]Erythromycin resistance gene
[e]Codes for alcohol dehydrogenase
[f]Codes for hygromycin phosphotransferase
[g]Codes for β-galactosidase
[h]Codes for HMG-CoA reductase
[i]Tryptophan synthetase

Lipps 2008). Replicons for the host are based on extrachromosomal elements and are sometimes based on the replication origin from the host (Contursi et al. 2004; Farkas et al. 2011).

The need for host-vector systems has led to a search for extrachromosomal elements from thermophiles. However, there are limited numbers of reports concerning the phage infecting thermophilic bacteria where only the phages infecting *T. thermophilus*, *Rhodothermus marinus*, and *G. stearothermophilus* to our knowledge have been studied (Yu et al. 2006; Minakhin et al. 2008; Blondal et al. 2005; Liu and Zhang 2008; Wei and Zhang 2010). In contrast, many thermophilic bacterial strains containing plasmids are reported.

There are many reviews concerning archaeal (thermophilic archaeal) viruses and plasmids (Prangishvili et al. 2006; Lipps 2006, 2007; Pina et al. 2011); hence, we will describe only the latest. Garrette and his coworkers studied genome sequences associated with unidentified viruslike particles (VLPs) using metagenomic analyses where two novel viral genomes and four plasmids were assembled (Garrett et al. 2010). They investigated potential hosts for the viruses and plasmids by matching their genome sequences to spacer sequences using the CRISPR/Cas system (van der Oost et al. 2009). Four novel viruses infecting *Sulfolobus* and *Acidianus* were isolated and characterized (Redder et al. 2009), and a novel virus, TPV1, infecting *Thermococcus*, was isolated (Gorlas et al. 2012). Additionally, *Aeropyrum pernix* bacilliform virus 1 (APBV1) and *A. pernix* proviruses, APSV1 and APOV1, were isolated (Mochizuki et al. 2010, 2011). APBV1 are the first virus from the genus *Aeropyrum* with the smallest prokaryotic dsDNA viral genome at 5.2 kb. Three new plasmids, pTN2 (~12 kb) from *Thermococcus nautilus* sp.30-1, pT26-2 (21.5 kb) from *Thermococcus* sp. 26-2, and pP12-1 (~12 kb) from *Pyrococcus* sp. 12-1 p, were isolated (Soler et al. 2010). These extrachromosomal elements may provide new tools for host-vector systems in thermophilic archaea.

13.2.4 Hosts (Recipient Cells)

When we compare plating efficiency, transformation frequency, genetic stability, and restriction/modification activity (etc.) within the same species, it becomes obvious that there are large differences depending on the strain. Therefore, screening a variety of strains is one useful strategy in developing a host-vector system (Narumi et al. 1992). For example, some *R. marinus* isolates have shown variable plating efficiencies or failed to produce colonies on solid media (Bjornsdottir et al. 2006). Some *Clostridium thermocellum* isolates exhibit large differences in transformation efficiency (Tyurin et al. 2004). *S. solfataricus* P1 and P2 do not recombine foreign DNA into their chromosome (Jonuscheit et al. 2003), whereas *S. solfataricus* PBL2025 originating from *S. solfataricus* 98/2 is shown to be capable of recombining foreign DNA into its chromosome (Schelert et al. 2004).

13.2.4.1 Genetic Stability

In genetic experiments, problems arise when large parts of the host genome can be changed within a few generations. For example, transposable elements that are present in very high numbers in the genomes of *S. solfataricus* P1 and *S. solfataricus* P2 (She et al. 2001; Brügger et al. 2004) that are widely used can influence the results of genetic experiments (Martusewitsch et al. 2000; Redder and Garrett 2006; Schleper et al. 1994). Apparent mutation frequencies vary by more than five orders of magnitude between different *Sulfolobus* strains (Berkner and Lipps 2008). Therefore, with organisms that lack genomic stability such as *S. solfataricus* P1 and P2, handling should be considered avoiding long-term cultivation.

13.2.4.2 Restriction/Modification

The presence of the restriction/modification system is an important property of host strains to establish host-vector systems. Enhancement of transformation efficiency was achieved using specific methylation in several *Clostridium* species (Chen et al. 1996; Jennert et al. 2000). Conversely, methylation of plasmid DNA did not influence the transformation efficiency in the thermophilic bacterium *B. coagulans* (Rhee et al. 2007). And, with *Thermoanaerobacterium thermosaccharolyticum* HG-8 that did not exhibit restriction activity under the condition examined, increased transformation efficiency by two orders of magnitude was reported when the transforming plasmid was isolated from *T. thermosaccharolyticum* as compared to the DNA isolated from *E. coli* cells. This was not the result of the restriction activity but also may be explained by other factors where a possible alternative explanation is the differing topology of the DNAs (Klapatch et al. 1996).

13.3 Critical Review and Analysis; Application

For industrial processes, the applications of host-vector systems in thermophiles offer enormous possibilities. In the first part of this section, we present the tools for applications of host-vector systems, and in the second part of this section, we present the typical applications for host-vector systems in thermophiles.

13.3.1 Tools for Application

13.3.1.1 Expression Vectors and Reporter Gene Systems

Expression vectors are generally constructed by combining a suitable promoter with multiple cloning sites and ribosome binding sites (RBS) and a terminator sequence included in a shuttle vector (Fig. 13.1). Especially for *T. thermophilus*, a number of

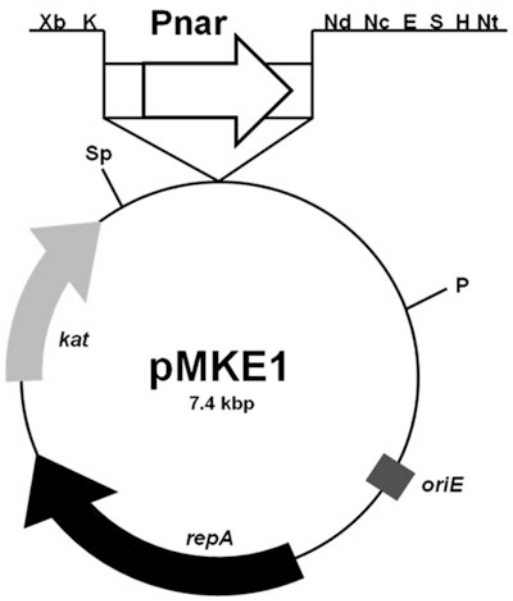

Fig. 13.1 *Thermus* expression vectors pMKE1 (Moreno et al. 2003). Kat: kanamycin nucleotidyltransferase; Pnar: promoter of the nitrate reductase operon; OriE: *E. coli* replication origin; Xb: *Xba*I; K: *Kpn*I; Nd: *Nde*I; Nc: *Nco*I; E: *Eco*RI; S: *Sal*I; H: *Hind*III; Nt: *Not*I; Sp: *Sph*I; P: *Pst*I

expression vectors were developed (Averhoff 2006). However, study of RBS and terminator sequences in thermophiles is limited (Lasa et al. 1992; Santangelo et al. 2008). Changing the RBS sequence of *T. kodakaraensis* affected the expression of products considerably (Santangelo et al. 2008).

Several useful promoters, particularly inducible promoters, were found and used for protein expression in some thermophiles (see Sect. 13.3.2.1). Requirements for an inducible promoter are a low basal activity combined with a high activity upon induction. The inducer should be easy to handle and stable under culture conditions and should not negatively affect the host. To characterize promoters, reporter gene systems for thermophiles were developed in several thermophilic bacteria and archaea. In addition, they were selected to be used with selectable marker genes (*pyrE*, *pyrE*, *kat*, etc.) as reporters (Maseda and Hoshino 1995); in addition, a few genes coding for easily detectable and quantitatively assayable thermostable enzymes have been used as reporters in thermophiles. In *T. thermophilus*, the β-galactosidase gene from *Thermus* spp. and *T. thermophilus* HB27 (Koyama et al. 1990; Moreno et al. 2003; Cava et al. 2007) is used widely with several reporter gene systems. Other genes used or potentially used as reporter genes are the malate dehydrogenase from *Thermus flavus* gene (Kayser et al. 2001), the α-galactosidase gene from *Thermus* spp. (Koyama et al. 1990) and *G. stearothermophilus* (Fridjonsson et al. 2002), and the periplasmic hyperalkaline phospherase from *T. thermophilus* (Moreno et al. 2003).

The known reporter genes for thermophilic archaea are limited. Thermostable β-galactosidase from *S. solfataricus* encoding *lacS* was used as a reporter gene for transcription analysis in *S. solfataricus* (Jonuscheit et al. 2003) and *S. islandicus*

(Deng et al. 2009). In *T. kodakaraensis*, β-glycosidase was used as a reporter gene (Santangelo et al. 2009, 2010).

The fluorescent proteins are also extremely useful for reporter gene systems. There are some thermostable fluorescent proteins, e.g., superfolder GEP (sGFP) and CGP (Pedelacq et al. 2006; Kiss et al. 2009), where sGFP is able to fold and to fluoresce properly in *T. thrermophilus* at 70°C and modified CGP is able to fold and to fluoresce properly in *S. acidocaldarius* at 76°C, respectively (Cava et al. 2008; Henche et al. 2012).

13.3.1.2 Targeted Gene Disruption (Replacement)

This tool was recently developed especially for the thermophilic archaea, *T. kodakaraensis* (Matsumi et al. 2007; Santangelo et al. 2010; Sato et al. 2005), *P. furiosus* (Lipscomb et al. 2011), *S. solfataricus* (Albers and Driessen 2007; Schelert et al. 2004; Worthington et al. 2003), *S. acidocaldarius* (Kurosawa and Grogan 2005; Grogan and Stengel 2008), and *Sulfolobus islandicus* (Deng et al. 2009; Zheng et al. 2012; Zhang and Whitaker 2012). For thermophilic bacteria, the tool was used in *T. thermophilus* (reviewed by Cava et al. 2009), used in some low-G+C thermophilic gram-positive bacteria (Mai and Wiegel 2000; Tyurin et al. 2006), and used to engineer these microorganisms (see Sect. 13.3.2.2).

The simplest strategy is the direct replacement of a gene with a selectable marker by homologous recombination. However, since the number of selectable markers for thermophiles is very limited, this method has the disadvantage that the selectable marker cannot be reused (Mevarech and Allers 2007). To overcome this disadvantage, alternative methods are used to select using marker-free deletion mutants (pop-in/pop-out and MCI, marker circularization and integration, methods) that were developed in some thermophiles (Fig. 13.2). This method has been used successfully for *T. thermophilus* (Blas-Galindo et al. 2007; Laptenko et al. 2007; Tamakoshi et al. 1999), *T. kodakaraensis* (Santangelo et al. 2010; Sato et al. 2005), *P. furiosus* (Lipscomb et al. 2011), and *S. islandicus* (Deng et al. 2009; Zheng et al. 2012; Zhang and Whitaker 2012).

13.3.2 Case Studies

13.3.2.1 Thermophiles as Cell Factories

Current development of structural genomic programs for the thermophilic bacteria and archaea shows about 40% of the protein from thermophiles cannot be expressed or are expressed in inactive forms in mesophilic hosts (Jenney and Adams 2008). The difference in GC content and codon bias has been shown to disturb the efficient gene expression of thermophilic proteins in mesophiles (Ishida et al. 2002). The different growth temperatures cause improper folding of the products (Hidalgo et al.

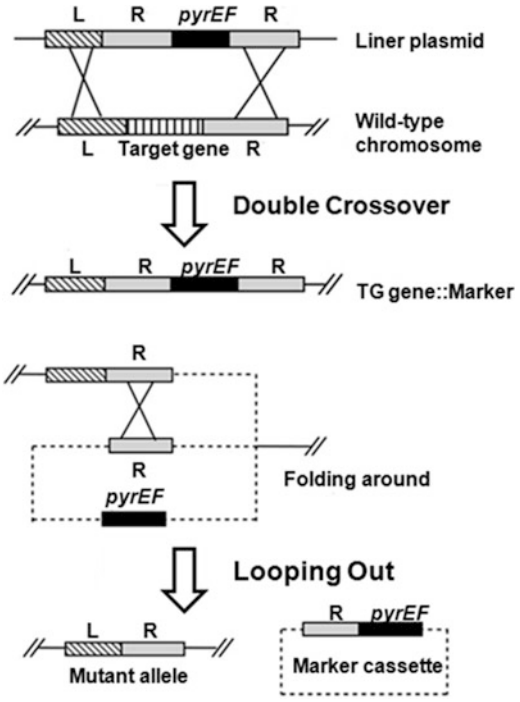

Fig. 13.2 Two-Step Markerless Gene Deletion Using *pyrEF* Marker (She et al. 2009). Knockout plasmid was digested with a unique enzyme, resulting in a linear plasmid for transformation. Transformants containing the target gene: marker locus grown on uracil-free plates (uracil-dropout selection) and then the markerless gene knockouts are selected with 5-FOA (counter selection). L: L-arm; R: R-arm and TG gene: target gene

2004). And the most severe problem is instability that may be due to a requirement for a complex maturation process only present in thermophiles. For these reasons, using a thermophilic cell factory to overcome these problems was clearly required.

There are some examples of constitutive and induced overexpression of heterologous and homologous genes from thermophiles using thermophiles, primarily in *T. thermophilus*, *Sulfolobus*, and *T. kodakaraensis*.

Thermus Thermophilus as a Cell Factory

There are a number of examples of constitutive and induced expressions of proteins in *T. thermophilus* (Moreno et al. 2003, 2005; Hidalgo et al. 2004; Park et al. 2004). *Thermus thermophilus* HB27 or its derivatives were used because it is plasmid-free and has higher transformation efficiency. Constitutive expression was performed frequently using a *slpA* promoter (Cava et al. 2007) where respiratory complex I (Pnqo) and ribosome protein promoters have been used. Inducible expression was performed using nitrate reductase (Pnar), NRC complex (Pnrc), dnaK, or GroE/L promoters.

When the Mn-dependent catalase gene from *T. thermophilus* HB27 and other strains were overexpressed in *E. coli*, the products were expressed in an inactive form (Hidalgo et al. 2004). They reported the backbone of the product was folded

in a way similar to the native holoenzyme where the absence of activity in the product was not functional possibly lacking the Mn^{2+} in its active center. Conversely, the genes were overexpressed in an active form in *T. thermophilus* HB8 and HB27 using the PMKE1 vector under the control of the Pnar from *T. thermophilus* HB8 (Hidalgo et al. 2004).

Heterologous genes from a variety of diverse species can be functionally expressed in *T. thermophilus* HB27 including the genes from the hyperthermophilic archaea and cofactor-requiring and multicomponent enzymes from heterologous species (Park et al. 2004). However, not all heterologous genes can be readily expressed in *T. thermophilus*. For example, the chloramphenicol acetyltransferase (CAT) gene from *Staphylococcus aureus* was not functionally expressed.

Sulfolobus as a Cell Factory

For *S. solfataricus*, *S. acidocaldarius*, and *S. islandicus*, functional expression of heterologous and homologous genes was demonstrated (Albers et al. 2006; Berkner et al. 2010; HasenÖhrl et al. 2008; Zaparty et al. 2010). Inducible expression was performed using arabinose-binding protein promoter (ParaS), chaperonin TF55 promoter (Ptf55), and maltose-/martoliose-binding protein promoter (PmalS).

Albers et al. reported production of the Fe-S protein of the ABC class proteins (SSO0287) and membrane-associated ATPases (SSO2680) from *S. solfataricus* using the pSVA vector under the control of Ptf55 and ParaS (Albers et al. 2006). Attempts to obtain correct assembly of Fe-S clusters of SSO0287 using expression in *E. coli* failed, whereas the produced protein in *S. solfataricus* contained the Fe-S clusters. When the SSO2680 gene from *S. solfataricus* was expressed in *E. coli*, most of the product was recovered from inclusion bodies, and only a small fraction was isolated from the soluble fraction (Albers et al. 2006). Conversely, when the gene was expressed in *S. solfataricus*, the product was purified from detergent solubilized membranes of the host. Further, the specific activity of the protein produced and purified from *S. solfataricus* was six times higher than the enzyme isolated from *E. coli* that indicated a more native conformation in the homologously expressed protein. In addition, the stable production of the proteins in large-scale fermentations was performed (45 L).

T. kodakaraensis as a Cell Factory

Functional expression of heterologous and homologous genes was demonstrated in *T. kodakaraensis* using constitutive glutamate dehydrogenase promoter (P-*gdh*) and cell surface glycoprotein promoter (P-*csg*). For example, α-1,4 glucan phosphorylase from *S. solfataricus* and endogenous pantoate kinase were successfully expressed in *T. kodakaraensis*, while these failed to be expressed in *E. coli* (Mueller et al 2009; Yokooji et al. 2009). Recently, gene expression and efficient protein secretion system

was successfully constructed (Takemasa et al. 2011). Two protease genes with putative signal sequence were overexpressed under the control of P-*csg*. As a result, the product was found exclusively in the culture supernatant and was not detected in the soluble and membrane fraction of the cells. In addition, this engineered strain had significantly greater protein degradation capability than that of wild-type strain.

Others

Induced heterologous expression was observed in the thermophilic bacterium *R. marinus* (Bjornsdottir et al. 2007). Genes encoding α- and β-galactosidase from *Thermus brockianus* were expressed under the control of *dnaK* and *dnaJ* promoters. Heterologous expression of hydrolytic enzymes (mannanase, endoglucanase, exoglucanase, and cellobiohydrolase) was observed in *Thermoanaerobacterium saccharolyticum* (Mai and Wiegel 2000). To increase ethanol yields, in some thermophilic ethanologens, several enzymes have been expressed (Thompson et al. 2008; Yao and Mikkelsen 2010). For example, glycerol dehydrogenase and pyruvate decarboxylase from *Z. mobilis* and *Sarcina ventriculi* were used (See Sect. 13.3.2.2).

In the hyperthermophilic archaeon, *P. furiosus*, functional expression of homologous and heterologous genes was demonstrated (Waege et al. 2010; Chandrayan et al. 2012; Basen et al. 2012). It should be noted that homologous overproduction of an affinity-tagged hydrogenase was achieved. In the overexpression strain, transcription of the hydrogenase operon genes were under the control of a strong constitutive promoter. Additionally, a *Strep*-tag II was added to the N terminus of one subunit.

13.3.2.2 Metabolic Engineering in Thermophiles

The potential application of thermophiles themselves for industrial application is enormous and has particular application in biofuel production (Barnard et al. 2010). There are a number of advantages for using thermophiles in industrial applications; however, there are the negative aspects, as mentioned above. To overcome the negative aspects, metabolic engineering was recently developed in thermophiles. Several heterotrophic hyperthermophiles have been focused upon to their abilities to produce hydrogen. Of these, *T. kodakaraensis* was engineered to enhance hydrogen production (Santangelo et al. 2011). Here, we will describe metabolic engineering in some thermophilic ethanologenes.

Bioethanol Production

Ideal microbe strains for bioethanol production should produce high yields of ethanol, with few side products, and have high ethanol tolerance. However, the production of high ethanol yields with high ethanol tolerance is typically lacking in the thermophilic ethanologens, e.g., thermophilic *Clostridia*, *Thermoanaerobacterium,*

and *Thermoanaerobacter* (Dien et al. 2003; Zaldivar et al. 2001). Low product yields are often the result of mixed acid fermentation that reduces the yield of the metabolite of interest. These problems have been solved through genetic modification of the metabolism (metabolic engineering). Genetic modification tools have been developed in some thermophilic ethanologenes and used for insertion, deletion, or overexpression of the targeted genes.

Thermophilic Clostridia

Using UV mutation, a *C. thermocellum* lactate dehydrogenase mutant with higher ethanol tolerance (4% v/v) and ethanol production (~12.5 g/L) was constructed (Tailliez et al. 1989a, b). The acetate kinase (Ack)- and the lactate dehydrogenase (ldh)-disrupted strain of *C. thermocellum* tolerated up to 60 g/L of ethanol and was also able to produce as much as 26 g/L of ethanol (van Lynd et al. 2005).

Thermoanaerobacterium

To increase ethanol yield in *T. saccharolyticum*, metabolic engineering of the end product metabolism was performed where a knockout mutant for *ldh* showed fourfold increase in ethanol yield (Desai et al. 2004). Recently, *T. saccharolyticum* strain ALK1 was engineered to produce ethanol as the only organic product using knockout genes encoding ldh and Ack (Shaw et al. 2008). Strain ALK1 was cultivated in continuous culture with higher concentrations of xylose, glucose, mannose, and galactose and produced 33 g/L ethanol in continuous culture and 37 g/L in fed-batch cultures. To date, these are the highest yields of ethanol production by thermophilic anaerobes reported.

Thermoanaerobacter

Burdette and Arni reported ethanol tolerance in *T. ethanolicus* seemed to be linked to the function of the alcohol dehydrogenase (Burdette et al. 1997). A *T. ethanolicus* mutant in this gene was tolerant to 8% ethanol (v/v) (Burdette et al. 2002). In addition, a *T. mathranii* BG1L1 *ldh* mutant has resistance to 8.3% ethanol (Georgieva et al. 2007). Recently, Yao and Mikkelsen created a recombinant strain BG1G1 expressing a heterologous NAD^+-dependent glycerol dehydrogenase (GLDH) in combination with the deletion of *ldh*. Strain BG1G1 produced ethanol at high yields and acquired the capability to utilize glycerol as an extra carbon source in the presence of xylose (Yao and Mikkelsen 2010).

Geobacillus

The functional expression of the pyruvate decarboxylase (Pdc) from *Z. mobilis* in *G. thermoglucosidasius* was developed. Although functional expression was

demonstrated in *G. thermoglucosidasius*, Pdc activity was not stable above 54°C (Thompson et al. 2008). A mutant strain with knockouts in the *ldh* and pyruvate formate lyase genes combined with upregulation of Pdc was constructed where this mutant could ferment pentose and hexose sugars, producing ethanol efficiently as the major product (Cripps et al. 2009).

13.3.2.3 Directed Evolution in Thermophiles

Prediction of the changes to be made in a specific protein to increase its thermostability is a difficult task even if the three-dimensional structure is known (Chautard et al. 2007). Therefore, directed evolution methods were developed especially for thermostable enzymes. In addition to antibiotic resistance genes, some useful metabolic enzymes have been adapted using host-vector systems in thermophiles. We described thermo-adaptation of the antibiotic genes in the section of "Selectable Markers" (See Sect. 13.2.2) and will not discuss this further here.

The application of this method to metabolic enzymes is much more difficult than resistance genes because it requires the genetic modification of the organisms in order to make its growth dependent on the activity of the enzyme during the selection conditions. Thermo-adaptation of a 3-iso propylmalate dehydrogenase (LeuB) from *E. coli* and *Saccharomyces cerevisiae* using the *T. thermophilus* host-vector system was reported (Tamakoshi et al. 1995, 2001). Thermo-adaptation of α-galactosidase from *G. stearothermophilus* in a *T. thermophilus* mutant was also reported (Fridjonsson et al. 2002). It is not always possible to make the organism dependent on the selectable activity, and this method is inapplicable to proteins without a known enzymatic activity. Further, this method requires a high-throughput robotic format with high costs and is time-consuming. To overcome these limitation and problems, the activity-independent THR method for selection of thermostable mutants in *T. thermophilus* was developed (Chautard et al. 2007). Using this method, they demonstrated thermo-adaptation of human interferons.

13.4 Future Perspectives

There is considerable progress in the development of host-vector systems for several thermophiles. However, in comparison with the host-vector systems in *E. coli* or yeast, the tools available for thermophiles are very limited. There is need for further improvements to apply these host-vector systems for thermophiles to industrial field use. For example, host-vector systems in thermophiles lack highly controlled repression and induction systems as used for T7 expression systems. Further understanding the infecting mechanisms of phage and the viruses of thermophilic bacteria and archaea would remove many methodology problems where this system would allow

for controlled and conditional overexpression of proteins in thermophiles more efficiently (Allers et al. 2010). A detailed study concerning thermophilic phages and viruses will make further progress in the thermophilic host-vector systems. Recently, metagenomics-based functional screening was successfully used to isolate novel biocatalysts from various environments and deserves considerable attention from industry (Tuffin et al. 2009). However, the lack of host-vector systems for functional screening in thermophiles has delayed efficient screening of metagenomic libraries from thermophilic environments. More recently, the *E. coli-T. thermophilus* shuttle fosmid system permitting the use of thermophilic bacterial hosts for the activity screening assay was developed (Angelov et al. 2009). In the near future, a fosmid vector system in thermophiles will be required to search for novel thermostable enzymes.

(Hyper)thermophiles are defined simply by their growth temperatures. However, other than the fact that they all thrive at high temperatures, (hyper)thermophiles span an extremely diverse group of organisms (Atomi et al. 2012). A number of recent studies have revealed several novel enzymes and metabolic pathways in thermophiles, which had not yet been recognized in mesophilic model organisms. Today, thermophiles are focused upon as a source of thermostable version not only of established enzymes but also of novel enzymes. Thermophiles have been also focused upon to their abilities to ferment structurally complex carbohydrates and to produce hydrogen in biofuel production. Additionally, several thermophiles are becoming aware of their (heavy) metal resistance and abilities to carry out specific metal transformation in bioremediation and other applications, such as bioleaching (Bini 2010). Further industrial application of thermophiles and their enzymes will require the development of host-vector systems in thermophiles.

13.5 Conclusions

Host-vector systems in thermophiles have been established in several model microorganisms. For these microorganisms, various tools are available. For example, shuttle, reporter, expression vectors, and targeted gene replacement systems were developed. For industrial application, using these tools, thermophiles have been used as cell factories for the expression of thermophilic enzymes and used for the selection of thermophilic mutants of mesophilic proteins through directed evolution. In addition, these tools were used to metabolic engineer in these organisms to improve desirable cell properties.

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Chapter 14
Molecular Chaperones in Thermophilic Eubacteria and Archaea

Muhamad Sahlan and Masafumi Yohda

Abstract Thermophilic organisms tolerate or adapt to high temperatures by making their proteins thermostable or thermophilic. Even though, thermophilic bacteria have their own optimal growth temperatures, and heat-shock responses are still induced at the temperatures higher than optimal temperatures. The molecular chaperone systems of thermophilic eubacteria are very similar to those of mesophilic eubacteria. On the contrary, the molecular chaperone system of hyperthermophilic archaea is much simpler than those of other organisms. Within the hyperthermophilic archaea, only the following six kinds of chaperones have so far been identified: group II chaperonins, prefoldin, small heat-shock proteins, peptidyl-prolyl cis-trans isomerases, AAA proteins, and NAC. These archaea lack the Hsp70 chaperone system as well as Hsp90 and Hsp100, though these are thought to be indispensable chaperones in all other organisms. Since group II chaperonins are highly induced at elevated temperatures and related to the stress response of hyperthermophilic archaea, this manuscript focuses on the group II chaperonin and its cofactor, prefoldin, in addition to sHsps that are ubiquitous in thermophilic eubacteria and archaea. The limited number of molecular chaperones in hyperthermophilic archaea might be due to the relatively high stability of their proteins. The molecular chaperones in

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hyperthermophilic archaea might contribute to the protection of only a limited number of relatively unstable proteins.

Keywords Chaperone • Chaperonin • Prefoldin • Small heat-shock protein • Folding • Aggregation

14.1 Introduction

Thermophilic organisms tolerate or adapt to high temperatures by making their proteins thermostable or thermophilic (Ladenstein and Antranikian 1998). Even though, thermophilic bacteria have their own optimal growth temperatures, and heat-shock responses are still induced at the temperatures higher than optimal temperatures. Several heat-shock proteins are induced during the heat-shock response. These proteins are named Hsp104 (ClpB), Hsp90 (HtpG), Hsp70 (DnaK), Hsp60 (Chaperonin, GroEL), Hsp40 (DnaJ), Hsp27 (sHsp), and Hsp10 (GroES) according to their molecular weights (Buchner 1996). Most of them are molecular chaperones that assist in the folding of nascent or unfolded polypeptides into mature conformations or protect denatured proteins from aggregation. The molecular chaperone systems of thermophilic eubacteria are very similar to those of mesophilic eubacteria (Table 14.1). The best-studied thermophilic chaperone system is that of *Thermus thermophilus* HB8. Chaperonin from *T. thermophilus* HB8 was first isolated and characterized in 1991 (Taguchi et al. 1991). Because of its high stability, detailed structural and functional characterizations of this chaperonin have been performed (Taguchi and Yoshida 1998). The stability of *Thermus* chaperonin also renders it amenable to nanobiotechnology. It captured a CdS quantum dot in its cavity and released it in an ATP-dependent manner (Ishii et al. 2003). Characterizations of other *T. thermophilus* HB8 heat-shock proteins, including ClpB, HtpG, Trigger Factor, and those of the DnaK/DnaJ and GrpE systems, have also been performed (Motohashi et al. 1994, 1999; Suno et al. 2004).

The molecular chaperone system of thermophilic archaea is significantly different from that of thermophilic eubacteria (Table 14.1). The acidothermophilic archaeon *Sulfolobus* sp. strain B12 (*S. shibatae*) has been shown to exhibit an acquired thermotolerance response (Trent et al. 1990). Cell survival during a temperature elevation from 70°C to the lethal temperature of 92°C was enhanced by as much as 6 orders of magnitude by preheating at 88°C. Thermotolerance correlated with preferential synthesis of one major protein (with a molecular weight of about 55 kDa) and, to a much lesser extent, two minor proteins (28 and 35 kDa). These smaller species could not be identified as known heat-shock proteins. The 55 kDa protein, TF55, was found to be an oligomeric complex composed of two stacked 9-membered rings, closely resembling the 7-membered ring complex of the chaperonin GroEL. The TF55 complex, similar to many molecular chaperones, binds unfolded polypeptides in vitro and has ATPase activity. The primary structure of

Table 14.1 Types of chaperones in thermophilic eubacteria and archaea

Chaperones	Thermophilic eubacteria	Thermophilic archaea
Hsp 100s/AAA proteins	ClpB, ClpA, ClpC, HslU	Absent (Genes of ClpA/B homologues were found in the genome of *Methanothermobacter thermautotrophicus*)
Hsp90s	HtpG	Absent
Hsp70s	DnaK	Absent
Hsp60s	GroEL	Group II Chaperonin (Thermosome)
Hsp40s	DnaJ	Absent
shsp	IbpA, IbpB, sHsp	sHsp
Hsp10	GroES	Absent
Prefoldin	Absent	Prefoldin
Other	Trigger Factor	NAC
	PPIase	PPIase

TF55, however, is not significantly related to that of the chaperonins. On the other hand, it is highly homologous to a ubiquitous eukaryotic protein, t-complex polypeptide-1 (TCP1), with which it shares 36–40% identity. It was therefore suggested that TF55, which is present in archaea, and TCP1, which is present in the eukaryotic cytosol, are members of a new class of molecular chaperones (Trent et al. 1991). The archaeal and cytosolic chaperonins were later categorized as group II chaperonins (Gutsche et al. 1999).

The molecular chaperone system of hyperthermophilic archaea is much simpler than those of other organisms. Within the hyperthermophilic archaea, only the following six kinds of chaperones have so far been identified: group II chaperonins, prefoldin (PFD), small heat-shock proteins (Kim et al. 1998b), peptidyl-prolyl cis-trans isomerases (Maruyama et al. 2004), AAA proteins (ATPases associated with various cellular activities), and NAC (Conway de Macario and Macario 2003; Macario and Conway De Macario 2001). These archaea lack the Hsp70 chaperone system as well as Hsp90 and Hsp100, though these are thought to be indispensable chaperones in all other organisms (Laksanalamai et al. 2004; Macario and Conway de Macario 1999).

Since group II chaperonins are highly induced at elevated temperatures and related to the stress response of hyperthermophilic archaea, this study focuses on the group II chaperonin and its cofactor, prefoldin, in addition to sHsps that are ubiquitous in thermophilic eubacteria and archaea.

14.2 Chaperonins

The chaperonins exist in all kingdoms of life; they have an essential role in mediating the folding of newly synthesized and stress-denatured proteins in an ATP-dependent manner (Fenton and Horwich 2003; Frydman 2001; Hartl and Hayer-Hartl

2002). They form double-ring assemblies composed of seven to nine subunits (each weighing about 60 kDa) per ring. Based on their amino acid sequences and oligomeric structure, the chaperonins are divided into two groups (Gutsche et al. 1999; Horwich et al. 2007). Group I chaperonins are found in the bacterial cytosol (e.g., GroEL), eukaryotic organelles, such as mitochondria (e.g., mHsp60) and chloroplasts (e.g., Rubisco-subunit-binding protein), and the cytosol of some archaea (Klunker et al. 2003). Group II chaperonin are present in cytosol of eukaryote (TCP1) ring complex (TRiC) or chaperonin containing TCP1 (CCT) (Frydman et al. 1992; Kubota et al. 1994) and are also present in the in archaea cytosol (Trent et al. 1991). Group I chaperonins generally exist as double-homoheptameric rings of about 800 kDa and functionally cooperate with Hsp10 (GroES), which forms the lid of the ring cage (Brinker et al. 2001; Mayhew et al. 1996; Weissman et al. 1996). Group II chaperonins consist of eight- or nine-member rings. They are independent of Hsp10 (GroES), the functions of which are supplied by (1) an α-helical extension in the upper ring and (2) the molecular chaperone prefoldin (also known as GimC (for genes involved in microtubule biogenesis complex) in yeast (Hansen et al. 1999; Leroux et al. 1999; Vainberg et al. 1998)).

14.2.1 Subunit Compositions of Archaeal Group II Chaperonins

The archaeal group II chaperonins are double-ring complexes composed of identical or unlike subunits. Two chaperonin genes are present in most complete archaeal genomes. Some thermophilic methanogens (e.g., *Methanopyrus kandleri*, *Methanococcus jannaschii* (also known as *Methanocaldococcus jannaschii*), and *Methanococcus thermolithotrophicus*) have homo-oligomeric chaperonins (Andra et al. 1996; Furutani et al. 1998; Kowalski et al. 1998). Other archaea are equipped with more than two chaperonin subunits. *Sulfolobus* spp. and *M. burtonii* contain three different types of subunits. Recently, it was found that there are five chaperonin subunits (Hsp60-1, -2, -3, -4 and -5) in *Methanosarcina acetivorans* (Maeder et al. 2005). Among them, Hsp60-1, Hsp60-2, and Hsp60-3 have orthologs in *Methanosarcinae*, but the others, Hsp60-4 and Hsp60-5, occur only in *M. acetivorans*. In most archaeal group II chaperonins, the subunit composition is thought to be constant. However, it changes according to the growth temperature in several thermophilic archaeal chaperonins: *S. shibatae*, *Thermococcus* sp. strain KS-1 (T. KS-1), and *T. kodakaraensis* (Izumi et al. 2001; Kagawa et al. 2003; Yoshida et al. 2001). T. KS-1 chaperonin is composed of two highly homologous subunits, α and β. In contrast to the constitutive expression of the α subunit, the expression of the β subunit is thermally induced. Furthermore, the β subunit shows higher thermostability than the α subunit (Yoshida et al. 2002). This difference in thermostability derives from differences in the *C*-terminal 20 amino acid residues, which give the *C*-terminal a flexibility that is not observed in the crystal structure of group II chaperonins (Yoshida et al. 2006). The natural chaperonin isolated from T. KS-1 is a hetero-oligomer and accommodates itself to the growth temperature by changing its subunit composition (Yoshida et al. 2001). The group II chaperonin obtained from

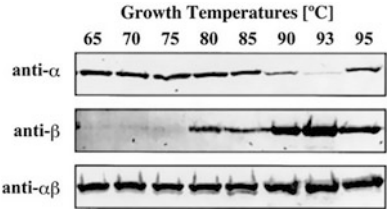

Fig. 14.1 Effect of growth temperature on the expression of *Thermococcus* chaperonin subunits. Western blot analysis of chaperonin subunits in *Thermococcus* sp. KS-1 cells grown at different temperatures. The chaperonin subunits were detected with the anti-α- (*top*), anti-β- (*middle*), and anti-αβ-subunit antibodies (*bottom*) (From Yoshida et al. 2001)

T. KS-1 cells grown at 60°C is mainly composed of α subunits, but at 90°C, the β subunit content is significantly increased (Fig. 14.1). The thermal stability of the chaperonin complex correlates with the β subunit content. Both the recombinant α and β subunits form stable double-ring homo-oligomers, which are termed Cpnα and Cpnβ. Both Cpnα and Cpnβ exhibit high ATP-dependent protein folding activity for denatured GFP and citrate synthase (CS) (Yoshida et al. 2002). Cpnα functions better at lower temperatures, such as 60°C, than Cpnβ. The group II chaperonin of *S. shibatae* is composed of three different subunits: α, β, and γ (Kagawa et al. 2003). The expression of α and β genes is increased by heat shock (86°C) and decreased by cold shock (60°C). Expression of the γ subunit is undetectable at heat-shock temperatures and low at normal temperatures (75–79°C) but is induced by cold shock. The denaturation temperatures for α, β, and γ subunits were determined to be 95.7, 96.7, and 80.5°C, respectively, by scanning calorimetry. *Sulfolobus* chaperonin complexes containing the γ subunit were less stable than those with α and/or β subunits only. *Sulfolobus* chaperonin does not have a fixed geometry in vivo. The α and β subunits form homo-oligomeric chaperonins in vitro, and mixtures of α, β, and γ form hetero-oligomers.

14.2.2 Structure of the Chaperonins

Chaperonin consists of two stacked rings of seven to nine subunits each, which together form a large cylindrical protein complex of approximately 1 MDa. Crystal structures of GroEL (Braig et al. 1994) and archaeal group II chaperonins (Ditzel et al. 1998; Shomura et al. 2004) indicate that they share a similar domain arrangement. Each subunit is comprised of three domains: the apical, intermediate, and equatorial domains (Fig. 14.2). The equatorial domain contains the ATP-binding site and is involved in intra- and inter-ring contacts. The apical domain is involved in binding to substrate proteins. The intermediate domain connects the equatorial and apical domains of each subunit and transfers the ATP-induced conformational changes from the equatorial to the apical domain. The most striking structural difference between the two classes of chaperonins is the lid of the central cavity of the chaperonin complex. The co-chaperonin GroES serves as a lid for the group I

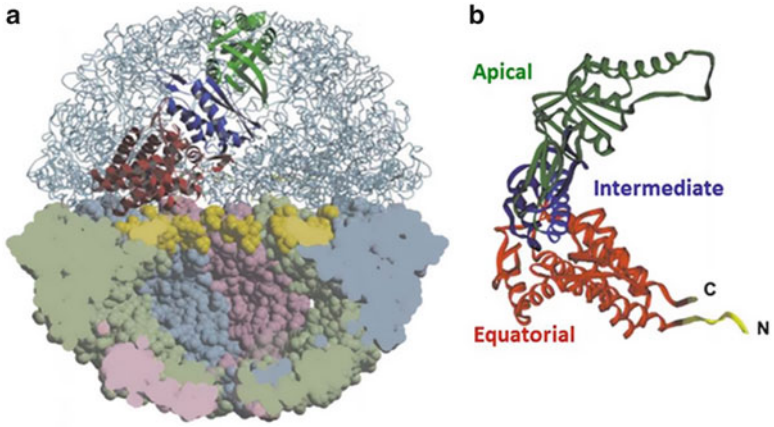

Fig. 14.2 Structure of an archaeal group II chaperonin from *Thermococcus* sp. KS-1. (**a**) Side view of the crystal structure of *T.* KS-1 CPN. One subunit has been marked to illustrate the domain structure, as shown in (**b**), (**b**) Domain arrangement of a subunit. The subunit has three distinct domains: equatorial (*red*), intermediate (*blue*), and apical (*green*) (From Shomura et al. 2004)

chaperonin, forming a heptameric dome-like structure. GroES interacts with one or both GroEL rings in an ATP-regulated fashion, thereby sealing the cavity from the outside. On the other hand, group II chaperonins function without the assistance of a cofactor corresponding to GroES. The crystal structure of the group II chaperonins suggests that the function of GroES is performed by long helical protrusions from the apical domain, known as the helical protrusion region (Ditzel et al. 1998). The region is strictly conserved among group II chaperonins but absent in group I chaperonins (Gutsche et al. 1999). In the crystal structure, the helical protrusions overhang the central cavity. The closed cavities have a hydrophilic surface implicated in protein folding (Fig. 14.2a). Structural and biochemical analyses have revealed that the protrusion acts as a built-in lid for the central cavity, substituting for a detachable lid. All the crystal structures of group II chaperonins obtained so far are in conformations with the built-in lid closed (Fig. 14.2a) (Ditzel et al. 1998). Despite many efforts to determine the structure of the group II chaperonin in the open conformation, no such structure has been obtained until now. It is quite likely that the chaperonins are locked in a "lid-closed" conformation under the crystallization conditions (Iizuka et al. 2003, 2004).

14.2.3 Substrates of the Archaeal Chaperonins

Recent analyses of group I and group II chaperonins from a methanogenic archaeon, *M. mazei*, show that chaperonin-interacting proteins in the archaeon can provide insights into the differential substrate specificities of the two chaperonin systems

(Hirtreiter et al. 2009). These coexisting chaperonins interact with partially overlapping subsets of proteins, making available parallel and intersecting pathways for protein folding and structural stability. The substrates of *M. mazei* chaperonins compose ~13% of the soluble proteome. This value is comparable to that seen in *E. coli*, where ~10% of the soluble proteome interacts with chaperonins (Kerner et al. 2005). The GroEL found in *M. mazei*, MmGroEL, appears to function differently from *M. mazei*'s group II chaperonin, MmCPN. The substrates for MmGroEL and MmCPN differ in size, hydrophobicity, electrostatic properties, and preferred fold types (Hirtreiter et al. 2009). The size distribution of MmGroEL substrates is very similar to that of the GroEL substrates in *E. coli*. The sequences of GroEL-selective substrates are more hydrophobic and less charged than those of the average soluble protein. Moreover, the GroEL interactors, such as the stringent GroEL substrates in *E. coli*, are enriched in structurally complex α/β domains of relatively large size (Kerner et al. 2005). The MmCPN-selective substrates follow more closely the size distribution of the complete soluble proteome and are less hydrophobic and more negatively charged, on average, than MmGroEL interactors. They include many small single-domain proteins that are generally absent from the set of MmGroEL interactors. In addition, large multi-domain proteins are enriched among the MmCPN-selective substrates relative to the substrates for MmGroEL. Some of these proteins dramatically exceed the size limit of the chaperonin cavity, suggesting a folding mechanism involving partial or domain-wise protein encapsulation by the flexible built-in lid segments of CPN. Substrate partitioning between MmGroEL and MmCPN may occur as well. The greater structural diversity of CPN substrates and their decreased hydrophobicity compared with the GroEL interactors suggests that the predominant function of MmCPN is not to prevent aggregation but to actively promote folding. Such a role would be facilitated by the hetero-oligomeric architecture of MmCPNs, which consist of three paralogous subunits that differ in their substrate-binding domains (Hirtreiter et al. 2009).

14.2.4 Reaction Mechanisms of Archaeal Group II Chaperonins

A key feature of group II chaperonins is their ability to close their cavity and encapsulate the bound substrate, thus providing a favorable environment for protein folding. The conformational cycle involving the opening and closing of the folding chamber is driven by ATP binding and hydrolysis. Based on the structural information available from electron microscopy, X-ray crystallography and small-angle X-ray scattering, three conformational states have been identified in the archaeal group II chaperonins: an open conformation, a spherical closed conformation, and an asymmetric bullet-shaped conformation (Ditzel et al. 1998; Iizuka et al. 2004; Nitsch et al. 1998; Schoehn et al. 2000a, b). These forms are hypothesized to exist at various points during the functional cycles of group II chaperonins. The model for the ATP-dependent conformational change of archaea group II chaperonins is shown in Fig. 14.3 (Kanzaki et al. 2008); in this model, ATP binding/hydrolysis

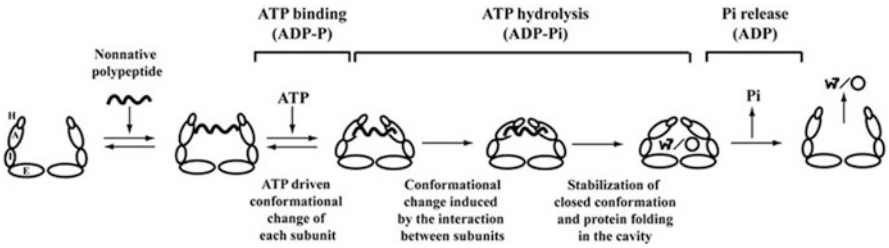

Fig. 14.3 Model for ATP-dependent conformational changes in group II chaperonins. Only one ring is shown. *A*, *I*, *E*, and *H* represent the apical domain, intermediate domain, equatorial domain, and helical protrusion, respectively (From Kanzaki et al. 2008)

induces conformational change in each subunit (Iizuka et al. 2003, 2004). These conformational changes seem to be independent and do not occur in a cooperative manner. The ATP-induced conformational changes bring the helical protrusions into close proximity. However, the conformation at this stage does not yet facilitate protein folding, perhaps because it is too transient. For protein folding to occur in the cavity, a further conformational change, which is induced by the interaction between helical protrusions, is required. The interactions among all of the helical protrusions at the lid of the cavity are likely to be important for the stabilization of the closed conformation (Fig. 14.3). The chaperonin complex is likely to be more stable in the closed conformation than in the open conformation. Therefore, energy from ATP hydrolysis might be required for the change from the closed conformation to the open conformation (Iizuka et al. 2005; Kanzaki et al. 2008).

14.3 Prefoldin

Prefoldin is a ~90 kDa molecular chaperone and is also known as GimC (genes involved in microtubule biogenesis complex). This chaperone was first identified in archaea in *Methanobacterium thermoautotrophicum* (Leroux et al. 1999). Prefoldin is expressed in archaea regardless of the presence of actin or tubulin. Sequence database searches reveal that prefoldin genes exist in nearly all of the eukaryotic and archaeal genomes sequenced so far. It has been suggested that prefoldin captures an unfolded protein substrate and transfers it to a group II chaperonin (Vainberg et al. 1998).

14.3.1 Structure of Prefoldin

The crystal structures of prefoldins from *M. thermoautotrophicum* and *Pyrococcus horikoshii* (PhPFD) have been resolved to 2.3 and 3.0 Å resolutions, respectively

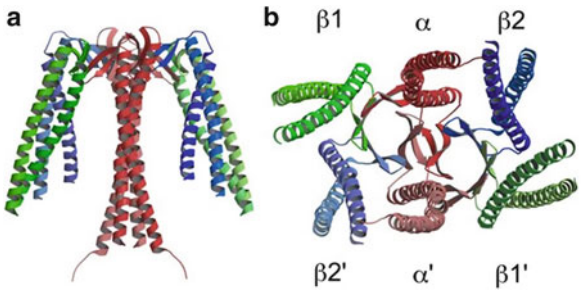

Fig. 14.4 Crystal structure of *Pyrococcus horikoshii* PFD, Overall structure of the α2β4 hexameric complex of PhPFD showing side (**a**) and *bottom* (**b**) views, as visualized using the program MOLSCRIPT. The crystal structure of PhPFD is composed of one α subunit and two β subunits that form an asymmetrical unit. The structure of the biologically active β hexamer was derived by applying crystallographic symmetry. The α subunit and two β subunits are shown in *red* (α subunit), *green* (β1 subunit), and *blue* (β2 subunit). The symmetrical molecules are shown in *light colors* (From Ohtaki et al. 2008)

(Ohtaki et al. 2008; Siegert et al. 2000). The quaternary structure of prefoldin is a hexamer composed of two α and four β subunits. Strikingly, prefoldin resembles a jellyfish (Fig. 14.4); its body consists of a double-barrel assembly with six long tentacle-like coiled coils protruding from it. The distal regions of the coiled coils expose hydrophobic patches. Each of the α and β subunits contains two or one central β-hairpins, respectively; these are *N*- and *C*-terminally flanked by coiled-coil helices. The coiled-coil helices within each subunit assemble into an antiparallel conformation, and these double-stranded coiled coils form the cavity (Ohtaki et al. 2008; Siegert et al. 2000).

Unlike other archaea, which express only one pair of α and β subunits, the hyperthermophilic archaea *Thermococcus* spp. expresses two pairs of prefoldin subunits genes. Genome analysis has revealed that *Thermococcus kodakaraensis* KOD1 has two pairs of prefoldin subunit genes, consisting of TK1005 and TK1121 (α subunits) and TK0643 and TK1122 (β subunits) (Danno et al. 2008; Fukui et al. 2005). *T.* KS-1 also expresses two pairs of prefoldin subunits genes: two α subunit genes, pfdα_1 and pfdα_2, and two β subunit genes, pfdβ_1 and pfdβ_2. The four recombinant *T.* KS-1 prefoldin complexes (PFDα1-β1, PFDα1-β2, PFDα2-β1, and PFDα2-β2) have similar heterohexameric structures and chaperone activity that was able to suppress thermal aggregation (Iizuka et al. 2008). Studies of *T. kodakaraensis* showed that PFDα1–β1 and PFDα2–β2 complexes were predominantly expressed in cells (Danno et al. 2008). Transcriptional and translational analyses showed that the β1 subunit of *T. kodakaraensis* is constitutively expressed under normal and heat-stressed conditions and that β2 expression is strongly induced at elevated temperatures.

The crystal structure of the *T.* KS-1 PFDβ1 subunit was determined at 1.7 Å resolution (Kida et al. 2008). *T.* KS-1 PFDβ1 subunits form a tetramer with four coiled-coil tentacles that resemble the jellyfish-like structure of heterohexameric

prefoldins. The β-hairpin linkers of β1 subunits assemble to form a β-barrel "body" around a central fourfold axis. Recently, the crystal structure of *T.* KS-1 PFDβ2 subunit was determined (Sahlan et al. 2010a). It also forms a tetramer similar to that of PFDβ1.

14.3.2 Substrates of Prefoldin

Detailed in vitro studies have revealed that archaeal prefoldins are capable of stabilizing a wide range of denatured, nonnative proteins such as actin, citrate synthase, conalbumin glucose dehydrogenase, dihydrofolate reductase (DHFR), firefly luciferase, green fluorescent protein (GFP), lysozyme, and rhodanese (Leroux et al. 1999; Lundin et al. 2004; Okochi et al. 2002, 2004), suggesting that archaeal prefoldins have a general role in recognizing and assisting in the production of nonnative proteins in the cell. Archaeal prefoldins are also effective at preventing thermally induced aggregation of such proteins.

The crystal structure of PhPFD indicates that this archaeal prefoldin exists as a hexamer with coiled-coil tentacles protruding from a double β-barrel (Fig. 14.4). Each subunit has hydrophobic residues at the very tips of the tentacles. Truncation studies show that the distal ends of the coiled-coil tentacles contribute to binding of nonnative proteins in a concerted manner (Lundin et al. 2004; Okochi et al. 2004; Siegert et al. 2000). The tentacles of prefoldin are approximately 80 Å long, and they bind and prevent the aggregation of denatured substrate proteins of varying sizes (14–75 kDa). EM images of GFP-bound PhPFD suggest that prefoldin functions as a molecular clamp, since the tentacles move ~12° outward upon binding. Because the tentacles may move independently, it is likely that prefoldin can grasp a range of nonnative proteins with different sizes and shapes using these independent clamps (Lundin et al. 2004). Martin-Benito et al. investigated the molecular strategies that archaeal prefoldins use to capture nonnative proteins. Their cryo-EM study of complexes of PhPFD and substrate proteins of different sizes shows that PhPFD selectively uses an increasing number of subunits to interact with substrate proteins of larger sizes. For example, lysozyme (14 kDa) interacts with a pair of β subunits and GFP (27 kDa) with a pair of β subunits and one α subunit. The much larger substrate protein, conalbumin (75 kDa), interacts with all six PhPFD subunits. These results also suggest that β subunits are more important for binding than α subunits, which is consistent with our results using truncation mutants (Martin-Benito et al. 2007).

14.3.3 Interaction Between Prefoldin and Group II Chaperonin

To understand the mechanism of substrate transfer from a prefoldin to a group II chaperonin, structural analysis of the prefoldin-chaperonin complex is essential.

Martin-Benito et al. showed, in a cryo-EM study, that eukaryotic prefoldin binds to CCT like a "pot lid"; their study also confirms the physical interaction between the two chaperones (Martin-Benito et al. 2002). In the three-dimensional reconstruction model, prefoldin seems to interact with the apical domain of CCT via its tentacle tips. Importantly, a structurally similar complex involving archaeal prefoldin and chaperonin was also observed by EM, confirming that the transfer mechanism is conserved between the eukaryotic and archaeal systems. It should be noted, however, that there is a significant difference between these complexes. The eukaryotic prefoldin can bind one or both rings of CCT simultaneously, whereas the PhPFD was observed to form only asymmetric complexes with *Pyrococcus* chaperonin (PhCPN), possibly due to differences between the models of conformational change, as described above (Martin-Benito et al. 2002, 2007). The two-dimensional average EM images of the eukaryotic prefoldin-chaperonin complexes showed that the interaction between the two chaperones involves binding of the outer regions of the prefoldin tentacles to the inner surface of the apical domains of the chaperonin. Eukaryotic prefoldin seems to insert its tips into the CCT cavity. Furthermore, a three-dimensional reconstruction also revealed that prefoldin interacts with two specific subunits in each of the CCT rings in a 1–4 arrangement. In archaea, the interaction between the two chaperones has been characterized in more detail by biochemical experiments and EM analysis. The two-dimensional average EM images of the archaeal prefoldin-chaperonin complex (PhPFD-PhCPN) showed that, as in the eukaryotic system, the tentacles of PhPFD use their coiled-coil tips to interact with the PhCPN apical domains. However, the PhPFD tentacles do not penetrate into the PhCPN cavity as much as those of the eukaryotic prefoldin, implying that there are differences between the transfer mechanisms used in archaea and in eukaryotic systems (Martin-Benito et al. 2007). Recent studies using subunit linkage mutants of *T.* KS-1 chaperonin suggest that two adjacent β tentacles of prefoldin coordinately interact with the two helical protrusions of chaperonin in the archaeal prefoldin-chaperonin complex (Sahlan et al. 2010b). To identify the sites of interaction and important amino acid residues in archaeal prefoldin and chaperonin in more detail, interaction studies using various mutants have been carried out. Truncation analyses of PhPFD subunits demonstrated that the *N*- and *C*-terminal regions of the outer β subunits are more important for chaperonin interactions than the two central β subunits, consistent with the EM data (Kurimoto et al. 2008; Okochi et al. 2004). The tentacles of the PhPFD β subunits are also important for substrate binding. Kinetic analyses suggest that prefoldin-chaperonin binding is multivalent and does not fit a simple one-to-one binding model, consistent with multiple binding sites between the two chaperones (Okochi et al. 2004; Zako et al. 2006). Interestingly, there is likely to be cooperation between the α and β subunits in the interaction with chaperonin, suggesting that the PhPFD α and β subunits bind to chaperonin in a concerted manner. On the other hand, a chaperonin mutant missing the helical protrusion demonstrates that this region is important for the interaction with prefoldin, consistent with the EM data. Biochemical and thermodynamic studies have indicated that the interaction between prefoldin and chaperonin is

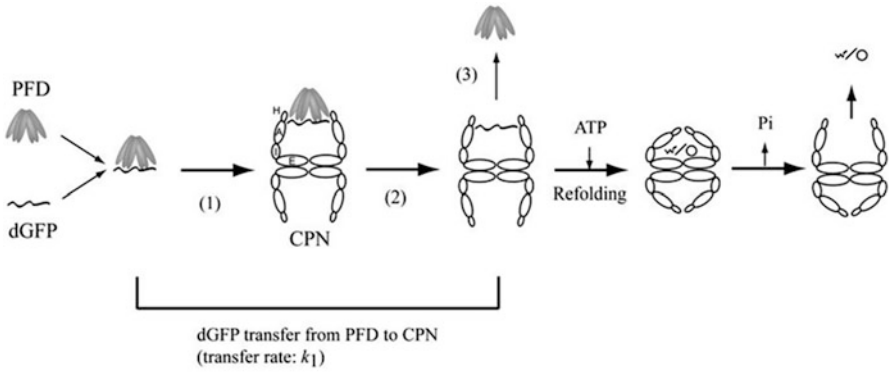

Fig. 14.5 Model of the structure of the complex of *Pyrococcus* prefoldin and *Thermococcus* group II chaperonin. The *Pyrococcus* prefoldin crystal structure is superimposed on the model of the open structure of *Thermococcus* chaperonin based on the sites of interaction between the chaperonin helical protrusion and the β subunit of prefoldin. Only one ring of the *Thermococcus* chaperonin is shown

driven by hydrophobic and electrostatic interactions (Sahlan et al. 2010a; Zako et al. 2006). Figure 14.5 shows a model of the structure of the complex of prefoldin and group II chaperonin.

14.3.4 Folding Activities of Prefoldin

Previous studies on archaeal prefoldin have shown that prefoldin possesses only holdase activity and is unable to fold unfolded proteins by itself (Okochi et al. 2002; Vainberg et al. 1998). Recently, it was demonstrated that PhPFD exhibits refolding activity for denatured lysozyme at lower than physiological temperatures. The interaction between PhPFD and denatured lysozyme at various temperatures was investigated using a surface plasmon resonance sensor. Although PhPFD had a strong affinity for denatured lysozyme at high temperatures, it exhibited relatively weak interactions at lower temperatures. The protein folding seems to occur via binding to and release from PhPFD due to this weak affinity. The results also imply that that prefoldin might be able to contribute to the folding of some cellular proteins whose affinity for prefoldin is weak (Zako et al. 2010).

14.3.5 Cooperation Between Prefoldins and Group II Chaperonins

Figure 14.6 shows a schematic reaction mechanism for the transfer of a substrate protein from prefoldin to group II chaperonin and its subsequent refolding. First, a

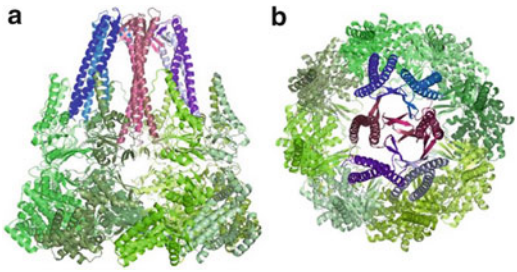

Fig. 14.6 Schematic model of the mechanism of substrate protein transfer from prefoldin to group II chaperonins. A, I, and E refer to the apical, intermediate, and equatorial domains, respectively. H represents the helical protrusion (Zako et al. 2006)

denatured protein is captured by prefoldin, possibly via hydrophobic interactions. The interaction between prefoldin and the substrate protein is characterized by a dynamic equilibrium between association and dissociation. Prefoldin binds to chaperonin like "the lid of a pot" (Martin-Benito et al. 2004). Archaeal prefoldin may utilize electrostatic and hydrophobic interactions to bind to the helical protrusion region of a group II chaperonin (Sahlan et al. 2010a; Zako et al. 2006). Binding of the chaperonin accelerates the dissociation of the substrate by about fivefold. Consequently, the released substrate proteins should then enter the cavity of chaperonin. According to the model, prefoldin would be released from chaperonin simultaneously with the release of substrate from prefoldin into chaperonin because the ternary complex of substrate protein, prefoldin, and chaperonin is thought to be unstable and transient. Subsequently, the binding of ATP favors the closed conformation of chaperonin, in which productive refolding occurs. Consequently, the release of the γ–phosphate generated by ATP hydrolysis in the active ring triggers the opening of the lid and release of the substrate (Zako et al. 2006).

14.4 Small Heat-Shock Protein

Small heat-shock proteins (sHsps) are the most ubiquitous of all chaperones; they exist in all types of organisms, including thermophilic organisms (Narberhaus 2002). They bestow thermotolerance upon cells (Plesofsky-Vig and Brambl 1995; van den Ijssel et al. 1994), protect proteins from thermal aggregation, and, in some cases, promote the renaturation of proteins (Horwitz 1992; Jakob et al. 1993; Muchowski and Clark 1998).

14.4.1 Sequence and Structure of sHsps

In comparison with other chaperones, sHsps are relatively heterogeneous in terms of their sequences and sizes. They are grouped together based on a conserved domain, the α-crystallin domain, which is named after the α-crystallin of

the vertebrate eye lens (Caspers et al. 1995). The α-crystallin domain is preceded by a highly variable *N*-terminal region and is followed by a short, partially conserved *C*-terminal extension (de Jong et al. 1998). Although all sHsps exist as large oligomeric complexes, their quaternary structures are diverse and are composed of between 9 and 40 subunits (Buchner et al. 1998). Structures of two sHsps in large oligomeric conformations have been revealed by X-ray crystallography (Kim et al. 1998a; van Montfort et al. 2001). The sHsp from a hyperthermophilic archaeon, *Methanocaldococcus jannaschii*, (MjHsp16.5) forms a spherical oligomer with 4-3-2 symmetry and consists of 24 subunits (PDB-ID: 1SHS). The diameter of the particle is approximately 120 Å. On the other hand, the sHsp from wheat, wHsp16.9, forms a double-ring-shaped oligomer consisting of 12 subunits with 3-2 symmetry (PDB-ID: 1GME). The oligomers consist of dimerized sHsps, despite diversity in their oligomeric structures as well as their amino acid sequences. The α-crystallin domain folds into a compact α-sandwich fold. The peripheral *N*- and *C*-terminal regions protrude from the α-crystallin domain. Although the *C*-terminal regions of the sHsps are very diverse in their amino acid sequences and lengths, the short IXI/V motif at the *C*-terminus is completely conserved in all homologous proteins. The IXI/V motif has a propensity to participate in inter-subunit interactions. These interactions are important for the structure and function of α-crystallins (Pasta et al. 2004). The recombinant sHsp of *Sulfolobus tokodaii* strain 7, StHsp19.7, forms a filamentous structure (Usui et al. 2004). Although StHsp19.7 does not exhibit chaperone function in vitro, fractionation of *Sulfolobus* extracts by size-exclusion chromatography combined with Western blotting indicates that StHsp19.7 exists as a filamentous structure in vivo.

14.4.2 Genes and Heat-Shock Induction of sHsps in Thermophilic Bacteria

The copy number of sHsp genes is variable. There exist three sHsp genes in the genome of *T. thermophilus* HB8. Thermophilic and hyperthermophilic archaea contain one, two, or three genes corresponding to sHsp homologues. Due to the relatively weak sequence identity among sHsps, it is often difficult to identify sHsp genes in genomic sequences.

Western blotting of proteins extracted from *T.* KS-1 cells using an antibody against sHsp of *T.* KS-1 has shown that the expression is not detectable at 65, 75, and 85°C (the optimal growth temperature) but is highly induced at 93 or 95°C (Fig. 14.7; (Usui et al. 2001). The amount of *T.* KS-1 sHsp in the cell at 93°C was estimated to be on the order of 0.1% of the total soluble protein. Although expression of chaperonins is induced at high temperatures, chaperonins are present at all temperatures. Thus, this sHsp plays a role only in the thermotolerance of *T.* KS-1, while chaperonin is important not only for thermotolerance but also for protein folding

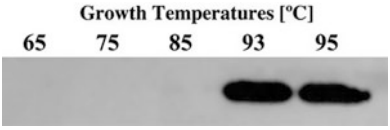

Fig. 14.7 The expression of *Thermococcus* sHsp is markedly increased under conditions of heat stress. The cellular content of *T.* sHsp in *Thermococcus* sp. KS-1 cells grown at different temperatures was determined using Western blot analysis (From Usui et al. 2001)

under normal conditions. *Pyrococcus furiosus* also expresses an sHsp in response to exposure to extreme temperatures (above 105°C; (Laksanalamai et al. 2001).

14.4.3 Functional Mechanisms of sHsp

The chaperone potential of sHsps is latent when they exist as large oligomeric structures under physiological conditions. At elevated temperatures, the large oligomeric structures dissociate into small oligomers (probably dimers). Therefore, we can argue that the hydrophobic sites are controlled by transitions among different oligomeric states (Shashidharamurthy et al. 2005; Yang et al. 1999). Basha and colleagues have shown that substrate-binding sites comprise both the newly exposed hydrophobic surfaces on the α-crystallin domain of the sHsp and the segments of hydrophobic residues on the *N*-terminal arm (Basha et al. 2006). In the presence of denatured proteins, dissociated sHsps form a large stable complex to protect the guest protein from aggregation. However, cross-linking experiments using Hsp26 have shown that dissociation into dimers is not required for chaperone function (Franzmann et al. 2005). Dissociation into dimers at higher temperatures is no longer observed for the cross-linked Hsp26, yet chaperone activity remains unaffected. Basha and colleagues have also shown that stability of the oligomer does not correlate with substrate protection ability (Basha et al. 2006).

An sHsp from *S. tokodaii* strain 7 with a molecular mass of 14 kDa (StHsp14.0) was shown to protect isopropyl malate dehydrogenase derived from *T. thermophilus* HB (TIPMDH) from thermal aggregation. Although TIPMDH is much more stable against thermal denaturation than mesophilic enzymes are, it denatures and aggregate at 87°C (as detected using light scattering). The presence of StHsp14.0 completely inhibited the thermal aggregation of IPMDH at 87°C at a 24:1 ratio of StHsp14.0 monomer to IPMDH monomer. Thus, the StHsp14.0 24-mer prevented the aggregation of IPMDH monomer at an almost equimolar ratio. On the contrary, about a 40- to 80-fold excess of Hsp16.5 from *M. jannaschii* or the T KS-1 sHsp 24-mer was required to prevent the thermal aggregation of the CS monomer at 40–45°C (Kim et al. 1998b; Usui et al. 2001). StHsp14.0 was also ineffective at suppressing the aggregation of citrate synthase at 45°C, and a 32-fold excess of the StHsp14.0 24-mer over the CS monomer was required for complete suppression. Therefore, the sHsps of hyperthermophilic archaea

are active around, or at temperatures greater than, the optimal growth temperatures of these archaea. StHsp14.0 protected denatured TIPMDH by forming large complexes corresponding to spherical particles of 17–18 nm or spread particles of 17–39 nm in diameter, which are larger than the spherical particles of the StHsp14.0 oligomer (Usui et al. 2004). Since hyperthermophilic sHsp oligomers dissociate at very high temperatures and easily reassemble at moderate temperatures, it was difficult to observe oligomer dissociation. The dissociation of the StHsp14.0 oligomer was observed to involve subunit exchange (Usui et al. 2004). Mutation of the IXI/V motif at the *C*-terminus of StHsp14.0 (IKI) affects the stability of the oligomers (Usui et al. 2004). StHsp14.0 with its *C*-terminus deleted or with mutations of the IIe residues in the IXI/V motif to Ala, Ser, or Phe residues was unable form large oligomers and lost its chaperoning activity. StHsp14.0WKW, in which the Ile residues in the IXI/V motif are changed to Trp, exists as an oligomer, such as the wild-type protein. However, it dissociates into small oligomers and exhibits chaperone activity at a relatively low temperature. These results clearly support the idea that thermophilic sHsps also dissociate into small oligomers at the elevated temperatures that correlate with the chaperone function.

14.4.4 Cooperation with Other Chaperones

As described above, sHsps protect proteins from aggregation by forming large substrate-sHsp complexes. The complexes are dissociated to be able to renature by the DnaK system or, more efficiently, by the DnaK and ClpB systems working together (Ehrnsperger et al. 1997; Lee and Vierling 2000; Mogk et al. 2003). A similar type of cooperation between the sHsp and DnaK/ClpB systems is expected to exist in *Thermus thermophilus* HB8 because the DnaK/ClpB system has a high folding activity toward proteins from aggregates (Motohashi et al. 1999) and one sHsp gene lies close to the DnaK/ClpB operon. However, such a system does not exist in hyperthermophilic archaea. Reconstitution of a protein refolding pathway in archaeal hyperthermophilic archaea has been described (Laksanalamai et al. 2006). Denatured Taq DNA polymerase was reactivated at 100°C in an ATP-dependent manner by a mixture of sHsp or prefoldin with group II chaperonin from *P. furiosus*. The cooperative protein salvage pathway depends on the presence of a group II chaperonin and ATP. Prefoldin and sHsp appears to play similar roles in this system. They both act to capture denatured proteins and transfer them to group II chaperonins, albeit by different mechanisms (Fig. 14.8).

14.5 Conclusions

Although thermophiles adapt to high temperatures by making their proteins thermostable or thermophilic, they have to prepare for elevations in temperature by the induction of heat-shock proteins/molecular chaperones such as mesophilic

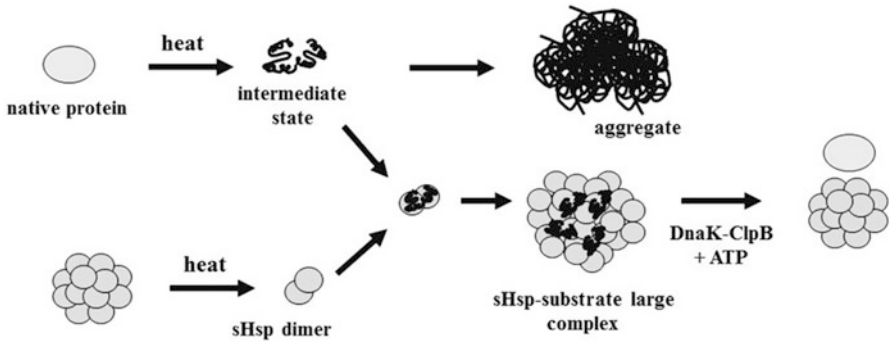

Fig. 14.8 A schematic model of the function of sHsps and their cooperation with other chaperones

organisms. The thermophilic eubacterial molecular chaperone system is very similar to those of mesophilic bacteria. However, the molecular chaperone system of archaea is very different. Notably, hyperthermophilic archaea have a limited number of molecular chaperones, which might be due to the relatively high stability of the proteins in hyperthermophilic archaea. The molecular chaperones in hyperthermophilic archaea might contribute to the protection of only a limited number of relatively unstable proteins. However, identification of the substrates of molecular chaperones of hyperthermophilic archaea has not been performed due to the difficulty of the experiments required, which must be performed at extremely high temperatures (around 100°C). To reveal the roles of molecular chaperones in hyperthermophilic archaea, it will be necessary to develop proteomic analysis methods for studying interactomes in hyperthermophilic archaea.

Thermophilic proteins are advantageous for various applications because of their stability. However, thermophilic molecular chaperones are less useful for refolding mesophilic proteins because of their high optimal temperatures. The stability of thermophilic molecular chaperones makes them potential tools in the field of nanoscale biotechnology. *Thermus* chaperonin was shown to capture a CdS quantum dot in its cavity and release it in an ATP-dependent manner (Ishii et al. 2003). McMillan et al. fabricated nanoscale ordered arrays of metal and semiconductor quantum dots by binding preformed nanoparticles onto crystalline protein templates made from genetically engineered hollow double-ring structures derived from *Sulfolobus* group II chaperonin (McMillan et al. 2002).

Molecular chaperones have important roles in the maintenance of protein systems, even in thermophilic organisms. Although they have almost the same roles as those from mesophilic organisms, some chaperones seem to function differently. Therefore, further investigation will be required to reveal the roles and function of these molecules, whose extreme stability makes them very advantageous in some applications.

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Chapter 15
Heterologous Production of Thermostable Proteins and Enzymes

Haruhiko Sakuraba and Toshihisa Ohshima

Abstract In the last decade, the genes encoding hyperthermophilic proteins and enzymes have been extensively expressed in heterologous organisms such as *Escherichia coli*, and their good productions have been achieved. However, some difficulties are often encountered when attempting to produce these proteins in the mesophilic hosts. This chapter focuses on the recent efforts made to overcome problems in heterologous production of hyperthermophilic enzymes: (1) successful production of hetero-oligomeric dye-linked L-proline dehydrogenases by use of effective promoters, (2) a typical procedure for the in vitro refolding of inclusion bodies composed of several hyperthermophilic enzymes (malate dehydrogenase, lysine dehydrogenase, and agmatinase), and (3) heat-induced structural conversion of hyperthermophilic glutamate dehydrogenase. This information could be useful in successful production of hyperthermophilic proteins and enzymes.

Keywords Hyperthermophilic proteins and enzymes • Heterologous expression • Inclusion bodies • Mesophilic hosts

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15.1 Introduction

Thermophilic enzymes have been attracting much interest from industry because they offer several major biotechnological advantages over their mesophilic counterparts: (1) once produced in mesophilic hosts, they are easy to purify through heat treatment, which could provide a comparatively inexpensive supply of proteins and enzymes; (2) they have a higher resistance to a variety of denaturants, including organic solvents and detergents; and (3) they show greater stability under various storage conditions. Moreover, their higher stability permits more detailed examination of their structural features and functionality. The knowledge gained from such studies could lead to the development of a wide range of biotechnological applications. One of the early successes in the area of industrial enzymes was the application of thermostable amino acid dehydrogenases to the enantiomeric-specific synthesis and analysis of amino acids (Ohshima and Soda 1989, 2000).

Hyperthermophiles are a group of microorganisms that exhibit optimum growth at temperatures above 80°C (Stetter et al. 1990; Adams 1993). Almost all of these organisms are classified as archaea, the third domain of life (Woese et al. 1990), and show a strong potential to serve as a new source of enzymes that are much more stable than their counterparts from mesophiles or moderate thermophiles (Vieille and Zeikus 2001). In addition, structural comparison of hyperthermophilic and mesophilic enzymes is expected to provide us with a better understanding of the mechanisms by which highly thermostable proteins are stabilized. As genomic sequence data for hyperthermophiles have accumulated over the last decade, their genes have been extensively expressed in heterologous organisms such as *Escherichia coli*. Construction of a heterologous expression system is particularly important for expressing genes from hard-to-grow hyperthermophiles; however, difficulties are often encountered when attempting to produce these proteins in *E. coli*. These difficulties include weak expression of the gene of interest due to ineffective promoters, formation of inclusion bodies, and improper folding of the protein at low temperatures. Considerable effort has been made to overcome these problems, and interesting findings dealing with heat-induced protein folding in vitro have been reported. In this chapter, we address these recent efforts with an emphasis on the methods for successful production of hyperthermophilic enzymes.

15.2 Production of Hetero-Oligomeric Dye-Linked L-Proline Dehydrogenase

Dye-linked dehydrogenases (dye-DHs) catalyze the oxidation of various amino acids, sugars, organic acids, amines, and alcohols in the presence of artificial electron accepters such as 2,6-dichloroindophenol (DCIP) and ferricyanide (Fig. 15.1). Dye-DHs generally function as mediators of electron transfer from a reduced substrate to an electron transfer system used to produce energy for the cell. Within cells, these

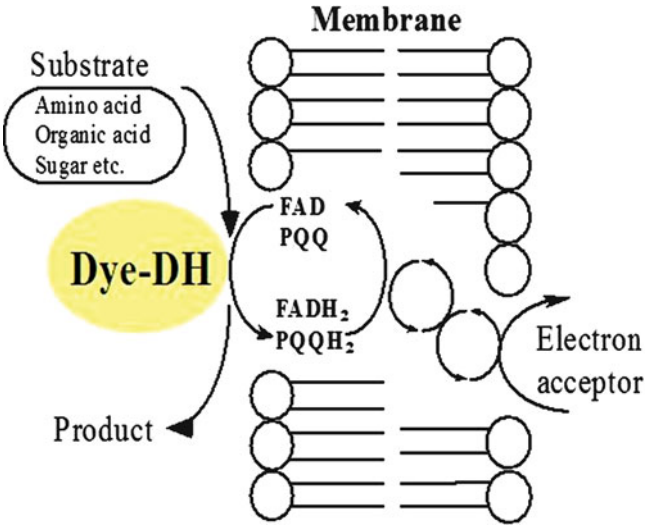

Fig. 15.1 Schematic diagram of dye-DHs

enzymes usually present in a membrane-associated or membrane-bound form, as a component of a complex containing several other proteins. Many types of dye-DHs have been identified in mesophilic microorganisms, and it has been suggested that they have the potential for use as specific elements in biosensors (Frew and Hill 1987). So far, however, their instability has limited the practical application of mesophilic dye-DHs. If dye-DHs are present in hyperthermophiles, their high stability would increase our ability to obtain useful information about their structures and functions and make them more amenable to practical application.

For several years, we have been extensively screening hyperthermophiles for dye-DHs. During that period, we have identified several dye-DHs in hyperthermophilic archaea, including a dye-linked L-proline dehydrogenase (LPDH) (Sakuraba et al. 2001; Kawakami et al. 2004, 2005; Satomura et al. 2010, 2011), a dye-linked D-proline dehydrogenase (Satomura et al. 2002), a dye-linked D-lactate dehydrogenase (Satomura et al. 2008), and a quinoprotein aldose sugar dehydrogenase (Sakuraba et al. 2010). We found that these enzymes remain stable for prolonged periods under a variety of conditions and some are applicable to a novel type of electrochemical detector for L-proline and D-proline (Tani et al. 2008, 2009; Zheng et al. 2010). Among these dye-DHs, LPDHs identified in the order Thermococcales have unique hetero-oligomeric structures.

LPDH catalyzes the oxidation of L-proline to Δ^1-pyrroline-5-carboxylate (P5C) in the presence of an artificial electron accepter. Two different types of LPDH, PDH1 and PDH2, have been identified in the anaerobic hyperthermophile *Pyrococcus horikoshii* OT-3 (Kawakami et al. 2005). PDH1 is a heterooctameric complex ($\alpha 4\beta 4$; molecular mass, 440 kDa) containing FAD, FMN, Fe, and ATP (Kawakami et al. 2005), while PDH2 of a tetrameric complex ($\alpha\beta\gamma\delta$; molecular mass, 120 kDa).

Structural analysis of the PDH1 complex showed the enzyme to be a unique diflavin dehydrogenase containing a novel electron transfer system totally different from that of the PDH2 complex, which contains L-proline dehydrogenase, NADH dehydrogenase, a ferredoxin-like protein, and a protein of unknown function (Kawakami et al. 2004; Tsuge et al. 2005). Proteins homologous to PDH1 and PDH2 are widely distributed among the hyperthermophilic archaea that belong to the phylum Euryarchaeota, including *Thermococcus profundus* (Sakuraba et al. 2001; Kawakami et al. 2004, 2005). For detailed analysis of the structures and functions of these LPDHs, heterologous expression of their genes in *E. coli* is required.

15.2.1 Identification and Cloning of the Gene Encoding PDH1

To identify organisms that produce LPDHs, we screened for enzymes using native PAGE coupled with activity staining. When we ran the crude extract of *P. horikoshii* OT-3 on a polyacrylamide gel, we detected two distinguishable activity bands for LPDH. This means that at least two types of LPDH (PDH1 and PDH2) are produced by this organism. After purification, PDH1 was found to be composed of two different subunits (α1 and β1) with molecular masses of 56 and 43 kDa, respectively. The native molecular mass of PDH1 is 440 kDa, indicating PDH1 has an α4β4 structure.

The N-terminal amino acid sequence of the α1 subunit was determined to be MRPLDLTEKR, which corresponds to the underlined amino acid sequence in ML<u>MRPLDLTEKR</u> from the putative protein encoded by the open reading frame (ORF; PH1363) predicted by the genome analysis. This means that the ATG situated 7 bp downstream from the 5′-terminus of the predicted ORF is the proper initial codon for the *α1* gene. The N-terminal amino acid sequence of the β1 subunit was determined to be MLPEKSEIVV, which corresponds to that of the predicted PH1364 gene product. The *α1* and *β1* genes are arranged in tandem (α1-β1) and were estimated to encode proteins with molecular masses of 55,316 and 42,685 Da, respectively. Similar gene clusters have been observed in the genomes of *P. furiosus* (PF1245–PF1246) and *P. abyssi* (PAB1842–PAB1843), suggesting that homologues of this enzyme are widely distributed within the order Thermococcales in the archaeal domain.

To avoid nucleotide incorporation errors, we did not use the PCR method to clone the PDH1 gene. Instead, an oligonucleotide probe was synthesized based on the DNA sequence in the *P. horikoshii* genome database, after which the probe was labeled with ^{32}P and used as a specific probe for southern and colony hybridizations. To prepare the PDH1 gene, genomic DNA was digested with SphI and KpnI, and the resultant fragments were separated on agarose gels. Approximately 8.0 kbp of fragments were extracted from the gel and inserted between the SphI and KpnI sites of plasmid pUC19. The recombinant plasmid was then used to transform *E. coli* JM109 cells growing on a Luria-Bertani (LB) plate containing ampicillin. Thereafter, the transformants were subjected to colony hybridization,

which enabled the plasmid pPDH1, containing the PDH1 gene (insert length, 8.1 kbp), to be obtained.

15.2.2 Expression of the PDH1 Gene and Purification of Its Product

We initially attempted to express the PDH1 gene using the plasmid pPDH1, but no functional product was obtained. We therefore introduced the gene into pET11a (Novagen) and were then able to successfully express the gene. As mentioned, PDH1 forms a complex comprised of α1 and β1 subunits. As a first step, separate systems for expressing the *α1* and *β1* genes were developed, and then a double expression system for the genes was constructed. Two sets of PCR primers were prepared to construct the expression plasmids for *α1* and *β1*. In each set, the forward primer introduced a unique NdeI restriction site that overlapped the 5′-initiation codon, and the reverse primer introduced a unique SmaI or BamHI restriction site proximal to the 3′-end of the termination codon. PCR was carried out using pPDH1 as the template, after which the amplified fragments were digested with NdeI and SmaI for *α1* or with NdeI and BamHI for *β1*. For ligation to *α1*, plasmid pET11a was digested with BamHI, blunted, and then further digested with NdeI. For ligation to *β1*, plasmid pET11a was digested with NdeI and BamHI. The *α1* and *β1* gene fragments were introduced into pET11a after linearizing the plasmid with NdeI and blunted BamHI to generate pET11a/*α1*, and with NdeI and BamHI to generate pET11a/*β1*. To construct the double expression system, pET11a/*α1* was digested with ClaI, blunted, and further digested with SphI. The resultant fragment containing *α1* and the T7 promoter were introduced into pET11a/*β1* digested with SphI and BglII (the BglII site had already been blunted) to generate the expression plasmid pEPDH1 (Fig. 15.2), which was then used to transform *E. coli* strain BL21 CodonPlus RIL (DE3) cells (Stratagene).

The transformed cells were grown for 8 h at 37°C in SB medium (1.2% tryptone peptone, 2.4% yeast extract, 1.25% K_2HPO_4, 0.38% KH_2PO_4, and 0.5% glycerol) containing ampicillin, after which isopropyl β-D-thiogalactopyranoside (IPTG) was added to 1 mM, and cultivation was continued for an additional 4 h. The transformants exhibited a high level of LPDH activity, which was not lost upon incubation at 90°C for 20 min. The enzyme was purified to homogeneity from the cell extract using heat treatment followed by successive Butyl Toyopearl and Superdex 200 gel filtration column chromatography steps. About 50 mg of the purified enzyme was obtained from 2 L of *E. coli* culture. The purified PDH1 showed the same mobility as the native enzyme on native PAGE, and the N-terminal amino acid sequences of the recombinant α1 and β1 subunits were confirmed to be identical to those of the native enzyme. These results suggest that *α1* and *β1* were properly expressed in *E. coli* only when they were cloned under the control of a strong promoter, but that the heterooctameric complex of the enzyme can be successfully produced in *E. coli*.

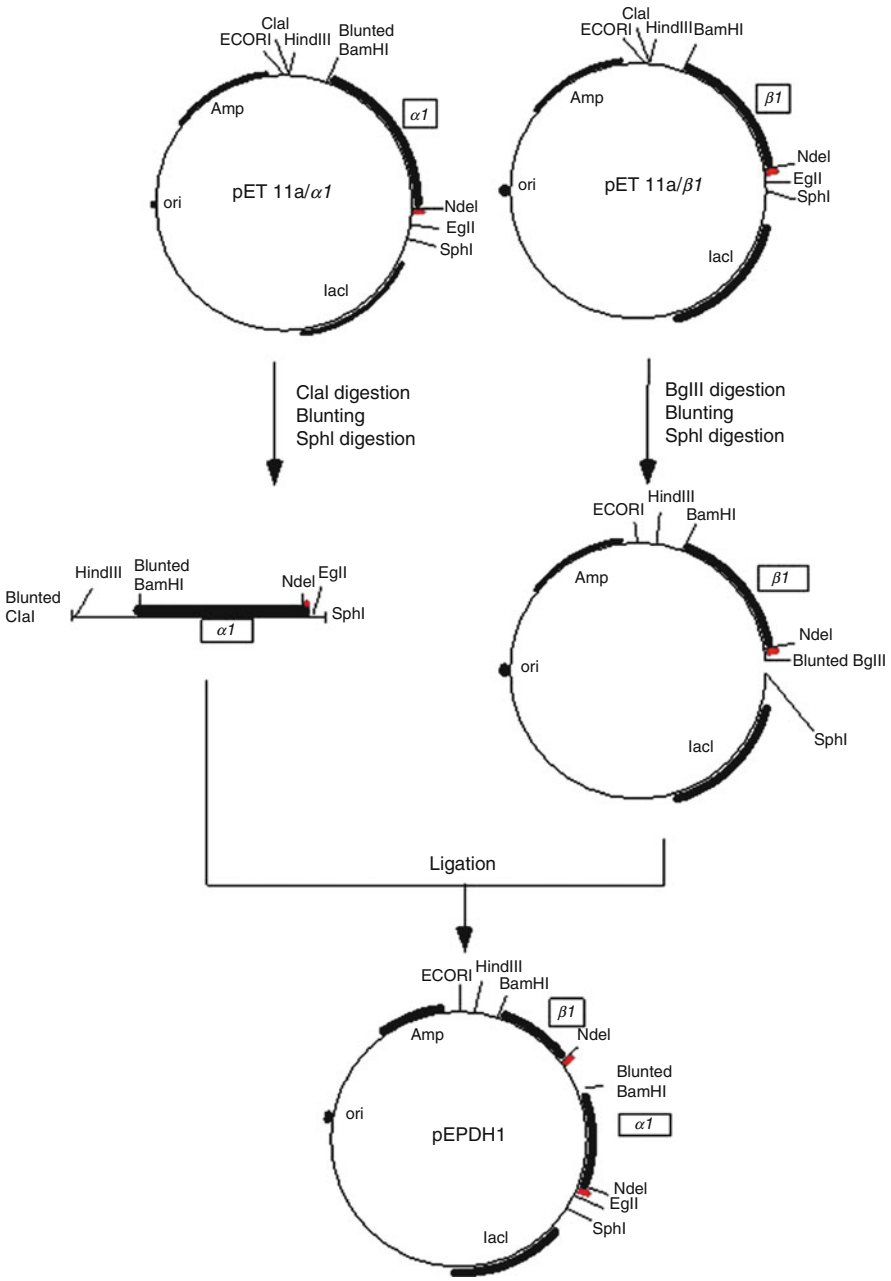

Fig. 15.2 Construction of a double expression system for the *α1* and *β1* genes encoding the PDH1 subunits

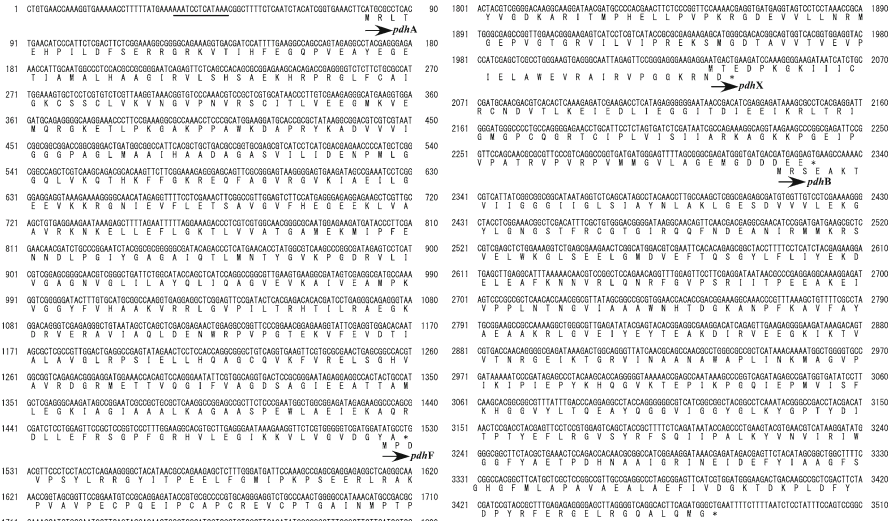

Fig. 15.3 Nucleotide and deduced amino acid sequences of a 3.6-kbp DNA fragment containing *pdhA*, *pdhF*, *pdhX,* and *pdhB* from *T. profundus*. The 3′-ends of *pdhA*, *pdhF,* and *pdhX* partially overlap the 5′-ends of *pdhF*, *pdhX,* and *pdhB*, respectively. A deduced promoter sequence is *underlined*. The figure cited in reference Kawakami et al. (2004) was modified in part

15.2.3 Expression of the Gene Encoding Heterotetrameric LPDH

The gene encoding the heterotetrameric LPDH from *T. profundus* (αβγδ; a PDH2 homolog) forms an operon comprised of *pdh*A, *pdh*F, *pdh*X, and *pdh*B, in that order (Fig. 15.3). Within the operon, the 3′ parts of *pdh*A, *pdh*F, and *pdh*X partially overlap the 5′ parts of *pdh*F, *pdh*X, and *pdh*B, respectively. To clone this gene cluster, degenerate oligonucleotides were designed to be DNA probes for the genes, based on an amino acid sequence determined to be part of the N-terminal sequence of the α subunit of the natural LPDH, and were labeled with ^{32}P. The genomic DNA was then digested with PstI, and the resultant fragments were separated on agarose gels. An approximately 6-kb PstI fragment, which gave a positive signal on southern hybridizations labeled with the probe, was inserted into the PstI site of plasmid pUC18, which was then used to transform *E. coli* JM109 cells. The transformants were selected by colony hybridization on an LB plate containing ampicillin, which enabled us to obtain a plasmid, pUPDH19, containing the heterotetrameric LPDH gene (insert length; 5.8 kbp).

The pUPDH19 was digested with NruI and ligated to produce the expression vector pUPDH, which was used to transform *E. coli* JM109 cells. The cells were then grown for 12 h at 37°C in SB medium containing ampicillin and IPTG. The transformants exhibited highly thermostable LPDH activity, and the recombinant enzyme was purified to homogeneity from the cell extract using heat treatment

followed by successive Butyl Toyopearl and Red Sepharose CL-4B column chromatography steps. The N-terminal amino acid sequences of the α, β, γ, and δ subunits of the purified enzyme coincided with those deduced from the respective gene sequences, and the α:β:γ:δ molar ratio was calculated to be about 1:1:1:1 based on SDS-PAGE analysis. These results indicate that, in contrast to the *P. horikoshii* PDH1, the gene cluster for the *T. profundus* LPDH was directly expressed using its own promoter and the heterotetrameric enzyme complex was successfully produced in *E. coli*.

Because archaeal transcription systems are generally closer to eukaryal systems than to bacterial ones, it is not surprising that most archaeal genes are expressed in *E. coli* only when they are cloned under the control of strong promoter such as the T7 promoter. In the order Thermococcales, however, *E. coli* consensus promoter-like sequences are often found within intergenic regions, and some of the genes can be expressed in *E. coli* without introduction of strong promoters. Upstream of the start codon in *pdh*A, which encodes the α subunit of the *T. profundus* LPDH, a promoter-like sequence, AAATCCTCATAAA, was identified (Fig. 15.3). The presence of this region might explain why *T. profundus* LPDH gene is directly expressed in *E. coli*.

15.3 In Vitro Refolding of Hyperthermophilic Enzymes

In *E. coli*-based expression systems, the recombinant protein is often produced as an insoluble aggregate called an inclusion body. The formation of such inclusion bodies results from an anomaly in the protein folding. To produce the protein in a soluble form, a co-expression or fusion system that includes the target protein and a molecular chaperone has to be developed. For hyperthermophilic enzymes, an expression system entailing the fusion of the target protein with an archaeal FK506 binding protein (FKBP) has recently been reported (Ideno et al. 2004). The archaeal FKBP from a hyperthermophilic archaeon, *Thermococcus* sp. KS-1 (TcFKBP18), possesses both peptidyl-prolyl *cis-trans* isomerase activity and chaperone-like activity that increases the refolding yield from unfolded protein by suppressing irreversible protein aggregation (Iida et al. 1998; Ideno et al. 2001). For example, a putative rhodanese from the aerobic hyperthermophilic archaeon *Aeropyrum pernix* forms insoluble aggregates when expressed alone in *E. coli*. However, by modifying its gene (APE_2595.1) so that the rhodanese was expressed as a fusion protein, connecting to the *C*-terminal end of TcFKBP18 significantly increased its production in the soluble fraction. Using this approach, the percentage of the soluble form of the expressed protein reached 28% of the host soluble proteins (Ideno et al. 2004). That said, it is still unclear whether this FKBP fusion system is sufficient to reduce inclusion body formation with other hyperthermophilic enzymes. In addition, it has been reported that co-expression of APE_2595.1 with TcFKBP18 gene does not improve protein

production in the soluble fraction of *E. coli* (Ideno et al. 2004). This may indicate that intermolecular interaction between the refolding protein and TcFKBP18 enhances neither the proper folding nor the soluble production of the target protein, although intramolecular interactions do.

On the other hand, inclusion bodies composed of several hyperthermophilic enzymes have been properly refolded, and soluble enzyme preparations have been successfully obtained through the use of conventional protein-folding procedures, which entail solubilization of the aggregate using a denaturant, refolding through removal of the denaturant, and purification of the refolded protein. Although these conventional protocols are insufficient for use in all the cases, careful optimization of the folding conditions can often enable efficient refolding of the target proteins. In this section, we introduce a typical procedure for the in vitro folding of a hyperthermophilic enzyme.

15.3.1 Cloning and Expression of the Gene Encoding Malate Dehydrogenase

Tartrate oxidation activity was found in the crude extract of *A. pernix*, and the enzyme was identified as (S)-malate dehydrogenase (ApeMDH). Because there were no earlier reports of an MDH exhibiting strong catalytic activity toward tartrate, we endeavored to express the ApeMDH gene (APE_0672.1) in *E. coli* and characterize the purified enzyme (Kawakami et al. 2009).

The gene was amplified by PCR using a set of oligonucleotide primers in which a unique NdeI restriction site that overlapped the 5′-initiation codon was introduced in the forward primer and a unique BamHI restriction site proximal to the 3′-end of the termination codon was introduced in the reverse primer. Chromosomal *A. pernix* DNA was used as the template. The amplified 0.9-kb fragment was digested with NdeI and BamHI and ligated with the expression vector pET11a linearized with NdeI and BamHI to generate pET/ApeMDH. *E. coli* BL-21 CodonPlus (DE3)-RIL cells were then transformed with pET/ApeMDH, after which the transformants were cultivated for 8 h at 37°C in LB medium supplemented with ampicillin. Thereafter, expression was induced by adding 1 mM IPTG to the medium, and cultivation was continued for an additional 3 h at 37°C.

In the *E. coli* transformants, ApeMDH was present in both the soluble and insoluble fractions but was found mainly as an inclusion body. The MDH activity in the soluble fraction was unaffected by incubation at 90°C for 10 min, confirming that APE_0672.1 does indeed encode a hyperthermostable MDH. However, the protein in the soluble fraction migrated as an aggregate on native PAGE. Because changing the cultivation temperature or the conditions under which the cells were disrupted did not prevent this aggregation, we decided to carry out refolding of the inclusion body.

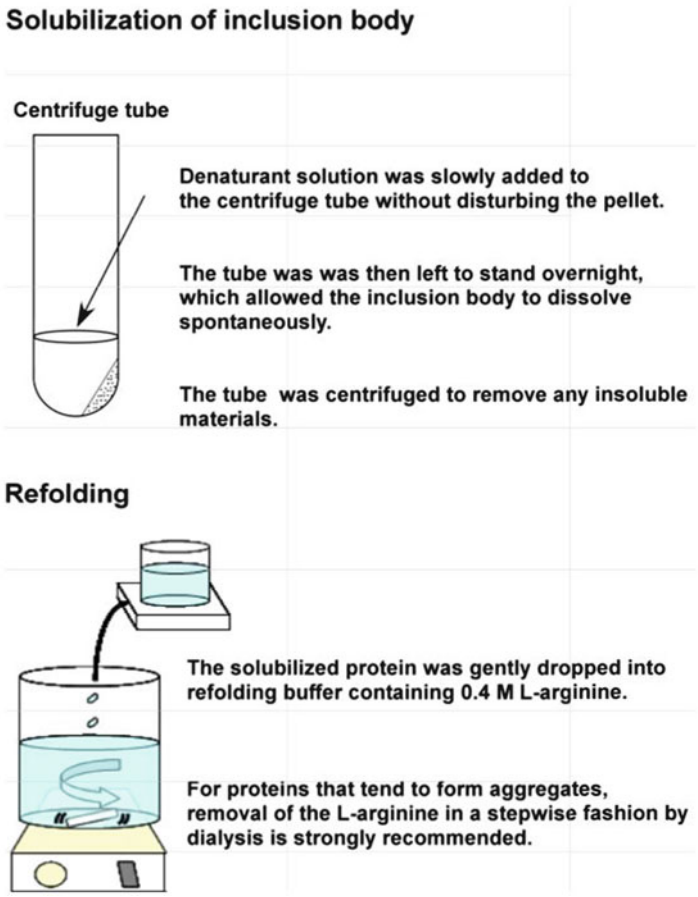

Fig. 15.4 Scheme of the procedure for refolding ApeMDH. Essentially the same method has been used for the refolding of agmatinase and LysDH from *P. horikoshii*

15.3.2 Refolding and Purification of the Recombinant MDH

The scheme for a typical refolding procedure is illustrated in Fig. 15.4. To purify ApeMDH from the inclusion bodies, *E. coli* transformants were collected (20 g wet weight from 2 L of culture), washed with 0.85% NaCl, suspended in 100 ml of 10 mM Tris-HCl buffer (pH 7.5), and disrupted by sonication. The homogenate was centrifuged at 20,000 ×g for 10 min at 4°C, and the pellet was resuspended by sonication in 100 ml of 10 mM Tris/HCl (pH 7.5) buffer containing 1 mM EDTA and 4% Triton X-100. The suspension was then incubated for 30 min at room temperature and centrifuged again. This procedure was repeated twice. The resultant pellet was washed twice with 200 ml of Milli Q water, and then the denaturant solution

(10 ml; 50 mM Tris-HCl buffer (pH 7.5) containing 6 M guanidine HCl, 0.2 M NaCl, and 1 mM EDTA) was slowly added to the centrifuge tube without disturbing the pellet. The tube was then left to stand overnight at 4°C, which allowed the inclusion body to dissolve spontaneously, after which the tube was centrifuged at 20,000 ×g for 10 min at 4°C to remove any insoluble material. The protein concentration of the resultant supernatant was calculated based on the absorbance at 280 nm (Gill and von Hippel 1989) and adjusted to 1 mg/ml by dilution with the denaturant solution.

The solubilized ApeMDH (200 mg of enzyme in 200-ml solution) was gently dropped into refolding buffer (1.5 L of 0.1 M Tris/HCl (pH 7.5) containing 2 mM EDTA and 0.4 M L-arginine) and then incubated for 36 h at 4°C. The resultant enzyme solution (1.7 L) containing the refolded ApeMDH was then concentrated to a volume of 50 ml using Vivaflow 50 ultrafiltration modules (30000 MWCO module×6) (Sartorius Stedim Biotech), dialyzed against 50 mM Tris/HCl (pH 7.5) buffer containing 0.2 M NaCl, and centrifuged at 20,000×g for 10 min at 4°C to remove the insoluble material. The supernatant was then further concentrated to a volume of 15 ml using an Amicon Ultra Centrifuge Filter (30000 MWCO) (Millipore), and an aliquot (5 ml) of the resultant solution was applied to a Superdex 200 gel filtration column (2.6×60 cm, GE Healthcare) equilibrated with 50 mM Tris/HCl (pH7.5) buffer containing 0.2 M NaCl. The eluate was fractionated, and the active fractions were pooled and used for biochemical and structural experiments. All solutions used for the refolding procedures were filtered through 0.45-μm membrane filters (Advantec) to remove dust and any other impurities.

The protein in the washed inclusion body was over 95% pure, as judged from the SDS-PAGE, and the amount of protein solubilized by the guanidine HCl was estimated to be about 200 mg based on the absorbance at 280 nm. The refolded enzyme catalyzed malate dehydrogenation, confirming that active ApeMDH was successfully recovered from the inclusion body. After further purification using gel chromatography, the mobility of the refolded ApeMDH in native PAGE was the same as that of the natural ApeMDH. With this procedure, about 70 mg of purified ApeMDH were obtained from 2 L of culture.

15.3.3 Refolding of Other Hyperthermophilic Enzymes

Agmatinase and L-lysine 6-dehydrogenase (LysDH) from *P. horikoshii* were refolded from inclusion bodies using a method that was essentially the same the one described above (Goda et al. 2005b; Yoneda et al. 2010). The agmatinase inclusion bodies were solubilized in a denaturant solution composed of 50 mM Tris/HCl (pH 8.0), 6 M guanidine HCl, 200 mM NaCl, and 1 mM EDTA. The solubilized enzyme was then added to refolding buffer (0.1 M Tris/HCl (pH 8.0), 0.4 M L-arginine, 2 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)) at 4°C and further incubated for about 40 h. The resultant enzyme solution was concentrated and dialyzed to remove L-arginine, and the active enzyme

was successively recovered in the supernatant after centrifugation. The efficiency of the refolding was calculated to be 67% based on the absorbance at 280 nm. After the refolded agmatinase was further purified using a gel filtration column, about 45 mg of soluble homogeneous enzyme was obtained from 200 ml of *E. coli* culture.

To refold the LysDH from *P. horikoshii*, the inclusion bodies were solubilized in 50 mM Tris-HCl buffer (pH 7.5) containing 5 mM β-mercaptoethanol, 6 M guanidine HCl, 0.2 M NaCl, and 1 mM EDTA. The solubilized LysDH was added to a refolding buffer containing 0.1 M Tris-HCl (pH 7.5), 5 mM β-mercaptoethanol, 0.4 M L-arginine, 2 mM EDTA, and 0.1 mM PMSF and incubated for 36 h at 4°C, after which the resultant enzyme solution was concentrated and dialyzed to remove L-arginine. The refolded LysDH solution was then heated at 80°C for 10 min and clarified by centrifugation. The protein in the supernatant was then further purified by running it on a gel filtration column. In the *E. coli* transformants, LysDH was present in both the soluble and insoluble fractions but was found mainly as an inclusion body. When soluble enzyme was purified from the crude extract of the transformant cells using heat treatment and Ni^{2+}-chelating affinity chromatography, only 1.7 mg of the purified enzyme was obtained from 7.3 g of cells (wet weight). However, the refolding procedure increased the yield to 7.8 mg of the purified enzyme from the same starting material.

In all cases, 0.4 M L-arginine was added to the refolding buffer to prevent irreversible protein aggregation. For proteins that tend to form aggregates, removal of the L-arginine in a stepwise fashion by dialysis is strongly recommended. If the native protein contains metal ions, EDTA should be omitted from the denaturant solution and refolding buffer. Alternatively, an excess of the respective metal ion should be added to the refolding buffer. Similarly, addition of other ligands that bind to the native protein is required.

15.4 Heat-Induced Structural Conversion of Hyperthermophilic Enzymes

When the properties of native and recombinant hyperthermophilic enzymes are compared, a majority of the hyperthermophilic enzymes expressed in *E. coli* retain all of the native enzyme's biochemical properties, including its thermostability and its optimal pH and optimal activity at high temperatures. Thus, most proteins from hyperthermophiles are thought to be able to fold properly even at temperatures much lower (e.g., room temperature) than their physiological temperatures (80–100°C). On the other hand, over the last decade, there have been several reports showing that recombinant enzymes derived from hyperthermophiles and expressed in *E. coli* are not present in the same form as the corresponding native enzymes.

Glutamate dehydrogenases (GDHs; EC 1.4.1.2-4) are a widely distributed group of oligomeric oxidoreductases whose structural characteristics and structure-function relationships have been well studied. Some GDHs derived from hyperthermophiles

are expressed as inactive forms in *E. coli*, but in vitro application of heat is sufficient to convert the inactive recombinant form to one more similar to the active native form. This suggests that high temperature has a crucial effect on the folding, oligomerization, and subunit arrangement of hyperthermophilic GDHs. DiRuggiero and Robb (Diruggiero and Robb 1995) originally described the heat-induced activation of inactive recombinant *P. furiosus* GDH. In that case, the enzyme was expressed in *E. coli* as a mixture of both monomeric and hexameric forms, but the inactive monomers in the solution assembled into the active hexameric form upon application of heat. Similar heat-induced activation of hexameric GDH was reported for the recombinant GDH from *T. kodakaraensis* (formerly *Pyrococcus* sp. KOD1)(Abd Rahman et al. 1997). Heating the enzyme at 90°C in vitro induced the conversion from a low-activity form to a high-activity form, but the specific activity of the heat-activated enzyme was still not as high as that of the native enzyme (Abd Rahman et al. 1997), suggesting an additional factor is required for the full activation of *T. kodakaraensis* GDH. Although similar heat-induced structural conversions of several other thermostable proteins have been reported (Schultes and Jaenicke 1991; Rehaber and Jaenicke 1992; Siddiqui et al. 1998; Schroder et al. 2004), the mechanisms of these conversions are not yet fully understood. To obtain more detailed information, we cloned and expressed the gene encoding GDH from the hyperthermophilic archaeon *Pyrobaculum islandicum* (pis-GDH) in *E. coli* (Kujo et al. 1999). Unlike the two aforementioned enzymes, the recombinant pis-GDH always assumed a hexameric form but exhibited extremely low specific activity, nevertheless. Heating the inactive enzyme increased its activity to a level comparable to that seen with the native enzyme. This suggests that the mechanism underlying the heat-induced activation of recombinant pis-GDH differs from those underlying the activation of *P. furiosus* and *T. kodakaraensis* GDHs. We therefore used small-angle X-ray scattering (SAXS) and 1-anilinonaphthalene-8-sulfonic acid (ANS) fluorescence analyses to examine the structural changes that accompany activation of recombinant pis-GDH (Goda et al. 2005a).

15.4.1 Preparation of Recombinant pis-GDH

Native pis-GDH was purified from *Pb. islandicum* cells using previously described procedures (Kujo and Ohshima 1998). In addition, the enzyme was expressed in *E. coli* as follows. The expression vector pKGDH1 was constructed as described previously (Kujo et al. 1999), after which the *pis-gdh* gene in pKGDH1 was subcloned into a pET11a vector to obtain a better producer. The DNA fragment containing *pis-gdh* was then amplified by PCR using two primers containing NdeI and BamHI restriction sites, after which the PCR product was inserted into the NdeI and BamHI sites of pET11a, yielding pEGDH2. *E. coli* BL21 (DE3)-codon plus-RIL cells carrying the recombinant plasmid were grown at 37°C in LB medium (2.1 L) containing ampicillin. After cultivation for 9 h, expression of the enzyme was induced by addition of 1 mM IPTG, and the cultivation was continued for an additional 3 h. The cells were then collected (11.6 g wet weight); suspended in 10 mM potassium

phosphate buffer (pH 7.2) containing 10% glycerol, 1 mM EDTA, and 0.1 mM DTT (buffer A); and disrupted by sonication. After centrifugation (16,000 × *g* for 15 min), the soluble fraction was used as the crude extract. Further purification of the recombinant enzyme was carried out at 4°C to prevent heat-induced activation of the enzyme. The crude extract was applied to a Red Sepharose CL-4B column (Ohshima and Sakuraba 1986) previously equilibrated with buffer A. After washing the column with buffer A, the enzyme was eluted with the same buffer containing 0.5 M NaCl, and the active fractions were pooled. The enzyme solution was then dialyzed against buffer A and applied to a DEAE-Toyopearl column (Tosoh) equilibrated with buffer A. When the column was washed with the same buffer, the enzyme was found in the eluate, as it was not adsorbed onto the column. The enzyme solution was therefore applied to a hydroxyapatite column (GIGAPITE K-100S) (Seikagaku Kogyo) equilibrated with buffer A. After being washed with the same buffer, the enzyme was eluted with a linear gradient of 10–300 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.2) containing 10% glycerol, 1 mM EDTA, and 0.1 mM DTT. The active fractions were pooled, dialyzed against buffer A, and used as the purified enzyme preparation. At each step, an aliquot of the enzyme solution was dialyzed against buffer A and heated at 90°C for 15 min to assess the heat-treated enzyme activity.

15.4.2 Properties of Recombinant pis-GDH

The extract from *E. coli* expressing recombinant pis-GDH exhibited a low level of enzyme activity that was markedly increased by heating at 90°C for 30 min. Likewise, the specific activity of the purified recombinant enzyme was much lower than that of the native enzyme, but heating (from 37 to 110°C) enhanced its activity. The effect of heat was both temperature and time dependent, with heating to 90°C for 15 min resulting in an increase in the enzyme's activity to a level comparable to that seen with native pis-GDH (Table 15.1). Gel filtration of the recombinant pis-GDH showed an activity peak corresponding to a molecular mass of 280 kDa. In addition, the recombinant enzyme migrated as a single band on SDS-PAGE, from which the molecular mass was calculated to be 47 kDa. Thus, like native pis-GDH, the most active recombinant enzyme was a hexamer (Kujo and Ohshima 1998). The kinetic parameters for oxidative deamination by the native and recombinant GDHs are summarized in Table 15.1. Note that the Km values of the heat-activated enzyme for NAD and L-glutamate are similar to those of the native enzyme.

15.4.3 Structural Differences Between Inactive and Heat-Activated Recombinant pis-GDHs

Measurements of SAXS were carried out with inactive and heat-activated recombinant pis-GDHs, and the values obtained before and after activation are summarized in Table 15.2. The radius of gyration, $R_{g,z}$ (Kratky et al. 1951; Guinier and Fournet 1955)

Table 15.1 Kinetic properties of native and heat-activated recombinant pis-GDHs

	Specific activity	Km (mM)	
pis-GDH	(μmol/min/mg)	L-glutamate	NAD
Native	3.51	0.16	0.019
Heat-activated	3.65	0.16	0.021

Table 15.2 Structural parameters of inactive and heat-activated recombinant pis-GDHs determined by SAXS

State	Rg,z (Å)	Mw,w (kDa)	Dmax (Å)
Inactive	54.6 ± 0.1	280 ± 26	145 ± 3
Heat-activated	46.5 ± 0.1	299 ± 14	124 ± 3

reflects the molecular shape and size, while D_{max} gives the maximum particle dimension. In addition, the weight average molecular mass, $M_{w,w}$, was estimated as described previously (Glatter and Kratky 1982; Kajiwara and Hiragi 1996). The $R_{g,z}$ values for the inactive and heat-activated enzymes were determined to be 54.6 Å and 46.5 Å, respectively. That the radius of gyration of the inactive enzyme is 8 Å larger than that of the activated form means that heat can cause the nascent recombinant pis-GDH to undergo a change in its quaternary structure from a loose assembly of subunits to a much more compact one. Correspondingly, D_{max} decreased from 145 Å for the inactive enzyme to 124 Å for the heat-activated enzyme, confirming that heat causes the enzyme molecule to become substantially smaller. On the other hand, the values of $M_{w,w}$ were the same, within the experimental error, clearly indicating that the molecular masses of the two proteins were unaffected by heat. These values are nearly the same as the molecular mass of hexameric pis-GDH (280 kDa).

Although the crystal structure of heat-activated pis-GDH (PDB entry 1V9L) has been described previously (Bhuiya et al. 2005), the structure of the inactive recombinant enzyme remains unknown. We therefore used DAMMIN to carry out ab initio modeling of the low-resolution structure of the recombinant enzyme from the scattering curve (Svergun 1999). Because DAMMIN allows one to apply symmetry restrictions to a model's solution, we restored the low-resolution structure of the heat-activated pis-GDH assuming 32-point symmetry, like that of the crystal structure (Bhuiya et al. 2005), and the resultant model was well fitted to the crystal structure (Fig. 15.5a, b). We found that the heat-activated enzyme has one threefold axis passing through two identical trimers, which face one another, yielding a whole molecule having a cylindrical shape (Fig. 15.5a, b, lower row) with one trimer stacking on the other and forming a symmetrical interface (Fig. 15.5a, b, upper row). By contrast, the ab initio modeling of the inactive enzyme yielded better results with no internal symmetry than with 32-point symmetry. This suggests the inactive form may have no internal symmetry, despite the same total number of subunits; consequently, no subunit from one trimer superimposes on the corresponding subunit of the other (Fig. 15.5c, upper row). Another structural feature of the inactive pis-GDH is that it is not cylindrical. It is instead more spread out, especially

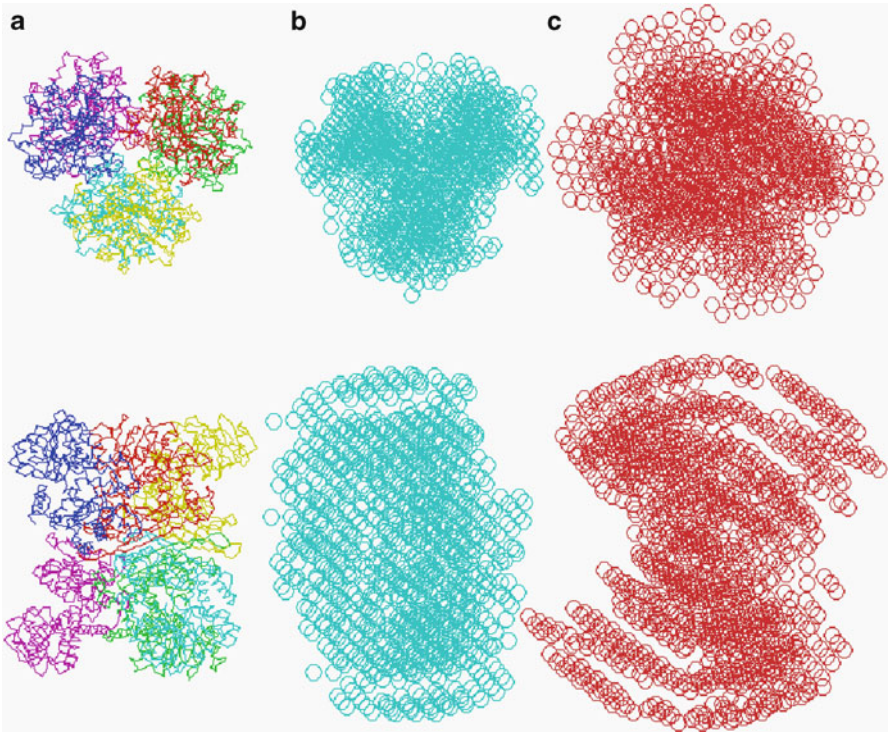

Fig. 15.5 Crystal and low-resolution structures of pis-GDH. (**a**) Crystal structure of heat-activated pis-GDH. (**b**) Low-resolution structure of the heat-activated enzyme with 32-point symmetry. (**c**) Low-resolution structure of the inactive enzyme with no symmetry. The *upper row* is the hexameric model viewed down the threefold axis. The *lower row* are views down the twofold axis. The figure cited in reference Goda et al. (2005a) was modified in part

at the surface of the molecule (Fig. 15.5c, lower row). This also suggests that the inactive enzyme is comprised of a loose and improper arrangement of subunits. The relative hydrophobicity of the recombinant proteins was evaluated as a function of ANS fluorescence intensity. When inactive pis-GDH was incubated with ANS, the resultant fluorescence spectrum (excitation wavelength, 350 nm) exhibited intense emission with a maximum at 446 nm. By contrast, the fluorescence spectra of the ANS-modified native and heat-activated enzymes were nearly identical and far weaker than that of the inactive enzyme. This means that there are a greater number of exposed hydrophobic residues on the surface of the inactive enzyme than on the native or heat-activated enzyme and suggests that activation causes those hydrophobic residues to be moved to the interior of the oligomer. The presence of buried hydrophobic residues at the intersubunit interface in the crystal structure (Bhuiya et al. 2005) of the active enzyme strongly suggests that the major source of stability against extremely high temperature is the much stronger hydrophobic interaction of these residues. The crystal structures thus substantiate the notion that, upon heat

activation, each subunit of the enzyme refolds, and the hydrophobic residues on the surface move to the intersubunit interface.

Overall, the process of heat-induced activation of pis-GDH expressed in *E. coli* can be summarized as follows: (1) the inactive enzyme is comprised of a loose and unstable arrangement of subunits that is perturbed by heat, leading the protein to settle into a tighter and more stable arrangement; (2) during this process, hydrophobic residues on the surface are brought to the interior interface between subunits where they promote the hydrophobic association of the subunits within the oligomer. These results strongly suggest that subunit rearrangement – i.e., a change in the quaternary structure of the hexameric recombinant pis-GDH – is essential for activation of the enzyme.

15.5 Conclusions

Hyperthermophilic enzymes have become model systems with which to study enzyme stability mechanisms and protein structure-function relationships. Moreover, these enzymes are now attracting a great deal of interest due to their potential to serve as both analytical tools and as biocatalysts for application. The recent advances in this field have been facilitated by the cloning and expression of hyperthermophilic genes in mesophilic hosts, although difficulties are still encountered with heterologous production of some hyperthermophilic enzymes. In this chapter, we presented an overview of several recently developed protocols for the proper production of these enzymes in *E. coli*, as well as protein-folding protocols. As additional related information leads to the production of still more recombinant proteins at high yields, the functional and structural analysis of hyperthermophilic enzymes and their industrial application will be greatly accelerated.

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Chapter 16
Discovery of Thermostable Enzymes from Hot Environmental Samples by Metagenomic Approaches

Norio Kurosawa

Abstract Besides continuous progress in molecular biological techniques, metagenomic approach has become one of the routine experiments to examine the microbial diversity, ecology, and enzymes. This approach basically does not require the cultivation of microbes, which has a strong bias to a population of microbes and allows to have a glimpse of uncultured microbes and to find novel biomolecules. In the last 15 years, nearly 20 papers describing properties of thermostable enzymes discovered from hot environments by metagenomic strategies have been published. Most of them attempted to find and analyze hydrolytic enzymes such as cellulases, amylase, xylanase, esterase, lipase, and others because such enzymes are relatively easy to detect their activities in the function-based screening procedure. On the other hand, sequencing-based approach does not rely on the activity measurement and aids in discovering novel thermostable enzymes employing recently improved DNA sequencing technology and cloning vectors. The spectacular progress made in molecular biological techniques facilitates comprehensive metagenomic analysis for discovering novel biomolecules from the major fraction of the uncultured microbes in nature.

Keywords Thermostable enzymes • Thermophiles • Metagenome • Hot spring • Hydrothermal vent • Thermozyme • Extremozymes

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16.1 Introduction

Microbial enzymes have been isolated from cultivated microbial cells or supernatants of cultures. This study started in 1897 when Eduard Buchner showed the ability of yeast extracts to ferment sugar despite the absence of living yeast cells, followed by a large number of studies for purification and characterization of enzymes, and recent techniques for gene cloning and expression in an appropriate host (Buchner 1897). These traditional strategies have been made great success for analysis and application of microbial enzymes. However, many novel microbial enzymes may still exist in nature since most naturally occurring microbes have never before been cultivated or characterized in the laboratory. At present, the traditional cultivation experiment is not the only way to discover the microbial enzymes from the nature. The enzymes are of course encoded in the DNA, and the DNA can be extracted from not only cultivated cells but also uncultivated cells presenting with various media or substrates in the environments. In the latter case, the extracted DNA is a mixture of genomes and plasmids derived from individual species including uncultivated microbes. Therefore, we possibly find the genes of novel enzymes in the DNA directly extracted from the environmental samples. This kind of mixed DNA is called "environmental DNA" or "metagenomic DNA." The term metagenome is firstly used by Handelsman et al. (1998) and is also sometimes used for mixture DNA extracted from mixed cell cultures such as enrichment cultures.

The study of metagenome is called "metagenomics" including sequencing, expression, comparative, and bioinformatic analysis and had started from the pioneering work by Norman Pace and colleagues to reveal the naturally occurring microbial population (1985). They used small subunit ribosomal RNA gene as a molecular marker, and this strategy has been widely used by many researchers to reveal the microbial diversity and community structures of every conceivable habitats and given countless research papers so far. Once we realized the potential of environmental mixture DNA, we have tried to develop a variety of applications of broad-sense metagenomics such as transcriptosome, proteome, metabolome analysis, and also expression of enzyme using environmental DNA. At the same time, great improvements of DNA sequencing technology and cloning vectors, such as BAC (bacterial artificial chromosome, Kim et al. 1992; Shizuya et al. 1992), rendered the recovery and sequencing of large DNA inserts from single environmental sample a routine laboratory process (DeLong 2004). This "mega-metagenomics" relying on the "massive sequencing" has now contributed to understanding the nature of microbes and also to discovery of novel microbial enzymes.

The enzymes from extremophiles, called extremozymes, expanded a limit of biocatalysis and need not be constrained to mild conditions and can be considered at pH, temperature, pressure, ionic, and solvent environments long thought to be destructive to biomolecules (Adams et al. 1995). And the metagenomic approaches also expanded a limit of discovery of extremozymes from the nature. In this chapter, I describe roughly the general procedure of extraction of metagenomic DNA, screening strategies, and library construction and then review the metagenomic approaches for discovery of thermostable enzymes from hot environments.

16.2 Extraction of Metagenomic DNA

Metagenomic approach normally skips cultivation of microbes, resulting that we can get DNA from both culturable and unculturable microbes. This fact sometimes leads a kind of misunderstanding which is "all the cells in a sample can be analyzed" to researchers. However, in fact, it is not possible to extract DNA equally from all the cells in the environmental sample. Some cells may be hiding in microholes of substrates and may show some resistance to cell lysis. Also, the DNA extraction efficiencies must be different among the individual species which have different surface structures from each other. Therefore, it is important to be aware of such difficulties and some biases and to choose carefully the procedure for extraction of metagenomic DNA.

When the target microbial consortium is a free-living (planktonic) community in a water sample (i.e., hot springs or hydrothermal vents water) or mixed cell cultures, the cells need to be collected by filtration or centrifugation prior to extraction of metagenomic DNA. However, in these cases, no special technique will be required, and standard DNA extraction methods for microbial cultures can be used for preparation of metagenomic DNA. On the other hand, various procedures including manufacture's kits have been described for extraction of metagenomic DNA from sediment and soil samples where some or most microbial cells attach to the sample matrix. For example, UltraClean™ Soil DNA Isolation Kit and related kits (MO BIO Laboratories, Inc.) have been widely used in the studies for microbial diversity of hot environments such as hot springs and hydrothermal vents. In the case of my laboratory, we usually (not always of course) recover ~20-ng DNA from 1-g mud sample derived from acidic and sulfuric hot springs where they are relatively low biomass habitats by using the UltraClean reagents, and the extracted DNA can be used for PCR experiments without any further purification procedures. SoilMaster™ (Epicentre Biotechnologies), Meta-G-Nome™ DNA Isolation Kit (Epicentre Biotechnologies), FastDNA™ Spin Kit for Soil (MP Biomedicals), and ISOIL (Nippon Gene) are also commercially available for isolation of metagenomic DNA. Few researchers have compared the efficiency of commercial kits with respect to the DNA yield from soil and its purity, but the efficiencies of commercial kits reported by various researchers are inconsistent (Rajendhran and Gunasekaran 2008). The DNA extraction protocol of those commercial kits is based on the "direct DNA extraction procedure" firstly described by Ogram et al. (1987), in which the cells are lysed within the sample matrix and the DNA molecules are separated from the matrix and cell debris. Various modifications of Ogram's method have been described to improve yield and purity of DNA and also to simplify the procedure. A simple and rapid method for extraction of metagenomic DNA reported by Zhou et al. (1996) has been relatively preferred to be used for metagenomics of hot environments. On the other hand, the humic acids (humic compounds) compete with nucleic acids during purification of DNA (Rajendhran and Gunasekaran 2008) and also inhibit PCR amplification of DNA (Tsai and Olson 1992; Watson and Blackwell 2000). Various

procedures have been developed to avoid the humic acids' inhibition (Rajendhran and Gunasekaran 2008), and those improved methods may be useful for extraction of metagenomic DNA from hot humic sediments and high-temperature compost.

In contrast to the "direct DNA extraction procedure," the "indirect DNA extraction procedure" in which the cells are dispersed and separated from environmental materials prior to the cell lysis was firstly reported by Holben et al. (1988). In many cases, the amounts of DNA recovered by this strategy are significantly lower than the direct method (Rondon et al. 2000; Gabor et al. 2003). However, this method is generally contributed to yield DNA of higher molecular mass and greater purity than the direct lysis procedures. Furthermore, much lower amounts of eukaryotic DNA were co-extracted in the cell extraction-based methods, resulting that the indirect method allows the construction of environmental gene banks of superior quality (Gabor et al. 2003). Although the direct DNA extraction procedure has been mainly used for the hot environmental metagenomics so far, the Gabor's indirect method was applied in the discovery of novel thermostable poly(DL-lactic acid) depolymerases from compost (Mayumi et al. 2008). This improved indirect DNA extraction procedure may be useful in the BAC library construction and "massive sequencing-based metagenomics" (described in Sect. 16.3) because of its advantage of recovery of higher molecular mass DNA.

Other miscellaneous procedures regarding extraction and purification of metagenomic DNA are reviewed in more detail in the article by Rajendhran and Gunasekaran (2008).

16.3 Screening Strategies and Libraries

The screening strategies of enzymes toward the metagenomic libraries have been classified into the sequence-based and function-based strategies. However, it is not so simple and has become a little complicated because of recent progress of methodologies for metagenomics. I have tried to classify and summarize the strategies and procedures in Fig. 16.1.

The typical, or can be called traditional, sequence-based strategy is PCR-based approach that relies on the identification of highly conserved amino acid/nucleotide sequence motifs for design of PCR primers. The partial gene of enzyme can be found by PCR amplification from metagenomic DNA. Then the full-length gene is recovered by using a technique such as genomic walking and is usually expressed in heterologous host for enzymatic characterization. The highly conserved motif sequences are also used to design the oligonucleotide probes which are used for hybridization experiments for screening homologous genes in a metagenomic library constructed with the plasmid or phage/phagemid vectors. Although the PCR or hybridization-based approaches contributed to the identification of various new enzymes, it has the major limitation that it relies on known protein structures (Ferrer et al. 2009). Another sequence-based strategy is, established in the past decade,

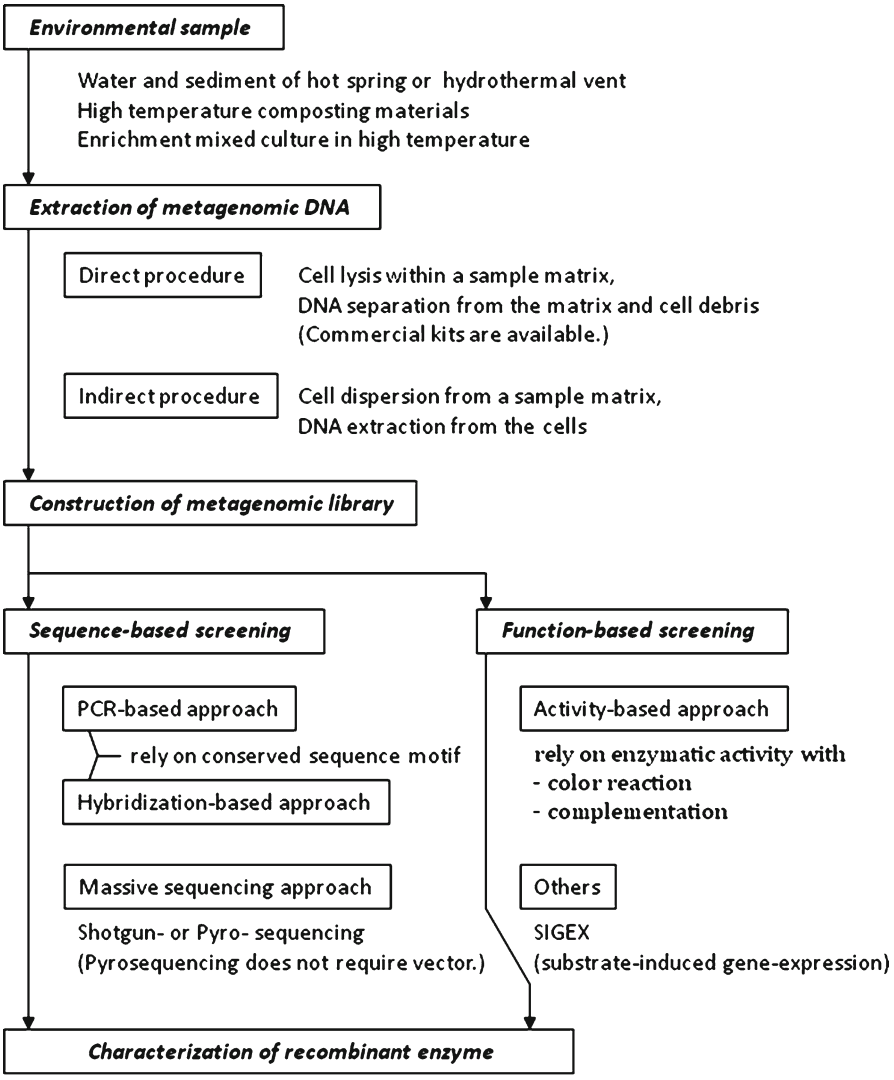

Fig. 16.1 Metagenomic strategies for enzyme discovery

whole/partial sequencing of metagenomic DNA with subsequent (homology-based) screening of enzyme genes. For this massive-sequencing procedure, cosmid, fosmid, and BAC vectors are necessary to clone larger DNA fragments of approximately 30–300 kbp.

In contrast to the sequence-based strategies, function-based screening does not rely on amino acid/nucleotide sequence data and have potential to find novel enzymes which have no sequence similarity with known enzymes. The most simple and traditional method of function-based metagenome screening is activity-based

approach involving plasmid/phagemid/cosmid/fosmid vectors and color reaction mediated by the target enzyme. Once a target activity is isolated, the enzyme is usually reexpressed in an appropriate host and is then purified and characterized. This approach also made great success to discover a number of novel enzymes (Ferrer et al. 2009; Uchiyama and Miyazaki 2009). For example, from a metagenomic library created from the seawater:brine interface of the Urania West Basin, a hypersaline anoxic deep-sea basin off the coast of Crete, five distinct groups of esterases were obtained, two of which exhibited no homology to known esterases (Ferrer et al. 2005). On the other hand, in the activity-based approach, the heterologous gene expression in *E. coli* sometimes fails because of incompatibility of transcriptional/translational machinery between *E. coli* and uncultivated microbe from which the clone is derived. To solve this problem, alternative host, such as *Bacillus* spp., *Pseudomonas putida*, *Streptomyces lividans* (Lorenz and Eck 2005), and *Rhizobium leguminosarum* (Li et al. 2005), has been investigated.

The SIGEX (substrate-induced gene-expression screening), developed by Uchiyama et al. (2005), is an alternative tool to discover novel enzymes from metagenomic libraries. This approach is based on the function of regulatory elements of catabolic genes and can be included in the broad-sense function-based screening but is not an activity-based approach. In this method, the "operon-trap *gfp*-expression vector" is used for high-throughput screening (Uchiyama et al. 2005).

Additional improvements of both sequence and function-based metagenome screening have been achieved and reviewed (Schmeisser et al. 2007; Medini et al. 2008; Steele et al. 2009; Singh et al. 2009; Ferrer et al. 2009; Simon and Daniel 2009; Uchiyama and Miyazaki 2009; Lee et al. 2010; Xu 2010). These approaches combining with the standard procedure mentioned above might be complemented with each other for efficient discovery of novel enzymes from the nature.

16.4 Discoveries of Enzymes from Hot Environments

As described in Chap. 1, various hot habits and artificial reactors can be targeted for sampling for mining of thermostable enzymes. The enzymes derived from thermophiles are sometimes called thermozymes (Vieille et al. 1996) and have attracted the broad interests, such as industrial application, structure and thermostability of proteins, and evolution, of many researchers involving microbiologists, biochemists, protein engineers, biophysicians, bioinformaticians, evolutionally biologists, and so on. Needless to say, thermostable DNA polymerases represented by *Taq* and *Pfu* DNA polymerases which were derived from thermophilic bacteria and hyperthermophilic archaea, respectively, made great success as commercial thermozymes and have also been given enormous contribution to the broad range of scientific achievements as the PCR enzyme so far. The discovery of those great enzymes also let us learn that the study themes directly regarding human life are not only important subjects but also fundamental studies, for example, isolation and describing

novel microbes, which also have a great potential to lead academic revolutions. Although it is less conspicuous in behind of the PCR enzymes, many extremozymes have been discovered by both cultivation-dependent and cultivation-independent approaches. These discoveries were already well reviewed and summarized in the previous papers (Hough and Danson 1999; Niehaus et al. 1999; Demirjian et al. 2001; Atomi 2005; Egorova and Antranikian 2005; Podar and Reysenbach 2006; Ferrer et al. 2007). Here I review the thermozymes discovered from hot environment by metagenomic approaches. In this case, it should be noted that there are two kinds of sense of discovery. The one includes confirmation of catalytic activity of enzyme(s) interested. All the enzymes found by using activity-based approach should be classified in this category. However, sequence-based strategy provides just prediction of enzyme genes at first stage. The following examinations of heterologous expression and enzymatic characterization have not been always achieved depending on a purpose of the study. In this chapter, I should focus on the activity-confirmed thermozymes discovered by the metagenomic studies.

The activity-confirmed thermozymes from metagenome are summarized in Table 16.1. The first example of published thermozymes derived from metagenome was probably cellulase (endoglucanase, CelA protein) presented in anaerobic digester maintained at 55°C on lignocelluloses (Healy et al. 1995). This achievement was based on the activity-based approach using plasmid pUC19 and host *E. coli* strain ER1647. The metagenomic DNA was extracted by using also traditional method. The DNA insertion length was ranged 2–12 kb, and the cellulase activity was indicated by using carboxymethyl cellulose (CMC). This CelA was distantly different in the primary sequence from all the previously reported orthologues (<40% identity). The expressed CelA exhibited temperature optima at 60–65°C and pH optima at 6–7. This is one of the pioneering works not only for thermozymes but also for all the kinds of enzymes derived from metagenome since only a few metagenome-derived enzymes have been described in the 1990s (Vakhlu et al. 2008).

Richardson and his colleagues (2002) demonstrated high-throughput screening of microbial DNA libraries to identify three α-amylase genes related to the orthologues from hyperthermophilic archaea *Thermococcus*, and the expressed amylases exhibited high activity at 105°C and pH 4.5. The initial screening was done by using λ phage vector and standard protocol. However, subsequently, in order to optimize the α-amylase productivity and expression phenotypes to better suit the commercial process for starch liquefaction, the "gene reassembly" was performed using the three wild-type genes as parental sequence. Finally, they obtained a reassembled (chimeric) α-amylase which had enhanced thermostability and was employed in a continuous 2-gallon/min pilot reactor to liquefy cornstarch at pH 4.5. In this study, they used genomic DNA libraries which not only were derived from hot environmental metagenome but also included other metagenomes and DNA from isolates and enrichment cultures. Therefore, it is not sure that the three wild-type genes come from hot environmental metagenome. However, it is no exaggeration to say that the present work surely displayed one of the advanced procedures for discovery of novel enzymes from metagenome.

Table 16.1 List of thermostable enzymes discovered by metagenomic approach

Enzyme	T_{OPT}	Source of metagenomic DNA	Vector	Screening method	References
Amylase	(105)[a]	Various hot environments	Fosmid	Activity/PCR	Richardson et al. (2002)
Cellulase	60–65	Anaerobic digester (55°C, pH 6.5–7)	Plasmid	Activity	Healy et al. (1995)
Cyclodextrinase	55	Hot spring (60–65°C, pH 6–8)	Plasmid	PCR	Labes et al. (2008)
Cyclomaltodextrinase	50–55	Hot spring (55°C, pH 6.0)	Plasmid	PCR	Tang et al. (2006)
DNA polymerase	70	Hydrothermal vent (polychaete tubes)	Fosmid	Sequencing	Moussard et al. (2006)
Esterase	50	Hot spring (70°C, pH 7.0–7.5)	Fosmid	Activity	Kim et al. (2005)
Esterase	95	Hot spring (80–95°C, pH 4–6)	Fosmid	Activity	Rhee et al. (2005)
Esterase	70	Hot spring (70°C, pH 7)	Plasmid	Activity	Tirawongsaroj et al. (2008)
Lipase	60	Fed-batch bioreactor (50–70°C)	Cosmid	Activity	Meilleur et al. (2009)
Lipase	60	Fed-batch bioreactor (50–70°C)	Cosmid	Activity	Côté and Shareck (2010)
Neopullulanase	65	Hot spring (60–65°C, pH 7–8)	Plasmid	PCR	Labes et al. (2008)
Phospholipase (patatin-like)	70	Hot spring (70°C, pH 7)	Plasmid	Activity	Tirawongsaroj et al. (2008)
Poly(DL-lactic acid) depolymerase	70	PLA disks buried in compost (65°C)	Plasmid	Activity	Mayumi et al. (2008)
Pullulanase	75	Hot spring (55°C, pH 6.0)	Plasmid	PCR	Tang et al. (2008)
Superoxide dismutase (Fe-SOD)	(80)[b]	Hot spring (85–90°C, pH 7.0)	Plasmid	Sequencing	He et al. (2007)
Xylanase	100	Hot pool (78°C, pH 7.5)	Plasmid	PCR	Sunna and Bergquist (2003)
Xylanase	55	Hot spring (55°C, pH 8)	Plasmid	PCR	Helianti (2007)

[a]One the of discovered enzymes, BD5063, showed greater amylase activity at 105°C than that of *Bacillus licheniformis*
[b]The enzyme retained >85% of the activity after treatment at 80°C for 3 h

On the other hand, Labes et al. (2008) achieved a unique "in situ enrichment" procedure prior to preparation of metagenomic DNA when they isolated starch and pullulan-modifying enzymes of the α-amylase family. In situ enrichments were performed using permeable polyethylene flasks containing untreated hot spring water and 0.1% starch. The flasks were closed to prevent medium and biomass loss, but the gases and ions from the hot spring were allowed to flow into and out of the flask. The flasks were maintained with 500 g of weight in the hot springs (60–65°C, pH 6–8) in Iceland. The metagenomic DNA recovered from the enrichments was applied for PCR-based approach using consensus primer sets for glycoside hydrolase family 13 in which most of the known starch-modifying enzymes have been classified. They successfully isolated novel bacterial-type cyclodextrinase and neopullulanase genes, and the recombinant enzymes expressed in *E. coli* exhibited maximal activities at 55 and 65°C, respectively.

Sunna and Bergquist (2003) discovered another polysaccharide-degrading enzyme, xylanase, by PCR-based approach using hot pool metagenome in New Zealand. The temperature of the pool was 78°C. They obtained a partial gene for 1,4-β-xylanase, *xynA*, at first, using a degenerated PCR primer set, then successfully got full-length *xynA* by genomic walking. The resulting XynA amino acid sequence did not show high similarity with any known XynA sequences. This XynA expressed in *E. coli* exhibited optimum activity at 100°C and pH 6.0 and was extremely thermostable at 90°C and showed 27% of maximum activity at 125°C. Helianti (2007) also isolated 1,4-β-xylanase gene from metagenomic library derived from muddy water sample of Pawan Hot Spring (55°C), Indonesia, although this enzyme was less thermophilic than the one reported by Sunna and Bergquist.

In 2005, two independent papers describing metagenome-derived esterases were published by Korean teams. Both teams conducted almost the same activity-based approaches involving fosmid vectors and *E. coli* hosts. The sampling sites were hot springs in Indonesia in both studies. However, interestingly, one team isolated archaeal-type esterases (Rhee et al. 2005) and the other isolated bacterial ones (Kim et al. 2005). The archaeal esterase named EstE1 found by Rhee et al. showed relatively high similarity with *Pyrobaculum calidifontis* esterase (64%) and *Sulfolobus solfataricus* lipase (63%), and the recombinant EstE1 degraded *p*-nitrophenyl esters at temperature ranging from 30°C up to 95°C in a pH range of 5.5–7.5. The other bacterial-type esterase named EstMa by Kim et al. was the closest (33%) to the LipC produced by *Mycobacterium avium*, and the recombinant EstMa degraded *p*-nitrophenyl esters maximally at 50°C and pH 6.5. Both archaeal- and bacterial-type enzymes prefer to degrade *p*-nitrophenyl caprate (C_6), suggesting that these enzymes were esterases and not lipases.

Tang et al. (2006) found a gene for novel cyclodextrin- and maltodextrin-degrading enzyme, *cda13A*, from metagenomic DNA extracted from sediment of hot springs in Thailand by using PCR-based approach. Their first achievement to express the *cda13A* in *E. coli* failed because of the expression of insoluble protein. However, remarkably, they successfully obtained recombinant enzyme with yeast, *Pichia pastoris*, as expression host. By using this host, the purity of secreted CDA13A in the culture medium reached more than 90% before

purification. The recombinant cyclomaltodextrinase showed optimal temperature and pH at 50–55°C and 6–7, respectively. They also isolated a gene encoding a novel thermophilic pullulan-hydrolyzing enzyme from the same metagenome sample (Tang et al. 2008). The isolated enzyme, designated Npu193A, was most likely a "neopullulanase-like enzyme" firstly reported by Oslancová and Janećek (2002). The recombinant Npu193A was also obtained by using *P. pastoris* as expression host and hydrolyzed various polysaccharides including pullulan, starch, and γ-cyclodextrin optimally at 75°C and pH 7.

He et al. (2007) sequenced a metagenomic library constructed from the metagenomic DNA recovered from Tengchong Hot Spring (85–90°C, pH 7.0) in China and found a full-length functional Fe-superoxide dismutase (Fe-SOD) gene. The deduced amino acid sequence of their enzyme showed 85–86% identity to Fe-SODs from the hyperthermophilic bacteria *Aquifex* spp. The recombinant Fe-SOD, designated tcSOD, expressed in *E. coli* was highly stable at 80°C and retained 50% activity after heat treatment at 95°C for 2 h. It also showed striking stability across a wide pH span from 4 to 11.

The lipolytic enzymes such as lipases and phospholipases are also important biocatalysts and have a potentially broad spectrum of biotechnological uses. In recent years, some novel lipases isolated from metagenomic libraries derived from hot environment have been reported. Tirawongsaroj et al. (2008) found two genes for novel lipolytic enzymes, designated PLP and Est1, from metagenomic DNA extracted from soil of hot spring (70°C, pH 7) in Thailand. They used activity-based approach to find them, and the recombinant enzymes were reexpressed in *E. coli* and characterized. Both PLP and Est1 showed highest activity at 70°C and, interestingly, displayed both esterase and lipase activities, in which the latter acts only toward long-chain monoacylglycerol. Shareck and his colleagues also reported two novel lipases discovered from the biomasses of a sequencing fed-batch reactors (SFBR, 50–70°C, pH 7–8.5) by using activity-based metagenomics with cosmid vectors (Meilleur et al. 2009; Côté and Shareck 2010). The first one is alkali-thermostable lipase, designated LipIAF5.2, found from gelatin enriched SFBR. The maximal activity of the recombinant LipIAF5.2 was not too high (60°C), but this enzyme had a remarkable thermostability that contributed no decrease of activity after 4 h of incubation at 90°C. The other was moderately thermostable isolated from meat extract enriched SFBR. This enzyme, designated LipIAF1-6, was more novel than the first one because the amino acid sequence of this enzyme showed less than 31% identity to the previously reported lipases. Both enzymes were successfully highly expressed in *Streptomyces lividans*.

The poly(lactic acid) (PLA) is one of the biodegradable aliphatic polyesters, and many microbes degrading PLA have also been isolated. Mayumi et al. (2008) have firstly reported the PLA-degrading enzymes, PLA depolymerases, by metagenomics in which the metagenomic library was derived from adherent biomass with PLA disks buried in compost. One of the discovered PLA depolymerases, PlaM4, showed highest activity at 70°C and degraded not only PLA but also various aliphatic polyesters, such as tributyrin, and p-nitrophenyl esters.

I should describe one more example of discovering thermozymes from metagenomic library, even though this study was aimed to examine the temperature preference of an uncultured archaeon, and not mainly for industrial application. The members of the lineage of the deep-sea hydrothermal vent Euryarchaeota 2 (DHVE2) (Takai and Horikoshi 1999) can be considered likely candidates to represent a group having a significant impact in hydrothermal habitats based on their ecological distribution. However, the phenotypic features of DHVE2 were unknown. Moussard et al. (2006) sequenced about 40-kbp insert of fosmid clone "Alv-FOS1" derived from metagenomic library of microbial assemblage associated with polychaete tubes at hydrothermal vent. They found a gene for family B DNA polymerase within the sequence, and the recombinant enzyme was expressed in *E. coli* and characterized. The Alv-FOS1 DNA polymerase activity was optimal at above 70°C, indicating that Alv-FOS1 and its relatives of the DHVE2 lineage harbor a thermophilic lifestyle (Moussard et al. 2006).

16.5 Future Perspectives

As shown in Table 16.1, most of the discoveries of thermozymes from hot environmental metagenomic libraries have been achieved by the PCR-based or activity-based approaches. Although, as mentioned, the PCR-based approach must involve, in most cases, cloning of full-length open reading flame (ORF) after the PCR amplification of gene fragment of target enzyme, this requirement does not seem to limit a potential of the PCR-based approaches because various techniques for cloning of intact ORFs from partial genes such as cDNA fragment derived from mRNA have already been established and regularly used in general molecular biological experiments. On the other hand, the other sequence-based strategy, sequencing-based approach has contributed only two isolations of thermozymes so far (Moussard et al. 2006; He et al. 2007). However, this approach does not rely on the conserved sequences of known enzymes, which is absolutely necessary for PCR-based approach, and may have great potential for discovery of really novel thermozymes having both novel structure and function. Recently, a new sequencing platform, a chip-based pyrosequencing (Margulies et al. 2005), has also been used for direct analysis of metagenomic DNA (Xu 2010), in which no vector is required. It should also be noted that various bioinformatic tools and databases for metagenomics have been established (Singh et al. 2009) and will enhance the advantages of sequence-based strategies for metagenomic analysis. The only serious hindrance in the progressed sequencing-based approaches may be a great deal of cost which has been constantly decreased in the past decade but is still too high to achieve such experiments in a small laboratory, and less cost may be expected.

As Ferrer et al. (2007, 2009) pointed out in their reviews, "the main challenges for mining enzymes from extreme environments is not only to develop a robust screening or selection system, but also to overcome the problem of the very low biomass yields and cell numbers that hinder high yields of DNA for cloning" (Abulencia et al. 2006). One of the solutions for this problem may be

multiple displacement amplification (MDA) with Φ29 DNA polymerase which allows amplification of sufficient amount of DNA for metagenomic analysis from less than 1-ng DNA. Even though this technique may be biased, yields rather short DNA fragments, and often produces artificial sequences, the MDA has contributed for metagenomic studies in which rare and very dilute DNA samples were used (i.e., Erwin et al. 2005; Abulencia et al. 2006; Yokouchi et al. 2006).

After Herley's discovery of novel thermostable cellulase (1995) by activity-based approach, for about 7 years, there is no publication (as a journal paper) of thermozymes identified by metagenomics. However, during this period, Gelfand and his colleagues of Roche Molecular Systems, Inc., presented a progressive work for design of new functional DNA polymerases at the international conference for extremophiles (Extremophiles 1998) in Brest (Wang et al. 1998). They amplified portions of the active sites of thermophilic DNA polymerase domains using degenerated PCR primers and metagenomic DNA recovered from hot springs. The PCR amplicons were then cloned into a modified *Taq* DNA polymerase expression plasmid to create "Chimeric Hot Spring DNA Polymerase Expression Libraries." Subsequent functional assay gave two kinds of chimeric DNA polymerases, both of which had an increased ability to incorporate dideoxy NTPs as compared to wild-type *Taq* DNA polymerase. This alternative PCR-based approach was a remarkable application of metagenomics to construct industrial enzymes with novel function.

Kanaya et al. (2010) discovered various thermostable ribonuclease H (RNase H) from leaf-and-branch compost (50°C) by metagenomic approach combining with complementally genetics. They used temperature-sensitive (ts) mutant strain as a heterologous host for screening the RNase H activity. This method also has a potential to expand the limitation of activity-based metagenomics.

The heterologous host, which is necessary not only for activity-based screening but also for final expression of recombinant enzyme in all the kinds of metagenomic approaches for enzyme discovery, should be paid more attention in the coming decade. As mentioned in the Screening Strategies and Libraries, some mesophilic bacterial species other than *E. coli* have already been tested and contributed to some metagenomic approaches of thermozymes. However, as described in Chap. 28 "Cloning and Expression of Thermostable Proteins and Enzymes," a number of thermozymes have failed to be expressed in mesophiles. To overcome this problem, Angelov et al. (2009) developed a two-host fosmid system for functional screening of genomic DNA library of thermophilic spirochaete, *Spirochaeta thermophila*. In this study, extreme thermophilic bacterium *Thermus thermophilus* was used as heterologous host to clone a thermostable xylanase of *S. thermophila*. Although, this system was a very good procedure for screening thermozymes, we really need more number of options for heterologous expression of thermozymes. Lack or insufficiency of thermophilic host-vector system undoubtedly will become a major limitation factor to metagenomics of thermophiles. Therefore, host-vector systems in thermophiles (discussed in Chap. 27) should be improved in both Archaea and Bacteria.

16.6 Conclusions

The technologies regarding metagenomics, that is, massive sequencing, have been improved at surprising speed and now provide a reconstructed complete genome sequence of uncultured microbe (Tyson et al. 2004). The resulting data gives us complicated metabolisms and physiological properties of such uncultivated microbe rather precisely, and the recombinant proteins also can be expressed in heterologous host and biochemically characterized. Moreover, Gibson et al. recently created a bacterial cell controlled by a chemically synthesized genome (2010). Do we no longer need to isolate and purify microbes? I, one of microbiologists loving classical microbial experiments, somehow feel sad for this situation. However, we may not have a time to sentimentalize about such change in the coming decades.

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Chapter 17
DNA Polymerases and DNA Ligases

Sonoko Ishino and Yoshizumi Ishino

Abstract DNA polymerase and DNA ligase are ubiquitous enzymes that synthesize complementary DNA strands according to the template DNA and ligate breaks in the backbone structure of DNA, respectively, in living organisms. Multiple enzymes have been identified from each organism, and the functional sharing of these enzymes has been investigated for both DNA polymerase and DNA ligase. In addition to their fundamental role in maintaining genome integrity during replication and repair, DNA polymerases and DNA ligases are widely used for genetic engineering techniques, including DNA cloning, dideoxy sequencing, DNA labeling, mutagenesis, and other in vitro DNA manipulations. Thermostable DNA polymerases and DNA ligases are especially important in PCR and LDR/LCR, which are indispensable techniques for gene manipulation. In this chapter, we have summarized the recent developments in DNA polymerases and DNA ligases from thermophilic microbes.

Keywords DNA replication • DNA repair • Polymerase chain reaction • Ligase chain reaction • Genetic engineering

17.1 Introduction

DNA polymerase and DNA ligase were originally discovered by basic molecular biology research on the molecular mechanisms of DNA replication in living organisms. It is now well known that DNA polymerases synthesize new DNA strands according to the template DNA, using deoxynucleotide triphosphates during DNA replication. DNA ligase works to join the Okazaki fragment during the lagging

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Table 17.1 Distribution of DNA polymerases from seven families in the three domains of life

		Archaea		Eukaryota
Family	Bacteria	Crenarchaeota	Euryarchaeota	(Mitochondrion)
A	Pol I			Pol θ (Pol γ)
B	Pol II	Pol I, Pol II	Pol I(B)	Pol α, Pol δ, Pol ε, Pol ζ
C	Pol III			
D			Pol II(D)	
E				
X				Pol β, Pol λ, Pol μ, Pol σ
Y	Pol IV, Pol V			Pol η Pol ι, Pol κ

strand synthesis in semiconservative DNA replication. For these essential functions in the DNA replication process, all living organisms have DNA polymerase and DNA ligase in their cells. Furthermore, these enzymes work in several processes to repair lesions in the DNA strand to maintain genome integrity, and most organisms have multiple DNA polymerases and DNA ligases that share the functions in DNA metabolism (Table 17.1).

DNA polymerase catalyzes phosphodiester bond formation between the terminal 3′–OH of the primer and the α-phosphate of the incoming triphosphate. DNA polymerases have been classified by amino acid sequence similarity, and seven families, A, B, C, D, E, X, and Y, are now widely recognized (Braithwaite and Ito 1993; Cann and Ishino 1999; Ishino and Cann 1998; Lipps et al. 2003; Ohmori et al. 2001). For example, *Escherichia coli* has five DNA polymerases, and Pol I, Pol II, and Pol III belong to families A, B, and C, respectively. Pol IV and Pol V are classified in family Y, as the DNA polymerases for translesion synthesis (TLS). In eukaryotes, the replicative DNA polymerases, Pol α, Pol δ, and Pol ε, belong to family B, and the translesion DNA polymerases, η, ι, and κ, belong to family Y (Loeb and Monnat 2008; Guo et al. 2009). The most studied enzyme in family X is Pol β, a gap-filling polymerase for DNA repair processes. The distribution of DNA polymerases in Archaea, the third domain of life, is interesting. Members of the Crenarchaeota, a subdomain of Archaea, have at least two family B DNA polymerases (Uemori et al. 1995; Cann et al. 1999). On the other hand, there is only one family B DNA polymerase in the Euryarchaeota, the other subdomain of Archaea. Instead, the euryarchaeotic genomes encode a family D DNA polymerase, which seemed to be specific for these Archaea and had been never found in any other living organisms until recently (Cann et al. 1998; Cann and Ishino 1999). The role of each DNA polymerase in the archaeal cells is unknown (Ishino and Ishino 2006), although the archaeal family B DNA polymerases are useful models to understand the structure-function relationships of the eukaryotic replicative DNA polymerases (Perler et al. 1996). Many researchers are working to elucidate the physiology and enzymology of DNA polymerases.

As shown in Fig. 17.1, the common three-dimensional core structure of polynucleotide polymerases resembles the human right hand and consists of three distinct domains: palm, finger, and thumb (Rothwell and Waksman 2005).

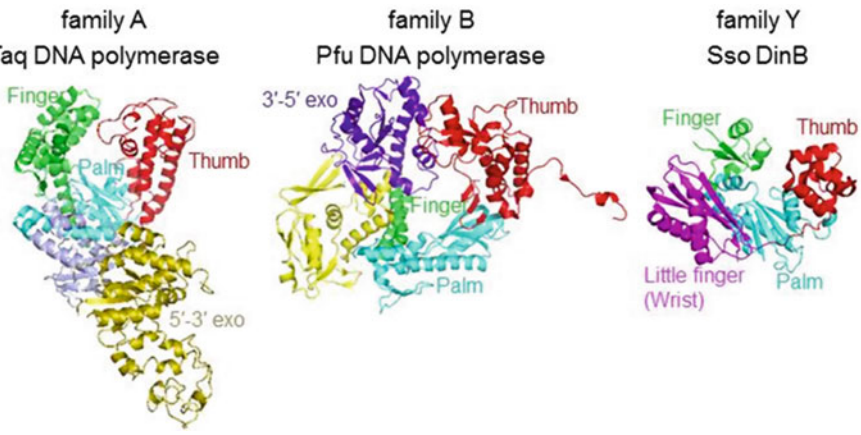

Fig. 17.1 Structural overview of DNA polymerases. Ribbon diagrams of the three DNA polymerases from family A, B, and Y are shown. Taq DNA polymerase (PDB code 1TAQ), Pfu DNA polymerase (PDB code 3A2F), and Sso DinB DNA polymerase (PDB code 1K1S) represented from each family. The three distinct domains of the DNA polymerases are shown in *different colors*. A unique C-terminal domain in Sso DinB is called the "little finger" or "wrist" domain

The palm domain has the catalytic site of the phosphoryl transfer reaction. The finger domain is important for incoming nucleotide recognition and binding. The thumb is involved in binding the DNA strand. A comparison of the *pol* gene-encoding amino acid sequences, including not only DNA polymerases but also RNA polymerases, reverse transcriptases, and RNA-dependent RNA polymerases, revealed three regions with highly conserved amino acid sequences, named motifs A, B, and C (Perler et al. 1996). The fundamental ability of DNA polymerases to synthesize a deoxyribonucleotide chain is conserved in relation to the structural conservation. However, the more specific properties, including processivity, synthesis accuracy, and substrate nucleotide selectivity, differ among the enzymes. The enzymes within the same family have basically similar properties.

DNA ligases catalyze nick-sealing reactions via three nucleotidyl transfer steps, as described in previous review articles (Wilkinson et al. 2001; Tomkinson et al. 2006). In the first step, DNA ligases form a covalent enzyme-AMP intermediate, by reacting with ATP or NAD^+ as a cofactor (step 1). In the second step, DNA ligases recognize the substrate DNA, and the AMP is subsequently transferred from the ligases to the 5′-phosphate terminus of the DNA to form a DNA-adenylate intermediate (AppDNA) (step 2). In the final step, the 5′-AppDNA is attacked by the adjacent 3′-hydroxy group of the DNA to form a phosphodiester bond (step 3). Generally, DNA ligases are grouped into two families, according to their requirement for ATP or NAD^+ as a nucleotide cofactor in the step 1 reaction (Table 17.2). ATP-dependent DNA ligases are widely found in all three domains of life (Eukaryota, Bacteria, and Archaea), whereas NAD^+-dependent DNA ligases exist in Bacteria, halophilic archaea (Poidevin and

Table 17.2 Distribution of DNA ligases in the three domains of life

Bacteria	Archaea	Eukaryota
ATP-dependent NAD^+-dependent	ATP-dependent NAD^+-dependent	ATP-dependent

MacNeill 2006), and some eukaryotic viruses (Benarroch and Shuman 2006). Three genes (*LIG1*, *LIG3*, and *LIG4*) encoding ATP-dependent DNA ligases have been identified in the human genome to date. Human DNA ligase I (Lig I) is a replicative enzyme that joins Okazaki fragments during the DNA replication process. In Archaea, a number of archaeal ATP-dependent DNA ligases, which are homologous to eukaryotic DNA ligase I, have been biochemically characterized (Shuman 2009). Interestingly, the genetic analysis of a DNA ligase from the halophilic euryarchaeon *Haloferax volcanii* revealed the existence of both ATP- and NAD^+-dependent DNA ligases in one organism (Zhao et al. 2006; Poidevin and MacNeill 2006). Based on a phylogenetic analysis, it was proposed that the NAD^+-dependent DNA ligase in *H. volcanii* was acquired by lateral gene transfer from bacteria.

In addition to their fundamental roles in maintaining genome integrity during replication and repair, DNA polymerases and DNA ligases are widely used for genetic engineering techniques, including DNA cloning, dideoxy sequencing, DNA labeling, mutagenesis, and other in vitro DNA manipulations. Thermostable DNA polymerases are particularly useful for PCR (polymerase chain reaction) and cycle sequencing. To develop DNA polymerases suitable for PCR, the enzyme's velocity, fidelity, and efficiency of primer usage for in vitro DNA strand synthesis are the critical factors to evaluate, and faster DNA polymerases, with better accuracy and higher efficiency, in addition to heat stability, are desired for practical usage (Barnes 1994). The fundamental ability to synthesize a deoxyribonucleotide chain is conserved in relation to the structural conservation of the DNA polymerases. However, the more specific properties, including processivity, synthesis accuracy, and substrate nucleotide selectivity, differ among the enzymes. The enzymes within the same family have basically similar properties. Currently, only the family A enzymes are commercially available for dideoxy sequencing, and the family A and B enzymes are practically used for PCR. None of the DNA polymerases from the other families are suitable for general use in genetic engineering experiments. The main difference between the family A and family B DNA polymerases is the absence or presence of a 3′–5′ exonuclease activity, which contributes to the proofreading of DNA strand synthesis. The 3′–5′ exonuclease activity is generally associated with the family B enzymes, but not with the family A enzymes, although some family A enzymes exceptionally have a weak 3′–5′ exonuclease activity (Joyce and Steitz 1994; Villbrandt et al. 2000). Based on these differences, family A is advantageous for the efficient amplification of a long DNA region, and family B is generally more suitable for the precise amplification of a shorter region by PCR (Eckert and Kunkel 1991).

Thermostable DNA ligase is also utilized in temperature-cycling reactions, called ligase detection reaction (LDR) and ligase chain reaction (LCR), and these techniques are employed for single-nucleotide polymorphism (SNP) detection. Currently,

thermostable DNA ligases from both bacterial (*Thermus*) and archaeal (*Pyrococcus* and *Thermococcus*) sources are commercially available.

In this chapter, we describe the current basic research topics of the thermostable DNA polymerases and DNA ligases and their applications to genetic engineering techniques.

17.2 Thermostable DNA Polymerases

17.2.1 History of Research on Thermostable DNA Polymerases

PCR technology became practically applicable because of Taq DNA polymerase (Saiki et al. 1988). This thermostable DNA polymerase is not inactivated by the heat-denaturing step during PCR, and therefore, the PCR procedure could be automated in the thermal cycler (the PCR instrument). Taq DNA polymerase was originally purified from *Thermus aquaticus* YT1, isolated from Yellowstone National Park in the USA. This strain grows at 75°C, and the DNA polymerase, working at an optimal temperature of 80°C (Chien et al. 1976), was adapted for PCR application. Subsequently, the Tth DNA polymerase from *Thermus thermophilus* was utilized for PCR. This enzyme is especially useful for one-step RT (reverse transcription) PCR because of its distinct reverse transcription activity (Myers and Gelfand 1991). A remarkable defect of these *Thermus* DNA polymerases is their low fidelity of in vitro DNA synthesis. Due to the lack of 3′–5′ exonuclease activity, which is needed for the proofreading function, these DNA polymerases have remarkably high error rates during the PCR amplification of the target DNAs, and therefore, a more accurate DNA polymerase was required. Thermophiles and hyperthermophiles are the source organisms for heat-stable DNA polymerases. Most of the known hyperthermophiles on the earth belong to Archaea, and therefore, searches for new, useful DNA polymerases have been focused on the hyperthermophilic archaea. We cloned the genes encoding the DNA polymerases from *Pyrococcus furiosus* (Uemori et al. 1993a, 1997), *Pyrodictium occultum* (Uemori et al. 1995), and *Aeropyrum pernix* (Cann et al. 1999) in the 1990s. The most interesting features discovered at the inception of this research area were that (1) the archaea indeed have the eukaryotic Pol α-like (family B) DNA polymerases (Uemori et al. 1993a); (2) some archaea (Crenarchaeota) have multiple Pol α-like DNA polymerases, as in eukaryotic cells (Uemori et al. 1995; Cann et al. 1999); and (3) some archaea (Euryarchaeota) have a very unique DNA polymerase, named Pol D and classified in a new family (family D) (Uemori et al. 1997; Cann and Ishino 1999).

Another noteworthy topic is that the intervening sequences that are spliced out after translation are encoded in many DNA polymerase genes in Archaea. This phenomenon was originally discovered in the *pol* gene in *Thermococcus litoralis*, and an active DNA polymerase was produced by removing the intervening sequences, called inteins (Perler et al. 1992). The splicing of the DNA polymerase precursor protein was experimentally demonstrated in vitro (Xu et al. 1993).

Subsequent genome sequence analyses of archaeal organisms revealed that one or two inteins are commonly inserted in the conserved motif A, B, or C in the family B DNA polymerases (Perler 2002).

The early stages of the research on the thermostable DNA polymerases have been summarized in a review article (Perler et al. 1996).

17.2.2 The Family A DNA Polymerases as the Most Popular PCR Enzymes

The family A DNA polymerases from bacteria, represented by Taq DNA polymerase, are useful for general PCR. The production of the *Thermus* DNA polymerase in recombinant *E. coli* cells is not straightforward because of inefficient gene expression, probably due to the high GC contents of their genome sequences (Lawyer et al. 1989). We modified six codons in the translational start region of the *pol* gene and succeeded in dramatically improving the production level of Taq DNA polymerase in *E. coli* cells (Ishino et al. 1994).

More powerful DNA polymerases from *Thermus* species have been sought. For example, the *pol* genes from 22 different *Thermus* strains were compared, and 8 *pol* genes, selected from each clade of the phylogenetic tree, were expressed in *E. coli.* The DNA polymerases thus produced were mostly comparable, suggesting that only limited natural variations in Taq-like DNA polymerase may be discovered (Gibbs et al. 2009).

Protein engineering of the Taq DNA polymerases has been actively pursued around the world. For example, one of the most successful mutations is the Phe at position 667, which is involved in substrate nucleotide selection, and the single mutation, F667Y, drastically decreased the discrimination against four dideoxynucleotides, thus generating a more suitable enzyme for dideoxy sequencing (Tabor and Richardson 1995). This mutant enzyme is commonly used for cycle sequencing (dideoxy sequencing coupled with PCR).

A cold-sensitive Taq DNA polymerase was developed with markedly reduced activity at 37°C, as compared with the wild-type enzyme, and this mutant is suitable for hot start PCR (Kermekchiev et al. 2003). Another target for DNA polymerase mutagenesis is to create an enzyme that is more resistant to PCR inhibitors in the blood or soil, such as hemoglobin and humic acid. A mutant Taq DNA polymerase with enhanced resistance to various inhibitors of PCR reactions, including whole blood, plasma, hemoglobin, lactoferrin, serum IgG, soil extracts, and humic acid, was successfully isolated (Kermekchiev et al. 2009). The molecular breeding of *Thermus* DNA polymerases by using a direct evolution technique, compartmentalized self-replication (CSR) (Ghadessy et al. 2001), also generated a PCR enzyme with striking resistance to a broad spectrum of inhibitors with highly divergent compositions, including humic acid, bone dust, coprolite, peat extract, clay-rich soil, cave sediment, and tar (Baar et al. 2011). Furthermore, enzymes with a broad substrate specificity spectrum, which are thus useful for

the amplification of ancient DNA containing numerous lesions, were also obtained by the CSR technique (Ghadessy et al. 2004; d'Abbadie et al. 2007). Mutational studies of Taq DNA polymerase in the O-helix produced enzymes with reduced fidelity, which may be useful for error-prone PCR (Suzuki et al. 1997, 2000; Tosaka et al. 2001).

DNA polymerases from the moderate thermophiles, including *Bacillus caldotenax* (Uemori et al. 1993b) and *B. stearothermophilus* (Phang et al. 1995), are not applicable to PCR because of their insufficient thermostability. However, these DNA polymerases provide more precise and beautiful sequencing ladders with less signal compression when used for dideoxy sequencing, as compared to the *E. coli* Klenow fragment and T7 DNA polymerase (Uemori et al. 1993b), and these enzymes are commercially available as Bca DNA polymerase and Bst DNA polymerase, respectively.

17.2.3 The Family B DNA Polymerases as High-Fidelity PCR Enzymes

The 3′–5′ exonuclease activity is commonly associated with the family B DNA polymerases. The family B DNA polymerases from hyperthermophilic archaea have the potential to be ideal enzymes for accurate PCR. Therefore, many research reports describing the DNA polymerases from various archaeal organisms have been published. Pfu DNA polymerase, from *P. furiosus*, is the representative enzyme for accurate PCR (Lundberg et al. 1991). A family B DNA polymerase was identified from *Thermococcus kodakarensis* KOD1, which is phylogenetically close to *P. furiosus,* and the amino acid sequence identity between the two polymerases is 79%. However, the latter enzyme, named "KOD DNA polymerase," has faster primer extension ability with high fidelity and is now a very popular PCR enzyme (Takagi et al. 1997). A comparison of the crystal structures of Pfu DNA polymerase (Nishida et al. 2009) and KOD DNA polymerase revealed some interesting differences (Hashimoto et al. 2001). One remarkable feature of KOD DNA polymerase is that it contains an abundance of basic residues (Arg and Lys) flanking the polymerization active site in the finger domain (Fig. 17.2). These basic residues are advantageous for capturing the incoming dNTP by electrostatic interactions. In addition, many arginine residues are located at the junction of the template binding and editing clefts, called the forked-point. This basic environment is probably suitable for stabilizing the melted DNA structure at the forked-point, resulting in high PCR performance (Imanaka 2011).

The most recent successful engineering of the DNA polymerase for PCR is the creation of a fusion protein by Pfu DNA polymerase and a nonspecific dsDNA-binding protein, Sso7d, from *Sulfolobus solfataricus* by genetic engineering techniques (Wang et al. 2004). Although Pfu DNA polymerase is highly accurate for DNA synthesis, it has low processivity. To overcome this weak point of the enzyme, a small protein with high affinity to the DNA strand was fused to the C-terminus of

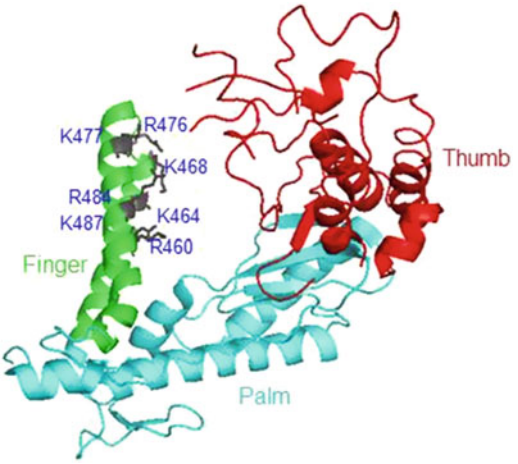

Fig. 17.2 Structure of the polymerase core domain of *T. kodakarensis* DNA polymerase. A ribbon diagram of the three domains for DNA polymerase activity is shown (PDB code 1WNS). Many basic residues are located at the side of the active site in the finger domain

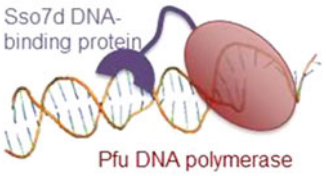

Fig. 17.3 A highly processive DNA polymerase engineered from a family B DNA polymerase. Pfu DNA polymerase fused with Sso7d DNA-binding protein became actually highly processive and is now commercially available

the DNA polymerase (Fig. 17.3). This artificial DNA polymerase possesses high processivity while maintaining accurate DNA synthesis ability. This enzyme is now commercially available as Phusion DNA polymerase.

The sliding clamp is the processivity factor that retains the DNA polymerase on the template DNA strand for processive DNA synthesis. The doughnut-shaped ring structure of the clamp molecules is conserved among biological organisms (Fig. 17.4). The eukaryotic and archaeal PCNAs (proliferating cell nuclear antigen) form a homotrimeric ring structure (Matsumiya et al. 2000), which encircles the DNA strand and anchors the DNA polymerase onto the DNA by binding it on one surface (front side) of the ring, as described below. We have been studying the structure and functions of the *P. furiosus* PCNA (Cann et al. 1999; Ishino et al. 2001; Matsumiya et al. 2002, 2003; Tori et al. 2007), and we successfully developed a novel processive PCR method, using the archaeal family B DNA polymerase with the help of a mutant PCNA (Ishino et al. 2012).

Protein engineering of the family B DNA polymerases has also been actively performed, to create more useful enzymes. Mutations in the conserved Y-GG/A motif, which is located between the N-terminal 3′–5′ exonuclease and the C-terminal polymerase domains, changed the balance between the synthesis and degradation of

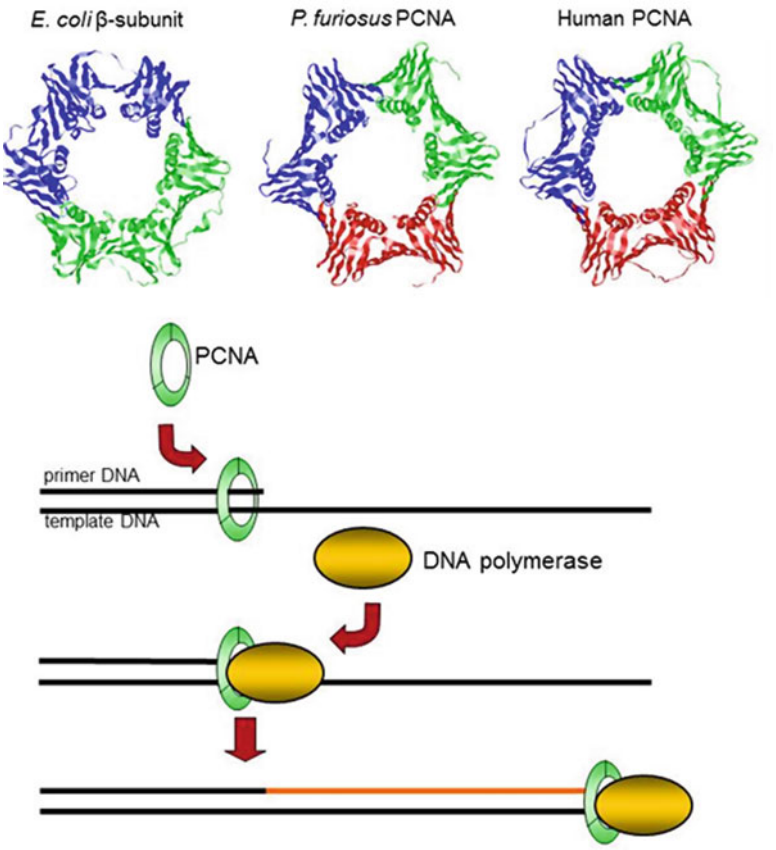

Fig. 17.4 Processive DNA synthesis by DNA polymerase-clamp complex. Crystal structure of clamp molecules from three domains of life, *E. coli* (PDB code 2POL), *P. furiosus* (PDB code 1GE8), and human (PDB code 1AXC), are shown on the *top*. Each subunit is shown in *different color*. PCNA clamps DNA polymerase to protect it from falling off the DNA strand

the DNA strand and directly affected the PCR performance (Böhlke et al. 2000). Mutations in the loop region between the N-helix and O-helix, consisting of the finger subdomain, affected the fidelity of DNA synthesis, because the conformational changes of the finger subdomain play an important role in dNTP recognition. Combined with additional mutations at the active sites of the 3′–5′ exonuclease activity, the loop mutation generated a DNA polymerase that is applicable for error-prone PCR under exactly the same conditions as those used for standard PCR (Biles and Connolly 2004).

Another remarkable feature of the archaeal family B DNA polymerases is their specific interactions with uracil and hypoxanthine, which stall the progression of the polymerases in DNA template strands (Connolly 2009). This phenomenon, called “read-ahead recognition” of deaminated bases, was originally

proposed to be a characteristic of hyperthermophilic archaea to protect them against increased deamination at high temperature. However, the family B DNA polymerase from the mesophilic archaeon, *Methanosarcina acetivorans*, also recognizes uracil and hypoxanthine as efficiently as the enzymes from hyperthermophiles (Wardle et al. 2008). The crystal structure of the DNA polymerase revealed that read-ahead recognition occurs by an interaction with the deaminated bases in a binding pocket located in the N-terminal region, found specifically in the archaeal family B DNA polymerases (Fogg et al. 2002). Due to this specific recognition of uracil, the archaeal family B DNA polymerases, including Pfu DNA polymerase and KOD DNA polymerase, cannot be applied to the carryover prevention PCR, using dUTP instead of dTTP. To overcome this defect, a point mutation (V98Q) was introduced into Pfu DNA polymerase. This mutant enzyme is completely unable to recognize uracil, while its DNA polymerase activity is unaffected (Fogg et al. 2002; Firbank et al. 2008), and it is applicable to uracil-excision DNA engineering (Nørholm 2010).

17.2.4 The Family D DNA Polymerases

The first family D DNA polymerase was identified from *P. furiosus*, by screening for DNA polymerase activity in the cell extract (Imamura et al. 1995). Three independent fractions showing deoxynucleotide incorporation activity were obtained from the anion-exchange chromatography. The family B DNA polymerase (Pol BI) was identified from one fraction, and the second DNA polymerase (Pol II), which was not a family B-like enzyme because of its resistance to aphidicolin (α-like DNA polymerase-specific inhibitor), was found in another fraction. The corresponding gene was cloned, revealing that this new DNA polymerase consists of two proteins, named DP1 and DP2, and that the deduced amino acid sequences of these proteins were not conserved in the DNA polymerase families (Uemori et al. 1997). This DNA polymerase, proposed as Pol D from the new family (family D), has only been identified in the euryarchaeal organisms to date (Cann et al. 1998; Cann and Ishino 1999). The biochemical properties of Pol B and Pol D from *P. furiosus* have been characterized (Uemori et al. 1997; Ishino and Ishino 2001). *P. furiosus* Pol D has efficient strand extension activity and strong proofreading activity. Other family D DNA polymerases were also characterized by several groups (Gueguen et al. 2001; Shen et al. 2001, 2003; Tang et al. 2004; Jokela et al. 2004; Henneke et al. 2005; Castrec et al. 2010).

A genetic study on *Halobacterium* sp. NRC-1 showed that these two DNA polymerases (Pol BI and Pol D) are essential for viability (Berquist et al. 2007). The functional roles of these two DNA polymerase in the cells are being analyzed. According to its biochemical properties, including the usage of an RNA primer and the presence of strand displacement activity, Pol D may take charge of lagging strand synthesis. An interesting issue is to elucidate whether Pol BI and Pol D work

together at the replication fork for the synthesis of the leading and lagging strands, respectively (Henneke et al. 2005; Ishino and Ishino 2006).

Archaea are divided into two phyla, the Crenarchaeota and Euryarchaeota, and the Pol D genes have been found only in Euryarchaeota, as described above. Environmental genomics and cultivation efforts have recently revealed two novel phyla/divisions in the Archaea: Thaumarchaeota, Korarchaeota, and Aigarchaeota. Thaumarchaeota and Aigarchaeota harbor the genes encoding Pol D and crenarchaeal Pol BII (Brochier-Armanet et al. 2008; Nunoura et al. 2011), and Korarchaeota encodes Pol BI, Pol BII, and Pol D (Elkins et al. 2008). In spite of accumulating information about the distributions of DNA polymerases among novel archaeal phyla, their functions have not been investigated, except for the PolBII from the psychrophilic marine thaumarchaeon, *Cenarchaeum symbiosum* (Schleper et al. 1997). Biochemical characterizations of these gene products are interesting, in terms of the evolution of DNA polymerases in living organisms. An evolutional investigation of the relationships between DNA polymerases of archaea and eukaryotes led to the proposal of a hypothesis, in which the archaeal ancestor of eukaryotes encoded three DNA polymerases, two distinct family B polymerases, and a family D polymerase, which all contributed to the evolution of the eukaryotic replication machinery, consisting of Pol α, δ, and ε (Tahirov et al. 2009).

The Pol Ds from the hyperthermophilic euryarchaea are also applicable for PCR, because of their sufficient heat stability (Ishino et al., unpublished). Based on their efficient strand extension activity and strong proofreading activity, Pol D is enzymatically sufficient as a candidate for a PCR enzyme.

17.2.5 The Family E DNA Polymerases

An interesting protein was identified in the plasmid pRN1, isolated from a *Sulfolobus* strain (Zillig et al. 1994). This protein, ORF904 (dubbed RepA), has primase and DNA polymerase activities in the N-terminal domain and helicase activity in the C-terminal domain (Lipps et al. 2003; Lipps 2009). The amino acid sequence of the N-terminal domain lacks homology to any known DNA polymerases or primases, although the C-terminal domain contains a sequence conserved in the superfamily three helicases, and therefore, family E was proposed as a new DNA polymerase family. Similar proteins are encoded by various archaeal and bacterial plasmids, as well as by some bacterial viruses (Lipps 2004). ORF904 is the founding member of the family E DNA polymerases, and recently one protein, tn2-12p, encoded in the plasmid pTN2 isolated from *Thermococcus nautilus*, was experimentally identified as a DNA polymerase in this family (Soler et al. 2010). The crystal structure of the primase/polymerase domain of ORF904 was solved. It revealed a structural fold that differs from all known DNA polymerases but is distantly related to that of the archaeal primases (Lipps et al. 2004). Further investigations of this family of DNA polymerases will be interesting from an evolutional perspective.

17.2.6 The Family Y DNA Polymerases

All organisms are exposed to UV sunlight radiation, a potent DNA-damaging agent known to cause DNA lesions, such as cis-syn thymine-thymine (TT) dimers and 6–4 photoproducts. Although replicative DNA polymerases synthesize DNA strands with high accuracy, most of these polymerases are incapable of replicating through UV-induced lesions. These lesions impede the progress of the DNA replication fork. The means by which the DNA polymerases acting at the replication fork overcome these blocking lesions were unknown, until the discovery of specialized DNA polymerases called translesion DNA polymerases. The amino acid sequences of the DNA polymerases for translesion synthesis (TLS) are similar, and a new family name, family Y, was proposed (Ohmori et al. 2001). Subsequently, these polymerases were shown to be present in cells across the three domains of life. The family Y DNA polymerases are considered to be recruited to temporarily replace the high-fidelity replicative polymerases, in order to replicate through DNA lesions.

E. coli possesses two family Y DNA polymerases, Pol IV (DinB) and Pol V (UmuD'2C). Pol V is clearly the major lesion bypass polymerase involved in damage-induced mutagenesis. Pol V can bypass multiple template lesions, including the TT (6–4) photoproduct, the TT cis-syn cyclobutane pyrimidine dimer (CPD), and abasic sites. In contrast, Pol IV exhibited no extension, although it incorporated nucleotides opposite the lesions. In eukaryotes, several Y-family polymerases have been identified in human cells, including Polη, Polι, Polκ, and Rev1. Polη plays a major role in the efficient bypass of CPD sites and inserts the correct bases opposite the lesion.

TLS in Archaea has served as an important paradigm, by providing significant insights into the molecular mechanisms of lesion bypass. Investigations of TLS have focused on the thermostable Y-family polymerases of the crenarchaeon, *Sulfolobus* species. In comparison, knowledge on the TLS DNA polymerases in the subdomain Euryarchaeota and the mesophilic archaea was lacking. Due to the rapid accumulation of genomic sequences in the databases, investigations of the evolutional relationships of these genes are now possible. A phylogenetic analysis revealed that the archaeal Pol Y proteins can be grouped into five clusters (Lin et al. 2010). Interestingly, two genes encoding Pol Y-like DNA polymerases were found in the genomes of the euryarchaeal mesophiles, *Methanosarcina mazei* and *M. barkeri*, whereas only one such gene is present in the other *Methanosarcina* species, including *M. acetivorans* C2A. Cluster I comprises the DinB homologs of the hyperthermophilic crenarchaea. Among these are the *Sulfolobus* spp. Dpo4 and Dbh, which are the most well-characterized DinB homologs in the archaeal domain. Cluster II includes the group designated as DinB-1 of the Methanosarcinales, with the *Methanosarcina* spp. and *Methanococcoides burtonii*. There are only two representatives in cluster III, and each protein is derived from members of the so-called mesophilic crenarchaea, a group with only one cultured member, *Nitrosopumilus maritimus*. Cluster IV comprises DinB homologs from the haloarchaea, which are known to exist in very high salt environments, and cluster V

contains the DinB-2 homologs of the Methanosarcinales and the DinB proteins from other euryarchaeotes, which are mostly methanogens. From the topology of the tree, the DinB proteins can also be clearly divided into the crenarchaeal cluster (cluster I) and the euryarchaeal cluster (clusters II, III, IV, and V). We cloned the gene-encoding DinB-1 from the *M. acetivorans* C2A genome and characterized the gene product, MacDinB-1. Unexpectedly, MacDinB synthesized distinctly long (~7.2 kb) DNA products in the presence of PCNA in vitro (Lin et al. 2010), in contrast to the results reported for *Sulfolobus* DinB homologs (Grúz et al. 2001).

The crystal structures of three family Y polymerases from *Sulfolobus*, alone (Zhou et al. 2001; Silvian et al. 2001) or complexed with DNA and a nucleotide substrate (Ling et al. 2001), revealed a conventional right-hand-like catalytic core, consisting of finger, thumb, and palm domains. However, the finger and thumb domains are unusually small, resulting in an open and spacious active site (Fig. 17.1). This structural feature accommodates mismatched base pairs as well as various DNA lesions. Furthermore, the family Y DNA polymerases possess a unique "little finger" domain, which may facilitate DNA association and catalytic efficiency (Yang 2003).

17.2.7 The Family X DNA Polymerases

The eukaryotic DNA polymerase β and terminal deoxynucleotidyl transferase are famous as family X DNA polymerases. In addition, DNA polymerases λ and μ were identified recently. The eubacterial Pol X also belongs to this family. The catalytic subunit of the eukaryotic and archaeal primases, which synthesizes a short primer on the template DNA, shares some sequence homology with those of the family X DNA polymerases (Kirk and Kuchta 1999). After the identification of Pol B and Pol D in *P. furiosus*, we found a gene encoding a sequence homologous to that of the catalytic subunit of the eukaryotic primase, p48 protein, and characterized the gene product. Unexpectedly, the archaeal p41 protein did not catalyze the synthesis of short RNA by itself but preferentially utilized deoxynucleotides to synthesize DNA strands up to several kilobases in length (Bocquier et al. 2001). We subsequently found that the gene neighboring the p41 gene encodes a protein with very weak similarity to the p58 subunit of the eukaryotic primase. The gene product, named p46, actually forms a stable complex with p41, and the complex can synthesize a short RNA primer in vitro (Liu et al. 2001), consistent with the fact that the short RNA primer was identified in *Pyrococcus* cells (Matsunaga et al. 2003). However, this primase complex also synthesizes DNA strands with several hundred nucleotide lengths in vitro (Liu et al. 2001), and therefore, it can also be called a DNA polymerase. Several reports describing the biochemical properties and structural analyses of primases from *Methanococcus jannaschii* (Desogus et al. 1999), *P. horikoshii* (Ito et al. 2003, 2007; Matsui et al. 2003), *P. abyssi* (Le Breton et al. 2007), and *S. solfataricus* (Lao-Sirieix and Bell 2004; Lao-Sirieix et al. 2005) have been published to date.

17.2.8 Are There More Powerful DNA Polymerases on Earth?

Several strategies are available to obtain DNA polymerases with superior properties as genetic engineering reagents for in vitro DNA strand synthesis, including sequencing and PCR. Screening for a suitable DNA polymerase activity from known organisms, which can be cultured, is the most conventional way to discover useful enzymes. However, it requires a relatively large cultivation scale to obtain sufficient amounts of cells for the purification of the DNA polymerase activity. Alternatively, protein engineering techniques, using site-specific or random mutagenesis, are powerful ways to create mutant enzymes from the known DNA polymerases. As introduced in the previous sections, several useful enzymes were successfully produced by these procedures. An artificial evolution procedure has attracted a great deal of attention for the creation of DNA polymerases with novel activities (Holmberg et al. 2005; Henry and Romesberg 2005; Brakmann 2005; Ong et al. 2006). These studies have contributed to the elucidation of the detailed structure-function relationships of DNA polymerases, in addition to the creation of novel enzymes with different substrate specificities, stabilities, and activities from those of the naturally evolved counterparts, as in the several examples described above.

We have developed a useful strategy that combines the advantageous points of the above two procedures. The experimental procedure includes (1) metagenomic DNA preparation from soil samples; (2) amplification of the DNA polymerase gene fragment from the metagenomic DNAs by PCR, without cultivation of the organisms; and (3) construction of chimeric *pol* genes in vitro, by substituting parts of the known *pol* gene with the amplified gene fragments (Fig. 17.5). This experimental strategy includes both natural evolution and artificial protein engineering and therefore has the advantageous potential to create novel DNA polymerases. This strategy also generates valuable experimental data about the structure-function relationships of the DNA polymerases to clarify the detailed molecular characteristics of the enzymes.

Three highly conserved amino acid sequences, motifs A, B, and C, as described above, are present in DNA polymerases. Motifs A and C possess catalytically essential carboxylates and are located within the palm domain. Motif B is located in the finger domain and is thought to bind the template strand and dNTPs. Based on these structural features, it would be possible to create degenerate primers based on the conserved amino acid sequences in motifs A and C for PCR amplification of the important region for nucleotide polymerization.

In terms of a genetic engineering reagent, only the family A enzymes are commercially available for dideoxy sequencing, and the family A and B enzymes are practically used for PCR. We focused on the DNA polymerases belonging to families A and B to create useful enzymes as genetic engineering reagents. As mentioned above, screening for a suitable DNA polymerase activity from organisms that can be cultured is commonly performed. However, large amounts of the cells must be cultured to prepare the extracts for biochemical analyses of the DNA polymerase

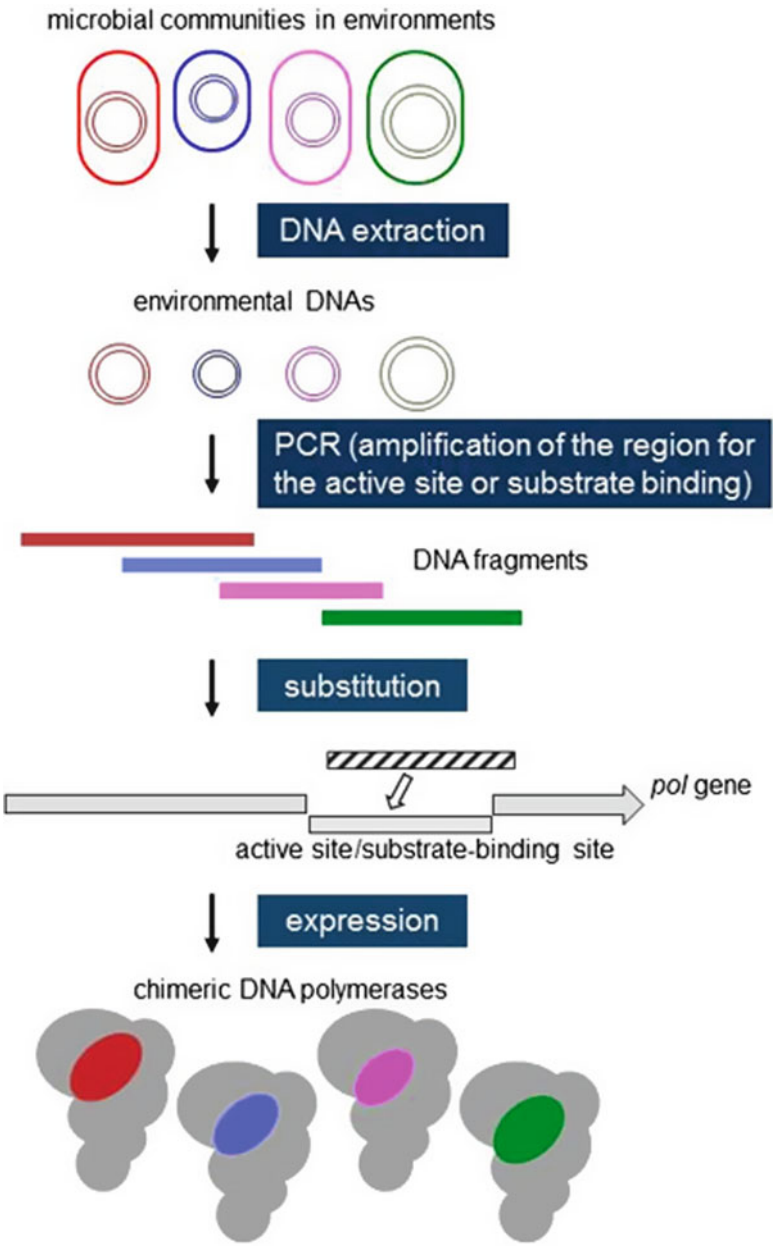

Fig. 17.5 Experimental procedure presented to create chimeric DNA polymerases. Environmental DNAs isolated from various places, including hot spring areas, are used for PCR amplification of the region that is directly involved in the activity of the DNA polymerases. Each isolated gene fragment is then used to substitute for the corresponding region of the wild-type *pol* gene on the expression plasmid. The resultant chimeric genes are expressed in *E. coli,* and the purified DNA polymerases are subjected to evaluations of various biochemical properties

activity (Imamura et al. 1995). Site-specific mutagenesis is now experimentally practical; however, rational design to choose the sites where the mutations should be introduced in the target polymerase genes is necessary to obtain useful enzymes. The type of mutation, substitution, deletion, or insertion, the amino acid residues to be substituted, and the lengths of deletions or insertions also have to be considered carefully.

Over 99% of the microbial organisms on the earth have not been identified (Amann et al. 1995), and therefore, huge amounts of useful genetic resources are available in our natural environment. Metagenomic analysis is a revolutionary technique for microbiological ecology (Kennedy et al. 2010) and biocatalytic applications (Fernandez-Arrojo et al. 2010). Amplification of a target gene from unidentified DNA (genomic DNA, plasmid, and phage DNA) is a very powerful method to obtain new proteins.

When we attempted to amplify part of the *pol* gene from microorganisms in the early 1990s, we designed degenerate primers for PCR based on the sequences of motifs A and C, DPNLQNIP and QVHDE(L/I)(V/L), to amplify the genes encoding the palm region of DNA polymerase from various bacteria (Uemori et al. 1993b). Using these primers, we successfully amplified the target regions of the family A *pol* genes from the bacterial DNAs that were available, including *E. coli, Bacillus subtilis, B. caldotenax, B. caldolyticus, Lactobacillus bulgaricus, L. homohiochii*, and *L. heterohiochii*, in addition to *Thermus aquaticus* and *T. thermophilus* (Uemori et al. 1993b). We also applied this strategy to the amplification of family B DNA polymerases. The degenerate primers were based on the sequences SLYPSII in motif A and VIYGDTD in motif C (Uemori et al. 1995). These experiments revealed for the first time that one archaeal organism encodes two different family B DNA polymerases in its genome. It was quite exciting to find two different family B DNA polymerase genes, when we successfully amplified part of the *polB* gene from *Pyrodictium occultum* (Uemori et al. 1995). It was also fascinating to find plural family B DNA polymerases in one archaeal cell, since at that time, this was similar to the situation in eukaryotic cells, which possess three family B DNA polymerases (Pol α, Pol δ, and Pol ε).

To isolate DNA from thermophilic organisms, environmental specimens were obtained from various hot areas in Japan, including Onikobe (Miyagi Prefecture), Hachimantai (Iwate Prefecture), Kirishima (Kagoshima Prefecture), Ibusuki (Kagoshima Prefecture), Beppu (Oita Prefecture), and Nasu (Tochigi Prefecture). The DNA was extracted from the environmental specimens by using the conventional phenol extraction method and was used as PCR templates.

The plasmids for the overexpression of Taq DNA polymerase (Ishino et al. 1994) and Pfu DNA polymerase (Komori and Ishino 2000), for example, were used for the expression of the chimeric DNA polymerase genes. To construct a system for the gene substitution, restriction sites were needed at appropriate sites in their structural genes, and therefore, silent mutations were introduced to create recognition sites to remove the substitution region. PCR primers containing the recognition sequences for the same restriction enzymes were synthesized, so that the PCR-amplified DNA fragment could be used for the substitution

of the structural genes by digestion with these restriction enzymes, to construct the chimeric genes directly on the Taq or Pfu DNA polymerase expression plasmid. The reconstructed plasmids were introduced into *E. coli*, and the chimeric genes were expressed under the same conditions as those used for the wild-type Taq and Pfu DNA polymerases. The detailed experimental procedures and the results showing some examples of family B DNA polymerases have been reported (Matsukawa et al. 2009).

The experimental strategy described here is clearly different from the artificial random mutagenesis of the same region (between motifs A and C). The introduced sequences that we manipulated originated from genes that naturally evolved, and therefore, unreasonable mutations should be eliminated. We expect that many DNA polymerases with novel properties, which are different from those created by other methods, will be obtained by this procedure.

17.2.9 Interactions with PCNA

The sliding clamp is the processivity factor that retains DNA polymerase on the template DNA strand for processive DNA synthesis, as described above. After clamp loading, DNA polymerase accesses the PCNA, and the polymerase-clamp complex catalyzes processive DNA synthesis (Fig. 17.4). Therefore, structural and functional studies of the DNA polymerase-PCNA interaction are important issues for elucidating the overall mechanisms of replication fork progression. Extensive studies of the PCNA-interacting proteins revealed that the proteins contain a small conserved sequence motif, called the PCNA-interacting protein box (PIP box), which binds to a common site on PCNA (Warbrick 2000). The PIP box consists of the sequence "Qxxhxxaa," where "x" represents any amino acid, "h" represents a hydrophobic residue (e.g., L, I, or M), and "a" represents an aromatic residue (e.g., F, Y, or W). Archaeal DNA polymerases have PIP box-like motifs in their sequences (Vivona and Kelman 2003), although few studies have experimentally investigated the function of the motifs. The C-terminal 50 amino acid fragments of the family B DNA polymerases from *Archaeoglobus fulgidus* and *Pyrococcus horikoshii*, including the putative PIP box-like sequence, generated positive signals in two-hybrid analyses, thus revealing that they interacted with PCNA (Motz et al. 2002). In the case of *Sulfolobus solfataricus*, a family B DNA polymerase (Pol B1) was unable to interact with PCNA after the deletion of the PIP box-like motif located at the N-terminus (Dionne et al. 2003). We demonstrated that both Pol BI and Pol D functionally interact with PfuPCNA via their C-terminal PIP boxes. The mutant Pol BI and Pol D enzymes lacking the PIP box sequence did not respond to the PfuPCNA at all in an in vitro primer extension reaction (Tori et al. 2007).

We then determined the crystal structure of *P. furiosus* Pol BI complexed with a Pfu monomeric PCNA mutant, which allowed us to construct a convincing model of the polymerase-PCNA ring interaction. Our structural study revealed

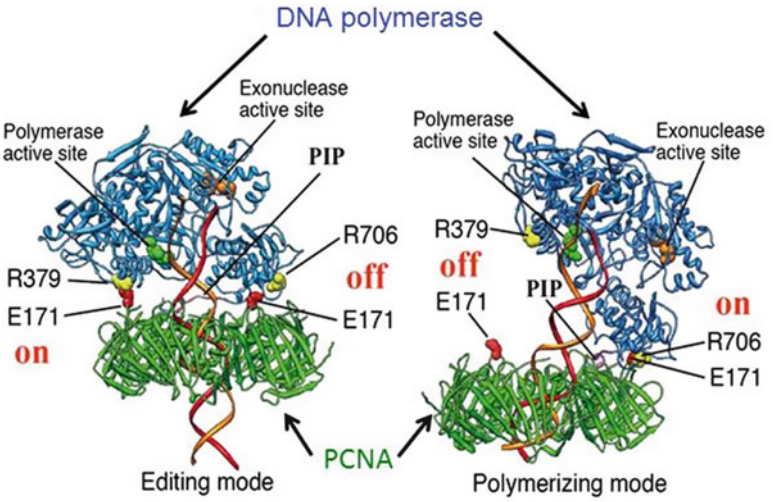

Fig. 17.6 Switching mechanism of DNA polymerase between the polymerase and exonuclease modes. Pol B and PCNA are colored in *blue* and *green*, respectively. The template and primer strands are shown in *orange* and *red ribbons*, respectively. The two E171 residues in the PCNA "switching hook" are shown in *red sphere*. R706 in the thumb domain and R379 in the palm domain of Pol B are represented in *yellow spheres*. The DIE motif in exonuclease active site and the DTDG motif in polymerase active site are shown in *orange* and *green sphere* representation. The interactions between the PCNA hooks and the Pol B counterparts are just in the inverse relation between the polymerizing mode and the editing mode

that a novel interaction is formed between a stretched loop of PCNA and the thumb domain of Pol B, in addition to the authentic PCNA-polymerase recognition site (PIP box). A comparison of the model structure with the previously reported structures of a family B DNA polymerase from RB69 phage, complexed with DNA (Shamoo and Steitz 1999; Franklin et al. 2001), suggested that the second interaction site plays a crucial role in switching between the polymerase and exonuclease modes, by inducing a favorable PCNA-polymerase complex configuration for synthesis over editing. This putative mechanism for fidelity control of replicative DNA polymerases is supported by experiments, in which mutations at the second interaction site enhanced the exonuclease activity in the presence of PCNA (Nishida et al. 2009). Furthermore, we presented the three-dimensional structure of the DNA polymerase-PCNA-DNA ternary complex, solved by electron microscopic (EM) single-particle analysis. This structural view revealed the entire domain configuration of the trimeric ring of PCNA and DNA polymerase, including protein-protein or protein-DNA contacts. Together with the finding of an unexpected contact between DNA polymerase and PCNA, this architecture provides clearer insights into the switching mechanism between the two distinct modes (editing and synthesis, respectively), as shown in Fig. 17.6 (Mayanagi et al. 2011).

17.3 Thermostable DNA Ligases

17.3.1 Applications to Technology

Due to their physiological importance and biochemical potential, DNA ligases from numerous living organisms have been studied since the enzyme was discovered in 1968. The identification of DNA ligases from thermophiles is also a fundamentally interesting research subject, and such thermostable DNA ligases, which maintain the catalytic activity for nick-joining reaction during temperature cycling, including the heat-denaturing step of duplex DNAs (90–100°C), are valuable as LDR/LCR enzymes. LDR/LCR is a technique to detect a single-base mutation in the DNA strand and is useful for the diagnosis of genetic diseases (Barany 1991b; Zirvi et al. 1999). Recently, this technique has also been applied for the detection of microRNAs (miRNAs), which play important regulatory roles in fundamental cellular processes through the formation of the RNA-induced silencing complex (RISC) with target mRNAs (Yang et al. 2010). LCR using thermostable DNA ligases also facilitated the elimination of the background caused by target-independent ligation. DNA modifications of LCR have been performed to improve the sensitivity and utility, including Gap-PCR (Abravaya et al. 1995), and, more recently, quantitative real-time LCR (Psifidi et al. 2011). DNA ligases from hyperthermophiles are especially applicable to LCR. DNA ligases from *T. aquaticus, T. thermophilus, T. filiformis, and T. scotoductus* (eubacteria) and *P. furiosus* and *Thermococcus* 9°N (archaea) are now commercially available.

17.3.2 DNA Ligases from Thermophilic Bacteria

A common feature of the main replicative DNA ligases in eubacteria is that their ligation activity is NAD^+ dependent (Table 17.3). The DNA ligase from *T. thermophilus* (Lauer et al. 1991) was originally used for the detection of a point mutation in the β-globin gene by LCR (Barany 1991a). DNA ligases from other *Thermus* species, *T. scotoductus* and *Rhodothermus marinus*, were also utilized for LCR (Housby and Southern 2002). Quantitative studies of most mismatch ligations on the 3′-side of the nick revealed that Tth DNA ligase is much more accurate than T4 phage DNA ligase, which is most commonly used for in vitro DNA manipulations. The fidelity of Tth DNA ligase was improved by site-directed mutagenesis at K294R and K294P (Luo et al. 1996). The amino acid sequences of the *Thermus* DNA ligases are highly homologous, ranging from 85 to 98% identities, and therefore, seven *Thermus* species were compared, and a superior enzyme for LCR was found from the *Thermus* species AK16D (Tong et al. 1999).

Table 17.3 Thermostable DNA ligases for archaea and bacteria characterized to date and their cofactor utilities

Species	Cofactor		References
Archaea			
Acidithiobacillus ferrooxidans	ATP		Ferrer et al. (2008)
Aeropyrum pernix	ATP	ADP	Jeon and Ishikawa (2003)
Ferroplasma acidarmanus	ATP		Jackson et al. (2007)
Ferroplasma acidophilum	ATP	NAD^+	Ferrer et al. (2008)
Methanothermobacterium thermoautrophicum	ATP		Sriskanda et al. (2000)
Picrophilus torridus	ATP	NAD^+	Ferrer et al. (2008)
Pyrococcus horikoshii	ATP		Keppetipola and Shuman (2005)
Pyrococcus furiosus	ATP		Kiyonari et al. (2006)
Staphylothermus marinus	ATP	ADP GTP	Seo et al. (2007)
Sulfophobococcus zilligii	ATP	ADP	Sun et al. (2008)
Sulfolobus acidocaldarius	ATP		Ferrer et al. (2008)
Sulfolobus shibatae	ATP		Lai et al. (2002)
Thermococcus fumicolans	ATP	NAD^+	Rolland et al. (2004)
Thermococcus kodakarensis	ATP	NAD^+	Nakatani et al. (2000)
Thermococcus sp.	ATP	NAD^+	Kim et al. (2006)
Thermococcus sp. 1519	ATP		Bezsudnova et al. (2009)
Thermoplasma acidophilum	ATP	NAD^+	Ferrer et al. (2008)
Bacteria			
Aquifex aeolicus VF5		NAD^+	Tong et al. (2000)
Aquifex pyrophilus		NAD^+	Lim et al. (2001)
Bacillus stearothermophilus		NAD^+	Singleton et al. (1999) and Timson and Wigley (1999)
Rhodothermus marinus		NAD^+	Thorbjarnardóttir et al. (1995)
Thermus filiformis		NAD^+	Lee et al. (2000)
Thermus scotoductus (Ts)		NAD^+	Thorbjarnardóttir et al. (1995)
Thermus species AK16D		NAD^+	Tong et al. (1999)
Thermus thermophilus HB8		NAD^+	Takahashi et al. (1984)
Zymomonas mobilis		NAD^+	Shark and Conway (1992)

17.3.3 DNA Ligases from Thermophilic Archaea

It was interesting that a gene encoding a eukaryotic ATP-dependent DNA ligase was found in the thermophilic archaeon, *Desulfolobus ambivalens* (Kletzin 1992). This fact supported the proposal that the genetic information system in Archaea is similar to that of Eukaryota but differs from the counterparts in Bacteria. Subsequent identifications of the DNA ligases from archaeal organisms revealed that these enzymes use ATP mainly as a cofactor. However, this classification may not be so strict now (Table 17.3). The utilization of NAD^+, as well as ATP, as the cofactor has been observed in several DNA ligases, including *T. kodakarensis* (Nakatani et al. 2000), *T. fumicolans* (Jeon et al. 2004) and *P. abyssi* (Rolland et al. 2004), *Thermococcus* sp.

NA1 (Kim et al. 2006), *Thermoplasma acidophilum*, *Picrophilus torridus*, and *Ferroplasma acidophilum* (Ferrer et al. 2008). The dual cofactor specificity (ATP/NAD^+) is interesting in terms of understanding the structures and functions of these DNA ligases, although ATP is evidently preferable in all of the cases. Another dual cofactor specificity exists in the archaeal DNA ligases, which use ADP as well as ATP. These enzymes are from *Aeropyrum pernix* (Jeon and Ishikawa 2003) and *Staphylothermus marinus* (Seo et al. 2007), and in the case of *Sulfobococcus zilligii,* GTP is also the functional cofactor (Sun et al. 2008). In terms of the cofactor specificity, sufficient biochemical data have not been obtained. It was pointed out that a thermostable *E. coli* adenylate kinase, which was present as a contaminant in the purified DNA ligases from the recombinant *E. coli* cell lysate, may convert ADP into ATP and AMP (Chen et al. 2009). The DNA ligases from *P. horikoshii* (Keppetipola and Shuman 2005) and *P. furiosus* (Kiyonari et al. 2006) have a strict ATP preference. Further biochemical and structural analyses are required to resolve the issue of dual cofactor specificity.

17.3.4 Interactions with PCNA

We have shown the physical and functional interactions between DNA ligase and PCNA from *P. furiosus,* although the detailed interaction mode between human Lig I and PCNA is somewhat unclear, as there are several controversial reports. The stimulatory effect of PfuPCNA on the enzyme activity of PfuLig was observed at a high salt concentration, at which a DNA ligase alone cannot bind to a nicked DNA substrate. Furthermore, we identified the amino acid residues that are critical for the PCNA binding, based on mutational analyses. Interestingly, the binding site is located in the middle of the N-terminal DNA-binding domain (DBD), and the binding motif, QKSFF, which we proposed as a shorter version of PIP box, is actually looped out from the protein surface (Kiyonari et al. 2006). We confirmed that Gln and the first Phe in the QKSFF motif are especially important for the stable binding with PCNA. It is also interesting that this motif is located in the middle of the protein chain, rather than the N- or C-terminal region, where the PIP boxes are usually located. To confirm that this motif is conserved in the archaeal/eukaryotic DNA ligases, we showed that the physical and functional interactions between *A. pernix* DNA ligase and PCNA depend mainly on the phenylalanine residue, which is located in the region predicted by the multiple sequence alignment of the ATP-dependent DNA ligases (Kiyonari et al. 2007).

17.3.5 Structural Dynamics of DNA Ligase for the Ligation Reaction

The human Lig I, complexed with DNA, was the first crystal structure of an ATP-dependent cellular DNA ligase (Pascal et al. 2004). The enzyme comprises the

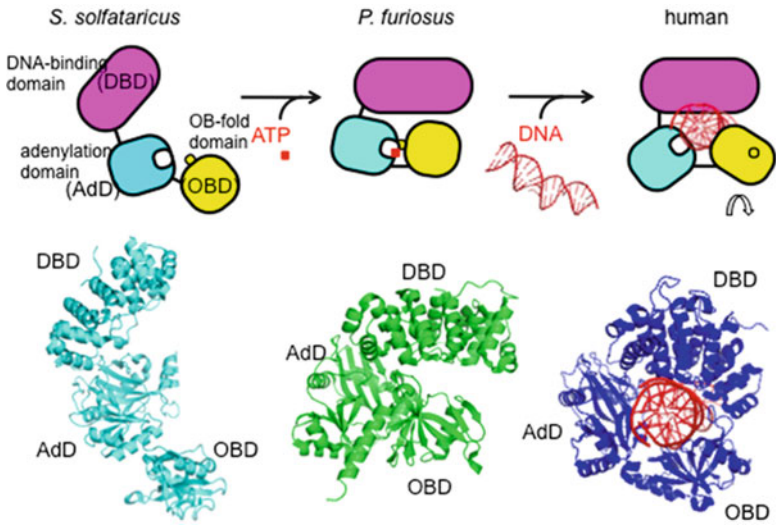

Fig. 17.7 Conformational change of the DNA ligase according to the process of ligation. The ATP-dependent DNA ligases consisting of three distinct domains change their conformation flexibly to capture the DNA strand and process ligation. Three crystal structures supporting these conformational changes are shown on the *bottom*. SsoLig (*cyan*, PDB code 2HIV), PfuLig (*green*, PDB code 2CFM), and human Lig I with DNA (*dark blue* and *red*, PDB code 1X9N)

N-terminal DNA-binding domain, the middle adenylation domain, and the C-terminal OB-fold domain. The crystal structure of Lig I (residues 233–919) in complex with a nicked, 5′-adenylated DNA intermediate revealed that the enzyme redirects the path of the double-stranded DNA to expose the nick termini for the strand-joining reaction (Fig. 17.7). Like PCNA, the N-terminal DNA-binding domain works to encircle the DNA substrate and stabilizes it in a distorted structure, positioning the catalytic core on the nick. We solved the crystal structure of the full-length DNA ligase from *P. furiosus* (PfuLig) (Nishida et al. 2006). The architecture of each domain resembles those of Lig I, but the domain arrangements strikingly differ between the two enzymes (Fig. 17.7). This domain rearrangement is probably derived from the "domain-connecting" role of the helical extension, conserved at the C-termini in the archaeal and eukaryotic DNA ligases. The DNA substrate in the open form of Lig I is replaced by motif VI at the C-terminus in the closed form of PfuLig. Both the shapes and electrostatic distributions are similar between motif VI and the DNA substrate, suggesting that motif VI in the closed state mimics the incoming substrate DNA. Subsequently, another crystal structure of the ATP-dependent DNA ligase was published (Pascal et al. 2006). The crystal structure of SsoLig from *Sulfolobus solfataricus* is the fully open structure, in which the three domains are high extended (Fig. 17.7). In this work, the SsoLig-PCNA complex was also analyzed by small-angle X-ray scattering (SAXS). SsoLig bound to the PCNA ring still retains an open, extended conformation. The closed, ring-shaped conformation observed in the Lig I struc-

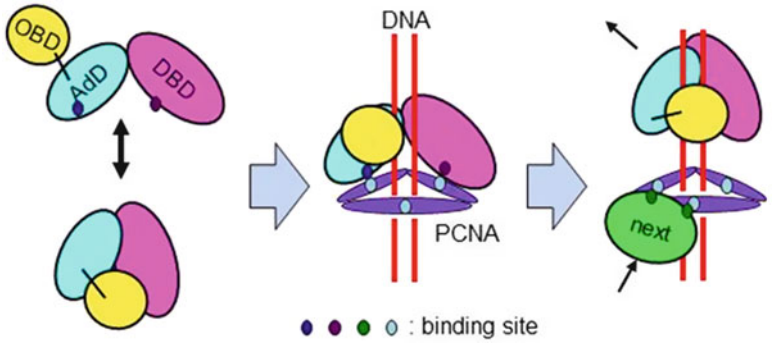

Fig. 17.8 Switching mechanism of the binding factors on PCNA. The DNA ligase adopts various conformations, such as the closed and extended configurations as observed in the PfuLig and SsoLig crystal structures, respectively. Upon binding to PCNA and DNA, the DNA ligase forms the crescent configuration, which embraces the DNA as observed from the EM single-particle analysis. Then, the DNA ligase grips the DNA to accomplish the ligation reaction, as observed in the crystal structure of human Lig I-DNA. Accordingly, the binding sites will be released, thereby enabling the PCNA ring to interact with the PIP of the next enzyme

ture is probably the active form to catalyze a DNA end-joining reaction, and therefore, it is proposed that the open-to-closed conformational change occurs for ligation, and the switch in the conformational change is accommodated by a malleable interface with PCNA, which serves as an efficient platform for DNA ligation, as illustrated in Fig. 17.8 (Pascal et al. 2006).

After the publication of these crystal structures, we presented the three-dimensional structure of the ternary complex, consisting of DNA ligase-PCNA-DNA, obtained by EM single-particle analysis (Mayanagi et al. 2009). In the complex structure, the three domains of the crescent-shaped PfuLig surround the central DNA duplex, encircled by the closed PCNA ring. The relative orientations of the PfuLig domains, which remarkably differ from those of the previous crystal structures, suggest that a large domain rearrangement occurs upon ternary complex formation. PfuLig contacted PCNA at two sites, the conventional PIP box and a novel second contact at the middle adenylation domain. It is also interesting that a substantial DNA tilt from the PCNA ring axis was observed. Based on the structural model, we considered a mechanism in which PCNA-binding proteins are bound and released sequentially. In fact, most of the PCNA-binding proteins share the same binding sites in the interdomain connecting loop (IDCL) and the C-terminal tail of the PCNA. The structural features exclude the possibility that the three proteins contact the single PCNA ring simultaneously, because DNA ligase occupies two of the three subunits of the PCNA trimer. In the case of our previous analysis of the RFC-PCNA-DNA complex, RFC entirely covers the PCNA ring, thus blocking the access of other proteins (Miyata et al. 2005). Our ternary complex appears to favor a mechanism involving the sequential binding and release of replication factors, as schematically represented in Fig. 17.8. In solution, a DNA ligase can adopt various conformations between the extended and closed forms, as described above. Our ternary structure could be regarded as an intermediate state, just before DNA ligation.

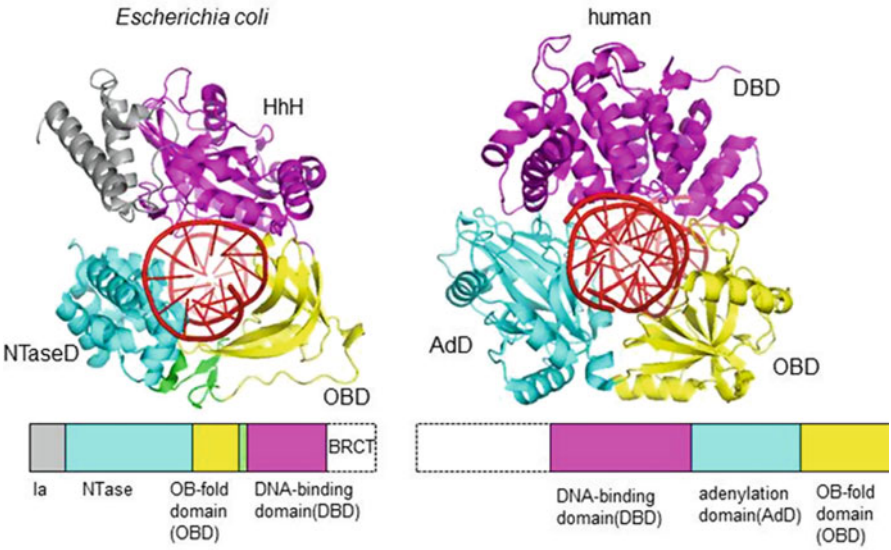

Fig. 17.9 Structural comparison of ATP- and NAD^+-dependent DNA ligases. Ribbon diagrams of DNA ligases from human (PDB code 1X9N) and *E. coli* (PDB code 2OWO), both complexed with DNA, are shown with schematic representation of the domain organizations. Each distinct domain is shown in *different color*

17.3.6 Drug Targets

Due to the differences in the cofactors between the major DNA ligases from Bacteria and Archaea/Eukaryota, as well as the progress in the structural analyses of many DNA ligases as described above, the enzyme is a good candidate as a drug target. The NAD^+-dependent DNA ligases are essential for the viability of many eubacteria, but no NAD^+-dependent DNA ligase has been identified in eukaryotic organisms to date. In addition, the bacterial enzyme has a distinctive domain structure as compared to the ATP-dependent enzymes, as shown in Fig. 17.9 (Pascal 2008), and therefore, inhibitors for the bacterial enzymes may be developed as new antibacterial drugs. Several reports describing the inhibitors have already been published (Brötz-Oesterhelt et al. 2003; Miesel et al. 2007; Meier et al. 2008; Srivastava et al. 2005, 2007).

17.4 Conclusions

DNA polymerases and DNA ligases from thermophiles have been contributing a great deal to our lives as useful reagents for genetic diagnosis, forensic DNA typing, and detection of bacterial and virus infections. They are also important for basic

molecular biology research using in vitro genetic manipulations. Numerous enzymes have been developed as commercial products from various companies around the world, and they are utilized daily in various processes. Furthermore, the recent progress in DNA sequencing technology is quite conspicuous. Newer methods, referred to as "next-generation sequencing," based on several technologies using different chemistries of DNA, have been developed and are now available as systems, including machines, reagents, and analysis software (Shendure and Ji 2008; Ansorge 2009). All of these technologies include DNA polymerase or DNA ligase from various sources, indicating that these DNA-related enzymes are extremely valuable for the development of life science technology. Furthermore, single-molecule detection, using dye-labeled modified nucleotides and longer read lengths, is now known as "third-generation DNA sequencing" (Metzker 2010). This technology requires DNA polymerases with the ability to incorporate various modified nucleotides. For instance, an engineered Pfu DNA polymerase with improved activity to incorporate bulky γ-phosphate-O-linker-dabcyl nucleotides has recently been reported (Hansen et al. 2011). Further efforts will provide valuable DNA polymerase and DNA ligase enzymes with specialized activities, to promote genetic engineering technologies, and thermostable enzymes with greater potential.

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Chapter 18
Molecular Diversity and Biotechnological Relevance of Thermophilic Actinobacteria

Satya P. Singh, Rushit J. Shukla, and Bhavtosh A. Kikani

Abstract Extremophilic organisms have attracted significant attention of the research community during the recent past, not only due to their survival and growth at extreme conditions but also due to their huge potential in various fields of biotechnology. Among the various groups of extremophiles, thermophilic actinomycetes have been less explored due to the difficulties in their isolation and maintenance in pure culture. Therefore, it largely remains to explore their diversity, molecular phylogeny, adaptive features, and biocatalytic and other biotechnological potentials. In order to study actinomycetes, morphological features and morphogenesis, antibiotic sensitivity and resistance, biochemical characteristics, and certain key molecular features have been taken into account to get insight into the actinomycetes, in general. The molecular approaches include sequence homology of 16S rRNA genes, nucleic acid hybridization, G+C% ratio, protein profiling, RFLP, DGGE, TGGE and ARDRA for the assessment of diversity, taxonomic status, and molecular phylogeny. The thermophilic actinomycetes reflect quite appealing and unique applications in various fields of biotechnology, viz., production of thermostable enzymes, antibiotics, and hormones and their role in bioremediation processes of recalcitrant compounds. Further studies on the diversity and phylogeny would enhance understanding of the unexplored thermophilic actinobacteria that will promote their applications.

Keywords Thermophilic actinomycetes • Diversity • Molecular approaches • Biotechnological potentials

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18.1 Introduction

Extremophiles such as thermophiles, halophiles, barophiles, psychrophiles, and acidophiles have provided a fascinating platform for researchers since the time of their discovery. Besides growth under the extreme conditions, production of industrially valuable compounds has focused attention during the recent years (Singh 2006; Austain 1988). Thermophilic bacteria and actinobacteria can optimally grow and produce economically beneficial compounds at high temperatures (Brock 1986). Thermophiles are further categorized on the basis of their temperature tolerance: for instance, facultative thermophiles can grow at temperatures between 50 and 65°C, but also grow at 37°C; obligate thermophiles have maximum growth temperatures of 65–70°C and will not grow below 40°C; extreme thermophiles can grow between 40 and 70°C with an optimal growth temperatures of about 65°C, while hyperthermophiles, mainly comprising of archaea, can grow over 90°C with optimal temperatures between 80 and 115°C.

Actinobacteria are a large and diverse group of Gram-positive bacteria that have morphological features similar to fungi, as they produce branching networks of filaments. Thermophilic actinobacteria occur in a wide range of habitats including soils, self-heating plant residues, manure, and composts. They also exist in highly specialized habitats like birds' nests, volcanic vents, and hot springs. Other groups of actinobacteria are known to occur at moderate or extreme cold temperatures.

Actinobacteria are traditionally known to produce a range of secondary metabolites, including some volatile organic compounds (VOCs). In this regard, they are more active than most fungi. Some of the VOCs commonly produced by actinobacteria are responsible largely for the moldy or musty odors associated with soils as well as damp basements. Actinobacteria are well-known for their ability to produce antimicrobial substances, and, in fact, many of the naturally produced antimicrobial drugs of common use today are derived from actinobacteria (e.g., streptomycin, nystatin, and tetracycline). However, exposure to cells and other materials colonized by actinobacteria has been associated with respiratory diseases such as hypersensitivity pneumonitis. This is particularly evident in agriculture where actinobacterial exposures are known to cause a disease known as farmer's lung.

Thermophilic actinobacteria are relatively less explored due to the difficulties associated with their isolation and maintenance in pure culture. Besides, their survival and production of stable, active enzymes and other bioactive molecules at high temperatures should be addressed. It largely remains to explore their diversity, molecular phylogeny, production of biotechnologically useful enzymes, and other compounds. Aspects relating to specific adaptive features are also interesting features to investigate. It appears that thermophilic organisms could provide answer to many basic questions relating to the evolution and stability of macromolecules, besides being a source of biotechnologically novel compounds. In this chapter, we have dealt with the diversity and biotechnological potentials of thermophilic actinobacteria.

18.2 Critical Review

18.2.1 Diversity of Thermophilic Actinobacteria

Actinobacteria are the group of organisms which have not been explored in great detail. The unexplored diversity of the thermophilic actinobacteria can be explored through conventional microbiological techniques along with various molecular tools. The assessment of the community diversity with nucleic acid-based methods is more appropriate than those based on cultured organisms (Ovreås and Torsvik 1998). It has been shown that culture-independent methods project greater complexity than realized by traditional approaches (Yang et al. 2001; McCaig et al. 2001). Obviously, the use of culture-independent approaches will remove the bias imposed by isolation in the laboratory. However, it does not take into account the differences in cellular activity (LeBaron et al. 2001). As many microorganisms can exist in dormant forms (Kell et al. 1998), it is important to measure activity so that ecologically relevant bacteria are assessed and not inactive cells that do not functionally contribute to the ecosystem.

Culture-independent methods have received particular attention because it is commonly held that only a small proportion of microbes in any environment will form colonies on laboratory media. Although methods and media improving the proportion of cultivable microorganisms from environmental samples have come to light (Janseen et al. 2002; Sait et al. 2002; Zengler et al. 2002), several investigations have suggested that the readily cultivable component of soil microbial communities may be important for both biomass and activity.

It has been shown that there are positive correlations between activity and cell size (Bernard et al. 2000), cell size and culturability (Bakken and Olsen 1987), and activity and culturability (Soderberg and Baath 1998). While they may carry a large proportion of the genetic material, the numerically dominant component of soil bacterial communities consisting of very small cells does not contribute greatly to biomass or metabolic activity (Bakken 1997; Janseen et al. 2002). In addition, differences in the proportion of microorganisms that are isolated from soil samples from increasing depths have been attributed to differences in bacterial activity (Sait et al. 2002). Therefore, it could be hypothesized that the readily culturable component of soil bacteria may have greater contribution to their ecosystem.

The diversity of the cultured and uncultured in the context of total communities has not been addressed in a focused manner until the last few years (Ellis et al. 2002; Purohit and Singh 2009; Siddhpura et al. 2010). Therefore, one of the objectives of this chapter is to address the correlation between the diversity of culturable and nonculturable communities.

18.2.1.1 Culture-Dependent Diversity

The culture-dependent approaches include the conventional microbiological methods to molecular techniques. The conventional approaches include enrichment and

isolation on various media, followed by their morphological analysis. This is further supplemented with biochemical characteristics and antibiotic sensitivity of the organisms. The morphological features of the organisms provide certain clues in identification and assessment of the diversity. The mycelial arrangements, sporangial structure, patterns, and characteristics of spores have been studied to a great extent.

The effect of temperature on the germination of spores of thermophilic actinobacteria was examined. At temperatures above and below the growth temperature of 55°C, marked changes in the germination patterns of spores were evident. High temperatures caused reduction in the germination of spores. However, spores regained original germinative activities when they were brought to 25°C. Recovery from the effect of heat on spore germination was also observed at 4°C, although at a much slower rate than at 25°C. Spores of few strains of thermophilic actinobacteria, grown and prepared at 55°C, failed to germinate. Storage of dormant (nonactivated) spore populations at different temperatures demonstrated a low temperature requirement for the activation of these spores. While only limited or no activation was evident at 55°C, rapid activation resulted at 25°C. Heating the spores at 80°C for 30 min slightly delayed the activation of spores at 25°C. However, the requirement of low temperature for spore activation was strain specific and influenced by the germination medium (Foerster 1978).

In view of the structural and physiological similarities among different bacterial spores, it is interesting to find temperature-dependent differences on their germination profiles. Heating activated spore suspensions of thermophilic actinobacteria at 15°C above the temperature of spores' production resulted in complete loss of germinative activity when tested immediately after heating. On the other hand, shifting nonactivated (dormant) spore suspensions to 30°C, below their sporulation temperature, led to significant increase in the germination of spore populations of thermophilic actinobacteria (Foerster 1978). Some studies on the spores of thermophilic actinobacteria have demonstrated many similarities between these spores and the endospores of *Bacillus* and *Clostridium* (Foerster 1978).

Thus, from the above literature, it is evident that thermal loss of germinative activity and low temperature activation are inducible characteristics of the spores of actinobacteria (Foerster 1978).

Identification is also based on some other parameters, such as biochemical characteristics and ability of the organisms to utilize different substrates. When *Thermoactinomyces candidus* was isolated from home environments and other sources, they were identified and compared using some of these parameters. *T. candidus* differs from *T. vulgaris* in that the former species hydrolyzes esculin and also splits arbutin but does not attack tyrosine, hypoxanthine, or starch, whereas the latter does. *T. candidus* differs from *T. sacchari* by producing fast-growing colonies, abundant aerial mycelia, and hemolysis in blood agar, by decomposing esculin and arbutin, and by failing to hydrolyze starch. The type strain identified was *T. candidus* T-106 (ATCC 27868) (Kurup et al. 1975). Thus, a scheme was developed for the identification of thermophilic actinobacteria associated with hypersensitivity pneumonitis based on the biochemical tests (Kurup et al. 1975). By employing this

approach, the strains of thermophilic actinobacteria, such as *Micropolyspora faeni*, *Saccharomonospora viridis*, *Thermoactinomyces candidus*, *T. vulgaris*, *T. sacchari*, and *T. dichotomica*, can be identified easily.

In addition to the cultural and microscopic examinations on various media, the biochemical tests are also important in identification. These tests include decomposition of tyrosine, xanthine, hypoxanthine, gelatin, casein, esculin, and arbutin. Using a rapid thin-layer chromatographic method, the isomer of diaminopimelic acid and sugar in the whole-cell hydrolysates were studied. The thermophilic actinobacteria can be identified in a reasonable period of time using a combination of these tests (Kurup and Fink 1975). Recently, the effect of bioaerosol on human health was carried out, where it was found that by burning poultry plant litter, the concentration of airborne thermophilic actinobacteria was raised up to 8.7×10^4 CFU/m^3 and this led to the increased number of pathogens causing extrinsic allergic alveolitis (Wultsch et al. 2009). Attempts are also being made to construct the 16S rDNA and 18S rDNA libraries using bioaerosol. In this context, 800 sequences of microorganisms including bacteria, fungi, and actinobacteria were analyzed, and microbial signature of aerosol was generated (Goff et al. 2010).

18.2.1.2 Culture-Independent Diversity

More than 99% of prokaryotes in the environment cannot be cultured in the laboratory, a phenomenon that limits our understanding of microbial physiology, genetics, and community ecology. One of the approaches to address this anomaly is metagenomics, the culture-independent cloning and analysis of microbial DNA extracted directly from an environmental sample. Recent advances in shotgun sequencing and computational methods for genome assembly have advanced the field of metagenomics to provide insight into the life of uncultured microorganisms (Purohit and Singh 2009; Siddhpura et al. 2010). Similar culture-independent approach was employed to study the microbial diversity from the industrial open sites (Goff et al. 2010).

18.2.1.3 Molecular Approaches to Explore Diversity

16S rRNA Sequencing

Carl Woese proposed the three-domain system of classification – Archaea, Bacteria, and Eucarya – based on rRNA gene sequencing. The rRNA is the most conserved (least variable) gene, and portions of the rDNA sequence from distantly related organisms are remarkably similar. This means that sequences from distantly related organisms can be precisely aligned, making the true differences easy to measure. For this reason, genes that encode the rRNA (rDNA) have been used extensively to determine taxonomy and phylogeny (evolutionary relationships) and to estimate rates of species divergence among various microorganisms. Thus, the comparison of 16S rDNA sequence can show evolutionary relatedness among microorganisms,

and one can diversify the organisms accurately at species level. For instance, mushroom worker's lung (MWL) is a hypersensitivity pneumonitis or allergic alveolitis caused by a type III IgG-mediated immunopathogenic inflammatory reaction in the host due to the inhalation of several thermophilic organisms, including *Thermoactinomyces* spp. It is difficult to distinguish phenotypically the eight species of this genus. Therefore, an improved molecular means of identifying *Thermoactinomyces* spp. associated with MWL by partial 16S rDNA PCR amplification and direct sequencing was employed. Hypervariable regions within the 16S rRNA gene, which could be employed as signature sequences of the eight individual species, were identified and employed with highly conserved flanking primers to allow initial PCR amplification. A novel 24-mer 16S rDNA oligonucleotide upstream primer was designed from *in silico* alignments of all *Thermoactinomyces* spp. and was employed in combination with downstream (reverse) 16S rDNA primers. This permitted the successful identification of all four isolates associated with mushroom worker's lung. The method may be useful in the identification of *Thermoactinomyces* spp. associated with allergic alveolitis or pneumonitis associated with occupational exposure in agricultural and horticultural environments (Xu et al. 2002).

In another study, the dominant filamentous actinobacteria occurring in water-damaged building materials were detected by observing the cultures and characterized by automated ribotyping and 16S rRNA gene sequencing. Few samples were taken from some water-damaged houses in four different countries. A total of 122 bacterial isolates were analyzed that indicated the presence of actinobacteria or thermoactinobacteria in 48% of the samples. The dominant genus was *Streptomyces* (58% of isolates), followed by *Thermoactinomyces* (23%), *Laceyella* (14%), *Nocardiopsis* (3%), *Pseudonocardia* (1%), and *Saccharomonospora* (1 The most frequently detected species was the thermophilic *Thermoactinomyces vulgaris* (14 samples/4 countries). The most common *Streptomycetes* were closely related to the heterogeneous species *Streptomyces microflavus* (7/2) or *Streptomyces griseus* (6/2). Automated ribotyping is a rapid tool for reliable characterization of these isolates. The spores of thermoactinobacteria and toxic substances of *Nocardiopsis* spp. and *S. griseus* may cause a risk for human health (Suihko et al. 2009).

A novel actinobacterium IMMIB L-1269 that originated from sputum was characterized using phenotypic and molecular methods. It showed cell wall chemotype III and phospholipid type PII (with phosphatidylethanolamine as the diagnostic phospholipid) and contained an unsaturated menaquinone with seven isoprene units (MK-7) as the predominant isoprenoid quinone. It has synthesized long-chain cellular fatty acids of the straight-chain saturated, monounsaturated, and iso- and anteiso-branched types (with iso-$C_{15:0}$, iso-$C_{16:0}$, and iso-$C_{17:0}$ predominating) and possessed a DNA G+C content of 49.3 mol%. On the basis of its morphological, biochemical, and chemical characteristics, strain IMMIB L-1269^T did not relate to any presently recognized taxon. Comparative analyses based on 16S rRNA gene sequences confirmed the distinctiveness of the isolate, as it displayed sequence-divergence values greater than 7.7% with respect to recognized Gram-positive taxa. Phylogenetic analysis highlighted that strain IMMIB L-1269^T was distinct from the recognized taxa, as it formed a relatively long subline branching within a 16S rRNA

gene sequence cluster that encompassed the genera *Thermoactinomyces*, *Laceyella*, *Mechercharimyces*, *Thermoflavimicrobium*, *Planifilum*, *Seinonella*, and *Shimazuella* of the family *Thermoactinomycetaceae*. On the basis of phenotypic and molecular phylogenetic evidence, strain IMMIB L-1269^T represents a novel genus and species, for which the name *Desmospora activa* gen. nov., sp. nov. was proposed. The type strain of *Desmospora activa* is strain IMMIB L-1269^T (=DSM 45169^T=CCUG 55916^T), belonging to the family Thermoactinomycetaceae (Yassin et al. 2006).

G+C (%) Ratio

GC content is variable with different organisms, which may be contributed to variation in selection, mutational bias, and biased recombination-associated DNA repair. The species problem in prokaryotic taxonomy has led to various suggestions in classifying bacteria, and the ad hoc committee on reconciliation of approaches to bacterial systematics has recommended the use of GC ratios in higher level hierarchical classification. For example, the actinobacteria are characterized as "high GC-content bacteria." In *Streptomyces coelicolor* A3, GC content is 72%, which in *Saccharomyces cerevisiae* is 38% and in *Arabidopsis thaliana* 36%. Because of the nature of the genetic code, it is virtually impossible for an organism to have a genome with a GC content approaching either 0 or 100%.

DNA-DNA Hybridization

DNA-DNA hybridization generally refers to a molecular biology technique that measures the degree of genetic similarity between pools of DNA sequences. It is often used to determine the genetic distance between two species. When several species are compared, the similarity values allow the species to be arranged in a phylogenetic tree.

DNA Microarray

It is a multiplex technology used in molecular biology. It consists of an arrayed series of thousands of microscopic spots of DNA oligonucleotides, called features, each containing picomoles (10–12 moles) of a specific DNA sequence, known as probes. This can be a short section of a gene or other DNA element that is used to hybridize a cDNA or cRNA sample (called target) under high-stringency conditions. Probe-target hybridization is usually detected and quantified by detection of fluorophore-, silver-, or chemiluminescence-labeled targets to determine relative abundance of nucleic acid sequences in the target. Since an array can contain tens of thousands of probes, a microarray experiment can accomplish many genetic tests in parallel. Therefore, arrays have dramatically accelerated many types of investigation.

DNA microarrays can be used to detect DNA (as in comparative genomic hybridization) or detect RNA (most commonly as cDNA after reverse transcription) that may or may not be translated into proteins. The process of measuring gene expression via cDNA is called expression analysis or expression profiling. Thus, DNA microarray can be used in gene expression profiling, single-nucleotide polymorphism (SNP) detection, and comparative genomic hybridization.

FAME Analysis

Since every microorganism has its specific fatty acid methyl ester (FAME) profile (microbial fingerprinting), it can be used as a tool for microbial source tracking (MST). The types and proportions of fatty acids present in cytoplasmic membrane and outer membrane (Gram-negative) lipids of cells are characteristics of the organism. Clinical analysis can determine the lengths, bonds, rings, and branches of the FAME. To perform this analysis, the fatty acids are extracted from a culture, which then are used to form methyl esters – the volatile derivatives, which are further analyzed by a gas chromatograph – and the patterns of the peaks help to identify the organism. This is widely used in characterizing new species and is useful for identifying various microbial strains.

The bacterial cell envelope has a structural role in maintaining cellular integrity and is also critical as the site of interaction and traffic between the cell and its environment. Thus, it is important that we understand fully the composition and organization of the cell envelope and how they differ across bacterial taxa.

In Gram-positive bacteria, the cell envelope consists of the plasma membrane, cell wall matrix (peptidoglycan and associated glycopolymers such as teichoic acids), and other components/layers (such as S-layers and capsules). The membrane-wall interface is of particular interest because this region may be considered directly analogous to the periplasm of Gram-negative bacteria and there is experimental evidence for the existence of a discrete periplasmic space. Thus, it can be envisaged that macromolecules on the outer leaflet of the plasma membrane will project into and occupy this space. Some components may also project further into the wall matrix, and thus, the membrane-wall interface in Gram-positive bacteria should be viewed in continuation. This Gram-positive periplasm likely includes the extra cytoplasmic domains of integral membrane proteins, bacterial lipoproteins, secreted proteins, and macroamphiphilic glycopolymers, i.e., structurally diverse polymers with covalently linked lipid anchor. The latter can typically be divided into two major classes, the lipoteichoic acids (LTA) and lipoglycans, although they can also be further categorized based on their electrostatic properties. Each of these classes can be further divided into various subtypes. LTA are defined as polymers with repeating units containing alditol phosphates, of which the polyglycerophosphate LTA (PGP-LTA) are the prototypical class. Lipoglycans are structurally more diverse, but several structural archetypes, such as lipoglucogalactans, lipomannans, and lipoarabinomannans, have been recognized. It has been suggested that LTA are representative of the Gram-positive bacterial class Firmicutes, whereas the cell

envelopes of members of the class Actinobacteria typically contain lipoglycans. However, it is now clear that there are significant exceptions to this pattern of distribution, with lipoglycans present in Mollicutes and LTA reported in representatives of two genera of actinobacteria. LTA has recently been shown to be essential for growth and cell division in *Staphylococcus aureus* (Grundling and Schneewind 2007).

The cell envelopes of thermophilic bacteria play an important role in adaptation at high temperatures toward the maintenance of membrane structure and function. While macroamphiphiles may represent as much as 10 mol% of the lipid in the outer plasma membrane in mesophilic bacteria, to our knowledge, there is no study on macroamphiphiles in the cell envelopes of thermophilic actinobacteria. *Thermobifida fusca* has considerable biotechnological significance as a source of novel heat-stable enzymes and has focused attention from a comparative-genomics perspective as a sporulating and filamentous actinomycete. *Rubrobacter xylanophilus* is a less intensively studied organism, although its genome sequence has recently been completed and is of interest with regard to the mechanisms of radiation resistance. Moreover, the phylogenetic position of the subclass Rubrobacteridae is of interest, as this lineage may represent one of the earliest branches of the class Actinobacteria. While *T. fusca* synthesizes a PGP-LTA, the cell envelope of *R. xylanophilus* does not have a macroamphiphile (Rahman et al. 2009).

RFLP

In RFLP analysis, the DNA is digested into pieces by restriction enzymes, and the resulting fragments are separated according to their lengths by gel electrophoresis. RFLP analysis was the first DNA profiling technique having widespread applications. In addition to genetic fingerprinting, RFLP was an important tool in genome mapping, localization of genes for genetic disorders, determination of risk for disease, and paternity testing.

ARDRA

Amplified rDNA restriction analysis (ARDRA) is the extension of the technique of RFLP to the gene encoding the small (16S) ribosomal subunit of bacteria. The technique involves an enzymatic amplification using primers directed at the conserved regions at the ends of the 16S gene, followed by digestion using tetracutter restriction enzymes. The pattern obtained is said to be representative of the species analyzed. Patterns from 3 or more restriction enzymes can be used to phylogenetically characterize isolates.

Hypersensitivity pneumonitis (HP) is a pulmonary disease characterized by inflammation caused by a subset of four thermophilic mycelial bacteria: *Saccharopolyspora rectivirgula*, *Saccharomonospora viridis*, *Thermoactinomyces sacchari*,

and *Thermoactinomyces vulgaris*. Air sampling analyses in highly contaminated environments are often performed to evaluate exposure to these species, which are difficult and fastidious to identify by conventional techniques. The study was conducted to use amplified ribosomal DNA restriction analysis (ARDRA) to develop a rapid and simple method of identification for those thermophilic organisms. Strains of four species *Saccharopolyspora rectivirgula*, *Saccharomonospora viridis*, *Thermoactinomyces sacchari*, and *Thermoactinomyces vulgaris* were obtained from the American Type Culture Collection (ATCC) and were characterized using biochemical tests and ARDRA patterns obtained on their partial-length amplified 16S rDNAs. To validate this approach, ARDRA with two restriction enzymes, *Taq* I and *Hha* I, was applied to 49 thermophilic actinomycetes. The results indicated that combining some cultural characteristics and biochemical tests, such as xanthine or hypoxanthine decomposition, growth in the presence of NaCl, lysozyme or novobiocin, and spore resistance over 100°C provided a rough identification and selection of the genera of interest. Consequently, target species could be confirmed by digestion of partial-length 16S rDNA with the use of *Taq* I and *Hha* I restriction enzymes leading to the specific restriction patterns. ARDRA analyses on the 49 environmental actinomycete-like organisms revealed the presence of 8 *Saccharopolyspora rectivirgula*, 2 *Saccharomonospora viridis*, and 15 *Thermoactinomyces vulgaris* strains; the other strains had restriction patterns different than those of the species of interest. These results would be applicable to other potential environments such as dairy barns, peat bogs, and compost plants (Harvey et al. 2001).

DGGE and TGGE

Temperature gradient gel electrophoresis (TGGE) and denaturing gradient gel electrophoresis (DGGE) are types of electrophoretic techniques using either a temperature or chemical gradient across a gel, typically made of acrylamide. TGGE and DGGE are useful techniques for studying nucleic acids, such as DNA and RNA, and sometimes for proteins.

The process of DGGE involves PCR amplification of the environmental DNA with PCR primers specific for 16S rRNA gene fragments of Bacteria and Archaea, and 18S rRNA gene fragments of Eukaryotes. Because these amplicons will have the same length, they cannot be separated from each other by normal agarose gel electrophoresis. However, sequence variations (i.e., differences in GC content and distribution) between different microbial rRNAs result in different denaturation properties of these DNA molecules. Sequence differences in otherwise identical fragments often cause them to partially melt at different positions in the gradient and therefore stop at different positions in the gel. By comparing the melting behavior of the polymorphic DNA fragments side by side on denaturing gradient gels, it is possible to detect fragments that have mutations in the first melting domain. Hence, DGGE and/ or TGGE band patterns can be used to visualize variations in of the richness and abundance of predominant microbial community members.

18.2.2 Biotechnological Potentials

18.2.2.1 Biocatalytic Potentials

Amylase

Starch is the most important renewable biological recourse, and like other polymers, it requires a combination of enzymes for its complete hydrolysis. The enzymes include α-amylases, glucoamylases or β-amylases, and isoamylases or pullulanases (Poonam and Dalel 1995). The enzymes are classified into endo-acting and exo-acting enzymes; α-amylase is an endo-acting enzyme and hydrolyzes linkages in a random fashion, leading to the formation of linear and branched oligosaccharides, while the rest are exo-acting enzymes and attack the substrate from the nonreducing end, producing oligo- and/or monosaccharides.

The starch hydrolytic enzymes comprise 30% of the world's enzyme consumption (Van der Maarel et al. 2002). The enzymatic conversion of starch includes gelatinization and saccharification, and thereby it is desirable that α-amylases should be active at the high temperatures of gelatinization (100–110°C) and liquefaction (80–90°C) to economize processes. Therefore, there has been a need for more thermophilic and thermostable α-amylases (Sidhu et al. 1997). The properties of α-amylases such as temperature profile, thermostability, pH profile, and pH stability must be compatible to application conditions.

On the other hand, one of the concerns of the starch industry is the calcium requirement of the α-amylases, which may lead to the formation of calcium oxalate, a substance that may block process pipes and heat exchangers. Therefore, the need for calcium-independent α-amylase is the demand of the day for starch-based industries.

The anticipated diversity of species of thermophiles within high-temperature environments (Huber and Stetter 1998) and the requirements of the new and improved enzymes with novel features (Emmanuel et al. 2000) are challenges to follow. Several thermophilic actinobacteria, for instance, *T. vulgaris*, have been found to produce amylase significantly (Tonozuka et al. 1993). *Streptomyces gulbargensis* produces extracellular alkali-thermotolerant amylase with optimal activity at pH 9.0 and 45°C (Dastager et al. 2007).

Protease

Proteases, generally classified into two categories (exopeptidases that cleave off amino acids from the ends of the protein chain and endopeptidases that cleave peptide bonds within the protein), have emerged as major industrial enzymes and constitute more than 65% of the world market (Rao et al. 1998).

Thermoactinomyces sp. HS682 secrets an alkaline protease stable at pH 11 and 70°C on a medium containing maltose, casamino acids, yeast extract, and sodium

carbonate at 15g/l (Tsuchiya et al. 1991). An alkalophilic *Streptomyces* had been isolated on soluble starch polypeptone yeast extract salts medium adjusted to pH 10.5. The enzyme was produced in a medium containing raw potato starch, casein, and 1% sodium carbonate. The protease was active at 60°C at pH 12 (Nakanishi et al. 1973).

Thermostable proteases from thermophiles have attracted considerable attention, and several proteolytic enzymes with high-temperature stability and resistance to autolysis have been identified. Cowan et al. (1985) reviewed proteases that are heat stable or produced by organisms growing at high temperatures, half-life being greater than one hour at 65°C or above 10 min at 75°C. Many of these enzymes are extensively used in food, pharmaceutical, leather, and textile industries (Cowan and Littlechild 1996; Srinivasan et al. 1999).

Proteolytic enzymes from microorganisms have been extensively investigated, and several comprehensive reviews have appeared (Kelly and Forgarty 1976; Morihara and Oda 1993). Increasing interest and attention has been focused on alkaline proteases as well as thermostable proteases due to their widespread potential applications in the industry.

Streptomyces fungicidicus MML1614, though being from a marine habitat, produced protease stable at pH 11 and temperature 60°C (Subramani 2009). However, *Streptomyces* sp. strain AB1 was studied for keratinase enzyme, using keratin azure as a substrate; the optimum pH and temperature values for keratinase activity were 11.5 and 75°C, respectively. This keratinase was stable between 30 and 60°C and pH 4–11 for 4 and 96 h, respectively. The thermostability was enhanced in the presence of 5 mM Mg^{2+} (Jaouadi et al. 2010).

Cellulase

Cellulose, the most abundant organic source of feed, fuel, and chemicals, consists of glucose units linked by β-1,4-glycosidic bonds in a linear mode. The difference in the type of bond and the highly ordered crystalline form of the compound between starch and cellulose make cellulose more resistant to hydrolyze. The enzymes required for the hydrolysis of cellulose include endoglucanases, exoglucanases, and β-glucosidases (Matsui et al. 2000). While cellulase is an endoglucanase that hydrolyzes cellulose randomly, producing oligosaccharides, cellobiose, and glucose, exoglucanases hydrolyze β-1,4-D-glucosidic linkages in cellulose, releasing cellobiose from the nonreducing end, while β-glycosidases of thermophilic origin, which have received renewed attention in the pharmaceutical industry, hydrolyze cellobiose to glucose.

Use of alkaline cellulose in detergents was pioneered in Japan with the introduction of a commercial product designated “Attack” which was produced by an alkalophilic *Bacillus* strain (Fukumori et al. 1985). The cellulases introduced in laundry detergents exhibit fabric softening and color brightening properties besides removing soil. The enzyme removes microfibrils that are formed on the surface of cotton fabrics due to repeated washing, restoring a smooth finish. However, the enzyme also weakens the fabric during repeated use (Grant et al.

1990). Cellulases active at alkaline pH find extensive applications in the manufacture of denim jeans and also "bio-polishing" of fabrics to achieve increased smoothness and softness of the finished product. The "stonewashed" effect to produce "faded" denim jeans earlier achieved by abrasive action of pumice stones on blue denims is now performed with cellulose enzymes which has been termed "biostoning." Since small doses of cellulose can replace huge amount of stones, the enzyme has gained considerable popularity in the textile industry. Alkaline cellulases also effectively prevent redeposition of the indigo blue dye on the garment, which is called "back staining." Enzyme treatment also results in low tear strength, and compared to treatment with pumice stones, the damage to the fabric as well as the processing equipment is minimal. In "bio-polishing," cellulose enzymes are used to weaken the cellulosic polymer and help in dislodging the weakened fiber by mechanical means, resulting in a softer and attractive surface to the textile material. Actinobacteria produced cellulase which was stable between 28 and 55°C and having pH range 4–8 (Al-Tai 1989).

Lipase

Lipases of microbial origin are the most versatile enzymes and are known to bring about a range of bioconversion reactions including hydrolysis, interesterification, esterification, alcoholysis, acidolysis, and aminolysis (Pandey et al. 1999; Kim et al. 2002a, b). Their unique characteristics include substrate specificity, stereospecificity, regioselectivity, and ability to catalyze a heterogeneous reaction at the interface of water-soluble and water-insoluble systems (Jaeger and Reetz 1998). The esters play a relevant role in the food industry as flavor and aroma constituents (Pandey et al. 1999). Long-chain methyl and ethyl esters of carboxylic acid moieties provide valuable oleochemical species that may function as fuel for diesel engines. Esters of long-chain carboxylic acid and alcohol moieties (waxes) have applications as lubricants and additives in cosmetic formulations (Linko et al. 1994). Other applications include the removal of the pitch from pulp produced in the paper industry, hydrolysis of milk fat in the dairy industry, removal of non-cellulosic impurities from raw cotton before further processing into dyed and finished products, drug formulations in the pharmaceutical industry, and removal of subcutaneous fat in the leather industry (Pandey et al. 1999). Most of the industrial processes in which lipases are employed function at temperatures exceeding 45°C. The enzymes, thus, are required with an optimum temperature of around 50°C (Sharma et al. 2002).

Among the desirable characteristics that commercially important lipases should mainly exhibit are thermostability and alkalitolerance. Although, few lipases exist which are able to operate at 100°C, their half-lives are short (Rathi et al. 2000). Therefore, it is necessary to search for sources of highly active lipolytic enzymes with stability at wider pH, higher temperatures, ionic strength, and organic solvents. Among different studies on thermophilic actinobacteria, *Streptomyces rimosus* R6-554W produced lipase which was highly active between 50 and 60°C and in alkaline pH 9–10 (Abrami et al. 1999).

Xylanase

Xylan, a polysaccharide found in plant, has D-xylose as the basic unit bonded with β-1,4 glycosyl bonds. In their native state, they are partially substituted with acetyl, 4-O-methyl, D-glucuronosyl, and L-arabinofuranosyl residues, forming complex heterogeneous polymer. Xylan is degraded by xylanase that is produced by the microorganisms. Cellulose-free xylanases are significantly useful for bleaching the paper pulp in place of chlorine compounds. Some thermophilic actinobacteria, such as *Streptomyces* and *Actinomadura*, produce xylanase (Holtz et al. 1990). The optimal pH and temperature ranged from 6.0 to 7.0 and 70 to 80°C for xylanase activity. The enzymes exhibited considerable thermostability at their optimum temperature, with half-lives at 75°C for 6.5 to 17 h. Hydrolysis of xylan by extracellular xylanases yielded xylobiose, xylose, and arabinose as principal products. Complete utilization of xylan is presumably achieved by β-xylosidase activities which are largely cell associated in three *Actinomadura* strains (Holtz et al. 1990).

The xylanase activity in the culture supernatants of strains representing *Saccharomonospora viridis* and *Thermomonospora* spp. was characterized by measurement of reducing sugars released from oat spelt xylan and analysis of degradation products by thin-layer chromatography. In all four species, xylanase activity was optimal within the temperature range 60–75°C and between pH 5 and 8 (Holtz et al. 1990). Culture supernatants of *Thermomonospora* strains incubated at 70°C for 60 min retained >80% of their activity, while that of *S. viridis* was totally inactivated (Holtz et al. 1990).

The culture supernatants initially hydrolyzed xylan to a mixture of oligomeric products, indicating that the main activity was of the endoxylanase type. Prolonged incubation for 24 h resulted in the hydrolysis of xylan to D-xylose by *T. curvata* and *T. fusca* xylanase preparations, indicating the additional presence of exoxylanase or β-xylosidase activity. Xylanase production was induced by growth on xylan, although low levels of activity were also detected in glucose-grown cultures. *Thermomonospora curvata* MT815 culture supernatant was most active and produced D-xylose from milled wheat straw in yields approximately 10% of those from oat spelt xylan (Mc Carthy et al. 1985).

18.2.2.2 Cloning and Overexpression of Enzymes

Among the Actinobacteria, *Streptomycetes* are considered interesting candidates for the production of heterologous proteins for several reasons, including their efficient secretion mechanism by which the secreted proteins are localized into the culture supernatant. In view of this potential, different aspects of gene expression and regulation in *Streptomyces*, using *Streptomyces lividans* as host for the secretion of heterologous proteins of prokaryotic and eukaryotic origin, have been investigated (Anne and Mellaert 1993).

Aculeacin A acylase (AAC), produced by *Actinoplanes utahensis*, catalyzes the hydrolysis of the palmitoyl moiety of the antifungal antibiotic, aculeacin A. Using

mixed oligodeoxyribonucleotide probes based on the N-terminal amino acid (aa) sequences of the two subunits of AAC, overlapping clones were identified in a cosmid library of *A. utahensis* DNA. After the sub-cloning of a 3.0-kb fragment into *Streptomyces lividans*, the recombinant produced AAC extracellularly. The nucleotide sequence of this fragment predicted an open reading frame of 2,358 bp with GTG start and TGA stop codons. The deduced 786-aa sequence should correspond to a single polypeptide chain, indicating that this polypeptide is processed to the active form that is composed of two subunits. Threefold more AAC was obtained from the *S. lividans* recombinant carrying the cloned gene than the original *A. utahensis* strain (Junji et al. 1992). *Streptomyces* species offer many potential advantages as hosts for the expression and secretion of eukaryotic gene products.

A gene encoding an extracellular lipase from *Streptomyces* sp. M11 was cloned in the high-copy-number vector pIJ486, using *S. lividans* 66 as host. A 28-kDa protein was secreted by *S. lividans* carrying pB13, which harbors a 6-kb insert, and identified as the product of the cloned gene. Comparison of the N-terminal amino acid (aa) sequence of the purified extracellular lipase with the nucleotide (nt) sequence of the *lip* gene revealed the presence of a 48-aa-long signal peptide. The nucleotide sequence also revealed the presence of a motif, Gly-His-Ser-Met-Gly, similar to the one found surrounding the active-site Ser in other lipases. The gene is most likely monocistronic. Sub-cloning experiments indicated that another gene might be required for high-level expression, since sub-cloning of the structural gene alone resulted in diminished extracellular lipase activity. The lipase gene promoter was identified by S1 mapping experiments and found to be similar to other *Streptomyces* vegetative promoters (Perez et al. 1992).

A broad-spectrum mercury resistance locus (mer) from a spontaneous chloramphenicol-sensitive (Cms), arginine auxotrophic mutant of *Streptomyces lividans* 1326 was isolated on a 6-kb DNA fragment by shotgun cloning into the mercury-sensitive derivative *S. lividans* TK64 using the vector pIJ702. The mer genes form part of a very large amplifiable DNA sequence present in *S. lividans* 1326. This element was amplified to about 20 copies per chromosome in the Cms arginine auxotrophic mutant and was missing from strains like *S. lividans* TK64, cured for the plasmid SLP3. DNA sequence analysis of a 5-kb region encompassing the whole region required for broad-spectrum mercury resistance revealed six open reading frames (ORFs) transcribed in opposite directions from a common intercistronic region. The protein sequences predicted from the two ORFs transcribed in one direction showed a high degree of similarity to mercuric reductase and organomercurial lyase from other Gram-negative and Gram-positive sources. Few, if any, similarities were found between the predicted polypeptide sequences of the other four ORFs and other known proteins (Sedlmeier and Altenbuchner 1992).

18.2.2.3 Antibiotic Production

Over 16,500 of the naturally occurring antibiotics discovered are synthesized by actinobacteria. Actinobacteria have provided many important bioactive compounds

of high commercial value and are being continuously screened for new bioactive substances. Some of these antibiotics target bacterial ribosomes and are used in treating respiratory infections, including Legionnaires' disease (tetracycline, erythromycin). Vancomycin attacks bacterial cell walls and deadly organisms such as MRSA (methicillin-resistant *Staphylococcus aureus*). Rifamycin targets bacterial RNA polymerase and is useful against tuberculosis and leprosy. Amphotericin is one of the few antibiotics that attack fungal membranes. These antibiotics generally do not affect human cells and therefore have few side effects. However, actinobacterial metabolites such as Adriamycin prevent DNA replication and are used in the treatment of cancer, while rapamycin is used to suppress the immune system to enable organ transplants (Radhakrishnan et al. 2007).

18.2.2.4 Environmental Applications

Some actinobacteria produce volatile organic compounds (VOCs). The odor of freshly turned soil is the result of geosmin, a volatile organic compound produced by actinobacteria. Geosmin is also produced by some cyanobacteria and produces an earthy taste in drinking water. Some fungi also produce geosmin, which can impart the same earthy taste to wine made from moldy grapes. Besides, some species of thermophilic actinobacteria are able to degrade compounds, such as rutin and keratin.

Many actinobacteria are well known as degraders of toxic materials and used in bioremediation. They are particularly well adapted to survival in harsh environments, and some are able to grow at higher temperatures (>50°C). Some members of genus *Actinodura*, *Microbispora*, *Streptomyces*, *Thermoactinomyces*, and *Saccharomonospora* are found to degrade bioplastic, such as PES (polyethylene succinate), PCL poly(ε-caprolactone), and PHB poly(3-hydroxybutyrate), by composting at high temperatures. Some mutagenic compounds, rutin and quercetin, are hydrolyzed by thermoactinobacteria such as *Thermoactinomyces vulgaris* PU18-2 (Yang et al. 2009).

18.2.2.5 Adverse Effects

Thermophilic actinobacteria have also some detrimental effects and are found to cause diseases. *Nocardia asteroides*, a thermophilic actinomycete and commonly found in soil, can cause infection via the respiratory route. Infection is opportunistic and relies on the weakened cell-mediated immunity. Other species of *Nocardia* may also be involved in hypersensitivity pneumonitis (HP). Thermophilic actinobacteria are the most common cause of HP. Farmer's lung disease is caused by the exposure to hay. Clouds of spores are released when farmers handle stored hay in winter and early spring. Common species include *Thermoactinomyces vulgaris*, *Saccharopolyspora rectivirgula*, and *Thermoactinomyces viridis* (Roberts et al. 1983).

The thermophilic actinobacteria are the most common etiological agents causing hypersensitivity pneumonitis. Antigen preparations of these organisms contain proteolytic activity. Further investigation of the proteinases of the thermophilic actinobacteria was undertaken to determine whether this activity may contribute directly to the pathogenesis of hypersensitivity pneumonitis and pulmonary mycotoxicosis. The presence of proteolytic activity in aerosolized dust from moldy silage was demonstrated, and antibodies to two proteolytic enzymes from *Thermoactinomyces candidus* were found in the blood of farmer's lung patients who had been sensitized to this organism. These two enzymes were isolated from culture filtrate antigen preparations that had been partially characterized with respect to the proteolytic activities and their interaction with human serum proteinase inhibitors. Both proteinases belonged to the serine class of endopeptidases. Neither proteinase was inhibited by alpha 1-proteinase inhibitor or alpha 1-antichymotrypsin. However, both proteinases were inhibited by alpha 2-macroglobulin. One of the proteinases had elastase activity. Inhalation of these proteinases may induce an inflammatory response in the lungs since they are not inhibited by the main proteinase inhibitors protecting the lung (Roberts et al. 1983).

18.3 Analysis

Attention has been focused on extremophiles in the last few decades due to their unique inherent properties and ability to produce a range of secondary metabolites. Further interest stems from the fact that they share morphological similarities with bacteria and fungi. Using several molecular approaches, the diversity of thermophilic actinobacteria has been studied and revealed many facts of thermophilic life. Large variations have been found in the cell and spore morphology in terms of their arrangement. Using several conserved DNA regions, the identification schemes have been developed for accurate identification of thermophilic actinobacteria. Some initial work has also been done to study the uncultivable actinobacteria using metagenomics.

Actinobacteria have exhibited good biotechnological potential, with reference to metabolites and biocatalysts. Thermophilic actinobacteria also degrade some recalcitrant compounds and thus may play significant role in bioremediation. Some thermophilic actinobacteria have detrimental effects and cause diseases.

In the larger context of exploring actinomycetes from extreme habitats, we have been involved with the salt-tolerant alkaliphilic actinomycetes from the saline habitats of Coastal Gujarat in India. Our research over the last several years has revealed their diversity, phylogeny, and enzymatic potential. Ability of their alkaline proteases to function under multitude of extreme conditions, such as salinity, broad pH range, and high temperatures, has attracted attention, both from molecular stability and biotechnological point of view (Thumar and Singh 2007a, b, 2009, 2011; Mehta et al. 2006). The thermodynamic analysis of a protease from salt-tolerant alkaliphilic actinobacteria has recently added to the knowledge of biocatalysis from extremophiles

(Gohel and Singh 2012a). Besides, the cloning and expression of protease genes from two actinobacteria into *E. coli* has opened new opportunities for recombinant enzymes (Gohel and Singh 2012b). Similarly, the work on the unexplored diversity of thermophilic bacteria and actinobacteria from thermal habitats of the Saurashtra region, Gujarat, India, has generated interesting findings on the diversity and biocatalytic potential (Kikani and Singh 2011; Kikani et al. 2010).

18.4 Future Prospectives

In order to explore diversity, certain molecular tools and culture-independent approaches should be used along with the conventional microbiological techniques. This would be coupled with the construction of clone libraries followed by the analysis of the clones with the help of various bioinformatic tools. Due to huge biotechnological relevance, functional-based metagenomic approaches can open new horizons. Potential strains can also be explored for cloning and overexpression of recombinant products at industrial scale to meet the increasing demands at reduced cost with better efficiency.

Further research can also focus on the comparison and correlation of diversity along with the biotechnological potentials of other extremophilic actinobacteria with thermophilic ones. Bioinformatic approaches can be useful in preparing databases on the cultural, phylogenetic, biocatalytic, and other biotechnological features.

18.5 Conclusions

Thermophilic actinobacteria are largely unexplored due to difficulties in their isolation and maintenance in pure culture. With the advancements in new molecular techniques that have potential to unravel total diversity of thermophilic actinobacteria, the studies have gained impetus. The molecular approaches in conjunction with the conventional microbiological techniques would highlight the diversity and phylogeny of the thermophilic actinobacteria in comprehensive manner. This may open up new horizons for developing new biotechnological applications.

In past various conventional methods such as staining, biochemical studies and morphological features have been mainly employed to study diversity of thermophilic actinobacteria. However, to explore total diversity, various modern techniques such as 16S rRNA sequencing, RFLP, ARDRA, and DGGE are being employed along with the conventional techniques.

Initial explorations have indicated that thermophilic actinobacteria have large application potential in the field of biocatalysis, environmental applications, and production of value-added chemicals. In this context, understanding the expression systems and production of heterologous proteins would also be interesting aspect for future studies.

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Chapter 19
Mechanisms of Thermal Stability Adopted by Thermophilic Proteins and Their Use in White Biotechnology

Jennifer Littlechild, Halina Novak, Paul James, and Christopher Sayer

Abstract Considerable interest has been generated in the mechanism which nature utilises to increase the stability of enzymes found in thermophilic and hyperthermophilic species. This has been the subject of many reviews, and our understanding has been enhanced by the increasing number of high-resolution thermostable enzyme structures that have been determined. Different species of bacteria and Archaea have used different mechanisms to achieve stability. A comparative approach has been used to carry out a detailed study of specific enzymes from a range of organisms in order to understand acquired stability at a structural level. This chapter will discuss the rules to increase protein thermostability that have been obtained from protein structural studies that are currently available. It will also examine other ways to stabilise existing proteins by lessons learnt from nature and by protein immobilisation.

Thermostable enzymes find applications in 'white biotechnology' including the biosynthesis of fine chemicals. This chapter will discuss specific examples of thermophilic enzymes already adopted for industrial applications. These include alcohol dehydrogenases for chiral alcohol production, aminoacylases for optically pure amino acids and amino acid analogues, transaminases for chiral amine production and gamma-lactamases for chiral gamma-lactam building blocks which are subsequently incorporated into carbocyclic nucleotides. A brief overview of other applications in biorefining, biofuel cells and detergents are also presented.

Keywords Thermostable proteins • Mechanisms • Stabilisation • White biotechnology • Biocatalysis

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19.1 Introduction to Thermophilic Enzymes

Thermophiles are classified as organisms that are able to grow at elevated temperatures (above 37°C) and can be further subclassified on the basis of their optimal growth temperatures into 'moderate' thermophiles (T_{opt} ~ 60°C), extreme thermophiles (T_{opt} ~ 75°C) and hyperthermophiles (T_{opt} ~ 85°C) (Segerer et al. 1993).

Many of the hyperthermophiles belong to the archaea. Currently the most hyperthermophilic microorganisms isolated are the archaeon *Pyrolobus fumarii* which grows optimally at 106°C and can survive at up to 113°C (Blochl et al. 1997) and more recently described the archaeon *Methanopyrus kandleri* which is reported to have an upper growth limit of 122°C (Takai et al. 2008). Thermophilic organisms have been identified from a number of different environments. Moderate thermophiles have been located in a wide range of warm habitats from hot desert soils to industrial waste water. The hyperthermophiles have been mainly isolated from natural geothermal features such as deep-sea hydrothermal vents (Bult et al. 1996), volcanic solfataras (Brock et al. 1972) and hot springs (Brock and Freeze 1969). The history of the discovery of the first thermophiles is clearly documented by the review of Karl Stetter (2006).

Analysis of nucleic acid samples collected from these habitats has indicated that the diversity of hyperthermophilic organisms extends much further than those species already isolated (Harmsen et al. 1997), and currently there are a large number of metagenomic projects being undertaken to look at complete microbial communities in these environments (Takami et al. 2012). A paper reported in 1999 that there were already more than 30 genera and 70 species of hyperthermophiles identified (Madigan and Oren 1999) and this number has continued to significantly increase.

The thermostable enzymes (sometimes referred to as thermozymes) found in these organisms have been the subject of extensive research (Bruins et al. 2001; Li et al. 2005). These thermozymes have been of great interest from the start as not only are they able to function at higher temperatures but often show increased stability to solvents, pH and proteolytic degradation. Stability of an enzyme is dependent on maintenance of a functional structure, and the stability of any protein is marginal and equivalent to a small number of molecular interactions (Daniel et al. 2008). This remains the case with a thermostable protein; the only difference being that the free energy of stabilisation is slightly higher than that of its mesophilic counterpart (Jaenicke and Bohm 1998).

Thermozymes have been shown to retain their thermophilic properties when cloned into a mesophilic host (e.g. *Escherichia coli*) and therefore can be easily purified from the mesophilic host in a heat-treatment step. This ease of production has allowed a number of thermophilic proteins to become readily available for a number of biocatalytic processes, some of which are outlined below.

The thermal stability of all enzymes can be measured in two different ways, either as a function of the temperature at which the protein undergoes reversible or irreversible unfolding. This typically uses circular dichroism (CD) or differential scanning fluorimetry (DSF) to visualise the conformational change (van Mierlo and

Steensma 2000). The other more widely used method is to look at the temperature at which the loss of function occurs (T_{inact}). Thermozymes have been important in the identification of the factors affecting thermal stability by comparison of these enzymes with their mesophilic counterparts.

19.2 Protein Stability and Denaturation

The active form of a protein is usually held together by a combination of non-covalent forces including hydrogen bonds, ion pairs, hydrophobic bonds and Van der Waals interactions. When these interactions are disrupted, for example, by elevated temperatures, both mesophilic and thermophilic proteins unfold into inactive but kinetically stable structures. Once unfolded in this manner, the protein is prone to aggregation and chemical modification. Aggregation occurs when the hydrophobic residues of a protein that have been exposed by unfolding interact with hydrophobic residues from other protein molecules, which usually follows immediately after unfolding. Chemical modifications of the protein include cysteine oxidation, deamination of asparagine and glutamine residues and peptide bond hydrolysis. The actual unfolding of the protein may be reversible or irreversible, with most larger proteins falling into the latter class. However, once aggregation and chemical modification have occurred, the correctly folded form of the protein is not usually regained.

The thermodynamic stability of a protein is described by its free energy of stabilisation, ΔG_{stab}, the energy difference between the correctly folded and denatured states. ΔG_{stab} is a function of temperature and is usually only a small value, approximately 5–15 kcal mol^{-1} at 25°C for a mesophilic protein (Jaenicke 1991). ΔG_{stab} measurements are difficult to obtain for any protein as the unfolded protein aggregates almost immediately, but particularly so for thermostable proteins because the conditions required to denature them are very harsh and often beyond the limits of the equipment available. For these reasons, the ΔG_{stab} values available tend to be from small globular proteins, and those of thermostable proteins are often measured in the presence of denaturing conditions such as low pH. The data that are available suggest that the difference in ΔG_{stab} between a hyperthermophilic protein and its mesophilic counterpart is frequently small, with values in the region of 5–15 kcal mol^{-1} (Nojima et al. 1978; Davies et al. 1993; Li et al. 1998). A plot of ΔG_{stab} against temperature for a hyperthermophilic protein may vary from that of the mesophilic protein in three ways: The maximum value of ΔG_{stab} may be higher, the peak may be at a higher temperature or the peak may be broader. In practice, the difference in stability between a hyperthermophilic protein and its mesophilic equivalent is frequently achieved by a combination of these effects (Vieille and Zeikus 2001).

The classical enzyme model describes the dependence of enzyme activity in relation to temperature. Using this model, the temperature optimum T_{opt} is calculated by analysing enzyme activity over an increasing temperature range for a set amount of time. Thermal denaturation is time dependent, meaning the resulting T_{opt} value calculated is dependent on the duration of the assay.

Reduced enzyme activity at high temperatures which could not be explained by thermal denaturation with numerous enzymes, along with evidence that reduced enzyme activity above the T_{opt} is reversible upon lowering the temperature to a value below the temperature optimum, was observed with a citrate synthase and a 3-phosphoglycerate kinase (Gerike et al. 1997; Thomas and Scopes 1998). These observations led to the development of the equilibrium model (Eisenthal et al. 2006) which incorporates reversible and time-dependent inactivation. The equilibrium model includes an inactive (E_{inact}) and active (E_{act}) form of the enzyme in reversible equilibrium which is described by the equilibrium constant (K_{eq}) with E_{inact} undergoing irreversible thermal inactivation to the denatured state (Eq. 19.1).

$$E_{act} \overset{K_{eq}}{\rightleftharpoons} E_{inact} \xrightarrow{K_{inact}} X \tag{19.1}$$

K_{eq} is characterised in terms of enthalpy of the equilibrium (ΔH_{eq}). T_{eq}, a new thermal parameter which is the temperature where E_{inact} and E_{act} are equal, allows the prediction of T_{opt}. The equilibrium model also includes the temperature-dependent inactivation rate constant K_{inact} which buffers the enzyme against irreversible inactivation. Eisenthal and co-workers applied the equilibrium model to approximately 30 enzymes, of which all followed the equilibrium model (Eisenthal et al. 2006). The incorporation of X, an inactive form of the enzyme, in the reversible equilibrium allows the T_{opt} at time zero to be calculated. The equilibrium model allows the relationship between activity and stability of an enzyme to be more fully understood.

19.3 Mechanisms to Increase Thermostability in Naturally Thermostable Enzymes

As more high-resolution structures of thermophilic and hyperthermophilic proteins become available, it is becoming increasingly apparent that there are no set of defined rules for thermostability. The mechanism of coping with life at high temperatures varies between species, organisms and even individual proteins. There are, however, a number of adaptations that are known to confer increased stability that have been experimentally observed, and most proteins from thermophilic and hyperthermophilic organisms appear to use a combination of these factors to achieve resistance to thermal denaturation. A summary of the factors reported by authors to make contributions to increased stability of thermophilic and hyperthermophilic proteins is shown in Fig. 19.1.

19.3.1 Primary Structure

Relative abundance of certain amino acids has been thought to contribute to the thermostability of a protein since some amino acids are more prone to chemical modification and other amino acids have advantageous properties with regard to

Summary of Methods of Protein Stabilisation

- Increase in ionic interactions. Often large ionic networks between subunit interfaces. Capping of ends of α-helices.
- Increased hydrophobicity.
- Increased packing – additional secondary structure and C-terminal extensions to fill internal cavities.
- Shortening of surface loops.
- Stabilisation of loops by interaction with metal ions.
- Increased proline content especially in *Thermus* bacterial species that have a high GC DNA.
- Reduction in amino acids that are unstable at high temperatures such as asparagines and cysteines except where they play an important catalytic role.
- Introduction of disulfide bonds into cytoplasmic proteins.

Fig. 19.1 Summary of methods of protein stabilisation

protein stability. In particular comparisons of thermophilic proteins with their mesophilic counterparts have shown trends for increasing incidence of charged residues and for replacement of glycine with alanine, and lysine with arginine. A higher proportion of charged residues within a thermophilic protein would increase the number of potential ionic interactions available to stabilise the structure (see below). A higher alanine content would stabilise α-helices, while arginine is suggested to be less susceptible to chemical attack at high temperatures than lysine (Vieille and Zeikus 2001). Cysteine is particularly susceptible to oxidation at elevated temperature and is often absent from thermophilic proteins unless it is required for activity or formation of disulfide bonds. Asparagine and glutamine residues are easily deaminated, and a reduction in the frequency of these residues has been noted in hyperthermophilic proteins of several families (Hess et al. 1995; Fleming and Littlechild 1997). Increased incidence of the aromatic residues, and of proline residues which will confer conformational stability through increased rigidity, has also been reported (Fleming and Littlechild 1997; Korkhin et al. 1999).

The adaptations in primary sequence to maximise thermostability vary between different proteins which is not unexpected since their different environments are within the varied tertiary structures. Recent and more extensive comparisons based on the genome sequences of mesophilic and hyperthermophilic organisms have shown only minor trends in amino acid composition (Vieille and Zeikus 2001). Primarily, an increase in the number of charged residues was observed, along with a decrease in polar, uncharged residues. A general reduction in the number of cysteines was observed, but the trend was shown to be highly dependent on species, with *Methanococcus jannaschii*, for example, containing a larger number than the average mesophile. It was noted, however, that particularly in the aerobic hyperthermophiles, cysteine residues were generally involved in essential interactions such as disulfide bridges, catalysis or metal coordination, with free cysteine residues being extremely rare.

19.3.2 Ion Pairs

An increase in the number of ion pairs in a protein has been shown to enhance thermostability (Davies et al. 1993). In accordance with this, a range of thermophilic and hyperthermophilic proteins are known to have significantly more ion pairs than their mesophilic counterparts (Davies et al. 1993; Yip et al. 1995; Aguilar et al. 1997). A comparison of lactate dehydrogenase enzymes from six different organisms showed a clear trend in increasing frequency of ionic interactions as optimum growth temperature of the organism increased (Auerbach et al. 1998). It is not just the number of ionic interactions that appears to be important in thermostability but also the arrangement of these bonds. Ion pairs are frequently seen in thermostable proteins to be clustered together, forming extended ionic networks involving as many as 24 residues (Yip et al. 1995). The contribution of such a network to the stability of the protein is significantly higher than the sum of an equal number of individual ionic bonds. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *Sulfolobus solfataricus* contains an ion-pair cluster involving 15 charged amino acids situated on the subunit interface and extending into the active site (Isupov et al. 1999). These amino acids are highly conserved in thermophilic GAPDH sequences but less so in mesophilic GAPDHs. Trends for increasingly large ionic networks with thermostability are also seen in glutamate dehydrogenases (Yip et al. 1995; Knapp et al. 1997; Rahman et al. 1998), alcohol dehydrogenases (Guy et al. 2003) and aminopeptidases (Tahirov et al. 1998) and in many other hyperthermophilic proteins.

19.3.3 Hydrogen Bonds

The number of hydrogen bonds present in a protein is thought to influence its thermostability. In particular, a strong correlation has been shown between the number of charged-neutral hydrogen bonds and thermophilicity of four GAPDH enzymes (Tanner et al. 1996; Littlechild et al. 2007). A charged-neutral hydrogen bond is defined as that formed between a side-chain atom of a charged residue and either a main-chain atom of any residue or a side-chain atom of a neutral residue, and is stronger than a hydrogen bond between two neutral residues. Increased numbers of charged-neutral hydrogen bonds have also been observed in *Thermotoga maritima* ferredoxin (Macedo-Ribeiro et al. 1996) and on the subunit interfaces of dihydrofolate reductase from the same organism (Dams et al. 2000).

19.3.4 Disulfide Bonds

Disulfide bonds are uncommon in cytosolic proteins due to the reducing environment frequently found within the cell. This observation, in addition to the increased susceptibility of disulfide bonds to reduction at high temperatures, has resulted in the assumption

that disulfide bridges were unlikely to contribute significantly to stability in thermophilic and hyperthermophilic proteins. Disulfide bonds are however frequently seen in extracellular proteins from mesophilic species and are known to contribute to their stability. Recently, disulfide bonds have been characterised in cytosolic proteins with optimum activity at and above 100°C (Cacciapuoti et al. 1994), suggesting that these bonds may contribute to protein stability. Some enzymes from the hyperthermophilic archaea are now known to contain disulfide bonds as observed from structural studies which include GAPDH from *Sulfolobus solfataricus* (Isupov et al. 1999) and pyrrolidone carboxyl peptidase (Pcp) from *Thermococcus litoralis* (Singleton et al. 1999a). In general, disulfide bonds in thermophilic proteins appear to be protected by their buried environment and inaccessibility to solvents. A study of genomic DNA sequences from hyperthermophilic archaea suggests that the intracellular proteins of these organisms may contain more disulfide interactions than previously thought, with up to 40% of the intracellular cysteines in *Aeropyrum pernix* proteins predicted to be present as disulfide bonds (Mallick et al. 2002; Beeby et al. 2005).

19.3.5 Hydrophobic Interactions

The folding of a protein in such a way as to bury hydrophobic residues within the core is known to play a major role in protein stability. An average stability increase of 1.3 kcal mol^{-1} has been calculated for each additional methyl group buried in protein folding (Pace 1992). Evidence for the importance of hydrophobic effects in protein folding has been supplied by numerous X-ray structures and from mutagenesis studies (Matsumura et al. 1988; Kirino et al. 1994).

An increase in hydrophobic interactions at subunit interfaces has been observed in the crystal structures of many hyperthermophilic proteins, for example, a lactate dehydrogenase from *Thermotoga maritima* (Auerbach et al. 1998) and the 3-isopropylmalate dehydrogenase from *Thermus thermophilus* (Kirino et al. 1994). Hydrophobic interactions are also thought to be one of the major contributors to thermostability of the Pcp from *Thermococcus litoralis* (Singleton et al. 1999b). The centre of this tetrameric protein contains a hydrophobic core consisting of two phenylalanine and two leucine residues from each subunit, which is not seen in the homologous enzyme from the mesophile *Bacillus amyloliquefaciens* (Odagaki et al. 1999). In addition the *Thermococcus litoralis* enzyme is stabilised by a disulfide bond at one interface of the dimer within the tetrameric Pcp. However, the Pcp enzyme from the hyperthermophilic *Pyrococcus furiosus* also has the disulfide present, but the central core of the tetramer is stabilised by ionic interactions (Sokabe et al. 2002). These differences in the Pcp enzymes from different species are illustrated in Fig. 19.2.

Aromatic clusters have been studied in a dataset of 24 protein structures where thermophilic and mesophilic homologues are known (Kannan and Vishveshwara 2000). The results show evidence for additional or enlarged aromatic clusters in 17 of the protein families where these are found in thermophilic homologues but not mesophilic counterparts.

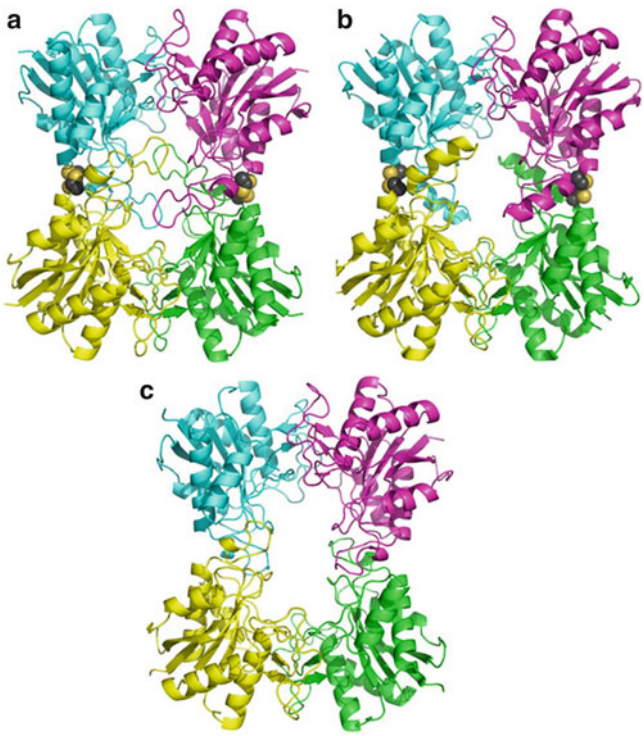

Fig. 19.2 A diagram showing the structures of three Pcp enzymes to illustrate the different mechanisms used by different species to stabilise the proteins. (**a**) The Pcp from the thermophilic archaeon *Thermococcus litoralis* (PDB code 1A2Z), (**b**) the Pcp from the hyperthermophilic archaeon *Pyrococcus furiosus* (PDB code 1IOF) and (**c**) the mesophilic Pcp from *B. amyloliquefaciens*. The disulfide bonds at one of the subunit interfaces of the tetramer are highlighted as *spheres* (Figure produced using PyMol (DeLano 2002))

19.3.6 Helix-Dipole Stabilisation

The directional nature of an α-helix produces an intrinsic dipole between the negatively charged *C*-terminus and the positively charged *N*-terminus. This dipole may be stabilised by the presence of negatively charged residues near the *N*-terminal end or positively charged residues near the *C*-terminus. Increased stabilisation of α-helices in this manner has been observed in thermostable proteins when compared to their mesophilic counterparts (Davies et al. 1993; Hennig et al. 1995).

19.3.7 Structural Elements

It is common to observe a reduction in the frequency and length of external loops in thermophilic proteins. Loops may also be better anchored to the rest of the structure (by ion pairs, hydrogen bonding or hydrophobic interactions) in a hyperthermophilic

protein than in its mesophilic counterpart (Vieille and Zeikus 2001). Similarly, the *N*- and *C*-termini may either be anchored to the core or may interact with each other to increase stability (Macedo-Ribeiro et al. 1996; Auerbach et al. 1997). Quaternary structure is also thought to influence thermostability. Thermophilic proteins appear to be more likely to adopt a higher oligomeric state than mesophilic proteins, and association between the subunits is often enhanced. This factor is thought to confer increased thermostability. Site-directed mutagenesis has been used to show the importance of dimerisation in phosphoribosylanthranilate isomerase from *Thermotoga maritima* (Thoma et al. 2000); monomeric mutants of the enzyme retained catalytic activity but showed significantly reduced thermal stability when compared to the wild type.

19.3.8 Specifically Bound Metals

Metals such as zinc and calcium are often found in proteins where they can stabilise a loop structure or hold secondary structures together as is the case for zinc that can co-ordinate cysteine and histidine amino acid side chains from different structural elements of a protein. An example of this is seen below for the case of *Aeropyrum pernix* alcohol dehydrogenase.

A summary of different factors making a contribution to stability as observed from 25 thermophilic protein structures published up to 2000 is shown in Fig. 19.3.

19.4 Extrinsic Factors

Many extrinsic factors such as high protein concentrations or the presence of the enzyme substrate, cofactor or certain salts have been shown to increase the stability of both thermophilic and mesophilic proteins. No evidence has been found to indicate that these factors are more important in thermostable proteins than in their mesophilic equivalents. It has been shown however that hyperthermophilic proteins can exhibit higher stability under moderately increased pressure (Robb and Clark 1999). This correlates with the high pressures naturally present in the environments from which many hyperthermophiles are isolated.

19.4.1 Stabilisation by Osmolytes

Under extreme conditions, microorganisms produce small organic compounds called osmolytes, which are zwitterionic, uncharged or anionic molecules (Lentzen and Schwarz 2006). Their primary function is to maintain intracellular homeostasis under high osmotic pressure in order to maintain cell turgor. Osmolytes are also produced by microorganisms in response to other environmental changes such as high temperatures and can accumulate at concentrations up to 2 M and

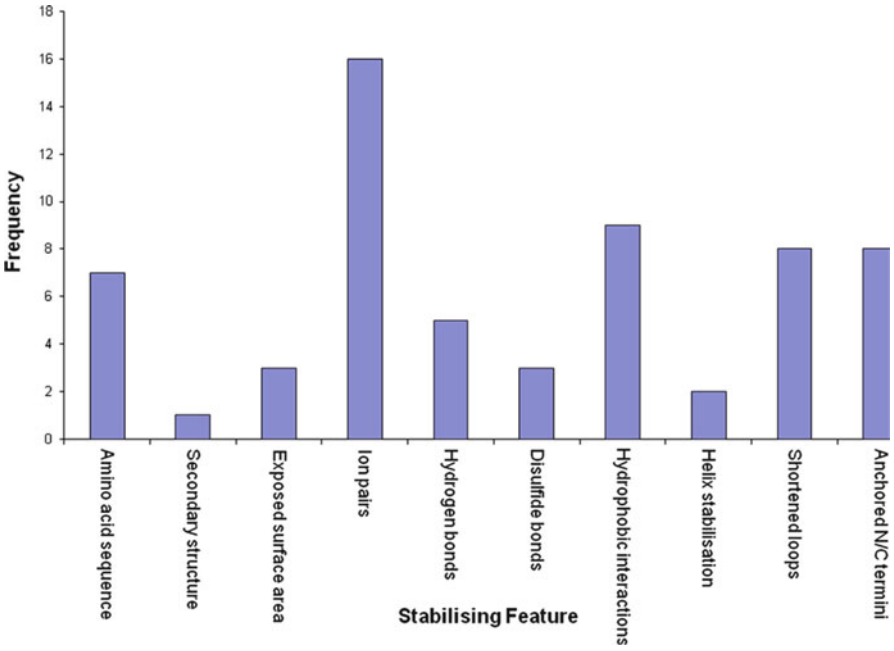

Fig. 19.3 Factors reported by the authors as making major contributions to increased stability as observed in the X-ray structures of 25 proteins from thermophilic sources (Guy 2004) (Data taken from Chan et al. 1995; Hennig et al. 1995; Korndorfer et al. 1995; Yip et al. 1995; Knochel et al. 1996; Macedo-Ribeiro et al. 1996; Aguilar et al. 1997; Auerbach et al. 1997; Ermler et al. 1997; Hennig et al. 1997; Knapp et al. 1997; Lim et al. 1997; Russell et al. 1997; Auerbach et al. 1998; Tahirov et al. 1998; Vonrhein et al. 1998; Zhu et al. 1998; Chi et al. 1999; Grabarse et al. 1999; Hashimoto et al. 1999; Hopfner et al. 1999; Isupov et al. 1999; Maes et al. 1999; Singleton et al. 1999b; Ursby et al. 1999; Dams et al. 2000)

therefore do not interfere with normal cellular activities (Brown 1990; da Costa et al. 1998; Lentzen and Schwarz 2006). Osmolyte production is tightly regulated, and in many cases multiple compounds are either produced or taken up from environmental surroundings to produce pools of osmolytes which regulate cell dynamics (Lentzen and Schwarz 2006).

Osmolytes found in mesophiles include amino acids, sugars, polyols, betaines, ectoine and hydroxyectoine. Over the last decade, studies on osmolytes from extremophiles have determined that alternative osmolytes are produced in extremophiles in comparison to mesophiles. Osmolytes from extremophiles have been named 'extremolytes' and include mannosylglycerate (MG), mannosylglyceramide (MGA), di-*myo*-inositol-1,1′-phosphate (DIP), diglycerol phosphate (DGP), di-mannosyl-di-*myo*-inositol phosphate and cyclic 2,3-bisphosphoglycerate (cBPG) (Borges et al. 2002). Osmolyte production is strongly associated with high temperatures, with an increasing amount being produced at temperatures above the optimal growth temperatures (Ciulla et al. 1994; Martins and Santos 1995; Silva et al. 1999). Halotolerant thermophilics such as *Pyrococcus furiosus* and *Thermotoga maritima*

accumulate DIP which can reach 20-fold in *Pyrococcus furiosus* at 101°C (Scholz et al. 1992; Ciulla et al. 1994; Martins and Santos 1995).

The stabilising properties of trehalose in comparison to disaccharides, polyols and betaine have shown that trehalose can have a much great effect of protein stability (Hottiger et al. 1994; Paiva and Panek 1996). Research by Canovas et al. (1999) and Lippert and Galinski (1992) showed that ectoine and hydroxyectoine stabilise enzymes at high temperatures. Research published by Borges et al. (2002) showed that MG and hydroxyectoine had a high stabilising effect on a lactate dehydrogenase in comparison to other solutes including trehalose, ectoine, mannosylglyceramide and di-*myo*-inositol phosphate. An increase in 4.5°C in the melting temperature in the presence of 0.5 M MG was observed in comparisons to an increase of 2.2°C in the presence of trehalose. This provides substantial evidence that intracellular pools of osmolytes stabilise proteins (da Costa and Santos 2001).

Osmolytes from microorganisms are being used in numerous applications, which are highlighted in a review by Lentzen and Schwarz (2006). Based on the preferential exclusion model by Arakawa and Timasheff (1985), the stabilising mode of action of osmolytes is determined by the fact that they are less likely to be found at the protein surface, which leads to the increased hydration of the protein. Protein hydration consequently reduces protein unfolding in the presence of osmolytes. Figure 19.4 highlights how this proposed mechanism works. The stabilising effects are due to modification of the water properties around the protein.

Taking this proposed mechanism into consideration, molecular dynamics simulations were performed with chymotrypsin inhibitor 2 in an ectoine-water mixture by Yu and Nagaoka (2004) that showed that solvent (water) diffusion slows down in a higher concentration of ectoine, which in turn stabilises the protein. Alternatively, results shown by Street et al. (2006) emphasise that there is no universal theory which explains the mechanism by which osmolytes and proteins interact to aid stabilisation. Their work highlights that osmolytes predominantly interact with the backbone of the protein.

Polysaccharides are also known to be produced by microorganisms, and in recent years the production of exopolysaccharides from extremophiles in response to environmental extremes has been studied (Manca et al. 1996; Nicolaus et al. 2000, 2002). Numerous *Haloferax* species are known to produce polysaccharides in response to environmental extremes in order to aid cell survival and have been found in numerous forms including linear, acidic, neutral and sulphated compounds (Nicolaus et al. 2004).

19.4.2 Stabilisation by Salt

Stabilisation of proteins by salts may occur either through a specific effect such an interaction with a metal ion or by causing a salting effect which affects the solvent (water) activity. The protein *Methanopyrus kandleri* formylmethanofuran:tetrahy

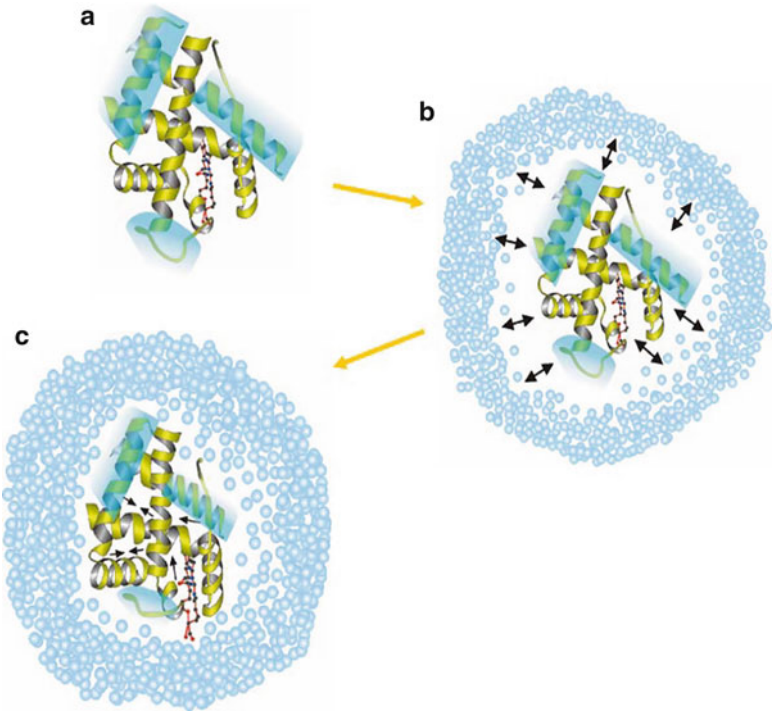

Fig. 19.4 A diagram showing the proposed mechanism of stabilisation of proteins by osmolytes, showing that the solute does not directly interact with the protein but that the stabilising effects are due to modification of the solvent properties. A protein molecule hydrated in aqueous solutions (**a**), which is stabilised by osmolytes (**b**), which form solute hydrate clusters which are preferentially excluded from the hydrate shell. The resulting protein has a more compact tertiary structure and a reduced surface area (**c**) (This figure was taken from Lentzen and Schwarz (2006))

dromethanopterin formyltransferase (MkFT) has been studied in a range of salt concentrations. The protein exists as a monomer and is inactive at low salt concentrations and adopts either a dimeric or tetrameric active form of the enzyme in higher salt concentration. MkFT is kinetically and thermodynamically more stable in the dimer or tetramer oligomeric states.

Two structural features of MkFT were associated with stabilisation in high salt concentrations. The first feature of the crystal structure is low surface hydrophobicity and a predominantly hydrophobic subunit interface, consequently meaning that lyotropic salts can reinforce subunit interactions by increasing hydrophobic interactions (Shima et al. 1998). The second feature is that the surface area of the tetramer contains an abundant number of negatively changed residues. Acidic residues are able to form strong hydrogen bonding, meaning that these amino acids can compete with inorganic cations or water (Ermler et al. 1997; Vieille and Zeikus 2001).

19.4.3 Stabilisation by Ligand Binding

The interaction of proteins with ligands is often coupled with an increase in melting temperature, often due to changes in the protein structure and a decrease in protein flexibility. The work by Gonzalez et al. (1997, 1999) describes the binding of biotin to Streptavidin from *Streptomyces avidinii* which increases the melting temperature (*Tm*) by 10°C. This increase in *Tm* is associated with an increase in the unfolding cooperative and a significant increase in the structural order.

Flexible regions of a protein can be identified by molecular dynamics computer simulations so that appropriate targets can be designed to aid protein stabilisation. The work by Sousa et al. (2009) highlights the ability to use solid-phase libraries which were rationally designed to identify affinity ligands that bind to flexible regions to improve the stability of a cutinase from *Fusarium solani*.

19.4.3.1 Protein Stabilisation by Immobilisation

A traditional method to stabilise proteins is by immobilisation. This is an area that has been well researched and many studies have been carried out. This continues very topical especially due to the increased use of enzymes in so-called white biotechnology and green chemistry. Many of the enzymes being marketed to date for commercial applications are immobilised in some manner. A review by Cowan and Fernandez-Lafuente (2011) discusses this area and the enhancement of functional properties of thermophilic enzymes by chemical modification and immobilisation. Enzymes can be immobilised onto solid supports which stabilises the enzyme and also makes its removal from the large-scale biotransformation relatively easy. Since the cost of the enzyme in such processes can be considerable, it is usually essential that the enzyme is recycled and used several times before disposal. The fact that the enzyme is immobilised makes this a more feasible procedure.

Another way to reuse the enzyme and to overcome the problems of substrate and product inhibition, often encountered when using enzymes for industrial-scale conversions where unnaturally high concentrations of substrate are used, is to utilise some kind of continuous-flow fluidic system where the substrate flows over an immobilised bed or column of the biocatalyst. This process can be carried out on different scales; however, a microreactor system can increase efficiency and make scale-up processes easier to control. Originally developed for chemical applications, microfluidic reactors offer advantages which include a reduction in cost, greater control of reaction parameters, hazard reduction and potential for scale-up. They also can be used for cascade reactions where a biocatalytic process is used in tandem with a chemical conversion, as required in the production of drugs in the pharmaceutical industries.

The microreactor system has been developed for two of the enzymes described above, the *Thermococcus litoralis* L-aminoacylase immobilised onto monoliths by amide coupling and the *Sulfolobus solfataricus* γ-lactamase made into cross-linked enzyme aggregates and packed into microreactor channels (Hickey et al. 2009).

As can be seen, different organisms and species use a variety of ways to stabilise their proteins. Some are of a general nature and others are specific for a particular protein. In general the increase in ion pairs within proteins is a general property associated with increases in thermostability. This is especially true for the hyperthermophiles which use this approach to stabilise their proteins often with multi-ion networks. An increase in hydrophobicity is also used as a general feature in thermophilic proteins but not seen as much in the hyperthermophiles since the contribution of hydrophobicity to stability decreases over 70°C.

In the case of the thermophilic bacterial *Thermus* species that have a high-GC content in their DNA, the proteins have evolved using an increase in the amino acid proline, with the codons CCC or CCG, at specific positions to make the protein more rigid and hence more thermostable. This is an example of co-evolution of high GC to stabilise the DNA and the introduction of proline to stabilise the proteins. We have to consider whether life originated at high temperatures and most of the proteins we know today have evolved to work at lower ambient temperatures. This idea is substantiated by the studies of Watanabe et al. (2006) who have developed a new method for designing thermostable proteins using phylogenetic trees of enzymes. In their study, they have investigated a method for designing proteins with improved stability using 3-isopropylmalate dehydrogenase (IPMDH) from *Thermus thermophilus* using mutant enzymes, each having an ancestral amino acid residue that was present in the common ancestor of bacteria and archaea.

19.5 Applications of Thermophilic Enzymes in White Biotechnology

Industrial biotechnology, known mainly in Europe as ‘white biotechnology’, is the application of biotechnology for industrial purposes.

19.5.1 Thermophilic Enzymes Important in Biocatalysis

The use of enzymes in industrial bio-processes is becoming increasingly important. This is to some extent driven by the pharmaceutical sector for the development of new therapeutic agents that are required to be enantiomerically pure compounds. Drug molecules often have several chiral centres and are difficult to synthesise by conventional chemistry. Their production relies increasingly on individual enzymatic steps which can be combined with chemical steps to make a more rapid and cost-effective process. In addition, when using enzymes, the chemistry is carried out in an environmentally friendly way using less harmful chemicals which need to be disposed of after use. The use of a biocatalytic step shows high performance under mild conditions minimising problems of isomerisation, racemisation, epimerisation and rearrangements reducing the amount of side products that occur during the process.

Enzymes are able to carry out reactions using both regio- and stereoselectivity. The number of chiral drug intermediates produced by enzymes was predicted to reach 70% by the end of 2010. In fact the number of industrialised biotransformations has doubled every decade since 1960.

One disadvantage of using enzymes rather than more traditional processes of chemical synthesis is that proteins are often not stable under the reaction conditions required for the industrial process. One approach to overcome this problem is to search for new novel enzymes from extremophiles, especially thermophilic and hyperthermophilic organisms that have increased stability not only to high temperatures but also to organic solvents and proteolytic enzymes. Although the rate of reaction is optimal for these enzymes at high temperatures, they usually have the ability to work at reduced rates at ambient temperature. The ability to carry out the biotransformation at elevated temperatures can be important especially when using non-natural substrates which can be insoluble at ambient temperatures.

There is a need to discover new activities from 'nature's catalysts' that are required for the chemical reactions that industry demands. The enzymes from thermophilic archaea often have novel activities that are distinct from their bacterial counterparts.

Several thermophilic archaeal enzymes have already been developed for industrial biocatalysis. The enzymes of interest can be cloned by screening of genomic libraries or by using direct PCR amplification from sequenced genomes. They can be over-expressed in *E. coli* in order to produce quantities that are required for biochemical characterisation and structural analysis. It is also possible to scale up production for industrial applications.

19.5.1.1 *Thermococcus litoralis* L-aminoacylase

Many pharmaceutically active structures are nitrogen-containing compounds which can be derived from either L- or D-amino acids. There is a large growth in the area of unnatural amino acids. A thermostable L-aminoacylase from *Thermococcus litoralis* has been cloned, sequenced and over-expressed in *E. coli* (Toogood et al. 2004). The *aminoacylase* gene was found upstream of a gene *pcp* coding for a novel cysteine protease pyroglutamyl carboxypeptidase (Singleton et al. 1999a, b, 2000) as described earlier. The aminoacylase enzyme is a homotetramer of 43 kDa monomers and has an 82% sequence identity to an aminoacylase from *Pyrococcus horikoshii* (Tanimoto et al. 2008) and 45% sequence identity to a carboxypeptidase from *Sulfolobus solfataricus* (Colombo et al. 1995). It contains one cysteine residue that is highly conserved among aminoacylases. Cell-free extracts of the recombinant enzyme have been characterised and were found to have optimal activity at 85°C in Tris-HCl pH 8.0. The recombinant enzyme is thermostable, with a half-life of 25 h at 70°C. Aminoacylase inhibitors, such as mono-tert-butyl malonate, had only a slight effect on activity. The enzyme was most specific for substrates containing *N*-benzoyl- or *N*-chloroacetyl-amino acids, preferring substrates containing hydrophobic, uncharged or weakly charged amino acids such as phenylalanine, methionine and cysteine (Toogood et al. 2002).

The thermostable archaeal L-aminoacylase from *Thermococcus litoralis* has been used in immobilisation trials to optimise its application in industrial biotransformation reactions (Toogood et al. 2002). Immobilisation techniques used included direct absorption and cross-linking of the enzyme onto solid supports, bioencapsulation and covalent bonding onto a variety of activated matrices. The most successful immobilisation methods were covalent binding of the enzyme onto glyoxyl-Sepharose and Amberlite XAD7. These methods yielded an average of 15 and 80 mg of protein bound per gram of support (wet weight for glyoxyl-Sepharose), respectively, with nearly 80% activity recovery in both cases. Enzyme immobilised onto glyoxyl-agarose was stabilised 106-fold under aqueous conditions and 142-fold in 100% acetonitrile when activity was measured after 24 h at 90°C. A column bioreactor containing the recombinant L-aminoacylase immobilised onto Sepharose beads was constructed with the substrate, *N*-acetyl-DL-Trp, continuously flowing at 60°C for 10 days. No loss of activity was detected over 5 days, with 32% activity remaining after 40 days at 60°C.

More recently the enzyme has been immobilised onto monoliths formed in microreactor channels (Ngamsom et al. 2010). The use of this microfluidic system is ideal for rapid substrate screening and overcomes any problems with substrate or product inhibition.

This enzyme is now used in a commercial biotransformation process (Taylor et al. 2004) by Dow Pharma web site and Dr. Reddy's dynamic resolution in cascade process with a racemase enzyme (Holt-Tiffin 2012; Baxter et al. 2012).

The recombinant *Thermococcus litoralis* L-aminoacylase has been purified to homogeneity. This zinc-containing enzyme has been crystallised in a form suitable for X-ray structural analysis. The crystals diffract to 2.8 Å resolution and belong to the rhombohedral space group R32 with unit cell parameters $a=b=102.4$, $c=178.5$ Å, $\gamma=120°$. The asymmetric unit contains one enzyme monomer. Two Synchrotron data sets have been collected at remote and maximum f′ wavelengths. The single zinc ion position has been identified in both the anomalous and isomorphous difference Patterson maps (Hollingsworth et al. 2002).

19.5.1.2 *Sulfolobus solfataricus* γ-lactamase

The resolution of the bicyclic synthon (racemic)-γ-lactam (2-azabicyclo[2.2.1]hept-5-en-3-one) is an important step in the synthesis of a group of chemotherapeutic agents known as carbocyclic nucleosides. The archaeon *Sulfolobus solfataricus* MT4 produces a thermostable γ-lactamase that was cloned, sequenced and overexpressed in *E. coli* (Toogood et al. 2004). It has a high sequence homology to the signature amidase family of enzymes. The enzyme was thermostable, with a half-life of 14 min at 80°C in the absence of the substrate. The γ-lactamase was found to selectively cleave the (+)-enantiomer of γ-lactam. It also exhibits general amidase activity by cleaving linear and branched aliphatic and aromatic amides. The enzyme catalyses the synthesis of benzoic hydrazide from benzamide preferentially to benzamide cleavage in the presence of excess hydrazine. This enzyme has potential for

use in industrial biotransformations in the production of both carbocyclic nucleosides and hydrazides.

Alignment of the amino acid sequences of the γ-lactamase from *Sulfolobus solfataricus* MT4 with four other amidases from *Pseudomonas chlororaphis* B23, *Rhodococcus* sp. N-771, *R. erythropolis* N-774 and *Rhodococcus rhodochrous* J shows it has a 41–44% sequence identity. These amidases belong to the signature amidase family as they all contain the sequence GGSS(S/G)GS. This family of enzymes hydrolyses amide bonds other than peptide bonds producing carboxylic acid and ammonia. The amino acid sequence of γ-lactamase contains the highly conserved putative catalytic residues aspartic and serine but not the highly conserved cysteine residue (Kobayashi et al. 1997).

This archaeal γ-lactamase has been purified to homogeneity (Waddell 2006). The molecular mass of the monomer was estimated to be 55 kDa by SDS-PAGE that is consistent with the calculated molecular mass of 55.7 kDa. Native molecular mass was determined to be 110 kDa by gel filtration indicating the enzyme exists as a dimer. The purified enzyme has been crystallised with a view to determining its three-dimensional structure.

The cross-linked, polymerised enzyme has also recently been packed into microreactors (Hickey et al. 2009). The thermophilic (+)-γ-lactamase retained 100% of its initial activity at the assay temperature, 80°C, for 6 h and retained 52% activity after 10 h, indicating the advantage of immobilisation. This high stability of the immobilised enzyme provided the advantage that it could be utilised to screen many compounds in the microreactor system.

19.5.1.3 *Aeropyrum pernix* Alcohol Dehydrogenase

Aeropyrum pernix is one of the most thermophilic aerobic archaeal species. The alcohol dehydrogenase (ADH) enzyme was amplified by PCR and over-expressed in *E. coli*. The *Aeropyrum pernix* ADH enzyme is a tetrameric, zinc-containing, type I ADH with a monomer size of 39.5 kDa (Guy et al. 2003). The sequence identity to horse liver ADH is 24%. The highest identity to a known structure is 39% to a medium-chain alcohol dehydrogenase from the hyperthermophilic archaeon *Sulfolobus solfataricus* (Esposito et al. 2002). The *Aeropyrum pernix* enzyme is highly specific for the cofactor NAD(H) and displays activity towards a broad range of alcohols, aldehydes and ketones, while appearing to show a preference for cyclic substrates. The enzyme is very thermostable with a half-life of 2 h at 90°C. The maximal activity is beyond 75°C; however, there is still 10% activity at 20°C. The enzyme is solvent stable with over 50% activity retained after incubation with 60% acetonitrile or dioxane. The enzyme is stabilised by an ion-pair cluster at the subunit interface, and a disulfide bond formed at a second zinc-binding site in the enzyme (Fig. 19.5). When the disulfide bond is not formed, then a bound zinc ion is able to stabilise the protein in this region. It has now been predicted that disulfide bonds do exist to stabilise many cytoplasmic proteins from *Aeropyrum pernix* (Mallick et al. 2002).

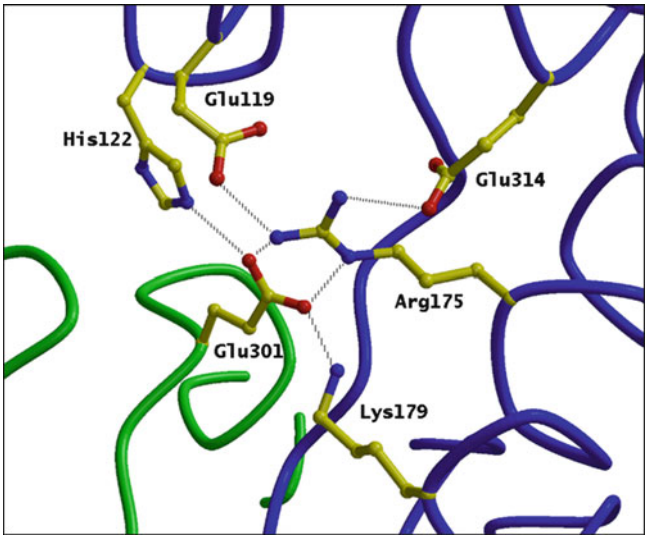

Fig. 19.5 The ionic bond network found in hyperthermophilic archaeon *Aeropyrum pernix* alcohol dehydrogenase (PDB code 1H2B) on the interface of subunits A (*blue*) and B (*green*) (Figure created using BobScript (Esnouf 1997) and rendered with Raster3D (Merritt and Bacon 1997))

The enzyme is active against primary and secondary alcohols with optimum chain length of C4–C5. It is most active to large cyclic alcohols such as cycloheptanol and cyclooctanol.

19.5.1.4 *Thermotoga maritima* Aldo-ketoreductase

A robust aldo-ketoreductase has been isolated and cloned from the thermophilic bacterium *Thermotoga maritima* (Willies et al. 2010). The aldo-ketoreductase can be used for production of primary alcohols using substrates including benzaldehyde, 1,2,3,6-tetrahydrobenzaldehyde and para-anisaldehyde. It is stable up to 80°C, retaining over 60% activity for 5 h at this temperature. The enzyme at pH 6.5 showed a preference for the forward, carbonyl reduction. The enzyme showed moderate stability with organic solvents and retained 70% activity in 20% (v/v) isopropanol or DMSO. These properties are favourable for its potential industrial applications.

19.5.1.5 *Thermus scotoductus* SA-01 Old Yellow Enzyme (OYE)

Despite having a conserved overall structure, there are mechanistic differences and a variation in substrate preference within the OYE family members (Brige et al. 2006; Chaparro-Riggers et al. 2007). Recently, a novel chromate reductase (CrS) from the thermophilic bacterium *Thermus scotoductus* SA-01, able to reduce the

carcinogen hexavalent chromium to the innocuous trivalent oxidation state through the oxidation of NAD(P)H (Opperman et al. 2008). *Thermus scotoductus* was isolated from the deep mines in South Africa. The CrS protein was found to be related to the OYE family, in particular the homologues YqjM and XenA. The structure of a thermostable OYE homologue in its holoform and its complex with pHBA has been reported (Opperman et al. 2010). This has provided information regarding its ability to reduce α,β-unsaturated carbonyl compounds.

Comparison of CrS with its closest mesophilic counterparts YqjM and XenA shows an increase in proline content (8.1%) for CrS, of which the majority was found within the loops and turns between the alternating core elements. This is typical for the *Thermus* bacterial species which have a high-GC content in their DNA (Wilquet and Van de Casteele 1999). The largest loop (40 amino acids) formed between helices b3 and a3 contains eight proline residues. An increase in intersubunit interactions through hydrogen bonding as well as ionic interactions is also observed for CrS. Three complex ionic networks are found at the dimerisation interface, of which two of these networks consist of five residues each and the third involving four residues (Opperman et al. 2010).

19.5.1.6 *Sulfolobus shibatae* Carboxylesterase

A thermostable carboxylesterase from *Sulfolobus shibatae* has been cloned, sequenced and over-expressed in *E. coli* (Toogood and Littlechild 2012). The enzyme has a 71–77% sequence identity to an esterase from *Sulfolobus tokodaii* and a carboxylesterase from *Sulfolobus solfataricus* strain P1, respectively (Morana et al. 2002). It contains the conserved putative catalytic triad residues Ser151, Asp244 and His274. Partially purified preparations of the enzyme have been characterised and found to have optimal activity towards *p*-nitrophenyl laurate at pH 6.5 at 85–90°C. The enzyme is almost completely inhibited by the serine hydrolase inhibitors phenylmethylsulfonyl fluoride and benzamidine and only partially inhibited by thiol reagents. The enzyme is thermostable, with no loss of activity detected after 24 h at 60°C. The enzyme was able to cleave a variety of *p*-nitrophenyl ester substrates, with the highest activity detected with *p*-nitrophenyl caproate.

The carboxylesterase was tested for its ability to cleave a variety of industrially relevant esters and diesters. It has a preference for substrates containing aromatic groups such as diethyl-2-benzyl malonate, benzyl acetoacetate and *Z*-phenylalanine methyl ester. However, it was also able to enantioselectively cleave 2-methyl-1,3-propanediol diacetate.

19.5.1.7 *Sulfolobus tokodaii* L-haloacid Dehalogenase

The enzyme has been cloned and over-expressed in *E. coli*. It has been characterised biochemically and structurally (Rye et al. 2007, 2009). The enzyme monomer has two domains. The core domain has a Rossmann fold with a six-stranded parallel

β-strand bundle surrounded by five α-helices and three 3_{10} helices. The subdomain is composed of α-helices. The active site is located between the two domains, and the native enzyme forms a dimer.

This enzyme has applications for chiral halo-carboxylic acid production and bioremediation. Chiral halo-carboxylic acids are important intermediates in the fine chemical/pharmaceutical industries. Many drugs are halogenated, and this is difficult to achieve chemically and can be carried out by haloperoxidase enzymes. Removal of the halogen group can be carried out by a dehalogenase. The *Sulfolobus* enzyme has the potential to resolve racemic mixtures of bromocarboxylic acids. The L-bromoacid dehalogenase catalyses the conversion of 2-halo-carboxylic acids to the corresponding hydroxyalkanoic acid. The *Sulfolobus tokodaii* dehalogenase has been shown to display activity towards longer-chain substrates than the bacterial *Xanthomonas autotrophicus* dehalogenase with activity seen towards 2-chlorobutyric acid which is due to a more accessible active site. The enzyme has a maximum activity at 60°C and a half-life of over an hour at 70°C. It is stabilised by a salt bridge and hydrophobic interactions on the subunit interface, helix capping, a more compact subdomain than related enzymes and shortening of surface loops. Other thermophilic enzymes of this family have addressed the problem of thermostability in different ways. *Pyrococcus* dehalogenase (29% sequence identity) solved from a structural genomics project is a monomeric structure stabilised by a disulfide bond (Arai et al. 2006).

19.5.1.8 *Sulfolobus solfataricus* Serine Transaminase

This pyridoxal phosphate-containing enzyme is involved in the non-phosphorylated pathway for serine synthesis which is not found in bacteria and is found in animals and plants. The transaminase reaction that the enzyme carries out is the conversion of L-serine and pyruvate to 3-hydroxypyruvate and alanine. Activity is also shown towards methionine, asparagine, glutamine, phenylalanine, histidine and tryptophan. The enzyme can be used in combination with transketolase for synthesis of chiral intermediates (Chen et al. 2006). The enzyme structure has been solved in the holo form of the enzyme and in complex with an inhibitor gabaculine and in a substrate complex with phenylpyruvate, the keto product of phenylalanine (Sayer et al. 2012). These studies have given some insight into the conformational changes around the active site of the enzyme that occur during catalysis and help to understand substrate specificity. The most related enzyme is the mesophilic yeast alanine-glyoxylate aminotransferase which shares 37% amino acid identity (Meyer et al. 2005). The yeast enzyme has 10 salt bridges compared to 21 salt bridges in the *Sulfolobus* serine transaminase enzyme which includes several 3–4 amino acid networks. There is a *C*-terminal extension in the *Sulfolobus* enzyme and shorter surface loops. The *Sulfolobus* transaminase dimer interface is hydrophobic in nature with few ionic interactions as shown in Fig. 19.6. The *Sulfolobus* serine transaminase is the first example of a thermophilic archaeal serine transaminase enzyme to be studied structurally and has implications for the commercial application of the enzyme for biotransformation reactions (Sayer et al. 2012).

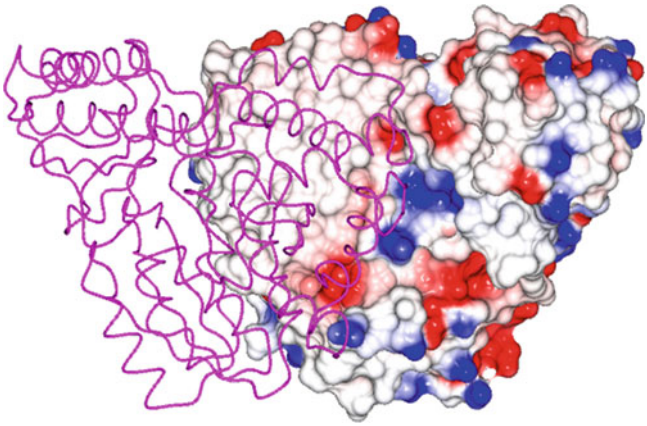

Fig. 19.6 A figure to show the mainly hydrophobic subunit interface of the dimeric thermophilic *Sulfolobus solfataricus* serine transaminase enzyme (PDB code 3ZRP). One subunit is shown in surface representation with the hydrophobic areas in *white*, the acidic areas in *red* and the basic areas in *blue*. The other subunit is shown in *purple C* α backbone representation (The figure was produced by CCP4 MG (Potterton et al. 2004))

19.6 Thermophilic Enzymes in Other Aspects of White Biotechnology

19.6.1 Biorefining

The conversion of biomass to ethanol in the production of biofuels has been enhanced by the utilisation of thermophilic enzymes organisms which can specifically breakdown lignocellulosic substrates (Blumer-Schuette et al. 2008; Miller and Blum 2010). The thermophilic *Anaerocellum thermophilum* has been shown to degrade cellulose, a major challenge to plant biomass degradation (Yang et al. 2009). *B*-glucosidase from *Pyrococcus horikoshii* was patented for the hydrolysis of cellulose in the synthesis of optically pure heterosaccharides (Antranikian et al. 2005).

The use of thermophilic enzymes in the paper and pulp industries has been described. Xylanase B from the hyperthermophile *Thermotoga maritima* is used in the biobleaching of wheat straw pulp, reducing the amount of chlorine needed in this process (Jiang et al. 2006). Recombinant laccase from *Thermus thermophilus* has also been applied to the biobleaching of wheat straw pulp with reduced amounts of hydrogen peroxide required (Zheng et al. 2012).

The starch-processing industry which converts starch into more valuable products such as glucose, fructose and trehalose has previously been carried out using mesophilic organisms. The use of thermophilic enzymes is particularly attractive as high temperatures are required to liquify starch to make it soluble for enzymatic hydrolysis. Numerous amylase, amylomaltase, glucoamylase, glucosidase,

pullulanase and pullulan-hydrolase enzymes have been identified from many thermophile and hyperthermophile organisms for industrial applications (Egorova and Antranikian 2005).

19.6.2 Biofuel Cells

Traditional fuel cells contain an anode where fuels such as hydrogen gas, methane and methanol are oxidised through a metal catalyst producing electrons. These electrons flow through an external circuit and are transferred to a cathode where they react with free protons to reduce oxygen to water. Biofuel cells are a potential candidate for clean and renewable energy conversion systems in which enzymes (whole organism or alone) are used instead of the metal catalyst. The biological catalysts are capable of performing natural oxidation reactions at the anode, resulting in electrons generating an electrical current (Bullen et al. 2006; Moehlenbrock and Minteer 2008). The use of thermophilic enzymes within such biofuel systems would allow them to operate under a wide variety of reaction conditions. The use of thermophilic recombinant enzymes from *Thermus thermophilus* in a fuel cell has been demonstrated using a pyrroloquinoline quinine-dependent glucose dehydrogenase and a laccase enzyme with glucose as the fuel source in the presence of oxygen. The electrodes consisted of single-walled carbon nanotubes with enzyme films showing enhanced biofuel-cell performance within a wide temperature range (Wang et al. 2012).

19.6.3 Detergents

Laundry detergents consist of water softeners, surfactants, oxidising agents, soaps, dirt suspension agents, brighteners and foam-controlling agents. The addition of enzymes to laundry detergents to aid cleaning has increased over the last couple of decades, with the detergent industry being the largest enzyme-consuming sector (Hasan et al. 2010). Methods and understanding in biotechnological applications have also improved, meaning it is now possible to modify protein structures to improve their stability. The most commonly used enzymes in detergents (Hasan et al. 2010) are proteases (to remove protein stains such as blood, grass, egg and sweat), amylases (to remove starch-based food), lipases (to remove fats) and cellulases (to remove cotton fibres and aid in fabric softening and colour brightening). Proteases are currently the most popular enzyme in the global industrial enzyme market constituting 60–65% of all enzymes produced (Kumar et al. 2008). These proteases are based on subtilisin mutant enzymes with increased thermostability. These enzymes originally come from a variety of *Bacillus* strains and have been engineered and are marketed by Novozymes (Savinase), Genecor (Properase, Purafect) and Henkel (BLAP S, BLAP X) as reviewed by Maurer (2004).

There are concerns over laundry components persisting in the environment and contaminate ground water. The replacement of standard laundry components with enzymes which are biodegradable, non-toxic and leave no harmful residues on clothes has allowed the detergent industry to move forwards to produce a more environmentally friendly and greener product (Kumar et al. 2008).

Laundry detergent formulations designed to work at high temperatures use relatively thermal stable lipases called Lumafast from the bacteria *Pseudomonas mendocina* and Lipolase from the fungus *Humicola lanuginosa* (Hoq et al. 1985; Jaeger et al. 1994). The lipolase enzyme was expressed in *Aspergillus oryzae* and was the first commercial lipase introduced by Nova Nordisk in 1994 (Hoq et al. 1985).

19.7 Conclusions

Our understanding of how nature stabilises proteins to high temperatures is increasing due to the rapidly increasing numbers of naturally thermostable proteins being studied at a structural level. Different species use difference mechanisms to address this problem. One of the most successful structural bioinformatics programmes has been carried out by attempting to solve the structures all of the proteins encoded by the *Thermus thermophilus* genome as reviewed by Yokoyama et al. (2000). Another structural bioinformatics project is being carried out with the hyperthermophilic archaeon *Methanococcus jannaschii* (Hwang et al. 1999). In fact thermophilic proteins, being more rigid, often crystallise more easily than mesophilic equivalents, and their structures are therefore used as models to provide a structural understanding for homologues from other species where crystallisation has been difficult or impossible. The DNA replication complexes and ribosome structures are important examples. The DNA replication apparatus of thermophilic archaea is similar to eukaryotic systems making structural information relevant to human enzyme systems.

The application of natural enzymatic processes to replace traditional chemistry and many other enzyme applications in biorefining, biofuel cells and detergents, for example, has driven the need for an increased understanding of enzyme stability and stabilisation. The use of enzymes for drug synthesis is advantageous not only for environmental issues but for the fact that the product of most enzyme reactions is optically pure and as such vital for the production of new safer drug molecules with fewer side effects.

Thermophilic proteins offer a source of new stable biocatalysts for many new developments in ‘white biotechnology’ worldwide.

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Chapter 20
Starch-Hydrolyzing Enzymes from Thermophiles

Skander Elleuche and Garabed Antranikian

Abstract Starch represents a ubiquitous molecule in plants and is composed of linear polymer amylose and branched polymer amylopectin. Due to its complex structure, it is insoluble in water and needs to be liquefied at high temperatures to make it a useable substrate for hydrolyzing biocatalysts. Hyper-/thermophilic microorganisms belonging to Archaea and Bacteria have been isolated from volcanically and geothermal-heated hydrothermal vent systems and were shown to be capable of utilizing natural polymeric compounds such as starch and cellulose as energy and carbon sources. During the last 25 years, considerable efforts have been made to shed light on structure-function relationships of starch-degrading thermoactive enzymes (extremozymes) and exploit these in various industrial processes. Mostly derived from Bacteria or Archaea, these biocatalysts are stable and highly active at temperatures up to 120°C even in the presence of high concentrations (99%) of organic solvents. A great portfolio of amylolytic enzymes enables these microorganisms to degrade polysaccharides into oligo- and monosaccharides. Such enzymes (e.g. amylases, glucoamylases, pullulanases and CGTases) have been employed in producing a series of valuable products. In this chapter, we will focus on starch-converting enzymes from thermophiles and their application in food, feed, textile, chemical, pharmaceutical and other industrial sectors.

Keywords Starch • Extremozymes • Thermophiles • Industrial application • Heterologous expression

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20.1 Introduction

Amylolytic hydrolases cleave glycosidic bonds between sugar molecules or between carbohydrate and non-carbohydrate moieties. Most of the biocatalysts (85%) with potential biotechnological applications belong to the enzyme class of hydrolases, and amylolytic enzymes (in particular α-amylases, glucoamylases and glucose isomerases) account for 30% of all bulk enzymes that are industrially produced. Thermostable starch-degrading enzymes play a role in the industrial production of glucose from starch, which is an energy-demanding two-step process (liquefaction and saccharification at high temperatures) and important in food, feed, pharmaceutical and chemical industries (Bertoldo and Antranikian 2002b).

There are two main strategies that are pursued to produce novel biocatalysts for possible applications: (I) the search for biocatalysts from microorganisms that are adapted to extreme environments and (II) the improvement of the process performance of enzymes that are not yet suitable for industrial application. Extremophilic microorganisms have developed various molecular strategies to survive under extremes of temperature (60–110°C), salinity (35% salt) or pH (0.5–11) and are therefore a valuable source of robust enzymes (extremozymes) (Eichler 2001). A challenge to the biotechnological application of extremozymes is to overcome the low production of these proteins in their natural hosts. The utilization of heterologous expression systems in mesophiles in most cases circumvents this problem and enables enzyme production in larger amounts. For this reason, the number of genes encoding heat-adapted starch-degrading enzymes provided to the industry has significantly increased in the last two decades. This chapter will briefly summarize the properties of thermoactive enzymes (active above 70°C) capable of starch degeneration and will focus on their production and application in various biotechnological processes.

20.2 Starch and Starch-Degrading Enzymes

Starch is one of nature's largest renewable carbon sources. It is a ubiquitous reserve compound in plants, deposited as large granules in the cytosol of cells and in seed. This polysaccharide is heterogeneous, composed of the two polymers amylopectin and amylose. Both are high-molecular-weight components, containing exclusively the single monomer α-D-glucose. Amylose accounts for 15–25% and is a polydisperse linear molecule composed of 1,000–6,000 α-1,4-linked glucopyranose residues, whereas the branched polymer amylopectin (75–85% of the polysaccharide) contains about 5% of α-1,6-linked branch points, occurring every 17–26 glucose units, in addition to α-1,4-glycosidic linkages. The compound amylopectin is composed of 6×10^6 molecules of glucose with the actual number of monomers depending on the plant source. Importantly, amylose chains are water insoluble and form hydrated micelles, while amylopectin is water soluble. As easily accessible

substrate, this polysaccharide is of tremendous value as a source of energy and nutrition. Genes encoding starch-degrading enzymes are widely distributed not only in microorganisms but also in animals and plants. Due to the complex structure of starch, it is obvious that cells require an appropriate portfolio of enzymes for its depolymerization and the production of small sugars, such as glucose and maltose.

Enzymes involved in starch degradation can be grouped into endo-acting enzymes (endohydrolases) and exo-acting enzymes (exohydrolases), respectively (Antranikian 1992). The group of glycosyl hydrolases is characterized by their ability to hydrolyze glycosidic bonds between two or more carbohydrates or between carbohydrate and non-carbohydrate moieties and is currently divided into 131 families based on amino acid similarities (Henrissat 1991). To date, starch-hydrolyzing enzymes have been assigned to glycoside hydrolase families 4, 13, 14, 15, 31, 49, 57, 63, 97, 119 and 122. Endohydrolases, such as α-amylases, catalyze the release of linear and branched intermediates of oligosaccharides, by acting on the inner part of the starch molecule in a random fashion, while exohydrolases produce defined end products by cleaving mono- or oligosaccharides from the nonreducing end of the starch molecule. Exohydrolases include glucoamylases, β-amylases, α-glucosidases and isoamylases. The final product glucose is widely used, e.g. for the industrial production of amino acids and ethanol or for the isomerization to fructose.

The ability to bind to the insoluble form of starch is of crucial importance for the performance of these enzymes. Moreover, the binding needs to be a reversible process. To meet these requirements, starch-binding domains (SBD) were evolved. These structural highly diverse protein parts can be either located at the N- or C-terminal region of the enzyme. Furthermore, SBDs have been described to enhance the enzymatic activity towards starch granules up to 100-folds, and they have been suggested to govern the thermostability of α-amylases (Iefuji et al. 1996).

20.3 Thermophiles and Their Repertoire of Starch-Hydrolyzing Enzymes

Extremophilic microorganisms survive in environments that are considered as extreme from a human point of view. Microbial communities have been found at extremes of temperature, acidity, alkalinity, pressure or salinity. Due to the cellular complexity, it is very likely that higher organisms are unable to live under these extreme conditions. The temperature range of microbial growth has been accurately defined as psychrophilic (below the freezing point of water to ~20°C), mesophilic (moderate temperatures) and thermophilic (above 45°C). The huge group of thermophiles mainly include members of the Bacteria and Archaea, while some moderate thermophiles (growing between 50 and 60°C) have also been identified within the eukaryotes belonging to fungi, protozoa and algae (Bertoldo and Antranikian 2002b). Additionally, microorganisms growing at temperatures between 60 and 80°C are defined as extreme thermophiles. Hyperthermophiles grow optimally above 80°C;

the highest growth temperature seems to be around 110°C. The hyperthermophiles are dominated by the Archaea, which are currently divided into the two main phyla: Crenarchaeota and Euryarchaeota, but also three further groups have been recently proposed: Korarchaeota, Nanoarchaeota and Thaumarchaeota (Gupta and Shami 2011). Interestingly, some members of the Archaea are also able to survive at two combined extreme conditions; the so-called thermoacidophilic microorganisms can stand high temperatures and low pH (50–90°C and pH 0–4). Thermoacidophilic species have been found within the genera *Picrophilus*, *Thermoplasma*, *Sulfolobus* and others (Egorova and Antranikian 2007).

Representatives of extremophiles usually provide an interesting enzyme system. In comparison to their mesophilic counterparts, the stability of their proteins may not only be increased towards high temperatures but also towards pressure, high or low pH and salinity. Furthermore, extremozymes are usually more stable in the presence of detergents, heavy metals and organic solvents (Egorova and Antranikian 2007). These enzymatic properties are attractive for the utilization of extremozymes in a variety of biotechnological applications in which extreme conditions prevail. During the last three decades, primarily thermophilic microorganisms have been proven to be capable of hydrolyzing polysaccharidic substrates as energy and carbon sources, and their enzymes have been intensively studied. Starch-degrading enzymes from thermophilic fungi have been shown to be catalytically active at temperatures up to 80°C. Members of extremophilic Archaea and Bacteria facilitate the enzymatic degradation of carbohydrates above 100°C, and the most heat-active enzymes display catalytic activity even at 140°C (Bertoldo and Antranikian 2001, 2002a).

20.3.1 α-Amylases

The α-amylases (EC 3.2.1.1, α-1,4-glucan-4-glucanohydrolase) are endo-acting enzymes, randomly cleaving α-1,4-linkages in the interior of the starch molecule and related substrates, which yield in the formation of branched and linear α-anomeric oligo- and polysaccharides of varying sizes. Members of the α-amylases have been grouped into the glycoside hydrolase families 13 and 57 and very recently into family 119 (Henrissat 1991). These enzymes are divided into three domains (A, B and C). Domain A is located at the N-terminal part of the full-length protein and contains a highly symmetrical $(\beta/\alpha)_8$-barrel domain. This barrel is made up of eight β-sheets encircled by eight α-helices, while domain B is composed of an extension between the third α-helix and β-sheet of the barrel. This specific domain is highly diverse among amlyases and has been proposed to be important for substrate specificity (Prakash and Jaiswal 2010). Finally, domain C forms a β-sandwich and is located at the C-terminal part of the protein. The catalytic acid (proton donor) and the catalytic base (nucleophile) in α-amylases comprise a glutamate and an aspartate residue. Only six further amino acid residues are invariant in the α-amylase

family (Egorova and Antranikian 2007). Using phylogenetic approaches, Leveque et al. demonstrated that archaeal α-amylases are evolutionary related to their counterparts of plant origin (Leveque et al. 2000).

Thermostable α-amylases have been identified both in pro- and eukaryotic species, including plants, fungi, animals and microbes (Prakash and Jaiswal 2010). Most of the characterized enzymes are generally active in a broad pH range between 3.5 and 9.0 and exhibit a pH optimum in a low acidic range (pH 5.0–6.0). The α-amylases from *Thermococcus* species usually display less thermophilic properties than their *Pyrococcus* counterparts, reflecting their preferred growth conditions (Leveque et al. 2000). Exceptions are also existing, the isozyme characterized from the mesophilic bacterium *Bacillus licheniformis* is optimally active at 92°C (Kindle 1983). The most heat-active α-amylases with optimal temperature between 90 and 100°C have been identified in species of the genus *Pyrococcus* and in *Methanococcus jannaschii* (Table 20.1). Enzymes with lower thermostabilities and temperature optima around 70–80°C have been isolated from various bacteria of the genera *Bacillus* and *Geobacillus* but also from lower eukaryotes (Burhan et al. 2003; Ezeji and Bahl 2006; Kelly et al. 1985; Kindle 1983; Petrova et al. 2000). In particular, representatives derived from species of the genera *Bacillus* and *Aspergillus* are commercially produced in bulk for industrial applications.

In 1990, an extracellular enzyme from the hyperthermophilic archaeon *Pyrococcus furiosus* with a temperature optimum around 100°C has been isolated and characterized (Koch et al. 1990). This α-amylase exhibits enzymatic activity at a temperature range between 40 and 130°C and was not able to attack short chain oligosaccharides, but it converts starch and glycogen to small linear and branched oligosaccharides. Moreover, the respective gene has been amplified and expressed in another heterologous host in *Bacillus subtilis*. In this report, the expression of a gene from an archaeal extremophile in *B. subtilis* has been described for the first time (Jorgensen et al. 1997). Since most of these amylolytic enzymes are extracellular, they are even more interesting for industrial applications, because they are not stabilized by intracellular factors. Nevertheless, the microbial degradation of starch is affected by a variety of abiotic factors influencing the enzymatic performance. In a further attempt, another enzyme has been purified from *P. furiosus*, and the gene encoding this intracellular α-amylase has been cloned and expressed in *Escherichia coli*. The heterologously produced enzyme displayed a comparable temperature profile to the purified protein from its natural host *P. furiosus* (Laderman et al. 1993a, b). Interestingly, the highly conserved regions usually found in α-amylases were not identified in this enzyme. In 2001, Kim et al. described the gene *MJ1611*, encoding a GH-family 57-like α-amylase MJA1 isolated from the hyperthermophilic archaeon *M. jannaschii* (Kim et al. 2001). This enzyme was partially purified after heterologous production in *E. coli* and displayed a temperature optimum of 120°C using amylose as substrate (temperature range between 50 and 140°C) and was extremely stable against denaturants. Unlike to mesophilic counterparts, no heat activation was observed for MJA1 and the activity was not dependent on calcium. The hydrolysis of large sugar polymers resulted in the production of oligomers of 1–7 glucose units (Kim et al. 2001).

Table 20.1 Thermoactive starch-degrading α-amylases from Bacteria and Archaea

Source	T_{opt} (°C)	pH	MW (kDa)	Reference
Bacillus amyloliquefaciens	70	7	43	Kikani and Singh (2011)
Bacillus sp. 11–15	70	2.0	54	Kindle (1983)
Bacillus sp. Ferdowsicous	70	4.5	53	Asoodeh et al. (2010)
Lipomyces kononenkoae	70	4.5–5.0	76	Prieto et al. (1995)
Lipomyces starkeyi	70	4.0	76	Kelly et al. (1985)
Thermoactin omyces vulgaris	70	5.0	–	Kindle (1983)
Thermomyces lanuginosus	70	5.0	58	Petrova et al. (2000)
Chloroflexus aurantiacus	71	7.5	210	Ratanakhanokchai et al. (1992)
Alicyclobacillus acidocaldarius	75	3.0	160	Schwermann et al. (1994)
Thermotoga neapolitana	75	6.5	48	Park et al. (2010)
Bacillus brevis	80	5.0–9.0	58	Tsvetkov and Emanullova (1989)
Bacillus mojavensis	80	6.5	58	Hmidet et al. (2010)
Bacillus sp. ANT-6	80	10.5	94.5	Burhan et al. (2003)
Bacillus stearothermophilus	80	5.2–6.5	58	Brumm et al. (1988)
Geobacillus sp. IIPTN	80	5	97	Dheeran et al. (2010)
Geobacillus sp. LH8	80	5.0–7.0	52	Mollania et al. (2010)
Geobacillus thermodenitrificans	80	5.5	58	Ezeji and Bahl (2006)
Thermococcus profundus	80	5.0–6.0	43	Chung et al. (1995)
Bacillus coagulans CUMC512	85	–	–	Kindle (1983)
Rhodothermus marinus	85	6.5–7.0	–	Gomes et al. (2003)
Thermotoga maritima	85–90	7.0	61	Liebl et al. (1997)
Bacillus licheniformis	90	9.0	28	Krishnan and Chandra (1983)
Dictyoglomus thermophilum	90	5.5	81	Fukusumi et al. (1988)
Thermotoga maritima	90	8.5	241	Ballschmiter et al. (2006)
Pyrococcus furiosus	90	4.5	76	Yang et al. (2004)
Thermococcus sp.	95	5.0	51	Wang et al. (2008)
Thermus filiformis	95	5.5–6.0	60	Egas et al. (1998)
Geobacillus thermoleovorans	100	8.0	26	Uma Maheswar Rao and Satyanarayana (2007)
Pyrococcus furiosus	100	5.5–6.0	100	Koch et al. (1990)
Pyrococcus woesei	100	5.5	68	Koch et al. (1991)
Methanococcus jannaschii	120	5.0–8.0	>100	Kim et al. (2001)

Another extracellular α-amylase from the anaerobic hyperthermophilic (optimal growth at 100–103°C) archaeon *Pyrococcus woesei* also exhibited comparable biochemical properties (Koch et al. 1991). The gene has been expressed in *E. coli* and *Halomonas elongata*, and the respective protein has been crystallized and constituted the first molecular structure of an α-amylase from a hyperthermophilic archaeon (Linden et al. 2000, 2003; Frillingos et al. 2000). The structure was used to identify molecular parameters supporting the adaptation to extreme environmental conditions. The enzyme is dimeric with a molecular mass of 50 kDa for each subunit. As it has been hypothesized from structures of other heat-adapted proteins, the α-amylase

Table 20.2 Thermoactive β-amylases from pro- and eukaryotes

Source	T_{opt}	pH	MW (kDa)	Reference
Ipomoea batatas	70	–	–	Germain and Crichton (1988)
Salimicrobium halophilum	70	10	81	Li and Yu (2012)
Clostridium thermosulfurogenes	75	5.5	210	Shen et al. (1988)
Bacillus stearothermophilus	80	6.9	57	Srivastava (1987)

exhibited an increase in salt bridges and showed a highly compact organization (Linden and Wilmanns 2004). Furthermore, a conserved calcium ion, which is known to be important for activity and thermostability in heat-active enzymes, is located at the interface between domain A and B. Since this calcium ion is too far from the catalytic region, it has been proposed to play solely a structural role. However, every time it has been achieved to remove this metal ion, related α-amylases lost their catalytic activities (Prakash and Jaiswal 2010). Several α-amylases have also been described that do not require calcium for catalytic activity and stability. The hyperthermostable maltogenic isozyme of the bacterium *Geobacillus thermoleovorans* has been shown to be optimally active at 100°C and pH 8.0 in a calcium-independent manner, but the enzymatic activity was even stimulated by the addition of several other metal ions (Table 20.1). *G. thermoleovorans* α-amylase was capable of utilizing dry and soluble starch as substrate. Under these conditions, the enzyme was stable for 10 h and the half-life increased in the presence of cholic acid (Uma Maheswar Rao and Satyanarayana 2007).

20.3.2 β-Amylases

The enzyme β-amylase (EC 3.2.1.2, α-1,4-D-glucan maltohydrolase or saccharogen amylase) attacks every alternate α−1,4-glucosidic linkage of the starch molecule, thereby producing the dimeric sugar compound β-maltose by an inversion of the anomeric configuration. β-amylase belongs to the group of exo-acting hydrolases, and all known isozymes are grouped into glycosyl hydrolase family 14, except for a family 57 β-amylase identified in *P. furiosus*. A microarray analysis for this heterotrophic, hyperthermophilic archaeon, grown on α-glucans as sole carbon source, led to the identification of the novel protein PF0870, which was most active on *p*NP-α-maltopyranoside (Comfort et al. 2008). In addition, PF0870 was capable of hydrolyzing maltotriose into maltose and glucose, but no activity has been observed using starch, glycogen, pullulan or large maltooligosaccharides as substrates. To date, only a few thermoactive β-amylases have been characterized (Table 20.2).

Most of the isozymes with industrial relevance are derived from plants and do not display heat adapted properties. They usually exhibit temperature optima in a moderate range with a few exceptions as shown for the β-amylase isolated from the eudicotyledonous plant *Ipomoea batatas*, which is optimally active at 70°C (Germain and Crichton 1988).

20.3.3 Glucoamylases

Glucoamylase (EC 3.2.1.3, amyloglucosidases, glucan 1,4-α-glucosidase or γ-amylase) follows an inverting mechanism. This enzyme is capable of successively releasing single β-D-glucose units from nonreducing ends by cleaving α-1,4-glycosidic bonds from starch or related oligo- and polysaccharides, indicating that it is an exo-glycosyl hydrolase. Moreover, glucoamylase hydrolyzes all other α-glycosidic linkages with lower efficiency between glycosyl residues except that of α,α-trehalose, which is not cleaved at all (Kumar and Satyanarayana 2009). This enzyme is also capable of hydrolyzing α-1,3- and α-1,6-gylcosidic bonds in high-molecular-weight polysaccharides. According to CAZy database, glucoamylases can be exclusively assigned to glycoside hydrolase family 15 (Henrissat 1991).

Although the crystal structures of glucoamylases from eukaryotes and the recently solved structure of the moderate thermoactive (optimal temperature range between 40 and 65°C) two-domain isozyme of the clostridial species *Thermoanaerobacterium thermosaccharolyticum* exhibit some differences, the catalytic domain is highly similar. It is composed of an $(\alpha/\alpha)_6$-barrel with a bottle-shaped active site at the C-terminus (Aleshin et al. 2003; Ganghofner et al. 1998). Two highly conserved glutamate residues act as the catalytic acid and base. These amino acid residues were found in glucoamylases of prokaryotic and yeast origin, indicating that the enzymes follow the same inverting reaction mechanism (Kumar and Satyanarayana 2009).

Most of the glucoamylases known so far are fungal enzymes, and some of them are thermoactive. The first gene encoding a glucoamylase has been identified in the filamentous fungus *Aspergillus niger* (Svensson et al. 1983). In the meantime, several fungal glucoamylases which exhibit high temperature optima and heat-adapted stability profiles have been described. The 599 amino acid glucoamylase from *Chaetomium thermophilum* shows an optimal temperature at 65°C and was completely stable at 60°C after heterologous expression in the yeast *Pichia pastoris* (Chen et al. 2007). These enzymes often display further characteristics that make them applicable at harsh conditions. Recently, a glucoamylase from *Aspergillus niveus*, which is optimally active at 65°C and pH 5.0–5.5, has been described to remain completely active in acetone and methanol and stable at 60°C for 4 h (da Silva et al. 2009). Moreover, the glucoamylase TGA from *Thermomucor indicae-seudaticae* has been extensively studied. This enzyme is optimally active at 60°C and at a pH of 7.0 (Kumar et al. 2010; Kumar and Satyanarayana 2003, 2009). The adaptation to neutral pH is a very rare feature among the glucoamylases from microorganisms, which are mostly active under acidic conditions (Table 20.3).

Interestingly, filamentous fungi often produce multiple glucoamylases, which are encoded by a single gene. These isoforms are the result of alternative splicing mechanisms or of limited proteolytic cleavage of an immature enzyme variant. In contrast to this, a group of unlinked glucoamylase encoding genes were found within the genomes of hemiascomycetous yeasts (Kumar and Satyanarayana 2009). Comparable to the yeast glucoamylase family, a set of three genes has also been found in the genomes of some Archaea, including the thermoacidophilic species

Table 20.3 Bacterial and archaeal glucoamylases active at elevated temperatures

Source	T_{opt}	pH	MW (kDa)	Reference
Clostridium thermosaccharolyticum	70	5.0	75	Specka et al. (1991)
Bacillus sp.	70	5.0	–	Gill and Kaur (2004)
Beta vulgaris	70	4.4	83	Masuda et al. (1988)
Hypocrea jecorina	70	–	–	Norouzian et al. (2006)
Myrothecium sp.	70	4.0	72	Ali et al. (1994)
Myrothecium sp.	70	4.0	96	Ali et al. (1994)
Scytalidium thermophilum	70	5.5	75	Aquino et al. (2001)
Streptosporangium sp.	70	4.5	–	Stamford et al. (2002)
Talaromyces emersonii	70	4.0–4.5	63	Nielsen et al. (2002)
Thermomyces lanuginosus	70	5.0	66	Thorsen et al. (2006)
Thermomyces lanuginosus F_1	70	6.0	70	Odibo and Ulbrich-Hoffmann (2001)
Thermomyces lanuginosus	70	4.4–5.6	75	Nguyen et al. (2002)
Trichoderma reesei	70	–	–	Norouzian et al. (2006)
Thermoanaerobacter tengcongensis	75	5.0	77	Zheng et al. (2010)
Thermoplasma acidophilum	75	5.0	140	Dock et al. (2008)
Methanococcus jannaschii	80	6.5	–	Uotsu-Tomita et al. (2001)
Sulfolobus solfataricus	90	5.5–6.0	250	Kim et al. (2004)
Picrophilus oshimae	90	2.0	140	Serour and Antranikian (2002)
Picrophilus torridus	90	2.0	133	Serour and Antranikian (2002)
Thermoplasma acidophilum	90	2.0	141	Serour and Antranikian (2002)

Picrophilus torridus (Fütterer et al. 2004). Although Archaea and Bacteria are usually a rich source of α-amylases, the abundance of glucoamylases seems to be very rare. Only a few prokaryotic glucoamylases have been investigated so far, and to the best of our knowledge, no heat-adapted isozyme of bacterial origin displays an optimal temperature above 75°C. The most thermostable bacterial glucoamylase described to date has been identified in *Thermoanaerobacter tengcongensis*. TtcGA is optimally active at 75°C and pH 5.0 on maltooligosaccharides with four monosaccharide units (Zheng et al. 2010). The first archaeal glucoamylases were purified from the thermoacidophilic species *Picrophilus oshimae*, *P. torridus* and *Thermoplasma acidophilum* (Table 20.3). These enzymes are more thermostable than the glucoamylases of bacterial and fungal origin described so far. They are secreted into the medium and are optimally active at 90°C. Since these organisms usually grow under extreme acidic pH conditions (*P. torridus* thrives at 65°C and pH 0), the purified enzymes are acid stable at pH 1.0 (Serour and Antranikian 2002). Additionally, these enzymes remain catalytically active even at 100°C and pH 0.5. Moreover, the *P. torridus* extracellular glucoamylase does not lose activity after incubation at 70°C and pH 1.0–4.0, while it was rapidly inactivated at pH 8.0. In contrast to most organisms that live at extreme pH, *P. torridus* does not maintain its internal pH in a neutral range but exhibits an intracellular pH value of pH 4.6

(Ciaramella et al. 2005). Therefore, it was not surprising that an intracellular glucoamylase could be purified after expression in *E. coli* that was optimally active at pH 5.0, but this enzyme was not as thermoactive as its extracellular counterpart and displayed a temperature optimum at 50°C (Schepers et al. 2006). Another gene (*taGA*) encoding a thermoactive glucoamylase has been identified within the fully sequenced genome of the thermoacidophilic euryarchaeon *T. acidophilum* and was heterologously expressed in *E. coli*. In addition to the previously described extracellular glucoamylase activity, *taGA* encodes a second isozyme, which remains to be located in the intercellular region. TaGA hydrolyzes a variety of polysaccharides, but maximal activity was measured towards amylopectin between 40 and 90°C and the temperature optimum is 75°C (Dock et al. 2008). This observation is in contrast to the majority of investigated glucoamylases isolated from Archaea and Bacteria, which usually have a preference towards smaller molecules (Kim et al. 2004; Uotsu-Tomita et al. 2001; Schepers et al. 2006). In the presence of calcium ions, TaGA was even capable of hydrolyzing amylopectin at 100°C, and the thermostability was increased at temperatures up to 90°C (Dock et al. 2008). Furthermore, third open reading frames encoding putative glucoamylases have been identified within the genomes of *T. acidophilum* and *P. torridus* (unpublished results). The extremely thermostable glucoamylase from *Sulfolobus solfataricus* was purified after expression in *E. coli*. This tetrameric enzyme has an optimal temperature of 80°C and prefers maltotriose as substrate (Kim et al. 2004).

20.3.4 α-Glucosidases

α-glucosidase (EC 3.2.1.20, α-D-glucoside glucohydrolase) is capable of sequentially breaking every α-1,4-glycosidic bond from the terminal nonreducing end of the starch molecule or of smaller polysaccharides, which are produced by the catalytic activity of other starch-degrading enzymes. Thereby this enzyme liberates free monomeric glucose with an α-anomeric configuration. α-glucosidase is involved in the last step of starch degradation. Unlike glucoamylases, di- or oligosaccharides (e.g. maltotriose) are favoured as substrates, while polysaccharides are only slowly or even not at all hydrolyzed. These enzymes have been assigned to glycosyl hydrolase families 4, 13, 31, 63, 97 and 122 (Henrissat 1991). Heat-active thermostable α-glucosidases are extremely diverse in their molecular weights, and with few exceptions, their optimal pH is fairly acidic (Leveque et al. 2000).

Highly active heat-stable α-glucosidases have been identified and characterized from several Archaea (Table 20.4). The *malA* gene from the hyperthermophilic archaeon *S. solfataricus* was the first open reading frame encoding an α-glucosidase to be expressed in the heterologous host *E. coli*. MalA hydrolyzed *p*-nitrophenyl-α-D-glucopyranoside at a temperature of 85°C (Rolfsmeier et al. 1998). A gene (*aglA*) encoding a putative α-glucosidase has also been found in the genome of *P. torridus*. Activity assays after expression of the gene in *E. coli* proved the enzyme to be capable of hydrolyzing maltose and longer α-1,4 linked maltooligosaccharides with

Table 20.4 Heat-stable α-glucosidases

Source	T_{opt}	pH	MW (kDa)	Reference
Geobacillus toebii	70	6.8	45	Cihan et al. (2012)
Thermoascus aurantiacus	70	4.5	83	Carvalho et al. (2010)
Bacillus sp. SAM1606	75	5.5	64	Nakao et al. (1994)
Bacillus thermoglucosidius	75	5.0–6.0	55	Suzuki et al. (1976)
Clostridium thermohydrosulfuricum	75	5.0–5.5	–	Saha and Zeikus (1991)
Sulfolobus solfataricus	75	–	310	Giuliano et al. (2004)
Bacillus thermoamyloliquefaciens	80	–	540	Kashiwabara et al. (2000)
Sulfolobus shibatae	85	5.5	313	di Lernia et al. (1998)
Picrophilus torridus	87	5.0	440	Angelov et al. (2006)
Thermotoga maritima	90	7.5	110	Raasch et al. (2000)
Thermus caldophilus	90	6.5	90	Nashiru et al. (2001)
Thermus thermophilus	90	6.2	132	Alarico et al. (2008)
Thermococcus sp. AN1	98	7.0	60	Piller et al. (1996)
Sulfolobus solfataricus	105	4.5	80	Rolfsmeier and Blum (1995)
Pyrococcus furiosus	105	5.0–6.0	125	Costantino et al. (1990)
Pyrococcus furiosus	105–115	5.5	125	Chang et al. (2001)
Pyrococcus woesei	105	–	–	Chang et al. (2001)
Thermococcus hydrothermalis	110	5.5	57	Leveque et al. (2000)

a temperature optimum of 87°C in an exo-type cleavage manner (Angelov et al. 2006). Another open reading frame, *TtGluA*, encodes a moderate thermostable α-glucosidase in the anaerobic thermophilic species *T. tengcongensis*. It has been shown that the enzyme is most active at 60°C on short chain maltooligosaccharides and displayed regioselectivity to produce high yields of α-1,6-linked isomaltooligosaccharides (Zhou et al. 2009). Several α-glucosidases have been directly purified from their host organisms as well. The extracellular α-glucosidase of the thermophilic archaeon *Thermococcus* strain AN1 was still active at 130°C (Piller et al. 1996). Recently, Carvalho et al. described a novel heat-active α-glucosidase purified from the industrial-relevant ascomycetous thermophilic fungus *Thermoascus aurantiacus* with maximum activity observed at 70°C, which is among the most thermoactive isozymes isolated from fungi at all. This α-glucosidase was remarkably stable against various mono- and divalent metal ions and in a pH range of 3.0–9.0. Moreover, the isozyme generates oligosaccharides by transglycosylation reactions (Carvalho et al. 2010).

20.3.5 Pullulanases

Enzymes attacking the α-glucosidase resistant α-1,6-glycosidic bonds in the linear α-glucan polymer pullulan are defined as pullulanases (EC 3.2.1.41). According to

CAZy, all known thermoactive pullulanases belong either to glycosyl hydrolase family 13 or 57, while mesophilic pullulanases exclusively belong to family 13 (Henrissat 1991). Pullulan is an exopolysaccharide produced by the ascomycetous fungus *Aureobasidium pullulans* and is composed of 480 maltotriose units. It is industrially produced and is used in food processing and allied industries (Wu et al. 2010). Two groups of pullulanases have been introduced and are grouped by their substrate specificities. Type I pullulanase (debranching enzyme) produces linear oligosaccharides in addition to maltotriose by the hydrolyzation of α-1,6-glycosidic bonds in pullulan and branched oligosaccharides. In contrast to this type, enzymes belonging to the group of type II pullulanases (amylopullulanase) exhibit a dual specificity towards α-1,6-linkages in pullulan and α-1,4-glycosidic bonds in linear and branched oligosaccharides. Since these enzymes exhibit multiple specificities, the degradation products of polysaccharides (in the absence of other starch-degrading enzymes) are small sugar compounds with depolymerization degrees between DP_1 and DP_4. Pullulanases usually seem to have a high molecular weight (approx. 100 kDa) with gene length usually ranging between 3,000 and 5,000 bp. The catalytic region of the GH57 family amylopullulanase from *Thermococcus hydrothermalis* has been intensely investigated. Amino acid residues Glu_{291} and Asp_{394} have been identified as the conserved nucleophile and proton donor and were validated by site-directed mutagenesis, resulting in catalytic inactivity. Furthermore, five highly conserved regions have been predicted and were postulated to be consensus sequences for glycoside hydrolase 57 family proteins (Zona et al. 2004). These data strongly imply that the bifunctional activity of amylopullulanase on starch and pullulan are clearly determined by a single catalytic domain (Egorova and Antranikian 2007).

Several heat-active thermostable pullulanases have been investigated from thermophilic microorganisms and most of the enzymes described so far belong to pullulanase type II. In general, this group of enzymes exhibits a pH optimum between 5.5 and 6.5 (Table 20.5). More than a decade ago, pullulytic activities with temperature optima between 90 and 100°C have been detected in strains belonging to the orders *Thermococcales* and *Thermoproteales* (Brown and Kelly 1993; Canganella et al. 1994). In the presence of calcium, thermostability was even enhanced and pullulytic activity was measurable at temperatures up to 140°C (Brown and Kelly 1993). Another extremely heat-stable pullulanase type II from *P. woesei* has been expressed in *E. coli* and was investigated in detail (Rüdiger et al. 1995; Schwerdtfeger et al. 1999). This monomeric enzyme exhibits a molecular mass of 90 kDa and is optimally active at 100°C, while temperature elevation up to 120°C still resulted in 40% remaining activity. Interestingly, the addition of calcium ions caused enzyme activation up to 370% and stabilized this pullulanase against thermal inactivation (Schwerdtfeger et al. 1999). Subsequently, a thermostable type II pullulanase-encoding gene, *apu*, has been identified in *Thermoanaerobacter ethanolicus,* and bifunctional activities of α-amylase and pullulanase were described for the recombinant amylopullulanase. Pullulan was identified as the preferred substrate, with the catalytic temperature optimum at 80°C and calcium mediated activation of enzymatic activity (Lin and Leu 2002). Recently, another glycosyl hydrolase family 57 amylopullulanase has been investigated from the deep-sea archaeon *Thermococcus siculi* and was shown to be active at 95°C. The optimal pH conditions for pullulanase and amylase activities were pH 6.0

Table 20.5 Pullulanases optimally active above 70°C

Source	T_{opt}	pH	MW (kDa)	Reference
Anaerobranca gottschalkii	70	8.0	96	Bertoldo et al. (2004)
Geobacillus thermoleovorans	70	6.0	160	Zouari Ayadi et al. (2008)
Thermoactinomyces thalpophilus	70	7.0	79	Odibo and Obi (1988)
Thermus thermophilus	70	5.5–6.0	80	Tomiyasu et al. (2001)
Thermus caldophilus	75	5.5	65	Kim et al. (1996)
Fervidobacterium pennavorans	80	6.0	93	Bertoldo et al. (1999)
Rhodothermus marinus	80	6.5–7.0	–	Gomes et al. (2003)
Thermoanaerobacter ethanolicus	80	6.0	109	Lin and Leu (2002)
Thermotoga neapolitana	80	5.0–7.0	93	Kang et al. (2011)
Clostridium thermohydrosulfuricum	85	5.5–6.0	–	Hyun and Zeikus (1985)
Desulfurococcus mucosus	85	5.0	132	Duffner et al. (2000)
Fervidobacterium pennavorans	85	6.0	190	Koch et al. (1997)
Bacillus sp. AN-7	90	6.0	106	Kunamneni and Singh (2006)
Clostridium thermohydrosulfuricum	90	5.0–5.5	136.5	Saha et al. (1988)
Thermoanaerobacter finnii,	90	5.0–6.0	–	Koch et al. (1987)
Thermobacteroides acetoethylicus	90	5.0–6.0	–	Koch et al. (1987)
Thermococcus celer	90	5.5	–	Canganella et al. (1994)
Thermotoga maritima	90	6.0	96.3	Bibel et al. (1998)
Thermococcus siculi	95	6.0	148.6	Jiao et al. (2011)
Pyrococcus furiosus	98	5.5	110	Brown and Kelly (1993)
Thermococcus litoralis	98	5.5	119	Brown and Kelly (1993)
Desulfurococcus mucosus	100	5.0	–	Canganella et al. (1994)
Pyrococcus woesei	100	6.0	90	Rüdiger et al. (1995)
Thermococcus aggregans	100	6.5	–	Canganella et al. (1994)

and 5.0, respectively. The functional N-terminal domain (773 amino acids) was produced in a heterologous host in *E. coli* to circumvent problems that have arisen by the expression of the intact gene encoding a protein of 1,342 amino acid residues. Moreover, truncation of the C-terminal region harbouring two carbohydrate binding domains and a threonine rich region even increased the thermostability and the optimal temperature (100°C) of the enzyme, indicating that the C-terminal domain might interfere with the thermoactivity (Jiao et al. 2011). Furthermore, the glycoside hydrolase family 13 amylopullulanase from *T. ethanolicus* has been successfully minimized as well. The wild-type protein contains 1,481 amino acid residues, while mutants TetApuR855 (855 amino acid residues) and TetApuQ818 (818 amino acid residues) exhibit a truncated N-terminal region that is not conserved among bacterial pullulanases and a deletion of the C-terminal fibronectin type III (FNIII) motif, without affecting its catalytic activity (Lin et al. 2008, 2012).

Thermostable pullulanase type I seems to be very rare among thermophilic prokaryotes and has not been identified in Archaea at all (Antranikian 1992). A member of this group has been purified from *Thermus caldophilus* in 1996; this enzyme was maximal active at 75°C, it debranched amylopectin, glycogen, dextrins and was able to completely hydrolyze pullulan to maltotriose (Kim et al. 1996). Investigation of the portfolio of starch-degrading enzymes in the anaerobic

thermophilic bacterium *Fervidobacterium pennivorans* revealed the presence of a debranching enzyme (pullulanase type I), which attacks exclusively α-1,6-linkages. This thermostable pullulanase produces long chain linear polysaccharides from amylopectin (Bertoldo et al. 1999).

The third class of pullulan-degrading enzymes contains the so-called pullulan hydrolases, which are not capable of attacking α-1,6-glycosidic bonds in branched oligosaccharides or pullulan, but they are active towards α-1,4-glycosidic linkages in starch, pullulan and amylose. Pullulan hydrolase type I (EC 3.2.1.35, neopullulanase) produces panose (α-6-D glucosylmaltose) from pullulan, while pullulan hydrolase type II (EC 3.2.1.57, isopullulanase) hydrolyzes pullulan to isopanose (α-6 maltosylglucose). According to BRENDA, no heat-adapted pullulan hydrolase type I or type II isozymes have been characterized from thermophilic or hyperthermophilic microorganisms (Schomburg et al. 2002). Another pullulan hydrolase, type III, attacks α-1,4- and α-1,6-glycosidic linkages in pullulan (Bertoldo and Antranikian 2002a). A member of the group of pullulan hydrolase type III has been identified in *Thermococcus aggregans* and was purified after heterologous expression in *E. coli*. *T. aggregans* pullulan hydrolase type III displayed a temperature optimum at 95°C and is active at temperatures up to 120°C (Niehaus et al. 2000).

20.3.6 Isoamylases

The enzyme isoamylase (3.2.1.68, glycogen 6-glucanohydrolase) specifically attacks α-1,6-linkages in the branched polysaccharides amylopectin, β-limit dextrin or glycogen, thereby debranching these complex compounds. Pullulan or branched oligosaccharides exhibiting α-1,6-linkages are not hydrolyzed by isoamylase (Bertoldo and Antranikian 2001).

TreX from *S. solfataricus* is an interesting candidate of a novel type of isoamylase, which displays specific 4-α-glucantransferase activity. This enzyme specifically hydrolyzes the α-1,6-branch portion of amylopectin and glycogen but also transfers α-1,4-glucan oligosaccharides from one molecule to another and produces cyclic glucans from amylopectin and amylose (Park et al. 2007). It has a temperature and pH optimum of 75°C and 5.5, respectively. Interestingly in a further study, the authors showed that both activities take place at the same catalytic site, while substrate specificity is closely associated with a structural lid that is generated by tetramerization of the subunits (Woo et al. 2008).

20.3.7 Cyclodextrin Glycosyltransferases

The enzyme group of cyclodextrin glycosyltransferases (EC 2.4.1.19, CGTases or α-1,4-D-glucan α-4-D-(α-1,4-D-glucano) transferase) catalyzes the conversion of polysaccharides to produce nonreducing cyclic dextrins by three main reactions: cyclization, coupling and disproportionation. CGTases are capable of using starch,

Table 20.6 CGTases from Bacteria and Archaea

Source	T_{opt}	pH	MW (kDa)	Reference
Bacillus stearothermophilus	80	8.0	66.8	Chung et al. (1998)
Thermococcus kodakaraensis	80	5.5–6.0	77	Rashid et al. (2002)
Thermoanaerobacter sp.	85	5.5	75.5	Alcalde et al. (1998)
Pyrococcus furiosus	95	5.0	65	Lee et al. (2007)
Thermococcus sp. B1001	90–100	7.0	83	Tachibana et al. (1999)

amylose or oligodextrins as substrates. Between six and eight glucose units are linked via α-1,4-linkages in α-, β- and γ-cyclodextrins. Due to their apolar interior, cyclodextrins are applied as hosts for other molecules (pharmaceutical important proteins or peptides) that are solubilized and stabilized. Most of the CGTases from Bacteria are primarily producing small and stable ring cyclodextrins CD_6 to $CD_{8,}$ and these different sizes make them suitable for various applications (Turner et al. 2007). Organic solvents are routinely used to rearrange the ratio of produced cyclodextrins. In particular CD_7 derivatives are mostly used as drug transporters in the pharmaceutical industry. CGTases generally share four conserved regions, which are relevant for catalytic activity and exhibit a common $(\alpha/\beta)_8$-barrel structure, assigning these enzymes to the superfamily of α-amylases (Bertoldo and Antranikian 2002b). Multiple site-directed and deletion analyses resulted in changes of product compositions and thereby confirmed the importance of the biomolecular structure of CGTases.

So far, CGTases are generally found in bacterial species, but recently some isozymes were discovered in Archaea as well. The first identified hyperthermophilic CGTase has been purified from *Thermoanaerobacter sp.* (Norman and Joergensen 1992). Another CGTase has been identified in the anaerobic archaeon *Thermococcus sp*. strain B1001 (Table 20.6). Although the growth range of this thermophilic species is between 60 and 95°C, the temperature optima for starch degradation and cyclodextrin synthesis is 110°C and 90–100°C, respectively. The main product of the enzymatic reaction is α-cyclodextrin with small amounts of β- and γ-cyclodextrins, when starch is used as substrate (Tachibana et al. 1999). According to BRENDA, this enzyme is the most heat-active CGTase described so far. Several heat-active CGTases have predominately been characterized from species of the genera *Bacillus*, *Geobacillus* and *Paenibacillus,* but the most heat-active isozymes with a temperature optimum ≥80°C were exclusively isolated and characterized from the bacterial genera *Geobacillus*, *Thermoanaerobacter*, *Thermoanaerobacterium* and within the archaeal genus *Pyrococcus* (Table 20.6).

20.4 Production of Heat-Stable Starch-Degrading Enzymes Derived from Thermophiles

An important prerequisite that has to be taken into account when selecting an industrial-relevant biocatalyst is the prospect of producing it in adequate amounts. Unfortunately, the broad application of extremozymes is limited by difficulties

associated with cultivation of their extremophilic hosts. Furthermore, the expression of genes encoding starch-degrading enzymes in thermophilic microorganisms is usually very low. The production of enzymes in heterologous expression systems by means of molecular biology techniques hence facilitates their application in various processes. It is generally possible to express genes encoding for heat-adapted enzymes in mesophilic pro- or eukaryotic host strains like *E. coli*, *B. subtilis* or *Saccharomyces cerevisiae*. Furthermore, it is well accepted that the activity of extremozymes relies on the same catalytic mechanism as of mesophilic enzymes and that the adaptation of heat-tolerant enzymes to high temperatures is genetically encoded (Eichler 2001). However, heterologous expression systems often fail because of differences in codon usage or improper folding of the produced proteins during modification processes in the host. In this context, thermostability means that the unique three-dimensional structure of a polypeptide chain is preserved at elevated temperatures. Although no universal mechanism that promotes thermostability has been proposed so far, it is expected that common features are contributing to stability. These include an increased number of hydrophobic interactions, disulfide bonds, ionic networks, salt bridges, metals bound to the structure, increased helix stability, preferred amino acid residues and shorter surface loops. A stabilization effect of the substrate has been described as well for starch-degrading enzymes (Prakash and Jaiswal 2010). Furthermore, it is of tremendous importance to produce soluble proteins to initiate purification. Since enzymes derived from extremophiles are stable and active under harsh conditions (e.g. temperature, detergents, organic solvents) and are more resistant to proteolytic attack compared to their mesophilic counterparts, the efficient separation of recombinant extremozymes can easily be achieved by mild heat denaturation or extraction with organic solvents (Egorova and Antranikian 2007).

Suitable vectors for the expression of extremozymes encoding genes in mesophilic host strains are beneficial, and the development of new systems is constantly increasing. Different strategies have been described to identify and clone unknown starch-degrading enzymes from thermophilic microorganisms. An adequate open reading frame can be amplified by polymerase chain reaction using degenerated oligonucleotides derived of conserved sequences from known proteins in closely related species. Another approach is based on the creation of a genomic library, either from non-sequenced organisms, which exhibit the specific enzyme activity or from metagenomes. These procedures have been established two to three decades ago but are still timely and routinely used for the identification of novel valuable biocatalysts (Bertoldo and Antranikian 2001; Egorova and Antranikian 2007; Ilmberger and Streit 2010; Rüdiger et al. 1995; Simon and Daniel 2009). The production of recombinant archaeal enzymes in bacterial or eukaryotic hosts can also be improved by the expression of a synthetic gene with an optimized codon usage. To overcome the limitations of different codon usage, few extremophilic archaeal expression systems have been developed as well. The virus-based shuttle vector pMJ05 for crenarchaeotal *S. solfataricus* is an available gene expression system in a hyperthermophilic archaeal species. Most genetic tools for Archaea are restricted by the lack of efficient transformation systems, but in case of pMJ05, the *Sulfolobus*

virus SSV1 has been used as plasmid backbone. Additionally, an effective selection marker and controllable promoters are also available for *S. solfataricus* (Angelov and Liebl 2010). An extracellular α-amylase from the hyperthermophilic archaeon *P. woesei* has been expressed in the moderate halophilic *H. elongata*, which lacks native amylase activity. This host offers several advantages, including the possibility to produce the archaeal protein under extreme salt conditions and to use simple nutritional requirements. In this approach, a derivative of the *H. elongata* small cryptic plasmid pHE1 has been used as shuttle vector for the cloning procedures (Frillingos et al. 2000). Furthermore, the gene encoding the extracellular *P. woesei* α-amylase has been heterologously expressed in the xanthan gum producing bacterium *Xanthomonas campestris*. The extracellular polysaccharide xanthan is used as thickener, stabilizer or as food additive in textile and food industry, and the authors developed a method to co-produce two industrially relevant products, namely, α-amylase and xanthan gum, in one step. Additionally, *X. campestris* is able to utilize industrial by-products such as molasses, corn syrup and cheese whey (Konsoula et al. 2008).

20.5 Improving Starch-Degrading Enzymes

Enzymes have been improved in matters of multiple properties, such as catalytic activity, thermostability, acid stabilization or resistance to oxidative agents (Khemakhem et al. 2009a; Liu et al. 2008; McDaniel et al. 2008). Stabilization of the enzymes can be increased by a variety of methods, including chemical modification, immobilization or by the addition of stabilizing agents. Traditional molecular and genetic engineering approaches such as random-mutagenesis/directed evolution and gene shuffling are also widely used to improve the catalytic performance of starch-degrading enzymes (Leveque et al. 2000). An advantage of the directed evolution approach is that no knowledge on the protein structure is required.

Genetic engineering of amylolytic enzymes has been mostly done in the heterologous hosts *E. coli* and *S. cerevisiae*. In vitro evolution strategies were often used to improve thermostability of genes encoding moderate thermostable or even thermolabile enzymes with desired catalytic properties as templates. The increased temperature is then used as selection pressure during the screening assay. The glucoamylases of *A. niger* and *Aspergillus awamori* have been repeatedly used as a target for directed evolution to improve the catalytic activity and thermostability (Liu and Wang 2003; Liu et al. 2000; McDaniel et al. 2008; Wang et al. 2006). *A. niger* glucoamylase has a complex structure, with a 440 amino acid catalytic N-terminal domain, which is coupled via a 68 amino acid linker to the 108 amino acid C-terminal starch-binding domain. The SBD is important for the hydrolysis of insoluble starch granules, but indispensable for soluble substrates. Recently, a glucoamylase variant with 16 independent amino acid replacements in the catalytic domain was generated by a combination of directed evolution and site-directed mutagenesis. This multi-mutated glucoamylase is

more thermostable (by 5 kJ mol^{-1} at 80°C) than the wild type and any mutant previously described (McDaniel et al. 2008).

Another possibility to improve biocatalysts is to confer known thermo-stabilizing features to less stable enzymes or to delete thermo-destabilizing regions (Al Khudary et al. 2010; Zhang et al. 2010). In this context, it is widely accepted that these stabilizing properties are usually associated with a decrease in structural flexibility. The α-amlyases from species of the genus *Bacillus* have been extensively investigated to improve heat adaptation. In former studies, novel stabilizing disulfide bonds have been introduced into proteins and hybrids of *Bacillus amyloliquefaciens* and *B. licheniformis* α-amylases were generated (Prakash and Jaiswal 2010; Conrad et al. 1995). Several parts of the proteins have been identified that were proposed to contain thermostability determinants. A highly conserved region that has been intensively investigated is the domain B in the α-amylase AmyUS100 from *Geobacillus stearothermophilus* and from related bacterial species. A loop created by five amino acid residues occurred between β-sheets β_{11} and β_{12} inside the domain B. The parallel deletion of either Gly_{213}-Ile_{214} or Ile_{214}-Gly_{215} pair dramatically improved the thermostability of AmyUS100, while the deletion of three amino acid residues decreased it (Khemakhem et al. 2009b). The CGTases of the mesophilic *Bacillus circulans* and the thermophilic *Thermoanaerobacterium thermosulfurigenes* also contain the highly conserved domain B. The replacement of two amino acid residues in *B. circulans* CGTase with those encoded in the *T. thermosulfurigenes* gene strongly increased the stability of the *B. circulans* mutant CGTase at 60°C. It has been shown that Asp_{188} and Arg_{192} are important for salt bridge formation in *T. thermosulfurigenes* and this stabilizing element could be successfully conferred to *B. circulans* (Leemhuis et al. 2004). Moreover, a CGTase from the bacterium *Paenibacillus* sp. A11 was recently enhanced in its thermostability, solvent tolerance and specific activity by the simple fusion of a thioredoxin-His_6-S-protein tag at its N-terminus together with a 30 amino acid residues proline-rich peptide at the C-terminal end of the protein. Moreover, the temperature optimum was increased from 60 to 65°C (Kaulpiboon et al. 2010).

20.6 Relevance for Biotechnology

Tailor-made enzymes have been proven to be powerful tools for a variety of industrial applications (Egorova and Antranikian 2007). The global annual enzyme market has been estimated to be around five billion Euros, and the market derived from enzymes is more than 30-fold. The annual growth rate of industrial-relevant biocatalysts has been predicted to be between 5 and 10%. At the turn of the twentieth century, α-amylases were first used to desize starch in the textile industry, a process that has been previously accomplished by acid hydrolysis, which is dependent on high temperatures and acidic pH. Nowadays, starch processing is predominantly a biocatalyst-based industry. In the case of thermophiles, a number of hydrolases have found their way to the market.

The starch-processing industry degrades starch into smaller, more valuable products, such as glucose, maltose, fructose or dextrins. Production of glucose is a complex process involving a series of amylolytic enzymes and numerous physical process steps. Due to their thermostability, starch-processing extremozymes from fungi and prokaryotes are of great interest. In all starch-converting processes, it is required to dissolve dry starch, a procedure that is dependent on high temperatures. An ideal biocatalyst should have a temperature optimum around 100°C and a pH optimum in the acidic range. Additionally, it should not require calcium or other metal ions for activity. A first heating step (105°C for 5 min and 95°C for 1 h) results in gelatinization and makes the substrate accessible to the hydrolytic activity of heat-stable α-amylases at 80–90°C (liquefaction); during this process starch becomes truncated to shorter-chain-length dextrins. High process temperatures have multiple advantages like an increase in solubilization velocity of starch, higher reaction rate, higher process yield, and the risk for microbial contaminations is drastically reduced (Dock et al. 2008). Since, the gelatinization of starch is incomplete in case of the temperature drops below 105°C, it is advantageous to use extremely heat-stable α-amylases during gelatinization and liquefaction. Among the Bacteria, α-amylases from species of the genera *Bacillus* and *Geobacillus* including *B. amyloliquefaciens, B. licheniformis, B. subtilis* and *G. stearothermophilus* are known to be valuable producers of heat-active starch-degrading enzymes and are widely used for industrial applications (Prakash and Jaiswal 2010). As mentioned above, extremozymes derived from hyperthermophilic Archaea also display promising and even better properties than bacterial isozymes, making them valuable candidates for industrial use. In a further reaction step, debranching enzymes are used to cleave α-1,6-glycosidic linkages. Glucoamylases are used to release glucose monomers from the low-molecular-weight polymeric dextrins under acidic pH during a second heating step at 60°C (saccharification). The enzyme β-amylase is usually applied in the production of maltose syrup. For biotechnological applications, β-amylase is generally derived from plants, while all other enzymes applied in starch saccharification are of fungal or prokaryotic origin. The saccharification reaction step usually takes several days to complete. The performance of starch liquefaction and saccharification at similar reaction conditions would drastically decrease the cost to industrially produced glucose. Although enzymes applied in the liquefaction step remain stable under the process conditions, no enzymes employed for saccharification and for debranching of amylopectin are sufficiently thermostable at the moment (Prakash and Jaiswal 2010).

Glucoamylases have also a long-standing history as biocatalyst relevant in food industry, where these enzymes are applied for the saccharification of partially processed starch to produce crystalline glucose units or glucose syrup. Most of the isozymes are produced in fungi, but also glucoamylases of prokaryotic origin have been reported. Fungal glucoamylases are mostly known to be thermolabile and not suitable for biotechnological processes because of their slow catalytic activity and acidic pH requirement. Additionally, various undesired side products are formed during the process. These circumstances have made fungal glucoamylases an important target for molecular biology-based improvement strategies to expand pH and

temperature dependent properties. Due to their stability under acidic and high temperature conditions, glucoamylases from thermoacidophilic Archaea are interesting candidates for future applications (Dock et al. 2008; Egorova and Antranikian 2007).

Moreover, glucose as the end product in starch saccharification is generally used in food and fermentation industries. The industrial production of fructose necessitates a third step (isomerization). This process usually takes place at 65°C and pH 8.0 (Egorova and Antranikian 2007). Since glucose has only half of the sweetness of fructose, bacterial glucose/xylose isomerase is used for the industrial production of fructose (Turner et al. 2007). The reaction results in final yields of up to 55% fructose after post-processing chromatographic enrichment methods (Kumar and Satyanarayana 2009).

Recently, a novel extracellular, heat- and acid-stable α-amylase with biotechnological relevance for the food industry, derived from *Bacillus acidicola* TSAS1, has been reported to be capable of using raw and soluble starch as substrate. This enzyme is active between 30 and 100°C with an optimal temperature at 60°C. Maltose, maltotriose, maltotetraose and maltopentose were shown to be the end products in starch hydrolysis, and a putative application in the production of high maltose syrups was proposed. Furthermore, this isozyme is independent on calcium, which eliminates the expensive addition and removal steps of this ion in industrial processes. The ion usually becomes removed from the product stream by the usage of ion exchangers (Sharma and Satyanarayana 2010, 2011, 2012).

Beside amylases, glucoamylases or α-glucosidases, several other starch-processing enzymes are of important biotechnological relevance. Cyclodextrins are valuable products used in pharmaceutical industry as mentioned above, but they are also in use as stabilizing or gelling agents in jelly deserts, dressing, dairy and meat products (Bertoldo and Antranikian 2002a). Usually, cyclodextrin production is a multistep procedure in which starch is processed by the usage of thermostable α-amylase, while a less heat-adapted CGTase usually from *Bacillus sp*. is added in the final production step. The use of thermostable CGTase from hyperthermophiles will allow the development of a more efficient one-step process (Egorova and Antranikian 2007; Bertoldo and Antranikian 2001).

20.7 Future Perspectives

The need for robust enzymes for countless environmentally friendly processes is increasing day by day. There is a growing demand for new biocatalysts in several fields such as food, feed, detergent, chemical and pharmaceutical industries and especially for the future bio-based industries (biorefinery). More efficiently and tailor-made biopolymer degrading enzymes such as amylases, cellulases, hemicellulases and laccases have to be produced in bulk amounts in order to develop future sustainable technologies. The advanced technologies in life sciences and engineering will provide a valuable tool for improving enzymatic processes for the production

of chemicals, biopolymers, materials and fuels from renewable resources. In order to meet the future challenges, innovative technologies have to be applied by exploiting nature's biodiversity.

20.8 Conclusion

Based on their unique properties as activity up to 120°C, pH 0–11, broad substrate specificity, enantioselectivity and stability in organic solvents, the biocatalysts from thermophiles represent the choice for future applications in industry. Enhanced functionality can even be obtained either by changing the structural properties of starch or by changing the physicochemical properties of starch-modifying enzymes. Additionally, the development of suitable expression systems for genes encoding extremozymes in mesophilic hosts, which can easily be cultivated, allows circumventing the problem of insufficient expression. Furthermore, fast growing thermophilic Archaea and Bacteria are also attractive candidates for recombinant enzyme production. The production of tailor-made enzymes by means of genomics, directed evolution, protein engineering and further modern techniques, such as synthetic biology, will pave the way for a broad application of enzymes with extreme properties for innovative and sustainable biotechnological applications.

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Chapter 21
Thermostable Archaeal and Bacterial Pullulanases and Amylopullulanases

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Abstract Pullulanase is one of the industrially important debranching enzymes, capable of hydrolyzing α-1, 6-glucosidic linkages in pullulan, starch, amylopectin, and other related oligosaccharides. It is widely used in starch industry for the production of various sugar syrups. Type I pullulanases specifically attack α-1, 6 linkages in branched oligosaccharides such as pullulan, starch, amylopectin, and glycogen, forming linear α-1, 4-linked oligomers, while the type II pullulanases (amylopullulanases) hydrolyze α-1, 6-glycosidic linkages in pullulan and branched substrates besides α-1, 4-glycosidic linkages in polysaccharides. With the advancements in biotechnology, the application of pullulanase has been extended to pharmaceutical chemistry as well as in automatic dishwashing detergents, baking industry, and production of cyclodextrins. Although pullulanases are ubiquitous in their occurrence in plants, animals, as well as microbes, the microbial sources are the most preferred ones for large-scale production and application. This chapter deals with the developments in production, characteristics, molecular aspects, and applications of microbial pullulanases and amylopullulanases.

Keywords Pullulan • Starch • Glycogen • Thermophiles • Thermostable • Pullulanase • Amylopullulanase • Sugar syrups • Site-directed mutagenesis

21.1 Introduction

There has been an increasing emphasis in the industrial sector on reducing energy consumption of key processes, limiting generation of waste and contaminants, and improving the quality of end products by applying sustainable technologies.

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By employing enzymes in industrial processes, consumption of energy and chemicals can be reduced and thus beneficial for the environment. In 1998, worldwide enzyme sales amounted to US $ 1.5 billion, and this grew at a double-digit rate to $ 5.1 billion in 2009 (Sanchez and Demain 2010). The enzyme reactions are specific in their action and therefore are less likely to produce unwanted by-products. They are biodegradable and thus cause less environmental pollution. The enzymes function under mild conditions (low temperature, neutral pH, and normal atmospheric pressure), leading to energy saving. The research is underway for developing new and faster enzymes which have better resistance to elevated temperatures, acidic or basic conditions, and organic solvents. The use of enzymes in specific industrial processes is widespread, including the dairy, pulp and paper, textiles, leather, energy, chemical, food, and mineral industries.

From the 1950s, acid hydrolysis of starch has been replaced by enzymatic process due to the availability of suitable enzymes and advantages with the latter. Enzymatic starch hydrolysis typically requires the coordinated action of different endo- and exo-amylases including α-amylase, glucoamylase, glucose isomerase, pullulanases, and others. Starch industry roughly accounts for 15–20% of the total industrial consumption of enzymes (Pandey 1995; Kumar and Satyanarayana 2009).

Pullulanase (EC 3.2.1.41) or pullulan-6-glucanohydrolase belongs to a family of 13 glycosyl hydrolases (GH), also called the α-amylase family, according to CAZy (Carbohydrate-Active enZymes) family classification system which is based on protein sequence and structural similarity (Cantarel et al. 2009; Janecek 1997; Matzke et al. 1997). The enzymes are categorized as endoacting enzymes which hydrolyze glycosidic linkages in a random fashion in the interior of polysaccharides to give products of low molecular weight such as glucose and smaller polymers composed of glucose units. The enzyme has been used as a biocatalyst for both starch liquefaction (with or without α-amylase) and saccharification. It improves the saccharification rate and yield (Norman 1988), when used in combination with other saccharifying amylases such as glucoamylase, fungal α-amylase, or β-amylase. Furthermore, the enzyme finds application in structural studies of carbohydrates (Whelan 1971).

Pullulanase (EC 3.2.1.41, pullulan 6-glucanohydrolase) was first reported by Bender and Wallenfels (1961) in a mesophilic bacterium, *Klebsiella pneumoniae* (formerly known as *Aerobacter aerogenes* or *Klebsiella aerogenes*). Later Plant et al. (1987a) reported the new class of pullulanases which cleave pullulan at α-1, 6 linkages and amylose at α-1, 4 linkages. Depending on their inability or ability to hydrolyze α-1, 4-glycosidic linkages in other polysaccharides, pullulanases are divided into two categories based on the substrate specificity, type I and type II pullulanases, respectively (Antranikian 1992).

Type I pullulanase (*α-Dextrin 6-Glucanohydrolase, EC 3.2.1.41*) specifically hydrolyzes α-1, 6 linkages in branched oligosaccharides, such as pullulan, starch, amylopectin, and glycogen, forming linear α-1, 4-linked oligomers. It is used in combination with other starch-hydrolyzing enzymes for the complete conversion of polysaccharides to low molecular weight compounds. The enzyme from plant

sources was formerly called R-enzyme (Lee and Whelan 1971), which causes complete conversion of pullulan to maltotriose. Its action on pullulan is commonly in a random endo-fashion. The smallest substrate that is attacked by pullulanase is 6^2-α-maltosyl maltose (Marshall 1973). Pullulanase type I also exhibits the reverse reaction of condensation. On incubation of high concentrations of pullulanase with maltotriose or maltose, various condensation products with α-1, 6-glycosidic linkages are synthesized (Abdullah and French 1970; Norman 1988).

Type II pullulanase (EC 3.2.1.1/41), unlike type I enzyme, is capable of hydrolyzing α-1, 4-glycosidic linkages in polysaccharides in addition to the α-1, 6-glycosidic linkages in pullulan and branched substrates (Bertoldo et al. 2004). It cleaves pullulan at α-1, 6 linkages and amylose at α-1, 4 linkages (Plant et al. 1987a). The enzyme causes the complete conversion of polysaccharides to small sugars without the requirement of other enzymes like α-amylase or β-amylase (Antranikian 1992). Due to its action pattern, this enzyme is also referred to as α-amylase-pullulanase (Melasniemi 1988; Kim and Kim 1995) or amylopullulanase (Saha et al. 1989). CAZy family classification system divided amylopullulanases into two groups, thermoactive amylopullulanases belonging to GH57 or GH13 family and mesophilic amylopullulanases often categorized under GH13 family (Lin et al. 2008; Zona et al. 2004).

Similar to type I, the end product of pullulan hydrolysis by amylopullulanase is maltotriose. The newly purified amylopullulanase from *Geobacillus stearothermophilus* L14 has been the only exception, where the enzyme produces glucose from pullulan along with maltose and maltotriose (Zareian et al. 2010). Amylopullulanases act randomly on starch and cleave α-1, 4 linkages to produce mainly DP2-DP4 products. The action of pullulanase type II on amylopectin leads to the formation of glucose, maltose, and maltotriose (Bertoldo and Antranikian 2001). All pullulanases present in hyperthermophiles are capable of cleaving both α-1, 6 and α-1, 4 linkages (Antranikian 1990; Melasniemi 1988; Saha et al. 1988; Spreinat and Antranikian 1990).

Both type I and type II pullulanases do not hydrolyze substrates such as dextran, isomaltose, and isomaltotriose, which are exclusively linked by α-1, 6-glycosidic bonds. The inability of these enzymes to hydrolyze these substrates suggests the requirement of α-1, 4 linkages in the vicinity of α-1, 6 bonds for enzymatic activity (Abdullah and French 1970; Antranikian 1992).

Other pullulan-hydrolyzing enzymes include the following: glucoamylase (EC 3.2.1.3) that hydrolyzes pullulan from the nonreducing ends to produce glucose but at a very slow rate (Marshall 1973; Saha et al. 1979); neopullulanase that hydrolyzes α-1, 4 linkages of pullulan to produce panose (Kuriki et al. 1988), also known as pullulan hydrolase type I; and isopullulanase (EC 3.2.1.57) that hydrolyzes α-1, 4-glucosidic linkages of pullulan to produce isopanose (Sakano et al. 1971), also known as pullulan hydrolase type II. This chapter is aimed at describing the sources, production and characterization, cloning and expression, and structure-function aspects of microbial pullulanases and amylopullulanases.

21.2 The Substrate Pullulan

Pullulan is an extracellular glucan synthesized by the fungus *Aureobasidium pullulans* (Kim et al. 1990). *Pullularia pullulans*, a bacterium, is also known to produce pullulan (Kim et al. 1990). It is composed of linearly polymerized maltotriose units linked by α-1, 6-glucosidic bonds (Bender et al. 1959), although the same can occur as a polymer of isopanose (Singh et al. 2008). It is a highly ordered and branched polysaccharide with 2:1 ratio of α-1, 4-glucano to α-1, 6-glucano bonds (Kim et al. 1990). The α-1, 6 linkages in pullulan are considered to mimic partially the α-1, 6-branch points of amylopectin. Hence, pullulan has been widely employed as a model substrate for assaying starch-debranching enzymes (Plant et al. 1986). Pullulan is insoluble in many solvents including methanol, ethanol, and acetone but soluble in water to form a transparent, colorless, and viscous adhesive solution (Shingel 2004). Pullulan has potential applications in food, pharmaceutical, and biomedical industries (Shingel 2004; Singh et al. 2008).

21.3 Microorganisms Producing Pullulanases

Pullulanases are produced by animals, plants, fungi, and bacteria. Among bacteria, many mesophilic, thermophilic and hyperthermophilic bacteria and archaea have been reported to produce pullulanases (Table 21.1).

Amylopullulanase is distributed mostly among thermophilic anaerobic bacteria (Coleman et al. 1987). Among thermoanaerobes, *Clostridium thermohydrosulfuricum* (Hyun and Zeikus 1985a; Melasniemi 1987, 1988), *Clostridium* sp. strain EM1 (now *Thermoanaerobacterium thermosulfurogenes*) (Antranikian et al. 1987b; Spreinat and Antranikian 1990), *Thermoanaerobium brockii* (Coleman et al. 1987), *Thermoanaerobium* Tok6-B1 (Plant et al. 1987a), *C. thermohydrosulfuricum* Z 21-109 (Saha et al. 1990), *Thermoanaerobacter ethanolicus* 39E (Mathupala and Zeikus 1993), *Thermoanaerobacter finni*, *Thermobacteroides acetoethylicus*, *T. ethanolicus* (Koch et al. 1987; Koch and Antranikian 1990), *Thermotoga maritima* (Bibel et al. 1998), *Thermoanaerobacterium thermosaccharolyticum* (Ganghofner et al. 1998), and *Thermococcus profundus* (Kwak et al. 1998) are known to produce amylopullulanase. Among aerobes, certain species of *Bacillus* and *Geobacillus* are identified as producers of amylopullulanase, most of which are thermophilic. *Bacillus* sp. 3183 (Shen et al. 1990), *Bacillus* sp. XAL 601 (Lee et al. 1994), *Bacillus circulans* F-2 (Saha and Zeikus 1989a; Kim et al. 1990; Kim and Kim 1995), *Bacillus* sp. TS-23 (Lin et al. 1996), *Bacillus subtilis* (Takasaki 1987), *Bacillus* sp. KSM-1378 (Ara et al. 1995a), *Bacillus* sp. DSM 405 (Brunswick et al. 1999), *Geobacillus thermoleovorans* NP33 (Noorvez et al. 2006), *Bacillus* sp. US149 (Roy et al. 2003), and *G. stearothermophilus* L14 (Zareian et al. 2010) have been reported to produce amylopullulanases. Plant et al. (1986) confirmed the presence of the enzyme in thermophilic aerobic eubacterium *Thermus aquaticus* YT-1. Hyperthermophilic archaea *Pyrococcus furiosus*, *P. woesei, Thermococcus litoralis*,

Table 21.1 The source and properties of pullulanases

Source	Molecular mass (kDa)	Opt. pH	Opt. temp (°C)	Type1	Reference
Mesophiles					
Bacillus cereus var. *mycoides*	Nd	6.5	30	I	Takasaki (1976b)
Bacillus sp. KSM-1876	128	10	50	I	Ara et al. (1992) and Hatada et al. (2001)
Bacillus sp. KSM-1378	210	10	50	II	Ara et al. (1995a) and Ara et al. (1996)
Bacillus circulans F-2	220	7–8.5	50	II	Sata et al. (1989), Kim et al. (1990), and Kim and Kim (1995)
Bacillus subtilis	450	7.0	30	II	Takasaki (1987)
Klebsiella aerogenes	Nd	7.2	30	I	Bender and Wallenfels (1961), Lee and Whelan (1971), Brandt et al. (1976), and Yamashita et al. (1997)
Klebsiella pneumoniae	145	7.0	30	I	Konishi et al. (1979), Chapon and Raibaud (1985), and Pugsley et al. (1986)
Thermophiles					
Anaerobranca gottschalkii	96	8.0	70	I	Bertoldo et al. (2004)
Bacillus acidopullulyticus	97	5.5	60	Nd	Stefanova et al. (1999)
Bacillus sp. DSM 405	126	6.0	70	I	Brunswick et al. (1999)
Bacillus sp. S-1	140	9.0	60	I	Lee et al. (1997)
Bacillus sp. US149	200	5.0	60	II	Roy et al. (2003)
Bacillus sp. AN7	106	6.0	90	I	Kunamneni and Singh (2006)
Bacillus strain 3183	Nd	5.0	62	II	Shen et al. (1990)
Bacillus sp. TS-23	140	9.0	55	II	Lin et al. (1996)
Bacillus thermoleovorans US105	Nd	5.6	75	I	Messaoud et al. (2002)
Clostridium sp. strain EM1	Nd	5.9	60	II	Antranikian et al. (1987b) and Spreinat and Antranikian (1990)

(continued)

Table 21.1 (continued)

Source	Molecular mass (kDa)	Opt. pH	Opt. temp (°C)	Type1	Reference
Clostridium thermohydrosulfuricum Z 21-109	136.5	6.0	65	I	Saha et al. (1988)
Clostridium thermosulfurogenes SV2	80	6.0	75	Nd	Reddy et al. (1999a) and Reddy et al. (1998)
Clostridium thermosulfurogenes SV9	Nd	7.0	60	I	Swamy and Seenayya (1996b)
Clostridium thermosulfurogenes DSM 3896	130kD	6.4–6.7	60	I	Burchhardt et al. (1999) and Madi and Antranikian (1989)
Clostridium thermohydrosulfuricum DSM 567	Nd	6.6	60	I	Antranikian et al. (1987c)
Clostridium thermosaccharolyticum	Nd	7.2	60	I	Koch et al. (1987)
Desulfurococcus mucosus	74	5.8	85	II	Canganella et al. (1994)
Fervidobacterium pennavorans	77	6.5	70	I	Canganella et al. (1994)
Fervidobacterium pennavorans Ven 5	Nd	6.8	65–70	I	Koch et al. (1997) and Bertoldo et al. (1999)
Geobacillus thermoleovorans NP33	Nd	7.0	80	II	Noorwez et al. (2006)
Geobacillus stearothermophilus	100	5.5	65	II	Zareian et al. (2010)
Micrococcus halobius OR-1	Nd	8.0	60	I	Devi and Yogeeswaran (1999)
Pyrococcus furiosus	89	5.5	100	II	Brown and Kelly (1993)
Pyrococcus woesei	90	6.0	100	II	Rudiger et al. (1995)
Rhodothermus marinus	Nd	6.5	65	Nd	Gomes et al. (2003)
Thermotoga maritima	89	5.9	90	I	Kriegshäuser and Liebl (2000)
Thermococcus hydrothermalis	Nd	5.5	95	II	Gantelet and Duchiron (1998)
Thermoactinomyces thalpophilus	79	7.0	70	I	Odibo and Obi (1988)
Thermoanaerobium Tok6-B1	120	5.6	70	II	Plant et al. (1987a)
Thermoanaerobacter strain B6A	450	5.0	75	II	Saha et al. (1990)
Thermococcus hydrothermalis	128	5.5	95	II	Erra-Pujada et al. (1999)
Thermus IM6501	80	6.0	70	I	Kim et al. (2000)
*Thermus caldophilus*GK-24	65	5.5	75	I	Kim et al. (1996)

and *Thermococcus hydrothermalis* are known to produce highly thermostable amylopullulanases (Koch et al. 1990; Brown and Kelly 1993; Dong et al. 1997; Rudiger et al. 1995; Erra-Pujada et al. 1999, 2001).

The type I pullulanase is produced by many mesophiles such as *Bacillus* sp. strain KSM-1876 (Hatada et al. 2001), *Bacillus* sp. strain KSM-1378 (Ara et al. 1995a), *Bacillus* sp. strain S-1 (Lee et al. 1997), *Bacillus* sp. US149 (Roy et al. 2003), *Micrococcus halobius* OR-1 (Devi and Yogeeswaran 1999), and *Klebsiella* (Michaelis et al. 1985; Eisele et al. 1972; Dupuy et al. 1992). Pullulanase has also been reported from the thermophiles *Bacillus thermoleovorans* US105 (Messaoud et al. 2002), *Anaerobranca gottschalkii* (Bertoldo et al. 2004), *Bacillus* sp. DSM 405 (Brunswick et al. 1999), and *Clostridium thermosulfurogenes* SV9 (Swamy and Seenayya 1996b) and in the hyperthermophiles *Fervidobacterium pennavorans* Ven5 (Bertoldo et al. 1999), *Rhodothermus marinus* (Gomes et al. 2003), *T. hydrothermalis* (Gantelet and Duchiron 1998), and *Thermus thermophilus* HB8 (Tomiyasu et al. 2001).

21.4 Physiology of Pullulanase Production

In view of the importance of pullulanase in starch-processing industry, the effects of physical and nutritional parameters on its production have been studied. Though much emphasis has been laid on cloning and expression of genes and continuous fermentation to achieve high titers, optimization of culture conditions still remains a useful way of increasing production.

21.4.1 Physiochemical Parameters

The role of various physicochemical parameters including carbon and nitrogen sources, surface-acting agents, metal ions, temperature, pH, and agitation for the enzyme production has been studied. The most important physical parameters that influence growth of organisms and production of enzymes are temperature, pH, agitation, dissolved oxygen or absence of it, and pressure. These are precisely the parameters, which have yielded a whole new group of enzymes called extremozymes, thermozymes being a part of them.

21.4.1.1 Temperature

Pullulanases have been reported from both mesophiles and thermophiles. More reports pertain to thermophilic bacteria because thermostable enzymes are needed in starch hydrolysis. Pullulanase has been produced at a much wider range of temperatures among bacteria. The impact of temperature on pullulanase production is

associated with the growth of the organism (Takasaki 1976a; Suzuki and Chishiro 1983; Wallenfels et al. 1966; Reddy et al. 2000; Mrudula et al. 2011). The extracellular pullulanases of *T. thalpophilus* (Odibo and Obi 1988) and *B. cereus* var. *mycoides* (Takasaki 1976a) were maximally synthesized in the late exponential phase of growth. In contrast, the enzymes of *B. stearothermophilus* (Suzuki and Chishiro 1983) and *A. aerogenes* (Wallenfels et al. 1966) attained a peak in enzyme production in the stationary phase. The maximum growth and pullulanase production by *C. thermosulfurogenes* SV2 occurred at 60°C (Reddy et al. 2000). A wide range of temperatures for the growth and amylopullulanase production was recorded in *C. thermosulfurogenes* SVM17, *C. thermosulfurogenes* SV9, *C. thermosulfurogenes* SV2, *C. thermosulfurogenes* 4B, and *C. thermosulfurogenes* EM1 (Mrudula et al. 2011; Reddy et al. 1999b; Schink and Zeikus 1983; Madi and Antranikian 1989). A significant pullulanase activity was observed in *Bacillus cereus* FDTA-13 at 40–70°C (Nair et al. 2007). Madi et al. (1987) reported that the growth of *Clostridium* strain was not affected by at different temperatures and thus did not influence the secretion of the amylolytic enzymes into the culture broth.

21.4.1.2 pH

Among the physical parameters, the pH of the growth medium has a profound effect on the production as well as stability and activity of extracellular enzymes. Amylopullulanase production in *Clostridium* sp. strain EM1 was very sensitive to the pH of the medium (Antranikian et al. 1987a). The pH greatly influenced the starch metabolism. The optimal pH for enzyme production was 5.9, while it fell drastically even at pH 6.2 or above and below 5.8. The concentration of amylase and pullulanase decreased from 1,000 and 2,200 UL^{-1} at pH 5.9 to 200 and 300 UL^{-1} at pH 6.2 (Antranikian et al. 1987a). In *T. finni*, the reduction of pH from 6.5 to 6.0 led to a decline in amylase activity from 3,000 to 1,300 UL^{-1} and pullulanase activity from 800 to 1,600 UL^{-1} at a dilution rate of 0.036 h^{-1}, respectively (Koch et al. 1987).

21.4.1.3 Agitation

Agitation intensity influences the mixing and oxygen transfer rates and thus has an impact on the growth of aerobes and thus may influence enzyme production. Agitation rates of up to 200 rpm have normally been employed for the production of pullulanase and amylopullulanase from various microbes. Pullulanase was maximally produced by *Thermoactinomyces thalpophilus* (Odibo and Obi 1988), *Bacillus cereus* var. *mycoides* (Takasaki 1976a), and *Bacillus* strain 3183 (Shen et al. 1990) at 180, 150, and 200 rpm, respectively. On the other hand, the anaerobe *P. furiosus* grew maximally under quiescent conditions (Brown et al. 1990), while *Clostridium thermohydrosulfuricum* DSM 567, another anaerobe, produced pullulanase optimally at 200 rpm (Antranikian et al. 1987b).

21.4.1.4 Carbon Sources

Different sources of carbon as well as their concentrations are important factors not only for optimal production of enzymes but also for determining their location, i.e., extracellular or cell bound. Starch has been the most preferred substrate for the production of pullulanases. One percent starch was optimal for the production of amylopullulanase by *B. subtilis* (Takasaki 1987). One percent starch was used for enzyme production by *Bacillus* sp. strain TS-23 (Lin et al. 1996), *Bacillus* sp. 3183 (Saha et al. 1989), *C. thermohydrosulfuricum* 39E (Mathupala et al. 1990), and *T. thermosaccharolyticum* (Ganghofner et al. 1998), while 0.5% starch was optimum for *Thermoanaerobacter* strain B6A (Saha et al. 1990), *C. thermohydrosulfuricum* (Saha et al. 1988), and *C. thermosulfurogenes* EM1 (Spreinat and Antranikian 1990).

Maltose has been used as a carbon source for the production of amylopullulanase from *T. litoralis* (Rinker and Kelly 1996), *Thermus aquaticus* YT-1 (Plant et al. 1986), *T. ethanolicus* 39E (Mathupala and Zeikus 1993), *T. profundus* (Kwak et al. 1998), *Bacillus* sp. DSM 405 (Brunswick et al. 1999), and *C. thermosulfurogenes* SVM17 (Mrudula 2010). Maltose has also been reported to be a better inducer than starch for amylase and/or pullulanase production by *Clostridium* sp. (Madi et al. 1987), *C. thermosulfurogenes* (Hyun and Zeikus 1985d), *C. thermocellum* (Swamy et al. 1994), *Thermococcus* sp. (Gantelet et al. 1998), *T. hydrothermalis* (Legin et al. 1998), and *T. profundus* (Kwak et al. 1998). In the extremely thermophilic eubacterium, *Rhodothermus marinus*, maltose was found to be most effective inducer for pullulanase production, which was followed by soluble maize starch, glycogen, and pullulan (Gomes et al. 2003). In *P. furiosus*, amylopullulanase production was shown to increase tremendously in the presence of added carbohydrates. The enzyme production accelerated with α-1, 4-linked saccharides and was not subject to catabolite repression (Brown et al. 1990). Amylopullulanases have shown to be induced by commercial maltooligosaccharide mixture in *P. furiosus* DSM1378 and *T. litoralis* DSM5473 (Brown and Kelly 1993).

Pullulan itself has been used as a carbon source for the production of amylopullulanase in *Bacillus* sp. KSM-1378 (Ara et al. 1995a) and various mesophilic marine bacteria (Arnosti and Repeta 1994). Cross-linked potato starch and highly soluble Zulkowsky starch also have been used for production by *B. circulans* F-2 and *T. thermosaccharolyticum*, respectively (Sata et al. 1989; Ganghofner et al. 1998; Melasniemi 1987).

Bacillus cereus FDTA-13 produced high titers of pullulanase in a medium containing branched polysaccharides (Nair et al. 2007). The addition of inducers like maltooligosaccharides (maltose to maltotetraose) resulted in varied levels of pullulanase in comparison with the control. Maltotriose has been reported as a potent inducer of maltose regulon, which regulates the release of pullulanase enzyme (Raibaud and Richet 1987). The enzyme production declined at high levels of inducer. Increased pullulanase activity was observed when grown under carbon-limited conditions with the inducers. In the presence of glucose, pullulanase activity diminished. In C. *thermosulfurogenes* SV9, soluble starch was shown to be the best substrate for pullulanase production (Swamy and Seenayya 1996a). Soluble starch

was also found to be the best carbon source for the production of thermostable pullulanase by *C. thermosulfurogenes* SV2 (Reddy et al. 1998). In submerged fermentation of *C. thermosulfurogenes* SV2, potato flour was shown to be the most suitable carbon source for pullulanase production (Ramesh et al. 2001). Similar observation was recorded in *C. thermosulfurogenes* SV9 from response surface plots that the yield of pullulanase increased with increase in the concentration of potato starch up to about 20%; the increase was more pronounced at lower concentrations of ferrous sulfate and pearl millet flour and higher concentrations of corn steep liquor (Swamy and Seenayya 1996b). Starch and dextrin stimulated pullulanase synthesis, while pullulan was inhibitory in *T. thalpophilus*, *B. stearothermophilus* KP1064 (Suzuki and Chishiro 1983), and *A. aerogenes* (Ohba and Ueda 1973). Ge et al. (1980) showed a preference for pullulan plus glutinous rice starch and dextrin for pullulanase enzyme synthesis in *A. aerogenes*.

Dextrin, Zulkowsky starch, pullulan, and maltose have been found to support good growth and pullulanase production in *C. thermohydrosulfuricum* E101-69 (Melasniemi 1987). Pullulanase activity was cell bound with less soluble starches, while the soluble small glucose polymers (dextrin) and Zulkowsky starch stimulated extracellular pullulanase production (Melasniemi 1987). In *Bacillus* strain 3183, starch, maltose, and pullulan supported good amylopullulanase production. Wheat flour has been reported to be a source of carbon and nitrogen for growth and enzyme production (Shen et al. 1990). Starch, dextrin, maltose, and glucose were effective carbon sources for *Bacillus cereus* var. *mycoides* (Takasaki 1976a). The pullulanase from *K. pneumoniae* was induced by maltose (Michaelis et al. 1985). Batch culture of *K. aerogenes* with excess of maltose as an inducer largely produced extracellular pullulanase, whereas under substrate-limited chemostat culture, pullulanase was shown to be firmly cell bound (Hope and Dean 1974).

21.4.1.5 Nitrogen Sources

The source of nitrogen in the culture medium is another important parameter that influences growth and enzyme production. Organic forms of nitrogen such as tryptone have been extensively used for the production of amylopullulanase by thermophiles, particularly hyperthermophiles. It was used as nitrogen source for enzyme production by *P. furiosus* and *T. litoralis* (Brown and Kelly 1993), *P. woesei* (Rudiger et al. 1995), *T. ethanolicus* 39E (Mathupala and Zeikus 1993), *T. thermosaccharolyticum* (Ganghofner et al. 1998), and *C. thermohydrosulfuricum* (Melasniemi 1987). Ammonium salts have also been used quite widely. Peptone served as the better nitrogen source for maximum production of amylopullulanase by *C. thermosulfurogenes* SVM17 (Mrudula 2010). Ammonium chloride and ammonium nitrate were used for the cultivation and enzyme production by *Bacillus* sp. 3183 (Saha et al. 1989) and *Bacillus* sp. DSM 405 (Brunswick et al. 1999), respectively. Ammonium sulfate was used as a nitrogen source for the production of enzyme by *Bacillus* sp. KSM-1378 (Ara et al. 1995b), *Bacillus* sp. strain TS-23 (Lin et al. 1996), *B. circulans* F-2 (Sata et al. 1989), and *G. thermoleovorans* NP33 (Noorvez et al. 2006). A complex

mixture of soybean meal, urea, and corn steep liquor was used by Takasaki (1987) for *Bacillus subtilis*, while Plant et al. (1986) used trypticase peptone for *T. aquaticus* YT-1. Wheat flour was a practical source of carbon and nitrogen for growth and enzyme production by *Bacillus* strain 3183 (Shen et al. 1990). The corn steep liquor in combination with yeast extract was a suitable nitrogen source for thermostable α-amylase and pullulanase production in submerged fermentation by *C. thermosulfurogenes* SV9 (Swamy and Seenayya 1996b), while peptone and yeast extract supported β-amylase and pullulanase production by *C. thermosulfurogenes* SV2 (Ramesh et al. 2001).

Nitrogen-limited medium resulted in a low pullulanase production, even with combinations of several maltosaccharides in *B. cereus* FDTA-13 (Nair et al. 2007). Polypeptone, meat extracts, and milk casein were the effective nitrogen sources for pullulanase production by *B. cereus* var. *mycoides* (Takasaki 1976a). Organic nitrogen source markedly increased pullulanase production in *T. thalpophilus* and *Bacillus stearothermophilus* (Takasaki 1976a; Suzuki and Chishiro 1983).

21.4.1.6 Trace Elements, Vitamins, and Surfactants

Besides carbon and nitrogen sources, trace elements and vitamins are important for the production of pullulanase, especially by thermoanaerobes (Antranikian et al. 1987b; Koch et al. 1990; Mathupala and Zeikus 1993; Swamy and Seenayya 1996b) as well as some Bacilli (Ara et al. 1996; Shen et al. 1990; Takasaki 1976a). Among hyperthermophiles, *T. aquaticus* YT-1 (Plant et al. 1986), *P. furiosus*, *T. litoralis* (Brown and Kelly 1993), *P. woesei* (Rudiger et al. 1995), *T. ethanolicus* 39E (Mathupala and Zeikus 1993), and *Bacillus* sp. 3183 (Saha et al. 1989) required the presence of trace elements and in some cases vitamins too. Mesophilic *B. subtilis* (Takasaki 1987) and *Bacillus* sp. KSM-1378 (Ara et al. 1995a) also had been reported to require trace elements for pullulanase production. There was no requirement of additional trace elements for enzyme production in case of *Thermoanaerobium thermosaccharolyticum* (Ganghofner et al. 1998), *Bacillus* sp. DSM 405 (Brunswick et al. 1999), *B. circulans* F-2 (Sata et al. 1989), and *Bacillus* sp. strain TS-23 (Lin et al. 1996).

Mg^{2+} and Fe^{2+} stimulated pullulanase production in *Aerobacter aerogenes* (Ohba and Ueda 1973). The pullulanase production decreased by 20–80% in the absence of each of these ions. Sr^{2+} enhanced pullulanase production by *T. thalpophilus* (Odibo and Obi 1988) but was inhibitory to pullulanase production by *Bacillus cereus* var. *mycoides* (Takasaki 1976a).

Surfactants like Triton X-100 (*t*-octylphenoxypolyethoxyethanol); Tween-80 (polyoxyethylene sorbitan monooleate) (nonionic surfactants); CHAPS (3-[(3-cholamidopropyl) dimethylammonia]-L-propane sulfonate), a zwitterionic surfactant; and sodium taurocholate (an anionic surfactant) individually enhanced synthesis and secretion of thermostable β-amylase and pullulanase by *C. thermosulfurogenes* SV2 (Reddy et al. 1998). These surfactants were also found to increase the stability of enzymes.

21.5 Fermentation Studies on Pullulanase Production

Higher productivity was attained in continuous fermentation than batch cultures even in chemostats (Antranikian et al. 1987a; Koch and Antranikian 1990). Antranikian et al. (1987a) cultivated *C. thermosulfurogenes* EM2 in continuous fermentation in a defined medium with 1% starch as carbon source and reported a 5- and 13-fold increase in amylase and pullulanase activities as compared to the production in batch cultures. *C. thermosulfurogenes* EM1 produced 320 and 250 UL^{-1} of amylase and pullulanase, respectively, under batch cultivation during the exponential growth phase. Under these conditions, 95% of amylase and 65% of pullulanase were found cell bound. The pH of the medium dropped sharply to 4.0 from 6.5. At 1% starch and dilution rate of 0.075 h^{-1} led to the formation of 1,450 UL^{-1} of amylase and 2,860 UL^{-1} of pullulanase. Under these conditions, 55 and 70% of these enzymes were released into the culture medium, respectively.

T. finni, *T. ethanolicus*, *T. acetoethylicus*, and *C. thermosaccharolyticum* were cultivated in continuous fermentation under starch limitation by Koch et al. (1987). Low levels of extracellular enzymes were reported under batch cultivation. It ranged between 100 and 580 UL^{-1} and 20 and 900 UL^{-1} for amylase and pullulanase, respectively. There was a significant increase in the titer of these enzymes when the strains were cultivated in continuous mode. *T. finni* produced a total of 3,800 UL^{-1} pullulanase and 3,000 UL^{-1} amylase at a dilution rate of 0.036 h^{-1}. At higher dilution rates up to 0.075 h^{-1}, enzyme production declined. *T. acetoethylicus* produced 3,200 and 4,100 UL^{-1} of pullulanase and amylase, respectively, at a dilution rate of 0.03 h^{-1}; more than 70% of the activities were released into the culture fluid.

Batch fermentation for the production of amylopullulanase has been reported in thermophiles such as *Bacillus* sp. 3183 (Saha et al. 1989), *P. furiosus*, *T. litoralis* (Brown and Kelly 1993), *P. woesei* (Rudiger et al. 1995), and the mesophilic *B. circulans* F-2 (Kim and Kim 1995).

In *C. thermosulfurogenes* SVM17, the maximum production of thermostable amylopullulanase was attained in solid-state fermentation than in submerged fermentation (Mrudula et al. 2010). Wheat bran was found to be the best substrate for maximal production of the enzyme followed by rice bran, groundnut cake, and black gram bran in solid-state fermentation of *C. thermosulfurogenes* SVM17 (Mrudula et al. 2011). In solid-state fermentation, the substrate supplies the nutrients for growth of the microorganisms and also provides anchorage to the cells (Lonsane et al. 1985; Babu and Satyanarayana 1996). The yield of amylopullulanase by *C. thermosulfurogenes* SVM17 enhanced with increase in moisture content from 30 to 75%. Moisture has been reported to cause swelling of the substrates, thereby facilitating better utilization of the substrate by microorganisms (Kim et al. 1985; Nagendra and Chandrasekharan 1996). The optimization of β-amylase and pullulanase production by response surface methodology was attempted in solid-state fermentation using has in *C. thermosulfurogenes* SV2

(Reddy et al. 2000). The potato flour was shown as the best substrate for the production of thermostable β-amylase and pullulanase in submerged fermentation by *C. thermosulfurogenes* SV2 (Ramesh et al. 2001). *Thermoanaerobacter* B6A has been reported to produce amylopullulanase constitutively (Saha and Zeikus 1990), while it was inducible in *T. ethanolicus* 39E and subjected to catabolite repression (Hyun and Zeikus 1985c). Tenfold higher extracellular enzyme production occurred at under maltose-limited conditions in continuous culture than in batch culture (Hope and Dean 1974).

Besides the production of enzyme, various other products of starch fermentation have also been reported. In case of *Clostridium* sp. strain EM1, the major products of fermentation were acetate, lactate, and ethanol. The concentration of ethanol and acetate remained constant over the dilution rates tested, but the concentration of ethanol rose at higher starch concentrations, and the ratio of ethanol to acetate increased from 3:1 (0.25% starch) to 6:1 (1.5–2% starch) in continuous fermentation (Antranikian et al. 1987a; Madi et al. 1987). *C. thermohydrosulfuricum* 39E alone or in coculture with *C. thermosulfurogenes* 4B yielded similar fermentation products (Hyun and Zeikus 1985b). The coculture resulted in an enhanced rate and yield of ethanol production. The lactate was totally consumed after depletion of sugar substrates. The maximum production rate was 15.0 mmol of ethanol per gram (cell weight) per hour. *Thermoanaerobacter thermohydrosulfuricus* converted the carbon sources into ethanol, acetate, lactate, formate, CO_2, and H_2 in addition to amylopullulanase. A mathematical model for the formation of these products was proposed by Heitmann et al. (1996).

Increased extracellular production of amylase and pullulanase has been reported in submerged fermentation as compared to solid-state fermentation using wheat bran in *Lactobacillus amylophilus* GV6 (Vishnu et al. 2006; Naveena et al. 2004). The presence of amylase and pullulanase activities has been shown to be beneficial for efficient direct conversion of starch to lactic acid (Vishnu et al. 2000; Naveena et al. 2005). In submerged fermentation, maximum amylolytic activity was found with amylopectin followed by soluble starch (Vishnu et al. 2006).

In batch culture, amylolytic activities in *C. thermohydrosulfuricum* 39E remained mainly cell-associated during growth up to late exponential phase (Hyun and Zeikus 1985c). Pullulanase was produced at high levels under starch-limited conditions but not under glucose- or xylose-limited conditions in chemostat cultures of both wild type and mutant strains. *C. thermohydrosulfuricum* strain DSM 567 produced high levels of pullulanase in a growth-dependent manner that occurred predominantly in the exponential phase (Madi et al. 1987). The enzyme was cell bound during growth of the organism with 0.5% starch, while the starch-limited continuous culture produced pullulanase and amylase extracellularly. High levels of pullulanase activity in chemostats under starch-limited conditions were also reported in *C. thermosaccharolyticum* and *T. ethanolicus* (Koch et al. 1987). Batch culture of *K. aerogenes* with excess of maltose resulted in predominantly extracellular pullulanase, whereas under substrate-limited chemostat culture, pullulanase was firmly cell bound (Hope and Dean 1974).

21.6 Purification of Microbial Pullulanases

The purified form of the enzyme is widely employed in studies to understand structure-function relationships and biochemical characterization. In most cases, the purification of pullulanases has been performed by the classical purification methods involving separation of the culture from the fermentation broth, selective concentration by precipitation using ammonium sulfate or organic solvents such as chilled acetone, chromatography usually affinity, ion exchange, and/or gel filtration (Tables 21.2 and 21.3). Cyclodextrin affinity chromatography was for the purification of pullulanases. The enzyme has been found to interact with cyclodextrins and the interaction was found to be occurring at the active site of the enzyme (Marshall 1973; Nakamura et al. 1989; Kimura and Horikoshi 1990; Smith and Salyers 1991; Iwamoto et al. 1993 and Ara et al. 1995b). The affinity for cyclodextrins could be explained by the fact that cyclodextrins structurally resemble the helical form of amylose (Jane et al. 1985).

21.7 Biochemical Characteristics of Pullulanases

21.7.1 Temperature

Most pullulanases from mesophiles are thermolabile (temperature optima between 45 and 60°C), which are not suitable for the current starch conversion process. Thus, research has been focused more on developing thermostable and thermoactive pullulanases which would be of immense value in the bioprocessing of starch, since the bioprocessing of starch at elevated temperature improves the solubility of starch, decreases its viscosity, limits microbial contamination, reduces reaction time, and becomes more economical (Brown and Kelly 1993). Most thermoactive pullulanases known to date belong to the type II group, which hydrolyze both α-1, 4- and α-1, 6-glycosidic linkages in polysaccharides. They have been purified from *P. furiosus* (Brown and Kelly 1993), *T. litoralis* (Brown and Kelly 1993), *T. hydrothermalis* (Gantelet and Duchiron 1998), and *Pyrococcus* strain ES4 (Schuliger et al. 1993).

The pullulanase from *B. circulans* F-2 and amylopullulanase from *Bacillus* sp. KSM-1378 were reported to be optimally active at 50°C (Sata et al. 1989; Ara et al. 1995b), while *C. thermosaccharolyticum* and *T. thalpophilus* pullulanases and *Bacillus strain* 3183 and *Bacillus* sp. strain TS-23 amylopullulanases were optimally active at 70–75°C (Koch et al. 1987; Odibo and Obi 1988; Lin et al. 1996; Shen et al. 1990). The pullulanases from *T. finni*, *T. ethanolicus*, and *T. acetoethylicus* had temperature optima at 90°C (Koch et al. 1987), and the amylopullulanases from *C. thermohydrosulfuricum* and *T. hydrothermalis* were optimally active at 90 and 95°C, respectively (Melasniemi 1987; Saha et al. 1988; Gantelet and Duchiron 1998), while *P. furiosus* amylopullulanase had an optimum temperature of over 100°C, representing the highest temperature range reported for any enzyme (Brown et al. 1990).

Table 21.2 Purification strategies employed for microbial pullulanases

Microorganism	Purification strategy	Fold purification/ yield (%)	Reference
Thermotoga maritima MSB8	Q-Sepharose HP column (pH 7.5), affinity chromatography using β-cyclodextrin (pH 6.0), and ultrafiltration	406/26	Kriegshäuser and Liebl (2000)
Clostridium thermohydrosulfuricum mutant Z 21-109	Thermal precipitation, streptomycin sulfate and $(NH_4)_2SO_4$ (30–60%), DEAE-sephacel, octyl-Sepharose, and pullulan-Sepharose chromatography	3,511	Saha et al. (1988)
Bacillus no. 202-1	DEAE-cellulose adsorption, acetone fractionation, $(NH_4)_2SO_4$ precipitation and DEAE-cellulose column chromatography, and Sephadex G-200 molecular sieve chromatography	290	Nakamura et al. (2003)
Bacillus sp. S-1	$(NH_4)_2SO_4$ (20–70% saturation), DEAE-Toyopearl 650M anion-exchange chromatography (pH 8.0), ultrafiltration, mono Q anion-exchange chromatography (pH 8.0), and Sepharose 4B	101.6/11.3	Kim et al. (1993)
Fervidobacterium pennavorans Ven5	Ultrafiltration, Q-Sepharose fast flow anion-exchange chromatography (pH 7.5), superose-12 gel permeation chromatography, and phenyl-Superose hydrophobic chromatography	65/22	Koch et al. (1997)
Thermus caldophilus GK-24	Ion-exchange chromatography and gel-filtration chromatography	431/13.2	Kim et al. (1996)
Bacillus acidopullulyticus	Macroaffinity ligand-facilitated three-phase partitioning (MLFTPP)	38/89	Mondal et al. (2003)
Thermococcus hydrothermalis	$(NH_4)_2SO_4$ (70% saturation), hydrophobic interaction chromatography on phenyl-Sepharose high-performance column (Pharmacia), Q-Sepharose high-performance anion exchanger (pH 8.5), maltotriose-Sepharose affinity column	97/8	Gantelet and Duchiron (1998)
Bacillus flavocaldarius KP 1228	$(NH_4)_2SO_4$ (20–60% saturation), diethylaminoethyl-Sephadex A-50 column, carboxymethyl-Sephadex C-50 column, ethyl agarose C2 column	844/1.3	Suzuki et al. (1991)

Table 21.3 Purification strategies employed for microbial amylopullulanases

Microorganism	Purification strategy	Fold purification/yield (%)		Reference
		Amy	Pul	
Bacillus sp. KSM-1378	Ultrafiltration, DEAE-cellulose (pH 8.0), α-cyclodextrin coupled with Sepharose 6B (pH 8.0), Sephacryl S-200	203/11	346/18	Ara et al. (1995b)
Bacillus circulans F-2	$(NH_4)_2SO_4$ (80% saturation), starch adsorption, DEAE-Toyopearl 650M ion-exchange chromatography(pH 8.0), hydrophobic interaction chromatographies using TSK Gel Phenyl-51AV (pH 7.5)	167/4	1,400/31.8	Sata et al. (1989)
Bacillus sp. DSM 405	Corn starch adsorption (pH 6.0), hydrophobic interaction chromatography using phenyl-Sepharose CL-4B (pH 7.0), ultrafiltration, and gel filtration	1,400		Brunswick et al. (1999)
Bacillus sp. US149 (Isopullulanase)	Vacuum evaporation, 80% $(NH_4)_2SO_4$, Bio-Gel P-2 column, FPLC Mono Q anion column (pH 9.5), gel-filtration column (pH 5.6), centricon with 5,000 kDa cutoff membrane, and HPLC TSK G2000 SW size exclusion column	3/13.3		Roy et al. (2003)
Clostridium thermosulfurogenes strain SV2	$(NH_4)_2SO_4$ (60% saturation), DEAE-cellulose column chromatography (pH 6.0), and gel filtration using Sephadex G-200.	86.5/9.5	98.1/8.8	Reddy et al. (1998)
Lactobacillus amylophilus GV6	$(NH_4)_2SO_4$ (pH 6.5) and Sephacryl S-200 column chromatography	11.4	10	Vishnu et al. (2006)

Thermoanaerobacter ethanolicus 39E (*Clostridium thermohydrosulfuricum* 39E)	Q-Sepharose, β-cyclodextrin Sepharose (pH 6.0), and ultrafiltration	Nd	2,400	Mathupala and Zeikus (1993)
Thermococcus litoralis	Q-Sepharose column (pH 8.0), ceramic hydroxyapatite (pH 8.0), α-cyclodextrin affinity (pH 5.6), and superdex 200 gel filtration	58	53	Brown and Kelly (1993)
Pyrococcus furiosus	Q-Sepharose column (pH 8.0), ceramic hydroxyapatite (pH 8.0), α-cyclodextrin affinity (pH 5.6), and superdex 200 gel filtration	6.4	82	Brown and Kelly (1993)
Geobacillus stearothermophilus L14	75% $(NH_4)_2SO_4$, Q-Sepharose column (pH 9.0), DEAE-Sepharose column (pH 8.5), ultrafiltration, and Sephadex G-100 gel-filtration column	10.1/30		Zareian et al. (2010)
Thermoanaerobacterium thermosaccharolyticum DSM 571	Butyl-Sepharose column, POROS ether, and superdex	140/1		Ganghofner et al. (1998)
Thermoanaerobacter strain B6A	DEAE-Sepharose CL-6B column chromatography, gel filtration using high-pressure liquid chromatography, and pullulan-Sepharose affinity chromatography.	Nd		Saha et al. (1990)

21.7.2 pH

Most pullulanases have pH optima ranging from acidic to neutral state, with only few alkaline pullulanases. The optimum pH range for pullulanases from *T. finni*, *T. ethanolicus*, *T. acetoethylicus*, and *C. thermosaccharolyticum* was found to be 5.0–6.0 (Koch et al. 1987). At pH 8.0, these enzymes got inactivated (Koch et al. 1987). Similar pH optimum was reported for pullulanase from *B. thermoleovorans US*105 (Messaoud et al. 2002). The pullulanases from *Bacillus* sp. DSM 405 and *Bacillus* sp. AN7 were optimally active at pH 6.0 (Brunswick et al. 1999; Kunamneni and Singh 2006). The *T. thalpophilus* pullulanase had a neutral pH optimum (Odibo and Obi 1988). The pH optimum for *B. cereus* var. *mycoides*, *P. furiosus*, *Fervidobacterium* sp., and *Clostridium* sp. strain EM1 pullulanases was found between 6.0 and 6.5 (Takasaki 1976b; Antranikian et al. 1987b; Brown et al. 1990; Canganella et al. 1994).

The amylopullulanases from *Bacillus* sp. 3183, *Thermoanaerobacter* strain B6A, *P. furiosus*, *Thermococcus litoralis*, and *Thermoanaerobium* ToK6-B1 were optimally active in the pH range of 5.0–6.5 (Brown and Kelly 1993; Plant et al. 1987a; Saha et al. 1989). The amylopullulanase from *Bacillus* sp. strain TS-23 is one of the rare alkali-stable enzymes acting optimally at pH 8.5 (Lin et al. 1996). The thermostable pullulanase from *B. acidopullulyticus* developed for commercial use works well at acidic pH (Norman 1988). Also, the purified pullulanase from C. *thermohydrosulfuricum* was optimally active in the pH range of 3.0–5.5. The pH optimum of pullulanase from another strain of *C. thermohydrosulfuricum* was pH 5.6 (Melasniemi 1987). The pullulanase from *C. thermosaccharolyticum* was most active between pH 5.0 and 6.0 (Koch et al. 1987).

21.7.3 Thermostability

Thermostable pullulanase activity has been found in a number of extreme and hyperthermophilic bacteria like *C. thermohydrosulfuricum* (Hyun and Zeikus 1985a; Melasniemi 1987), *C. thermosaccharolyticum* (Koch et al. 1987), *Clostridium* sp. (Madi et al. 1987), *C. thermosulfurogenes* (Reddy et al. 1999b), *T. aquaticus* YT-1(Plant et al. 1986), *Thermoanaerobium* Tok6-B1 (Plant et al. 1987b), *Thermoanaerobacter* (Saha et al. 1990), *T. maritima* (Kriegshäuser and Liebl 2000) *T. brockii* (Coleman et al. 1987), *T. finni* (Koch et al. 1987), *T. ethanolicus* (Koch et al. 1987), *T. acetoethylicus* (Koch et al. 1987), *T. thalpophilus* (Odibo and Obi 1988), *R. marinus* (Gomes et al. 2003), *B. stearothermophilus* (Wind et al. 1994), and *Bacillus flavocaldarius* KP 1228 (Suzuki et al. 1991).

The pullulanases from *T. finni*, *T. acetoethylicus*, and *T. ethanolicus* were stable at 70°C for 69 min under starch-limited and aerobic conditions (Koch et al. 1987). At 80°C, 90% of maximal activity was observed for the pullulanase from *T. ethanolicus*, which was found to be optimally active at 70°C. The enzyme was stable at 50 or 60°C for 30 min in the absence of polysaccharide substrate. Two and six percent

loss of activity occurred when preincubation was at 70 and 80°C, respectively, while preincubation at 90 and 100°C resulted in a drastic decrease of 45 and 68%, respectively. However, on preincubation with Ca^{++}, stability was enhanced with 72% of the original activity remaining after 30-min incubation at 100°C (Odibo and Obi 1988). The enzyme from *Thermoanaerobacter* Tok6-B1 was stable up to 80°C (Plant et al. 1987b). In case of *Thermococcus celer*, the pullulanase exhibited remarkable thermal stability. At 95°C, half-life of more than 6 h was observed, whereas 50% of the activity was lost after 1 h at 105°C. In case of *F. pennavorans*, 70% residual activity was detected after incubation for 1 h at 85°C while half-life at 95°C was 40 min (Canganella et al. 1994). The increased thermostability for pullulanase from *B. flavocaldarius* KP 1228 was shown to be associated with the increased proline content, on comparing the amino acid composition of the protein with pullulanases from *B. acidopullulyticus* and *K. pneumoniae* (Suzuki et al. 1991). The report provided evidence for the proline theory of increasing protein thermostability (Suzuki et al. 1987b; Suzuki 1989).

Likewise amylopullulanases also have very high thermostabilities. *P. furiosus* amylopullulanase has a half-life of approximately 1 h at 105°C, about 8 h at 98°C, and more than 24 h at 85°C (Brown et al. 1990). The optimum temperature for amylopullulanase activity from *Bacillus* strain TS-23 was 65°C; 70 and 60% of the maximum activity were retained at 70°C and 75°C, respectively (Lin et al. 1994). The amylopullulanase activity of *C. thermohydrosulfuricum* was maximum at 85–90°C for 15 min, while at higher temperatures, the activities declined sharply. The enzyme activity was stable at 65°C in the absence of substrate and Ca^{++} for 2 h. At 85°C, the enzyme activity diminished rapidly with only 30% of the activity after 10 min of incubation (Melasniemi 1988). Saha et al. (1988) reported high thermostability for amylopullulanase from *Clostridium thermohydrosulfuricum*. The enzyme was fairly thermostable in the absence of substrate up to 90°C, with essentially no loss of activity in 30 min. However, when the incubation was done at 95°C for 30 min, the enzyme lost about 40% of its activity. The amylopullulanase activity of *Thermoanaerobacter* strain B6A had an optimal temperature of 75°C and was stable up to 70°C for 60 min. Its half-life was about 5 h at 70°C in the absence of substrate (Saha et al. 1990). The *T. ethanolicus* 39E amylopullulanase activity was stable up to 90°C with a half-life of 40 min at that temperature. The activity was retained with no loss at 85°C for 1 h in the presence of 5 mM $CaCl_2$. The amylopullulanase from *Thermoanaerobium* ToK6-B1 had a half-life of 5 min at 90°C, 17 min at 85°C, and in order of hours at 80°C (Plant et al. 1987a). The *Bacillus* strain 3183 amylopullulanase had maximal activity at 75°C, while loss of activity was detected in the absence of substrate at temperatures above 75°C. A half-life of 96 h was observed at 70°C without substrate (Shen et al. 1990).

Thermostable and thermoactive pullulanases from extremophilic archaea have been found in *Thermococcus celer* (Canganella et al. 1994), *Desulfurococcus mucosus* (Canganella et al. 1994), *Thermococcus aggregans* (Canganella et al. 1994), *Thermococcus guaymagensis* (Canganella et al. 1994), *Thermococcus* sp. (Gantelet et al. 1998), *T. hydrothermalis* (Legin et al. 1998), and *T. profundus* (Kwak et al. 1998).

21.7.4 Effect of Metal Ions

Pullulanases from different microbial sources show diverse behavior towards metal ion requirement for their activity. Some enzymes require metal ions, some are inhibited by metal ions, while some are independent of their presence. Pullulanase from *T. thalpophilus* was found to be stimulated by 1 mM $CoSO_4$ by 61%. Other metal ions like Mg^{++}, Zn^{++}, Fe^{++}, and Cu^{++} inhibited its activity. Ca^{++} had no effect but it increased the thermostability of the enzyme (Odibo and Obi 1988). Ca^{++} was found to significantly enhance the activity of pullulanase from *T. finni* and *C. thermosaccharolyticum*, while Zn^{++} and Cu^{++} were found to be strongly inhibitory (Koch et al. 1987). EDTA was found to inhibit the activity in both cases. Ca^{++} was found to increase both activity and stability of pullulanases from *T. aquaticus* and *T. thalpophilus* at 100°C (Plant et al. 1986; Odibo and Obi 1988), while scarcely those of the *B. flavocaldarius* pullulanase (Suzuki et al. 1991). The pullulanases from *B. cereus* var. *mycoides*, *C. thermohydrosulfuricum* E101-69, *F. pennavorans*, *T. celer*, and *C. thermohydrosulfuricum* SV9 did not require any metal ions for their activity (Takasaki 1976b; Canganella et al. 1994; Melasniemi 1987; Swamy and Seenayya 1996b).

The amylopullulanase activity of *C. thermohydrosulfuricum* E101-69 was stimulated by Ca^{++} while EDTA reversed this activation. Almost full activation was obtained with 0.2 mM Ca^{++} (Melasniemi 1987). The effect of Ca^{++} on enzyme activity was found to be dependent on the substrate, in case of *T. litoralis* and *P. furiosus*. The starch-hydrolyzing activity of the *T. litoralis* enzyme was inhibited by about 14%, while the *P. furiosus* enzyme was inhibited by about 30%. In contrast, the pullulan-hydrolyzing activity of the enzymes was stimulated by about 25% in the presence of Ca^{++}. For both the enzymes, the presence of Ca^{++} had a significant positive effect on enzyme activity at temperatures above 120°C, extending the range at which activity can be measured up to 130–140°C (Brown and Kelly 1993). Stimulation of enzyme activity by Ca^{++} was seen in case of *Bacillus* sp. strain TS-23 (Lin et al. 1996), *Bacillus* strain 3183 (Shen et al. 1990), and *P. woesei* (Rudiger et al. 1995). The amylopullulanase of *Bacillus* sp. strain TS-23 was inhibited by Mg^{++}, Mn^{++}, Ni^{++}, Hg^{++}, and EDTA (Lin et al. 1996). The enzyme from *C. thermohydrosulfuricum* Z 21-109 did not require Ca^{++} for its activity. However, it is inhibited by EDTA, which indicates that Ca^{++} is probably important for its stability (Saha et al. 1988). Ca^{++} was found to be important in maintaining thermostability of the enzyme from *T. ethanolicus* 39E (Mathupala and Zeikus 1993) and *Thermoanaerobium* Tok6-B1 (Plant et al. 1987a). In presence of Ca^{++}, the *Thermoanaerobium* ToK6-B1 amylopullulanase activity was stimulated 100–110% compared with that measured in absence of Ca^{++}. Addition of EDTA reversed the effect suggesting that Ca^{++} is required for full activity (Plant et al. 1987a).

The amylopullulanase activity of *B. thermoamyloliquefaciens* KP 1071 was completely blocked by Hg^{++}, Pb^{++}, Cu^{++}, Zn^{++}, and Cd^{++}, and EDTA had no effect on this inhibition (Suzuki et al. 1987a). In case of amylopullulanase from *Bacillus* sp. KSM-1378, the pullulanase activity was strongly inhibited by Hg^{++}, Pb^{++}, Cd^{++}, and Mn^{++}, and the amylase activity was only slightly inhibited by these cations. Hg^{++} completely eliminated the pullulanase activity but had only a slight inhibitory effect

on amylase activity, while Co^{++} ions slightly stimulated the pullulanase activity (10%) but inhibited the amylase activity by 32% (Ara et al. 1995b).

21.7.5 Effect of Subst.rate

It is generally observed that the presence of substrate prevents thermal denaturation of enzyme at temperatures above the optimal temperature of activity as well as effect of specific enzyme inhibitors. In the absence of substrate, the activity of *Thermoanaerobium* Tok6-B1 amylopullulanase was reduced by 90.5% but by only 50.5% and 40.0% when pullulan or starch were present, respectively (Plant et al. 1987b). In case of *Bacillus* sp. DSM 405, the half-lives of both the α-amylase and pullulanase activities were increased fivefolds when either starch or pullulan was present (Brunswick et al. 1999). The *Bacillus* sp. strain TS-23 amylopullulanase was provided some protection against thermal denaturation by the substrate (Lin et al. 1996).

The substrate afforded protection to enzyme activity against N-bromosuccinimide, which completely abolished both the α-amylase and amylopullulanase activities of *Bacillus* sp. KSM-1378 (Ara et al. 1995a).

21.7.6 Effect of Inhibitors

Amylopullulanases are susceptible to the known inhibitors of α-amylase family. Various α-amylase inhibitors exhibited inhibitory action on the amylopullulanase of alkalophilic *Bacillus* sp. KSM-1378. The pullulanase activity was completely blocked by N-bromosuccinimide, whereas the amylase activity was inhibited by 76%. N-ethylmaleimide inhibited the amylase activity by 25% while there was no effect on pullulanase activity, and, in contrast, α-cyclodextrin eliminated the pullulanase activity by 75%, leaving the amylase activity untouched. The enzyme was also inhibited by maltotriose, isomaltitol, methyl α-D-galactoside and β-cyclodextrin (Ara et al. 1995b).

The *Thermoanaerobacter* strain B6A amylopullulanase did not show any metal activity, but both amylase and pullulanase activities were inhibited by β- and γ-cyclodextrins but not by α-cyclodextrin (Saha et al. 1990). The inhibition of amylopullulanase by cyclodextrins, EDTA, and N-bromosuccinimide was also reported by Saha et al. (1988) in case of *C. thermohydrosulfuricum*. β-cyclodextrin was also shown to inhibit the activity of *T. ethanolicus* 39E enzyme (Mathupala and Zeikus 1993). Marshall (1973) and Iwamoto et al. (1993) reported that the pullulanase of *K. pneumoniae* was competitively inhibited by cyclodextrins. Yu et al. (2011) have recorded the increase of fluorescence emission by β-cyclodextrin, which was possibly due to the formation of inclusion complexes between aromatic amino acid residues of pullulanase and β-cyclodextrin. The interaction of the cyclodextrins with amylopullulanases could be used to obtain information about the active site(s) on such enzymes.

For example, Nakamura et al. (1989) have shown that cyclodextrins competitively inhibited both the pullulanase and amylase activities of the amylopullulanase of *Thermus* sp. and thus found that a single active site was responsible for the dual activities. Contrary to this, Ara et al. (1995b) employed cyclodextrin affinity chromatography in the purification of the amylopullulanase of *Bacillus* sp. KSM-1378 and found that α- and β-cyclodextrins inhibited the pullulanase activity of the enzyme, suggesting separate active sites for the amylase and pullulanase activities.

High enzyme activity has been detected in the presence of reducing agents such as β-mercaptoethanol and dithiothreitol, while *N*-bromosuccinimide and α-cyclodextrin was inhibitory for amylopullulanase from *P. woesei* (Rudiger et al. 1995).

1-(3-dimethylaminopropyl)-3-ethylacarbodi-imide (EDAC), a modifier of the carboxyl group at the active site of the enzyme acts as inhibitor of enzymes containing this group in their active sites. EDAC acted as an inhibitor of the *Bacillus* DSM 405 amylopullulanase indicating the presence of carboxyl groups in its active site (Brunswick et al. 1999). Similar observations were reported in *Thermoanaerobium* Tok6-B1 and *B. circulans* F-2 (Kim and Kim 1995).

21.8 Molecular Properties

These enzymes are usually high molecular weight proteins, ranging from 80 to 250 kDa. Some of these are glycoproteins with attached carbohydrate moieties. Generally, the molecular weight of pullulanase type I is 70–80 kDa (Kim et al. 2000). The amylopullulanases are much larger proteins among other proteins of glycosyl hydrolases.

Enzyme sizes vary from <400 to >1,500 amino acid residues in length (Cantarel et al. 2009). The pullulanase from *T. thalpophilus* had a molecular mass of 79,000 (Odibo and Obi 1988). Two forms of amylopullulanase (I and II) were reported from the culture medium of *C. thermohydrosulfuricum* E 101-69 by gel filtration. Forms I and II had apparent molecular masses of 370 ± 85 kDa and 330 ± 85 kDa, respectively, as determined by native polyacrylamide gradient gel electrophoresis. Both forms were identified as dimers of two identical subunits. The molecular weights of these subunits were about 190 ± 30 kDa for enzyme I and 180 ± 30 kDa for enzyme II. Both the forms contained sugars identical with rhamnose, glucose, galactose, and mannose. Both had a pI of 4.25 (Melasniemi 1988). Multiple amylolytic and pullulanolytic activities were also reported by Koch et al. (1990). By using steric exclusion HPLC, Plant et al. (1987a) determined a molecular mass of 120 kDa for the enzyme from *Thermoanaerobium* Tok6-B1. Glycoprotein nature of amylopullulanase was demonstrated by Saha et al. (1990) in *Thermoanaerobacter* strain B6A, which was a 450 kDa glycoprotein composed of two identical subunits. A 220 kDa amylopullulanase with a pI of 4.13 was purified from *B. circulans* F-2 by Saha et al. (1989). The *T. ethanolicus* 39E amylopullulanase had a relative molecular weight of 140 kDa using SDS-PAGE and a molecular mass of 133 kDa

on gel-filtration chromatography. The pI of this enzyme was 4.0 (Mathupala and Zeikus 1993). The enzyme from a thermoalkalophilic *Bacillus* strain TS-23 had a molecular mass of about 140 kDa with a pI of 3.5 (Lin et al. 1996).

21.9 Molecular Cloning of Pullulanases and Amylopullulanases

Recombinant DNA technology has become the main stay for the development and production of industrial enzyme products at a competitive price. It has been shown that in most hyperthermophilic microorganisms, the starch-hydrolyzing enzyme titers are too low for biotechnological exploitation. The molecular cloning of the corresponding genes and their expression in homologous and heterologous hosts, which are genetically modified, circumvents the problem of inadequate expression in the natural host. Since pullulanases and amylopullulanases are generally produced in very low titers and often specific activity is also very low, attempts have been made for overproduction by the cloning and expression of genes encoding thermostable enzymes in mesophilic hosts and continuous cultivation (Canganella et al. 1994) (Tables 21.4 and 21.5).

The introduction of genetic modification can offer the following advantages in the production and/or quality of these enzymes:

- Higher production efficiency and thus less use of energy and raw materials and less waste
- Availability of enzyme products which for economic, occupational, or environmental reasons would otherwise not be available thus enabling new applications
- Technical improvement through higher specificity and purity of enzyme product

A large number of pullulanase genes have been cloned, expressed, and characterized (Tables 21.4 and 21.6). The pullulanase gene of *Klebsiella pneumoniae* has been cloned and introduced into the chromosome of *Escherichia coli* and *K. pneumoniae* (Katsuragi et al. 1987). The introduction of the pullulanase gene in a plasmid revealed a 20- to 40-fold higher pullulanase production of both hosts (Takizawa and Murooka 1985; Pugsley and Reyss 1990). Pullulanase production in *E. coli* was found to be maltose inducible, and the puLA gene was controlled by malT, the positive regulatory gene of the maltose regulon. The gene expression was not found in a malT-negative mutant and constitutive in a malTc strain. In *K. pneumoniae*, the equivalent of malT was supposed to regulate the pullulanase gene (Michaelis et al. 1985).

21.9.1 Cloning of Thermostable and Thermoactive Pullulanases and Amylopullulanases

Research on the pullulanases from thermophiles is interesting not only for understanding the mechanisms of enzyme stability but also for discovering improved enzymes with more efficient application in industrial starch hydrolysis process.

Table 21.4 Cloning of pullulanase genes

Organism	Molecular mass (kDa)	Host	Remarks	Reference
Anaerobranca gottschalkii	98	*E. coli*	The N-terminal sequence of the purified truncated recombinant pullulanase (70 kDa) was found 252 amino acids downstream from the start site, suggesting an alternative translation initiation or N-terminal protease cleavage by *E. coli*	Bertoldo et al. (2004)
Bacillus sp. KSM-1876	128	*E. coli*	The alkaline pullulanase showed very limited homology (63.2% identity) to previously reported debranching enzymes from prokaryotes and eukaryotes. It contained unique tandem repeats in both the N-terminal and C-terminal regions	Hatada et al. (2001)
Klebsiella pneumoniae W70	120	*E. coli* and *K. pneumoniae*	The precursor of the enzyme contained a signal peptide of 19 amino acid residues	Katsuragi et al. (1987)
K. pneumoniae UNF5023	140	*E. coli*	Pullulanase produced from *K. pneumoniae* was found to be a secreted lipoprotein. The secretion of pullulanase was reported to be induced by maltose. Secretion genes were found to be part of the maltose regulon	d'Enfert et al. (1987) and d'Enfert and Pugsley (1989)
Caldicellulosiruptor saccharolyticus	95	*E. coli*	The open reading frame of the gene was flanked by two other open reading frames. A truncated version of the gene which lacks 381 bp of 5′-sequence was found to exhibit pullulanase activity and thus concluded that the amino-terminal portion of the gene was nonessential for either activity or thermostability	Albertson et al. (1997)
Thermus thermophilus HB8	80	*E. coli*	Based on the nucleotide sequence and the flanking region analyzed by direct sequencing of the inverse PCR product, an expression vector was constructed	Tomiyasu et al. (2001)
Bacillus thermoleovorans US 105	80	*E. coli*	The pullulanase activity from the recombinant strain was improved 15-fold after cloning of the open reading frame under control of the lac promoter	Messaoud et al. (2002)

Bacillus stearothermophilus TRS128	75	*E. coli*	The nucleotide sequence was determined. Although the DNA sequence revealed only one large open reading frame, two possible pairs of SD sequence and initiation codon were found in the frame. To analyze the regulatory region, several mutations (deletion, insertion, and substitution of nucleotides) were introduced in the flanking region of the gene using site-directed mutagenesis	Kuriki et al. (1990)
Thermotoga neapolitana	93	*E. coli*	The pulA gene was subcloned and overexpressed in *E. coli* under the control of the T7 promoter. Thin-layer chromatography of the reaction products in the reaction with pullulan and aesculin showed that the enzyme had transglycosylation activity. Analysis of the transfer product using NMR and isoamylase treatment revealed it to be α-maltotriosyl-(1, 6)-aesculin. *T. neapolitana* pullulanase was the first reported thermostable type I pullulanase having α-1, 6-transferring activity	Kang et al. (2011)
Thermococcus aggregans	82.7	*E. coli*	The half-life of the recombinant enzyme was found to be 2.5 h at 100°C. The enzyme was able to hydrolyze both α-1, 6- and α-1, 4-glycosidic linkages in pullulan forming a mixture of maltotriose, panose, maltose, and glucose	Niehaus et al. (2000)
Bacillus acidopullulyticus	97	*Bacillus acidopullulyticus*	5M guanidinium chloride was found to completely unfold the pullulanase. Refolding was achieved by dilutions of the unfolding mixture. The optimal temperature, specific activity, Km, and fluorescence spectra of the renatured pullulanase were found similar to the native pullulanases	Stefanova et al. (1999)
Thermus IM6501	80	*E. coli*	The optimum temperature of 70ºC shifted to 75ºC in presence of 1 mM $CaCl_2$	Kim et al. (2000)

Table 21.5 Cloning of amylopullulanase genes

Organism	Molecular mass (kDa)	Host	Remarks	Reference
Bacillus sp. KSM-1378	211	*E. coli* and *Bacillus subtilis*	The amylase and the pullulanase domains were located in the amino-terminal half and carboxyl terminal half of the enzyme, respectively, being separated by a tandem repeat of a sequence of 35 amino acids. Asp550-Glu579-Asp645 and Asp1464-Glu1493-Asp1581 were recognized as catalytic triad for the amylase and pullulanase activity, respectively. The purified enzyme was found to be a "castanet-like" or "bent dumbbell-like" with transmission electron microscopy	Hatada et al. (1996)
Bacillus sp. strain XAL601	220	*E. coli*	The gene for alkaline α-amylase-pullulanase was found to overexpress in *E. coli* under the influence of lac promoter. The enzyme was analyzed for its binding efficiency to various carbohydrates and was found to have strong adsorption to crystalline cellulose (Avicel) and raw corn starch	Lee et al. (1994)
Bacillus stearothermophilus TS-23	223	*E. coli*	The amylopullulanase gene was expressed under the control of lac promoter in *E. coli*, and the product obtained was analyzed by α-amylase activity staining on SDS/PAGE. The products were found degenerate with the largest active polypeptide of 220 kDa and the smallest one had a molecular mass of about 105 kDa	Chen et al. (2001)
Clostridium thermohydrosulfuricum DSM 3783	165, 130, 100	*E. coli*	More than ten α-amylase-pullulanase-specific polypeptides were detected on immunoblotting. The largest polypeptide had a molecular weight of about 165 kDa, while the smallest enzymatically active polypeptide of about 100 kDa. The temperature optimum of the enzyme (80–85°C) was found 5°C lower and heat stability similar to that of enzyme produced from native host	Melasniemi and Paloheimo (1989)
Desulfurococcus mucosus	74	*E. coli* and *Bacillus subtilis*	The purified recombinant enzyme was shown to have wider substrate specificity, with the ability to hydrolyze α-1, 6-glycosidic linkages of pullulan and α-1, 4-glycosidic linkages in starch, amylose, amylopectin, and cyclodextrins. Divalent cations were found to have no influence on the stability or activity of the purified recombinant enzyme	Duffner et al. (2000)

Lactobacillus plantarum L137	200	*L. plantarum* NCL21	Amino acid sequence repeats were identified in the N- and C-terminal region of the recombinant enzyme. The C-terminal repeat region truncation of the recombinant amylopullulanase enhanced the enzyme production and specific activity of the enzyme	Kim et al. (2008) and Kim et al. (2009)
Thermoanaerobacter ethanolicus 39E	162	*E. coli*	Hydrophobic cluster analysis and site-directed mutagenesis studies revealed the possible involvement of 3 amino acids identified as Asp597, Glu626, and Asp703 in catalysis. Pullulan was identified as the best substrate for the enzyme	Mathupala et al. (1993) and Lin and Leu (2002)
Thermoanaerobacter saccharolyticum B6A-R1	140	*E. coli*	Hydrophobic cluster analysis and multiple sequence alignment of the amino acid sequence revealed highly conserved amino acid residues of the encoded protein	Ramesh et al. (1994)
Thermoanaerobium brockii	70–100	*E. coli* and *Bacillus subtilis*	Secretion of enzyme increased from 0.23U/ml (in *T. brockii*) to 0.80 to 1.0U/ml when *B. subtilis* was used as an expression host	Coleman et al. (1987)
Thermococcus hydrothermalis	128	*E. coli*	The enzyme was found to possess N-terminal signal peptide, a catalytic domain, a domain bearing S-layer homology-like motifs, a Thr-rich region, and a potential C-terminal transmembrane domain. The presence of these noncatalytic domains revealed that the protein may be anchored to the cell surface and O glycosylated	Erra-Pujada et al. (1999)
Pyrococcus furiosus	90	*E. coli*	The recombinant amylopullulanase was found to remain folded at temperatures of ≤70°C, and denaturing temperatures of above 100°C were required for complete unfolding. Ca^{2+} was shown to enhance the enzyme activity, thermostability, and substrate affinity	Dong et al. (1997)
Pyrococcus woesei	90	*E. coli*	The high rigidity of the purified heat-stable enzyme was demonstrated by fluorescence spectroscopic studies in the presence of denaturing agents such as sodium dodecyl sulfate. At temperatures above 80°C, the enzyme switches from the compact to the unfolded form with an apparent shift in the molecular mass from 45 to 90 kDa	Rudiger et al. (1995)

Table 21.6 Biochemical characteristics of recombinant pullulanases

Organism	Molecular mass (kDa)	Purification strategy	Opt. tem	Opt. pH	Specific activity (U/mg)	Fold purification/yield (%)	Inhibitors	Stabilizers	Additional properties	Reference
Thermotoga neapolitana	93	Heat treatment of the recombinant *E. coli* cells, subjected to nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography	80	5–7	28.7	95	Zn^{2+}, Ni^{2+}, Co^{2+}, and Mn^{2+}	Mg^{2+} and Ca^{2+}	The first thermostable type I pullulanase which has α-1,6-transferring activity.	Kang et al. (2011)
Anaerobranca gottschalkii	96	Heat treatment, source 15Q, phenyl-Sepharose	70	8	56	95/17	Zn^{2+}, Cd^{2+}, Hg^{2+}, Co^{2+}, Cu^{2+}	Sodium dodecyl sulfate, Tween, Triton X-100, iodoacetamide, iodoacetic acid, p-chloromercuribenzoate, α-, β- and γ- cyclodextrins.	Km: 0.75 mg/ml Vmax: 61 U/mg	Bertoldo et al. (2004)
Anaerobranca gottschalkii (N-terminal truncation)	70	Q-Sepharose, phenyl-Sepharose, Superdex S-200	70	8	43	95/3.3	Sodium dodecyl sulfate, Tween, Triton X-100	N-bromosuccinimide	Km: 0.83 mg/ml Vmax: 70 U/mg	Bertoldo et al. (2004)
Bacillus subtilis strain 168	81	Ammonium sulfate precipitation, hydrophobic chromatography and anion-exchange chromatography	310K	6	24.10	45.44/12.52	Nd	Nd	Km: 1.284 mg/ml Vmax: 27.609 U/mg	Malle et al. (2006)
Desulfurococcus mucosus DSM 2162	74	Q-Sepharose, phenyl-Sepharose, and Superdex S-200	85° C	5.0	26U/mg	76/8.2	Zn^{2+}, Cu^{2+}, and Fe^{2+}	*N*-bromosuccinimide	Km: pullulan 0.25 mg/ml soluble starch 5.88 mg/ ml	Duffner et al. (2000)

Table 21.7 Biochemical characteristics of recombinant amylopullulanases

Organism	Molecular mass (kDa)	Purification strategy	Opt. tem (°C)	Opt. pH	Specific activity (U/mg)	Fold purification/yield (%)	Inhibitors	Stabilizers	Additional properties	Reference
Pyrococcus furiosus	89	Ultrafiltration, Q-Sepharose column, α-cyclodextrin-Sepharose affinity chromatography	105	6.0	142.3	Nd	$Ni^{2+,}$ Cu^{2+}, and Zn^{2+}	Calcium ions, Triton X-100, Tween 80 and polyethylene glycol 8000	Km: pullulan 1.6mg/ml soluble starch 2.5 mg/ml Vmax: pullulan 133 U/mg starch 88 U/mg	Dong et al. (1997)
Pyrococcus woesei	90	Heat treatment of the recombinant *E. coli* cells, affinity chromatography on a maltotriose-coupled Sepharose 6B column, and anion-exchange chromatography on Mono Q	100	6.0	Nd	90/15	*N*-bromosuccinimide, α-cyclodextrin	Calcium ions, β-mercaptoethanol and dithiothreitol	Km: pullulan 1.3 mg/ml soluble starch 2.5 mg/ml maltodextrin 5 mg/ml Vmax: pullulan 35 U/mg starch 15 U/mg maltodextrin 3 U/mg	Rudiger et al. (1995)

(continued)

Table 21.7 (continued)

Organism	Molecular mass (kDa)	Purification strategy	Opt. tem (°C)	Opt. pH	Specific activity (U/mg)	Fold purification/yield (%)	Inhibitors	Stabilizers	Additional properties	Reference
Bifidobacterium breve UCC2003	174.9	Ni-nitrilotriacetic acid affinity chromatography	37	6	Nd	Nd	Nd	Nd	Nd	O'Connell Motherway et al. (2008)
Lactobacillus plantarum L137	215.6	Ultrafiltration, DEAE-Sepharose CL-4B and Superose 6	40–45	4.0–4.5	431	1.5/ 25	Hg^{2+}, Cu^{2+}, N-bromosuccinimide, guanidine-HCl, urea, moderately by α-cyclodextrin, γ-cyclodextrin	Co^{2+}	Km: pullulan 6.9 g/l Starch : 7.7 g/l Amylose 2.5 g/l Vmax: pullulan 37.9 U/mg Starch: 53.4 Amylose: 32.3	Kim et al. (2008)
Lactobacillus plantarum L137 (C-terminal truncation)	150–250	Ultrafiltration, DEAE-Sepharose CL-4B and Superose 6	40–45	4.0–4.5	596	1.5/29	Hg^{2+}, Cu^{2+}, N-bromosuccinimide, guanidine-HCl, and urea, moderately by α-cyclodextrin and γ-cyclodextrin	Co^{2+}	Km: pullulan 2.6 g/l	Kim et al. (2009)
Bacillus sp. strain XAL601	225	Ammonium sulfate precipitation, mono S HR5/5, Superdex 200HR 10/30	70	9.0	56.7 U/ml (for soluble starch) and 57.3 U/ml (for pullulan)	6.6	Nd	Nd	Nd	Lee et al. (1994)

Thermoanaerobacterium saccharolyticum B6A-RI,	142±2	Heat treatment of the recombinant *E. coli* cells, Q-Sepharose and β-cyclodextrin-coupled Sepharose affinity chromatography	65	6.0	498	17/15.2	Nd	Nd	Km: pullulan 0.49 mg/ml soluble starch 0.43 mg/ ml	Ramesh et al. (1994)
Thermoanaerobacter strain B6A	450	DEAE-Sepharose CL-6B column chromatography, gel filtration using high-pressure liquid chromatography, and pullulan-Sepharose affinity chromatography	75	5.0	215	Nd	*N*-bromosuccinimide, β and γ-cyclodextrins	Nd	Km: pullulan 0.43 mg/ml soluble starch 0.37 mg/ml	Saha et al. (1990)
Bacillus sp. KSM-1378	210	DEAE-cellulose, affinity chromatography on Sepharose 6B-a-cyclodextrin, and gel filtration on Sephacryl S-200	50	9.5	47 (for soluble starch) 84 (for pullulan)	Nd	Nd	Nd	Nd	Hatada et al. (1996)

(continued)

Table 21.7 (continued)

Organism	Molecular mass (kDa)	Purification strategy	Opt. tem (°C)	Opt. pH	Specific activity (U/mg)	Fold purification/yield (%)	Inhibitors	Stabilizers	Additional properties	Reference
Thermoanaerobacter ethanolicus 39E	109 (thermostable region)	Ni-nitriloacetic acid affinity purification using His-bind resin	90	6.0	Nd	Nd	EDTA, N-bromosuccinimide, and α-cyclodextrin	Ca^{2+}, Mn^{2+}, Ba^{2+}	Km: pullulan 3.79 mg/ml soluble starch 1.38 mg/ml Vmax:pullulan98 μmol/(min.mg) starch 39 μmol/(min.mg)	Lin and Leu (2002)

Nd Not determined

The number of genes from thermophiles encoding amylolytic enzymes that have been cloned and expressed in mesophiles has been increasing significantly (Bertoldo and Antranikian 2002; Niehaus et al. 2000; Sunna et al. 1996). In most cases, the thermostable proteins expressed in mesophilic hosts are found to maintain their thermostability, are correctly folded at low temperature, are resistant to host proteolysis, and can be easily purified by using thermal denaturation of the mesophilic host proteins. The level of enzyme purity obtained is generally suitable for most industrial applications.

Many thermostable amylopullulanases from thermophilic anaerobic bacteria have been cloned and characterized (Tables 21.5 and 21.7) (Mathupala et al. 1993; Melasniemi 1988; Ramesh et al. 1994; Saha et al. 1988; Spreinat and Antranikian 1990). The gene encoding amylopullulanase of *Thermoanaerobacter ethanolicus* 39E, formerly *C. thermohydrosulfuricum* 39E, has been cloned and expressed in *Escherichia coli* and *Bacillus subtilis* (Lee et al. 1993). In *E. coli*, the enzyme was found in the intracellular and periplasmic spaces, while in *B. subtilis* the recombinant enzyme was secreted to the culture supernatant. In case of *C. thermohydrosulfuricum* DSM 378, an α-amylase-pullulanase cloned in *Escherichia coli* revealed three peaks on gel filtration, each having α-amylase and pullulanase activities (Melasniemi 1988). Immunoblotting after SDS-PAGE identified more than ten α-amylase-pullulanase-specific polypeptides. The temperature optimum and the heat stability were found to be almost identical with that of the extracellular α-amylase-pullulanase produced by the native host. The enzyme of *T. saccharolyticum* purified from *E. coli* has been found to have the identical catalytic and thermal characteristics as the native glycoprotein from *T. saccharolyticum* B6A (Ramesh et al. 1994). Linear alignment and the hydrophobic cluster analysis of the amylopullulanase and other amylolytic enzymes revealed conserved amino acid residues. The amylopullulanase purified from *E. coli* was a monomer in contrast to the native enzyme purified from *T. saccharolyticum* B6A which was a dimeric glycoprotein. The amylopullulanase of *T. saccharolyticum* has a lower temperature optimum and degree of thermostability than the *T. ethanolicus* (Mathupala et al. 1993) enzyme. The purified *T. ethanolicus* amylopullulanase from *E. coli* has been found to lose its thermal stability at 85°C upon deletion of its N-terminal region.

Amylopullulanases were also cloned and purified from hyperthermophilic archaea, *P. furiosus* (Dong et al. 1997; Brown and Kelly 1993), *Pyrococcus* strain ES4 (Schuliger et al. 1993), *P. woesei* (Rudiger et al. 1995), and *T. litoralis* (Brown and Kelly 1993), growing at 90–100°C. The extreme thermostability of these enzymes, coupled with their ability to hydrolyze both α-1,4 and α-1,6 linkages, might improve the industrial starch hydrolysis process. Hyperthermophilic archaeal protein genes (Tiboni et al. 1993) contain preferably AGG and AGA arginine codons. The extreme thermostability of these enzymes is due to the low number of cysteine residues (DiRuggiero et al. 1993; Zwickl et al. 1990) and disulfide bridges, the protein structural elements found to be most sensitive to destruction at high temperatures (Volkin and Middaugh 1992).

The amylopullulanase of *P. furiosus* has been cloned and constitutively expressed in *E. coli* independently of the presence of isopropyl-β-D-thiogalactopyranoside,

the *lac* promoter inducer (Dong et al. 1997). The extreme thermostability of the enzyme is because of the complete absence of cysteine residues in the amylopullulanase peptide sequence. The recombinant amylopullulanase retains its globular structure after denaturation at temperatures of <70°C and shows an apparent molecular weight of 50 kDa in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Denaturating temperatures of above 100°C have been found to completely unfold the protein and display its actual molecular weight (90 kDa) in SDS-PAGE. The native amylopullulanase of *P. furiosus* is also shown to have the same migration behavior in SDS-PAGE suggesting that these properties depended on the enzyme's primary sequence (Brown and Kelly 1993). Similar behavior was also detected for the recombinant *P. woesei* amylopullulanase (Rudiger et al. 1995) and *P. furiosus* extracellular α-amylase (Dong et al. 1997). The *P. woesei* amylopullulanase, cloned and expressed in *E. coli*, has been found to shift from the compact globular to the unfolded form, with an apparent change in the molecular mass from 45 to 90 kDa. High enzyme activity has been detected in the presence of calcium ions and reducing agents such as β-mercaptoethanol and dithiothreitol, while *N*-bromosuccinimide and α-cyclodextrin were inhibitory. The amylopullulanase from *P. furiosus* was also cloned and heterologously expressed in the methylotrophic yeast, *Pichia pastoris* (Astolpho et al. 2011).

The low amounts of amylopullulanase activity in the cell-free broth of *T. brockii* led Coleman et al. (1987) to clone the gene into *E. coli* and *Bacillus subtilis*. Higher enzyme secretion has been detected from *B. subtilis* than in *T. brockii*; the secreted enzyme was, however, unglycosylated and had lower thermostability.

Another hyperthermostable pullulanase enzyme from *T. aggregans* was cloned and expressed in *E. coli* (Niehaus et al. 2000). Unlike all other pullulan-hydrolyzing enzymes described to date, the enzyme is able to attack α-1, 6- as well as α-1, 4-glycosidic linkages in pullulan, leading to the formation of a mixture of maltotriose, panose, maltose, and glucose. The enzyme is also able to hydrolyze starch, amylose, and amylopectin, forming maltotriose and maltose as major products.

Overexpression of a thermostable and alkaliphilic α-amylase-pullulanase of high molecular weight (220 kDa) from alkaliphilic *Bacillus* sp. XAL601 had been reported by Lee et al. (1994). Subcloning of the enzyme in *B. subtilis* ANA-l revealed two truncated forms of 85 and 135 kDa on an amylase activity staining, while recombinant *E. coli* expressed a single amylase with a molecular weight of 220 kDa, which corresponded with the molecular weight calculated from the open reading frame of amylopullulanase gene (Takagi et al. 1996). The amino acid sequence alignment has shown the truncation in C-terminal region of the protein.

A large number of thermostable pullulanases have also been cloned, expressed, and characterized. A heat-stable pullulanase from *Bacillus acidopullulyticus* was characterized with respect to its stability against thermal and chemical denaturation and its reactivation after complete chemical unfolding (Stefanova et al. 1999). Sucrose, polyols, and Na_2SO_4 were found to stabilize the enzyme against thermal inactivation. The gene encoding pullulanase from *Thermus* sp. AMD-33 and *B. stearothermophilus* TRS40 has been cloned in *E. coli* (Sashihara et al. 1988). Both pullulanases contained four consensus sequences coding for catalytic and/or substrate-binding sites.

Table 21.8 Four regions conserved among various amylolytic enzymes

Consensus	Organism	Region I DAVINH	Region II GFRLDAAKH	Region III EVID	Region IV FVDNHD
Pullulanase	*Klebsiella pneumonia*	600 DVVYNH	671 GFRFDLMGY	704 EGWD	827 YVSKHD
	Bacillus sp. KSM-1378	1396 DVVFNH	1460 GFRFDMMGD	1493 EGWV	1576 YIEAHD
	B. stearothermophilus TRS128	281 DVVYNH	348 GFRFDLMGI	381 EGWD	464 YVESHD
Amylopullulanase	*C. thermohydrosulfuricum*	487 DGVFNH	594 GWRLDVANE	627 ENWD	699 LLGSHD
	T. ethanolicus 39E	488 DGVFNH	593 GWRLDVANE	626 ELWG	698 LLGSHD
	Bacillus sp. XAL601	608 DGVFNE	743 GWRLDVANE	822 EEID	846 LIGSHD
Neopullulanase	*B. stearothermophilus*	242 DAVFNH	324 GWRLDVANE	357 EIWE	419 LLGSHD
Amylase	*Aspergillus oryzae*	117 DVVANH	202 GFRIDTVKH	230 EVLD	292 FVENHD
	B. stearothermophilus	101 DVVFDH	230 GFRLDAVKH	264 EYWS	326 FVDNHD
	B. amyloliquefaciens	98 DVVLNH	227 GFRIDAAKH	261 EYWQ	323 FVENHD
	B. subtilis	97 DAVINH	172 GFRFDAAKH	208 EILQ	264 WVESED
	Bacillus sp. KSM-1378	462 DVVLNH	546 YFRVDTVKH	579 EAWG	640 FLGSHD
	C. thermosulfurogenes EM1	161 DFAPNH	251 GIRLDAVKH	283 EWFL	349 FIDNHD
	Micrococcus sp. 207	469 DVVVNH	554 YFRVDTVKH	596 EAWG	652 FLGSHD
Cyclodextrin glucanotransferase (CGT)	*Klebsiella aerogenes*	130 DYADNE	219 AIRIDAIKH	257 EWFG	328 FMDNHD
	B. amyloliquefaciens	131 DFAPNH	221 GIRMDAVKH	253 EWFL	319 FIDNHD
	B. macerans	135 DFAPNE	225 GIRFDAVKH	258 EWFL	324 FIDNHD
	Bacillus sp.	135 DFAPNH	225 GIRVDAVKH	268 EYHQ	323 FIDNHD
Isoamylase	*Pseudomonas amyloderamosa*	291 DVVYNH	370 GFRFDLAHV	454 EWSV	502 FIDVHD

21.10 Conserved Regions in Pullulanases

The α-amylase family enzymes are multidomain enzymes. They share a common catalytic domain in the form of a parallel $(\alpha/\beta)_8$ barrel, which is a barrel of eight parallel β-strands surrounded by eight helices, the so-called domain A. The domain A also is the most conserved domain in all α-amylase family enzymes. The amino acid sequence of α-amylase family enzymes revealed four highly conserved regions in their primary sequence, which includes an active center and common substrate-binding sites (Table 21.8).

All type I pullulanases contain a highly conserved region consisting of seven amino acids YNWGYDP (Bertoldo et al. 2004). This conserved region was not found in the type II pullulanases (Bertoldo et al. 1999). Yamashita et al. (1997) proposed that the YNWGYDP motif might be involved in the degradation of α-1, 6-glycosidic linkages or substrate binding.

The amino acid sequence of amylopullulanase from alkaliphilic *Bacillus* sp. strain XAL601 has been shown to have a six-amino-acid sequence (Gly-Ser-Gly-Thr-Thr-Pro) repeated twelve times in the C-terminal region (Lin et al. 1994). The C-terminal region of amylopullulanase from *B. stearothermophilus* TS-23 has also a six-amino-acid sequence (Pro-Gly-Ser-Gly-Thr-Thr) repeated nine times (Chen et al. 2001). The N-terminal sequence of the purified α-amylase-pullulanase enzyme from *B. circulans* F-2 having dual activity with respect to glycosidic bond cleavage was Ala-Asp-Ala-Lys-Lys-Thr-Pro-Gln-Gln-Gln-Phe-Asp-Ala-Leu-Trp-Ala-Ala-Gly-ILe-Val-Trp-Gly-Thr-Pro-Asp-Gly-Phe (Kim and Kim 1995). The multiple amino acid sequence alignment of the GH57 amylopullulanases revealed that the N-terminal regions were highly conserved than the C-terminal regions (Y-L Jiao et al. 2011). The C-terminal domains of thermoactive amylopullulanases are composed of Thr-rich regions and S-layer motifs (Sa'ra et al. 1998). The Thr-rich regions have been predicted to have intensive O glycosylation, while the S-layer motifs have been speculated for anchoring to the cell envelope or increasing the substrate affinity (Chami et al. 1997; Olabarría et al. 1996). The C-terminal sequence of amylopullulanases from *T. hydrothermalis* (belonging to GH57), *T. ethanolicus* 39E (belonging to GH13), and *Thermoanaerobacterium saccharolyticum* NTOU1 (belonging to GH13) was found to be nonessential for catalytic activity (Y-L Jiao et al. 2011; Lin et al. 2008; Mathupala and Zeikus 1993; Lin et al. 2011).

21.10.1 Active Site

The amylopullulanases from different microbial sources contain either one or two active sites. The dual hydrolytic activity of several amylopullulanases such as those from *T. aquaticus* YT-1 (Plant et al. 1986), *Thermoanaerobium* Tok6-B1(Plant et al. 1987b), *Thermoanaerobacter ethanolicus* 39E (Lee et al. 1993), *C. thermohydrosulfuricum* (Melasniemi 1987; Saha et al. 1988), *B. subtilis* TU (Takasaki 1987),

Bacillus sp. strain XAL 601 (Lee et al. 1994), *Bacillus* sp. DSM 405 (Brunswick et al. 1999), *Bacillus* sp. 3183 (Saha et al. 1989), *P. furiosus*, *T. litoralis* (Brown and Kelly 1993), and *T. hydrothermalis* (Boussarsar et al. 2007; Zona et al. 2004) is due to a single catalytic site. When the enzyme from *Thermoanaerobium* Tok6-B1 was modified with 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide (EDAC) in the presence of pullulan, and amylase and residual pullulanase or α-amylase activity was determined, it was found that pullulan partially protected both the activities from EDAC modification. Similarly, amylose partially prevented inactivation of both the activities. This suggested that this dual activity occurred at a common EDAC sensitive site (Plant et al. 1987b). Kinetic experiments on competitive inhibition with mixed substrates and chemical modification with EDAC were used by Brunswick et al. (1999) to determine the number of active sites in the amylopullulanase of *Bacillus* sp. DSM 405. Both these approaches suggested the presence of a single active site possessing both hydrolytic activities at one site.

Dual hydrolytic activities associated with different active sites have been found in amylopullulanases/α-amylase-pullulanase from *B. circulans* F-2 (Saha and Zeikus 1989b), *Bacillus* sp. KSM-1378 (Hatada et al. 1996), and *Bifidobacterium breve* UCC2003 (O'Connell Motherway et al. 2008). Ara et al. (1996) showed the presence of two functional domains for α-1, 4- and α-1, 6- hydrolytic activity in amylopullulanase of *Bacillus* sp. KSM-1378 by means of partial hydrolysis with papain. Transmission electron microscopy analysis revealed amylose and pullulan-hydrolyzing polypeptides as a mixture of globular molecules of different sizes joined by a thin, short linker region. The involvement of acidic amino acids at the active site has been demonstrated by Plant et al. (1987b) and Mathupala and Zeikus (1993) for enzymes from amylopullulanases of *Thermoanaerobium* Tok6-B1 and *T. ethanolicus* 39E, respectively.

21.10.1.1 Hydrophobic Cluster Analysis and Site-Directed Mutagenesis Studies in Pullulanases and Amylopullulanases

Hydrophobic cluster analysis (HCA) is based on two-dimensional illustration of protein sequences. This is a more sensitive and efficient method than linear alignment in that information is provided about the similarity of the secondary structures in proteins (Lemesle-Varlot et al. 1990) and thus used as a means of comparing shapes and relative location of hydrophobic clusters in proteins. The shapes of the clusters are associated with secondary structures, and the similarity of the hydrophobic clusters reflects similarity in the polypeptide folding of the proteins (Woodcock et al. 1992).

The catalytic residues of amylopullulanases were tentatively identified by using hydrophobic cluster analysis (HCA) and further confirmed by site-directed mutagenesis studies. Using HCA plots, two conserved Asp residues and one conserved Glu residue, located within the well-conserved segments, have been found to play a catalytic role in TAk α-amylase A (Nagashima et al. 1992) and amylopullulanases

from *Bacillus* sp. KSM-1378 (Hatada et al. 1996), *T. ethanolicus* 39E (Mathupala et al. 1993), and *T. saccharolyticum* B6A-RI (Ramesh et al. 1994). The putative catalytic triads Asp550-Glu579-Asp645 for the amylase activity and Asp1464-Glu1493-Asp1581 for the pullulanase activity have been identified in *Bacillus* sp. KSM-1378 amylopullulanase. *T. ethanolicus* 39E and *T. saccharolyticum* B6A-RI amylopullulanases have Asp597-Glu626-Asp703 and Asp594-Asp700-Glu623 as catalytic triads, respectively, for both amylase and pullulanase activity. In *Thermococcus hydrothermalis*, Glu291, and Asp394 were identified (by site-directed mutagenesis) as the catalytic pair for both amylolytic and pullulanolytic activities (Zona et al. 2004).

Site-directed mutagenesis of three amino acid residues from *T. ethanolicus* 39E was putatively identified to be involved in catalysis that resulted in the loss of both α-amylase and pullulanase activities completely; this suggested the possible involvement of three residues in a catalytic triad (Mathupala et al. 1993). The three residues of a catalytic triad in *T. ethanolicus* 39E were individually modified to their respective amide form or the alternate acid form by single point base mutations of the amylopullulanase gene (Mathupala et al. 1993). These three residues are located within close proximity to each other, forming a single active site for the dual activities in both *T. ethanolicus* 39E and *T. saccharolyticum* B6A-RI, in contrast to the dual active sites proposed for the α-amylase-pullulanase of C. *thermohydrosulfuricum* El01 (Melasniemi et al. 1990), alkaline amylopullulanase from *Bacillus* sp. KSM-1378 (Hatada et al. 1996), and α-amylase-pullulanase of *B. circulans* F-2 (Sata et al. 1989).

The three acidic residues have been proposed as the catalytic residues for other glucosidases too, as in neopullulanase from *B. stearothermophilus* (Kuriki et al. 1991), cyclodextrin glucanotransferases from alkalophilic *Bacillus* sp. (Nakamura et al. 1992) and *B. circulans* (Klein and Schulz 1991), α-amylase from *B. stearothermophilus* (Holm et al. 1990) and *B. subtilis* (Takasaki et al. 1991), cyclodextrinase from *T. ethanolicus* 39E (Podkovyrov et al. 1993), endoglucanase from C. *thermocellum* (Chauvaux et al. 1992), and the branching enzymes (Takata et al. 1994; Kuriki et al. 1996) by site-directed mutagenesis. These results are consistent with the X-ray crystallographic analysis of α-amylases (Matsuura et al. 1984; Swift et al. 1991; Qian et al. 1993; Machius et al. 1995; Morishita et al. 1997), cyclodextrin glycosyltransferases (Klein and Schulz 1991; Kubota et al. 1991; Strokopytov et al. 1995), an oligo-1, 6-glucosidase (Watanabe et al. 1997a), and an isoamylase (Katsuya et al. 1998).

Mutagenesis studies in pullulanase type I from *Klebsiella aerogenes* at residues His607, Asp677, His682, and His833 located in the four conserved regions of the α-amylase family had shown that the amino acid substitutions for His607, Asp677, or His833 resulted in complete loss of enzyme activity (Yamashita et al. 1997). On the contrary, the mutation at His682 led to retention of pullulanase activity. The binding affinity of these variants for α- or β-cyclodextrin (CD), the competitive inhibitors for pullulanase, was measured using an α-CD Sepharose column. The mutations at His833 did not change the binding affinity for α-CD, whereas the mutations at His607 or Asp677 resulted in the loss of binding ability for pullulan. These

findings have suggested that in *Klebsiella* pullulanase, His607 and Asp677 participate in substrate binding and His833 is involved in catalysis, but His682 might not be in the active site.

There are also reports on the findings of new amino acid consensus sequences specific for starch-debranching enzymes in two oligo-1, 6-glucosidases, several pullulanases, and an isoamylase. Site-directed mutagenesis studies have also revealed the probability of two tyrosine residues to participate in cleaving α-1, 6-glucosidic linkages in starch-debranching enzymes. Two amino acid residues in the predicted consensus region of *Klebsiella* pullulanase, Tyr559 and Tyr564, were replaced by Ala or Phe (Yamashita et al. 1997). The mutations at Tyr559 resulted in complete loss of pullulanase activity without affecting the binding affinities for α-CD and pullulan and the Tyr564 variant dramatically decreased the activity.

21.10.1.2 Physicochemical Approach for the Analysis of Active Site(s)

Boussarsar et al. (2007) compared the action pattern of amylopullulanase from *T. hydrothermalis* with α-amylase (from *Aspergillus oryzae*) and glucoamylase (from *Aspergillus niger*). Various physicochemical approaches based on different principles (fluid mechanics, thermodynamics, and vibrational spectroscopy) were studied for determining the hydrolysis products of the three enzymes and to characterize the dual "'exo' and 'endo'" action patterns for the recombinant amylopullulanase from *T. hydrothermalis*. The reducing sugar measurements with the dynamic viscometric analysis have shown similarity between glucoamylase and amylopullulanase. The amylose hydrolysates of α-amylase significantly reduced the viscosity of medium, while the viscosity remained almost same with the glucoamylase and amylopullulanase hydrolysates. Glass transition temperatures of the enzymes were determined using differential scanning calorimeter; these were found different for α-amylase hydrolysates while almost similar for glucoamylase and amylopullulanase. Thin-layer chromatographic analysis of amylopullulanase hydrolysates revealed the presence of glucose and oligosaccharides (DP: 2-7), while the glucose was identified in the glucoamylase chromatograms. The analysis of the Fourier transform infrared (FTIR) spectra of amylopullulanase hydrolysate identified several intense peaks originating from short-chain oligosaccharides and confirmed the dual action pattern of the enzyme.

21.11 Fluorescence Emission and Circular Dichroism (CD) Spectrometry Analysis

Fluorescence spectroscopy is an effective method to study tertiary structure transitions in enzymes. The intrinsic fluorescence of protein is generally derived from aromatic amino acids such as tryptophan, phenylalanine, and tyrosine. Tryptophan is mainly responsible for the intrinsic fluorescence in many proteins or peptides

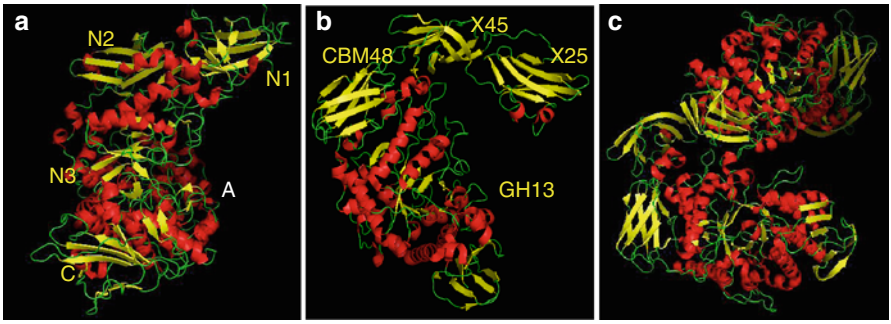

Fig. 21.1 3-D Macromolecular structures of microbial pullulanases: (**a**) Klebsiella pneumoniae complexed with glucose. N1 (*blue*), N2 (*green*), N3 (*cyan*), A (*white*), and C (*yellow*) domains, together with five calcium ions (*purple sphere*) and two G4 (*ball-and-stick*) in the active site are shown (Mikami et al. 2006); (**b**) Bacillus acidopullulyticus pullulanase, X45, X25, CBM48, and GH13 are the domains (Turkenburg et al. 2009); (**c**) Bacillus subtilis strain 168 complexed with maltose and α-cyclodextrin (Source: www.ncbi.nlm.nih.gov/structure)

because of low phenylalanine yield and easy fluorescence quenching of tyrosine. Besides, the intrinsic fluorescence of tryptophan is chiefly responsive to the polarity of microenvironment (Viseu et al. 2004). The 3-D macromolecular structures have been reported for the microbial pullulanases from *Klebsiella pneumoniae, Bacillus acidopullulyticus*, and *Bacillus subtilis* strain 168 (Fig. 21.1).

Lin et al. (2008) made the comparative and structural analysis of full-length amylopullulanase from *T. ethanolicus* 39E and its C-terminal truncated mutant using fluorescence emission and CD spectrometry. The enzymes were found to have almost similar overlapping fluorescence spectroscopic pattern upon denaturation with urea. The renatured enzymes have also revealed similar fluorescence spectra. Comparable enzymatic activities were determined for both the native and renatured enzymes. The protein secondary structures of full length amylopullulanase and its C-terminal truncated mutant were compared using far-UV CD spectroscopy. The intensity of CD spectra was not changed upon C-terminal deletion of up to 100 amino acid residues of full-length amylopullulanase. The overall structures of both enzymes were found identical as revealed from spectrometric data analysis.

Similar studies have been conducted for determining the structural integrities of full-length amylopullulanase from *Thermoanaerobacterium saccharolyticum* and its C-terminal truncated mutant (Lin et al. 2011). The fluorescence and circular dichroism spectrometric methods revealed highly indistinguishable structure for both enzymes. A similar active folding conformation was attained for both enzyme forms on fluorescence spectra. CD spectrometry revealed similar thermal unfolding and a one-step melting curve for both the enzymes. Far-UV spectroscopy was used to compare the protein secondary structures.

These results suggested that a large part of the C-terminal portion of amylopullulanases from *Thermoanaerobacter ethanolicus* 39E such as the C-terminal carbohydrate-binding module family 20, the second fibronectin type III, and a portion of the first fibronectin III motifs, and that of *Thermoanaerobacter saccharolyticum*

NTOU1 amylopullulanase such as C-terminal fibronectin type III (FnIII) motif could be removed without causing a serious aberrant structural change or a dramatic decrease in the hydrolysis of soluble starch and pullulan.

Fluorescence spectrometry has also been used for analyzing the conformational changes in *Bacillus acidopullulyticus* pullulanase (Stefanova et al. 1999). A fluorescence maximum of 342 nm was attained by the native pullulanase of *B. acidopullulyticus*, which indicates that the tryptophanyl residues are partially protected from the aqueous solvent (Schmid 1989). Unfolding of the native protein by guanidinium chloride has shifted the tryptophanyl fluorescence to 358 nm (red shift), suggesting the full exposure of tryptophan side chains to the solvent.

Yu et al. (2011) used fluorescence and circular dichroism spectroscopy to explore inhibition mechanisms of β-CD on pullulanase by detecting pullulanase microenvironment and secondary structural changes. The fluorescence spectroscopy revealed the formation of inclusion complex between β-CD and pullulanase, which caused a conformational change of pullulanase molecule. The circular dichroism spectra were further analyzed to examine whether changes in pullulanase secondary structure depended on the β-CD concentration. With increasing concentrations of β-CD, the ratio of α-helix to β-sheet in pullulanase declined, resulting in a loss of enzymatic activity.

21.12 Crystal Structure of Pullulanases and Amylopullulanases

The crystal structure of *Klebsiella pneumoniae* pullulanase and its complex with glucose (G1), maltose (G2), isomaltose (isoG2), maltotriose (G3), or maltotetraose (G4) have been refined at around 1.7–1.9 Å resolution by using a synchrotron radiation source at Spring-8 (Mikami et al. 2006). The crystals for X-ray diffraction experiments had been prepared by the hanging-drop and vapor-diffusion methods. The refined models of the enzyme were shown to possess 920–1,052 amino acid residues, 942–1,212 water molecules, four or five calcium ions, the bound sugar moieties, and five domains (N1, N2, N3, A, and C). The N1 domain was clearly visible only in the structure of the complex with maltotriose or maltotetraose. The N1 and N2 domains were characteristic of pullulanase, while the N3, A, and C domains have weak similarity with those of *Pseudomonas* isoamylase. The N1 domain was found to be a new type of carbohydrate-binding domain with one calcium site (CBM41). One glucose molecule binds at subsite K2, while two isomaltose molecules bind at K1wK2 and C2 wC1, two maltotriose molecules at K1wK3 and C2w00, and two maltotetraose molecules at K1wK4 and C2wK10. The two bound maltotriose and maltotetraose molecules in the active cleft are almost parallel and interact with each other. The subsites K1wK4 and C1wC2, including catalytic residues Glu706 and Asp677, are conserved between pullulanase and α-amylase, indicating that pullulanase strongly recognizes branched point and branched sugar residues, while subsites 00 and K10, which recognize the nonreducing end of main-chain α-1, 4 glucan, are specific to pullulanase and isoamylase.

The gene encoding pullulanase from the common spore-forming bacterium *B. subtilis* strain 168 cloned and overexpressed in *Escherichia coli* has also been purified and crystallized. X-ray crystallographic analysis of the pullulanase crystal showed that the crystal belongs to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 70.568, b = 127.68, and c = 189.25 Å. The crystal contained two molecules of pullulanase in the asymmetric unit, with a solvent content of 53.15%. The crystal was diffracted to 2.1 Å resolution at a synchrotron and therefore is suitable for structure determination.

Knapp et al. (1995) resolved the crystal structure of amylopullulanase from *P. woesei*. Crystals obtained by the vapor-diffusion method were in the form of hexagonal rods and diffracted to a maximum resolution of 3 Å. The X-ray crystallographic analysis revealed the trigonal lattice type of crystal with the space group *P3,21* or *P3,21*, respectively, and has the cell dimensions *a = b = 96.8* A, *c = 196.2* A (Y = p = go″, *y = 120″*). The crystals have a theoretical packing density of 2.7A31Da, assuming one molecule with a molecular weight of 88.8 kDa in the asymmetric unit.

21.13 Applications of Pullulanases and Amylopullulanases

Pullulanases are among the most important debranching enzymes widely employed in starch-processing industry. Pullulanase is used predominantly in combination with other amylolytic enzymes that break down starch. In the present day scenario, pullulanases find application in all the industrial processes such as in food as a baking enzyme, for the saccharification of starch into glucose and glucose syrup, maltose and maltotriose syrup, brewing industry, and detergent industry.

21.13.1 Bread and Baking Industry and as an Anti-staling Agent

Starch-modifying enzymes are of high market value in the baking industry. All undesirable changes like increase of crumb firmness, loss of crispness of the crust, decrease in moisture content of the crumb, and loss of bread flavor that occur upon storage of baked products are called staling. Staling limits the shelf life of baked products. Retrogradation of the amylopectin fraction of starch is considered very important in staling and the main cause for the firming rate of bread (Kulp and Ponte 1981; Champenois et al. 1999).

Starch-acting enzymes like α-amylases (De Stefanis and Turner 1981; Cole 1982), branching (Okada et al. 1984) and debranching (Carroll et al. 1987) enzymes, maltogenic amylases (Olesen 1991), β-amylases (Wursch and Gumy 1994), and amyloglucosidases (Vidal and Gerrity 1979) have been suggested to act as anti-staling agents. The use of α-amylases as anti-staling agent is not widespread because even a slight overdose of amylase results in sticky bread. The

increased gumminess of α-amylase-treated bread is associated with the production of branched maltodextrins of DP20-100 (De Stefanis and Turner 1981). Debranching enzymes such as thermostable amylopullulanases or a thermostable pullulanase and an α-amylase used together are claimed to decrease strongly the problems associated with the use of α-amylases as anti-staling agents in baking. The pullulanase hydrolyzes the branched maltodextrins produced by the α-amylase while having minor effect on the amylopectin (Carroll et al. 1987). Pullulanase thus specifically eliminates the compound responsible for the gumminess of α-amylase-treated bakery products.

21.13.2 Starch Liquefaction and Saccharification

The starch and alcohol industry are among the major users of pullulanases and amylopullulanases for hydrolysis of starch to useful products. The pullulanase has been used for saccharification of liquefied starch and amylopullulanases serve for one-step starch liquefaction-saccharification.

21.13.2.1 Production of Glucose, High-Maltose Conversion Syrups, Maltotriose Syrups

In dextrose (D-glucose) production, the pullulanase is used in combination with glucoamylase, which has been shown to increase the glucose yield by limiting the oligosaccharide content and allows reduced dosage of glucoamylase. In maltose production, saccharification is carried out with pullulanase, β-amylase, and maltogenic α-amylase. Addition of the pullulanase has been found to increase maltose yield and reduce the amount of branched oligosaccharides.

The current starch conversion processes suffer from number of limitations like the α-amylases, which are used for starch liquefaction to form soluble maltodextrins, are not active at a pH below 5.9. Thus the pH needs to be adjusted from the natural pH 4.5 of the starch slurry to pH 6 by adding NaOH. Also Ca^{2+} has to be added because of the Ca^{2+} dependency of these enzymes. The glucoamylase used in saccharification is specialized in cleaving α-1, 4-glycosidic bonds and slowly hydrolyzes α-1, 6-glycosidic bond present in maltodextrins. This will result in the accumulation of reversion products such as maltose and isomaltose at the expense of the amount of glucose. The pullulanase that is highly thermostable and compatible with respect to pH and other process parameters has been proved advantageous for the starch saccharification process, as it increases the concentration of glucose (about 2%) or maltose (about 20–25%), reduces reaction time (to 48 h), allows an increase in substrate concentration (to 40% DS), and allows a reduction in the use of glucoamylase (up to 50%). The highly thermostable amylopullulanases are useful for one-step starch liquefaction-saccharification and thus can replace other amylolytic enzymes like α-amylases and glucoamylase.

Thermostable enzyme mixes are now available on the market that contain both glucoamylase and pullulanase, e.g., OPTIMAX® from Genencor.

Maltose and maltooligosaccharides are widely employed in food, beverage, pharmaceutical, and chemical industries (Fogarty and Kelly 1990). Various maltose-containing syrups are used in the brewing, baking, soft drink, canning, confectionery, and other food industries. There are three types of maltose-containing syrups: high-maltose syrup (DE 35–50, 45–60% maltose, 10–25% maltotriose, 0.5–3% glucose), extra-high-maltose syrup (DE 45–60, 70–85% maltose, 8–21% maltotriose, 1.5–2% glucose), and high-conversion syrup (DE 60–70, 30–47% maltose, 35–43% glucose, 8–15% maltotriose). The production of these syrups from starch generally involves liquefaction and saccharification, as in the production of glucose. The liquefaction reaction is terminated when the DE is about 5–10 because a low DE value increases the final maltose content while glucose production requires partial hydrolysis of gelatinized starch to a dextrose equivalent (DE) of 10–15.

The production of high maltose syrups from starch has been reported from highly heat-stable pullulanase of *B. acidopullulyticus*. The temperature optimum (60°C) and pH optimum (5.0) of the enzyme was similar to that of *Aspergillus niger* glucoamylase (4.5) allowing both enzymes to be used for starch saccharification, which would result in glucoamylase savings and higher D-glucose levels (Jensen and Norman 1984). Besides, the thermostable pullulanase from *Klebsiella pneumoniae* has also been extensively utilized in the starch-processing industry (Norman 1979).

Satyanarayana et al. (2004) reported enhanced saccharification of starch to glucose by glucoamylase from *Thermomucor indicae-seudaticae* when pretreated with amylopullulanase from *G. thermoleovorans* NP33 as compared to that of α-amylase from *G. thermoleovorans* NP54.

Maltotriose rich syrups have been produced by cation-exchange resin chromatography of maltose syrups or by enzymatic hydrolysis of the polysaccharide pullulan using the debranching enzyme, pullulanase (Singh et al. 2010). Maltotriose syrup possesses excellent properties as low freezing point depression, mild sweetness, keeps in moisture, prevention of retrogradation of starch in foodstuffs, less color formation compared with maltose syrups, glucose syrups, or sucrose, good heat stability, low solution viscosity, high fermentability, and favoring glassy states. These properties are very useful in food and pharmaceutical industries (Zoebelein and Böllert 2001). High maltotriose syrup may be applied in the food industry for the manufacturing of desserts, baking and brewing, as well as in *the pharmaceutical industry for replacing glucose in intravenous feeding*.

21.13.2.2 Production of Biofuel

Ethanol production is usually produced via enzymatic hydrolysis of starch-containing crops such as wheat, barley, rice, sweet sorghum, and corn and root plants like potato

and cassava. The alcohol produced from food crops is called grain alcohol. The process of making alcohol from starch involves preparation of feedstock, fermentation of simple sugars to ethanol, and recovery of the alcohol.

The starch cannot be directly fermented to ethanol by conventional fermentation process. The macromolecular structure first broke down in to glucose. In this process, starch feedstocks are grounded and mixed with water to produce a mash that typically contained 15–20% starch. The mash is then boiled and treated subsequently with enzymes, amylase, pullulanase, and glucoamylase. The amylase cleaves starch molecules to short chains of glucose that liberates maltodextrin oligosaccharides by liquefaction process. The dextrin and oligosaccharides are further hydrolyzed by enzymes such as pullulanase and glucoamylase in a process known as saccharification. Saccharification converts all dextrans to glucose, maltose and isomaltose. The mash is then cooled to 30°C and yeast is added for fermentation (Shelley 2006). For the alcohol and brewing industries, use of pullulanase in addition to glucoamylase enzymes, increases the amount of fermentable sugars and may facilitate filtration steps. Pullulanase is also used to produce low carbohydrate (low calorie) "Lite beer"; it is added with fungal α-amylase or glucoamylase to the wort during fermentation.

21.13.2.3 Improving Starch Quality, Bioprocessing, and Nutritional Value in Rice Seeds

Transgenic rice seeds producing a thermostable and bifunctional starch hydrolase, amylopullulanase (APU) from *T. ethanolicus* 39E, have been generated (Chiang et al. 2005). Amylopullulanase from *T. ethanolicus* 39E is heat stable with a catalytic optimum at 90°C (Mathupala et al. 1993; Lin and Leu 2002). The use of transgenic rice seeds containing this thermostable and bifunctional enzyme would facilitate liquefaction and saccharification of starch simultaneously at high temperature without the need to add exogenous α-amylase and pullulanase. Transgenic rice seeds could be used directly or as a source of amylopullulanase, in the substitution of microbial amylolytic enzymes, to convert starch into liquefied starch hydrolysate, which can be further used for the industrial production of sweeteners or fermentation products.

Elevation in granule-bound pullulanase activity has been shown to correlate with the reduction of amylose in developing transgenic rice seeds. Amylose content affects rice quality such as palatability and industrial uses. It is a key determinant of the eating and cooking quality of rice; high-amylose levels are usually associated with dry, fluffy, and nonsticky rice grains (Juliano 1985). Such an application would be desirable for improving starch quality, bioprocessing of starch, and production of protein-enriched flour from rice seeds, thereby simplifying the sugar and protein production process and significantly reducing production cost. The protein-enriched rice flour has high nutritional value and is useful for the production of baby and health food (Hansen et al. 1981; Morita and Kiriyama 1993).

21.13.2.4 Preparation of Slowly Digestible Starch

Pullulanase debranching has been used to prepare slowly digestible starch (Guraya et al. 2001; Miao et al. 2009). Slowly digestible starch has potential applications on human health and is correlated with a low glycemic index for treatment and prevention of several diseases, such as cardiovascular diseases (Ells et al. 2005), non-insulin diabetes (Shin et al. 2005), and obesity (Wolf et al. 1999). It also provides sustained and stable energy for athletes (Eliasson 2004).

21.13.2.5 Preparation of Resistant Starch

During starch processing, the gelatinized starch may revert to a form that could be highly resistant to hydrolysis by α-amylase and is called resistant starch (RS) (Annison and Topping 1994). RS has attracted great interest in among the nutritionists and food industry, due to its reduced levels of plasma glucose and insulin, increased fecal bulk, and short-chain fatty acid (SCFA) production through fermentation in the large intestine (Mun and Shin 2006). RS has been manufactured by a heating-cooling process and chemical modification, respectively (Mun and Shin 2006). However, chemical modification may have safety problems, and RS content may be relatively low by heating-cooling process alone due to the structure of starch. Pullulanase has been found to increase RS content. Zhang and Jin (2011) produced high RS content product from maize starch upon hydrolysis with pullulanase.

21.13.2.6 Production of Branched Cyclodextrins (CDs)

Cyclodextrins (CDs) are cyclic oligosaccharides composed of six or more α-1, 4-linked glucose units. CDs of six, seven, and eight α-D-glucose residues are called α-, β-, and γ-CD, respectively. CDs possess the unique ability to form inclusion complexes with various chemicals and are useful in the food, cosmetic, pharmaceutical, and plastic industries as emulsifiers, antioxidants, and stabilizing agents. The CDs are industrially produced from starch or dextrins by cyclomaltodextrin glucanotransferase (CGTase) using intramolecular transglycosylation (cyclization reaction). Branched cyclodextrins contain one or more saccharide chains such as glucose, maltose, and other saccharides linked to cyclodextrins by an α-1, 6 bond (French et al. 1965).

Pullulanases have been used to produce branched cyclodextrins. Branched cyclodextrins such as 6-*O*-α-D-glucosyl-CDs (G_1-CDs) and 6-*O*-a-maltosyl-CDs (G_2-CDs) are generally more soluble in water and organic solvents than cyclodextrins which have no branches, and thus are likely to form more soluble inclusion complexes (Okada et al. 1988). The enzyme catalyzes the condensation of CDs and maltooligosaccharides (Kitahata et al. 1987). It has been demonstrated that *C. thermohydrosulfuricum* pullulanase (like other pullulanases) catalyzes

the production of condensation products from maltose and maltotriose (Saha et al. 1988). Thermostable pullulanases have been preferred for the production of branched cyclodextrins. Watanabe et al. (1997b) developed a novel method for the production of 6-*o*-α-D-glucosyl-α-cyclodextrins (G_1-α-CDs) such as G_I-α-CD and 6^I, 6^a-di-*O*-α-D-glucosyl-α-CDs ($(G_1)_2$-α-CDs). The process involved condensation of maltose (G_2) and α-CD by reverse-synthesis with pullulanase (from *Klebsiella pneumoniae*) and hydrolysis of intermediate Q-*O* -α-maltosyl-α-CD (G_2- α-CD) by glucoamylase.

21.13.2.7 Detergent Applications

Ara et al. (1992) had reported an alkaline pullulanase from alkalophilic *Bacillus* sp. KSM-1876 with pH optimum for activity at 10.0. This has been shown to have a potential application in dishwashing and laundry detergents under alkaline conditions.

21.14 Conclusions

Thermostable enzymes are receiving considerable attention because they are active under industrial process conditions. The important features of the enzyme that are important from industrial perspective include high thermostability, functioning in a wide range of pH and its independence from the requirement for calcium ions for activity. Amylopullulanases and pullulanases have been produced in submerged batch and continuous fermentations, purified, and characterized. Type I pullulanases are of 70–80 kDa, while amylopullulanases are larger proteins with molecular mass of 100–225 kDa. Since the enzyme titers are low in the native bacterial and archaeal hosts, several attempts have been made to clone and express in heterologous hosts such as *Escherichia coli*, *Bacillus subtilis*, and *Pichia pastoris*. Amylopullulanases have been used as catalysts for one-step liquefaction-saccharification process for the production of sugar syrups (high DP2 to DP4). The pullulanases, on the other hand, have been widely employed in starch saccharification as a debranching enzyme in order to enhance sugar yields.

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Chapter 22
Sugar Metabolic Enzymes

Kazuaki Yoshimune and Yutaka Kawarabayasi

Abstract Although some of the enzymes involved in carbohydrate metabolism in thermophilic archaea have been identified from genomic data, much less is known about the metabolic pathways in thermophilic archaea than in bacteria such as *Escherichia coli*. This is because many gaps still remain in most of the metabolic pathways constructed using data predicted from the genomic sequences of thermophilic archaea. In order to understand carbohydrate metabolism in thermophilic archaea, the proteins predicted to be carbohydrate metabolic enzymes from their genomic data have been expressed in *E. coli*, and the expressed proteins have been functionally analyzed. These analyses have suggested that the activities of novel and not seen in mesophiles could be detected in the thermostable enzymes from archaea. These observations enable novel pathways to be constructed based on the actual activities or functions of the enzymes obtained from thermophilic archaea. Furthermore, these investigations have confirmed that functional genomics is a powerful tool for studying the detailed features of microorganisms.

Keywords Thermophiles • Carbohydrate metabolism • Archaea • Functional genomics • Carbohydrate metabolic enzymes

22.1 Introduction

Most organisms express large numbers of different sugar metabolic enzymes to metabolize various sugars serving a range of important purposes. For example, the addition of polysaccharides to proteins can serve to increase their activity

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and stability (Skropeta 2009), their bioavailability of drugs (Otero-Espinar et al. 2010), and their capacity for self-recognition. In thermophilic microorganisms, polysaccharides serve structural functions in biofilm matrices (Vaningelgem et al. 2004), cell walls such as peptidoglycan and S-layer (Messner et al. 1997), and in parts of their flagella (Ng et al. 2006) and also serve as carbon and energy sources (Vanfossen et al. 2008). In this chapter, we describe the thermophilic enzymes that play important roles in the metabolism of glucose and the biosynthesis of nucleotide sugars, which are substrates for the construction of carbohydrate polymers. Particularly noteworthy is the great potential for application of these enzymes.

The genomic data from thermophiles provide a large amount of information about the genes predicted to encode sugar metabolic enzymes based on their similarity to known genes. Already, some of these genes have been expressed in *E. coli*, and the activities and functions of their gene products have been characterized. However, many genes involved in polysaccharide metabolism in thermophilic archaea have not yet been identified based on their similarity to known genes. It is thought that the reason for the missing of many genes in metabolic pathways is that thermophilic archaea may utilize unknown metabolic pathways and/or the sequences of the enzymes from thermophilic archaea are not conserved in the well-characterized mesophilic enzymes, even when same pathway is involved. Sugar metabolic enzymes from thermophilic archaea are known to exhibit unique features: for example, broader substrate specificity, multiple cation usage, and resistance to organic solutions, among others (Sato et al. 2007). Moreover, functional analysis of potentially relevant genes has shown that some sugar metabolic enzymes from thermophilic archaea possess unexpected and previously uncharacterized activities. This novel functionality suggests the presence of novel sugar metabolic pathways in thermophilic archaea, pathways that may be suitable for future applications involving the enzymatic synthesis of polysaccharides.

In this chapter, we summarize the enzymes which have so far been identified in thermophilic microorganisms and functionally characterized, including enzymes characterized after purification from the original host cell and expressed in a recombinant form in a heterologous host cell. In addition, a predicted nucleotide sugar biosynthetic pathway in *Sulfolobus tokodaii* is also briefly described as an example of a unique sugar anabolic pathway in thermophilic archaea.

22.2 Critical Review

Among various glucose metabolic pathways, the most common is the Embden-Meyerhof (EM) glycolysis pathway, which is comprised of enzymes that are highly conserved among bacteria and eukaryotes. Figure 22.1 shows the EM pathway, which includes the sugar phosphorylation reaction. The phosphorylation is necessary because activation of the sugar by phosphorylation supplies energy for its

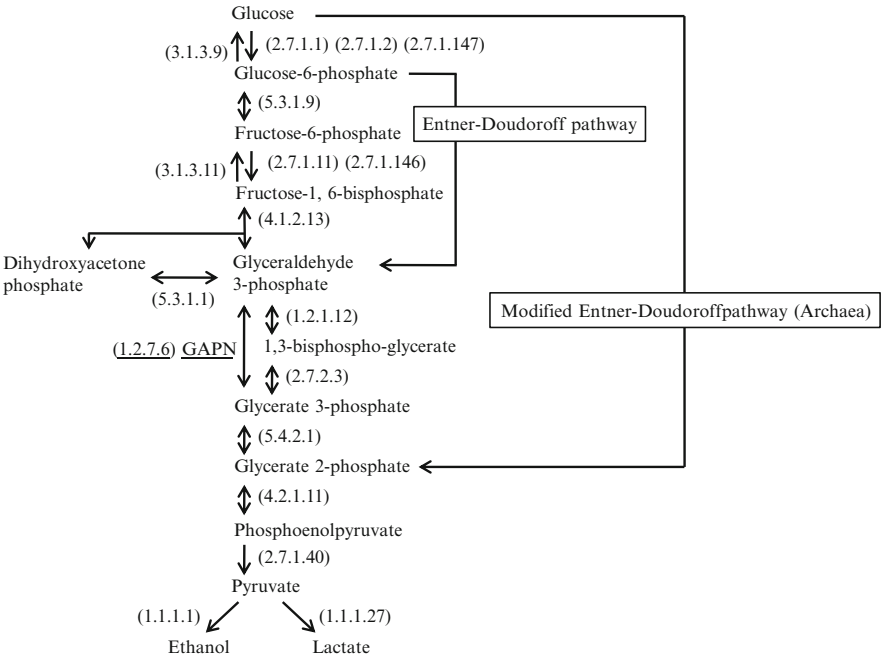


Fig. 22.1 The glycolysis pathways in thermophilic microorganisms. The enzymes in the forward and reverse Embden-Meyerhof pathway and the modified Embden-Meyerhof pathway are shown. In this and all of the other figures, the enzymes are shown with their Enzyme Commission (EC) numbers. The enzymes, indicated by their EC numbers, are also summarized in Table 22.1

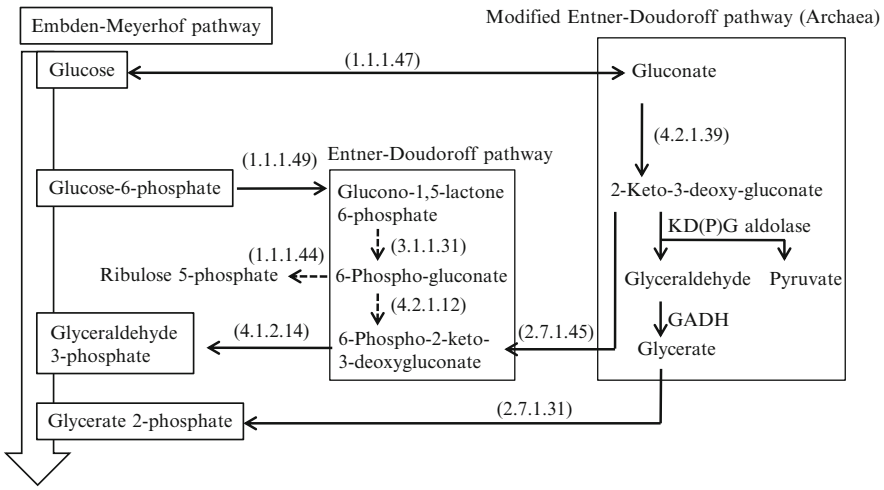


Fig. 22.2 The Entner-Doudoroff and modified Entner-Doudoroff pathways in thermophilic microorganisms. The enzymes, indicated by their EC numbers, are summarized in Table 22.2. The *dotted arrows* indicate uncharacterized reactions

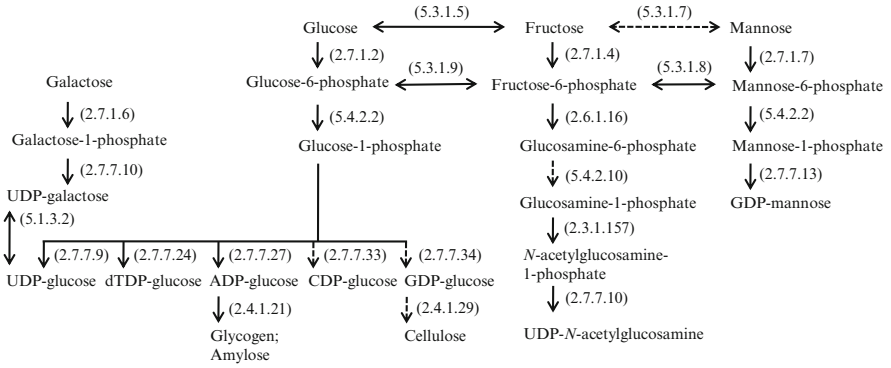


Fig. 22.3 Sugar nucleotide biosynthetic pathway in thermophilic microorganisms. The enzymes, indicated by their EC numbers, are summarized in Table 22.3. The *dotted arrows* indicate uncharacterized reactions

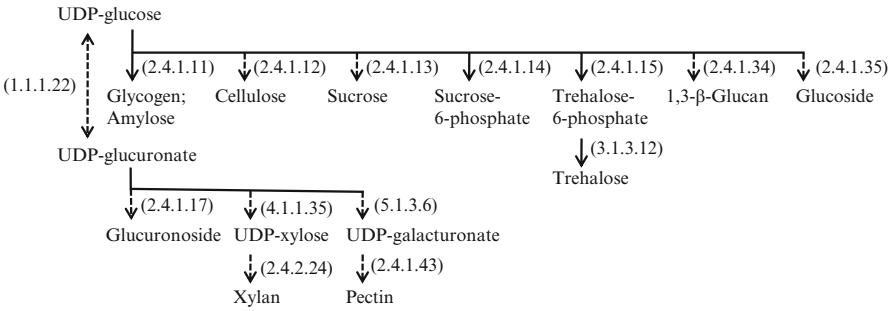


Fig. 22.4 Conversion pathway for UDP-glucose in thermophilic microorganisms. The enzymes, indicated by the EC numbers, are summarized in Table 22.4. The *dotted arrows* indicate uncharacterized reactions.

conversion and synthesis. Figure 22.2 shows an alternative glucose metabolic pathway, the Entner-Doudoroff (ED) pathway. Archaea possess unique sugar catabolic pathways that are modified versions of the EM and ED pathways. Although thermophilic enzymes have been identified in these pathways, only a few genes encoding enzymes for the conversion and synthesis of polysaccharides have so far been found in thermophiles. The major pathways for the conversion and the synthesis of polysaccharides are shown in Figs. 22.3, 22.4, 22.5, 22.6 and 22.7. The corresponding enzymes and their references are listed in Tables 22.1, 22.2, 22.3, 22.4, 22.5, 22.6 and 22.7. Listed are the thermophilic enzymes for which both the enzymatic activities and the nucleotide sequences (or at least their N-terminal sequences) have been reported. Bifunctional enzymes and enzymes with specificities for multiple substrates are listed for each independent enzymatic activity. In this section, we provide an overview of the thermophilic enzymes in the anabolic and catabolic pathways of

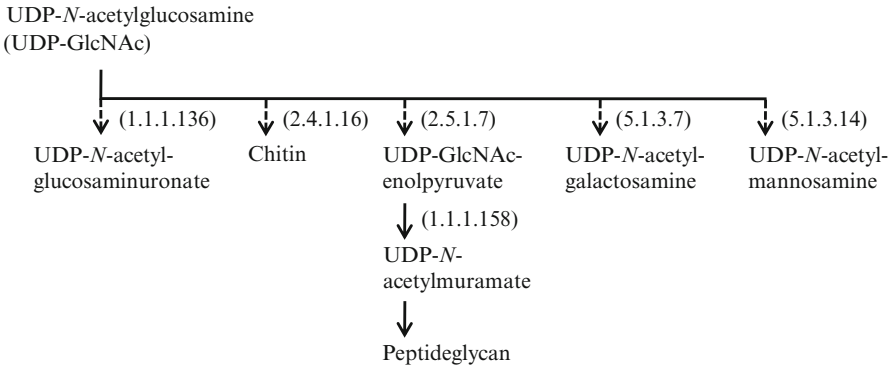


Fig. 22.5 Conversion pathway for UDP-N-acetylglucosamine in thermophilic microorganisms. The enzymes, indicated by their EC numbers, are summarized in Table 22.5. The dotted *arrows* indicate uncharacterized reactions.

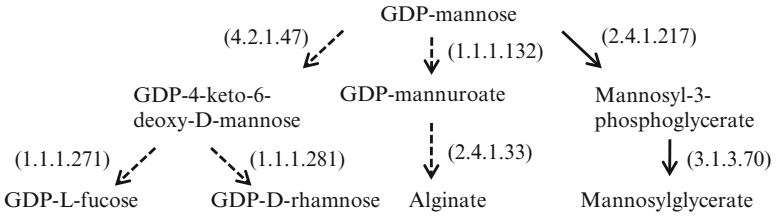
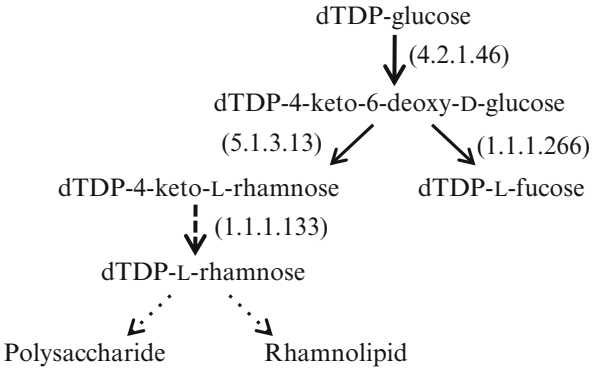


Fig. 22.6 Conversion pathway for GDP-mannose in thermophilic microorganisms. The enzymes, indicated by their EC numbers, are summarized in Table 22.6. The *dotted arrows* indicate uncharacterized reactions

Fig. 22.7 Conversion pathway for dTDP-glucose in thermophilic microorganisms. The enzymes, indicated by their EC numbers, are summarized in Table 22.7. The *dotted arrows* indicate uncharacterized reactions



thermophilic archaea, as well as the genes that encode the enzymes involved in the conversion and synthesis of polysaccharides in the thermoacidophilic archaeon *Sulfolobus tokodaii* strain7. Many of the genes in this pathway could not be identified through similarity searches against known enzymes.

Table 22.1 List of the thermophilic enzymes shown in Fig. 22.1

EC number	Enzyme name	Species name	Domain[a]	Reference
1.1.1.1	Alcohol dehydrogenase	*Aeropyrum pernix*	A	Hirakawa et al. (2005)
		Pyrococcus furiosus	A	van der Oost et al. (2001)
		Pyrococcus furiosus	A	Kube et al. (2006)
		Pyrococcus furiosus	A	Machielsen et al. (2006)
		Sulfolobus solfataricus	A	Ammendola et al. (1992)
		Sulfolobus tokodaii	A	Yanai et al. (2009)
		Thermococcus hydrothermalis	A	Antoine et al. (1999)
		Thermoanaerobacter ethanolicus	B	Holt et al. (2000)
		Thermoanaerobium brockii	B	Zhang et al. (1993)
1.1.1.27	L-Lactate dehydrogenase	*Methanococcus jannaschii*	A	Lee et al. (2001)
		Bacillus stearothermophilius	B	Zülli et al. (1987)
		Thermotoga maritima	B	Ostendorp et al. (1993)
		Thermus aquaticus	B	Machida et al. (1985)
		Thermus caldophilus	B	Taguchi et al. (1984)
1.2.1.12	Glyceraldehyde-3-phosphate dehydrogenase	*Haloarcula vallismortis*	A	Prüss et al. (1993)
		Methanocaldococcus jannaschii	A	Malay et al. (2009)
1.2.7.6	Glyceraldehyde-3-phosphate dehydrogenase (ferredoxin)	*Pyrococcus furiosus*	A	Mukund and Adams (1995)
2.7.1.1	Hexokinase	*Thermoproteus tenax*	A	Dörr et al. (2003)
2.7.1.2	Glucokinase	*Aeropyrum pernix*	A	Hansen et al. (2002b)
		Thermoproteus tenax	A	Dörr et al. (2003)
		Bacillus stearothermophilus	B	D'Auria et al. (2002)
2.7.1.11	6-Phosphofructokinase	*Aeropyrum pernix*	A	Hansen and Schönheit (2001)
		Aeropyrum pernix	A	Ronimus et al. (2001a)
		Desulfurococcus amylolyticus	A	Hansen and Schönheit (2000)
		Thermotoga maritima	B	Hansen et al. (2002a)
		Thermus thermophilus	B	Ishida et al. (1997)

2.7.1.40	Pyruvate kinase	*Aeropyrum pernix*	A	Johnsen et al. (2003)
		Archaeoglobus fulgidus	A	Johnsen et al. (2003)
		Pyrobaculum aerophilum	A	Johnsen et al. (2003)
		Thermoproteus tenax	A	Schramm et al. (2000)
		Thermotoga maritima	B	Ding et al. (2001)
		Thermotoga maritima	B	Johnsen et al. (2003)
2.7.1.146	ADP-specific phosphofructokinase	*Archaeoglobus fulgidus*	A	Hansen and Schönheit (2004)
		Methanococcus jannaschii	A	Tuininga et al. (1999)
		Methanococcus jannaschii	A	Sakuraba et al. (2002)
		Methanococcus jannaschii	A	Merino and Guixé (2008)
		Pyrococcus horikoshii	A	Currie et al. (2009)
		Thermococcus litoralis	A	Jeong et al. (2003b)
		Thermococcus zilligii	A	Ronimus et al. (2001b)
2.7.1.147	ADP-specific glucokinase	*Archaeoglobus fulgidus*	A	Labes and Schönheit (2003)
		Methanococcus jannaschii	A	Sakuraba et al. (2002)
		Pyrococcus furiosus	A	Kengen et al. (1995)
		Pyrococcus furiosus	A	Koga et al. (2000)
		Pyrococcus horikoshii	A	Tsuge et al. (2002)
		Thermococcus litoralis	A	Koga et al. (2000)
		Thermoproteus tenax	A	Dörr et al. (2003)
2.7.2.3	Phosphoglycerate kinase	*Methanothermus fervidus*	A	Fabry et al. (1990)
		Bacillus stearothermophilus	B	Davies et al. (1993)
		Thermus thermophilus	B	Bowen et al. (1988)
3.1.3.9	Glucose-6-phosphatase	None	–	
3.1.3.11	Fructose-bisphosphatase	*Archaeoglobus fulgidus*	A	Stieglitz et al. (2002)
		Methanococcus janaschii	A	Stec et al. (2000)
		Pyrococcus furiosus	A	Verhees et al. (2002)
		Sulfolobus tokodaii	A	Nishimasu et al. (2004)
		Thermococcus kodakaraensis	A	Rashid et al. (2002)
		Thermus thermophilus	B	Soulimane (2010)

(continued)

Table 22.1 (continued)

EC number	Enzyme name	Species name	Domain[a]	Reference
4.1.2.13	Fructose-bisphosphate aldolase	*Methanocaldococcus jannaschii*	A	Samland et al. (2008)
		Pyrococcus furiosus	A	Siebers et al. (2001)
		Thermoproteus tenax	A	Siebers et al. (2001)
		Bacillus stearothermophilus	B	De Montigny and Sygusch (1996)
		Thermus caldophilus	B	Lee et al. (2006)
4.2.1.11	Phosphopyruvate hydratase	*Methanococcus jannaschii*	A	Yamamoto and Kunishima (2008)
		Pyrococcus furiosus	A	Peak et al. (1994)
5.3.1.1	Triose-phosphate isomerase	*Methanocaldococcus jannaschii*	A	Gayathri et al. (2007)
		Pyrococcus woesei	A	Kohlhoff et al. (1996)
		Thermoproteus tenax	A	Walden et al. (2004)
		Bacillus stearothermophilus	B	Rentier-Delrue et al. (1993)
5.3.1.9	Glucose-6-phosphate isomerase	*Aeropyrum pernix*	A	Hansen et al. (2004 b)
		Methanococcus jannaschii	A	Rudolph et al. (2004)
		Pyrobaculum aerophilum	A	Swan et al. (2004)
		Pyrococcus furiosus	A	Hansen et al. (2001)
		Pyrococcus furiosus	A	Swan et al. (2003)
		Thermococcus litoralis	A	Jeong et al. (2003a)
		Thermoplasma acidophilum	A	Hansen et al. (2004)
5.4.2.1	Phosphoglycerate mustase	*Methanococcus jannaschii*	A	Graham et al. (2002)
		Methanococcus jannaschii	A	van der Oost et al. (2002)
		Pyrococcus furiosus	A	van der Oost et al. (2002)
		Pyrococcus horikoshii	A	Lokanath and Kunishima (2006)
		Sulfolobus solfataricus	A	Potters et al. (2003)
		Thermoplasma acidophilum	A	Johnsen and Schönheit (2007)
GAPN	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase	*Thermoproteus tenax*	A	Brunner et al. (1998)

The thermophilic enzymes of which genetic and functional data is available are listed

[a]A and B represents archaea and bacteria, respectively

Table 22.2 List of the thermophilic enzymes shown in Fig. 22.2

EC number	Enzyme name	Species name	Domain[a]	Reference
1.1.1.44	Phosphogluconate dehydrogenase	None	–	
1.1.1.47	Glucose 1-dehydrogenase	*Sulfolobus solfataricus*	A	Lamble et al. (2003)
		Sulfolobus tokodaii	A	Ohshima et al. (2003)
1.1.1.49	Glucose-6-phosphate dehydrogenase	*Aquifex aeolicus*	B	Iyer et al. (2002)
		Aquifex aeolicus	B	Nakka et al. (2006)
		Thermotogamaritima	B	Hansen et al. (2002c)
2.7.1.31	Glycerate kinase	*Picrophilus torridus*	A	Reher et al. (2006)
		Pyrococcus horikoshii	A	Liu et al. (2007)
		Sulfolobus tokodaii	A	Liu et al. (2009)
		Thermoproteus tenax	A	Kehrer et al. (2007)
		Thermotogamaritima	B	Yang et al. (2008)
2.7.1.45	2-Dehydro-3-deoxygluconokinase	*Sulfolobus solfataricus*	A	Kim and Lee (2006)
		Sulfolobus tokodaii	A	Ohshima et al. (2007)
		Thermotogamaritima	B	Mathews et al. (2008)
		Thermus thermophilus	B	Ohshima et al. (2004)
3.1.1.31	6-Phosphogluconolactonase	None	–	
4.1.2.14	2-Dehydro-3-deoxyphosphogluconate aldolase	*Sulfolobus solfataricus*	A	Ahmed et al. (2005)
		Thermoproteus tenax	A	Pauluhn et al. (2008)
		Thermotoga maritima	B	Griffiths et al. (2002)
4.2.1.12	Phosphogluconate dehydratase	None	–	
4.2.1.39	Gluconate dehydratase	*Sulfolobus solfataricus*	A	Ahmed et al. (2005)
		Sulfolobus solfataricus	A	Kim and Lee (2005)
		Thermoproteus tenax	A	Ahmed et al. (2005)

(continued)

Table 22.2 (continued)

EC number	Enzyme name	Species name	Domain[a]	Reference
GADH	Glyceraldehyde dehydrogenase	*Picrophilus torridus*	A	Reher and Schönheit (2006)
		Thermoplasma acidophilum	A	Jung and Lee (2006)
		Thermoplasma acidophilum	A	Reher and Schönheit (2006)
KD(P)G Aldolase	2-Keto-3-deoxy-gluconate aldolase	*Sulfolobus acidocaldarius*	A	Wolterink-van Loo et al. (2007)
		Sulfolobus solfataricus	A	Buchanan et al. (1999)
		Sulfolobus solfataricus	A	Lamble et al. (2003)
		Sulfolobus solfataricus	A	Ahmed et al. (2005)
		Sulfolobus tokodaii	A	Wolterink-van Loo et al. (2007)
		Thermoproteus tenax	A	Ahmed et al. (2005)

The thermophilic enzymes of which genetic and functional data is available are listed

[a]A and B represents archaea and bacteria, respectively

Table 22.3 List of the thermophilic enzymes shown in Fig. 22.3

EC number	Enzyme name	Species name	Domain[a]	Reference
2.3.1.157	Glucosamine-1-phosphate N-acetyltransferase	*Sulfolobus tokodaii*	A	Zhang et al. (2005, 2010)
2.4.1.21	Starch synthase	*Bacillus stearothermophilus*	B	Takata et al. (1997)
2.4.1.29	Cellulose synthase (GDP-forming)	None	–	
2.6.1.16	Glutamine-fructose-6-phosphate transaminase	*Thermus thermophilus*	B	Badet-Denisot et al. (1997)
2.7.1.2	Glucokinase	*Aeropyrum pernix*	A	Hansen et al. (2002b)
		Thermoproteus tenax	A	Dörr et al. (2003)
		Bacillus stearothermophilus	B	D'Auria et al. (2002)
2.7.1.4	Fructokinase	*Aeropyrum pernix*	A	Hansen et al. (2002b)
		Thermococcus litoralis	A	Qu et al. (2004)
		Thermoproteus tenax	A	Dörr et al. (2003)
2.7.1.6	Galactokinase	*Pyrococcus horikoshii*	A	Inagaki et al. (2006)
2.7.1.7	Mannokinase	*Aeropyrum pernix*	A	Hansen et al. (2002b)
		hermoproteus tenax	A	Dörr et al. (2003)
2.7.7.9	UTP-glucose-1-phosphate uridyltransferase	*Sulfolobus tokodaii*	A	Zhang et al. (2005)
2.7.7.10	UTP-hexose-1-phosphate uridyltransferase	*Methanococcus jannaschii*	A	Namboori and Graham (2008)
		Sulfolobus tokodaii	A	Zhang et al. (2005)
2.7.7.13	Mannose-1-phosphate guanylyltransferase	*Pyrococcus furiosus*	A	Mizanur and Pohl (2009)
		Sulfolobus solfataricus	A	Sacchetti et al. (2004)
2.7.7.24	Glucose-1-phosphate thymidylyltransferase	*Sulfolobus tokodaii*	A	Zhang et al. (2005)
		Aneurinibacillus thermoaerophilus	B	Graninger et al. (2002)
		Thermus caldophilus	B	Parajuli et al. (2004)
2.7.7.27	Glucose-1-phosphate adenylyltransferase	*Sulfolobus tokodaii*	A	Zhang et al. (2005)
		Bacillus stearothermophilus	B	Takata et al. (1997)
		Thermus caldophilus	B	Ko et al. (1996)
2.7.7.33	Glucose-1-phosphate cytidylyltransferase	*Sulfolobus tokodaii*	A	Zhang et al. (2005)
2.7.7.34	Glucose-1-phosphate guanylyltransferase	*Sulfolobus tokodaii*	A	Zhang et al. (2005)

(continued)

Table 22.3 (continued)

EC number	Enzyme name	Species name	Domain[a]	Reference
5.1.3.2	UDP-glucose 4-epimerase	*Thermus thermophilus*	B	Niou et al. (2009)
5.3.1.5	Xylose isomerase	*Caldanaerobacter subterraneus*	B	Kim et al. (2010)
		Clostridium thermohydrosulfuricum	B	Dekker et al. (1991a)
		Thermus thermophilus	B	Dekker et al. (1991b)
5.3.1.7	Mannoseisomerase	None	–	
5.3.1.8	Mannose-6-phosphate isomerase	*Aeropyrum pernix*	A	Hansen et al. (2004)
		Pyrobaculum aerophilum	A	Swan et al. (2004)
		Pyrococcus furiosus	A	Mizanur and Pohl (2009)
		Thermoplasma acidophilum	A	Hansen et al. (2004)
5.3.1.9	Glucose-6-phosphate isomerase	*Aeropyrum pernix*	A	Hansen et al. (2004)
		Methanococcus jannaschii	A	Rudolph et al. (2004)
		Pyrobaculum aerophilum	A	Swan et al. (2004)
		Pyrococcus furiosus	A	Hansen et al. (2001)
		Pyrococcus furiosus	A	Swan et al. (2003)
		Thermococcus litoralis	A	Jeong et al. (2003a)
		Thermoplasma acidophilum	A	Hansen et al. (2004)
5.4.2.2	Phosphoglucomutase	*Pyrococcus horikoshii*	A	Akutsu et al. (2005)
		Thermococcus kodakaraensis	A	Rashid et al. (2004)
5.4.2.10	Phosphoglucosamine mutase	None	–	

The thermophilic enzymes of which genetic and functional data is available are listed

[a]A and B represents archaea and bacteria, respectively

Table 22.4 List of the thermophilic enzymes shown in Fig. 22.4

EC number	Enzyme name	Species name	Domain[a]	Reference
1.1.1.22	UDP-glucose 6-dehydrogenase	None	–	
2.4.1.11	Glycogen synthase	*Pyrococcus abyssi*	A	Horcajada et al. (2006)
		Pyrococcus furiosus	A	Zea et al. (2003)
		Sulfolobus acidocaldarius	A	Cardona et al. (2001)
2.4.1.12	Cellulose synthase (UDP-forming)	None	–	
2.4.1.13	Sucrose synthase	None	–	
2.4.1.14	Sucrose-phosphate synthase	*Halothermothrix orenii*	B	Chua et al. (2008)
2.4.1.15	Trehalose-phosphate synthase	*Thermus thermophilus*	B	Silva et al. (2005)
2.4.1.17	Glucuronosyltransferase	None	–	
2.4.1.34	1,3-b-Glucan synthase	None	–	
2.4.1.35	Phenol b-glucosyltransferase	None	–	
2.4.1.43	Polygalacturonate 4-a-galacturonosyltransferase	None	–	
2.4.2.24	1,4-b-Xylan synthase	None	–	
3.1.3.12	Trehalose-phosphatase	*Thermus thermophilus*	B	Silva et al. (2005)
4.1.1.35	UDP-glucuronate decaroxylase	None	–	
5.1.3.6	UDP-glucuronate 4-epimerase	None	–	

The thermophilic enzymes of which genetic and functional data is available are listed
[a]A and B represents archaea and bacteria, respectively

Table 22.5 List of the thermophilic enzymes shown in Fig. 22.5

EC number	Enzyme name	Species name	Domain[a]	Reference
1.1.1.136	UDP-*N*-acetylglucosamine 6-dehydrogenase	None	–	
1.1.1.158	UDP-*N*-acetylmuramate dehydrogenase	*Thermus caldophilus*	B	Kim et al. (2007)
2.4.1.16	Chitin synthase	None	–	
2.5.1.7	UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase	None	–	
5.1.3.7	UDP-*N*-acetylglucosamine 4-epimerase	None	–	
5.1.3.14	UDP-*N*-acetylglucosamine 2-epimerase	None	–	

The thermophilic enzymes of which genetic and functional data is available are listed
[a]A and B represents archaea and bacteria, respectively

Table 22.6 List of the thermophilic enzymes shown in Fig. 22.6

EC number	Enzyme name	Species name	Domain[a]	Reference
1.1.1.132	GDP-mannose 6-dehydrogenase	None	–	
1.1.1.271	GDP-L-fucose synthase	None	–	
1.1.1.281	GDP-4-dehydro-6-deoxy-D-mannose reductase	None	–	
2.4.1.33	Alginate synthase	None	–	
2.4.1.217	Mannosyl-3-phosphoglycerate synthase	*Palaeococcus ferrophilus*	A	Neves et al. (2005)
		Thermus thermophilus	B	Empadinhas et al. (2003)
3.1.3.70	Mannosyl-3-phosphoglycerate phosphatase	*Palaeococcus ferrophilus*	A	Neves et al. (2005)
		Thermus thermophilus	B	Empadinhas et al. (2003)
4.2.1.47	GDP-mannose 4,6-dehydratase	None	–	

The thermophilic enzymes of which genetic and functional data is available are listed
[a]A and B represents archaea and bacteria, respectively

Table 22.7 List of the thermophilic enzymes shown in Fig. 22.7

EC number	Enzyme name	Species name	Domain[a]	Reference
1.1.1.133	dTDP-4-dehydrorhamnose reductase	None	–	
1.1.1.266	dTDP-4-dehydro-6-deoxyglucose reductase	*Geobacillus tepidamans*	B	Zayni et al. (2007)
4.2.1.46	dTDP-glucose 4,6-dehydratase	*Aneurinibacillus thermoaerophilus*	B	Pfoestl et al. (2003)
5.1.3.13	dTDP-4-dehydrorhamnose 3,5-epimerase	*Methanobacterium thermoautotrophicum*	A	Christendat et al. (2000)

The thermophilic enzymes of which genetic and functional data is available are listed
[a]A and B represents archaea and bacteria, respectively

22.2.1 Glucose Metabolic Pathway

The major glucose metabolic pathways are shown in Figs. 22.1 and 22.2. These pathways mainly include enzymes involved in catalyzing phosphorylated sugar molecules, though they are also suitable for application to the enzymatic synthesis of polysaccharides. Figure 22.1 shows the EM pathway, which is the most widely distributed and most studied glycolysis pathway. The reverse pathway is allowed and is important for maintaining appropriate sugar concentrations and for polysaccharide anabolism. Many thermophilic enzymes in the EM pathway, for example, glucokinase (Goward et al. 1986) and phosphofructokinase (Yoshida et al. 1971), were well studied before the genomes of any thermophiles were sequenced. Figure 22.2 shows the ED pathway, which is an alternate glycolysis pathway, and is found in mesophiles (Conway 1992). In archaea, glucose is metabolized via the modified EM or ED pathways illustrated in Figs. 22.1 or 22.2. Modified EM pathways are mainly found in anaerobic archaea (e.g., *Pyrococcus, Thermococcus, Desulfurococcus,* and *Archaeoglobus*), while modified ED pathways are found in aerobic archaea (e.g., *Sulfolobus* and *Thermoplasma*) (Ahmed et al. 2005).

22.2.2 Pathways for the Synthesis and Conversion of Nucleotide Sugars

Anabolic pathways include the activation of sugars by phosphorylation and intramolecular transposition of phosphates by mutases, followed by their conversion to NDP-sugars by different kinds of sugar-1-phosphate nucleotidylyltransferases. The activated precursors, NDP-sugars, are utilized for construction of polymeric carbohydrates. The characterized thermophilic enzymes involved in the synthesis of NDP-sugars, including UDP-glucose, UDP-GlcNAc, and dTDP-glucose, are summarized in Fig. 22.3. However, only a few of the genes encoding enzymes for conversion of NDP-sugars and synthesis of polysaccharides have been functionally identified, as is shown in Figs. 22.4, 22.5, 22.6 and 22.7. Further studies will be needed to elucidate in detail the characteristics of the enzymes catalyzing these reactions, which should be useful for the enzymatic synthesis of polysaccharides.

22.2.3 *Nucleotide Sugar Synthesis in* Sulfolobus tokodaii *Strain7*

The entire genome sequence of *S. tokodaii*, an aerobic thermoacidophilic crenarchaeon, was published about a decade ago (Kawarabayasi et al. 2001). Within the genome of this microorganism, only a limited number of the genes were

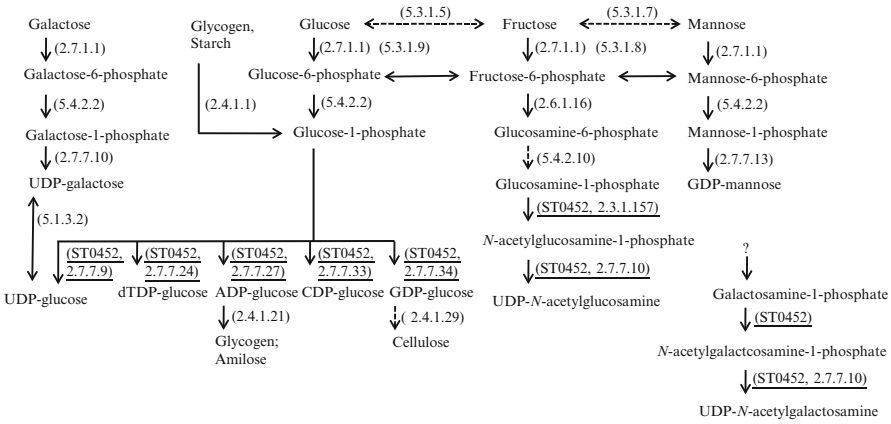


Fig. 22.8 The predicted synthetic pathway for nucleotide sugar in *Sulfolobus tokodaii*. The enzymes, indicated by their EC numbers and gene IDs, are summarized in Table 22.8. The *dotted arrows* indicate uncharacterized reactions. Reactions catalyzed by ST0452 are underlined

found to encode enzymes for the conversion and synthesis of polysaccharides; moreover, no genes for glucose-1-phosphate nucleotidylyltransferases (EC 2.7.7.9, 2.7.7.12, 2.7.7.24, 2.7.7.27, and 2.7.7.34) were predicted (Fig. 22.8). Nonetheless, characterization of the ST0452 protein, which was detected as a sugar-1-phosphate thymidylyltransferase, revealed the enzyme to have specificities for multiple substrates and to be capable of accepting TTP, dCTP, dGTP, dATP, and UTP with glucose-1-phosphate and TTP and UTP with *N*-acetyl-glucosamine-1-phosphate as substrates for production of the corresponding nucleotide sugars (Zhang et al. 2005). Furthermore, the ST0452 protein can also act as an independent bifunctional enzyme with glucosamine-1-phosphate *N*-acetyltransferase (EC 2.3.1.157) and *N*-acetylgalactosamine-1-phosphate uridyltransferase activities (Zhang et al. 2010). In contrast to the activities of similar enzymes from mesophilic microorganisms, the sugar-1-phosphate nucleotidylyltransferase activity of the ST0452 protein can utilize *N*-acetylgalactosamine-1-phosphate as a substrate, and the amino-sugar-1-phosphate acetyltransferase activity of the ST0452 protein can catalyze the acetyl transfer to galactosamine-1-phosphate. Figure 22.8 shows the pathway for the synthesis of nucleotide sugar molecules in *S. tokodaii*. The pathway was constructed based on the functionally determined activities of each gene product. All of the enzymes in Fig. 22.8 are also summarized in Table 22.8. As shown in Fig. 22.8, the ST0452 protein (Zhang et al. 2005, 2010) can catalyze nine independent reactions utilizing multiple substrates, which is a level of activity not seen in similar enzymes from bacteria and eukarya. Like the ST0452 protein, the PH0923 protein from *Pyrococcus horikoshii* can reportedly also catalyze intramolecular transposition of phosphate groups between two independent substrates: glucose-1- and -6-phosphate as well as mannose-1- and -6-phosphate (Akutsu et al. 2005).

Table 22.8 List of the thermophilic enzymes shown in Fig. 22.8

EC number	Enzyme name	Gene ID[a]	Reference
2.3.1.157	Glucosamine-1-phosphate N-acetyltransferase	ST0452	Zhang et al. (2005, 2010)
2.4.1.1	Phosphorylase	One candidate	None
2.4.1.21	Starch synthase	One candidate	None
2.4.1.29	Cellulose synthase (GDP-forming)	None	–
2.6.1.16	Glutamine-fructose-6-phosphate transaminase	Two candidates	None
2.7.1.1	Hexokinase	One candidate	Nishimasu et al. (2006)
2.7.7.9	Glucose-1-phosphate uridyltransferase	ST0452	Zhang et al. (2005)
2.7.7.10	Hexose-1-phosphate uridyltransferase	ST0452	Zhang et al. (2005)
2.7.7.13	Mannose-1-phosphate guanylyltransferase	One candidate	None
2.7.7.24	Glucose-1-phosphate thymidylyltransferase	ST0452	Zhang et al. (2005)
2.7.7.27	Glucose-1-phosphate adenylyltransferase	ST0452	Zhang et al. (2005)
2.7.7.33	Glucose-1-phosphate cytidylyltransferase	ST0452	Zhang et al. (2005)
2.7.7.34	Glucose-1-phosphate guanylyltransferase	ST0452	Zhang et al. (2005)
5.1.3.2	UDP-glucose 4-epimerase	One candidate	None
5.3.1.5	Xylose isomerase	None	–
5.3.1.7	Mannose isomerase	None	–
5.3.1.8	Mannose-6-phosphate isomerase	One candidate	None
5.3.1.9	Glucose-6-phosphate isomerase	One candidate	None
5.4.2.2	Phosphoglucomutase	One candidate	None
5.4.2.10	Phosphoglucosamine mutase	None	–

For the characterized enzyme, gene ID is shown. For the uncharacterized enzyme, number of candidate is shown

[a]The gene IDs shown is the original codes for identified genes in *S. tokodaii*

22.3 Functional Features of Thermophilic Enzymes

To functionally analyze thermostable and thermophilic enzymes, their activities must be assayed at high temperature. Consequently, their substrates and products must be stable at high temperature, if the assays are to be accurate. If a coupling reaction is utilized, it is recommended that the coupling enzymes are also stable at high temperature. If thermostable enzymes are not available, the designed coupling reaction should be performed carefully. Because thermophilic enzymes normally function at high temperature, they may not work at low temperatures (e.g., room temperature).

A noteworthy advantage of carrying out these reactions at high temperature is that it diminishes potential contamination by mesophilic microorganisms.

The following are some of the general features of identified thermostable enzymes.

1. They can accept a variety of metal ions, which are not usually utilized by bacterial enzymes but are utilized as cofactors by thermostable enzymes from thermophilic archaea. For example, metal ions other than Mg^{2+} and Mn^{2+} may enhance the enzymatic activities of the ST0452 protein.
2. Thermophilic enzymes generally have lower Michaelis constants (*K*m) for their substrates than enzymes isolated from mesophilic microorganisms, which indicates that thermostable enzymes can bind substrates more easily than mesophilic enzymes.
3. On the other hand, the catalytic constants (*kcat*) for reactions catalyzed by thermophilic enzymes are usually the same or lower than those for enzymes isolated from mesophilic microorganisms. This means that the turnover number for the reaction is the same or lower with thermophilic enzymes than with mesophilic enzymes (Zhang et al. 2007).
   From these observations, it can be hypothesized that thermophilic enzymes efficiently bind their substrates, but their turnover is not as efficient.
4. The enzymes from thermophilic archaea are usually able to utilize multiple compounds as substrates. This suggests that the genes in thermophilic archaea possess a capacity for multifunctionality, which likely reflects the limited number of genes and the small size of the genome.

22.4 Conclusions

In this chapter, we summarized the thermophilic enzymes catalyzing carbohydrate metabolism in thermophilic archaea and bacteria. The archaeal genes involved are usually able to be expressed in *E. coli*, so that the functional analyses are often carried out using enzymes expressed in heterologous host cells. These analyses indicate that thermophilic enzymes are absolutely thermostable, that they can utilize multiple metal ions and multiple substrates, and that their reactions proceed comparatively slowly. That these enzymes are generally able to utilize multiple substrates and metal ions can be explained based on the small sizes of the archaeal genomes. Within those genomes, many of the genes remain in ancient forms, which have not separated into more modern and specific ones. However the multifunctional enzymes they encode are potentially powerful and useful tools from the viewpoint of their application, and we anticipate that they will be successfully applied in a variety of contexts.

Furthermore, it is through functional genomics – that is, analysis of the activity of each protein encoded within a genome – that a full understanding of the information encoded in the genomes of thermophilic microorganisms will be gained. At present, a large number of unknown or hypothetical genes remain to be characterized.

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Chapter 23
Restriction Enzymes from Thermophiles

Prince Sharma, Ravinder Kumar, and Neena Capalash

Abstract Restriction endonucleases (REases) are enzymes that recognize and cleave DNA in a sequence specific manner. The recognition site consists of a sequence of nucleotides in the DNA duplex, typically four to eight base pairs long. Most of the commercially produced REases are isolated from the mesophilic bacteria. But the disadvantage of REases from mesophilic sources is that these enzymes are usually denatured at ambient and high temperature. As temperature produces opposite effects on both enzyme activity and stability, it is therefore a key variable in any biocatalytic process. Also, mesophilic enzymes are unstable, have low reactivity, lose activity during purification, and require refrigerated transport and storage. So, thermostable REases are preferred to circumvent these problems. This chapter deals mainly with thermophilic REases. The increasing interest in this field is reflected by the growing information on the discovery, purification, and characterization of REases from thermophilic sources. The properties associated with these enzymes offer additional advantages over their mesophilic counterparts.

Keywords Restriction endonucleases • DNA duplex • Methylation • Thermophiles • Thermostable enzymes

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23.1 Introduction

1953 was a historical year for biology because it marked the birth of the DNA helix which was unknown previously. This opened an entirely new field of research in understanding life and control of heredity. It was only after this discovery that scientists started to think about the manipulation of DNA and hence the properties of organisms. However, this was possible only after the discovery of enzymes that worked upon DNA molecules to cut them and join them or to synthesize new molecules from existing ones. The advancements in DNA enzymology (Arthur Kornberg and colleagues isolated DNA polymerase in 1955, B. Weiss and C.C. Richardson isolated DNA ligase in 1966, and Smith and Wilcox isolated and characterized the first sequence specific restriction nuclease in 1970) made it feasible to manipulate the genes in the DNA molecules. Out of these enzymes, restriction enzymes can be considered as the most important enzymes because of their high specificity of DNA recognition and cleavage of the DNA to fragments of manageable size. Researchers were quick to recognize that restriction enzymes offer themselves as remarkable new tools for investigating gene organization, function, and expression. This was the beginning of an era of genetic engineering or recombinant DNA technology.

In general, a restriction site is a 4- to 8-base-pair sequence that is generally a palindrome. Most of the enzymes cut the phosphodiester backbone of DNA at a specific position within the recognition site, resulting in a break in the DNA.

A major limitation of most restriction enzymes is their inactivation by even slight change of temperatures, pH, and the chemicals that may be present in the reaction mixture. Therefore, it will be fruitful to produce stable enzymes that can retain their activities under such conditions. Restriction enzymes presently used in molecular biology are obtained mostly from mesophilic bacterial sources. Enzymes from such sources are highly temperature labile and tend to lose their activity quite rapidly even if placed at 37°C for a few minutes. So, refrigeration of restriction enzymes is must to retain their activity. Almost all restriction enzymes have to be stored at −20°C in their recommended buffers. The cost of restriction enzymes is pushed up due to the following factors: (1) requirement of storage in small quantities, (2) transportation in refrigerated form, (3) low yield due to loss of activity during purification, and (4) loss of activity due to repeated freeze-thaw operations.

The problem of enzyme stability has been tackled from different perspectives. Temperature produces negative effects on enzyme activity and stability and therefore is a key variable in any enzymatic reaction. Enzyme thermostability allows a higher operation temperature which is clearly advantageous because of higher reactivity, higher stability, higher process yield, lower viscosity, and inactivation of contaminants from mesophilic sources. In addition, thermostable enzymes tend to demonstrate enhanced resistance to denaturation by chemical agents, such as urea and guanidine hydrochloride.

The ability of microorganisms to grow at extreme temperatures implies that their enzymes are inherently stable and active at these temperatures. Such enzymes are indeed more thermostable than the equivalents isolated from phylogenetically

related mesophilic organisms. Bacteria from different thermophilic genera, e.g., *Bacillus, Thermus, Rhodothermus, Aeropyrum, Methanocaldococcus, Thermococcus, Sulfolobus, Thermotoga, Pyrococcus, and Anoxybacillus* have been reported to produce thermostable REases.

REases have wide applications in molecular biology which involve DNA cloning, sequencing, mapping, restriction analysis, restriction fragment length polymorphism, and many more. Recently, the potential of thermostable restriction enzymes has been explored to improve techniques like pulse field gel electrophoresis and strand displacement amplification. They have also been incorporated into PCR. BstNI from *Bacillus stearothermophilus* has been used for the detection of point mutations at codon 12 of the *k-ras* gene and in real-time detection and quantification of nucleic acid amplification. BsoBI has application in isothermal strand displacement amplification to detect pathogens such as *Mycobacterium tuberculosis*. Thermostable restriction enzymes are potential candidates to determine the status of CpG islands in eukaryotes through MSP (methylation-specific PCR). Recent advances in single-molecule studies have enabled to study the mechanism of DNA cleavage by REases. For the first time single-molecule interactions with DNA of thermostable REases, PspGI and its isoschizomer SsoII, were demonstrated using transmission electron microscopy. Single-molecule real-time interaction of TspMI with lambda DNA was also studied by total internal reflection microscopy (Parashar et al. 2006).

So, there has always been a niche for thermostable REases. Advances in this area have been possible either with the isolation of a large number of thermophilic microorganisms from different exotic ecological zones of earth and the subsequent isolation of restriction enzymes from them or with genetic and protein engineering techniques for the commercial production of enzymes with improved stability to high temperatures. Cloning and expression of genomic information of a thermophile in a suitable and faster growing mesophilic host has also provided possibilities of producing specific thermostable enzymes.

23.2 Historical Perspective

The phenomenon of restriction and modification was first observed in 1952–1953 by Luria and Human and Bertani and Weigle. They referred to it as host-induced or host-controlled variation. They observed that several different bacteriophages varied in their ability to grow on different host strains. However, once growth was achieved in one strain, the phages could continue to grow unrestricted in this strain but were now restricted in their ability to grow in other strains.

It was seen that phage particles that grow well and efficiently infect one strain are often unable to grow well and infect other strains of the same bacterial species. In addition, phage particles that do succeed in infecting a second strain often show the opposite pattern. They are able to efficiently infect the second strain while growing only poorly in the original strain.

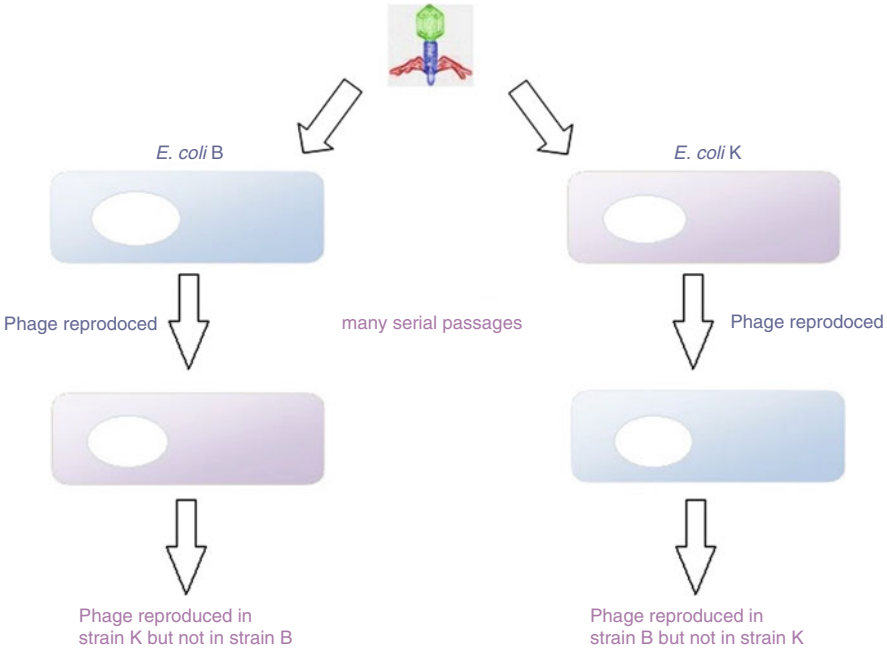


Fig. 23.1 Host restriction modification systems of *E. coli* K and B strains

A series of studies showed that phage particles that efficiently grow and infect host cells have DNA molecules that have been chemically modified by the addition of methyl groups to some of their adenine and/or cytosine bases, while the DNA of poorly infecting phage particles do not show or show a different pattern of chemical modification or "methylation." Phage particles with unmethylated DNA do not grow and are not able to infect efficiently because their DNA molecules are cleaved and degraded by enzymes of the host cell, while phages having methylated DNA are protected from this degradation. This phenomenon of degrading unmethylated DNA destroys the growth ability of the phage and is responsible for the pattern of growth restriction described above (Fig. 23.1).

The restriction enzyme of *E. coli* was the first to be isolated and studied (Meselson and Yuan 1968) but it showed a complex property of cleaving the DNA randomly. An important development came when H.O. Smith, K.W. Wilcox, and T.J. Kelley (1968) isolated and characterized the first restriction endonuclease (HindII) whose functioning was dependent on a specific DNA nucleotide sequence (Smith and Kelly 1970; Smith and Wilcox 1970).

Kathleen Danna and Daniel Nathans (1971) showed for the first time that the restriction enzyme discovered by Smith and Wilcox could be used to produce specific fragments of Simian virus 40 (SV40) DNA. This enzyme is presently known as HindII. Both genomics and the entire biotechnology industry owe, in great measure, to the discovery of restriction enzymes.

For his discovery Hamilton Smith shared the Nobel Prize in physiology and medicine in 1978 with Werner Arber and Daniel Nathans.

23.3 Restriction Modification Systems

The presence of restriction enzymes immediately begs the question of why they do not chew up the genomic DNA of their host. In almost all cases, a bacterium that makes a particular restriction endonuclease also synthesizes a companion DNA methyltransferase, which methylates the DNA target sequence for that restriction enzyme, thereby protecting it from cleavage. This combination of restriction endonuclease and methylase is referred to as a restriction modification system. The system comprises opposing intracellular enzyme activities: endonuclease (*ENase*) that recognizes and cleaves its target site and a methyltransferase (MTase) that transfers methyl group from S-adenosyl methionine (SAM) onto specific nucleobases within the target, thereby protecting it from the action of ENase. Methylation occurs either at adenine or cytosine, thus yielding N6-methyladenine (m6A), N4-methylcytosine (m4C), or C5-methylcytosine (m5C).

23.3.1 Types of REases

Restriction endonucleases are categorized into one of four general groups: Types I, II, III, and IV based on their subunit structure, cofactor requirements, specificity of cleavage, and associated methylase activity. Over 10,000 bacteria have been reported to be screened for producing restriction enzymes worldwide and approximately 3,967 different REases have been discovered. Out of these, 94 Type I, 3,849 Type II, 14 Type III, and 10 Type IV enzymes are reported as of 4 April 2011 (REBASE) and the list is growing every day. Type II restriction enzymes are the most important in the molecular biology because they cut the DNA at specific sites. This has been the reason for more commercial availability of these. Out of 3,849 Type II enzymes with 296 distinct specificities, 645 restriction enzymes with 240 specificities are commercially available. Till date more than 500 thermostable Type II restriction enzymes have been reported with optimum temperature range between 60 and 103°C (REBASE statistics 4/04/2011) (http://rebase.neb.com). A palindrome of 4 and 6 bp has 16 and 64 possibilities, respectively, out of which only about 50% have been discovered as targets for REases and are predominantly GC rich. So there is vast opportunity to find novel REases with undiscovered palindromic sites as target (Table 23.1).

Type II restriction enzymes cut DNA within the recognition site. Since the exact position of the cut is known, these are the restriction enzymes that find wide applications in genetic engineering. A high level of substrate specificity is often the key to biological function. Restriction endonucleases that cleave DNA at specific

Table 23.1 Characteristics and organization of the genetic determinants of different classes of R-M systems (Tock and Dryden 2005)

	Type I	Type II	Type III	Type IV
R-M system	EcoKI	EcoRI	EcoP1I	EcoMcrBC
Genes	*hsd*R, *hsd*M, *hsd*S	*eco*RIR, *eco*RIM	*mod, res*	*mcr*B, *mcr*C
Subunits	Three different subunits (R, M, and S) combine to form $R_2M_2S_1$ and M_2S_1	Two different subunits (R and M) combine to form R_2 and M_1	Two different subunits (Mod and Res) combine to form Mod_2Res_2	Two different subunits are present, McrB and McrC
Enzyme activities	REase, MTase, and ATPase	REase, MTase	REase, MTase, and ATPase	REase and GTPase
Cofactors required for DNA cleavage	ATP (hydrolyzed), S-adenosyl methionine (SAM), Mg^{2+}	Mg^{2+}	ATP (not hydrolyzed), S-adenosylmethionine (SAM), Mg^{2+}	GTP, Mg^{2+}
Cofactors required for methylation	SAM	SAM	SAM	No methylation
Recognition sequence	Asymmetric and bipartite, 5′AAC(N_6)GTGC	Mostly symmetric, 5′GAATTC	Asymmetric, 5′AGACC	Bipartite and methylated, 5′RmC($N_{30–4000}$) RmC
Cleavage site	Variable locations >1,000 bp from recognition site	Fixed location at or near the recognition site	Fixed location 25–27 bp from recognition site	Between methylated bases at multiple sites

Table 23.2 Subtypes of Type II restriction enzymes (Pingoud and Jeltsch 2001)

Type	Defining features	Examples	Recognition sequence/cleavage site
IIA	Recognition site recognized by a homodimeric enzyme, cleavage within or near recognition site	FokI	GGATG(9/13)
IIB	Cleaves both sides of target on both strands	BcgI	(10/12)CGANNNNNNTGC(12/10)
IIC	Symmetric or asymmetric target. R and M functions in one polypeptide	GsuI	CTGGAG(16/14)
IIE	Two sites required for cleavage, one serving as allosteric effector	EcoRII	CCWGG
IIF	Enzyme is homotetrameric, two sites required, both cleaved coordinately	SfiI SgrAI	GGCCNNNN/NGGCC CR/CCGGYG
IIG	Symmetric or asymmetric target. Affected by AdoMet	BsgI Eco571	GTGCAG(16/14) CTGAAG(16/14)
IIH	Symmetric or asymmetric target. Similar to Type I gene structure	AhdI	GACNNN/NNGTC
IIM	Required methylated target	DpnI	Gm6A/TC
IIP	Symmetric target and cleavage sites	EcoRI	G/AATTC
IIS	Asymmetric recognition site with cleavage occurring at a defined distance	MmeI	TCCRAC(20/18)
IIT	Composed of heterodimeric subunits	Bs1I	CCNNNNN/NNGG

recognition sequences discriminate between molecules that contain these recognition sequences and those that do not. Within the enzyme–substrate complex, the DNA sequence is modified in such a manner that creates a magnesium ion binding site between the enzyme and DNA. Structural studies have revealed that there may be nonspecific binding between the enzymes and DNA molecules, but such molecules are not distorted in a manner that could allow magnesium ion binding and, hence, catalysis. In this way the nonspecific sequences are not cut. Restriction enzymes are prevented from acting on the DNA of a host cell by the methylation of key sites within their recognition sequences. The added methyl groups block specific interactions between the enzymes and the DNA such that the distortion necessary for cleavage does not occur and hence preventing cleavage (Table 23.2).

FokI: GGATG(9/13) where the first numeral in the parentheses indicates the position of cleavage on the strand written and the second numeral indicates the cleavage position on the complementary strand.

In full double-stranded form this corresponds to:

5′GGATGNNNNNNNNN
3′CCTACNNNNNNNNNNNNN

23.3.1.1 Homing Endonucleases

Homing endonucleases are double strand-specific DNases that have large, asymmetric recognition sequences and coding sequences that are usually embedded in either introns or inteins. Introns are spliced out of the precursor RNAs, while inteins are spliced out of proteins. These have extremely rare recognition sites, e.g., an 18 base pair recognition sequence will occur only once in every 7×10^{10} base pairs of a random sequence. This is equivalent to 1 site in 20 mammalian-sized genomes. However, unlike standard restriction enzymes, homing endonucleases tolerate some sequence degeneracy within their recognition sequence. This means that a single base pair change does not prevent the cleavage, but reduce its efficiency. Their observed sequence specificity is typically in the range of 10–12 base pairs.

23.3.2 Nomenclature

The system for naming restriction endonucleases and their corresponding methylases is based on the genus and species of the source organism, the particular strain, and the order of discovery.

By convention, the first letter of the genus name and the first two letters of the species name are used to derive the basic enzyme name. Thus, *Escherichia coli* yields Eco. Then comes a designation, if any, of the particular strain (sometimes an enzyme is encoded by a plasmid and the plasmid designation is used). A common restriction endonuclease from *E. coli* comes from an R plasmid. Finally, a Roman numeral is applied to indicate the order of discovery. Thus, the first restriction enzyme from *E. coli* carrying an R plasmid is EcoRI.

There are certain rules, standard abbreviations, and definitions that are recommended for nomenclature of REases (Roberts et al. 2003).

1. "Restriction enzyme" and "restriction endonuclease" should be regarded as synonymous and the abbreviation REase (or, in some cases, R) is preferred. The abbreviation R-M should be used for restriction modification.
2. Methyltransferase is the preferred name for methylase since it correctly describes the activity. The abbreviation MTase (or, in some cases, M) should be the standard.
3. Italics shall not be used for the first three-letter acronym of the REase or MTase name. There is convention of naming different enzymes from the same isolate or the same organism with increasing Roman numerals, e.g., EcoRI and EcoRII, in the order of discovery.
4. Restriction enzyme names should not include a space between the main acronym and the Roman numeral. Furthermore, except for the single period or hyphen (in homing endonucleases) that is used to separate the prefix from the main part of the name, no punctuation marks, such as parentheses, periods, commas, or slashes, should be used in REase or MTase names. According to

these rules, the enzymes from *Nostoc* species C have been changed from their original Nsp(7524)I to NspI and many others have also changed. The most recent is Bst4.4I, which has changed to Bst44I.

5. Three main types of REases are designated as Type I, Type II, and Type III, with the capital "T" preferred. Type IV includes REases that cleave only methylated DNA as their substrate and show only weak specificity, such as the McrA, McrBC, and Mrr systems of *E. coli*.
6. The sequence databases containing genes that are excellent candidates to encode DNA MTases and REases, based on sequence similarity, should be named according to the same guidelines as are used for biochemically characterized enzymes but carry the suffix P to indicate their putative nature. Once they have been characterized biochemically and shown to be active, the P should be dropped and their names should be changed to a regular name with the next Roman numeral that is appropriate.
7. The standard abbreviations for methylated bases should be 5-methylcytosine (m5C), N4-methylcytosine (m4C), and N6-methyladenine (m6A). It is not necessary to use a superscript for the number.
8. The solitary MTases (i.e., not associated with an REase) such as the Dam and Dcm MTases of *E. coli* and the eukaryotic MTases such as Dnmt1 and Dnmt3a are named systematically in accordance with the general rules established for the prokaryotic enzymes.
9. In the case of Type I enzymes, an acronym for the source organism should be followed by the traditional gene designations, *hsdS*, *hsdR,* and *hsdM*. Thus, the three genes of the EcoKI restriction system would be *ecoKIhsdM*, *ecoKIhsdR,* and *ecoKIhsdS*. However, it will be acceptable to omit the EcoKI where appropriate.
10. Homing endonucleases should be abbreviated HEases and are named using conventions similar to those of restriction enzymes with intron-coding endonucleases containing prefix "I" and intein-coding endonucleases containing the prefix "PI," e.g., I-CreI is intron-coded HEase from *Chlamydomonas reinhardtii* and PI-TliII is the second enzyme encoded by an intein found in *Thermococcus litoralis*.
11. For two genes that are required for a single enzyme activity, effectively encoding two subunits, the two genes and their products should carry a suffix A and B. For example, BbvCI is a heterodimeric REase. The two gene products should be called R.BbvCIA (or just BbvCIA) and R.BbvCIB (or just BbvCIB), and the active holoenzyme should be BbvCI. The two separate MTases of this system would be M1.BbvCI and M2.BbvCI. For enzymes like Eco57I, which have both endonuclease and MTase activity in the same polypeptide chain, the endonuclease would be referred to as RM.Eco57I, but the second MTase activity associated with this system would be called M.Eco57I. For MTases, an example is M.AquI, which has one gene encoding the N-terminal region up to the middle of the variable region of this m5C MTase and a second gene encoding the remaining C-terminal region. In this case, the two parts of this protein should be referred to as M.AquIA and M.AquIB and the genes as *aquIAM* and *aquIBM*.

23.3.3 Recognition Sites and Patterns of Cleavage

Restriction enzymes recognize a specific sequence of nucleotides and produce a double-stranded cut in the DNA. While recognition sequences vary between 4 and 8 nucleotides, many of them are palindromic (read same from 5′→3′ direction on both strands). The inverted repeat palindrome is also a sequence that reads the same forward and backwards, but the forward and backward sequences are found in complementary DNA strands (i.e., double stranded) as in GTATAC (notice that GTATAC is complementary to CATATG).

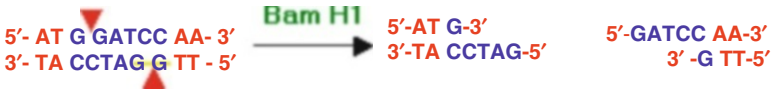
The recognition site of a restriction enzyme can be unambiguous or ambiguous. The enzyme BamHI recognizes the sequence GGATCC and none other. This is what is meant by unambiguous. In contrast, HinfI recognizes a 5 bp sequence starting with GA, ending in TC, and having any base in-between (GANTC). HinfI has an ambiguous recognition site. XhoII also has an ambiguous recognition site. It cuts at PuGATCPy. Py stands for pyrimidine (T or C) and Pu for purine (A or G), so XhoII will recognize and cut sequences AGATCT, AGATCC, GGATCT, and GGATCC.

Most enzymes cut within the recognition sequence but some also cut outside. MnlI recognizes CCTC but cuts 7 bp downstream (CCTC 7/7). Other examples are BbvI (GCGGC 8/12) and HgaI (GACGC 5/10).

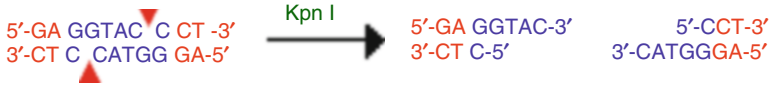
23.3.3.1 Patterns of Cleavage

Restriction enzymes break the bond between deoxyribose sugar and phosphate group. This leaves a phosphate group on the 5′ ends and a hydroxyl on the 3′ ends of both strands. Most restriction enzymes cut within their recognition sites and generate one of three different types of ends:

- 5′ overhangs: The enzyme cuts asymmetrically within the recognition site leaving a short single-stranded segment extending from the 5′ ends, e.g., BamHI.



- 3′ overhangs: The enzyme cuts asymmetrically within the recognition site, but the result is a single-stranded overhang from the 3′ ends, e.g., KpnI.



- Blunt ends: An enzyme that generates blunt ends, e.g., SmaI.

The 5′ or 3′ overhangs generated by enzymes that cut asymmetrically are called sticky ends or cohesive ends because they will readily stick or anneal with their partner by base pairing.

REases interact with specific sequences of nucleotides, usually comprising four to eight defined nucleotides. For example, Sau3AI recognizes 4 bp sequence, EcoRI recognizes 6 bp sequence, whereas NotI recognizes 8 bp sequence. They can be continuous or interrupted, symmetric or asymmetric, unique or degenerate.

Also, there are enzymes with multiple recognition sites, e.g., EcoRII (/GCWGG) where W = A or T and HindII (GTY/RAC) where Y = T or C and R = A or G. These increase the frequency of cutting and yield nonidentical ends.

The length of recognition sequence determines the probability of frequency of cleavage of a particular sequence. The enzymes with *n* bp recognition site cuts approximately after every 4^n bases provided that DNA has 50% GC content and recognition site contains all four types of bases. The frequency of cutting a sequence of bases

- 4 bp = 4^4 = 256 e.g., Sau3AI (/GATC)
- 6 bp = 4^6 = 4,096 e.g., EcoRI (G/AATTC)

In case of 6 bp cutters with only 2 types of bases (SmaI, CCCGGG), the frequency of cleavage is $4^6 \times 4^2$ = 65,536 bp in a DNA with 50% GC content and $4^6 \times 4^2 \times 4$ = 262,144 bp in a DNA with 25% GC content.

The restriction enzymes which recognize the same sequence and cleave at same position are called isoschizomer. The first example discovered is called a prototype and all subsequent enzymes that recognize the same sequence are isoschizomers of the prototype. Neoschizomers are that subset of isoschizomers that recognize the same sequence but cleave at different positions from the prototype. Thus, AatII (recognition sequence, GACGTC) and ZraI (recognition sequence, GACGTC) are neoschizomers of one another, while HpaII (recognition sequence, CCGG) and MspI (recognition sequence, CCGG) are isoschizomers. These often have different optimum reaction conditions, stabilities, and cost.

There are also some differences in terms of sensitivity towards methylation of a sequence. Both MboI and Sau3A cut GA*TC when this sequence is methylated at adenine (GA*TC). But if methylation is at cytosine (GATC*), Sau3AI cuts it but MboI fails to cut it. This is similar to the case of HpaII and MspI. Both enzymes recognize the sequence CCGG but when methylated at second cytosine CC*GG, MspI cuts but HpaII does not.

23.3.4 Structure of Type II Restriction Enzymes

There are two particular aspects of restriction enzymes that have attracted the attention of structural biologists. These are the primary sequence (i.e., the subunit amino acid sequence) and the secondary structure of the protein. Earlier it was thought that

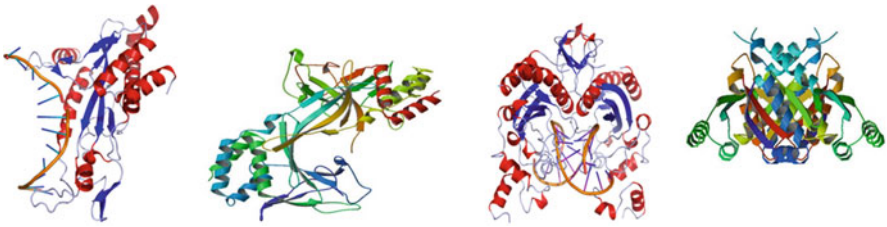


Fig. 23.2 Crystal structures of REases (Pictures adopted from Protein Data Bank, http://www.rcsb.org/pdb/home/home.do) (PabI and BstYI are from thermophiles)

the restriction enzymes have very less sequence homology. This conviction began to lose credibility by the observation of statistically high correlation between the amino acid sequence and recognition sequence of restriction enzymes. The second and probably most remarkable aspect of restriction enzymes which is based on the secondary structure is their specificity. A single base pair change in the recognition sequence can lead to millionfold reduction in activity of the enzyme (Roberts and Halford 1993).

The structural information on restriction enzymes comes from crystallographic studies on restriction enzymes. With the development in knowledge of crystal structures of more and more restriction enzymes, it became clear that although restriction endonucleases show little sequence similarity, they all share a highly similar α/β core. This core carries the active site (catalytic center) and the residues that contact DNA in the major groove. These include enzymes that produce 5′ overhangs (BglII, BsoBI, EcoRI, BamHI, MunI), 3′ overhangs (BglI), or blunt ends (EcoRV), as well as subtypes of Type II restriction enzymes.

The core comprises of a five-stranded mixed β-sheet flanked by α-helices (Venclovas et al. 1994); the second and third strands of the β-sheet serve as a scaffold for the catalytic residues of the PD-(D/E)XK motif. The fifth β-sheet can be parallel (as in EcoRI family) or antiparallel (as in EcoRV family) to the fourth strand. Based on the structural differences, in particular the topology of secondary structure elements and the arrangement of subunits, the enzymes of PD-(D/E)XK superfamily are divided into EcoRI and EcoRV families (Huai et al. 2000). Enzymes that belong to the EcoRI family (BamHI, BglII, BsoBI, Cfr10I) usually approach the DNA from the major groove, recognize the DNA mainly via α-helix, and in general produce 5′ staggered ends. Enzymes from EcoRV family (BglI, EcoRV, HincII, NaeI, MspI) usually approach the DNA from minor groove, use β-strand for DNA recognition, and in general produce blunt or 3′ staggered ends.

The Type II enzymes vary in size from 157 amino acids (PvuII) to 1,250 amino acids (CjeI) and even beyond. They have some sequence similarity in the so-called “twilight” or “midnight” zone of similarity (Sebastian et al. 2005). In this zone the sequences of homologous proteins are identical to the extent of <10–15% (Fig. 23.2).

23.3.5 Catalytic Mechanism of Type II REases

23.3.5.1 Target Site Location

All REases have to find their specific recognition site in the presence of a huge excess of nonspecific targets, to which they can also bind although the nonspecific binding event results in lower binding affinity. There are three different mechanisms that can account for the efficiency of target site location by REases: (1) sliding, (2) jumping or hopping, and (3) intersegment transfer (Jeltsch and Urbanke 2004).

Sliding (also called linear or one-dimensional diffusion) implies that the protein stays bound to the DNA after the first encounter and moves along the DNA by a random movement, following the pitch of the double helix until it finds its specific site or dissociates (Sun et al. 2003). During linear diffusion the nonspecific binding mode is not given up and the water layer around DNA and protein, characteristic for the nonspecific binding mode, remains largely intact. For EcoRI, it was estimated that in the transition from nonspecific to specific complex, approximately 100 water molecules are released at the protein–DNA interface (Sidorova and Rau 1996).

Jumping or hopping is normal (three-dimensional) diffusion that takes into account that the chance of reassociation of a DNA-binding protein to the DNA molecule close to the site it has dissociated from is much greater than associating with another DNA molecule or a distant site on the same molecule. During jumping or hopping the nonspecific binding is given up and the water layer characteristic for the free DNA and free protein is reformed. Jumping and hopping do not follow the pitch of the double helix, meaning that specific site along the DNA can be overlooked, depending on the step size.

Intersegment transfer is only possible for proteins that have two DNA-binding sites. If the DNA is released from one binding site, the enzyme still remains bound to the DNA with the other binding site and can bind to the same DNA molecule at a distant location via its free DNA-binding site. Binding of REase to both DNA-binding sites will produce loops in the DNA. Intersegment transfer is particularly efficient way of covering large distances exceeding the persistence length of DNA.

Due to steric constraints, intersegmental transfer is not an effective means for covering small distances in search of a specific site. REases normally use facilitated diffusion to locate their target site and the relative contributions of sliding and hopping presumably depend on the conditions, in particular Mg^{2+} concentration, ionic strength (Jeltsch and Pingoud 1998), and structure of the DNA-binding site of the REase.

23.3.5.2 Recognition

The recognition process in general consists of conformational adaptations of protein and DNA with water and counterion release at the protein–DNA interface (Sidorova and Rau 2004). Inspection of the available cocrystal structures of specific

REase–DNA complexes concluded that specific DNA binding is accompanied by more or less pronounced distortions of the DNA that bring functional groups of the DNA into positions required for optimal recognition and also position the scissile phosphates vis-a-vis the catalytic center and the 3′ proximal phosphates such that they can support phosphodiester bond hydrolysis. Specific DNA binding involves conformational changes in the protein that involve structuring regions that are unstructured in the free enzyme or in the nonspecific complex. These changes often involve a repositioning of the subunits and of the subdomains. In the specific examples, the DNA is partially (e.g., PvuII, 157 amino acid residues) or as in most cases fully (e.g., BsoBI, 323 amino acid residues) encircled by the REase. BsoBI is an extreme case as it forms a tunnel around its DNA substrate (van der Woerd et al. 2001). Unique among REase–DNA complexes is the intercalation of amino acid residues into the DNA double helix, as observed for HincII, where glutamine side chains (one from each subunit) penetrate the DNA on either side of the recognition site (Horton et al. 2002). Also, the formation of highly cooperative hydrogen bonds network is a characteristic feature of the specific protein–DNA complex of REases. The hydrogen bonds network comprises contacts to the bases as well as to the sugar phosphate. A majority of the possible hydrogen bonds are formed to the edges of the bases in the major groove (e.g., BamHI, 14/18; BglII, 12/18) and often in the minor groove (e.g., BamHI, 6/12; BglII, 10/12); most of them are direct, but a few are water mediated. In addition, van der Waals interactions and hydrophobic contacts are formed to the bases of recognition sequence. In general, several noncontiguous chain segments of a REase are involved in direct and indirect readout. Whereas most of the specific contacts are between one subunit and one half-site of the palindromic recognition sequence, a few are directed to the other half-site. A characteristic feature of the recognition process is its high redundancy, making the change of specificity by rational protein design difficult (Lanio et al. 2000; Alves and Vennekol 2004).

23.3.5.3 Coupling Between Recognition and Catalysis

Coupling between recognition and catalysis means coordination of the two catalytic centers. It is one of the least understood aspects of the enzymology of REases. It addresses how the residues involved in direct and indirect readout communicate with the catalytic centers and trigger conformational changes required for the initiation of phosphodiester bond cleavage. Experiments with EcoRV showed that the substitution of an amino acid residue responsible for base recognition in one half-site of the palindromic recognition sequence dramatically reduces the cleavage activity of the heterodimeric variant (Stahl et al. 1996). This means there is a cross talk between amino acid residues involved in a base-specific contact in one subunit with the catalytic centers of both subunits that guarantees that DNA cleavage is initiated only when all base-specific contacts have been made. In contrast, substitution of an amino acid residue of the catalytic center did not affect the activity of the catalytic center of the other subunit (Simoncsits et al. 2001).

Intersubunit cross talk in EcoRI is mediated by Glu144 and Arg145 (Grigorescu et al. 2004). These residues are a part of the 137–145 segments that is responsible for most of the base-specific contacts in EcoRI. Arg145 in each subunit (A) is hydrogen bonded through its guanidinium group to Glu144 of the other subunit (B) forming a ringlike structure involving the peptide backbones 144A–145A and 144B–145B and the other side chains Arg145A–Glu144B and Arg145B–Glu144A (Kurpiewski et al. 2004). A very similar arrangement is seen in the cocrystal structure of MunI with Glu120 and Arg121 (Diebert et al. 1999).

23.3.5.4 Mechanism of Phosphodiester Bond Cleavage

Phosphodiester bond hydrolysis by Type II REases follows an SN2-type mechanism, which is characterized by inversion of configuration at phosphorus (Connolly et al. 1984; Grasby and Connolly 1992). The general mechanism of phosphodiester hydrolysis comprises three steps:

1. Preparation of the attacking nucleophile by deprotonation

$$\mathrm{H_2O + B + R' - O5' - (PO_2) - - - O3' - R''} \rightarrow \mathbf{OH^-} + \mathrm{BH^+ + R'\text{-}O5' - (PO_2) - -O3' - R''}$$

2. The nucleophilic attack of the hydroxide ion on the phosphorus leading to the formation of the *pentavalent transition state*

$$\mathrm{OH^- + R - O5' - (PO_2) - O3' - R''} \rightarrow \mathbf{R - O5 - (PO_2\,OH)^{2-} - -O3 - R}$$

3. The departure of the *3′ hydroxyl leaving group*

$$\mathrm{O5' - (PO_3)^{2-} - O3' - + H_2O} \rightarrow \mathrm{R' - O5' - (PO_2) - O^- - +} \mathbf{HO3 - R} + \mathrm{OH^-}$$

To achieve efficient catalysis, all three steps require an assisting group: (1) a base (B) to deprotonate the water molecule, (2) a Lewis acid that stabilizes the pentavalent transition state with two negative charges, and (3) an acid that protonates the leaving 3′ oxyanion (Figs. 23.3 and 23.4).

Lysine is often considered as a general base candidate (Newman et al. 1994; Lukacs et al. 2000). The catalytic centers of the Type II REases generally contain a PD-D/ExK motif and the negatively charged side chains serve to ligate a divalent metal ion cofactor, usually Mg^{2+}. There is some controversy regarding the mechanism of DNA cleavage by REases about the number of divalent metal ions involved in the catalytic process. Three mechanistic models for DNA cleavage by REase are based on the number of metal ions involved in the reaction. However, a uniform mechanism can be applied for all REases of PD-D/ExK family. This mechanism requires at least one divalent metal ion. The presence of a second divalent ion can improve catalysis, though the cleavage reaction can be performed in its absence as

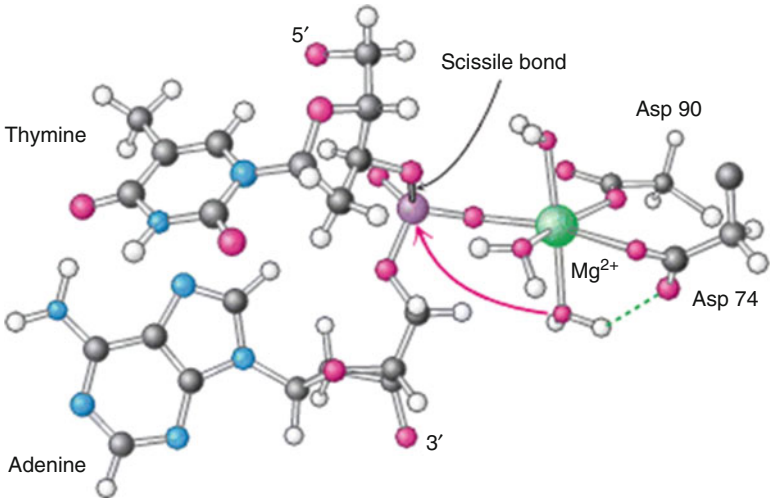


Fig. 23.3 Magnesium ion binding site in EcoRV endonuclease. The magnesium ion helps to activate a water molecule and positions it so that it can attack the phosphate (Berg et al. 2002)

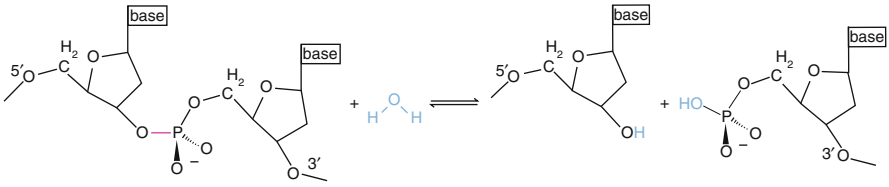


Fig. 23.4 Hydrolysis of a phosphodiester bond. All restriction enzymes catalyze the hydrolysis of DNA phosphodiester bonds, leaving a phosphoryl group attached to the 5′ end. The bond that is cleaved is shown in *red* (Berg et al. 2002)

well. The single- and double-ion mechanisms represent two alternative ways to facilitate phosphodiester bond hydrolysis (Fothergill et al. 1995).

If a single metal ion is present, it is responsible for catalyzing both reaction steps, stabilizing the OH nucleophile as well as the pentavalent transition state. To perform this task, the ion has to be able to move during the reaction.

If two metal ions are available, the tasks can be divided, so less movement is required. In any case the attacking molecule is proximal to the divalent metal ion held in place by two carboxylates of the PD-D/ExK motif and a main-chain carbonyl ("x" of the PD-D/ExK motif). The proton from the attacking water is transferred to a water molecule nearby (which acts as general base) and eventually to the bulk solvent. The water molecules are hydrogen bonded to the 3′ phosphate and the lysine/glutamic acid/glutamine of the PD-D/ExK motif. The metal ion

provides a favorable contribution to the stabilization of the nucleophile. The key role of the divalent metal ion is to stabilize the pentavalent transition state. After phosphodiester bond cleavage, the 3′ oxyanion is likely to associate itself with a divalent metal ion from the bulk solution as seen in postreactive complexes of the REase.

23.3.6 Single-Molecule Interaction of REase with DNA

In the past few years, methods have been developed that allow direct observation of binding of a protein molecule to a single binding site in a large DNA molecule. The basic procedure for this involves attaching colloidal particles ~1 nm diameter to the ends of the long DNA chain; these particles act as handles for micromanipulations. By manipulating the particles at the ends of the DNA (with laser tweezers), one can move the molecule around or stretch it out to any degree.

Magnetic particles also allow for twisting the DNA with magnetic tweezers into supercoiled configurations (Strick et al. 2000). Fluorescence techniques are also used for tracking single protein molecules in solutions. Proteins are attached to fluorescent particles and then their progress is tracked along a large DNA molecule and thus, its translocation path is observed directly. By studying the association/dissociation processes and the apparent sliding motions on DNA molecules in different states of extension, it may be possible to directly observe the sliding length and the dissociation time. The single-molecule interactions of PspGI, a thermostable REase, and its isoschizomer SsoII with oligonucleotide substrates containing one and two recognition sites showed that PspGI formed 1:1 complex, with the one-site substrate and 2:1 complex with two-site substrate. SsoII, in contrast, with one-site DNA not only formed 1:1 complex but frequently formed pairs of complexes, i.e., X-like structures, presumably induced by protein–protein interaction of SsoII-DNA complexes. On the two-site substrate, SsoII formed the complexes containing two DNA molecules held together by SsoII, forming X-like as well as bubble-like structures (Pingoud et al. 2003).

In another approach, phage λ DNA was biotinylated, stained by incubation with the fluorescent intercalating dye SYBR Green, and coupled to streptavidin-coated microspheres. Single molecules were then observed by fluorescence microscopy. Upon addition of enzyme, successive cleavages are observed beginning at the distal end and proceeding towards the site of bead attachment.

Another study involved labeling of the REase (TspMI) with Cy™3-OSu and labeling unmethylated λ DNA with YOYO-1. The reaction mixture was loaded on to a glass slide. The single molecules were then observed using an inverted microscope (Parashar et al. 2006). Absence of any loop formation in the DNA by the enzyme in all fields observed indicated that TspMI may not require two sites for activity and belongs to Type IIP subclass.

23.3.7 Star Activity

It has been observed that under extreme nonstandard conditions, restriction endonucleases are capable of cleaving sequences which are similar but not identical to their defined recognition sequence. It should be noted that typically the sites recognized under star conditions (high glycerol concentration, low ionic strength, high pH, presence of organic solvents, and substitution of Mg^{2+} with other divalent ions) differ from the canonical recognition sequence at one position. Thus, under star conditions, EcoRI (canonical recognition sequence, GAATTC) will probably cleave NAATTC, GNATTC, GANTTC, GAANTC, GAATNC, and GAATTN. This altered or relaxed specificity is called STAR activity.

23.4 Thermostable Restriction Enzymes

In 1966, Thomas Brock discovered that microorganisms were growing in the boiling hot springs of Yellowstone National Park. Since then, thermophiles have been discovered in geothermal features all over the world. This was a remarkable discovery because this presented a source of highly thermostable enzymes which could work at high temperatures thus widening the range of their applications. For example, two thermophilic species *Thermus aquaticus* and *Thermococcus litoralis* are used as sources of DNA polymerase, for the polymerase chain reaction (PCR).

Thermophiles are microorganisms with optimal growth temperatures between 60 and 108°C. They survive at such high temperatures because they have thermostable enzyme systems and hence are promising sources of thermostable enzymes. Since these enzymes tend to remain stable in the presence of denaturing agents such as detergents and organic solvents, they are being exploited in a number of biotechnological bioprocesses. Thermophiles have been screened for a variety of enzymes including restriction enzymes. Table 23.3 enlists some examples of REases from thermophiles.

23.4.1 Thermostability of REases

Thermostability seems to be a property acquired by a protein through a combination of many small structural modifications that are achieved with the exchange of some amino acids for others and the modulation of the canonical forces (e.g., hydrogen bonds, ion-pair interactions, hydrophobic interactions) found in all proteins (Scandurra et al. 1998). Factors contributing to stability include additional intermolecular interactions (e.g., hydrogen bonds, electrostatic interactions, hydrophobic interactions, disulfide bonds, metal binding) and good general conformational structure (i.e., more rigid, compact packing; conformational-strain release; stability of α-helix; reduced entropy of unfolding).

Table 23.3 Thermostable restriction enzymes

Restriction enzyme	Source	Recognition sequence	Temperature (°C)
BstI503	*Bacillus stearothermophilus*	5′G/GATCC3′	60–65
PspGI	*Pyrococcus* sp.	5′/CCWGG3′	65–85
BslI	*Bacillus* sp.	5′CCNNNNN/NNGG3′	55
TspM1	*Thermus* sp.	5′C/CCGGG3′	75
BfI1	*Anoxybacillus flavithermus*	5′CCNNNNN/NNGG3′	60
BstP1	*Bacillus stearothermophilus*	5′G/GTNACC3′	55–65
Tsp49I	*Thermus* sp.	5′ACGT/3′	65
Tsp1DSI	*Thermus* sp.	5′ACGT/3′	65
Tsp1WAM 8AI	*Thermus* sp.	5′ACGT/3′	65
SuaI	*Sulfolobus acidocaldarius*	5′GG/CC3′	70
Bse1I	*Geobacillus stearothermophilus*	5′ACTGGN/N3′	60

23.4.1.1 Hydrogen Bonds

Hydrogen bonds occur not only within and between polypeptide chains but with the surrounding aqueous medium. Thermostability of proteins is correlated with the number of hydrogen bonds and the fractional polar surface that results in increased density of hydrogen bonding with the surrounding water molecules (Vogt et al. 1997).

23.4.1.2 Electrostatic Interactions

Electrostatic forces are mainly of three types: charge–charge, charge–dipole, and dipole–dipole. Typical charge–charge interactions between oppositely charged residues are known as salt bridges. Both at the overall genomic level and in the helices, the amounts of Glu, Lys, and Arg are higher in thermophilic proteins than in mesophilic proteins. This increase in charged residues suggests that there are more salt bridges in thermophilic proteins than in equivalent proteins of mesophiles (Das and Gerstein 2000).

23.4.1.3 Hydrophobic Bonds

Hydrophobicity of a protein molecule is expressed as the ratio of buried nonpolar surface area to the total nonpolar surface area of the molecule. Hydrophobic forces are major contributors to molecular folding and thermostability (Goodenough and Jenkins 1991). Introduction of a methyl group in the cavity increased hydrophobic interaction within the protein core and therefore enhanced protein stability (Ishikawa et al. 1993).

23.4.1.4 Disulfide Bond

Disulfide bridges are believed to stabilize proteins mostly through an entropic effect, by decreasing the entropy of the protein's unfolded state (Matsumura et al. 1989). Cys residues were introduced at positions 61 (Gly) and 98 (Ser) in subtilisin E by site-directed mutagenesis. The half-life of the mutant enzyme was two to three times longer than that of the wild-type enzyme and Tm of the mutant increased by 4.5–8°C relative to the wild type. Differential scanning calorimetry data further confirmed the stabilizing effect (Takagi et al. 1990).

23.4.1.5 Metal Binding

Major stabilizing forces are associated with the presence of metals in the holoenzyme. The Tm values of apoenzyme, Mg^{2+}, Co^{2+}, and Mn^{2+} enzyme were 50.3, 53.3, 73.4, and 73.6°C, respectively. The energy of activation for irreversible inactivation was also strongly influenced by the metal present. The activation energy ranged from 342 (apoenzyme) to 1166 (Co^{2+} enzyme) kJ/mol (Vieille et al. 2001).

23.4.1.6 Good Conformational Structure

1. More Rigid, Less Flexible
   Increased rigidity is essential for preserving the catalytically active structure at elevated temperature. Increased rigidity protects a protein against unfolding. Overall enzyme rigidity increases through α-helix stabilization, electrostatic-interaction optimization, conformational-strain reduction, etc. Enhanced rigidity is demonstrated by reduced hydrogen–deuterium exchange rates, lower susceptibility to proteolytic degradation and chemical denaturants, and reduced thermally induced unfolding.
2. Higher Packing Efficiency, Fewer Cavities
   Compactness can be achieved by shortening one or more loops; increasing the number of atoms buried in the molecule and hence hidden from solvent; optimized packing of side chains in the interior; and elimination of unnecessary cavities. A number of hyperthermophilic proteins show significantly reduced hydrophobic ASA (accessible surface area) relative to their mesophilic counterparts.
3. Reduction of the Entropy of Unfolding
   In the protein's unfolded state, Gly is the residue that has the highest conformational entropy. In the folding process, much more energy is required to restrict the configuration of Gly than for any other residue. Pro residue, with its pyrrolidine ring, can only adopt a few configurations and has the lowest conformational entropy. Presence of a Pro residue restricts the configurations allowed for the preceding residue. Thus, Gly to Pro mutations should decrease the entropy of a protein's unfolded state and stabilize the protein, so long as

the engineered residue does not introduce unfavorable strains in the protein structure. Prolines were introduced in that loop to make it less susceptible to unfolding. The reduction in entropy of unfolding can also be achieved by shortened loops, anchoring of loose ends, introduction of disulfide bonds, and docking of the N- and C-termini (Vieille and Zeikus 2001).

4. Reduction of Conformational Strain
   When not stabilized by intramolecular noncovalent interactions, non-Gly residues in proteins with a left-handed helical conformation often cause instability (non-Gly residues with a left-handed helical conformation are less stable than the right-handed conformation, by 0.5–2.0 kcal/mol). Furthermore, the close contact between the H-carbon and carbonyl oxygen within the left-handed residues creates conformational strain on the protein structure (Vieille et al. 1996).
5. α-Helix Stabilization
   It can be achieved by substituting residues with a low helical propensity with residues that have high helical propensity. Residue substitution usually takes place when a residue's side chain is not well accommodated in the α-helix. β-branched residues (Val, Ile, Thr) were found to destabilize α-helix. The only factor occurring significantly in the thermostable proteins was the lack of H-branched residues (Val, Ile, Thr) in α-helix. The α-helix carries a considerable dipole moment that can be stabilized by negatively charged residues (e.g., Glu) near their N-terminal end as well as by positively charged residues (e.g., Lys) near their C-terminal end. Introducing these favorable residues at the end of α-helices can stabilize enzymes. In some proteins, common antiparallel arrangement of adjacent helices confers a stabilization of 5–7 kcal/mol. In contrast, a similarly packed array of parallel helices is relatively destabilized by 20 kcal/mol (Sheridan et al. 1982). In α-helix, NH donors of the first four residues and CO acceptors of the last four residues lack interhelical hydrogen bond partners. There are statistical preferences at the N-cap position of an α-helix for the residues Asn, Ser, Asp, Gly, and Thr. Conversely, Ala, Leu, Val, Ile, Trp, Arg, Gln, and Glu are not preferred. Apart from Gly, all the preferred residues have side-chain atoms that can form a hydrogen bond to the main-chain NH at the N3 position, thereby offering additional stability (Richardson and Richardson 1988).

23.4.2 Advantages of Thermostable Restriction Enzymes

Most of the restriction enzymes that are used in molecular biology come from mesophilic sources. As expected these are highly temperature sensitive and loose activity quite rapidly even at 37°C. So, these enzymes require refrigeration on storage and transportation which pushes up the cost. Also, the enzymes from mesophilic sources have a limited temperature range of activity which makes them unsuitable for use in various applications which are performed at high temperatures.

The properties and the respective advantages of thermostable restriction enzymes are:

Efficient Cleavage
Less secondary structures are formed in the substrate DNA due to high temperature (Raven et al. 1993). This makes the DNA readily accessible to restriction enzymes and the cleavage is efficient.
Amount of Enzyme
As with increase in temperature, rate of reaction also increases, less amount of restriction enzymes is required in case of thermostable restriction enzymes as compared to their mesophilic counterparts.
Reduced Nonspecific Cleavage
The nonspecific agents like nucleases from mesophilic sources are taken care by the high temperature at which the thermostable enzymes work and the nucleases from mesophiles are inactivated. So, there are fewer chances of nonspecific cleavages by contaminants.
Enzyme Stability
Thermostable enzymes are more resistant to activity loss by repeated freeze-thaw operations. BstVI, a thermostable isoschizomer of XhoI, is not affected by repeated freeze-thaw cycles. These also exhibit stability and resistance to protein denaturants like urea and guanidinium (Lobos and Vasquez 1993).
Higher Yield
Thermostable restriction enzymes are more resistant to proteolytic degradation. So, there is an increased purification yield due to less degradation during purification process.
Stability During Transportation
The need of −20°C during storage and transportation of mesophilic restriction enzymes pushes up the cost, whereas thermostable ones can be stored and transported at 4°C or even ambient temperature.
Economical
All these factors discussed above lead to decrease in overall cost of manufacturing, a key aspect in industries.

23.5 Sources of Thermostable Restriction Enzymes

Thermostable restriction enzymes can be obtained directly from the microorganisms that thrive at high temperatures. The growth at high temperatures implies that their enzyme systems are stable and active at these temperatures. However, there have been studies in which the genes from thermophiles encoding REases are cloned in mesophilic microorganisms (e.g., *E. coli*). The expressed REase, then, can be purified from these microorganisms. Also, generation of mutants/variants and protein engineering of the REases are also being attempted for producing thermostable restriction enzymes. Thus, on the basis of origin of thermostable REases, these can be divided into three broad categories,

Table 23.4 REases from different geothermal habitats

Site of isolation	REase	Organism	Reference
Geothermal regions of northern Himalayas, India	BflII	*Anoxybacillus flavithermus*	D'souza et al. (2004)
	TspMI	*Thermus* sp.	Parashar et al. (2006)
Yellowstone National Park, USA	BclI	*Bacillus caldolyticus*	Bingham et al. (1978)
	SuaI	*Sulfolobus acidocaldarius*	Prangishvili et al. (1985)
	Taq52I	*Thermus* sp.	Welch (1995)
Azores, Iceland	Tsp45I	*Thermus* sp.	Raven et al. (1993)
	Tsp32I	*Thermus* sp.	Welch and Williams (1995a, b)
	Tsp49I	*Thermus* sp.	Welch and Williams (1996)
The Great Artesian Basin, Australia	FgoI	*Fervidobacterium gondwanense*	Andrews et al. (1998)
Oil-contaminated desert soil, Kuwait	BstB7SI	*Bacillus stearothermophilus*	Al-Awadhi et al. (1998)
Hot springs of Matsue, Japan	PlaI, PlaII	*Phormidium lapideum*	Ochiai et al. (1989)
Oceanic hydrothermal vent	PspGI	*Pyrococcus* sp.	Morgan et al. (1998)
Burning refuse pile at the Friar Tuck coal mine, Indiana	ThaI	*Thermoplasma acidophilum*	McConnell et al. (1978)

1. REases isolated from thermophiles
2. REases from thermophiles cloned into mesophiles
3. REases from mutants exhibiting better thermostability/engineered REases with increased thermostability

23.5.1 REases from Thermophiles

A number of geothermal habitats have been screened for the isolation of thermophiles and their REases. The environments typically inhabited by thermophiles include deserts, thermal vents, volcanoes, and terrestrial hot springs. Hot springs are widely distributed in different parts of the world and hot springs of Yellowstone National Park (USA), Himalayas (India), the Great Artesian Basin (Australia), New Zealand, Azores (Iceland), mainland Portugal, and Matsue (Japan) are some from where isolation of thermostable restriction enzymes have been reported (Table 23.4). TaqI was the first REase from a thermophile (*Thermus aquaticus*).

23.5.1.1 Procedure for the Isolation of Type II REases from Thermophiles

A generalized procedure for the isolation, purification, and characterization of thermostable REases is discussed here which includes the following basic steps.

Isolation and Screening of Thermophiles for Endonucleolytic Activity

The soil and water samples for thermostable REases are generally collected from hot springs, thermal vents, or deserts which are geothermally active regions. The temperature and pH of the water samples at the time of collection are noted so as to formulate pH of growth medium and growth temperature. Soil and water samples are collected from the edges of the thermal pools.

There are various methods for the isolation of the thermophiles. M162 medium has been successfully used for the isolation of thermophiles from different types of habitats (Degryse et al. 1978). The medium can be used with several modifications such as addition of antibiotics and adjusting pH of medium so as to optimize growth of thermophiles other than *Bacillus* spp. Before isolation, it is better to enrich the samples for thermophiles (Brock 1978). Enrichment of soil and water samples is done by growing in the temperature range of 60–80°C where extreme thermophiles can be isolated due to selective advantage. For enrichment, the soil samples are added to M162 medium prepared with filter sterilized spring water (in case the samples are from hot springs) and incubated overnight under shaking conditions at temperature corresponding to the temperature of the source of samples.

For the isolation of organisms that grow at temperatures more than 70°C, M162 medium (1X), containing 3% agar, is cooled to 50–55°C before preparing the media plates. After solidification, the plates are incubated inverted at 37–40°C for a minimum of 3 days. These preincubated plates are then used for spread plating and streaking with enriched medium and incubated at optimum temperature. The isolates obtained are further grown in M162 broth (D'souza et al. 1996).

The cells are harvested and lysed by sonication and cell extract is used as enzyme source for screening for restriction endonuclease activity. Different DNA substrates (e.g., λ-DNA, pBR322, pUC18) are digested with the crude enzyme in the reaction buffer for different intervals of time for obtaining digestion patterns. If reaction mixture contains only Mg^{2+} as the catalytically important cofactor and no ATP, Type I and Type III activities can be ruled out.

λ-DNA is the substrate of choice for screening purposes being of a large size (48.5 kbp) and thus offering a higher probability of finding restriction sites (interrupted or uninterrupted) even for rare-cutting endonucleases. pUC18 is a circular dsDNA offering many unique sites for different REases in MCS (multiple cloning site) and whose cleavage ensures presence of endonuclease activity. Once pUC18 is cut by endonuclease, exonuclease(s) if present may start cutting the DNA from ends generated.

On digestion with crude extract, one may get clear banding patterns or smear which may be inconsistent as crude extracts have many types of nucleases and DNA-binding proteins, nonspecific endonucleases, or presence of more than one restriction enzymes. One step purification using Cibacron blue helps in preliminary screening as it results in some purification and removal of nonspecific nucleases and other DNA-modifying enzymes along with sonicated DNA.

It is necessary to provide nuclease-free conditions for restriction digestion because nonspecific nucleases (from hands of the person putting up reaction and solutions/glassware/plasticware) may lead to misleading results.

Purification of REases

The crude extracts of the isolates indicating restriction endonucleolytic activity are further selected to purify the REase by column chromatography. A number of matrices are available that are used for purification including Cibacron blue, phosphocellulose, Q-sepharose, heparin Sepharose, and DEAE cellulose. An important advantage of REases from thermophiles is that the purification need not be done at low temperature thus reducing the cost of purification.

Cibacron blue F3GA, a sulfonated polyaromatic blue dye, when covalently bound to activated agarose can act as "pseudoaffinity adsorbent" (Lowe et al. 1986). Cibacron blue is specific for binding super secondary structure called "dinucleotide fold." Restriction enzymes and those enzymes which possess nucleotide-binding sites have affinity for Cibacron blue, whereas cellular DNA comes out in the void volume because it does not bind to it. The use of Cibacron blue makes sure that precipitation of nucleic acids from the crude lysates with polyethyleneimine or streptomycin is not needed.

Q-Sepharose FF is a strong anion exchanger containing quaternary ammonium groups. The matrix is composed of highly cross-linked agarose (6%). Most of the restriction enzymes bind anion exchangers at 0.4 M NaCl (Pirrotta and Bickle 1980). Anion exchangers have been used in the purification steps for a number of restriction enzymes, e.g., BcoI and BstLVI. At times, anion exchangers have been used to remove proteins other than restriction endonucleases, e.g., BstVI (Vasquez 1985), RsaI (Lynn et al. 1980), and SanDI (Simcox et al. 1995). In such cases, the enzyme does not bind to the column matrix and hence, the washthrough containing the restriction endonuclease, but devoid of a large number of proteins, is collected and is then passed through a different column matrix.

Diethylaminoethyl cellulose (DEAE or DEAE-C) is a positively charged resin used for ion exchange chromatography for protein and nucleic acid purification/separation. Gel matrix beads are derivatized with DEAE and lock negatively charged proteins or nucleic acids into the matrix, until released by increasing the salt concentration of the solvent.

Heparin sepharose, a type of pseudoaffinity matrix, is also used for purification of restriction enzymes. Proteins which bind to nucleic acids, like restriction enzymes, apparently recognize heparin as a nucleic acid analogue and bind to the heparin agarose column because of this affinity. Restriction enzymes usually bind to heparin more tightly than nonspecific nucleases and consequently are eluted at high salt concentrations.

SP sepharose is a strong cation exchanger having sulfopropyl group as ion exchange group which remains charged and maintains consistently high ionic capacity.

Because restriction enzymes are isolated from widely different bacterial sources, each containing a unique set of proteins, a general purification procedure does not exist for all restriction enzymes. Most of the purification systems for restriction enzymes have been aimed at quickly obtaining a preparation that may not be homogeneously pure but is functionally pure, i.e., free from other DNA-modifying enzyme like nonspecific nucleases, kinases, phosphatases, nickases, recombinases, and ligases. Most of the commercially available restriction enzymes are partially

purified preparations which have passed quality control assays to ensure complete absence of nonspecific enzymes. However, in some studies, particularly for the characterization of the recognition sequences and where the cut segments are needed to be ligated, homogeneous purification is necessary.

Characterization of Purified REases

Enzyme characterization usually involves detailed analysis of the kinetic parameters and mechanistic features of the reaction catalyzed, together with some description of the protein, its molecular weight and subunit composition. It is also imperative to elucidate the common physicochemical properties such as pH and temperature optima. These studies in relation to activity and stability, star activity, and requirement for divalent (Mg^{2+}) and monovalent (Na^{+}/K^{+}) cations for a newly discovered restriction enzyme are needed to obtain complete digestion of the substrate DNAs. An important first step in the characterization of new restriction enzymes involves description of the digestion fragment patterns obtained on different DNA substrates (λ-phage DNA, adenovirus DNA phiX174, M13mp18, pBR322, and pUC18). Comparison of fragments with those obtained using enzymes of known specificity can indicate whether the new enzyme recognizes a novel sequence or is likely to recognize a sequence identical to the one recognized by an already characterized enzyme. For most practical purposes, it is the recognition sequence that is the fundamental property of restriction enzymes to characterize by sequencing.

Unit of Restriction Enzyme

One unit of restriction enzyme is defined as the amount of enzyme required to digest 1 μg of λ DNA completely in 1 h at its optimum temperature.

In Silico Analysis of Recognition/Cleavage Sequence of a New REase

REBASE (http://rebase.neb.com/rebase/rebase.html)

REBASE is a database which stores all the available information regarding restriction enzymes, DNA methyltransferases, homing endonucleases, and related proteins involved in restriction modification. It contains updated information about recognition and cleavage sites, isoschizomers, commercial availability, crystal structures, and sequence data. Additionally, REBASE contains complete and up-to-date information about the methylation sensitivity of restriction endonucleases.

Restriction Digestion of Different DNA Substrates

Different DNA substrates (fully sequenced and commercially available viz. unmethylated λ, pUC18, pBR322, M13mp18, and phiX174) are used to get digestion patterns on agarose gel after digestion with enzyme.

NEBcutter

NEBcutter (http://tools.neb.com/NEBcutter2/index.php)

NEBcutter is a program used to compare the digestion patterns obtained by in silico digestion of different substrate DNAs with known restriction enzymes with the digestion patterns obtained on different DNA substrates with a new restriction enzyme in laboratory.

REBpredictor

REBpredictor is a program used to predict the recognition sequence from a given fragment pattern obtained experimentally for a DNA substrate. For each fragment, its length (bp) along with % deviation (variation from mean value of fragment size from the lowest and highest values obtained in similar experiments) is required.

Runoff Sequencing

The most important characterization of a REase is in terms of the recognition/cleavage sequence. For this, the substrate DNAs are cut with the enzyme and the fragments generated are sequenced so as to determine the recognition/cleavage site of the REase.

23.5.1.2 Generic Distribution of Thermophiles Producing Restriction Enzymes

Thermophiles exist in a broad range of habitats and in different types of global ecosystems which are geothermally active. These habitats include geysers, black smokers, oil wells, deep-sea hydrothermal vents, as well as decaying plant matter such as peat bogs and compost. The thermophilic habitats have been screened for thermophiles and restriction enzymes from these thermophiles. On an average every third microorganism produces restriction enzymes. Different thermophiles which produce REases belong to genera *Bacillus, Geobacillus, Anoxybacillus, Thermus, Meiothermus, Acidothermus, Rhodothermus, Thermococcus, Methanobacterium, Methanococcus, Sulfolobus, Pyrococcus, Pyrobaculum* spp. These genera vary in their geographical distribution, growth conditions, and enzyme properties. A few of the genera which are important and known to produce majority of REases are discussed.

Bacillus

Bacillus is the most important genus from which more restriction enzymes than any other genus have been isolated. Different *Bacillus* spp. which produce thermostable REases have been isolated from various geothermal habitats like hot springs,

thermal vents, or deserts. The thermophilic *Bacillus* from which restriction enzymes have been isolated grow in a temperature range of 55–70°C. The pH of the habitats varies from acidic to alkaline. Some of the species from this genus which produce restriction enzyme are *B. stearothermophilus, B. thermoglucosidasius, B. subtilis, B. schlegelii, B. smithii, and B. globigii.* A maximum number of restriction enzymes have been isolated from different strains of *B. stearothermophilus*. There are more than 300 Type II restriction enzymes only from this genus.

Thermus

Thermus is the second most important genus known to produce restriction enzymes. *Thermus* is a Gram-negative eubacterium with typical rotund bodies and growth temperature ranging from 60–80°C, optimum being 72°C. Most of the *Thermus* spp. thrive at alkaline pH (7.5–8.0) though some strains have been isolated from habitats with as low as 5.1 and as high as 9.5 pH. Different species that produce restriction enzymes are *T. aquaticus, T. thermophilus, T. filiformis, T, scotoductus, and T. flavus*. *Thermus* strains were first isolated from neutral and alkaline hot springs in Yellowstone National Park and since then have been isolated from different parts of world. *Thermus* spp. for restriction enzymes have been isolated from various geysers of Yellowstone National Park (US), Azores (Iceland), Himalayas (India), and the Great Artesian Basin (Australia). 76 strains of different *Thermus* spp. are known to produce restriction enzymes. TaqI, isolated from *T. aquaticus*, works optimally at 65°C. More than 250 restriction enzymes have come from *Thermus, Meiothermus,* and *Acidothemus* spp.

Pyrococcus

Pyrococcus is Gram-negative, anaerobic, and hyperthermophilic archaebacterium growing at 95–103°C and at neutral pH. This genus represents the most thermophilic bacteria known to produce restriction enzymes. There are 24 restriction enzymes from *Pyrococcus* spp. Different species of *Pyrococcus* which produce restriction enzymes are *P. abyssi, P. furiosus, P. horikoshii,* and *P. kodakaraensis*. PabI, isolated from *P. abyssi,* is a highly thermostable enzyme working even at boiling temperature. PspGI was isolated from *Pyrococcus* sp. growing in an oceanic hydrothermal vent. Other than these, PhoI and PfuI are also obtained from *Pyrococcus* spp.

Rhodothermus

Rhodothermus is Gram-negative rod-shaped eubacterium growing in a temperature range of 65–75°C. Thirty-eight restriction enzymes have been isolated from different strains of *Rhodothermus marinus* which work optimally between

65–70°C. As *R. marinus* is a thermohalophilic organism, restriction enzymes from this organism are also salt resistant. This property is important because salts inhibit or hinder the activity of restriction enzymes which are not salt resistant. Some restriction enzymes have been isolated from thermophilic strains obtained from culture collection centers.

Sulfolobus

These are Gram-negative, irregularly lobed spherical bacteria which grow in temperature range of 70–80°C and pH 2.0–3.0 in sulfur-rich habitats. Yellowstone National Park, Mt. St. Helens, Iceland, Italy, and Russia are only a few to name from where *Sulfolobus* spp. have been isolated and screened for restriction enzymes. The species which produce restriction enzymes include *S. acidocaldarius, S. islandicus, S. solfataricus,* and *S. tokodaii*. There are 56 restriction enzymes isolated from different strains of these species.

Methanogenic Bacteria

Thermophilic methanogenic bacteria which grow in a temperature range 48–94°C, optimum being 85°C, and belonging to different genera are also known to produce restriction enzymes. Methanogens have been found in several extreme environments on earth, living in hot, dry desert soil and marshy habitats. These include *Methanobacterium, Methanococcus, Methanocaldococcus, Methanopyrus, Methylovorus*. All these genera contribute approximately 100 different restriction enzymes.

23.5.2 Cloning of REases from Thermophiles

Extensive use of REases in molecular biology and biotechnological applications makes high-level production of REases inevitable. The commercial incentive is even more in case of thermostable REases. Isolation and purification of thermostable REases from their native thermophilic hosts require high fermentation costs and results in low yields. Therefore, cloning of REases from thermophiles in mesophiles is considered to be beneficial for high-level expression of REases for high yields and economical fermentation process. In several cases, the native organism produces more than one restriction enzyme. Cloning of restriction enzymes from such organisms ensures greater purity with no cross contamination. Furthermore, having access to cloned and sequenced genes would offer opportunities to increase our basic knowledge about these enzymes and lead to the possibility of improving their properties. Also, it is possible to carry out structural studies and elucidate the mechanism of cleavage by these enzymes and determination of catalytic residues.

Table 23.5 REases cloned from thermophiles in *E. coli*

REase	Source organism
PhoI	*Pyrococcus horikoshii* OT3
PspGI	*Pyrococcus* sp.
MwoI	*Methanobacterium wolfeii*
BclI	*Bacillus caldolyticus*
BstNI	*Bacillus stearothermophilus* N
BsoBI	*Bacillus stearothermophilus*
BslI	*Bacillus* sp.
TaqI	*Thermus aquaticus*
Tsp45I	*Thermus* sp. YS45
Tsp32I	*Thermus* sp. SM32

REases are intracellular enzymes meant for host defense that are produced in very small amounts. However, cloning of these enzymes and their expression, both cytoplasmic and extracellularly, are reported in some studies (Barany 1988; Barany et al. 1992). *E. coli* has been used as the host organism for cloning REases in most studies due to ease of transformation and expression. Moreover, purification of recombinant proteins is well established in *E. coli* than thermophiles. The first restriction modification (R-M) system cloned for commercial availability was PstI (New England Biolabs). EcoRI, MspI, HindIII, and TaqI were soon to follow. As the number of researchers working in this area grew, BamHI, FokI, and BglII were cloned, as well as the important and useful 8-base cutters SfiI, NotI, and PacI. Presently, NEB, a leading company producing restriction enzymes, supplies more than 260 restriction enzymes, over 230 of which are available in recombinant form. The following Table 23.5 shows a few REases from thermophiles that were cloned into *E. coli* for overexpression.

23.5.2.1 Methods of Cloning Restriction Enzymes

There are three general methods for cloning of restriction endonuclease genes.

1. Phage Restriction Method
   In the first method, phages are used to select clones which carry endonuclease gene. The transformed *E. coli* cells are plated on a medium containing phages. If the transformed cells contain the endonuclease gene, they resist the infection by phages and hence are able to survive. However, the phage challenge method requires expression of the endonuclease gene to an appropriate level to allow restriction of incoming phages.
2. Methylase Selection Method
   The second method involves selection of an active methylase gene. As restriction and modification genes are often closely linked, both genes can be cloned simultaneously if the segment of DNA cloned is large enough. Although a majority of endonuclease genes have been obtained using the methylase selection method, the method sometimes yields only the methylase genes.

3. Endo-Blue Method
   An *E. coli* strain (*dinD1*::*lacZ*) deficient in methylation-dependent restriction systems (McrA-, McrBC-, Mrr-) has been used for direct cloning of restriction endonuclease genes from thermophiles (New England Biolabs, USA). The methylase activity of the system protects self-DNA from cleavage by own restriction enzyme. As the methylase activity is absent, the DNA is not protected against restriction activity. The method is based on the principle that when *E. coli* cells are not fully protected by methylase, the cognate restriction enzymes cloned from thermophiles cause nicks in the DNA in vivo in response to which *dinD1* gene (damage inducible gene of SOS repair system) is induced resulting in the expression of fused β-galactosidase gene, and hence, blue colonies are formed on indicator plates containing X-gal by those transformants in which a thermostable REases has been cloned (Fomenkov et al. 1994).

   Briefly, this method involves preparation of total DNA (plasmid plus genomic) of the thermophilic bacterium from which the REase gene is to be cloned. Genomic DNA is restricted with enzyme like Sau3AI and is ligated to BamHI digested and dephosphorylated vector, e.g., pBR322. The ligation mixture is transformed into competent *E. coli* cells. The cells that have got REase gene through plasmids form blue colonies on X-gal containing LB agar medium. Individual blue colonies are picked and further purified for assessing the REase activity.

Novel Restriction Enzymes from Metagenome

A new approach involves REases from metagenome. It is advantageous if the soil samples for isolation of metagenomic DNA are from thermophilic habitats, so as to isolate and clone genes encoding thermostable REases. The soil samples are collected and DNA is directly isolated from the soil. The DNA fragments from restricted metagenome are ligated to plasmids and transformed into *E. coli* cells and the transformants are checked for endonucleolytic activity by Endo-Blue method.

23.5.3 Thermostable REases from Mutants/Variants

Various studies have focused on the generation of mutants which exhibit properties of more thermostable enzymes. These include generation of mutants by spontaneous mutations, chemical mutagenesis, cloning of mesophilic genes in thermophiles, and protein engineering of restriction enzymes. However, there has been little success for producing thermostable restriction enzymes using these methods.

A method for rapidly generating thermostable enzyme variants is to introduce the gene coding for a given enzyme from a mesophilic organism into a thermophile, *Bacillus stearothermophilus*. Variants that retain the enzymatic activity at higher growth temperatures of the thermophile are then selected.

A mutant of *B. stearothermophilus* was obtained by spontaneous mutation which produced three times more thermostable restriction enzyme. The in vivo heat inactivation of wild-type Bst31 in strain NUB31 can be demonstrated using efficiency of phage plating. The ability of the strain to resist infection by phages was assayed on strain NUB31 at various temperatures. As the assay temperature was raised from 66 to 70°C, strain NUB31 was less efficient in the restriction of phages. These results indicate that strain NUB31 contained a relatively thermolabile Bst 31 that was inactivated at elevated temperatures. Optimal conditions for the isolation of spontaneous mutants that contain a more thermostable restriction enzyme were established using strain NUB311.

Lately, protein engineering of restriction enzymes is being tried to generate thermostable variants of restriction enzymes. There have been substantial efforts to engineer known REases to endow them with novel properties, by random mutagenesis as well as rational, i.e., structure-guided design. These properties include change in sequence specificity (Nasri and Thomas 1986); change in metal ion cofactor preference (Vipond et al. 1996), using modified substrates over their natural substrates (Heitman 1993); and increase in catalytic activity (Nath 1981). So, these studies provide substantial evidence favoring the use of protein engineering to increase thermostability of REases as is done in case of some other enzymes.

23.6 Specific Applications of Thermostable REases

The thermostability associated with REases from thermophiles makes them able to be used in several new applications and widens the range in which thermolabile REases are used. Thermostable REases are potentially useful in applications that require restriction enzymes to be incorporated into PCR.

23.6.1 Methylation-Specific PCR

Aberrant promoter methylation is a major mechanism for silencing tumor suppressor genes in cancer. Detection of hypermethylation is used as a molecular marker for early cancer diagnosis, as a prognostic index, or to define therapeutic targets for reversion of aberrant methylation. *MS-FLAG* (methylation-specific fluorescent amplicon generation) provides a new, quantitative, high-throughput method for detecting gene promoter methylation and is a convenient alternative to agarose gel-based MSP for screening methylation. MSP (methylation-specific PCR) uses primers that bind to and amplify bisulfite-converted sequences only if CpG dinucleotides on these sequences remain unaffected by the chemical treatment (i.e., the cytosines are methylated). Sodium bisulfite converts unmethylated cytosines into uracils while leaving methylated cytosines relatively intact, thus creating sequence differences between genomes that originally differ only in their CpG methylation pattern (Fig. 23.5).

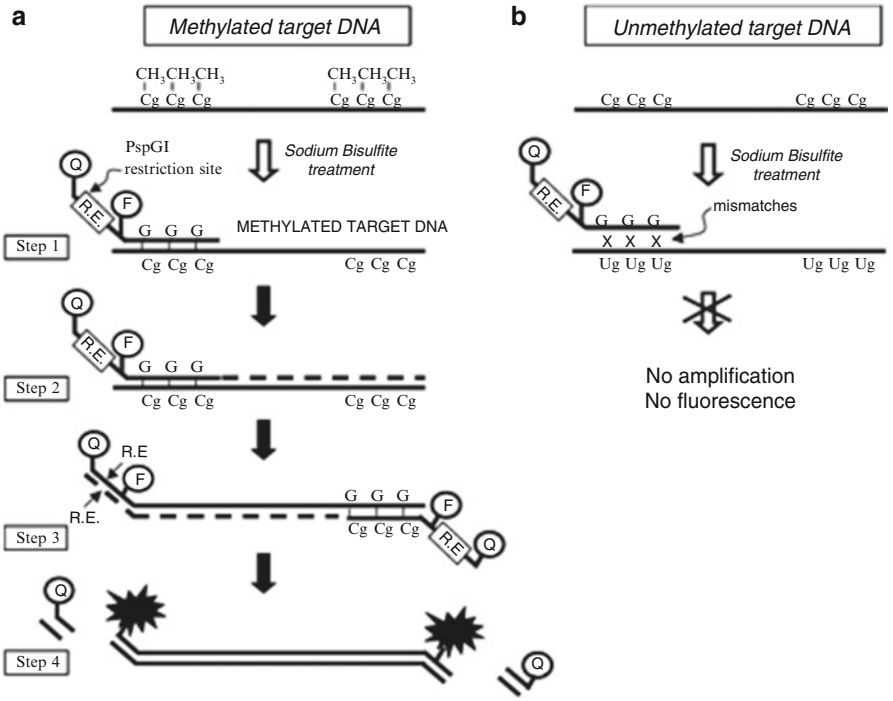


Fig. 23.5 Principle of signal generation via MS-FLAG. MS-FLAG primers are designed to anneal to regions harboring methylated CpG sites and contain an oligonucleotide 5′ tail carrying a quencher and a fluorophore separated by the recognition sequence of the PspGI endonuclease. The highly thermostable PspGI enzyme is present during the PCR reaction. (**a**) MS-FLAG forward primer anneals to methylated target DNA (*Step 1*) and is extended by the DNA polymerase (*Step 2*). The reverse primer synthesizes the opposite strand (*Step 3*) and generates a double-stranded recognition sequence for PspGI. Cleavage by PspGI (*Step 4*) leads to separation of the quencher and fluorophore, generating fluorescence. (**b**) If the interrogated target DNA is not methylated, the binding of the primers is inefficient and no PCR product or fluorescence is generated (Adapted from Bonanno et al. 2007)

FLAG is a novel signal generation technology for real-time PCR that includes the endonuclease PspGI in the reaction. Amplification is performed using primers that have a target-specific 3′ region and a 5′ oligonucleotide tail containing a fluorophore–quencher pair separated by nucleotides carrying the recognition sequence of an exceptionally thermostable restriction endonuclease, PspGI. PspGI has a half-life of 2 h at 95°C (Morgan et al. 1998) and remains active throughout the PCR reaction. At each amplification cycle, the complete double-stranded recognition site for this enzyme is generated at both ends (primer tails) of the amplified product. Cleavage of the primer tails by the endonuclease results in an increase in fluorescence due to loss of fluorescence resonance energy transfer quenching. Because only the introduced 5′ tail of the PCR amplicons is digested, amplicons retain their primer-binding regions, which serve as templates for primer binding and

amplification. MS-FLAG primers are specific for methylated sequences such that fluorescence signals are generated only if the interrogated sample contains methylated CpG DNA.

23.6.2 Strand Displacement Amplification

Strand Displacement Amplification (SDA) is an isothermal, in vitro nucleic acid amplification technique based upon the ability of restriction enzymes to nick a hemimodified restriction endonuclease recognition site by the enzyme and the ability of exonuclease-deficient Klenow (exo$^-$ Klenow) to extend the 3′ end at the nick and displace the downstream DNA strand.

The SDA reaction originally reported is typically conducted at a temperature between 35 and 45°C, and is capable of 10^8-fold amplification of a target sequence in about 2 h. Recently, SDA has been adapted for higher reaction temperatures (45–65°C-"thermophilic SDA" or "tSDA"). tSDA is capable of producing 10^9–10^{10}-fold amplification in about 15–30 min at 50–60°C. In addition to increased reaction speed, there is a significant reduction in nonspecific background amplification in tSDA as compared to conventional SDA.

23.6.3 Detection of k-ras Mutations in Cancer

Restriction endonuclease-mediated selective (REMS)-PCR allows detection of point mutations, deletions, and insertions. In this strategy, the reactions require concurrent activity of a REase and a DNA polymerase, both of which must be sufficiently thermostable to retain activity during thermocycling. The thermostable REase present in the REMS-PCR mixture inhibits amplification of sequences that contain its recognition site, thus producing selective amplification of amplicons that lack its recognition site. This strategy has been used to detect mutations in *k-ras* gene in which the concurrent activity of the REase BstNI and Taq polymerase allowed the amplification of mutant *k-ras* while inhibiting the formation of wild-type product. REMS-PCR is a sensitive, rapid, and robust assay for the detection of point mutations in a variety of clinical samples.

23.7 Future Prospects

A major challenge for future research is the structure prediction and homology modeling of REases from both thermophiles and mesophiles because REases lack the success of sequence similarity database search to understand their structure, function, and evolution.

To provide the best possible description of endonuclease catalytic mechanisms, each enzyme must be studied with a combination of crystallographic and biochemical techniques. Combining biochemical characterization and the structure determination using the rapidly developing computational methods will help understand how endonucleases and other enzymes work. The generation of mutants to determine the amino acid residues specifically important for thermostability of REases from thermophiles may pave a way towards increasing thermostability of REases from mesophilic organisms. Also, the structural analysis of REases from mesophiles and thermophiles and differences between them may help in understanding the important features required for thermostability. This information can be used for designing the REases with better and desired properties.

The combination of crystallographic analysis, structural modeling and large-scale mutational studies has made us understand the physical interactions within individual protein–DNA complexes. Even if structure-guided protein engineering still frequently falls short of providing fully active enzymes with new properties, progress made recently in this challenging task seems to be promising. The availability of a large number of designed nucleases with new specificities will undoubtedly prompt the development of multiple new applications of these enzymes and improve our abilities to analyze and manipulate the DNA both in vitro and in vivo.

23.8 Conclusions

Restriction enzymes have revolutionized molecular biology and genetic engineering due to their wide applications. Most of the REases in use are from mesophilic organisms. However, REases from thermophiles provide improved and additional properties over REases from mesophiles. A lot of REases from thermophiles have been isolated and commercialized. However, there is still a great niche for discovering REases with new specificities from thermophiles.

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Chapter 24
Microbial Chitinases: Natural Sources, Mutagenesis, and Directed Evolution to Obtain Thermophilic Counterparts

Pullabhotla Venkata Subba Rama Narsimha Sarma, Jogi Madhu Prakash, Subha Narayan Das, Manjeet Kaur, Pallinti Purushotham, and Appa Rao Podile

Abstract Chitin is the second most abundant polysaccharide, next to cellulose, occurring nature in the fungal cell walls, insect exoskeletons, while the shells of crustaceans contribute significantly to the availability of renewable biopolymer. Several enzymes are known to degrade different forms of chitin mostly produced by bacteria, fungi, and plants. Deacetylated polymer of chitosan and chitin is chemically hydrolyzed to generate oligomers and monomers for variety of applications that include pharmaceutical, environmental, agricultural, and cosmetic sectors. It would be possible to select from natural sources or modify the natural sources of chitinases to develop industrial processes that could replace the chemical processes for production of the chitooligomers, dimers, and monomers. Thermostable chitinases would give an added advantage for such industrial processes, and therefore there is a need to identify sources of such chitinases. In this chapter we have examined the availability of microbial sources of chitinases with a special attention to the thermostable chitinases. The approaches used in modifying chitinases and other related enzymes have been discussed to present the possible biotechnological approaches to generate novel thermostable enzymes. However, there was limited information available for chitinases indicating the need to focus research in that direction.

Keywords Chitin • Chitosan • Thermostable chitinases • Chitooligomers • Directed evolution • Protein engineering methods

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24.1 Introduction

Chitin, an unbranched homopolymer of β1, 4-linked *N*-acetyl-D-glucosamine (GlcNAc), is the second most abundant and renewable polysaccharide (after cellulose) on Earth. About 10^{11} tons of chitin is produced annually in the aquatic biosphere alone (Haki and Rakshit 2003). Fungal cell walls, insect exoskeletons, and shells of crustaceans are the main sources of chitin (Majeti and Kumar 2000). Chitinases are chitin-degrading enzymes and hydrolyze the β-(1,4) linkages of chitin. Chitooligomers produced by enzymatic hydrolysis of chitin have been of interest in the past few decades due to their broad range of medical, agricultural, and industrial applications. In agriculture, chitinases have been projected to play a useful role in control of selected fungal diseases of plants (Neeraja et al. 2010). The lytic activities of chitinases inhibit specific developmental changes in fungi either by hydrolysis or physical binding to the chitin and glucan components of cell wall. There is an urgent need to develop appropriate chitinase formulations to function broadly in a range of environments for a wide variety of applications including the biocontrol of phytopathogenic fungi, for preparation of chitooligosaccharides, and for degradation of chitinous waste. The interest in the utilization of chitin and its derivatives for a wide range of large-scale applications suggested the need to look for inexpensive, reliable sources of active and stable chitinase preparation(s). However, the current limitation on the use of industrial enzymes is due to the cost of isolation of enzymes from natural resources, short half-life at elevated temperature, and activity within narrow temperature and pH ranges. The activity and stability of enzymes at elevated temperatures largely determine the economic feasibility of applying chitinases in industrial processes. Research efforts were focused on microorganisms from extreme environments such as hot springs and geothermal areas in the search for thermostable chitinases. Apart from searching for thermostable chitinases from natural sources, improvements of thermostability of the available chitinases by protein engineering methods were also employed. In this chapter, the importance of thermostable chitinases from industrial perspective and various possible approaches to improve and obtain such enzymes are discussed. The experimental findings related to the improvement of thermostability of chitinases are scanty, and therefore we made an attempt to refer to similar strategies used for other hydrolytic enzymes for improvement of thermostability with a hope that such approaches would be adopted for chitinases in the future.

24.2 Physical and Chemical Properties of Chitin and Chitosan

Chitin is crystalline, intractable, highly hydrophobic, and insoluble in water and organic solvents. It is soluble in hexafluoroisopropanol, hexafluoroacetone, chloroalcohols in conjugation with aqueous solutions of mineral acids (Madhavan 1992), and dimethylacetamide containing 5% lithium chloride. Chitin is a nontoxic, nonallergenic, antimicrobial, and biodegradable polymer. It has a strong positive charge

which allows it to bind with negatively charged surfaces or materials including metals, skin, and macromolecules such as proteins. Many current methods to modify chitin and transform it to useful carbohydrate products employ harsh chemical treatments that incur problems of undesirable by-products that limit further usage of the products. The limitation in using the chemical treatments has lead to the development of efficient bioconversion process based on the exploitation of chitinases. Chitin in nature is available in two conformations: arrangement of individual polymeric chains as antiparallel fashion (α chitin) or as parallel fashion (β chitin). Its degree of crystallinity or its context with other polysaccharides or proteins leads to the evolution of numerous hydrolytic enzymes and proteins that need to specifically recognize it (Fig. 24.1).

Chitosan, a deacetylated derivative of chitin, is soluble in dilute acids like acetic acid and formic acid. Chitosan has numerous biotechnologically useful properties. In nature, chitosan is present in the cell walls of a limited group of fungi belonging to the order Mucorales, as well as during endophytic development of some biotrophic plant pathogenic fungi (El Gueddari et al. 2002). Chitosan and its derivatives have been employed to protect crop plants from phytopathogens, also used in tissue and paper coating due to its antimicrobial properties. The ability of chitosan to bind to heavy metals and proteins has been exploited in drinking water purification and in waste water treatment, respectively. Chitosan is also used extensively in the wound dressings to promote scar-free healing. Chitin and chitosans are partially or fully depolymerized to yield N-acetylglucosamine and glucosamine oligomers or monomers, respectively.

24.3 Microbial Chitinases are Diverse Across Several Genera

Chitinases can be classified into two major categories (Fig. 24.1b). Endo-chitinase (EC 3.2.1.14) cleave chitin randomly at internal sites, generating low molecular mass multimers of GlcNAc, such as chitotetraose, chitotriose, and diacetylchitobiose. Exo-chitinases that cleave from the termini could be divided into two subcategories: chitobiosidases (EC 3.2.1.29), which catalyze the progressive release of diacetylchitobiose from the nonreducing end of chitin microfibril, and β-(1,4) *N*-acetyl glucosaminidases (EC 3.2.1.30), which cleave the oligomeric products of endo-chitinases and chitobiosidases, generating monomers of GlcNAc (Sahai and Manocha 1993). An alternative pathway involves the deacetylation of chitin to chitosan, which is finally converted to glucosamine residues by the action of chitosanase (EC 3.2.1.132).

Based on amino acid sequence similarity, chitinolytic enzymes are grouped into families 18, 19, and 20 of glycosyl hydrolases (Henrissat and Bairoch 1993). Family 18 is diverse in evolutionary terms and contains chitinases from bacteria, fungi, viruses, animals, and some plant chitinases. Family 19 consists of plant chitinases (classes I, II, and IV) and some *Streptomyces* chitinases (Hart et al. 1995). The chitinases of the two families, that is, 18 and 19, do not share amino acid sequence

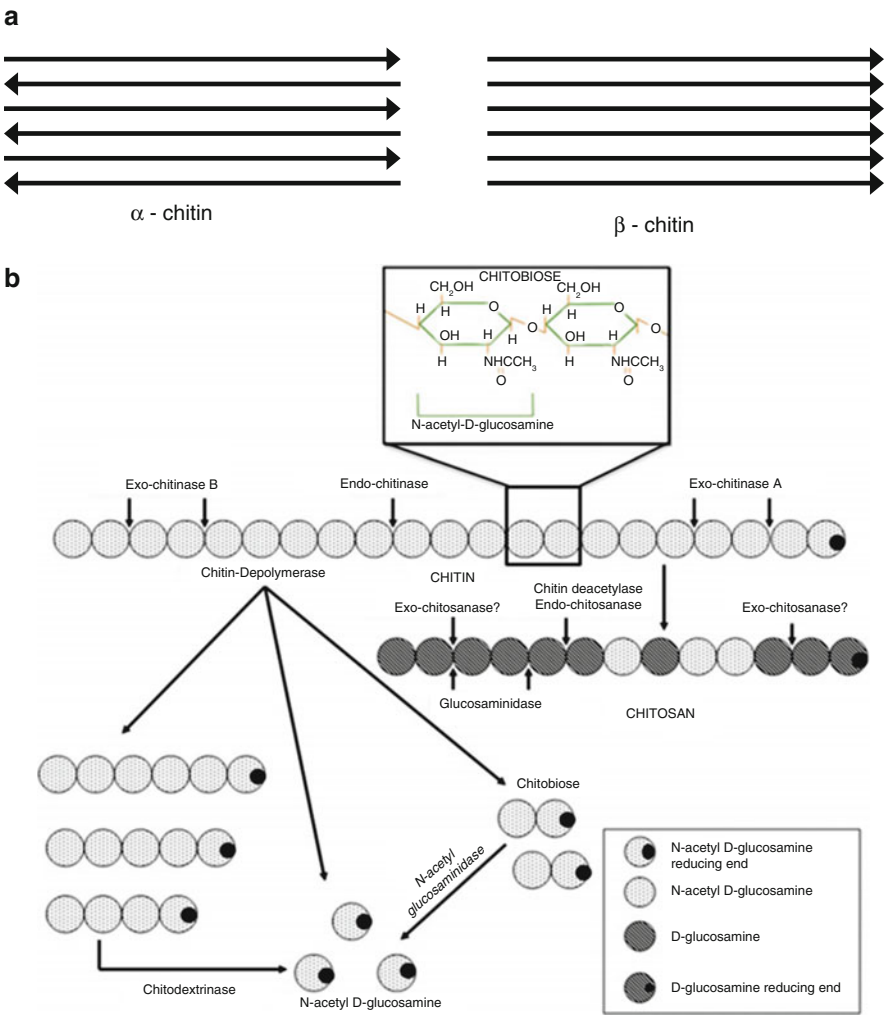

Fig. 24.1 Diagrammatic representation of different forms of chitin and the enzymology in chitin degradation. (**a**) Arrangements of chitin in alpha (antiparallel) and beta (parallel) forms and (**b**) structure of chitin and the multiple enzymes involved in chitin modification/degradation. Chitin polymer made up of *N*-acetyl-D-glucosamine residues can be cleaved by random-acting endo-chitinanse, exochitinase A from the reducing end, or exochitinase B from nonreducing end to produce oligomers/chitobiose. Chitin can be deacetylated by chitin deacetylase resulting in chitosan, which can be cleaved by endo-/exo-enzymes. Chitin oligomers and chitobiose can be further hydrolyzed by chitodextrinase and N-acetylglucosaminidase, respectively, to generate *N*-acetyl-D-glucosamine (part 'b' adopted from Neeraja et al. 2010)

similarity. They have completely different 3-D structures and molecular mechanisms and are therefore likely to have evolved from different ancestors (Suzuki et al. 1999). Family 20 includes the β-N-acetyl hexosaminidases from bacteria, *streptomycetes*, and humans.

Bacterial chitinases are clearly separated into three major subfamilies, A, B, and C, based on the amino acid sequence of individual catalytic domains (Watanabe et al. 1993). Subfamily A chitinases have a third domain corresponding to the insertion of an $\alpha+\beta$ fold region between the seventh and $(\alpha/\beta)_8$ barrel. On the other hand, none of the chitinases in subfamilies B and C have this insertion. Several chitinolytic bacteria like *Serratia marcescens* (Suzuki et al. 1999), *Bacillus circulans* WL-12 (Alam et al. 1995), and *Streptomyces coelicolor* A3 (2) (Saito et al. 1999) that possess chitinases belonging to different subfamilies are known.

24.3.1 Natural Sources and Production of Thermostable Chitinases

Thermostable enzymes function at higher temperatures often beyond 60°C. Thermostable enzymes are utilized in both industry and biomedical research in assays where critical steps of the assay are performed at considerably elevated temperatures. Enzymes from thermophilic microorganisms found in hot springs and volcanic origin such as eubacteria and archaebacteria are the natural sources which are being continuously exploited for several decades. An overview of the characteristics of some thermophilic chitinases is provided in Table 24.1. Thermostable chitinases have been isolated from hyperthermophilic archaeon, such as *Thermococcus chitonophagus* (Andronopoulou and Vorgias 2003), *T. kodakaraensis* (Tanaka et al. 2003), and *Pyrococcus furiosus* (Gao et al. 2003), and thermophilic bacteria, such as *Clostridium thermocellum* (Zverlov et al. 2002), *Streptomyces thermoviolaceus* (Tsujibo et al. 2003) and *Bacillus* sp. (Takayanagi et al. 1991; Bhushan 2000), *Pseudomonas aeruginosa* K-187 and *Bacillus* strain MH-1 (Sakai et al. 1998), *Pantoea dispersa* (Gohel and Naseby 2007), *Aeromonas* sp. DYU 007 (Lien et al. 2007), *Microbispora* sp. V2 (Nawani and Kapadnis 2005), *Streptomyces thermoviolaceus* OPC-520 (Hiroshi et al. 1993), *Streptomyces* RC1071 (Gomes et al. 2001), and *Bacillus stearothermophilus* CH-4 (Sakai et al. 1994), but there are only a few reports on chitinases from thermophilic fungi that are *Thermoascus aurantiacus* var. *levisporus*, *Chaetomium thermophilum* (Li et al. 2010), *T. lanuginosus* (Guo et al. 2005; Guo and Li 2006), and *Talaromyces emersonii* (McCormack et al. 1991). Thermostable chitinase from *Bacillus licheniformis* was reported of multiple kinds, I (98 kDa), II (76 kDa), III (66 kDa), and IV (59 kDa), with optimum temperatures of 70–80°C (Takayanagi et al. 1991; Tantimavanich et al. 1998). The thermophilic *Bacillus licheniformis* strain JS isolated from a bed of mushrooms, *Pleurotus sajor-caju*, produces a novel, single-component, 22-kDa thermostable chitobiosidase with an optimum temperature at 55°C (Waghmare and Ghosh 2010). The thermostability of this enzyme is important in the bioconversion of chitinous waste and for the production of chitooligosaccharides.

One fascinating aspect of purification of the heat stable enzymes is that while other heat-labile proteins denature at elevated temperature, the thermostable enzyme retains its active conformation during the enzyme purification (Yuli et al. 2004). The number of genes from thermophiles that have been cloned and expressed in

Table 24.1 An overview of the thermostable chitinases of microbial origin

Organism	Source	Gene/protein	Substrate	pH	Temperature (°C)	Reference
Thermostable chitinases of fungal origin						
T. aurantiacus var. levisporus	Isolated from dung	Tachit1	Colloidal chitin	8.0	50	Li et al. (2010)
C. thermophilum	Isolated from dung	Chhit1	Colloidal chitin	5.5	60	Li et al. (2010)
Thermomyces lanuginosus	NK	Chit	Colloidal chitin	5.5	60	Guo and Li (2006)
	NK	Chit	Colloidal chitin	4.5	55	Guo et al. (2005)
Talaromyces emersonii	NK	Chitinase	Colloidal chitin	5.0–5.5	65	McCormack et al. (1991)
Thermostable chitinases of bacterial origin						
Oerskovia xanthineolytica NCIM 2839	National Collection of Industrial Microorganisms (NCIM), Pune, India	Chi C1 ChiC2	Collodial chitin, pNP-GlcNAc	7.5 and 8.0	50 and 55	Shailesh et al. (2010)
Bacillus cereus I.21	Chilli Rhizosphere	Chitinase	Colloidal chitin	7.0	50–55	Mubarik et al. (2010)
Paenibacillus sp. *D1*	Effluent treatment plant of seafood processing industries	Chitinase	Colloidal chitin	5.0	50	Singh and Chhatpar (2010)
Pseudomonas aeruginosa K-187	NK	Chitinase	Colloidal chitin	8.0	50	Wang et al. (2010)
Bacillus licheniformis SK-1	NK	Chi72	Colloidal chitin	6 and 8	55	Kudan and Pichyangkura (2009)
Bacillus licheniformis A2 and A35	Red Palm Weavils' gut	Chitinase	Colloidal chitin	7.0	50	Khiyami and Masmal (2008)
Pantoea dispersa	Sea dumps containing crustacean wastes	Chi I Chi II Chi III	Acid-swollen chitin	8.0	30–60	Gohel and Naseby (2007)

Aeromonas sp. *DYU-Too7*	Beach sand	Chitinase	Colloidal chitin	5.0	70	Lien et al. (2007)
Rhodothermus marinus	Prokaria strain collection	ChiA	73% DDA chitosan	4.5–5	70	Hobel et al. (2005)
Bacillus licheniformis Mb-2	Tompaso geothermal springs	Chi-67	Colloidal chitin, glycol chitin, chitosan, and glycol chitosan	6.0	70	Toharisman et al. (2005)
Ralstonia sp. *A-471*	Composting system of chitin-containing waste	Chitinase	Chitosan 70% DDA	5.0	70	Sutrisno et al. (2004)
Bacillus sp. *13.26*	Hot spring	Chitinase	Colloidal chitin	7.0–8.0	60	Yuli et al. (2004)
Thermococcus chitonophagus	Hydrothermal vent	Chi70	Colloidal chitin	7.0	70	Andronopoulou and Vorgias (2003)
Pyrococcus furiosus	Hydrothermal vent	PF-ChiA PF-ChiB	Colloidal chitin	6.5	95	Gao et al. (2003)
Thermococcus kodakaraensis KOD1	Volcanic steam vent	Tko-ChiA	Colloidal chitin	5.0	85	Tanaka et al. (2003)
Bacillus thuringiensis	NK	ChiA74	Colloidal chitin		57.2	Corona et al. (2003)
Microbispora sp. *V2*	Hot springs	Chitinase	pNP-chitibiose	3–10	60	Nawani and Kapadnis (2005)
Clostridium thermocellum	Hot springs	Chi18A	pNP-chitotriose	4.5–6.5	60	Zverlov et al. (2002)
Streptomyces RC1071	Acidic orthic ferralsol soil	Chitinase	Glycol chitin	2.0–9.0	30–70	Gomes et al. (2001)
Bacillus sp. *BG-11*	Soil	Chitinase	Colloidal chitin	6.0–9.0	50	Bhushan (2000)
Pyrococcus kodakaraensis KOD1	Hydrothermal vents	Chi A	Colloidal chitin	5.0	85	Tanaka et al. (1999)
Bacillus MH-1	Compost of fermenting of citrus peels	Chi L Chi M Chi S	Chitohexaose	6.5 5.5 5.5	75 65 75	Sakai et al. (1998)

(continued)

Table 24.1 (continued)

Organism	Source	Gene/protein	Substrate	pH	Temperature (°C)	Reference
Cellulomonas flavigena NTOU 1	NK	Chitinase	Colloidal chitin	10.0	50	Chen et al. (1997)
Bacillus stearothermophilus CH-4	Compost	Chitinase	Chitobiose to chitopentose	6.5	75	Sakai et al. (1994)
Streptomyces thermoviolaceus OPC-520	NK	Chi40	Colloidal chitin	8.0–10. 0	70–80	Hiroshi et al. (1993)
B. licheniformis X-7u	NK	Chitinase	Colloidal chitin		70–80	Takayanagi et al. (1991)

NK not known

mesophiles has increased over the past couple of decades for industrial applications. The majority of proteins produced in mesophilic hosts are able to maintain their thermostability. Such proteins are correctly folded at low temperature, not hydrolyzed by host proteases and can be purified by using thermal denaturation of the mesophilic host proteins. The degree of purity of the enzymes obtained through this process was adequate for most of the industrial applications.

24.3.2 Thermostable Chitinases from Bacteria

Due to their ecological role and growing interests in biotechnology, a large number of bacterial chitinases have been isolated, and their respective genes have been cloned and characterized (Neeraja et al. 2010). Chitinases with greater stability under harsh conditions are desirable for industrial processes, which can be achievable by searching for thermostable enzymes in the extreme environments. Collection of diverse types of thermophilic chitinolytic strains would help in isolating highly desirable thermostable chitinases having several biotechnological applications. Thermostable chitinases from bacterial sources have lesser resistance to high temperatures in comparison to chitinases isolated from hyperthermophilic archaea. *Thermococcus chitonophagus* was the first chitinase-producing hyperthermophilic archaeon isolated from a deep sea hydrothermal vent environment (Huber et al. 1995). Subsequently, two other hyperthermophilic archaea, *Thermococcus kodakaraensis* KOD1 (Tanaka et al. 1999) and *Pyrococcus furiosus* (Gao et al. 2003), were also shown to grow on chitin. A bacterial chitinase isolated from *Rhodothermus marinus* retained 100% of activity after 16 h incubation at 70°C (Hobel et al. 2005).

T. kodakaraensis KOD1 is the first archaeon whose chitinase was structurally and biochemically characterized in great detail. It produces an extracellular chitinase, Tk-ChiA, of molecular mass of 134 kDa with optimal temperature and pH of 85°C and 5.0, respectively. Structural studies of Tk-ChiA showed unique features among known bacterial and eukaryal chitinases. It has two catalytic domains, one at the N-terminal region and the other at C-terminal region, and three chitin-binding domains (CBDs). Both the catalytic domains of Tk-ChiA are classified into family 18 of glycosyl hydrolase. Interestingly, both the catalytic domains are independently functional as chitinase, and these two domains act in a cooperative manner in the degradation of chitin as evidenced from the analysis of degradation products from colloidal chitin by HPLC. The N-terminal catalytic domain liberated diacetylchitobiose, whereas the C-terminal domain led to N-acetyl chitooligosaccharides of various chain lengths suggesting the N-terminal and C-terminal catalytic domains behave as exo- and endochitinase, respectively.

Cooperation or synergism between multiple chitinases secreted by bacteria is a well-known phenomenon. Synergistic effect among chitinases of *Serratia marcescens* has been observed as the rate of degradation of colloidal chitin got increased by combining ChiA and ChiB (Brurberg et al. 1996). Mining the genome of *Pyrococcus furiosus*, a hyperthermophile with an optimum growth temperature of 98–100°C,

resulted in identification and characterization of two family 18 chitinase genes *chiA* and *chiB*. The pH optimum of both the enzymes was 6.0 with a broad range of temperature optimum between 90 and 95°C (Gao et al. 2003). Between these two chitinases, ChiB is extremely thermostable with a melting temperature of 114°C.

Thermostable chitinases, with mesophilic origin, from *Bacillus* sp., showed stable activity ranging from 45 to 70°C. Yuli et al. (2004) characterized a chitinase from *Bacillus* sp. 13.26 with an optimum temperature 60°C at a pH range 7–8 and active after incubation for 5 h at 70°C. Similarly, chitinases from *Bacillus* sp. BG-11, *Bacillus* sp. DAU101, *Bacillus* strain MH-1, and *Bacillus licheniformis* SK-1 showed stable half-life (Kudan and Pichyangkura 2009; Sakai et al. 1998; Bhushan and Hoondal 1998). But a chitinase from *Bacillus* sp. strain CK4 showed optimum activity at 55°C and gradually reduced to 85% after 30 min and to 66% after 60 min, when treated at 80°C, and activity was completely lost after 60 min at 90°C (Yoon et al. 2000).

24.3.3 Thermostable Chitinases from Fungi

Thermophilic fungi are potential sources of enzymes of scientific and commercial interest particularly chitinases. As such, the chitinases play important biological and physiological roles in growth and development of fungi, being involved in autolytic, nutritional, morphogenetic, and parasitic roles (Adams 2004; Li 2006). For example, an endochitinase from *Trichoderma harzianum* conferred a high level of resistance against phytopathogenic fungi when expressed in tobacco, apple, and potato (Lorito et al. 1998; Bolar et al. 2000). Overexpression of the *Bbchit1* gene from the entomopathogenic fungus *Beauveria bassiana* enhanced the virulence of this fungus against aphids (Fang et al. 2005). More details on fungal chitinases are available in a review by Li (2006), but very few reports are available on thermostable chitinases. A chitinase of *Talaromyces emersonii* had maximum activity at 65°C and a half-life of 20 min at 70°C (McCormack et al. 1991). A chitinase of *T. lanuginosus* exhibits optimum catalytic activity at 55°C. The half-life of the enzyme at 65°C was 25 min (Guo et al. 2005). Li et al. (2010) cloned and expressed two novel thermostable chitinase genes (*TaCHIT1* and *CtCHIT1*) in *Pichia pastoris* from two thermophilic fungi, *T. aurantiacus var. levisporus* and *C. thermophilum*. The optimum temperature for activity was 50°C for *TaCHIT1* and 60°C for *CtCHIT1*, and upon incubation for 60 min, *TaCHIT1* retained 95.3% and *CtCHIT1* retained 96.7% of activity. Based on further studies on the half-life and stability at varied temperatures, it was concluded that *CtCHIT1* is more thermostable than *TaCHIT1*.

24.4 Approaches to Engineer Proteins for Thermal Stability

Enzyme thermostability is an intrinsic property determined by the primary structure of the protein and is of great importance to develop designed biocatalysts. External environmental factors including cations, substrates, coenzymes, modulators, polyols,

and proteins often increase enzyme thermostability (Ward and Moo-Youg 1988). While enzymes are capable of catalyzing reactions with exquisite specificity and selectivity, they are often limited by insufficient stability. Improvements in enzyme activity through protein engineering often come at the cost of reduced stability. This is likely a result of both natural drift and a trade-off that often exists between activity and stability for many single residue substitutions as exemplified by thermophilic organisms and demonstrated by laboratory evolution.

It is possible to improve enzyme stability without sacrificing activity. There are three major and principally different approaches followed to obtain enzyme variants with improved stability: (1) isolating enzyme variants from organisms living in appropriate extreme environments, (2) rationale-based mutagenesis (classical protein engineering), and (3) directed evolution. While directed evolution is based on generation of diversity followed by selection/screening, classical protein engineering utilizes growing knowledge of enzyme structure and function to make one or more amino acid changes that are predicted to elicit the desired improvements to enzyme function and further on the molecular basis of stability to rationally predict stabilizing mutations. But the most efficient stabilization strategies are often based on the simultaneous use of all these three methods.

24.4.1 Classical Protein Engineering Methods for Thermostability

Thermostability is often a primary goal to improve the properties of an industrial enzyme, since high temperatures in industrial processes provide benefits of working with increased substrate solubility, decreased viscosity of the medium, and a lower risk of microbial contamination. Thermostable enzymes in general and chitinases in particular are of considerable biotechnological interest since their enhanced stability could greatly reduce enzyme replacement costs or permit processes to be carried out at high temperatures working with chitin that poses problems of solubility.

One way of attaining thermostability is introducing some important properties of the thermostable chitinases (or other proteins) isolated from hyperthermophiles into the target protein which has its own limitations like poor information about the genome of thermophiles. Mutagenesis could be followed for creating thermostable proteins. Two major ways of mutagenesis include random mutagenesis and site-directed mutagenesis. Random mutagenesis/PCR-based *mis*-incorporation of oligonucleotides needs an efficient screening procedure of the mutants and also it is difficult to predict the chances of attaining thermostability. Site-directed mutagenesis (SDM) is yet another option for rational engineering and design for thermostable chitinases. Table 24.2 describes the list of SDM and the targeted amino acids for the improvement of chitinases properties.

Rational engineering includes approaches like improvement of the hydrophobic core, stabilization of α-helix dipoles, introduction of disulfide bridges, and point mutations aimed at reducing the entropy of the unfolded state (Burg and Eijsink 2002). However, the use of these techniques is limited to the enzymes whose three

Table 24.2 Targeted mutagenesis of bacterial chitinase genes

Organism	Targeted residues	Location	Property altered	References
Bacillus circulans	W687A	Binding site	Binding face Low chitin binding	Hardt and Laine (2004)
	E688K/P689A	Binding site	Altered substrate specificity	
	H681A		Increased affinity	
	H681W		Misfolding of protein	
	E688Q	Binding site	Negative charge not required for binding	
	P689A		No change in binding	
	T682A, P693F		Binding affinity increased but binding capacity decreased	
	P680A			
	W122A	Surface of the catalytic domain	Reduced hydrolyzing activity to β-chitin	Watanabe et al. (2001)
	W134A		”	
Serratia marcescens	Double mutant G188A/A234P	Surface-exposed region of the enzyme	Ten-fold increase in half-life, more active than wild type	Gaseidnes et al. (2003)
	A234G		Reduced in stability	
	W253E		Increased in stability	
	T26P	”	Minor effect on the stability	
	G104A	”		
	G153A	”		
	G188A	,,		
	A189P	,,		
	A200P	,,		
	A234P	,,		
	W252P	,,		
	E253P	,,		
	S282P	,,		
	G301P	,,		
	A371P	,,		
	A382P	,,		
	A425P	,,		
	A463P	,,		

S. marcescens 2170	Y481A, W479A W252A, Y240A	Catalytic cleft	Decreased the binding activity, hydrolyzing activity toward β-chitin, no reaction to soluble substrates	Katouno et al. (2004)
S. marcescens	W69A, W33A	N-terminal domain	Reduced binding activity	Uchiyama et al. (2001)
	W245A	Catalytic domain	Reduced binding activity	
	F232A	Catalytic domain	No effect on binding, guide the chitin chain into the catalytic cleft	
Aeromonas caviae	E315D	Active site	Abolished the enzyme activity	Lin et al. (1999)
	E315Q	”	Impaired the catalytic activity	
	D313E	”	Retained 50% activity and 90% relative activity	
	D313N	”	Toward substrates 4-MU-(GlcNAc)2 and 4-MU-(GlcNAc)3, respectively	
	D391E	”	Retained 37.5% relative activity	
	D391N	”	Retained 20% relative activity	
B. circulans	E204Q	Catalytic domain	Abolished the enzyme activity, identified as the proton donor in the hydrolysis reaction	Watanabe et al. (1993)
Vibrio carchariae	W70A Y245W	Chitin-binding domain	Decrease in the binding activity ”	Suginta et al. (2007)
	S33W	Catalytic cleft	Increase in the binding activity	
	W231A	”	”	
	Trp231	Catalytic cleft	Chitin hydrolysis	
	Trp245	”	”	
	Trp275	Active-site residue	Binding selectivity of the enzyme to soluble substrate	Suginta et al. (2007)
	Trp397	”	”	

(continued)

Table 24.2 (continued)

Organism	Targeted residues	Location	Property altered	References
Bacillus thuringiensis WB7	F201L	Active-site residues	Loss of enzyme activity	Cai et al. (2009)
	G203D	,,		
	D207E	,,		
	D205N	,,		
	D207N	,,		
	W208R	,,		
	W208C	,,		
	E209D	,,	Narrow pH active range	
	F201Y	,,		
	D205E	,,	Broader active pH range	
	G203A	,,		
	E209Q	,,		

dimensional structures have been determined. More recently, highly powerful bioinformatic tools have become available to build models of high accuracy to overcome this limitation.

24.4.1.1 Introduction of Disulfide Bridges

Disulphide bonds can significantly stabilize the native protein structure by decreasing the configurational chain entropy of the unfolded polypeptide. Matsumura et al. (1989) constructed several mutants of T4 lysozyme having cross-linked residues between 3–97, 9–164, and 21–142. Reversible thermal denaturation studies showed that the ΔTm values resulting from the individual disulphide bonds are approximately additive (22.2°C) and were nearly equal to the ΔTm of the triple mutant (3–97/9–164/21–142) which was 23.4°C higher than the wild-type lysozyme.

24.4.1.2 Improving the Hydrophobic Core

Globular proteins often contain phenylalanine, tyrosine, and tryptophan with aromatic side chains that occupy a substantial volume of the hydrophobic core and engage in specific, energetically favorable interactions. Such interactions can maintain the peptides in characteristic compact conformations, even in aqueous solution, thereby stabilizing the protein structure. In the study with λ repressor protein, replacement of glutamine with tyrosine at position 33 created an increase in the melting temperature (ΔTm) of about 6°C higher than wild-type protein (Burley and Petsko 1985).

24.4.1.3 Stabilization of α-Helix Dipoles

This approach describes one way of thermostabilizing proteins by the introduction of charged amino acid residues at specific sites where interaction with α-helix dipoles is possible. Nicholson et al. (1988) carried out aspartic acid substitutions at helical N-terminals of T4 lysozyme and generated a double mutant (S38D/N144D) which showed 4°C increase over the wild-type melting temperature (ΔTm), proved through denaturation studies monitored with the change in dichroism at 223 nm.

24.4.1.4 Reducing the Entropy of Unfolded State

Use of entropic effects could be another way of increasing thermostability of chitinases with known three dimensional structures. For example, glycine lacks a β-carbon and has more backbone conformational flexibility than alanine, in turn having greater configurational entropy than alanine. Subsequently, additional free energy is required during the folding process to restrict the conformation of glycine than alanine. Residues such as valine, isoleucine, and threonine with branched β-carbons

restrict the backbone conformations more than unbranched residues. Similarly the pyrrolidine ring of proline restricts this residue to fewer conformations. Taking these points into consideration, Matthews et al. (1987) generated the mutant T4 lysozyme G77A which showed 50–60% increase in the thermodynamic stability at pH6.5 in comparison with the wild type. Igarashi et al. (1999) reported for the first time that thermostability of α-amylase is improved by proline substitution. To increase thermostability of novel semi-alkaline enzyme, α-amylase (LAMY) from alkaliphilic *Bacillus* sp. strain KSM-1378, site-directed mutagenesis was carried out wherein Arg124 in LAMY was replaced with proline. The wild-type and engineered LAMYs were very similar with respect to specific activity, kinetic values, pH-activity curve, and degree of inhibition by chelating reagents. Fluorescence measurements confirmed the increase of thermostability and structure stiffness of LAMYs by the proline substitution. By this mutation it is assumed that the loop region is stabilized involving amino acid residues from 122 to 134. Gaseidnes et al. (2003) generated 15 mutants of ChiB from *Serratia marcescens*, which showed activity matching with wild type. Interestingly, many of these mutants showed only marginal stability effects, but a double mutant G188A/A234P showed ten-fold increase in half-life at 57 and 4.2°C rise in apparent Δ*T*m. These results showed that entropic stabilization works well and giving scope for utilizing other approaches in for the improvement of chitinases.

24.4.2 Directed Evolution of Enzyme Thermostability

In the case of enzymes for which extensive information concerning structure and function is not available, directed evolution is a powerful tool for studying or engineering thermostability and catalytic activity of the enzymes. Advances in molecular biology and genetic engineering have led to rapid methods for improving protein and enzyme properties (Williams et al. 2004). Directed evolution mimics the natural selection process, where genes are evolved through recursive rounds of mutation, recombination, and selection (Farinas et al. 2001). DNA shuffling allows a much larger spectrum of diversity to be generated than by natural recombination or mutational mechanisms; two or more homologs from multiple species in different rations are used for recombination (Kurtzman et al. 2001).

The improvement of enzymes in terms of thermostability also was done by DNA shuffling coupled with efficient screening. Liao et al. (1986) observed a >200-fold increase in half-life at 60–65°C. Similarly for subtilisin E, a 50-fold increase in half-life at 65°C was obtained (Zhao et al. 1998). Song and Rhee (2000) reported 11°C increase in thermal stability without compromising catalytic activity of phospholipase A1 from *Serratia* sp. strain MK1. By using DNA shuffling, thermostability of maltogenic amylase from *Bacillus thermoalkalophilus* ET2 was improved. The generated two highly thermostable mutants, III-1 and III-2, showed 10°C higher optimal temperature (70°C) than that of wild type (Tang et al. 2006).

The use of directed evolution technology for the improvement of chitinases for thermostability, bioremediation, and efficient bioconversion of chitin waste is attractive for industrial applications. Fan et al. (2007) used directed evolution through

DNA shuffling and screening to enhance the catalytic ability of *Beauveria bassiana*, chitinase, and *Bbchit1*. A total of 1,50,000 variants after three rounds of error-prone PCR and DNA shuffling were generated and expressed in *E. coli* using PelB (signal peptide) for efficient secretion of chitinase. Only two mutants SHU-1 and SHU-2 were positive after screening for chitinase activity and also reported that amino acid alterations in two chitinase variants occurred outside of the two putative substrate binding sites and the catalytic region. It is emphasized that rational design for predicting the effect of a specific mutation on enzyme properties is intricate, thereby making directed evolution a better choice.

Improvement of chitinase by directed evolution by Songsiriritthigul et al. (2009) generated and screened a library of mutant chitinases of two highly similar chitinases (*chiA13* and *chiA8785*) from *Bacillus licheniformis*. Further screening of 517 colonies for activity on colloidal chitin containing culturing plates and activity towards the chitotriose analogue (pNP-chitobiose) identified only one mutant which demonstrated improved catalytic efficiency (k_{cat}/K_m) by 2.3-fold than that of wild types. Sequencing analysis revealed that the mutant contains two mutations in the catalytic domain.

Rationalizing the outcome of direct evolution experiments is often difficult. Selected mutants with beneficial properties often contain several mutations with no obvious structural and functional implications. Clearly, the selection for nonobvious but useful mutation is one of the powers of directed evolution.

24.4.3 Designing Thermostable Proteins by Ancestral Mutation Method

Watanabe et al. (2006) developed a new method for designing thermostable proteins using phylogenetic trees of enzymes. Their study, based on hyperthermophilic universal ancestor hypothesis, investigated a method for designing proteins with improved stability using 3-isopropylmalate dehydrogenase (IPMDH) from *Thermus thermophilus* as a model enzyme. Among the 12 mutant enzymes designed, each has an ancestral amino acid residue that was present in the common ancestor of bacteria and archaea. At least six of the ancestral mutants tested showed thermal stability higher than that of the original enzyme. The effect of ancestral residues on IPMDHs of several organisms and on the related enzyme isocitrate dehydrogenase was summarized and analyzed. The effect of an ancestral residue on thermostability did not depend on the degree of conservation of the residue at the site, suggesting that the stabilization of these mutant proteins is not related to sequence conservation but to the antiquity of the introduced residues. Their results also suggest that this method could be an efficient way of designing mutant enzymes with higher thermostability based only on the primary structure and a phylogenetic tree. Similarly, thermostability of *Bacillus circulans* β-amylase, a mesophilic enzyme was improved. Eighteen mutants containing ancestral residues were designed, expressed in *E. coli* and purified. Several of these mutants were more thermostable than that of the wild-type amylase. Notably, one mutant had both greater activity and thermostability. It is

necessary to conserve the residues surrounding an ancestral residue if thermostability is to be improved by the ancestral mutation method (Yamashiro et al. 2010). As more information is gained in understanding the evolution of chitinases (Henrissat 1991), it should be possible to use this approach to obtain thermostable chitinases.

24.5 Immobilization for Thermostability of Enzymes

Industrial applications of chitinases have been governed mainly by key factors such as cost of production, shelf-life stabilities, and improvement in enzyme properties by immobilization. Chitinase was immobilized on swollen chitosan beads adopting the method developed by Mitani et al. (1995). Seven percent (w/v) of chitosan flakes (87% degree of deacetylation) were dissolved in 7% (w/v) acetic acid solution containing chitinase (200 U ml^{-1}). The resulting viscous solution was dropped into an alkaline-coagulating solvent (water, methanol, NaOH 4:5:1 w/w) with the aid of a syringe and needle to prepare highly swollen chitosan beads of different sizes (1–3 mm). The beads were washed with deionized water. In a second method, chitinase was immobilized on calcium alginate beads. A 2.5% sodium alginate solution was made up in the aqueous solution containing 200 chitinase U ml^{-1}.

Enzyme properties such as pH and thermostability were studied by placing a measured amount of beads in the respective environment for different time periods, and then assaying the residual chitinase activity under standard conditions. The immobilization of chitinase on chitosan beads led to a 35% increase in activity compared with that of free enzyme. This may be due to an improved stabilization of the active center via a non-covalent link between the amino group in the chitosan backbone and the enzyme (Dumitriu and Chornet 1991). Moreover, chitosan beads may provide a favorable and more protective microenvironment for the enzymatic activity. The temperature and pH optima of the immobilized chitinase remained the same as those of free enzyme, that is, 45–55°C and 7.5–9.0, respectively (Bhushan and Hoondal 1998), whereas pH stability and thermostability were improved significantly. The immobilized chitinase was stable from pH5.0 to 10.0, retaining more than 90% of its activity, whereas free enzyme was stable between pH6.0 and 9.0. The half-life of immobilized chitinase at 70, 80, and 90°C was 90, 70, and 60 min, respectively, which was higher than the half-life of free enzyme, that is, 30, 20, and 5 min at 70, 80, and 90°C, respectively.

24.6 Conclusions

Chemical processes have been developed to produce oligomers, dimers, and monomers of chitin and chitosan either through organic synthesis or by hydrolysis of chitin/chitosan polymers (Jeon et al. 2000). The environmental concerns on the use of harsh chemical processes and release of wastes prompt the use of enzymatic approaches in

generating the chitin oligomers for various large-scale applications. Use of cloned sources of microbial chitinases is a possible alternative to produce chitin oligomers from defined substrates. The concerns on the availability of the appropriate source and stability of such enzymes are daunting for an economically feasible process. Biotechnology offers several options, discussed above, to artificially create chitinases that can tolerate high temperature and survive for long periods in large-scale processes. Thermostable chitinases from natural or genetically modified sources are scanty, and therefore it would be of interest to focus on searching/developing such resources to generate chitin oligomers in environmentally safe biological processes.

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Chapter 25
Phytases and Phosphatases of Thermophilic Microbes: Production, Characteristics and Multifarious Biotechnological Applications

Bijender Singh and Tulasi Satyanarayana

Abstract Thermophilic microbes have been considered as good sources of thermostable enzymes with high catalytic activity, greater resistance to denaturing agents and lower incidence of contamination. Thermostable enzymes are receiving considerable attention because of their usefulness in high-temperature catalysis of various enzymatic industrial processes. Phytases (*myo*-inositol hexakisphosphate phosphohydrolase) are the phosphatases, which catalyse the hydrolysis of phytic acid to inorganic phosphate and *myo*-inositol phosphate derivatives, while phosphatases are able to hydrolyse a wide variety of esters and anhydride phosphoric acids, releasing phosphate, and are also able to perform transphosphorylation reactions. The phosphorus thus liberated is used in metabolic pathways. The phosphatases have been considered to be one of the most versatile groups of hydrolases because of their adaptability under different environmental extremes such as high-temperature regimes and regulate phosphate metabolism for maintaining phosphorus economy of the cell for fulfilling its growth as well as bioenergetic requirements. The reduction of phytic acid content in the foods and feeds by enzymatic hydrolysis using phytase is desirable, because the physical and chemical methods of phytate removal negatively affect their nutritional value. These enzymes, therefore, have potential applications in food and feed industries for mitigating their phytic acid content to liberate available inorganic phosphate and improve digestibility as a result of elimination of antinutrient characteristics. In this review, the attention is focused on the production, characteristics and potential biotechnological aspects of phytases and phosphatases from thermophilic microbes.

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25.1 Introduction

Phosphorus is an important constituent of cell and its components. It is a major essential macronutrient for biological growth and development. The biggest reserves of phosphorus are rocks and other deposits. Phytic acid is the principal storage form of phosphorus present (1–5% by weight) in cereals, legumes, oil seeds and nuts (Wodzinski and Ullah 1996; Vohra and Satyanarayana 2003; Vats and Banerjee 2004; Singh et al. 2006, 2011; Greiner and Konietzny 2006; Singh and Satyanarayana 2011a). The phytic acid present in the plant-derived foods acts as an anti-nutritional factor, since it causes mineral deficiency due to efficient chelation of metal ions, forms complexes with proteins which are difficult to digest and also inhibits some enzymes (Maga 1982; Harland and Morris 1995; Tyagi et al. 1998) (Fig. 25.1). Phytate phosphorus is largely unavailable to monogastric animals due to the lack of adequate levels of phytate-degrading enzymes in their gastrointestinal tract (Wodzinski and Ullah 1996; Vohra and Satyanarayana 2003; Vats and Banerjee 2004; Greiner and Konietzny 2006; Singh and Satyanarayana 2011a; Singh et al. 2011). Since phytic acid cannot be digested, feeds for monogastric animals are commonly supplemented with inorganic phosphate in order to meet their phosphorus requirement. The supplemented inorganic phosphorus and the excreted phytate phosphorus impose global ecological problems (eutrophication) when it enters into rivers, lakes and other water bodies, resulting in algal blooms, hypoxia and death of marine animals (Naqvi et al. 2000). Because of these problems, there is a considerable

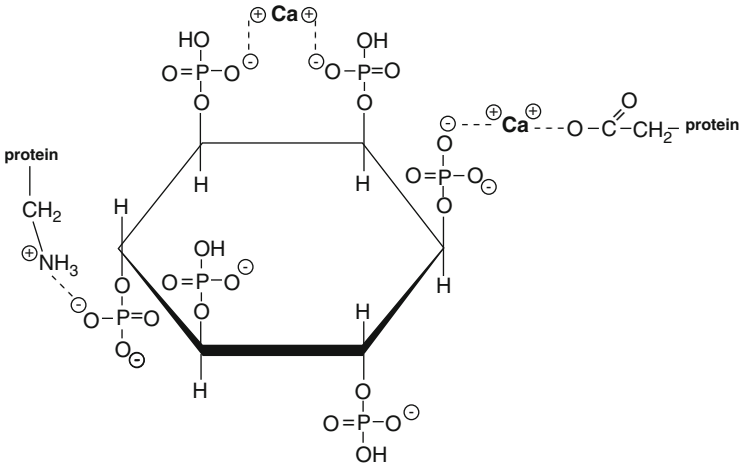

Fig. 25.1 Interaction of phytic acid with metals and proteins

interest in phytate-degrading enzymes and other phosphatases which can hydrolyse organic source of phosphorus, thereby eliminating the anti-nutritional properties of phytic acid and making the phosphorus available to the organisms. The supplementation of phytase and phosphatases in food will improve the phosphorus bioavailability and reduce phosphorus excretion in the areas of intensive livestock production (Singh et al. 2006, 2011; Singh and Satyanarayana 2011a). Moreover, phytases have become potential candidates for the production of special isomers of various lower phosphate esters of *myo*-inositol, some of which are considered to be pharmacoactive and important intracellular secondary messengers (Greiner and Konietzny 1996). Thus, for both environmental as well as economic reasons, phytase- and phosphatase-producing microbes are receiving immense attention.

The research on thermophilic microbes has been encouraged by secreting microorganism capable of secreting novel enzymes with unique properties, which are desirable characteristics of enzymes for commercial applications. There are many advantages in the use of thermostable enzymes in food-processing industries such as high reaction rates at elevated temperatures, low contamination risks and stability. Thus, thermostable enzymes from thermophiles appear to be suitable for use in biotechnological/industrial processes (Kelly et al. 1986; Reilly 1999; Maheshwari et al. 2000). The ability of these microorganisms to secrete large quantities of proteins, and their simple growth requirements, has made them a better option in industrial applications. The potential uses of thermophilic microorganisms have been recognised in the recent past with regard to basic academic research and industrial applications (Satyanarayana et al. 1992; Johri et al. 1999; Maheshwari et al. 2000; Satyanarayana and Singh 2004; Singh and Satyanarayana 2011a; Singh et al. 2011). In this chapter, an attempt has been made to describe the production, characteristics and potential biotechnological applications of phytases and phosphatases of thermophilic microbes.

25.2 Phosphatases and Phytases

The term phosphatase is commonly used for the enzymes which catalyse the hydrolysis of a variety of phosphomonoesters and thus degrade complex organic phosphorus compounds releasing phosphate. Phosphatases are widely distributed in nature and generally they show broad substrate specificity and thus, are able to hydrolyse a wide variety of esters and anhydride phosphoric acids, releasing phosphate. They are able to perform transphosphorylation reactions from phosphoesters of phenol, *p*-nitrophenylphosphate and others to various receptors such as glucose and pyridoxine (Guimaraes et al. 2004). They have been involved in translocation and regulatory processes involved in sugar metabolism for enhancing growth (Famurewa and Olutiola 1994). Acid phosphatases are also involved in signal transduction and cell regulation (Guimaraes et al. 2004). Phosphatases have been conventionally classified into alkaline phosphatases (EC 3.1.3.1) and acid phosphatases (EC 3.1.3.2) on the basis of their pH optima. The classification has been revised that categorises

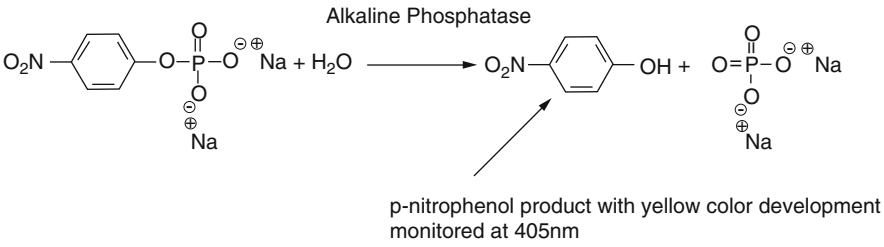

Fig. 25.2 Reaction catalysed by a phosphatase

phosphatases into five distinct families such as alkaline phosphatases, purple acid phosphatases, low-molecular-mass acid phosphatases, high-molecular-mass acid phosphatases and protein phosphatases. Acid phosphatase plays an important role is the hydrolysis of external phosphate esters, which do not penetrate the plasma membrane. Low- and high-molecular-mass acid phosphatases generally hydrolyse phosphomonoesters according to a two-step mechanism in which the enzyme-bound substrate produces a covalent phospho-enzyme intermediate and an alcohol; hydrolysis of the intermediate results in the formation of inorganic phosphate. The reaction catalysed by a phosphatase on *p*-nitrophenylphosphate is given in Fig. 25.2.

Phytases (*myo*-inositol hexaphosphate phosphohydrolase EC 3.1.3.8) are a class of acid phosphatases which hydrolyse phytic acid to *myo*-inositol and inorganic phosphates through a series of *myo*-inositol phosphate intermediates (Mitchell et al. 1997). There are two phytases as classified by Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB): **3-phytase** (EC 3.1.3.8), which first hydrolyses the ester bond at the 3 position of *myo*-inositol hexakisphosphate and is typical for microorganisms, and **6-phytase** (EC 3.1.3.26), which first hydrolyses the ester bond at the 6 position of *myo*-inositol hexakisphosphate. It is typical for plants. Recently, 6-phytase has been reported in some fungi (Lassen et al. 2001). The occurrence of 4- and 5-phytase has also been recently reported (Greiner and Konietzny 2006). Phytases have been classified by Mullaney and Ullah (2003) into HAP (histidine acid phosphatase), BPP (β-propeller phytase) and PAP (purple acid phosphatase) depending upon their catalytic properties. The phytase and acid phosphatase enzymes can work in coordination, where phytase can split the molecule of phytate in a selective manner, while acid phosphatase can attack the inositol phosphate intermediates independently, and as a result accelerate the total dephosphorylation process (Zyta 1993).

25.3 Sources and Production of Phosphatases

There are many reports of alkaline and acidic phosphatases of thermophilic microorganisms. Thermophilic bacteria are studied in more detail as compared to moulds for phosphatase production (Table 25.1). The production of phosphatase by a fungus

Table 25.1 Production of phytase and phosphatase by thermophilic microorganisms

Microbial source	Fermentation	Reference
Phytase		
Aspergillus fumigatus SRRC 322	SmF	Mullaney et al. (2000)
A. fumigatus WY-2	SmF	Wang et al. (2007)
Thermoascus aurantiacus	SmF	Nampoothiri et al. (2004)
T. aurantiacus	SSF	Hassouni et al. (2006)
Myceliophthora thermophila	SSF	Hassouni et al. (2006)
M. thermophila	SmF	Mitchell et al. (1997)
Rhizomucor pusillus	SSF	Chadha et al. (2004)
Sporotrichum thermophile	SmF, SSF	Singh and Satyanarayana (2006a)
Thermomyces lanuginosus	SmF	Berka et al. (1998)
T. lanuginosus TL-7	SSF	Gulati et al. (2007a)
Talaromyces thermophilus	SmF	Pasamontes et al. (1997b)
Emericella nidulans	SmF	Pasamontes et al. (1997b)
Bacillus laevolacticus		Gulati et al. (2007b)
Phosphatase		
Humicola lutea 120-5	SmF	Aleksieva and Micheva–Vitevaa (2000)
Scytalidium thermophilum	SmF	Guimarães et al. (2001)
Thermoactinomyces vulgaris	SmF	Singh (2007)
T. vulgaris	SmF	Lalwani et al. (1997)
Thermus thermophilus	SmF	Pantazaki et al. (2008)
T. thermophilus	SmF	Castán et al. (2002)
T. yunnanensis sp. Nov.	SmF	Gong et al. (2005)
Thermus sp. Rt41A	SmF	Hartog and Daniel (1992)
Meiothermus ruber	SmF	Yurchenko et al. (2003)
Bacillus stearothermophilus	SmF	Mori et al. (1999)
B. licheniformis	SmF	Pandey and Banik (2011)

Humicola lutea 120-5 was investigated by Aleksieva and Micheva–Vitevaa (2000). The enzyme synthesis was controlled by the presence of inorganic phosphate in the medium as the reduction of phosphate concentration resulted in five-fold improvement in phosphate production. The highest acid phosphatase production was achieved in 72-h shake-flask culture using phosphate-free medium containing glucose and casein. An extracellular (conidial) and an intracellular (mycelial) alkaline phosphatase from the thermophilic fungus *Scytalidium thermophilum* were purified and characterised by Guimarães et al. (2001). Both acid and alkaline phosphatases were reported in *Thermoactinomyces vulgaris* which were optimally active at 65 and 70°C, respectively (Singh 2007). An alkaline phosphatase of the PSTS system was purified to homogeneity from the thermophilic bacterium *Thermus thermophilus* (Pantazaki et al. 2008). An alkaline phosphatase was cloned from *Meiothermus ruber* and its nucleotide sequence was determined (Yurchenko et al. 2003). The presence of a periplasmic space within the cell envelope of *Thermus thermophilus* was analysed due to the expression of phosphatase (Castán et al. 2002). An alkaline phosphatase from *B. stearothermophilus* was purified by a combination of chromatographic techniques (Mori et al. 1999). *Thermoactinomyces vulgaris* also

secretes a phosphatase (Lalwani et al. 1997). A thermostable alkaline phosphatase with high specific activity and thermal resistance was purified from a novel bacterium *Thermus yunnanensis* sp. Nov. (Gong et al. 2005). A thermostable phosphohydrolase from *Thermus* sp. Rt41A has been purified 400-fold to give a specific activity of 25 U mg^{-1} at 60°C in IM diethanolamine (Hartog and Daniel 1992). The partitioning of *B. licheniformis* alkaline phosphatase in different aqueous two-phase systems composed of different molecular weight of PEG with salts and polymers was studied in detail by Pandey and Banik (2011). The effects of various physicochemical factors (pH, temperature and production media) were evaluated for partitioning of alkaline phosphatase. PEG 4000 (9.0% (w/v)) and dextran T500 (9.6% (w/v)) system supported high alkaline phosphatase production by *B. licheniformis*. The two-phase system supported higher enzyme titres than the control.

25.4 Sources and Production of Phytases

Phytases have been derived from a host of sources including plants, animals and microorganisms (Vohra and Satyanarayana 2003; Vats and Banerjee 2004; Singh and Satyanarayana 2011a, b; Singh et al. 2011). Among the microbes, more emphasis has been given on mesophilic fungi and bacteria and less work has been done on thermophilic microorganisms (Mitchell et al. 1997; Berka et al. 1998; Pasamontes et al. 1997a, b; Chadha et al. 2004; Nampoothiri et al. 2004; Singh and Satyanarayana 2006a, b, 2008a, b, 2009, 2010, 2011a, b) (Table 25.2). Mostly thermophilic moulds are better studied in detail for phytase production as compared to thermophilic bacteria unlike phosphatases (Tables 25.1 and 25.2). Thermophilic moulds are best studied for phytases in submerged and solid-state fermentations (Table 25.2). Phytase from *Aspergillus fumigatus* was cloned and over-expressed (Pasamontes et al. 1997a). A *phy*A gene encoding an extracellular phytase from a thermophilic mould *T. lanuginosus* was cloned and over-expressed heterologously in *Fusarium venenatum* (Berka et al. 1998). *Chaetomium thermophilum* ATCC 58420, *Rhizomucor miehei* ATCC22064, *Thermomucor indicae-seudaticae* ATCC28404 and *Myceliophthora thermophila* ATCC48102 are also known to produce phytases (Mitchell et al. 1997). *Rhizomucor pusillus* secreted phytase optimally at 50°C and pH 5.5 in SSF using wheat bran (Chadha et al. 2004), while *Thermoascus aurantiacus* produced phytase in semi-synthetic medium using glucose, starch and wheat bran (Nampoothiri et al. 2004), and *Talaromyces thermophilus* produced phytase was cloned (Pasamontes et al. 1997b). Several thermophilic moulds (*R. pusillus, Scytalidium thermophilum, Melanocarpus albomyces, Chaetomium thermophile* and *Thermomyces lanuginosus*) have been isolated from composts and soils and screened for phytase production (Chadha et al. 2004). Among these, *R. pusillus* produced a high titre of phytase on wheat bran supplemented with basal medium containing asparagine and corn steep liquor as nitrogen source along with some micronutrients. Maximum phytase production was achieved after 48 h of incubation at pH 6.0 and 50°C. Further optimisation of phytase production using

Table 25.2 Optimised culture conditions for the production of phytase by various thermophiles

Microbial strain	pH_{opt}	T_{opt}	Fermentation conditions		Reference
			Carbon source	Nitrogen source	
A. fumigatus SRRC 322	5.0	37	Hylon starch	$NaNO_3$	Mullaney et al. (2000)
Thermoascus aurantiacus	5.5	45	Starch, glucose, wheat bran	Peptone	Nampoothiri et al. (2004)
T. aurantiacus	6.0	45	Sugarcane bagasse	$NaNO_3$	Hassouni et al. (2006)
Rhizomucor pusillus	8.0	50	Wheat bran	Asparagine, CSL	Chadha et al. (2004)
Myceliophthora thermophila	5.5	45	Glucose	$NaNO_3$	Mitchell et al. (1997)
M. thermophila Lomy 713	6.0	45	Sugarcane bagasse	$NaNO_3$	Hassouni et al. (2006)
Sporotrichum thermophile	5.0	45	Starch, glucose	Peptone	Singh and Satyanarayana (2008a)
S. thermophile	5.0	45	Sesame oil cake, glucose	$(NH_4)_2SO_4$	Singh and Satyanarayana (2006a)
Thermomyces lanuginosus	6.0	37	Maltodextrin	Urea, asparagine	Berka et al. (1998)
T. lanuginosus TL-7	5.5	45	Wheat bran	$NaNO_3$	Gulati et al. (2007a)
Bacillus laevolacticus	–	–			Gulati et al. (2007b)

Box-Behnken design resulted in improved phytase production. *Thermoascus aurantiacus* TUB F43 secreted phytase in the semi-synthetic medium containing glucose and starch as carbon and peptone as nitrogen source at 45°C 150 rpm and pH 5.5 after 72 h of fermentation (Nampoothiri et al. 2004). Among various thermophilic moulds isolated, four thermophilic fungal strains – two strains of *Sporotrichum thermophile* and two of *Humicola lanuginosa* – were selected and screened for phytase production on sesame oil cake and wheat bran in solid-state fermentation (Singh and Satyanarayana 2006a). Phytase secretion by *S. thermophile* was the highest in sesame oil cake followed by wheat bran and mustard oil cake at 45°C, at substrate to moisture ratio of 1:2.5 and a_w of 0.95 after 120 h.

The thermophilic mould *S. thermophile* also secretes phytase in submerged fermentation in synthetic medium containing starch, glucose, peptone and phytic acid along with micronutrients (Singh and Satyanarayana 2008a) and in a cost-effective cane molasses medium supplemented with ammonium sulphate as nitrogen source at 45°C and pH 5.0 after 5 days of fermentation (Singh and Satyanarayana 2006b). Optimisation of phytase production using statistical designs (Plackett-Burman and Response surface Methodology) resulted in two-fold improvement in phytase production.

Among ten different strains of *Thermomyces lanuginosus*, isolated from composting soils, the strain CM was found to produce maximum amount of phytase (Gulati et al. 2007a). The UV mutant of this strain (TL-7) showed 2.3-fold increase in phytase production as compared to the wild strain. Statistical optimisation resulted in improved phytase production by mutant TL-7. A novel phytase producing thermophilic strain of *Bacillus laevolacticus* insensitive to inorganic phosphate was isolated from the rhizosphere soil of leguminous plant *Medicago falcata* (Gulati et al. 2007b). The culture conditions for production of phytase by *B. laevolacticus* under shake-flask culture were optimised to attain high levels of phytase. *Aspergillus fumigatus* secreted a heat-stable phytase, which was highly thermostable (Pasamontes et al. 1997a). Pasamontes et al. (1997b) reported cloning of a phytase gene from *Talaromyces thermophilus*, which shares 61% sequence homology with *A. niger*.

25.5 Characteristics of Phytases and Phosphatases of Thermophiles

Both phytases and phosphatases from thermophile have been purified by the conventional methods using chromatographic techniques. A gene-encoding alkaline phosphatase (AP) from thermophilic *Geobacillus thermodenitrificans* T2 was cloned and sequenced (Zhang et al. 2008). The deduced protein comprises 424 amino acids and shares a low homology with other known AP, while it exhibits the conservation of the active site and structure element of *Escherichia coli* AP. This protein was over-expressed in *E. coli* and purified as a hexa-His-tagged fusion protein. The enzyme was optimally active at pH 9.0 and 65°C. The enzyme retained a higher activity at 45–60°C as compared to 80°C with a half-life of 8 min at 70°C. The K_m and V_{max} for pNPP were determined to be 31.5 μM and 430 μM/min. It was a metal-dependent enzyme preferring a combination of Mg^{2+} and Co^{2+} or Zn^{2+}. The enzyme was strongly inhibited by 10 mM EDTA and vanadate but highly resistant to urea and dithiothreitol.

An alkaline phosphatase gene from *Talaromyces thermophilus* XM was cloned and sequenced (Li et al. 2007). It is 1,506 bp long encoding a protein of 501 amino acid residues with a molecular mass of 54.7 kDa. This phosphatase was expressed in *E. coli* and its enzymatic properties were characterised after purification. The recombinant enzyme was optimally active at pH 12 and 75°C. The enzyme was highly thermostable stimulated by 0.1 mM Co^{2+}, Fe^{2+}, Mg^{2+} or Mn^{2+} and strongly inhibited by 2.0 mM Fe^{2+}. The K_m value for hydrolysis of *p*-nitrophenyl-phosphate was 0.034 mM.

The nonspecific acidic and alkaline phosphatases of *Thermoactinomyces vulgaris* were found to be optimally active at 65 and 70°C, respectively (Singh 2007). Mg^{2+} stimulated the enzyme activity and decreased the activation energy of both acid and alkaline phosphatases. Phosphate transport in bacteria occurs via a phosphate-specific transporter system (PSTS) that belongs to the ABC family of

transporters, a multisubunit system, containing an alkaline phosphatase (Pantazaki et al. 2008). An alkaline phosphorolytic enzyme of the PSTS system was purified to homogeneity in *T. thermophilus*. The enzyme had a molecular mass of 40 kDa with optimal activity at pH 12.3 and 70°C.

An alkaline phosphatase gene was cloned from the thermophilic bacterium *M. ruber*, and its nucleotide sequence was determined (Yurchenko et al. 2003). The protein was composed of 503 amino acid residues with a molecular mass of 54.2 kDa. The protein was expressed in *E. coli* and its enzymatic properties were characterised. Its activity simulated Mg^{2+} ions, while Zn^{2+} ions had an inhibitory effect. It was optimally active at pH 11.0 and 62°C. The enzyme was thermostable with a K_m value of 0.055 mM for 4-nitrophenylphosphate. The presence of a periplasmic space within the cell envelope of *T. thermophilus* was analysed in a mutant defective in the regulation of its S-layer (surface crystalline layer) (Castán et al. 2002). The gene encoding an alkaline phosphatase from *T. thermophilus* HB8 was over-expressed in the mutant. Most of the enzyme activity was found as a soluble component of intercellular fraction which indicated that *T. thermophilus* has a periplasmic space which is functionally similar to that of Proteobacteria.

An extracellular (conidial) and an intracellular (mycelial) alkaline phosphatases of *S. thermophilum* were purified by DEAE-cellulose and Concanavalin A-Sepharose chromatography (Guimarães et al. 2001). These enzymes showed allosteric behaviour either in the presence or absence of divalent cations. Both enzymes were present in dimeric form with a monomeric molecular mass of 63 (conidial) and 58.5 kDa (mycelial). *p*-nitrophenylphosphate was the preferred substrate for both the enzymes. The optimum pH for the conidial and mycelial alkaline phosphatases was 10.0 and 9.5, and both enzymes were glycoprotein with carbohydrate contents of 54 and 63%, respectively. The optimum temperature was 70–75°C for both enzymes. An alkaline phosphatase from *B. stearothermophilus* was purified by a combination of chromatographic techniques (Mori et al. 1999). The purified enzyme had specific activity of 4.43 U/mg of protein with a molecular mass of 32 kDa. Its apparent K_m for p-nitrophenylphosphate was 1.114 mM. The enzyme was optimally active at pH 9.0 and at 60–70°C. Its activity was stimulated by Mg^{2+}. Its partial *N*-terminal sequence TFSIVAFDPATGELGIAVQ did not show any homology with the existing phosphatases.

The phosphatases from an extreme thermophile *T. thermophilus* were separated by ion exchange, hydrophobic, pseudoaffinity and affinity chromatography (Pantazaki et al. 1998). Among all phosphatases, four had optimum pH between 5.0 and 11.5 using *p*-nitrophenylphosphate, while other two were optimally active in a pH range between 7.0 and 11.0, with 32P-casein as substrate. This hyperalkaline phosphatase was produced maximally during stationary phase and is co-purified with alkaline phosphatase with optimum pH of 10.2. The enzymes showed molecular masses of 65 and 58 kDa, respectively, with similar properties. These enzymes were inhibited by EDTA, pyrophosphate and molybdate. Among the metals tested, Hg^{2+} was the strongest inhibitor.

A thermostable alkaline phosphatase with high specific activity and thermal resistance was purified from a novel bacterium *T. yunnanensis* sp. Nov (Gong et al. 2005).

The enzyme was a homodimer with a monomeric mass of 52 kDa. The optimal pH and temperature for its activities are pH 8.0–10.0 and 70–80°C, respectively. The enzyme was metal dependent and Mg^{2+}, Zn^{2+} and Co^{2+} were the main activators. A thermostable phosphohydrolase from *Thermus* sp. Rt41A has been purified 400-fold to give a specific activity of 25 U mg^{-1} (Hartog and Daniel 1992). The enzyme has a molecular mass of 160 kDa and is trimeric. The enzyme exhibits broad substrate specificity with half-life of 5 min at 85°C. The enzyme activity is reduced to half by 20 mM Ca^{2+} or Mg^{2+}. The K_i for phosphate, EDTA disodium salt and arsenate were 1.2, 1.6 and 4 mM, respectively.

Phytases are widely present in a variety of microorganisms and plants and in some animal tissues. Most thermophilic phytases belong to acidic phytases having an optimal pH range of 4.5–5.5. Mostly phytases are monomeric proteins with a few exceptions. The molecular masses of the enzymes are quite variable, within the range 38–500 kDa. Phytases are optimally active in the temperature range of 30–80°C. Among the thermophilic fungi, *T. lanuginosus* phytase had an optimum temperature of 65°C (Berka et al. 1998), while phytase of *R. pusillus* was optimally active at 70°C (Chadha et al. 2004). Phytase produced by *T. aurantiacus* was thermostable with temperature optimum of 55°C (Nampoothiri et al. 2004). An extremely thermostable phytase from *A. fumigatus* has been reported by Pasamontes et al. (1997a), which was able to withstand temperatures up to 100°C for 20 min with a loss of only 10% of its activity and it was optimally active at 70°C. Phytase of the thermophilic mould *Sporotrichum thermophile* is optimally active at 60°C (Singh and Satyanarayana 2006a, b).

Most thermophilic mould phytases are active in acidic range (pH 4.5–6.0), and the stability of enzyme decreased dramatically at pH values lower than 3 or higher than 7.5. Phytases from fungal origin show pH optimum between 4.5 and 5.5. Phytase of thermophilic moulds are acidic in nature, except *T. lanuginosus*, which was optimally active between pH 3.0 and 7.5 (Berka et al. 1998). Phytases of the thermophilic mould *S. thermophile* (Singh and Satyanarayana 2006a, b) and *T. aurantiacus* (Nampoothiri et al. 2004) were optimally active at pH 5.0. Phytase of *R. pusillus* was optimally active at pH of 5.4 (Chadha et al. 2004). The phytase of *A. fumigatus* hydrolysed phytic acid optimally at pH 5.5–6.5, whereas *p*-nitrophenylphosphate was hydrolysed at pH 5.0 (Pasamontes et al. 1997a). The requirement of ions for phytase activity varies from source to source. There is so far no known report of metal ion requirement by the phytases of thermophilic moulds. However, inhibition of phytase by the presence of metal ions has been reported.

Phytases from thermophiles exhibited broad substrate specificity, with the highest affinity for phytate. Phytase can be distinguished from acid phosphatase, due to its ability to degrade phytic acid. Phytase from *A. fumigatus, E. nidulans* and *M. thermophila* exhibited broad substrate specificity (Wyss et al. 1999a, b). Similarly, phytase from *T. lanuginosus* (Berka et al. 1998) and *S. thermophile* (Singh and Satyanarayana 2009) showed broad substrate specificity. The phytase of *R. pusillus* hydrolysed riboflavin phosphate, ATP, ADP, AMP, pNP phosphate, phenyl phosphate and phosphoenolpuruvate (Chadha et al. 2004).

Phytases are high-molecular-weight proteins ranging from 40 to 500 kDa. In case of thermophilic moulds, the molecular sizes of the phytases are generally small as compared to bacteria, yeasts and mesophilic moulds. The phytase of *S. thermophile* is a 456-kDa glycosylated protein (Singh and Satyanarayana 2009). The phytase of *E. nidulans* sequence consists of 463 amino acids and has a molecular mass of 51.785 kDa. The protein deduced from the *T. thermophilus* sequence consisted of 466 amino acids corresponding to a molecular weight of 51.45 kDa (Pasamontes et al. 1997b). The molecular mass of the phytase of *A. fumigatus* was 48.27 kDa.

A simple two-step purification (40.75-folds) of phytase from mutant TL-7 of *T. lanuginosus* was achieved by anion exchange and gel-filtration chromatography. The purified phytase of 54 kDa was optimally active at pH 5.0 and 70°C. The phytase showed broad substrate specificity with a K_m value of 4.55 μM and V_{max} of 0.833 μM/min/mg against sodium phytate. The partially purified phytase of *B. laevolacticus* was optimally active at 70°C and between pH 7.0 and pH 8.0 (Gulati et al. 2007b). The enzyme exhibited thermostability with approximately 80% activity at 70°C and pH 8.0 for up to 3 h in the presence/absence of 5 mM $CaCl_2$. The phytase from *B. laevolacticus* showed high specificity for phytate salts of $Ca^{2+} > Na^{+}$. The enzyme showed an apparent K_m 0.526 mM and V_{max} 12.3 μmole/min/mg against sodium phytate. The important characteristics of the phosphatases and phytases of thermophiles have been included in Table 25.3.

25.6 Cloning and Over-expression of Phytases and Phosphatases

The recombinant DNA technology led to improvement in the production and characteristics of thermophilic microbial enzymes. Though there are several reports of cloning and over-expression of phytases and phosphatase of thermophiles, Pasamontes et al. (1997b) cloned the phytases of the thermophilic moulds *E. nidulans* and *T. thermophilus*. Both predicted amino acid sequences exhibited high identity to known phytases. The *phyA* gene encoding an extracellular phytase from the thermophilic fungus *T. lanuginosus* was cloned and expressed in *F. venenatum* (Berka et al. 1998). Phytase showed limited sequence identity with that of *A. niger*. A 1.4-kb phytase gene of *A. fumigatus* phytase was expressed in *P. pastoris* as an extracellular phytase. The enzyme was resistant to pepsin digestion, but was degraded by high levels of trypsin (Rodríguez et al. 2000). The phytase gene (*phyA*) encoding a heat-stable phytase was cloned from *A. fumigatus* and over-expressed in *A. niger* (Pasamontes et al. 1997a). A novel phytase from a *M. thermophila* was isolated and over-expressed in *A. niger* (Mitchell et al. 1997). The encoded *phyA* phytase protein showed 48% identity to *phyA* of *A. niger* and has 21–29% identity compared to other histidine acid phosphatases.

A gene-encoding alkaline phosphatase from *G. thermodenitrificans* T2 was cloned and sequenced by Zhang et al. (2008). An alkaline phosphatase gene from

Table 25.3 Characteristics for phytases and phosphatases produced by thermophiles

Source	MW (kDa)	T_{opt}	pH_{opt}	K_m (mM)	V_{max} (U mg^{-1} protein)	Reference
Phytases						
Aspergillus fumigatus	88	55	5.5	0.114	0.114	Wang et al. (2007)
Thermomyces lanuginosus	60	65	7.0	0.11	–	Berka et al. (1998)
Thermoascus aurantiacus	–	55	–	–	–	Nampoothiri et al. (2004)
Rhizomucor pusillus	–	70	5.4	–	–	Chadha et al. (2004)
Myceliophthora thermophila	–	37	6.0	–	–	Mitchell et al. (1997)
Sporotrichum thermophile	456	60	5.5	0.15	0.156	Singh and Satyanarayana (2009)
T. lanuginosus TL-7	54	70	5.0	0.005	0.833	Gulati et al. (2007a)
Bacillus laevolacticus	–	70	7.0	0.526	12.3	Gulati et al. (2007b)
Phosphatases						
Scytalidium thermophilum	63, 58.5	70–75	9.5–10	–	–	Guimarães et al. (2001)
Geobacillus thermodenitrificans	46	65	9.0	0.0315	430	Zhang et al. (2008)
Thermoactinomyces vulgaris	–	65–70	10.0	–	–	Singh (2007)
Thermus thermophilus	40	70	12.0	–	–	Pantazaki et al. (2008)
T. thermophilus	54.7	75	12.0	0.034	–	Li et al. (2007)
T. yunnanensis sp. Nov.	104	70–80	8.0–10.0	–	–	Gong et al. (2005)
Thermus sp. Rt41A	160	60	11.0	–	–	Hartog and Daniel (1992)
Meiothermus ruber	54.2	62	11.0	0.055	–	Yurchenko et al. (2003)
Bacillus stearothermophilus	32	60–70	9.0	1.114	–	Mori et al. (1999)

T. thermophilus XM was cloned and sequenced (Li et al. 2007). It is 1,506 bp long encoding a protein of 501 amino acid residues with a molecular mass of 54.7 kDa. An alkaline phosphatase gene was cloned from the thermophilic bacterium *M. ruber*, and its nucleotide sequence was determined (Yurchenko et al. 2003). The protein was composed of 503 amino acid residues with a molecular mass of 54.2 kDa. The gene encoding an alkaline phosphatase from *T. thermophilus* HB8 was over-expressed in the mutant (Castán et al. 2002).

25.7 Applications of Phytases and Phosphatases of Thermophiles

25.7.1 Biotechnological Potential of Phytases

Basically, phytases have been used in the hydrolysis of phytic acid in the diets of monogastric animals. Besides their application as food and feed additives, they can also be used in pharmaceuticals, soil amendment and in synthesis of peroxidase and in plant growth promotion. Following are the major application of phytases from thermophiles reported so far.

25.7.1.1 Applicability in Dephytinisation of Food Ingredients

Dephytinisation is a process of degrading the phytic acid content of a food ingredient with phytase. Phytase of a thermophilic mould, *S. thermophile,* dephytinised wheat flour, sesame oil cake and soy milk efficiently with reduction in phytic acid and liberating inorganic phosphate (Singh and Satyanarayana 2006a, b, 2008a, b). Dephytinisation of food ingredients was higher at 60°C as compared to 37°C, because of optimal activity of phytase at 60°C.

25.7.1.2 Role in Bread Making

Phytic acid is present in wheat as stored organic form of phosphorus. Dough supplementation with phytase from *S. thermophile* resulted in liberation of higher inorganic phosphate, higher reducing sugars and higher soluble protein than control bread, made with commercial enzymes (Singh and Satyanarayana 2008b). Addition of α-amylase and phytase to dough further improved quality and properties of bread as compared to control bread prepared using commercial enzymes. Phytic acid content of wheat flour was also reduced with phytase supplementation (Singh and Satyanarayana 2008b).

25.7.1.3 Plant Growth Promotion

Phosphorus occurs in inorganic and organic form in soil. Rocks are the biggest reservoir of inorganic phosphorus, whereas organic form of phosphorus is mostly contributed by phytic acid, phospholipids, nucleic acid and other organic phosphates (Singh and Satyanarayana 2011b). Phytic acid occurs in soil either in adsorbed form or as insoluble phytates (Tang et al. 2006; Singh and Satyanarayana 2010, 2011b). There are a large number of reports explaining the role of phytase in improving the growth of the plants and reducing the phosphorus pollution. But first report came in 2010 when phytase of *S. thermophile* released inorganic phosphate from insoluble phytates (Singh and

Satyanarayana 2010). However, phosphate liberation, reported by Tang et al. (2006) for mesophilic microbial phytases, was lower than that of *S. thermophile* phytase.

Both phytase and mould promoted the growth of wheat seedlings (Singh and Satyanarayana 2010). Growth and inorganic phosphate content of plants were better than control.

Sodium phytate at a concentration of 5 mg per plant was adequate for liberating enough phosphorus for seedlings growth. Plant growth, root/shoot length and inorganic phosphate content of test plants were better than control plants. An enzyme dose of 20 U per plant was sufficient to liberate enough amount of inorganic phosphate required for supporting plant growth. Plant growth, root/shoot length and inorganic phosphate content of test plants were higher than control plants (Singh and Satyanarayana 2010). Compost prepared by combined action of native microflora of wheat straw along with *S. thermophile* promoted growth of plants (Singh and Satyanarayana 2010). Inorganic phosphate content of wheat plants was also high as compared to those cultivated on compost prepared either with only native microflora or *S. thermophile* (Singh and Satyanarayana 2010).

25.7.2 Applications of Phosphatases

Both acidic and alkaline phosphatases are useful in many biotechnological processes. They are used in removing phosphate monoester to prevent self-ligation (Maxam and Gilbert 1980). Alkaline phosphatase has been used as a tool in molecular biology laboratories, since DNA normally possesses phosphate groups on the 5′ end. Removing these phosphates prevents the DNA from ligating (the 5′ end attaching to the 3′ end), thereby keeping DNA molecules linear until the next step of the process for which they are being prepared; also, removal of the phosphate groups allows radiolabelling (replacement by radioactive phosphate groups) in order to measure the presence of the labelled DNA through further steps in the process or experiment. An important use of alkaline phosphatase is as a label for enzyme immunoassays. Another application of phosphatases is the use in the dairy industry is as a marker of pasteurisation in cow milk as it is denatured by elevated temperatures found during pasteurisation and can be tested for via colour change of a para-nitrophenylphosphate substrate in a buffered solution (Aschaffenburg Mullen Test) (Aschaffenburg, and Mullen 1949). Raw milk would typically produce a yellow colour within a couple of minutes, whereas properly pasteurised milk should show no change.

25.8 Conclusions and Future Perspectives

Phosphorus is an important component of cell and its constituents which plays an important role in growth and metabolism of organisms. Organic form of phosphorus is mainly broken down by phosphatases including phytases. Phytases are specific for

phytic acid and its salts but also found to degrade a wide range of organic phosphorus compounds. These enzymes are useful in food and feed industries for amelioration of nutritional status of food and combating environmental phosphorus pollution. To use phytases as animal feed additives, thermostability of the enzyme is a highly desirable property during the animal feed-pelleting process (80–100°C). Thermostability of phytase is considered to be an important and useful criterion for its industrial applications in food and feed industries. Thermostable phytases from different sources have shown different degrees of heat stability and half-life at 80°C and above, which is required during feed-pelleting process. Being useful in food and feed industries, they should be thermostable and with broad substrate specificity. Therefore, thermophiles would be a better candidate for production of these enzymes.

Thermophilic microorganisms are less exploited for phosphatases than mesophilic microbes. Phytases are now being increasingly recognised for their beneficial environmental role in reducing the phosphorus levels in manure and thereby minimising the need to supplement phosphorus in diets. Their usage as an animal feed additive is fast growing since it is eco-friendly, and there is an immense potential application for phytase in human nutrition as well. Further research is, however, called for exploiting thermophilic moulds and bacteria for the production of phytases and phosphatises.

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Chapter 26
Pectinases of Thermophilic Microbes

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Abstract Represented by archeal, bacterial, and fungal species, thermophilic organisms have been isolated from all types of terrestrial and marine hot environment. Pectinase from these organisms (thermophilic pectinase) developed unique structure–function properties of high thermostability and optimal activity at higher temperature. The advantage of using thermostable enzymes for various industrial applications is of course the intrinsic thermostability, and hence low activity losses during the raw material pretreatment at the elevated temperatures. Industrial applications of thermophilic pectinolytic enzyme have drawn a great deal of attention for use as biocatalysts because most of the industrial processes are carried out at higher temperature zone. Their potential to carry out myriads of biochemical reactions even at stringent conditions makes their use eco-friendly and best alternative to polluting chemical technologies. The role of acidic pectinases in extraction and clarification of fruit juices is well established. Recently, these have emerged as suitable candidate for biobleaching of wood pulp, desizing and bioscouring of cotton, degumming of plant fibers and biomass conversion, etc.

Keywords Thermophilic • Pectinase • Biobleaching • Desizing • Bioscouring

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26.1 Introduction

Pectinase is a general term for enzymes, such as pectolyase, pectozyme, and polygalacturonase, commonly referred to in brewing as pectic enzymes. These break down pectin, a polysaccharide substrate that is found in the cell walls of plants. Microbial pectinases account for 25% of the global food enzymes sales, and are used extensively for fruit juice clarification, juice extraction, manufacture of pectin-free starch, refinement of vegetable fibers, degumming of natural fibers, wastewater treatment, curing of coffee cocoa and tobacco, and as an analytical tool in the assessment of plant products. Pectin is a heteropolysaccharide composed of 1,4-linked galacturonate chains with a high percentage of methyl esterification. Complete degradation of pectin requires the action of cocktail of enzymes which can be broadly classified into two groups: (1) Methylesterases; which removes the methoxyl groups from pectin backbone and (2) depolymerases; which are responsible for the cleavage of bonds between different galacturonate subunits. This major group mainly includes the lyases and hydrolases. Lyases cleave the glycosidic bonds between the various subunits through β-elimination and give rise to unsaturated products (Soriano et al. 2000). On the basis of pH requirement for optimum enzymatic activity, pectinases can be broadly classified into acidic and alkaline pectinases (Ahlawat et al. 2007a, b). However, on the basis of mode of action, pectinase family can be classified into polygalacturonases (EC. 3.2.1.15), pectin esterases (EC. 3.1.1.11), pectin lyase (EC. 4.2.2.10), and pectate lyases (EC. 4.2.2.2).

Among these enzymes, pectin lyases show specificity for methyl esterified substrates (pectin) while pectate lyase is specific for unesterified polygalacturonate (pectate). Pectate lyases are widely distributed among microbial plant pathogens and have been the focus of several studies to ascertain their role as virulence factors (Barras et al. 1994). They have also been found in saprophytic microorganisms, including the genus *Bacillus* (Nasser et al. 1990), and in some thermophilic bacteria (Kozianowski et al. 1997). Multiple sequence alignment of pectate lyases has been used to classify these enzymes into several subfamilies and to identify consensus sequences of some of them (Henrissat et al. 1995), although recent sequencing and characterization of novel enzymes has resulted in the proposal of new groups of pectate lyase (Shevchik et al. 1997). On the basis of substrate specificity, different classes of pectinases are arranged in Table 26.1.

For some of the application, it is desirable to use thermostable enzymes, particularly when using substrates (which can also be other naturally occurring glycoside containing molecules with similar linkages as in pectin) that are poorly soluble at ambient temperatures, such as naringin and rutin, present in fruits (Birgisson et al. 2004). Many enzymes are involved in pectin degradation, but are referred to by several different names, which can be quite confusing. They may be acting either by hydrolysis or by trans-elimination; the latter performed by lyases (Kashyap et al. 2001a, b). Polymethylgalacturonase, (endo-)polygalacturonase, pectin depolymerase, pectinase, (EC. 3.2.1.15), exo-polygalacturonase (EC. 3.2.1.67),

Table 26.1 Classification of pectic enzymes acting on pectins or pectic acids

EC suggested name	Common name	EC number	Substrate	Action pattern
De-esterifying enzymes				
Polymethylgalacturonate (PMGE)	Pectinesterase	3.1.1.11	Pectin	Random
Depolymerizing enzymes (hydrolases)				
Endo-polygalacturonase (Endo-PG)	Polygalacturonase	3.2.1.15	Pectate	Random
Exo-polygalacturonase1 (Exo-PG1)	Polygalacturonase	3.2.1.67	Pectate	Terminal
Exo-polygalacturonase2 (Exo-PG2)	Polygalacturonase	3.2.1.82	Pectate	Penultimate bond
Endo-polymethylgalacturonase (Endo-PG)	Pectin hydrolase	–	Pectin	Random
Exo-polymethylgalacturonase (Exo-PG)	Pectin hydrolase	–	Pectin	Terminal
Depolymerizing enzymes (lyases)				
Endo-polygalacturonatelyase (Endo-PGL)	Pectate lyase	4.2.2.2	Pectate	Random
Exo-polygalacturonatelyase (Exo-PGL)	Pectate lyase	4.2.2.9	Pectate	Penultimate bond
Endopolymethylgalacturonatelyase (Endo-PMGL)	Pectin lyase	4.2.2.10	Pectin	Random
Exo-polymethylgalacturonatelyase (Exo-PMGL)	Pectin lyase	–	Pectin	Terminal

Adapted from Alkorta et al. (1998)

and exo-polygalacturanosidase (EC. 3.2.1.82) hydrolyzing the polygalacturonic acid chain by addition of water, are all classified under GH28 family, and are the most abundant among all the pectinolytic enzymes (Jayani et al. 2005). α-L-rhamnosidases (EC. 3.2.1.40, in GH family 28, 78, and 106) hydrolyze rhamnogalacturonan in the pectic backbone. α-L-arabinofuranosidases (EC. 3.2.1.55, α-L-AFases found in five different GH families) hydrolyze the L-arabinose side chains and endo-arabinase (EC. 3.2.1.99, GH43) act on arabinan side chains in pectin (Takao et al. 2002). These two enzymes operate synergistically in degrading branched arabinan to yield L-arabinose (Spagnuolo et al. 1999). Polysaccharide lyases, which like GH have been classified under sequence related families, cleave the galacturonic acid polymer by β-elimination and comprise, for example, polymethylgalacturonatelyase (pectin lyase, EC. 4.2.2.10), polygalacturonatelyase (pectatelyase, EC. 4.2.2.2), and exopolygalacturonatelyase (pectate disaccharide-lyase, EC. 4.2.2.9). Pectin esterase (pectinmethyl esterase, EC. 3.1.1.11) de-esterify the methyl ester linkages of the pectin backbone (Jayani et al. 2005). Mode of action of different pectinolytic enzymes is given in Fig. 26.1. Thermostable pectinases are not so frequently described, but reports show a few thermostable α-L-rhamnosidases, for example, from *Clostridium stercorarium* (Zverlov et al. 2000) and from a strain closely related to *Thermomicrobium* (Birgisson et al. 2004). Major commercial suppliers of the pectinase enzymes are summarized in Table 26.2.

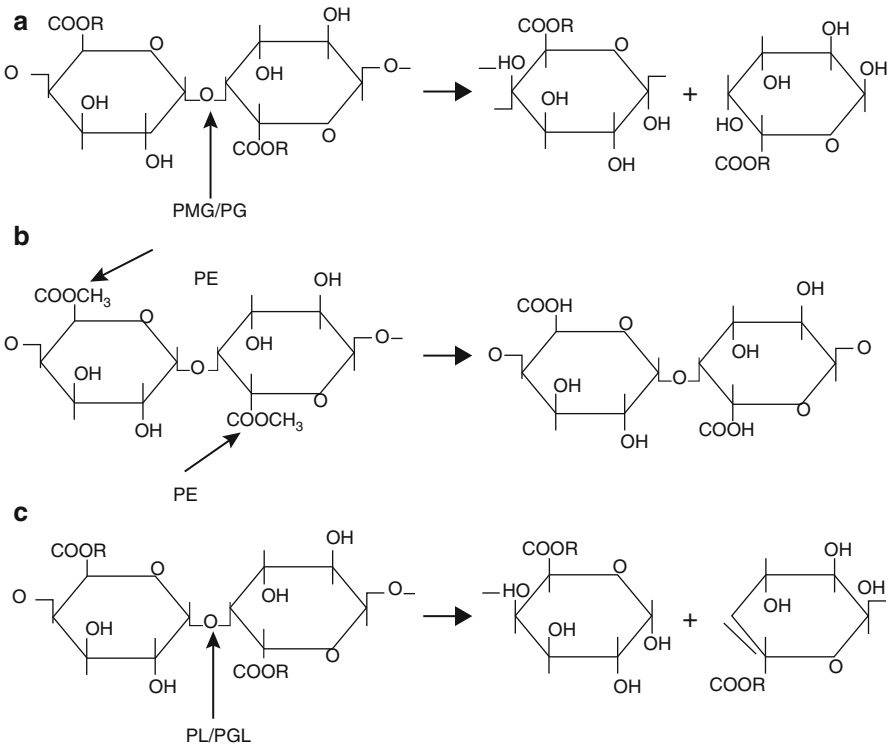

Fig. 26.1 Mode of action of pectinases. (**a**) R = H for PG and CH3 for PMG; (**b**) PE; and (**c**) R = H for PGL and CH3 for PL. The *arrow* indicates the place where the pectinase reacts with the pectic substances. *PMG* polymethylgalacturonases, *PG* polygalacturonases (EC 3.2.1.15), *PE* pectinesterase (EC 3.1.1.11), *PL* pectin lyase (EC-4.2.2.10) (Adapted from Jayani et al. 2005)

26.2 Thermophiles

Thermophiles are the organisms capable of survival and optimal growth at high temperatures (Bertoldo and Antranikian 2002). Generally, they have been isolated from terrestrial and marine habitats having high temperature. The most common habitats are hot springs and submarine hot vents. On the basis of their ability to tolerate different temperature range, they have been categorized into three classes: (1) Moderate thermophiles, having the property to grow optimally at temperature ranging from 40 to 70°C; (2) extreme thermophiles, adapted to grow optimally at temperature between 55 and 85°C; (3) hyperthermophiles, which are adapted to grow successfully at temperatures between 75 and 113°C (Baker et al. 2001). However, thermophilic microorganisms are taxonomically related to the mesophilic species. The thermophiles anatomy, ultrastructure, respiration, and metabolic processes are very similar when compared with those of mesophilic organisms.

Table 26.2 List of commercial suppliers of the pectinase enzyme

Sr. no.	Enzyme supplier	Product
1.	C.H. Boehringer Sohn; West Germany	Panzyme
2.	Ciba–Geigy, A.G; Switzerland	Ultrazyme
3.	Grinsteelvaeket; Denmark	Pectolase
4.	Kikkoman Shoyu Co; Japan	Sclase
5.	Schweizerische Ferment, A.G; Switzerland	Pectinex
6.	Societe Rapidase, S.A; France	Rapidase; Clarizyme
7.	Wallerstein, Co; USA	Klerzyme
8.	Rohm, GmbH; West Germany	Pectinol; Rohament

Adapted from Kashyap et al. (2001a, b)

On the other hand, depending upon their growth profile, thermophiles can also be classified as (1) obligate thermophiles, which do not grow below 40°C, and (2) facultative thermophiles, which can also able to grow at temperatures around 37°C (Hughes and Williams 1977). Ecological studies have shown that both aerobic and anaerobic species and many morphological and physiological types of microorganisms can exist in thermophilic environments (Madigan et al. 1997). Extreme thermophiles are mostly distributed among the genera of *Bacillus*, *Clostridium*, *Thermoanaerobacter*, *Thermus*, *Thermotoga*, and *Aquifex*. Most hyperthermophiles on the other hand include the two kingdoms of archaea: Crenarchaeota (*Sulfolobus*, *Pyrodictium*, *Pyrolobus*), Euryarchaeota (*Thermococcus*, *Pyrococcus*), and also include methanogens (*Methanocaldococcus*, *Methanobacterium*), sulfate reducers, and halophiles (Bertoldo and Antranikian 2002).

26.2.1 Thermophilic Enzymes

On the basis of evolutionary studies, it has been confirmed that thermophiles and mesophiles have evolved from common ancestors. Hence, it has been concluded that in physical properties, the enzymes from thermophilic organisms will behave like the enzymes extracted from the mesophilic organisms (Ljungdahl and Sherod 1960). On the other hand, it has also been observed that thermophiles produce specific proteins known as chaperonins which helps in the refolding of the denatured protein to attain their native active structure. Saturated fatty acids will constitute the major portion of the cell membrane of thermophiles and also creates the necessary hydrophobic environment for metabolic activities. Similarly, in case of cell membrane of hyperthermophiles, interaction between the lipid moieties and ether groups will maintain the structure.

The DNA of thermophiles has also been reported to have a reverse DNA gyrase producing positive supercoils in the DNA. Since this difference increases the melting

point of DNA, the stability at high temperatures is achieved (Haki and Rakshit 2003). No general strategy of stabilization has yet been established (Jaenicke and Bohm 1998). Alterations in the amino acid composition of proteins bring about additional electrostatic interactions, formation of hydrogen, disulfide bonds, and enhancement of hydrophobic interactions or compaction of the structure. There are only a few cysteine residues in thermophilic enzymes or they are completely absent. Since the fact that inactivation is often caused by oxidation of SH groups, lower cysteine content could enable the protein protected against the oxidation type of inactivation. In some cases, localization of cysteine residues is also important. For example, although *Bacillus stearothermophilus* alcohol dehydrogenase has the same number of cysteine residues as its mesophilic analogue, its SH groups are localized inside the protein globule.

Many significant substitutions in thermophilic enzymes as Lys to Arg, Ser to Ala, Ser to Thr, and Val to Ile have been reported (Scandurra et al. 1998). These substitutions cause an increase in the internal hydrophobicity. Thermophilic proteins generally show a decreased flexibility and increased hydrophobicity within the α-helical regions. The amino acids responsible for decreased flexibility are located in the helices at non-buried or surface positions (Jaenicke and Bohm 1998). Increased number of ion pairs, decreased hydrophobic area, increased helicity, less cavity volume, large number of additional salt bridges, stabilization of α-helices, replacement of conformationally strained residues by glycine, strong docking of N-terminal methionine, and increase in the number of hydrogen bonds are some of the reasons for the resistance of thermophilic proteins towards the higher temperature. Furthermore, helix-favoring residue, arginine, occurs more frequently, whereas helix-disfavoring residues cysteine, histidine, and proline have lower frequencies in thermophilic proteins (Kumar et al. 2000). Some thermophilic enzymes having proteolytic or amylolytic activities are stabilized by Ca, Mg, Zn, and other ions. The mechanism of stabilization is through the binding of cations to the labile parts of the globule. It is also known that some mesophilic enzymes are also stabilized by metal ions (Kumar and Takagi 1999).

Hydrophobic interactions are considered the main driving forces in protein folding. In thermophilic proteins, buried apolar surface areas are larger than in mesophilic proteins (Scandurra et al. 1998). However, it has been suggested that hydrophobicity shows little quantitative differences between thermophiles and mesophiles (Kumar et al. 2000). Difference in the number of hydrogen bonds and salt bridges may also be another factor for stability. Changes in the number of hydrogen bonds change the secondary structure of a protein. It has been observed that the main players of thermal stability were salt bridges and hydrogen bonds. The salt bridges around the active site may help to keep the active site region together by opposing disorder due to greater atomic mobility at high temperatures (Kumar et al. 2000). It has also been suggested that deletion or shortening of loops may increase the thermal stability and that oligomerization can be another contributing factor.

Microorganisms are the major source for thermophilic enzymes. Thermostable pectinases have been reported from many microorganisms like *Clostridium thermosulfurogenes* (Schink and Zeikus 1983), (*Sporotrichum thermophile* (Kaur et al. 2004), *Aspergillus fumigatus* (Phutela et al. 2005), and *Thermomucor indicae-seudaticae*

Table 26.3 List of alkalophilic thermostable pectinases from *Bacillus* species

Microorganism	Type of pectinase	Optimum pH	Optimum temperature (°C)
Bacillus sp. RK-9	PGL	10.0	75
Bacillus sp. NT-33	PG	10.5	75
Bacillus polymyxa	PG	8.4–9.4	45
Bacillus pumilus	PATE	8.0–8.5	60
Bacillus sp. DT-7	Pectate lyase	10.25	70
Bacillus subtilis	PAL	8.5	60–65
Pseudomonas syringae	PAL	8.0	40
Bacillus P-4-N	PG	10–10.5	65
Bacillus stearothermophilus	PATE	9.0	70

Adapted from Kashyap et al. (2001a, b)

N13 (Martin et al. 2010). Birgisson et al. (2004) produced a thermostable polygalacturonase from a thermophilic mold, *Sporotrichum thermophile*, optimally active at 55°C which is relevant for the fruit juice industry. *Aspergillus* is used mainly for the industrial production of thermophilic pectinolytic enzymes (Naidu and Panda 1998), though many species of *Bacillus* are also used for the production (Table 26.3) of thermophilic pectinases.

Cultivation of thermophiles-producing pectinases at high temperature is technically and economically interesting as it reduces the risk of contamination, reduces viscosity, thus making mixing easier and leads to a high degree of substrate solubility. However, compared to their mesophilic counterparts, the biomass achieved by these organisms is usually disappointingly low. The low cell yield poses problems for both large and small scale production, making extensive studies of thermophilic pectinase difficult. This has triggered considerable research aiming to cell yield for producing thermophilic pectinases. To date, several reports on media compositions and culture optimization of different thermophiles are available (Krahe et al. 1996). Special equipments and specific processes have been developed to improve fermentation processes of thermophiles and hyperthermophiles (Schiraldi and De Rosa 2002). However, due to factors such as requirement of complex and expensive media, low solubility of gas at high temperature, and low specific growth rates and product inhibition, large-scale commercial cultivation of thermophiles for enzyme production remains an economical challenge. The high cost of large-scale fermentation processes to produce enzymes by thermophiles and hyperthermophiles is justifiable only for very few specific applications.

26.2.2 Stability of Thermophilic Enzymes

In industrial applications with thermophiles and thermostable enzymes, isolated enzymes are today dominating over microorganisms. An enzyme or protein is called thermostable when a high-defined unfolding (transition) temperature (T), or a long half-life at a selected high temperature, is observed. A high temperature should be a

temperature above the thermophile boundary for growth (>55°C). Most, but not all proteins from thermophiles are thermostable. Extracellular enzymes generally show high thermostability, as they cannot be stabilized by cell-specific factors like compatible solutes (Santos and da Costa 2002). In addition, a few thermostable enzymes have also been identified from organisms growing at lower temperatures. Fundamental reasons to choose thermostable enzymes in bioprocessing is of course the intrinsic thermostability, which implies possibilities for prolonged storage (at room temperature), increased tolerance to organic solvents (Kristjansson 1989), reduced risk of contamination, as well as low activity losses during processing (when staying below the T_m of the enzyme) even at the elevated temperatures often used in raw material pretreatments.

Discovery and use of thermostable enzymes in combination with recombinant production and development using site directed and enzyme evolution technologies have erased some of the first identified hinders (*e.g.*, limited access and substrate specificity) for use in industrial biocatalysis. Today, a number of biotechnology companies are continuously prospecting for new, and adapting existing enzymes to reactions of higher volumes and more severe process conditions (OECD 2001).

Most of the commercially produced acidic pectinases are from the molds of genus *Aspergillus* such as *A. niger*, *A. oryzae*, *A. wentii*, and *A. flavus* for application in food processing industry, *whereas alkaline* pectinases are used widely in the fabric industry, for retting of plant fibers such as flax, hemp and jute, biopreparation of cotton fabrics, enzymatic polishing of jute, cotton-blended fabrics in paper industry to solve the retention problem in mechanical pulp bleaching, in the treatment of pulp and paper mill effluents, and for improving the quality of black tea. Thus, pectinases are today one of the upcoming enzymes of commercial sector.

26.2.3 Property Advantages in Process

Thermostability results in tolerance to high temperatures, organic solvents, high and low pH; also, there is increase in shelf life of the enzyme. Reaction at elevated temperatures increases availability of poorly soluble compounds, decreases viscosity thereby causing better mixing. Further at higher temperature, the mass transfer and diffusion rate increases, leading to acceleration in reaction rates, also there are fewer chances of microbial contaminations.

26.3 Biotechnological Applications

Pectinases are some of the first enzymes to be used in homes. Their first ever commercial application was reported in 1930 for the preparation of wines and fruit juices. Microbial pectinases contribute to almost 25% of global food enzyme sales and estimated to increase further (Tari et al. 2007). In the production of fruit juices,

extracts, and concentrates, pectinases are very important in maceration and solubilization of fruit pulps and in clarification (Naidu and Panda 1998). These enzymes are being used in several other areas including textile processing and bioscouring of cotton fibers, pulp and paper industry, degumming of plant bast fibers, retting of plant fibers, pretreatment of pectic wastewaters, coffee and tea fermentations, poultry feed, etc.

Cost-effective bulk production is required to commercialize any enzyme for industrial applications (Battan et al. 2007). Most of the commercial thermophilic enzymes are preferred to be produced through solid state fermentation (SSF) process. SSF is defined as any fermentation process performed on a non-soluble material that acts both as physical support and source of nutrients in absence of free-flowing liquid (Dhiman et al. 2009). The low moisture content means that fermentation can only be carried out by a limited number of microorganisms, mainly yeasts and fungi, although some bacteria have also been used (Battan et al. 2006). In addition, costs are much lower due to the efficient utilization and value addition of wastes (Robinson and Nigam 2003). Pectinases produced by SSF showed more stable properties; they had a higher stability for variation in pH and temperature and were less affected by catabolic repression than pectinases produced by SmF (Kaur et al. 2004).

The use of SSF for pectinase production has been proposed using different solid agricultural and agro-industrial residues as substrates such as wheat bran (Singh et al. 1999), soy bran (Castilho et al. 2000), cranberry and strawberry pomace (Zheng and Shetty 2000), coffee pulp and coffee husk (Antier et al. 1993a), husk (Antier et al. 1993b), cocoa (Schwan et al. 1997), orange bagasse, sugarcane bagasse and wheat bran (Martins et al. 2002), sugarcane bagasse (Acuna-Arguelles et al. 1995), and apple pomace (Hours et al. 1988a, b). Also, Bai et al. (2004) produced pectinase from *A. niger* by SSF using sugar beet pulp as a carbon source and wastewater from monosodium glutamate production as nitrogen and water source. Pectinase production under SSF not only reduced the production costs but also the method is less polluting (Baker and Wicker 1996).

One of the most attractive attributes of thermophiles is that they produce enzymes capable of catalyzing biochemical reactions at temperatures higher than those of mesophilic organisms (Demirjian et al. 2001). The property of higher thermal stability and tolerance to most of the chemical denaturants, for example, organic solvents enables them to resist harsh process conditions. They also show high catalytic activity at high temperatures and longer shelf life as commercial products (Aguilar et al. 1998). The increase of temperature in biotechnological processes has an influence on the bioavailability and solubility of organic compounds such as polyaromatic, aliphatic hydrocarbons, and polymeric substances. The elevation of temperature is accompanied by a decrease in viscosity and an increase in the diffusion coefficient of organic compounds. Consequently, higher reaction rates due to smaller boundary layers are expected (Niehaus et al. 1999).

Biological processes where high operational temperatures above 60°C employed, the risk of contamination by other organisms is also become substantially reduced (Adams and Kelly 1998). Furthermore, in large-scale fermentations with heat sensitive microorganisms, extensive efforts must be given for cooling the fermentation process

and as much as 10% of the energy cost of a microbial fermentation may be for heat transfer. Thermophilic fermentations, on the other hand, need not to be cooled (Niehaus et al. 1999). Thermostable pectinases have major use in textile processing and bioscouring of cotton fibers, pulp and paper industry, and fruit processing.

26.3.1 Textile Industry

Cotton remains the international fiber of choice among the world's expanding population, with as much as 62 billion pounds of this fiber consumed annually in textile use. There are, however, severe environmental costs associated with the widespread use of cotton. Unlike man-made cellulosic fibers such as rayon, cotton must be thoroughly prepared for subsequent wet processing treatments such as mercerizing, bleaching, dyeing, printing, or finishing. The purpose of the preparation step is to make the textile substrate uniformly absorbent, and the traditional method to achieve such absorbency is to scour the substrate with strong solutions of sodium hydroxide at elevated temperatures. Such scouring results in significant contributions to the effluent on a worldwide basis. About 75% of the organic pollutant level arising from textile finishing is derived from the preparation of cotton goods

It is useful, therefore, to discuss an alternative method that is commercially viable and cost-effective for preparing cotton fiber substrates. Such a method is based on the use of alkaline pectinase, a newly discovered enzyme. The cotton fiber primary wall is responsible for the lack of water absorbency of the fiber when no wetting agents are included in the various aqueous processing baths. The primary wall is about 0.1 μm thick and comprises only about l% of the total thickness of cotton fiber. This thin wall consists of about 52% cellulose in which 12% pectins, 7% wax, 12% proteins, 3% ash, and 14% other organic compounds are dispersed. The exact percentage of each component of the primary wall is determined by the type of the cotton plant, its origin, the growth conditions, and the degree of maturity.

The primary wall is a source of the hydrophobic nature of unscoured cotton. This wax consists of fatty alcohols, fatty acids, their esters, cholesterin, and other hydrocarbons. In addition to waxes, insoluble calcium, magnesium, iron, and other salts of polygalacturonic acids (pectins) contribute to the hydrophobic nature of unscoured cotton fiber. These pectate salts also act as biological glue, binding the noncellulosic components of the primary wall within the cellulosic matrix. In fact, it is more likely that the concentration of these components increases as the outer zone of the wall is approached.

26.3.1.1 Desizing of Fabric

Prior to weaving of yarn into fabric, the warp yarns are coated with a sizing agent to lubricate and protect the yarn from abrasion during weaving. Historically, the main sizing agent used for cotton fabrics has been starch because of its excellent film-forming capacity, availability, and relatively low cost. Desizing involves removal of adhesive

substance known as size from the warp threads, and the process must be carried out by treating the fabric with chemicals such as acids, alkali, or oxidizing agents at high temperature. It results in a degradation of the cotton fiber resulting in destruction of the natural soft feel, or "hand," of the cotton. The use of enzymes such as pectinases in combination with amylases, lipases, cellulases, and other hemicellulolytic enzymes to remove sizing agents has decreased the use of harsh chemicals to the environment, improving both safety of working conditions for textile workers and the quality of the fabric. Thermostable pectinase produced by *B. subtilis* was used for desizing of cotton and micropoly fabric (Ahlawat et al. 2009). Maximum desizing was observed at an enzyme dosage of 5 U/g after 120 min of retention time at a temperature of 65°C and at pH 9.5 in case of both cotton and micropoly fabric. At this optimized condition, substantial liberation of reducing sugar was observed. Remarkable increase in whiteness index was observed for cotton (19.79%) and micropoly fabric (35.33%) corresponding to maximum desizing efficiency as compared to control. For efficient desizing with integrated bioscouring process, the availability of an enzyme with broad operational range is desirable.

26.3.1.2 Bioscouring

Conventionally, scouring of cotton has been done with caustic alkaline solution at high temperature to achieve uniform dyeing and finishing. Although very effective in removing impurities, the process needs a huge amount of water for rinsing once the process is complete, has a high energy requirement and yields waste products that can be damaging to the environment. Excessive usage of water and water contamination are expensive and unacceptable. Moreover, the application of these hazardous chemicals results in high COD (chemical oxygen demand), BOD (biological oxygen demand), and TDS (total dissolved solid) in the wastewater.

Recently, alkaline pectinases have made very encouraging in roads in the degumming and retting of fiber crops for the preparation of good quality textile material (Hartzell and Hsieh 1998). Wet textile processes consists of desizing, scouring, and bleaching. Traditionally scouring has been carried out at higher temperature with caustic soda solutions, which has accompanying disadvantages such as fiber deterioration, high energy consumption, and large amount of strongly basic wastewater pollution (Li and Hardin 1998). In scouring, the desired hydrophilicity of fabric with high and even wettability can be achieved by removing noncellulosic material from the cotton fabric, especially from the cuticle (waxes and fats) and the primary wall (pectin, protein, and organic acids) so that it can be bleached and dyed successfully (Etters 1999). Nowadays, research has been directed towards the discovery of environmental-friendly enzymes that replace these chemical processes of scouring in textile industry as they have high specificity for their substrates, the reaction conditions are mild and there is no substrate loss due to chemical modifications (Traore and Buschle-Diller 1999).

Pectinase appeared to be the most efficient enzyme for optimal scouring result (Wang et al. 2007) being capable of depolymerizing the pectin, breaking it down to low-molecular water-soluble oligomers (Dhiman et al. 2008) and thereby improving the absorbency and whiteness of the textile material. It gives a fabric with a high and

even wettability so that it can be bleached and dyed successfully. An additional asset of this process is that besides being energy conservative and more environmentally friendly, enzymes used for bioscouring do not affect the cellulose backbone, thus drastically limiting fiber damage. Pectinases to be used for bioscouring are selected based on their pH and temperature compatibility taking into account the required time of treatment, end-product quality, water absorbency, whiteness, and residual pectin. In bioscouring, pectinase depolymerizes the pectin which adheres to cellulose and hydrophobic waxy materials as cements, and thereby improving the absorbency and whiteness of the textile material which further interfere with aqueous chemical processes on cotton, such as dyeing and finishing (Dhiman et al. 2008). In contrast to drastic alkaline conditions conventionally used, treatment with pectin degrading enzymes would not affect the cellulose backbone and thus avoid fiber damage (Rouette 2001).

Thermostable pectinase produced by *B. subtilis* was used for bioscouring on desized cotton fabric at an enzyme dose of 5 U/g at 65°C in 100 mM glycine-NaOH buffer (pH 9.5) for 2 h. The remarkable effect in weight loss of cotton (1.65%) was observed when along with enzyme, EDTA, and wetting agents were used. The enzymatic bioscouring resulted in enhancement of various physical properties of fabrics, namely, whiteness (1.2%), tensile strength (1.6%), and tearness (3.0%) over conventionally alkaline scoured fabrics.

After desizing and bioscouring process, the fabric showed better wetting and penetration properties apart from improving the physical properties of the fabric, making subsequent bleach process easy and resultantly giving much better dye uptake.

The study of the cotton fabric through electron microscopy (Fig. 26.2a, b) clearly depicted the enzymatic action during desizing and bioscouring process. The pectinase treated fabrics were much softer and showed strength and weight loss as compared to the untreated fabric, because of the removal of pectins, noncellulosic impurities from the fabric surface by the enzymatic action.

26.3.1.3 Degumming

Plant fibers are long, narrow, thick-walled, and lignified sclerenchymatous cells, which are dead and therefore serve a purely mechanical function of giving strength and rigidity to the plant body. These stem fibers are excellent source of natural textile materials. Conventionally, these fibers are obtained by a retting process involving mixed population of pectinolytic organisms. Subsequently, the fibers are treated with an alkaline solution (NaOH, 12–20%, w/v) containing wetting and reducing agents to remove the residual gummy substances rich in pectin. Pectinases play a significant role in deguming of bast fibers. Bast fibers such as jute, flax, ramie, and sunn hemp are lignocellulosic fibers containing three main categories of chemical compounds, namely, cellulose (58–63%), hemicellulose (20–24%), and lignin (12–14%), and some other small quantities of constituents such as fats and pectin. Utilization of thermostable polygalacturonase from *Bacillus* sp. for degumming of sunn hemp and ramie revealed the removal of noncellulosic

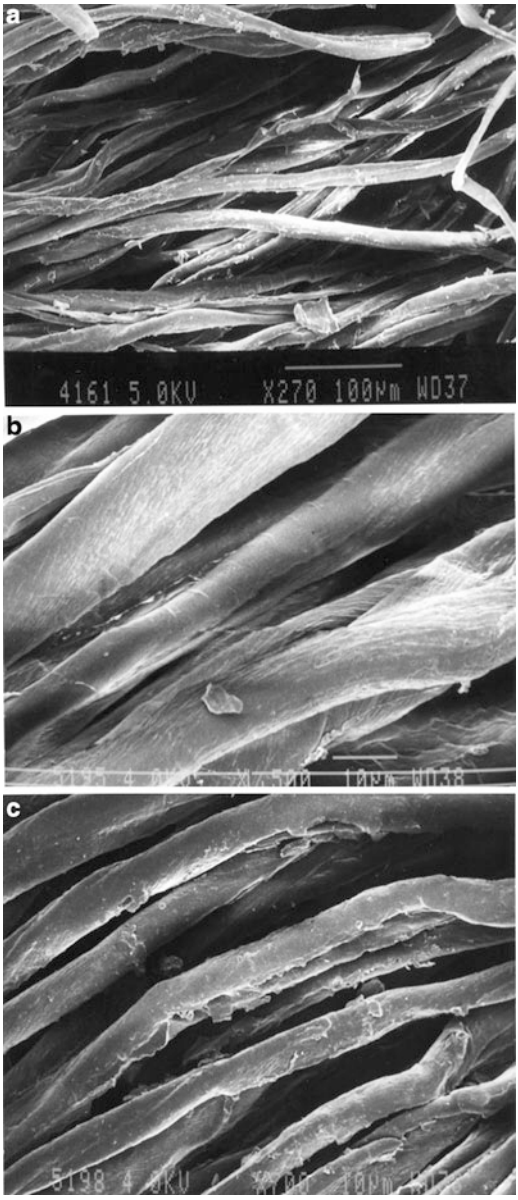

Fig. 26.2 (**a**) Scanning electron micrograph of untreated control cotton fabric. (**b**) SEM of enzymatically desized cotton fabric (**c**) SEM of bioscoured cotton fabric using alkaline pectinase

gummy material (Kapoor et al. 2001). Alkaline–thermotolerant pectinase of *Bacillus* sp. DT7 effectively removed pectic substances from buel (*Grewia optiva*) bast fibers. A combined (chemical and enzymatic) treatment resulted in release of galacturonic acid (575 µmol g^{-1} dry fibers) and a decrease in dry weight (43%) of these fibers (Kashyap et al. 2001a, b).

26.3.2 Pulp and Paper Industry

In pulp and paper industry, pulping is a step during which cellulose fibers are broken apart and most of the lignin is removed. The remaining lignin is then removed by a multistep bleaching process using chlorine (C), chlorine dioxide (C_D), and NaOH (E) treatments (Yadav et al. 2010). The organic chlorine by-products generated during chemical processing are toxic, mutagenic, bioaccumulating, and thus cause numerous harmful disturbances in the biological systems (Dhillon et al. 2000). Due to the increased environmental awareness and pressure of government, paper industries are currently changing practices to reduce organic pollutant load on the environment (Battan et al. 2007).

Among the new bleaching technologies, the use of enzymes to specifically modify the pulp by environmentally safe processes was first proposed by Viikari et al. (1986). Mechanical pulp is treated with pectinase in order to degrade pectins on the fibers thus weakening the bond between lignin and cellulose and further refining the pulp before bleaching. Pectinases depolymerize polygalacturonic acid and consequently decrease the cationic demand in the filterate from peroxide. Most of the researchers have used xylanase in pulp and paper industry for bleaching but there have been only a few reports so far utilizing pectinase for bleaching alone or in combination with xylanase.

Demand is increasing to replace these traditional processes such as chemical bleaching and textile pretreatment processes with biotechnological processes involving microorganisms and enzymes, which not only provide an economically viable alternative but are also more environmentally friendly. Pectinases to be used for bioscouring are selected on their pH and temperature compatibility taking into account the required time of treatment, end-product quality, water absorbency, whiteness, and residual pectin.

Pulping and bleaching process are performed at high temperatures and at high pH. Therefore, the paper industry needs enzyme that is thermostable and preferably active at neutral and alkaline pH. Pretreatment of paper pulp requires the use of cellulase-free pectinase, since cellulase may adversely affect the quality of the paper pulp by destroying the structure of cellulose and thus diminish the quality of the pulp. Pectinase from *Bacillus subtilis SS* is stable in neutral to alkaline pH range at 70°C; therefore, its suitability in pulp and paper industry was investigated (Ahlawat et al. 2007a, b). The enzyme pretreatment process at a pectinase dose of 5 IU/g of oven-dried pulp (10% consistency) at pH 9.5, temperature 70°C after 150 min of treatment gave the best pretreatment to the pulp. An increase of 4.3% in brightness along with an increase of 14.8 and 65.3% in whiteness and fluorescence, respectively, whereas a 15% decrease in the yellowness of the pretreated pulp were observed. There was a 5.85% reduction in kappa number and 6.1% reduction in permanganate number along with a reduction in the chemical oxygen demand value. A synergistic action of thermostable xylano-pectinolytic enzymes from the same bacterial isolate *Bacillus pumilus* was evaluated for the prebleaching of kraft pulp (Kaur et al. 2010). The enzymatic prebleaching of kraft pulp resulted in 8.5% reduction

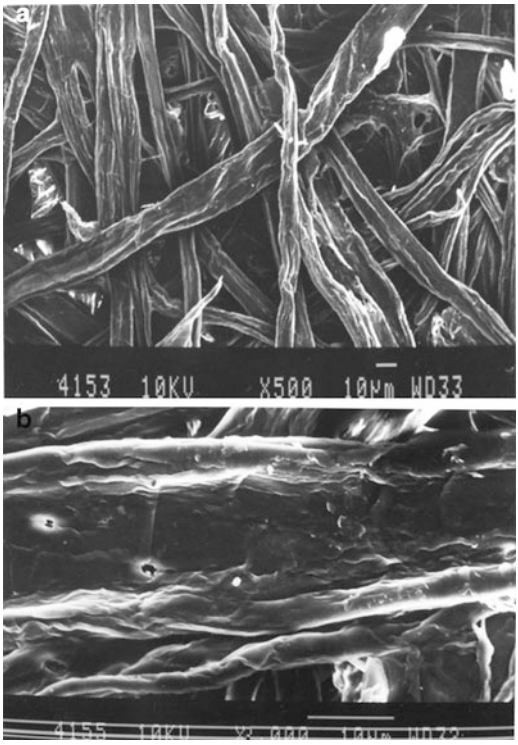

Fig. 26.3 (**a**) Effect of biobleaching using pectinase on kraft pulp fiber. (**b**) Scanning electron micrograph of untreated kraft pulp fiber

in kappa number of the pulp, showing remarkable delignification with the enzyme treatment. This approach resulted in 25% reduction in active chlorine consumption in subsequent bleaching stages without any decrease in brightness. Increase in burst factor (9%), tear factor (4.6%), breaking length (4.4%), double fold number (12.5%), Gurley porosity (4%), and viscosity (11.8%) of enzyme treated pulp reflected the significant improvement in pulp properties.

The results of the biobleaching of kraft pulp using the cellulase-free pectinase were also confirmed through scanning electron microscopic (SEM) studies. SEMs revealed that pectinases introduced greater porosity, swelling up, and separation of pulp microfibrils and pulp fibers (Fig. 26.3a) compared to smooth surfaces of untreated pulp (Fig. 26.3b). When the pulp fibers were subjected to enzymatic treatment, swelling, separation, and loss in compactness in the pulp fibers was observed. The enzymatic treatment of pulp renders the pulp fibers more accessible to the chemical bleaching agents, thereby, reducing the requirement of chlorine and chlorine compounds in the subsequent bleaching process (Beg et al. 2001).

Ricard and Reid (2004) demonstrated that a thermostable pure pectinase from the commercial enzyme mixture Novozym 863 lowered the cationic demand of peroxide-bleached mechanical pulp. It was also able to enhance the ability of cationic polymers to increase fines and ash retention. A second cloned polygalacturonase

I purified pectinase from the fungus *Aspergillus aculeatus*, also produced these effects. This research proved that pectinase can be used for both reducing cationic demand and improving the retention of peroxide-bleached thermomechanical pulps.

26.3.3 Fruit Juice Industry

The largest industrial application of pectinases is in fruit juice extraction and clarification. Pectin contributes to fruit juice viscosity and turbidity. A mixture of pectinases and amylases is used to clarify fruit juices. Pectinases have extensive applications in extraction and clarification of both clear and cloudy juices, in liquefaction and maceration of plant tissues.

26.3.3.1 Clarification

One of the major problems encountered in the preparation of fruit juices and wine is cloudiness, primarily due to the presence of pectins. One liter of juice with a dry matter content of 13% can contain 2–5 g of pectin. The pectin can be associated with other plant polymers and the cell debris. The cloudiness that these cause is difficult to remove except by enzymic depectinization. Thermostable pectinases can be added to the juice and incubated typically at 40–50°C. The dose should be determined in trials depending on the degree of depectinization desired and the type of fruit used. Addition of pectinase lowers the viscosity and causes cloud particles to aggregate to larger units (break), which sediment and is removed easily by centrifugation. The actions of purified pectin lyase from *Bacillus pumilus* and Pectinex 100 L Plus (Novozyme) were analyzed on apple, orange, carrot, and banana (Nadaroglu et al. 2010). These fruits were peeled, deseeded, and blended to get a homogenous fruit pulp. The pH of the pulp was adjusted to 6 and was incubated with enzyme preparation (purified pectin lyase and Pectinex 100 L Plus) for 5 h in a shaking water bath in 50°C. There was an increase, compared to the control, in the volume of juice of apple, banana, carrot, and peach and best results were bananas as eight times higher yield than the control was obtained.

26.3.3.2 Liquefaction

Total or whole fruit liquefaction refers to a process in which the entire fruit is subjected to enzymatic treatment. As a practical matter, liquefying the entire fruit is not possible because of the skin, seeds, and stems. In practice, the use of the term “total liquefaction” describes the action of enzymes on fruit pulp after (in the case of apples), seeds, skin, and stems are removed. It is process in which pulp is liquefied enzymatically, so pressing is not necessary.

Apple pomace when subjected to enzyme treatment with pectinases and cellulases results in enhanced juice yield. Pomace liquefaction may also be used to obtain value-added foods as it offers the opportunity of releasing apple polyphenols and polysaccharides to a greater extent and hence can be obtained preparatively. However, high concentration of polyphenols and pectic polysaccharides can lead to technological problems (Will et al. 2000).

Pectinase powder with pectinesterase and polygalacturonase activities of 1.15 and 6.68 units per gram, respectively, was used for liquefaction of pulps. An enzyme concentration of 0.5% was found optimum to liquefy apricot and plum pulps at 45°C in 5 h to obtain maximum juice yields of 78 and 82%, respectively. An enzyme concentration of 0.9% and incubation at 45°C for 6 h gave maximum juice yield of 59% from mango pulp. Juices obtained after liquefaction of pulps had higher moisture, total soluble solids, total sugars, reducing sugars and acidity and lower crude fiber, vitamin C, and pH than pulps (Chauhan et al. 2001).

Extraction of banana juice is most important step for banana syrup production, but banana is too pulpy and pertinacious to yield juices. Here, one of the most effective ways is enzymatic liquefaction at high temperature. The optimized process with 0.15% commercial pectinase and extraction for 2 h at 50°C resulted in about 60% increase in banana juice yield (Tadakittisarn et al. 2007). The use of pectinolytic enzymes not only resulted in higher yield of juice but also preserves the nutrients, original color, and flavor.

Swain and Ray (2010) used a thermostable exopolygalacturonase by *Bacillus subtilis* CM5 for carrot liquefaction along with commercially available Pectinex (Novozyme, Denmark). The yield of carrot juice was 70 and 95% more in Pectinex and *B. subtilis* PG treated samples, respectively, over control (no enzyme treatment) after 8 h of incubation. The higher juice yield might be due to thermostable activity by the *B. subtilis* PG enzyme and the presence of other extracellular enzymes (such as amylase and cellulase).

26.3.4 Improved Biomass Utilization

Biomass conversion for bioethanol is of global importance as an alternative to fossil fuels as sole energy sources. Biomass is the most important renewable energy source in terms of technical and economical feasibility. Fernandes et al. (2008) demonstrated feasibility of using thermostable hydrolytic enzyme for bioconversion of untreated sugar beet plants (including tops and stalks) to fermentable sugar syrup. *Talaromyces emersonii* produced enzyme cocktails relevant to hydrolysis of cellulose, hemicelluloses, and pectin when grown on different substrates. These cocktails were used to generate sugar rich syrup from untreated sugar beet plants by conducting hydrolysis at 71°C. Maximum level of sugar beet hydrolysis was obtained in enzyme cocktails induced with sorghum/beet pulp (68%) and sugar beet plant (56%). The principal monosacchrides released were glucose, xylose, and arabinose with minor amount of galactose and galacturonic acid.

Cassava pulp is the solid waste produced as a consequence of starch production. This pulp contains a high starch content (50–60% dry basis), causing an environmental problem with disposal. In order to recover this starch, physical or biological treatment of the material must be employed. Pulp can be treated either by sonic or incubation with a multienzyme mixture of cellulase and pectinase. Both methods were found to improve efficiency of starch extraction by disrupting the complex structure of polysaccharides associated with and entrapping starch granules. In the enzymatic treatment, the content of cellulase and pectinase for high efficiency of starch extraction determined the yield of liberated starch (Sriroth et al. 2000). Use of either cellulase or pectinase alone reportedly failed to effectively improve starch extraction. It was found that cellulase concentration seemed to have a greater effect on efficiency of starch yield than pectinase concentration. Treatment of pulp with 15 Novo cellulase units (NCU) of cellulase and 122.5 polygalacturonase (PG) units of pectinase per g dry pulp for 60 min resulted in 40% starch recovery. Quality characteristics of the liberated starch, including paste, viscosity, and thermal properties were reportedly comparable to a primary starch obtained by root extraction.

26.4 Conclusions and Future Perspectives

Thermostable enzymes and microorganisms are the topics of current research during the last two decades. The advantage of using thermostable enzymes for various industrial applications is of course the intrinsic thermostability, and hence low activity losses during the raw material pretreatment at the elevated temperatures (Turner et al. 2007; Martin et al. 2010). During the enzymatic pretreatments, it is simple to adjust the pH but difficult and expensive to control the temperature. Therefore, to reduce the cost of industrial processes, it becomes prerequisite to use the thermostable enzymes.

Pectinases are among the most important industrial enzymes and are of great significance in the current biotechnological arena with wide ranging applications. Pectinases are being used in clarification of fruit juice and in bringing down the bitterness of fruit juices, textile processing, degumming of plant fibers, treatment of pectic wastewaters, paper making, coffee and tea fermentation, animal feed, etc. Due to vast range of environmentally friendly application of pectinases, it is a leading enzyme of the industrial world.

New pectinases for use in commercial applications with desirable physicochemical characteristics and low-cost production have been the focus of much research (Da Silva et al. 2002; Malvessi and Silveira 2004; Phutela et al. 2005). Enzyme producing companies constantly improve their products for more widespread use. The exploration of knowledge of the various factors/amino acid residues/non-covalent interactions, that is, hydrogen bonding, electrostatic interactions, hydrophobic, and van der Waals forces contributing towards the stability of an enzyme could be beneficial for producing the enzymes with higher thermostability through protein/enzyme engineering techniques.

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Chapter 27
Developments in Thermostable Gellan Lyase

Margarita Kambourova and Anna Derekova

Abstract Thermostable carbohydrate-degrading enzymes are of special interest for many industrial applications as the solubility of carbohydrates at elevated temperatures sharply increases. Gellan is among the microbial exopolysaccharides found recently extensive use in food, microbial cultivation media, and pharmaceutical industries. Enzyme modification of gellan could change its molecular weight, hardness of its gel, and its elasticity and in such a way might broaden its current spectrum of application. As gellan is soluble at temperatures higher than 60°C, an industrial need in a thermostable gellan lyase is clearly outlined. Several reports on mesophilic bacterial strains producing gellan lyases are known, and only one thermophilic bacterial producer, *Geobacillus stearothermophilus* 98, was reported up to now. In this chapter, the source microorganism and properties of the thermostable gellan lyase are discussed in relation to those of mesophilic producers. Even though the accumulated knowledge on the structural and catalytic properties of the gellan lyase is still very limited, the results obtained clearly demonstrate that it is a new enzyme with interesting characteristics, which could add to the commercial value of gellan as an emulsifier, stabilizer, gel agent, thickener and suspending agent, and application in the future are also suggested.

Keywords Thermophilic microorganisms • Thermostable enzymes • Gellan lyase • Microbial exopolysaccharides • Gellan

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27.1 Introduction

Biopolymers are one of the most multifunctional and industrially interesting compounds employed in food (Harvey and McNeil 1998). The restricted volume of plant polysaccharides poses a problem in many industrial areas of modern society that suggests a successful replacement of plant with microbial polysaccharides, and research in this area has led to the production of several microbial polysaccharides on an industrial scale. Some microbial exopolysaccharides like xanthan gum (4.6% of annual polysaccharide consumption) already have gained a honorable places between hydrocolloids used in the food industry (Kohajdova and Karovicova 2009); however, their use is still in the very beginning phase. Despite the fact that gellan was comparatively recently found, its interesting properties imposed its deserved place in many applications, particularly in the food, pharmaceutical, and biomedical fields. Many interesting properties were reported for gellan and especially its deacetylated form, and a number of applications were suggested; however, the highly viscous properties of the polymer have largely limited its utility. Polymer treating by gellan lyase will lead not only to preparing low-viscosity and low-molecular-mass gellan for novel physiological and food technological functions but also to exploit new areas for the application of gellan in biopolymer-based industries.

Gellan lyase (EC 4.2.2.25) is an enzyme that degrades the microbial polysaccharide gellan. It cleaves the polymer by endo type of action in the D-glucose–D-glucuronic acid site (Fig. 27.1) in the repeating tetrasaccharide structure of this polysaccharide.

Several gellan lyases active at temperatures up to 45°C were isolated from mesophilic Gram-positive and Gram-negative bacteria (Kennedy and Sutherland 1994; Hashimoto et al. 1996, 1997; Jung et al. 2006). However, as the temperature for gellan resolving is higher than 60°, it is evident that an effective enzyme process for gellan depolymerization could be developed by using a thermostable enzyme.

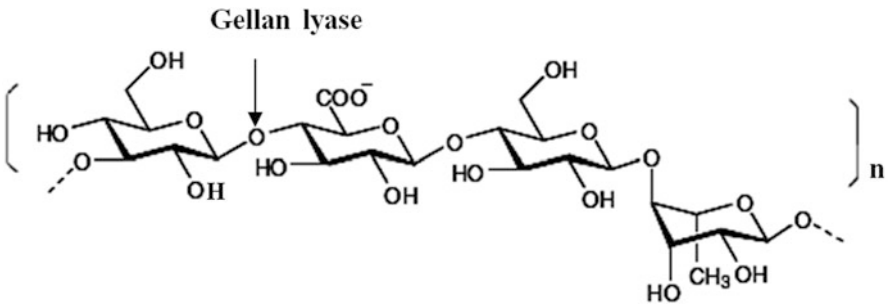

Fig. 27.1 Depolymerization of gellan by gellan lyase

27.2 Gellan as Substrate for Gellan Lyase Action

A wide range of chemical structures for microbial polysaccharides are possible with the range of available monosaccharide combinations, linkage types, and noncarbohydrate substituents. This structural heterogeneity determines variety in their properties and suggests high potential for application of exopolysaccharides and their depolymerization products in different industries. This is the case of sphingans, a series of eight, structurally closely related bacterial products, synthesized by the representatives of the genus *Sphingomonas*. Main structural differences between them refer to the nature and location of the monosaccharide and disaccharide side-chain groups and in some of the polymers (wellan), the presence of L-mannose as an alternative to L-rhamnose in the main-chain structures. Despite of gellan, other sphingans do not form gels, although they can form viscous aqueous solutions (Campana et al. 1992).

The native gellan has properties similar to xanthan-locust gum mixtures and, thus, rather limited uses. Only the deacylated form exhibits gelation properties useful for various food and nonfood applications. Gellan is a multifunctional gelling agent produced aerobically in high yields by the nonpathogenic industrial strain *Sphingomonas paucimobilis* ATCC 31461 (formerly *Pseudomonas elodea* ATCC 31461) (Jansson et al. 1983; Kang and Veeder 1981; Sanderson and Clark 1983; Pollock 1993), although the members of this bacterial genus are pathogenic as a rule. Gellan was discovered in 1978; it was approved for use in food in Japan since 1988, later in the USA, and more recently also in Europe (Sworn 2000). It received FDA approval for use in foods on November 25, 1992 due to its nontoxicity and safety (Pszczola 1993). Gellan hydrogels express interesting properties like high viscosity and high thermo- and acido resistance. Native gellan (Fig. 27.2) consists of a backbone of a repeating unit of D-glucose (D-Glc), L-rhamnose (L-Rha),

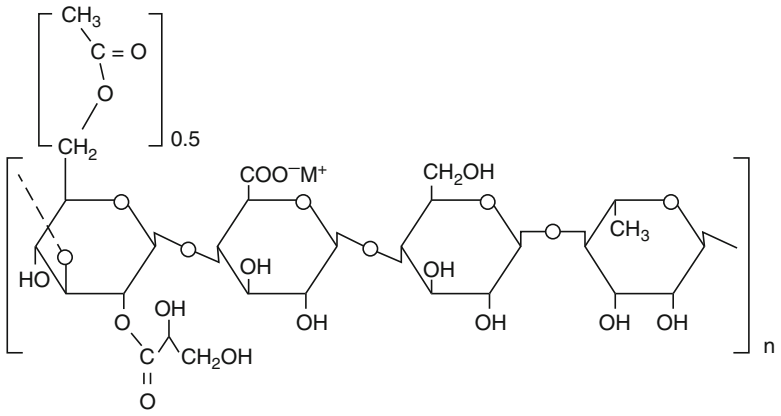

Fig. 27.2 Structure of native (highly acetylated) gellan

Fig. 27.3 Formula of Gelrite

and D-glucuronic acid (D-GlcA) to form a tetrasaccharide structure [→3)-β-D-Glcp-(1→4)-β-D-GlcpA-(1→4)-β-D-Glcp-(1→4)-α-L-Rhap-(1→] in the ratio of 2:1:1 (Khan et al. 2007) esterified with acyl substituents (1 mol of glycerate and 0.5 mol of acetate per unit). O-acetyl and L-glyceryl moieties are linked to the same D-glucosyl residue adjacent to the D-glucuronyl residue as the side chain (Banik et al. 2000).

The influence of the acetate groups on the aggregation of gellan molecules was suggested to be weak, while glycerate residues are responsible for their crystal packaging (Chandrasekaran and Thailambal 1990). The degree of ester substitution directly influences the gellan properties. A chemical deacylation of the native gellan results in a change from soft, elastic, thermoreversible gels to harder, more brittle gels, especially in presence of cations as Na^+, K^+, Ca^{2+}, and Mg^{2+} (Fialho et al. 1999; Rinaudo and Milas 2000). Gellan has an average molecular mass of about 500 kDa and has been shown to adopt a double-helical structure in solution (Bemiller 1996). In commercial gellan, Gelrite or Kelcogel, the acetyl groups are removed correspondingly fully or partly by alkaline treatment. It is commercially available in three forms: no, low, and high acyl content with the respective denominations of Gelrite® (Fig. 27.3), Kelcogel® F, and Kelcogel® LT100.

27.3 Critical Review of Thermophilic Gellan Lyase in Comparison with Mesophilic Ones

Thermostable enzymes, which have been isolated mainly from thermophilic organisms, have found a number of commercial applications (Demirijan et al. 2001). The biotechnological interest to the thermostable enzymes is due to the fact that their enzymes are better suited for harsh industrial processes, the risk of contamination is reduced, the reaction rates are higher because of a decrease in viscosity and an increase in diffusion coefficient of substrates, and process yield is higher based on the increased solubility of substrates and products (Haki and Rakshit 2003). In the field of industrial polysaccharide degradation, thermostable enzymes are much more preferable due to the higher substrate solubility at elevated temperatures. While some thermostable enzymes like amylases are widely used in industry, some comparatively new enzymes are just on the starting line of development. In the present review, an attempt is made to document first steps in the development of the thermostable gellan lyase investigations. The need for thermostable catalyst, the optimum conditions for an efficient enzyme synthesis and catalytic activity of the enzyme, and its properties and advantages are presented in the light of comparison with mesophilic enzymes.

27.3.1 Gellan Lyase Production

The reports on gellan degradation are scant. Only few mesophilic gellan lyase producers were announced (Table 27.1), and *G. stearothermophilus* 98 is the first known thermophilic producer.

The strain 98 able to degrade gellan was isolated after screening of 132 environment samples taken from water, soil, and algobacterial mat samples of various hot springs in Bulgaria (Derekova et al. 2006). It forms Gram-positive, spore-forming nonmotile rods 1.68–3.1 μm × 0.37–0.53 μm in size. The strain was facultative anaerobe. Its characteristics were typical for *Geobacillus stearothermophilus* (Nazina et al. 2001). The affiliation of the strain 98 to this species was confirmed by sequencing of its 16S rDNA. It was able to grow in a pH range of 5.5–8.5 at high temperatures (50–82°C). The optimal values for growth determined by their maximal growth rate were pH 7.0 and 55°C.

Gellan lyase synthesis was established to be growth associated. A decrease in cultivation temperature with only 5°C (from 55 to 50°C) resulted in 50% decrease of growth and enzyme synthesized. As can be seen from the data presented, mainly two groups of microorganisms possess the ability for gellan degradation: shingomonad group and *Bacillus senso lato* group to which also *G. stearothermophilus* belongs to (Ziegler 2001). In both groups, the enzymes were identified as extracellular, like other carbodegrading enzymes (alfa-amylases, xylanases, cellulases), which should degrade the high-weight polymer out of cells to oligomers that could be further uptake by the cells. The registered-specific enzyme activities for the thermostable enzyme (Derekova et al. 2006) and for the mesophilic one from *Bacillus* sp. GL1 (Hashimoto et al. 1997) were similar (correspondingly 0.11 and 0.139). However, when the thermophilic strain was cultivated in continuous cultures at low dilution rate, it increased tenfold and enzyme productivity was almost sixfold higher. Similar enhancement was reported for some carbodegrading enzymes (Becker et al. 1997; Emanuilova et al. 1999), and no other reports on continuous processes for gellan lyase production are available.

Typical for bacilli is the inducible character of the enzyme synthesis. When gellan was replaced by other carbohydrates like starch, xanthan, pullulan, and glucose, a good strain growth was observed; however, no enzyme activity was registered. The inducible enzyme activity was reported for other *Bacillus* representatives, *Bacillus* sp. GL1 (Hashimoto et al. 1996), and *Bacillus* sp. YJ-1 (Jung et al. 2006). However, the enzymes secreted by *Bacillus* sp. GL1 were induced not only by gellan but also by xanthan (Hashimoto et al. 1998b). A lack of enzyme activity in glucose-grown cells was reported for Gram-negative strains (Kennedy and Sutherland 1994). A number of *Sphingomonas* strains able to gellan production possessed a constitutive enzyme activity (Sutherland and Kennedy 1996). When gellan-producing strains of *Sphingomonas* were cultivated in media where gellan was the sole carbon source, no substrate utilization took place. This means that the enzyme role is not to utilize gellan as an energy source for the cells (Sutherland and Kennedy 1996). Contrary, when gellan was used as a carbon source in a defined medium, *G. stearothermophilus* 98 growth was the best and enzyme production was 50% higher than in a complex medium with gellan.

Table 27.1 Conditions for gellan lyase production by thermophilic and mesophilic producers

Microorganism	Growth conditions			Maximum activity in culture liquid (U/mg)	References
	Optimal temperature, °C	Optimal pH	Incubation period		
G. stearothermophilus 98	55	7.5	20 h	0.11[a]	Derekova et al. (2006)
Gram-negative bacterial strains	30	–	10–12 days		Kennedy and Sutherland (1994)
Mixed culture	30	–	5–6 days	2.3×10^{-3}[b]	Hashimoto et al. (1996)
Bacillus sp. GL1	30	7.2	72 h	0.139[b]	Hashimoto et al. (1997)
Bacillus sp. YJ-1	37	7.5	7 days	96.5[c]	Jung et al. (2006)

[a]One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μM of reducing sugars per minute
[b]One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μM mmol of b-formylpyruvic acid per minute
[c]One unit of enzyme activity was defined as the amount of enzymes that increases 1 optical density at 548 nm per 1 h

A short fermentation process is another advantage in using the thermophilic strain for production. Maximum gellan lyase activity was reached after 20 h of cultivation at the end of the exponential phase, while the fermentation processes with mesophiles continue from 72 h to 10–12 days (Table 27.1).

27.3.2 Gellan Lyase Purification

Together with the thermostable gellan lyase (Derekova et al. 2006), two mesophilic enzymes were purified (Hashimoto et al. 1996, 1997). A common feature of the gellan lyase purification roots is the several stages involved and the comparatively low enzyme yield. This value varied from 40.5% for *G. stearothermophilus* 98 (Derekova et al. 2006) to 5% for the enzyme from mixed culture (Hashimoto et al. 1996) and 1.54% for the gellan lyase protein from *Bacillus* sp. GL1 (Hashimoto et al. 1997). The probable reason for enzyme deactivation during the purification stages could be the high molecular weight of all gellan lyases causing easy aggregation and denaturation of the enzyme. Considerably higher yield for the thermostable enzymes reflects inherent stability of the thermostable protein.

27.3.3 Mechanism of Gellan Depolymerization

Polysaccharide lyases commonly recognize uronic acid residues in polysaccharides, catalyze a β-elimination reaction, and produce unsaturated saccharides with C=C double bonds in uronic acid residues at the newly formed nonreducing terminus (Ochiai et al. 2009), and a series of oligosaccharides ranging in size from a degree of polymerization (DP) of 2 to 3–5 are obtained (Sutherland 1999). The cleavage of polysaccharide chains containing 1,4 uronosyl linkages by eliminative rather than a hydrolytic mechanism is favored due to the possible lower energy requirement (Sutherland 1995). An exception of xanthan lyase, all other investigated polysaccharide lyases are endo-acting polysaccharide lyases. The lyase character of the enzyme from *G. stearothermophilus* 98 was confirmed spectrophotometrically by continuously increasing the absorbance at 235 nm due to the accumulation of the double bonds. Lyases are characterized by high specificity of action – only the polysaccharides which have been modified by chemical deacetylation are substrates. The removal of the side chains is required to obtain completely exposed carboxylate groups, which allows the enzyme to cleave at its recognition site (Sutherland and Kennedy 1996), which in Gelrite is unsubstituted. Similar effect of inhibition by the acyl groups was observed for other lyases (Kennedy et al. 1992); the only exception is the exolyting xanthan lyase depolymerizing xanthan whether or not acetyl or pyruvate ketal groups are present (Sutherland 1987). Gellan lyases degrade deacylated gellan through eliminase type of action to the tetrasaccharide (DGlcA–Glc–Rha–Glc) and cleave the sequence at the linkage between a neutral monosaccharide and the C4 of a uronic acid with simultaneous introduction of a double bond at the C4 and C5 of the uronic acid (Sutherland 1998) but exhibit

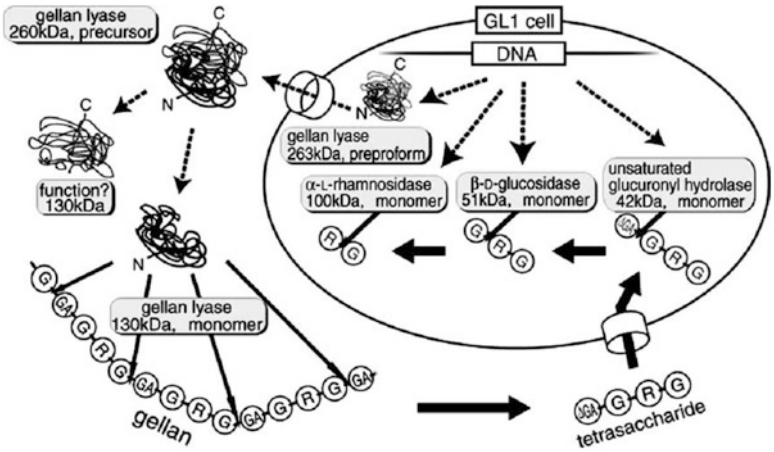

Fig. 27.4 Gellan metabolism in strain GL1. G, D-glucose; GA, D-glucuronic acid; R, L-rhamnose; Δ GA, unsaturated glucuronic acid (Miyake et al. 2004)

negligible activity against the native acylated gellan polysaccharide. It appears that the acetyl compounds inhibit enzyme activity, and the same effect results from the presence of side chains, especially when these are bound to the glucosyl residue attached to the reducing end of the glucuronic acid (Sutherland and Kennedy 1996). The unusually high specificity of the gellan lyase toward gellan was confirmed by the comparatively high K_m value (0.21 μM) for gellan depolymerization by the enzyme from *G. stearothermophilus* 98, and lack of activity to other tested substrates was observed (Derekova et al. 2010). Gellan lyases produced by Gram-positive mesophilic or thermophilic microorganisms, they are highly specific to deacetylated gellan and other gellan-related polysaccharides such as S-88, welan, rhamsan, and S-198 being inert as substrates. The enzyme produced by Gram-negative bacteria (Sutherland and Kennedy 1996) is partially active on deacetylated rhamsan gum (max. 47% activity). The product derived from gellan degradation through the reaction of the purified thermostable gellan lyase was a tetrasaccharide (Derekova et al. 2010) like the tetrasaccharide from *Bacillus* sp. GL1 (Hashimoto et al. 1999a). After the action of partially purified gellan lyases from Gram-negative bacteria, two major gellan-degrading products were obtained – tetrasaccharide and trisaccharide (Kennedy and Sutherland 1994). This assumption is apparently distinct from the result for bacilli enzymes but also could be due to the fact that the enzyme fraction could contain some traces of β-D-glucuronidase, the enzyme that converts the tetrasaccharide to trisaccharide by liberation of unsaturated glucuronyl residue. Tetrasaccharide received after gellan lyase action is further degraded by three exo-acting enzymes. They were isolated from a culture liquid of the enzyme producer *Bacillus sp.* GL1 (Hashimoto et al. 1998a, 1999b, c). When incubated in cell extracts of *Bacillus* sp. GL1, tetrasaccharide was further hydrolyzed to monosaccharides by successive actions of intracellular enzymes unsaturated glucuronidase, β-D-glucosidase, and α-L-rhamnosidase (Hashimoto et al. 1999a). These enzymes also expressed unusually high substrate specificity and do not act on oligosaccharides derived from other sphingans. The route for gellan depolymerization in *Bacillus* sp. GL1 is presented in Fig. 27.4.

Table 27.2 Enzyme activity of the intracellular exoglycosidases, produced by *Bacillus* sp. GL1 (Hashimoto et al. 1996) and *Geobacillus stearothermophilus* (unpublished results)

	Bacillus sp. GL1		*G. stearothermophilus* 98	
Exoglycosidases	Intracellular (U/g)	Extracellular (U/g)	Intracellular (U/g)	Extracellular (U/g)
β-D-glucuronidase	–	–	0.2	4.21
β-D-glucosidase	1.10	0.99	0.31	6
α-L-rhamnosidase	52.0	3.10	2.3	30

Unsaturated glucuronyl hydrolase from *Bacillus* sp. GL1 (Hashimoto et al. 1999b) was a monomer with a molecular mass of 42 kDa, most active at pH 6.5 and 45°C. It removes unsaturated glucuronic acid at the nonreducing end of the tetrasaccharide produced by gellan lyase. β-D-glucosidase has a molecular mass of 51 kDa and is most active at pH 6.0 (Hashimoto et al. 1998a). It hydrolyzes the trisaccharide (Glc–Rha–Glc) obtained after the action of unsaturated glucuronidase to glucose and a disaccharide. α-L-rhamnosidase is a monomer with a molecular mass of 100 kDa, most active at pH 7.0 and 50°C (Hashimoto et al. 1999c). It releases rhamnose from the disaccharide (Rha–Glc). A presence of β-D-glucuronidase and β-D-glucosidase periplasmic activities was reported for *Sphingomonas* strains (Sutherland and Kennedy 1996). Two spots were visualized as products derived from gellan by the action of the unpurified thermostable enzyme in the supernatant and the formation of only one product after the action of electrophoretically homogeneous gellan lyase was observed (Derekova et al. 2010). Identification of the enzymes β-D-glucuronidase, β-D-glucosidase, and α-L-rhamnosidase in the cell debris from *G. stearothermophilus* 98 (Table 27.2) suggests a common enzymatic route for gellan depolymerization with mesophilic microbial systems.

27.3.4 Molecular Weight of Gellan Lyases

Thermostable gellan lyase from *G. stearothermophilus* 98 was observed to exist in two forms – high-molecular-weight protein 216 kDa, determined by both capillary and SDS-electrophoresis, and low molecular weight of 120 kDa (Derekova et al. 2010). The authors working on *Bacillus* GL1 enzyme (Hashimoto et al. 1998b; Miyake et al. 2004) reported its existence in two forms – a large precursor protein (260 kDa gellan lyase after removing of 3 kDa signal protein) and a mature form (140 kDa) obtained after a posttranslationally processing through excising C-terminal peptide of about 120 kDa in the self-cleavage reaction in vitro, suggesting an activity of the precursor together with the main lyase activity (Hashimoto et al. 2005). Gellan lyase produced by a certain Gram-negative bacterium is roughly estimated to be a single polypeptide of 135 kDa (Kennedy and Sutherland 1994), which value is close to the mature form weight. Cloned in *E. coli* enzyme from *Bacillus* sp. GL1 was expressed as a protein with a molecular mass of about 260 kDa (Hashimoto

et al. 1998b). Posttranslational processing has also been observed in the case of other polysaccharide lyases: xanthan lyase in *Bacillus* sp. GL1 (Hashimoto et al. 2001), alginate lyase in *Sphingomonas* sp. A1 (Hisano et al. 1994), and hyaluronate lyase in *Streptococcus agalactiae* (Gase et al. 1998; Li et al. 2000). The sphinganase with a molecular weight of 110 kDa transformed to an active polypeptide of 51 kDa after storage at 4°C for 3 weeks (Mikolajczak et al. 1994).

Both purified enzymes from *Bacillus* sp. GL1 and *G. stearothermophilus* 98 showed about twofold higher specific activity for the low molecular weight (9.34 U/mg for 140 kDa protein and 5.24 U/mg for 260 kDa protein from *Bacillus* sp. GL1 and correspondingly 167 and 95 U/g for the enzyme from *G. stearothermophilus* 98). These differences in specific activity are thought to be caused by the molecular masses of the enzymes, suggesting that the catalytic ability of gellan lyases is the same whether posttranslational processing occurs or not.

27.3.5 Gellan Lyase Structure Investigations

Gellan lyase refers to the group of polysaccharide lyases. Polysaccharide lyases are classified in 21 families (1–22, family 19 has been moved to glycosyl hydrolases) based on their sequences, with ~50 singletons awaiting further classification (Garron and Cygler 2010), within the CAZy database (http://www.cazy.org/) based on recognizable sequence homologies. Presently known families belong to six general folds suggesting that this group of enzymes have been invented more than once during evolution from totally different scaffolds (Lombard et al. 2010). The number of families is expected to grow with the rapid progress in sequencing of new genomes.

Gellan lyase has not been classified into any of the described polysaccharide families since it shows no significant homology with other polysaccharide lyases. The sequence results for the only sequenced per now gellan lyase gene (Hashimoto et al. 1998b) revealed that its open reading frame (ORF) consists of 7,425 bp which can encode a huge polypeptide of 2,475 amino acids with a molecular weight of 263 030 Da. The N-terminal amino acid sequences of the 260 and 130 kDa enzymes were found to be identical. Determination of the C-terminal amino acid of the 130 kDa enzyme indicated that the 260 kDa enzyme is cleaved between the 1,205 Gly and 1,206 Leu residues to yield the mature form (130 kDa) of the gellan lyase. Therefore, the mature enzyme consists of 1,170 amino acids (36 Ala–1,205 Gly) with a molecular weight of 125,345 (Miyake et al. 2004). The attempts for the sequencing of the thermophilic gene were unsuccessful up to now (personal data), and further work should be carry out to reveal the actual differences in primary structure of two enzymes. The comparison between amino acid composition for the enzymes from *Bacillus* GL1 (Hashimoto et al. 1998b) and *G. stearothermophilus* 98 (Derekova et al. 2010) (Table 27.3) revealed that the charged amino acids were represented in higher number of residues in the thermostable gellan lyase molecule

Table 27.3 Amino acid composition of gellan lyases from *Geobacillus stearothermophilus* 98 and *Bacillus* sp. GL1

	G. stearothermophilus 98 gellan lyase		*Bacillus* sp. GL1 gellan lyase (Prot Param082833_BACSP)	
Amino acids	Residues number	(%)	Residues number	(%)
Asx	48	2.3	281	11.4
Glx	136	6.5	225	9.1
Ser	42	2.0	157	6.3
Gly	126	6.0	190	7.7
His	317	15.2	34	1.4
Arg	58	2.8	89	3.6
Thr	30	1.4	174	7.0
Ala	257	12.3	378	15.3
Pro	132	6.3	112	4.5
Cys	24	1.2	0	0
Tyr	16	0.8	91	3.7
Val	211	10.1	174	7.0
Met	16	0.8	46	1.9
Ile	141	6.8	95	3.8
Leu	80	3.8	232	9.4
Phe	46	2.2	64	2.6
Lys	368	17.7	101	4.1
Trp	35	1.7	32	1.3
Total	2,083	100	2,475	100

in comparison with mesophilic enzyme. The increased quantity of charged amino acids (Lys, His, Pro) in the case of *G. stearothermophilus* 98 enzyme suggests additional hydrogen and ion bonds into the protein molecules leading to inherent protein thermostability (Vielle and Zeikus 2001). These amino acids were generally detected with higher frequency in thermophilic proteins compared to mesophilic ones. In the case of the endo-ß-1,4-xylanase from *Geobacillus stearothermophilus* 236, such an increased frequency of charged amino acids was reported to improve a network of intramolecular interactions thus influencing and augmenting thermostability (Jeong et al. 2007). In some cases, this fact is connected to the predominance of G+C in their codons (Pro) or to the enhanced ability to create hydrogen bonds (His). The presence of high amount of Lys residues (17.7%) leads to the assumption for the basic character of *G. stearothermophilus* 98 enzyme molecule in contrast to the increased number of negatively charged residues (Asp+Glu – 10.8%) of *Bacillus* sp. GL1 enzyme (Hashimoto et al. 1998b). The increased presence of His and Pro residues reflects their significant role for β-structure formation, confirmed by its circular dichroism (CD) spectrum, and the Gly residue content for U-turns in the molecule. The significant amount of the hydrophobic amino acids Ala and Val in both enzymes could be related to the tendency of the enzyme molecule to aggregate.

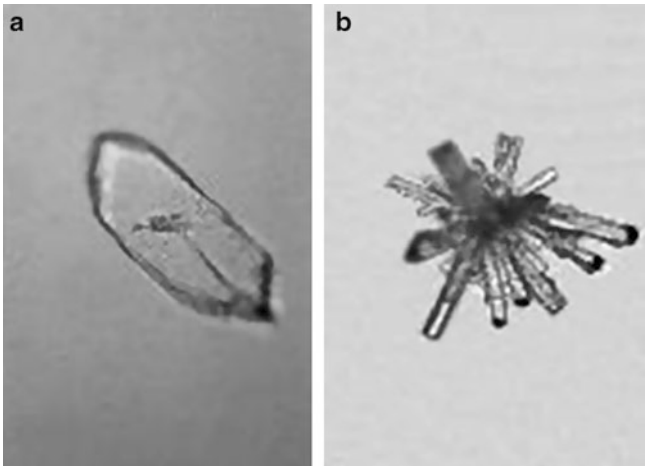

Fig. 27.5 Gellan lyase crystals from *G. stearothermophilus* 98. (**a**) Platelike single crystal of gellan lyase. (**b**) Bundle-like crystal roads of gellan lyase

The secondary structural elements for the enzyme from *Bacillus* sp. GL1 were predicted (Miyake et al. 2004) on the base of its primary structure (38.0% helices, 30.0% strands, and 32.0% coils and turns). The results from the analysis of circular dichroism (CD) spectrum revealed 45% β- type structures, about 54% for turns/coils and practically lack of α-spiral domains (Derekova et al. 2010). Predominance of β-structures and small amount of α-helixes confirmed the observation that polysaccharide lyases so far analyzed have α- or β-barrel structure (Miyake et al. 2004) and distinguished that enzyme from *Bacillus* sp. GL1 one.

Two types of crystals well reproducible were received for the thermostable gellan lyase and could be successfully utilized for further X-ray structure determinations (Fig. 27.5).

Presently, 19 of 21 PL families have a known three-dimensional structure of at least one representative (Garron and Cygler 2010), and the structure and functional relationships of enzymes such as lyases for polygalacturonan (families PL-1, -3, and -10) (Yoder et al. 1993; Akita et al. 2001; Charnock et al. 2002), alginate (family PL-5) (Yoon et al. 1999), chondroitin (families PL-6 and -8) (Huang et al. 1999; Féthiere et al. 1999), hyaluronan (family PL-8) (Li et al. 2000), and xanthan (family PL-8) (Hashimoto et al. 2003) have been demonstrated. The crystal characterization of the polysaccharide lyases is still in an opening stage. Crystallographic analysis should be further performed for the determination of three-dimensional structure of gellan lyase from both thermophilic and mesophilic source. Miyake et al. (2004) successfully expressed the enzyme from *Bacillus* sp. GL1 in aim to facilitate further X-ray crystallographic analysis. The expressed in *E. coli* protein exhibited gellan lyase activity and showed similar enzyme properties, such as optimal pH and temperature, thermal stability, and substrate specificity, to those of the 130 kDa gellan lyase.

Table 27.4 Properties of gellan lyases synthesized by thermophilic and mesophilic bacteria

Microorganism	Enzyme properties			References
	Optimal temperature, °C	Optimal pH	Thermostability, half-life	
G. stearothermophilus 98		5.0–8.0		Derekova et al. (2006)
Mixed culture	45	7.5	50/10 min	Hashimoto et al. (1996)
Gram-negative bacterial strain	35–40	6.5–7.5		Kennedy and Sutherland (1994)
Bacillus sp. YJ-1	37	7.5		Jung et al. (2006)
Bacillus sp. GL1	45	7.5		Hashimoto et al. (1997)

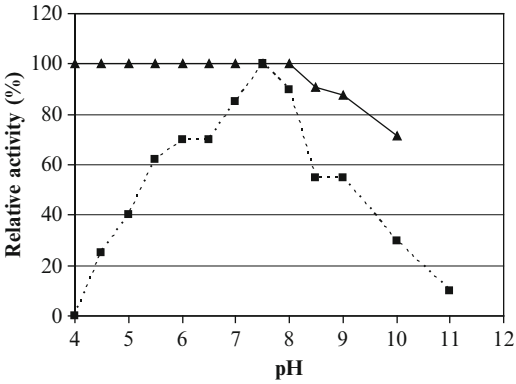

Fig. 27.6 pH optimum for gellan lyase from: ▲ *G. stearothermophilus* 98; --■-- *Bacillus* sp. GL1

27.3.6 Physicochemical Properties

As can be seen from Table 27.4, physicochemical properties of gellan lyases from different sources, the thermostable enzyme from *G. stearothermophilus* 98 was active in the largest pH area (5.0–8.0) which in fact is almost the whole area in which the gellan gum exhibits good stability (3.5–8.0), suggesting the suitability of the enzyme for gellan degradation at different pH according to different industrial demands. Mesophilic enzymes were active at around neutral pH. Comparison of pH profile of activity for the enzymes from *G. stearothermophilus* 98 and *Bacillus* sp. GL1 presented in demonstrates the universality for use at different pH for the thermostable enzyme (Fig. 27.6).

G. stearothermophilus 98 enzyme was active in the range of 50–80°C with temperature optimum at 70°C, and its activity at 55°C was 50% of the activity at 70°C. Temperature profile of the enzymes from *G. stearothermophilus* 98 and *Bacillus* sp. GL1 clearly demonstrates thermophilic character of the thermostable enzyme (Fig. 27.7).

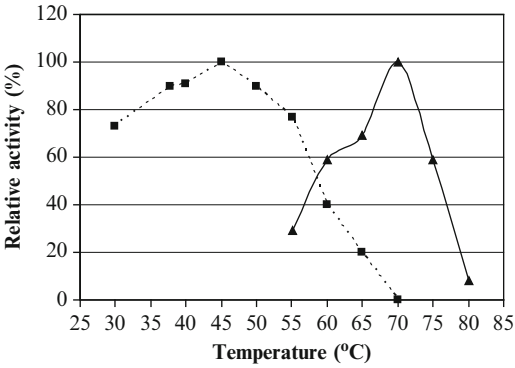

Fig. 27.7 Temperature optimum for gellan lyase from: ▲ *G. stearothermophilus* 98; --■-- *Bacillus* sp. GL1

As can be seen from Table 27.4, all mesophilic enzymes expressed maximum activity up to 45°C.

The thermophilic enzyme expressed unusual high thermostability incomparable with previously described enzymes. Its thermal denaturation curve investigated by means of circular dichroism studies showed a highly cooperative transition with a midpoint at about 75°C. The CD signal in the near-UV range also showed a transition step within the same temperature range, indicating that the tertiary and secondary structure unfolding was parallel (Derekova et al. 2010). The thermal stability of the gellan lyase characterized by differential scanning calorimetry (DSC) expressed large exothermic aggregation near T_m (75°C), rendering the unfolding transition irreversible. Provided calorimetric and circular dichroism spectroscopic data demonstrate valuable thermokinetic evidence for the stability of the enzyme. It retained 100% of its activity after being heated at 60°C for 24 h. Its half-life at 70°C was 50 min in an absence of a substrate. The presence of the substrate additionally stabilized the enzyme, and its residual activity after 2.5 h at 70°C was 100%. Residual activities of other enzymes (Hashimoto et al. 1996) were 100% after 10-min treatment with temperatures up to 40°C. Mesophilic lyase from *Bacillus* sp. GL1 lost 50% of the activity after incubation of the enzyme at 50°C and for 10 min (Mikolajczak et al. 1994). Having in mind that gellan is resolved at temperatures above 60°C, the indicated thermostability of *G. stearothermophilus* 98 gellan lyase determines the enzyme as an ideal candidate for application in the biotechnological processes.

27.3.7 Effect of Metal and Other Compounds on Gellan Lyase Activity

When the influence of different ions (K^+, Li^+, Co^{2+}, Na^+, Ca^{2+}, Fe^{3+}, Fe^{2+}, Mn^{2+}, Mg^{2+}, Hg^{2+}) on enzyme activity was investigated, a stimulating effect for almost all ions tested (up to 38% for K^+ and Ca^{2+}) was observed for the thermophilic gellan lyase

(Derekova et al. 2006). Similar effect for some metal ions was observed for other thermostable enzymes (Rahman et al. 1994; Adinarayana et al. 2004). These results suggest an active role of metal ions in preserving the active enzyme conformation at high temperature by including of additional ion bonds what was confirmed by the action of helatic agents on the enzyme activity. However, while a complete inhibition of gellan lyase occurred when EDTA (ethylene diamine tetra-acetic acid) or EGTA (ethylene glycol-bis-aminoethyl tetra-acetic acid) was added to the mesophilic enzyme solution (Mikolajczak et al. 1994), the thermostable enzymes expressed only a partial inhibition by EDTA (20%), demonstrating once more the inherent stability of the thermostable enzyme molecule. Divalent metal ions such as Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mo^{2+}, Mg^{2+}, and Zn^{2+} showed no effect on the activity of the mesophilic enzyme from *Bacillus* sp. GL1 (Miyake et al. 2004). Unlike the last enzyme, the activity of the enzyme from *Sphingomonas* sp. was slightly (10%) enhanced by the addition of Ca^{2+}. Thermostable enzyme was sensitive to all inhibitors used with highest degree of inhibition toward N-bromosuccinimide, indicating the presence of tryptophan (histidine) in the active center of the enzyme what was further confirmed by Cu^{2+} inhibition. The mercury ion (Hg^{2+}) significantly inhibited the mesophilic enzyme from *Bacillus* sp. GL1 and sulfhydryl agents such as dithiothreitol, glutathione (reduced form), 2-mercaptoethanol, and N-ethylmaleimide (1 mM) exhibited no effect on this enzyme (Miyake et al. 2004). Different modes of inhibition of thermophilic and mesophilic enzymes prove the differences in their active sites.

27.3.8 Enzyme Influence on Viscosity of Gellan Solution

Like all endolytic enzymes, gellan lyases significantly decrease the viscosity of the gellan solution with time due to the polysaccharide depolymerization to tetrasaccharide units. Very effective process of polymer degradation in comparatively short period was observed for polymer degradation by the thermostable enzyme – after just 20 or 24 h of incubation with the substrate at the optimal temperature of 70°C, undegraded gellan represented only 22 and 12% correspondingly (Derekova et al. 2006). Described by Kennedy and Sutherland (1994), gellan lyase degraded slower the gellan at 30°C, and 38% residual gellan was registered after 25 h of incubation.

27.4 Future Perspectives for Gellan Lyase Impact on Gellan Application

Microbial polysaccharides are of high interest for biotechnology due to their rheological properties. As food additives, they may act as thickeners in solutions, gelling agents, and stabilizers in multiphase solutions, or contribute to food quality as lubricants, flocculants, or flavor enhancers. Compared with other polysaccharides, gellan

has many advantages such as an excellent thermal and acid stability, adjustable gel elasticity and rigidity, high transparency, water solubility, compatibility with other polymers, and good flavor release (Giavasis et al. 2000). Gels can be formed with Gelrite in concentrations as low as 0.05%. High-clarity gellan (highly deacetylated and clarified) is suitable for some confectionary products where clarity is a crucial quality issue. Food products that typically incorporate gellan as stabilizer, texturizer, and film-forming and suspending agent include dessert gels, icings and glazes, sauces, puddings, and microwavable foods. Kelcogels are food-grade gellans used as gelling agents in foods (it has E number E418) and personal care applications (lotions, creams, and toothpastes), being mainly used as a stabilizer and suspending agent in a wide variety of applications in the food industry (Sutherland 2001). Gellan can be utilized in confectionery and bakery products (Camelin et al. 1993; Kohajdova and Karovicova 2009), in pet food for preserving of the structured form after heating (Shand et al. 1993), and in dairy products to reduce the loss of solids (Kanombirira and Kailasapathy 1995). Finally, the addition of gellan in fruit and milk beverages has also proven very useful in forming stable, homogeneous products (Duran et al. 1994). The major advantage of gellan here is the pronounced reduction of the setting time of the product.

Gellan has been adopted as one of the most perspective bacterial exopolysaccharides for food industry with a high degree of versatility. A gel of intermediate strength or firmness is required in many industrial applications of gellan, and the structural modification by decreasing the molecular sizes may enhance its physical and biological properties (Mikolajczak et al. 1994). A different consistency is required for different food products in which gellan is used as a gelling agent. Methods for depolymerization were sought to prepare low-viscous gellan. Instead of using chemicals, the use of gellan lyases could be very useful to regulate the viscosity of such gels in ecological friendly processes. A few number of mesophilic gellan lyases were reported in the 1990s. As most of polysaccharides, gellan is soluble at temperatures higher than 60°C, and its modification by a thermoactive enzyme can result in an effective and short process. Depolymerization of gellan by gellan lyase and especially by thermostable gellan lyase results in low-viscosity and low-molecular-mass gellan for novel physiological and food technological functions but also to exploit new areas for the application of gellan (alone or in combination with other hydrocolloids) in biopolymer-based industries.

Gellan applications in the biomedical field include hydrocolloid for slow drug release (Gal and Nussinovitch 2007). Preliminary enzyme treating of gellan for already established pharmaceutical applications like nasal and eye drops (Murano 1998; Jansson et al. 2005; Sultana et al. 2006) will result in more body-friendly consistency of the gel. Hydrocolloid beads based on gellan have been shown to be useful for slow drug release (Gal and Nussinovitch 2007). A speed of drug delivery could be controlled by an enzyme change of the polymer viscosity. The same is valid for the gellan film implanted for insulin delivery and further development of protein delivery systems (Li et al. 2001). Due to its stability, biocompatibility, and biodegradability, several recent patents have concerned applications attempted to obtain gellan capsules to be used as a drug delivery vehicle (Fialho et al. 2008).

Furthermore, possible uses of gellan in the production of capsules, miscellaneous films, and fibers, as well as dental and personal care products could be enlarged after its enzyme treatment and obtaining of versatile products (Kelco 1995). The gellan lyase may become a useful agent to liquefy gellan biofilm formed in patients infected with Sphingomonad as has been documented for bacterial alginate lyase used in *Pseudomonas aeruginosa* infections (Murata et al. 1993).

The possible physiological function of oligosaccharides derived after lyase action was considered by different authors. Hashimoto et al. (2002) observed that they promote the proliferation of human epithelium cells and could be used as epidermal growth factors. The enzyme could be used for improving the construction of poly- and oligosaccharides with great potential for advanced biotechnological uses (Hashimoto et al. 2005). The specific oligosaccharide fragments, which are amenable to NMR and other analytical techniques proved to be extremely useful in providing information about microbial polysaccharide structures.

Gelrite® is used as a substitute of agar for the culture of thermophilic bacterial species and in plant tissue culture media (Lin and Casida 1984). It is the only gelling agent that could be used for Petri dish cultivation at temperatures higher than 70°C. Gelrite also has potential environmental applications such as in the biodegradation of gasoline (Moslemy et al. 2004) and for the transportation of gel-encapsulated bacteria for bioaugmentation of contaminated aquifers (Moslemy et al. 2003). Endoglycanases might be used to resolve and remove the gellan gels from cultured plant tissues when gellan is used as a replacement for agar and agarose in culture media. Gellan was also proved to be a suitable material for the construction of 3D scaffolds for tissue engineering as it supports functional activity of cells immobilized on Gelrite matrix (Ciardelli et al. 2005; Bertoni et al. 2006; Smith et al. 2007). It could be used as an impregnant in paper industry in combination with starch.

27.5 Conclusions

Although gellan is interesting for many commercial applications, still the costly downstream processing steps impair the economic viability of gellan production, and it is the most expensive among food gums (Kohajdova and Karovicova 2009). From the other hand, its highly viscous properties have sometimes largely limited its utility, particularly in the food industry.

Thermostable gellan lyase exploitation will improve significantly gellan conversion not only because of its solubility at high temperature but also because of the decreased viscosity of the reaction mixture at elevated temperature. The first-described thermostable gellan lyase paves the way to establish highly effective processes for gellan modification followed by enlarging of area for its application. The experimental platform revealed a number of advantages in exploiting this enzyme. A fermentation process for enzyme production continues only 20 h, and enzyme productivity could be increased significantly in conditions of continuous cultures. The enzyme showed incomparable with other gellan lyases pH activity and stability

in a large pH area coinciding with the area for gellan stability and the area of almost all food products. This enzyme expressed unusual high thermostability that could suggest effective processes of gellan depolymerization at temperatures higher than 60°C at what temperature the substrate is soluble. The investigations on the thermostable gellan lyase produced by *Geobacillus stearothermophilus* 98 clearly showed that it is a new enzyme with interesting properties. First reported thermostable enzyme (Derekova et al. 2006) clearly showed that it is a new enzyme with interesting properties. Some of the described properties, like secondary structures, circular dichroism behavior, and crystal formation (Derekova et al. 2010), cannot be compared with other gellan lyases as similar results are still not available.

Although believed to provide tremendous economical benefits, production of the enzyme to the level required by the industries has remained a challenge. The way to obtain substantial enzyme amount is to clone the gene; however, the huge enzyme molecule hinders handling with this enzyme.

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Chapter 28
The Lignocellulolytic System of Thermophilic Fungi and Actinomycetes: Structure, Regulation, and Biotechnological Applications

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Abstract Thermophilic fungi and actinomycetes have been extensively studied in vegetal biomass bioconversion processes for the formulation of industrial enzymatic pools and as gene donors for the heterologous expression of thermostable enzymes. The production of second-generation biofuels and the application in industries such as the textile are of particular interest. In this chapter, we have reviewed the gene structure, gene regulation, biochemical properties, and biotechnological applications of lignocellulolytic enzymes and other potential industrial hydrolases of thermophilic fungi and actinobacteria. Besides *Humicola grisea* var. *thermoidea*, the object of study of our group for several years, we focus the following fungi: *Humicola insolens*, *Aureobasidium pullulans*, *Candida peltata*, *Chaetomium thermophilum*, *Coprinopsis cinerea*, *Ganoderma colossum*, *Malbranchea pulchella var. sulfurea*, *Melanocarpus albomyces*, *Rhizomucor pusillus*, *Myceliophthora thermophila*, *Myriococcum thermophilum*, *Penicillium duponti*, *Sporotrichum pulverulentum*, *Sporotrichum thermophile*, *Stilbella thermophila*, *Talaromyces emersonii*, *Thermoascus aurantiacus*, *Thermomyces lanuginosus*, and *Thielavia terrestris*. Among the actinomycetes, we explored *Acidothermus cellulolyticus*, *Cellulomonas* spp., *Streptomyces* spp., *Thermobifida fusca*, and *Thermomonospora curvata*.

Keywords Fungi • Actinobacteria • Thermophilic hydrolytic enzymes • Lignocellulosic residues • Biomass conversion

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28.1 Introduction

28.1.1 History and Applications

Particular interest in cellulolytic microorganisms began during World War II, in the Solomon Islands (Southeast Pacific), when the troops' clothes, tents, and other cellulosic fabrics were often degraded by the fungus latter classified as *Trichoderma reesei*. Soon, what was initially seen as an economical problem became a promising researching area, aiming the conversion of cellulose in products such as recycled paper, animal feed, and plant fertilizers. In the textile industry, cellulases have been employed in the formulation of detergents and in the biostoning and biopolishing processes; since textile industry processes normally take place at 60°C (Cavaco-Paulo 1998), thermostable enzymes produced by thermophilic microbes are particularly useful.

The crude oil crises in the mid-1970s draw the attention to the studies on alternative and renewable energy sources. Second-generation bioethanol production from lignocellulosic residues is at the moment the main driving force for cellulolytic microorganism research. Since our research group has been involved in investigating the cellulolytic system of the thermophilic deuteromycete *Humicola grisea* var. *thermoidea* in order to generate fermentable sugars from sugarcane bagasse, we will further stress this topic.

Numerous efforts have been made in order to decrease the petroleum dependence. In the last 50 years, the global consumption of liquid petroleum has tripled (Lovins et al. 2004). The energy demand is projected to grow more than 50% by the year 2025 due to the fast growth rates and progress of many developing nations. Furthermore, fossil petroleum resources will possibly be serious compromised in the next 50–100 years (Hood et al. 2007). Nowadays, the dependence of petroleum is considered a factor that undermines the economical strength and threatens the national energy security of many countries around the globe (Ransom et al. 2007).

Another important question concerning the use of petroleum derivatives is the sustainability issue. Many governments aim the substantial reduction of the petroleum derivatives employment and the decrease of greenhouse gas emissions. The US Department of Energy and the European Union have fixed goals such as replacing part of liquid petroleum transportation fuel for biofuels and the gradual substitution of industrial organic chemicals by biomass-derived chemicals up to the year 2025 (Ragauskas et al. 2006; Hoffert et al. 2002).

The fuel ethanol industry has been expanding worldwide, particularly in the United States, Brazil, India, and in many European Union countries (Ragauskas et al. 2006). Biomass offers a renewable, abundant, and inexpensive source of carbon. The use of lignocellulosic biomass represents an extraordinary petroleum's substitute which enhances energy security, can reduce greenhouse gas emissions, improves the economy, and diminishes problematic solid wastes such as sugarcane bagasse, corn stover, and wheat and rice straw. In this view, industrial residues, such as pulp and paper processing wastes, are also important (Wyman 2003).

Instead of using petroleum as raw material, biorefineries use biomass composed primarily of renewable polysaccharides and lignin in order to produce transportation fuels, coproducts, and direct energy (Ragauskas et al. 2006). At first, biorefineries extract high-value chemicals already present in the biomass, such as fragrances, flavoring agents, food-related products, and high-value nutraceuticals which provide health and medical benefits. The process continues with the separation of less valuable products and is finally shifted to biofuel production.

At the present day, the major problem of using lignocellulosic biomass for biofuel production is the high cost. Biomass conversion to fuel ethanol comprises stages such as transportation of the biomass material to the site of treatment, pretreatment of the biomass (chemical and/or physical), hydrolysis of the raw material into fermentable sugars (saccharification) and its subsequent transformation into innumerable fuels and chemicals (Knauf and Moniruzzaman 2004). Due to the costs, the production of hydrolytic enzymes is still an impediment to the large-scale use of vegetal biomass for producing energy, chemicals, or fuel.

The discovery of new microbial hydrolytic enzymes is of great interest for the bioethanol industry. A suitable enzyme for this purpose should be resistant to the pretreatment stages, be resistant to pH and temperature oscillations which may occur during the fermentation process, be accessible, cheap, and have a good catalytic efficiency.

Recently, much attention has been directed to thermophilic microorganisms which synthesize extracellular hydrolases – particularly cellulases, xylanases, pectinases, ligninases, and chitinases. Thermophilic microbe proteins are great alternatives for the enzymes currently in use. These proteins remain active even at high temperatures and acidic pH which are usual during the biomass pretreatment or the fermentation process.

28.1.2 The Fungal Cellulolytic System

Cellulose is the most abundant biopolymer in nature. It is composed by glucose units linked by β-1,4-glycosidic bonds. In spite of this simple chemical composition, the physical structure is highly complex, since from 100 to 15,000 linear chains are held together by hydrogen bonds and by van der Waals forces, thus creating rigid and insoluble microfibrils. In nature, cellulose occurs associated with xylan and lignin and often presents a heterogeneous crystallinity, with amorphous regions embedded in highly ordered crystalline regions (for review, see Klemm et al. 2005; Shen and Gnanakaram 2009; Park et al. 2010). Due to this complex physical structure, different enzymatic activities are necessary in order to achieve the cellulose complete saccharification into glucose molecules.

It is commonly accepted that cellulose degradation by fungi is achieved by the synergistic action of three major classes of enzymes: endoglucanases (3.2.1.4), exoglucanases or cellobiohydrolases (EC 3.2.1.91), and β-glucosidases (3.2.1.21). These enzymes act in combination to hydrolyze crystalline cellulose to smaller oligosaccharides

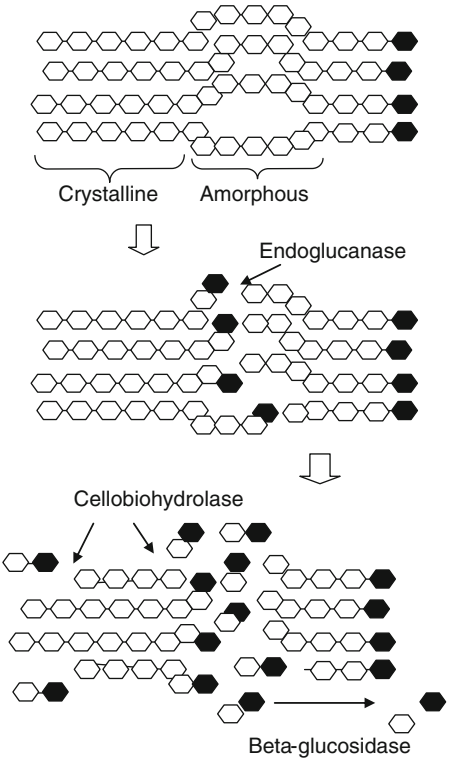

Fig. 28.1 Cellulolytic enzymes synergism. Glucose residues are shown as *hexagons*, and reducing ends are *black*. The *arrows* show cellulases action in degrading cellulose (Modified from Béguin and Aubert 1984)

and finally to glucose. Endoglucanases normally catalyze random cleavage of internal bonds of the amorphous regions of the cellulose chain, thus providing new chain ends for the action of cellobiohydrolases. These late enzymes act in a processive manner from the reducing or nonreducing ends of the polysaccharide chains, liberating either glucose or cellobiose as the major products. β-Glucosidases complete the process through the hydrolysis of cellobiose and other cello-oligosaccharides to glucose monomers (Eriksson 1981; Knowles et al. 1987; Ilmén et al. 1997) (Fig. 28.1).

28.2 Critical Review and Analysis of Fungi and Actinomycetes Thermophilic Enzymes Involved in Lignocellulose Degradation

28.2.1 Filamentous Fungi and Yeasts

28.2.1.1 *Humicola grisea* var. *thermoidea*

Humicola grisea var. *thermoidea* was isolated from Brazilian soil by Dr. Dayson Olsany, University of Viçosa (UFV), Viçosa-MG, Brazil (Araújo et al. 1983).

Table 28.1 Glycosyl hydrolase-encoding genes from *H. grisea* var. *thermoidea*

Gene	Gene product	Structural features	References
cbh1.1	CBH1 – cellobiohydrolase	catalytic domain, CBM[a]	Azevedo et al. (1990)
cbh1.2	CBH1.2 – cellobiohydrolase	catalytic domain, without CBM	Poças-Fonseca et al. (1997)
egl1	EGL1 – endoglucanase	catalytic domain, without CBM	Takashima et al. (1996)
egl2	EGL2 – endoglucanase	catalytic domain, CBM	Takashima et al. (1997)
egl3	EGL3 – endoglucanase	catalytic domain, CBM	Takashima et al. (1999a)
egl4	EGL4– endoglucanase	catalytic domain, without CBM	Takashima et al. (1999a)
bgl4	BGL4 – β-glucosidase	catalytic domain, without CBM	Takashima et al. (1999b)
xyn1	XYN1 – xylanase	catalytic domain, CBM	Iikura et al. (1997)
xyn2	XYN2 – xylanase	catalytic domain, without CBM	De-Faria et al. (2002)

[a]Carbohydrate-binding mobile

Our research group has been investigating the molecular genetics of *H. grisea* var. *thermoidea* lignocellulolytic system since late 1980s, and we have demonstrated that this fungus is a potent cellulase producer (Azevedo et al. 1990; Poças-Fonseca et al. 1997, 2000).

H. grisea presents a considerable potential for agricultural wastes bioconversion processes. Its hydrolytic system is able to perform the complete saccharification of lignocellulose from different kinds of substrates, including sugarcane bagasse, ball-milled straw (De-Paula et al. 1999, 2003; Rossi et al. 2007), brewers' spent grain, and wheat bran (Mandalari et al. 2008). *H. grisea* thermostable enzymes have already been employed as bleaching agents for *Eucalyptus* kraft pulp (Medeiros et al. 2002) and organosolv (ethanol/water and acetosolv) sugarcane bagasse pulps (Moriya et al. 2005). Moreover, enzyme preparations from *H. grisea* were successfully employed in enhancing the β-glucosidase activity of cellulases mixtures from *Trichoderma viride* (Takashima et al. 1999a, b) and from the *Trichoderma reesei* hyperproducer mutant RP-98 strain (Nascimento et al. 2010).

Major efforts in gathering scientific knowledge towards the promising utilization of *H. grisea* for biomass conversion focus three main streams: enzymatic activities characterization, gene cloning, and heterologous expression.

H. grisea produces a complex hydrolytic system which comprises several enzymatic activities, such as endoglucanase (Hayashida and Mo 1986), β-glucosidase (Peralta et al. 1990; Ferreira-Filho 1996; Nascimento et al. 2010), trehalase (Zimmermann et al. 1990; Lúcio-Eterovic et al. 2005), glucoamylase (Campos and Felix 1995), xylanases (Monti et al. 1991; Almeida et al. 1995; Silva et al. 2000; Lucena-Neto and Ferreira-Filho 2004), and feruloyl esterase (Mandalari et al. 2008). These enzymatic activities display high thermostability, which renders to *H. grisea* secreted enzymes interesting characteristics for industrial purposes.

The lack of wide genome information for *H. grisea* prompted the molecular cloning of several glycosyl hydrolase genes, mainly cellulases (Table 28.1). These data are relevant in order to better understand this fungus enzymatic pool. The existence

Table 28.2 Some properties of *H. grisea* var. *thermoidea* recombinant glycosyl hydrolases

Enzyme	Host	Optimal pH	Optimal temp (°C)	Thermostability (min)	Mol mass (KDa)	References
rCBH1.1	*A. oryzae*	5.0	60	10 (55°C)	67	Takashima et al. (1996)
rEGL1	*A. oryzae*	5.0	55–60	10 (60°C)	58	Takashima et al. (1996)
rEGL2	*A. oryzae*	5.0	75	10 (75°C)	55	Takashima et al. (1997)
rEGL3	*A. oryzae*	5.0	60	10 (80°C)	43	Takashima et al. (1999a)
rEGL4	*A. oryzae*	6.0	75	10 (80°C)	25	Takashima et al. (1999a)
rEGL4CBD[a]	*A. oryzae*	6.0	55	10 (80°C)	44	Takashima et al. (1999a)
rBGL4	*A. oryzae*	6.0	55	10 (50°C)	57	Takashima et al. (1999b)
rBGL4	*S. cerevisiae*	6.0	40	60 (40°C)	57	Benoliel et al. (2010)
rXYN2[b]	*T. reesei*	NR	NR	NR	23	De-Faria et al. (2002)

[a]Consists of a catalytic domain of EGL4 and the C-terminal region of EGL3, the carbohydrate-binding mobile
[b]Not purified

of several gene products belonging to the same hydrolase category, in spite of bearing different structural features, corroborates our initial experimental data that pointed out to a highly complex enzymatic system, composed by multigenic families (Poças-Fonseca et al. 1997).

Cellulase production by fungi is mainly regulated at the transcriptional level. Substrates such as cellulose, lactose, and sophorose act as inducers, while glucose normally acts as gene repressor. The presence of a low-level constitutive enzyme is required to initiate cellulose degradation, thus generating soluble inducers that can enter the cell and activate major cellulase gene transcription (Kubicek et al. 1988; El-Gogary et al. 1989).

In this chapter, we present the state of the art on cellulolytic fungi and actinomycetes research from a biotechnological perspective. A special attention will be paid to our traditional model of study, the thermophilic deuteromycete *Humicola grisea* var. *thermoidea*.

Gene cloning efforts opened the possibility of using *H. grisea* as gene donor for alternative producing hosts. The first attempts of heterologous expression were made in *Aspergillus oryzae,* aiming the refined biochemical characterization of the thermostable activities. So far, *cbh1.1*, *egl1*, *egl2*, *egl3*, *egl4,* and *bgl4* have been expressed in this microorganism (Takashima et al. 1996, 1997, 1999a, b). De-Faria et al. (2002) successfully produced in *T. reesei* a recombinant version of *H. grisea* xylanase 2 (*xyn2*) under the control of the *T. reesei* main cellobiohydrolase gene (*cbh1*) promoter. A summary view of *H. grisea* recombinant glycosyl hydrolase properties is presented in Table 28.2.

Saccharomyces cerevisiae has been used to co-express fungal exo/endocellulases and β-glucosidases aiming the production of ethanol from lignocellulosic materials (van Rensburg et al. 1998; Fujita et al. 2002). As a first step in the development of a *S. cerevisiae* strain capable of producing ethanol from cellobiose, our group reported the heterologous expression and biochemical characterization of *H. grisea* BGL4, a β-glucosidase (Benoliel et al. 2010). The most remarkable feature of this enzyme is a high resistance to glucose inhibition (inhibition constant (*K*i) of 70 mM), which renders BLG4 an appropriate enzyme for saccharification processes. Nascimento et al. (2010) reported that the wild-type purified enzyme activity is stimulated up to twofold by glucose concentrations up to 200 mM.

H. grisea cellulase gene molecular cloning also prompted gene regulation studies. Efforts have been made to identify *cis*-acting elements and *trans*-acting factors involved in these genes' expression. The understanding of how hydrolase genes are regulated may help the enhancement of enzyme production in the natural host by nutritional approaches and also the improvement of heterologous proteins expression under the control of hydrolase genes promoters.

Our group had previously shown, by means of a RT-PCR approach, that *H. grisea cbh1.1* and *cbh1.2* genes are induced by a complex carbon source (0.1% ball-milled straw) (Poças-Fonseca et al. 1997). *H. grisea creA* transcription repressor was isolated, and it seems to be related to the repression of *egl1* and *cbh1.2* (*exo1*) genes (Takashima et al. 1998b). In this last work, a Northern blot analysis revealed that both genes were downregulated when mycelium was grown for 15 h in 1% glucose. Another study performed a time-course analysis of cellulases transcript accumulation and reveled, also by Northern hybridization, that *egl1*, *egl2*, *egl3*, *egl4,* and *cbh1.2* are highly induced by 1% Avicel, with the *cbh1.2* gene presenting an earlier and stronger induction (Takashima et al. 1999b). In another Northern blotting time-course transcript accumulation analysis, performed by our group (De-Paula et al. 1999), we detected the early presence of the *cbh1.1* mRNA (6 h) when mycelium was grown in 0.1% sugarcane bagasse as the sole carbon source, whereas no transcript could be detected when 0.1% glucose was employed. In spite of the useful information obtained from Northern blotting experiments, this technique is less sensitive than PCR-based approaches, and thus, mRNAs produced in the earlier periods of induction might not have been detected.

More recently, we established the early time-course (0.5–18 h) expression profiles for several *H. grisea* hydrolase-encoding genes when the fungus was grown at different culture medium pH values and distinct carbon sources (Mello-de-Sousa et al. 2011). We observed a remarkable and parallel increase in mRNA accumulation at 2 h for *cbh1.1*, *cbh1.2*, *egl1*, *egl2*, *egl3*, *bgl4,* and *xyn1* genes at an alkaline milieu (pH 8.0) and with sugarcane bagasse as the sole carbon source. A distinct profile was observed for *egl4*, whose mRNA preferably accumulated in acidic conditions. When glucose was used as the sole carbon source, gene expression was repressed and the culture medium pH value had no effect on transcript accumulation. These data clearly demonstrate that *H. grisea* cellulolytic system is indeed subject to glucose repression, as it was previously suggested (Takashima et al. 1998a; De-Paula et al. 1999; Poças-Fonseca et al. 2000). Nonetheless, glucose did not lead to a complete shutdown of hydrolase gene transcription, since this sugar

total consumption was not sufficient to trigger gene expression. In this view, a mere derepression mechanism could be ruled out, indicating that *H. grisea* also seems to require a lignocelluloses-derived inducer.

We have also investigated the influence of the transcription factors involved in pH regulation and carbon repression mechanisms (PacC and CreA, respectively) on the expression of *H. grisea* glycosyl hydrolase-encoding genes (Mello-de-Sousa et al. 2011). Through in vitro pull-down DNA assays and EMSA analyses employing PacC and CreA DNA-binding domains (DBDs), we gathered information which supported the CreA-mediated carbon repression and the PacC-related pH regulation of *H. grisea* cellulase and xylanase genes. Moreover, EMSA analyses suggested a role for CreA on *PacC* transcription regulation, thus establishing a regulatory cross talk.

28.2.1.2 *Humicola insolens*

The thermophilic and saprophytic fungus *H. insolens* is a suitable producer of hydrolytic enzymes. It produces at least seven different cellulases and two xylanases (Schülein 1997). These seven cellulase genes were cloned and expressed in the noncellulolytic host *A. oryzae*, aiming the catalytic activity characterization and specificities confirmation. Schülein (1997) employed carboxymethyl cellulose (CMC) to determine the pH activity profiles of the five endoglucanases (EG I-V), whereas cellotriose was used to determine the pH activity profiles of cellobiohydrolase I (CBH I) and CBH II. All the EGs showed optimal activity between pH 7 and pH 8.5, while CBH I and CBH II activity peak was determined around pH 5.5 and pH 9, respectively. More recently, Luo et al. (2012) expressed in *P. pastoris* a thermostable and alkali-tolerant mannanase, Man5A, which exhibits optimal activity at pH 5.5 and at 70°C. Forty-five and thirty-six percent of the maximal activity was maintained at pH 8.0 and 9.0, respectively. These data suggest Man5A as a suitable enzyme for usage in the kraft pulp industry.

Due to the highly thermostable enzymatic activities (Hayashida and Yoshioka 1980), special attention has been dispensed to determine the 3D structures of several *H. insolens* celullases, aiming the refined understanding of these enzyme action mechanisms (Mackenzie et al. 1998; Otzen et al. 1999; Varrot et al. 1999a, b, 2003 Davies et al. 2000). An interesting example of how such data can help the improvement of new degradating systems is the development of a multifunctional GH43 arabinofuranosidase enzyme by the modification of the rim of the active site, which introduced an endo-xylanase activity (McKee et al. 2012).

H. insolens biotechnological potential prompted some efforts towards the optimization of enzymatic cocktails aiming the crystalline cellulose (Boisset et al. 2001), brewer's grain, wheat bran (Faulds et al. 2004), and whole vinasse (Sørensen et al. 2007) degradation.

In addition to the classical employment of cellobiohydrolases, endoglucanases, and beta-glucosidases, other enzymes have been considered in helping the complete breakdown of lignocellulosic substrates. Langston et al. (2011) proposed the usage

of a cellobiose dehydrogenase (CDH; EC 1.1.99.18) from *H. insolens*. The authors demonstrated that a combination of CDH with the glycoside hydrolase 61 from *Thermoascus aurantiacus* (GH61A) enhanced the bioconversion of microcrystalline, although the specific biochemical mechanism by which CDH and GH61A cooperate remains unknown.

28.2.1.3 *Aureobasidium pullulans*

A. pullulans is a world-spread yeastlike fungus, also known as black yeast due to its melanin production (de Hoog and Yurlova 1994). It comprehends three varieties: *A. pullulans* var. *pullulans*, *A. pullulans* var. *melanigenum*, and *A. pullulans* var. *aubasidani* (Yurlova and de Hoog 1997). These can be found in environments such as soil, water, wood and other plant-derived materials, rocks, limestone, hypersaline habitats, coastal water, and deep sea (Urz'i et al. 1999; Gunde-Cimerman et al. 2000; Li et al. 2007).

In the past 20 years, *A. pullulans* lignocellulolytic enzymes have been exploited for biotechnological purposes. *A. pullulans* color variants overproduce extracellular xylanases (Leathers 1986) and cellulases (Kudanga and Mwenje 2005). This fungus also presents thermostable starch-degrading activities such as α-amylase, glucoamylase, and α-glucosidase (Saha et al. 1993). Efforts have been made aiming the purification and biochemical characterization of *A. pullulans* strains glycosyl hydrolases (Table 28.3).

A. pullulans cellulase- and hemicellulase-encoding genes were expressed in different hosts; *S. cerevisiae* and *Pichia pastoris* seem to be the most common choices. Li and Ljungdahl (1996) expressed *xynA* from *A. pullulans* NRRL Y-2311-1 in *S. cerevisiae*, producing an active xylanase when the natural XynA signal peptide was employed. An alfa-L-arabinofuranosidase gene (*abfA*) was also expressed in *S. cerevisiae* (de Wet et al. 2008). The hyperproducing yeast *P. pastoris* served as host for the expression of an *A. pullulans* var. *melanigenum* (ATCC 20524) xylanase (*xynII*) and a β-xylosidase (*xylI*) gene (Tanaka et al. 2006; Ohta et al. 2010). Another xylanase gene (*xyn1*) from the same strain was expressed in *S. cerevisiae* (Ohta et al. 2001); although the recombinant enzyme was not completely characterized, it presented a lower specific activity when compared to the native enzyme. *Aspergillus niger* has also been employed as heterologous host: Tambor et al. (2012) expressed the *A. pullulans* NRRL Y-2311-1 Cel5A endoglucanase gene and obtained a recombinant enzyme with a hydrolysis rate on carboxymethyl cellulose 4M five times higher than the one presented by *T. reesei* Cel5A.

Considering *A. pullulans* gene expression regulation, some transcription factor-binding sites were reported in the 5′ noncoding gene regions. Potential binding sites for the *A. niger* xylanase transcription activator XlnR (van Peij et al. 1998) were observed in *xynI* (Ohta et al. 2001) and *xylI* genes (Ohta et al. 2010). Binding sites for the carbon catabolite repression protein CreA were also reported in *xynI* (Ohta et al. 2001), *xynII* (Tanaka et al. 2006), and *xylI* (Ohta et al. 2010) regulatory regions. Moreover, possible *cis*-acting elements for the pH regulation-related transcription

Table 28.3 Some properties of glycosyl hydrolases isolated from *A. pullulans* strains

Strain	Enzymatic activity	Optimal pH	p*I*	Optimal temp (°C)	Thermostability (min)	Mol mass (kDa)	References
ATCC 20524	Xylanase	2.0	6.7	50	30 (50°C)	24	Ohta et al. (2001)
ATCC 20524	Xylanase	6.0	8.9	70	30 (70°C)	39	Tanaka et al. (2006)
ATCC 20524	β-Xylosidase	3.5	NR	70	30 (60°C)	88.5	Ohta et al. (2010)
ER-16	Endoglucanase	4.0–4.5	NR	60	60 (50°C)	NR	Leite et al. (2007)
ER-16	β-Glucosidase	4.0–4.5	NR	75	60 (75°C)	NR	Leite et al. (2007)
ER-16	Xylanase	5.0	NR	50	60 (50°C)	NR	Leite et al. (2007)
NRRL Y-2311-1 (CV)	Xylanase	4.0–5.0	NR	35–50	NR	20–21	Leathers (1986)
NRRL Y-2311-1 (CV)	Xylanase	4.8	9.4	54	30 (55°C)	25	Li et al. (1993)
NRRL Y-2311-1 (CV)	Feruloyl esterase	6.7	6.5	60	NR	210	Rumbold et al. (2003)
NRRL Y-2567 (TP)	Xylanase	4.0–5.0	NR	35–50	NR	20–21	Leathers (1986)
NRRL Y-I2974 (CV)	α-Amylase	5.0	NR	55	60 (45°C)	NR	Saha et al. (1993)
NRRL Y-I2974 (CV)	Glucoamylase A	4.5	NR	50–60	60 (45°C)	NR	Saha et al. (1993)
NRRL Y-I2974 (CV)	Glucoamylase B	4.5–5.0	NR	65	60 (45°C)	NR	Saha et al. (1993)
NRRL Y-I2974 (CV)	α-Glucosidase	4.5	NR	65	60 (60°C)	NR	Saha et al. (1993)
Y-I2974 (CV)	β-Glucosidase	4.5	NR	75	60 (60°C)	340	Saha et al. (1994)
NRRL Y-I2974 (CV)	α-L-arabinofuranosidase	4.5–5.0	NR	75	30 (75°C)	210	Saha and Bothast (1998)

[a]Abbreviations: *CV* color variant, *TP* typically pigmented, *NR* not reported

factor PacC were found for *xynII*, and quantitative real-time polymerase chain reaction analysis revealed that *xynII* transcription levels at pH 8.0 were 22-fold higher than at pH 2.7 (Tanaka et al. 2006).

28.2.1.4 *Candida peltata*

Until recently more studied for xylitol production purposes (Saha and Bothast 1999; Park et al. 2005), the yeast *C. peltata* had previously been quoted for cellulases screening. Saha and Bothast (1996) described the purification of a β-glucosidase characterized as a monomeric protein, presenting optimum activity at pH 5.0 and at 50°C, being stable for up to 30 min at 45°C. This enzyme presented high tolerance to glucose (*K*i 1.4 M) during pNPG hydrolysis, which enables its utilization for the enzymatic conversion of cellulosic biomass to glucose.

28.2.1.5 *Chaetomium thermophilum*

The thermophilic ascomycete *C. thermophilum* also awakes interests for biotechnological applications. In order to determine the enzymatic properties, these fungus glycosyl hydrolases-encoding genes have been cloned and expressed in *P. pastoris* and *T. reesei* (Table 28.4). Cellobiohydrolase II (CBHII)-encoding gene was subject to in vitro directed evolution (Wang et al. 2012) aiming the thermostability increasing. In addition to an enhanced thermal stability, the two CBHII mutant versions produced in *P. pastoris* also presented higher optima temperature and pH values (60°C, pH 5–6) in comparison to the wild-type enzyme (50°C and pH 4).

Not only thermostability accounts for *C. thermophilum* enzymes' advantages. Voutilainen et al. (2008) reported that the acidic cellobiohydrolase Cel7A produced in *P. pastoris* was more thermostable (by 10°C) and more active (by fourfold) in the hydrolysis of microcrystalline cellulose when compared to *T. reesei* Cel7A, thus representing a competitive choice for industrial purposes. Another interesting example of such a versatility is xylanase Xyn11A produced in *T. reesei* (Mäntylä et al. 2007). Tests performed at pH 7.0 and at 70°C indicated that this enzyme could be commercially feasible for industrial-scale bleaching of kraft pulp at high temperatures.

28.2.1.6 *Coprinopsis cinerea*

C. cinerea (formerly *C. cinereus*) is a multicellular basidiomycete which presents a typical mushroom form and undergoes a complete sexual cycle. This fungus secretes a complex array of proteins. When *C. cinerea* was grown in liquid complex medium containing glucose, yeast extract, and mineral salts, it secreted several proteins that could be identified by 2-D gel electrophoresis and mass spectrometry analyses: glyoxal oxidases, β-glucosidases, β-1,3-glucanases, glucoamylases, metallo-peptidases and serine-peptidases (Hoegger et al. 2008).

Table 28.4 Some properties of recombinant glycosyl hydrolases from *C. thermophilum*

Enzyme	Host	Optimal pH	Optimal temp (°C)	Thermostability (min)	Mol mass (KDa)	References
Glucoamylase	*P. pastoris*	4.0–5.0	65	60 (60°C)	66	Chen et al. (2007)
CBH3 (cellobiohydrolase)	*P. pastoris*	5.0	60	60 (60°C)	48	Li et al. (2009)
CHIT1 (chitinase)	*P. pastoris*	5.5	60	60 (60°C)	47.3	Li et al. (2010)
Xyn11A (xylanase)	*P. pastoris*	6.5	70	10 (80°C)	24	Ghaffar et al. (2010)
BGL (β-glucosidase)	*P. pastoris*	5.0	60	55 (65°C)	119	Xu et al. (2011)
Xyn11A (xylanase)	*T. reesei*	6.0	70	60 (70°C)	27	Mäntylä et al. (2007)
Xyn11B (xylanase)	*T. reesei*	6.0	70	60 (70°C)	23	Mäntylä et al. (2007)
Cel7A (cellobiohydrolase)	*T. reesei*	4.0–5.0	65–70	[a]	54.6	Voutilainen et al. (2008)

[a]The thermostability of Cel7A was determined by temperature-induced unfolding studies employing circular dichroism analysis

C. cinerea glycosyl hydrolase activities were recently further studied. Five genes encoding family 6 enzymes (GH6), *CcCel6A*, *CcCel6B*, CcCel6C, *CcCel6D,* and *CcCel6E,* were cloned and their transcripts levels were estimated in cultures containing various distinct carbon sources (Yoshida et al. 2009). Transcription of four genes was clearly stimulated in the presence of cellulose and inhibited by glucose. The exception was *CcCel6C,* which seems to be expressed in constitutive levels. *CcCel6A* transcript was the most abundant in cellulose-grown cultures. Cellobiose also strongly induced the *CcCel6A* gene transcription, but weakly induced transcription of the other genes. CcCel6A was predicted to be a cellobiohydrolase, while the other CcCel6s have been mapped to a distant region in the enzyme evolutionary tree.

CcCelA, CcCel6B, and CcCel6C were expressed in *E. coli,* and the enzymatic properties were investigated (Liu et al. 2009). These enzymes exhibited cellobiohydrolase activity towards Avicel, and CcCelA showed the highest activity. On the other hand, CcCel6B and CcCel6C were able to hydrolyze carboxymethyl cellulose (CMC), whereas CcCelA was not. This indicates a possible endoglucanase activity for CcCelB and CcCelC. This observation was corroborated by the CcCelC crystal structure analysis (Liu et al. 2010).

Another example of *C. cinerea* as donor of genes for heterologous expression is the production in *P. pastoris* of CcAbf62A, a putative extracellular arabinofuranosidase (Hashimoto et al. 2011). The recombinant enzyme presented optimal conditions at pH 7.0 and at 45°C, releasing L-arabinose from both wheat arabinoxylan and oat-spelt xylan.

28.2.1.7 *Ganoderma colossum*

Considered as a rare species in the *Ganodermataceae* family, the basidiomycete *G. colossum* has not been mostly explored for biotechnological applications. This fungus was employed in a high-density hardwood (silver leaf oak) and a conifer (white fir) decaying study (Adaskaveg et al. 1991). In comparison to another *Ganoderma* species, *G. colossum* presented similar oak delignification rate (67.9%). However, *G. colossum* presents additional features, such as sexual reproduction, large chlamydospores production quantities, rapid growth, and best delignification rate at 40°C (Adaskaveg et al. 1995), thus being quoted as an excellent white rot fungus for lignocellulose delignification studies and for industrial applications of delignifying fungi, such as in biopulping and bioremediation.

28.2.1.8 *Malbranchea pulchella* var. *sulfurea*

The thermophilic ascomycete *M. pulchella var. sulfurea* glycosyl hydrolases applications have been poorly explored. Its untapped potential has already been subject of few studies, specially focusing hemicellulases. In a series of papers from 1977 to 1985, Matsuo and collaborators reported the purification of a β-xylosidase

and a xylanase from *M. pulchella*. The β-xylosidase exhibited optimum activity at pH 6.2–6.8 and at a temperature of 50°C (Matsuo et al. 1977a) and acted on aryl β-D-xylosides and xylan, but showed no activity on alkyl β-D-xylosides. This enzyme was also reported as being devoided of glucosidase and transglycosidase activities (Matsuo et al. 1977b). On the other hand, the purified xylanase presented an optimum pH in the range between 6.0 and 6.5, and an optimum temperature at 70°C, being active on xylotriose, xylotetraose, xylopentaose, and xylan. Xylobiose and phenyl β-D-xyloside were not hydrolyzed by this xylanase (Matsuo and Yasui 1985).

28.2.1.9 *Melanocarpus albomyces*

The ascomycete *M. albomyces* is an important source of lignocellulolytic enzymes. It produces at least seven thermostable xylanases, presenting optimal activity at pH 5.5–8.0 and at temperatures from 55 to 70°C (Saraswat and Bisaria 2000). Due to the possible employment of *M. albomyces* enzymes in the paper industry, studies aiming the production of high-titer xylanases have been performed both in solid-state fermentation (Narang et al. 2001) as in stirred tank bioreactor (Biswas et al. 2010).

M. albomyces is also a promising candidate to provide industrial cellulases. These fungus culture supernatants presenting two endoglucanases (20 and 50 KDa) and one cellobiohydrolase (50 kDa) activities were employed for denim fabrics indigo dye release (biostoning) (Miettinen-Oinonen et al. 2004). The purified 20-kDa endoglucanase (Cel45A) seemed to be the main responsible for the denim fabric biostoning effect at neutral pH, presenting an optimum temperature of 70°C. Addition of the purified 50-kDa endoglucanase (Cel7A) or of the 50-kDa cellobiohydrolase (Cel7B) to the 20-kDa endoglucanase mixture decreased backstaining in the biostoning process. These same cellulases were successfully expressed in *T. reesei*. Cel7B cellobiohydrolase presented the highest activity against crystalline cellulose, whereas Cel45A and Cel7A endoglucanases were more active on an amorphous substrate (phosphoric acid swollen cellulose) (Szijártó et al. 2008). Moreover, Cel7B was subject to thermostability and activity improvement by the introduction of an additional tenth disulfide bridge to the catalytic module and by the fusion with the cellulose-binding module (CBM) and a linker region from *T. reesei* Cel7A (Voutilainen et al. 2009).

Another important enzymatic activity described for *M. albomyces* is laccase. Since the purification of an unusual thermostable and neutral laccase (MaL) (Kiiskinen et al. 2002), several efforts have been made in order to further characterize this enzyme. The MaL three-dimensional structure was solved (Hakulinen et al. 2002, 2006, 2008; Andberg et al. 2009; Kallio et al. 2009, 2011), and the enzyme was produced in *T. reesei* (Kiiskinen and Saloheimo 2004; Frasconi et al. 2010; Moya et al. 2011), *S. cerevisiae* (Kiiskinen et al. 2004), and transgenic rice (de Wilde et al. 2008).

28.2.1.10 *Mucor pusillus*

More employed for the production of proteases used in curdling milk for cheese production (Aikawa et al. 1992), the thermophilic fungus *M. pusillus* presents a so far not explored cellulolytic potential. When Somkuti et al. (1969) were carrying out the purification of the *M. pusillus* acid protease, they noticed that some fractions presented a cellophane membrane-weakening factor. This activity was attributed to a hall of hydrolytic enzymes which attacked native cellulose, acid-swollen cellulose, carboxymethyl cellulose, and cellobiose. Moreover, Somkuti (1974) reported the inducing properties of wheat bran over the *M. pusillus* cellulolytic system and also a glucose repression mechanism.

28.2.1.11 *Myceliophthora thermophila*

Enzymes from the thermophilic ascomycete *M. thermophila* have been successfully employed for the complementation of a benchmark blend of a commercial cellulase product derived from *T. reesei* (Celluclast) and also of a β-glucosidase derived from *A. niger* (Novozym 188). This complementation enhanced the glucose release from steam-pretreated barley straw (Rosgaard et al. 2006). The production of β-glucosidases by *M. thermophila* is highly induced by *p*-nitrophenyl-*p*-D-glucoside (pNPG) and cellobiose, while a carbon repression mechanism was detected in the presence of glucose (Roy et al. 1988). Additionally, Roy et al. (1990) purified a thermostable 100-kDa endoglucanase whose optima pH and temperature were 4.8 and 65°C, respectively. Additional enzymatic activities explored so far include a recombinant feruloyl esterase produced in *P. pastoris* (Topakas et al. 2012) and a purified extracellular aldonolactonase which could possibly relieve the inhibition of cellulases and β-glucosidases by glucono-δ-lactone and cellobiono-δ-lactone, by-products of the lignocellulose degradation (Beeson et al. 2011).

Although *M. thermophila* presents potential as a cellulolytic microorganism, it is the lignin degradation property that aroused most interest. A *M. thermophila* laccase (MtL) was cloned and expressed in *A. oryzae*. The recombinant protein presented optimal activity at pH 6.5 and retained nearly 100% of activity when incubated at 60°C for 20 min (Berka et al. 1997). The same enzyme was also expressed in *S. cerevisiae* in a directed evolution approach, which resulted in mutants with higher thermostability and enhanced activity (Bulter et al. 2003). Claus et al. (2002) tested the MtL capacity of decolorizing synthetic dyes. Even though MtL presented the lower decolorizing activity, in comparison to laccases produced by the basidiomycetes *Trametes versicolor* and *Polyporus pinisitus*, it still represents an alternative for the treatment of effluents from textile, dye, or printing industries, as well as for pulp bleaching processes. Babot et al. (2011) reported the successful employment of this enzyme for the delignification of eucalypt pulp in the presence of lignin-derived phenolic mediators. The low quantity of enzyme used may stimulate the establishment of a suitable and cost-effective

bleaching process for industrial purposes. Another interesting application of MtL is at the degradation of endocrine-disrupting chemicals, such as estrogenic compounds, which are considered environmental pollutants found in rivers, industrial wastewaters treatment plants, and landfill leachates (Lloret et al. 2011, 2012).

M. thermophila genome was recently sequenced (Berka et al. 2011). The outcome of a pool of 205 putative glycoside hydrolase-encoding genes points out to this fungus as an additional alternative for the prospection of enzymatic activities.

28.2.1.12 *Myriococcum thermophilum*

Also known as *Papulospora thermophila, Myriococcum thermophilum* is a thermophilic ascomycete found mainly in mushroom composts in association with other thermophilic fungi, predominantly from the family *Chaetomiaceae* (Prodromou and Chapman 1974; Zámocký et al. 2008). It was first isolated in a compost pile in Switzerland (Fergus 1971). *M. thermophilum* presents an impressive lignocellulose degradation ability. As consequence, *M. thermophilum* promotes the growth of the white button mushroom *Agaricus bisporus* in compost piles, which consist basically of wheat straw, horse and chicken manure, and gypsum (Straatsma et al. 1994a, b). To date, only one *M. thermophilum* enzyme was described.

In 2008, Zámócký et al. described the isolation and heterologous expression in *P. pastoris* of a gene encoding a thermostable cellobiose dehydrogenase (CDH) from *M. thermophilum*.

Cellobiose dehydrogenases (CDH; E.C. 1.1.99.18; cellobiose:[acceptor] 1-oxidoreductase), formerly called cellobiose oxidases, are extracellular enzymes produced upon cellulases induction conditions by a number of wood degrading and phytopathogenic fungi (Ayers et al. 1978; Zamócký and Dunand 2006). CDH is a two-domain enzyme with a catalytic domain containing FAD and a second domain containing a heme *b* group as cofactors (Zámócký et al. 2006). CDH is a promising enzyme for the development of biosensors (Stoica et al. 2006) and biofuel cells (Tasca et al. 2008). This is due to its ability to electronically communicate directly with electrodes, thus accelerating the oxidation of specific sugars on the electrode without any mediators according to the principle of direct bioelectrocatalysis (Gorton et al. 1999). The heme domain seems to be responsible for the direct electron transfer (DET) communication with the electrode (Freire et al. 2003).

M. thermophilum CDH has an optimal temperature of 60°C and presents the highest activity when lactose or cellobiose is used as carbon source (Zámocký et al. 2008). For biocatalytic processes, this enzyme may offer either a greater operational stability at a given process temperature or the possibility of a higher temperature for the same process. Applications in biosensors, where actual measurement times can be very brief, will benefit from the expected higher shelf life of a thermostable enzyme at ambient temperatures. Therefore, *M. thermophilum* CDH presents relevant biotechnological potential (Zámocký et al. 2008).

28.2.1.13 *Penicillium duponti*

Also known as *Talaromyces thermophilus*, this fungus was first isolated from a compost sample in Japan, at a temperature of 45°C. *P. duponti* is not able to grow at temperatures below 27°C or above 60°C. The optimum growth temperature is between 45 and 50°C (Hashimoto et al. 1972).

P. duponti produces beta-hydrolases with interesting features for industrial use. Guerfali et al. (2009) analyzed the effects of immobilization of a *P. duponti* beta-xylosidase: the enzyme immobilization on chitosan pretreated with glutaraldehyde altered some characteristics such as optimal temperature and pH in comparison to the free enzyme. The maximal activity achieved for the immobilized enzyme was at pH 8.0 and at 53°C, whereas for the free enzyme the optima conditions were at pH 7.0 and at 50 °C.

Enzyme immobilization on various water-insoluble supports has been reported to improve biocatalyst long-term thermostability, to allow the enzyme reusability and the application in continuous operations and also to minimize the time and cost associated with industrial processes (Buchholz et al. 2005).

β-Xylosidase (EC 3.2.1.37) is one component of *P. duponti* hemicellulase complex. It catalyzes the hydrolysis of xylooligosaccharides, such as xylobiose and xylotriose, to xylose by recognizing the xylosyl residue at the nonreducing end and by cleaving the beta-1,4 glycosidic bonds (Rodionova et al. 1983). Therefore, this enzyme has a great potential in many biotechnological applications, particularly in paper pulp, food, beverage, and animal feed bioconversion industries (Sunna and Antranikian 1997).

In 2009, Maalej et al. reported the purification and characterization of a *P. duponti* cellulase-free secreted thermostable xylanase belonging to the glycosil hydrolase family G11. This enzyme presented high activity between pH 4 and 10 (optimum pH around 7–8). Optimal temperature was determined at 75°C. The enzyme showed high thermal stability at 50°C (for 7 days) and the half-life at 100°C was 60 min. These are promising characteristics for many industrial applications.

A *P. duponti* alfa-L-arabinofuranosidase was purified and characterized by Guerfali and collaborators in 2011. This enzyme has a wide range of applications, such as conversion of hemicellulosic biomass into fuels and chemicals, animal feed production, and hydrolysis of grape monoterpenyl glycosides during wine fermentation. This enzyme exhibited optimum pH and temperature at 6.0–7.0 and 55°C, respectively, and a half-life of 2 h at 60°C. Enzyme activity was inhibited in the presence of ions such as Hg^{2+} and Cu^{2+} and stimulated in the presence of Mn^{2+}. It was also reported that this enzyme can act synergistically with xylanases and hence improves the production of xylobiose and monosaccharides.

28.2.1.14 *Sporotrichum pulverulentum*

Also known as *Phanerochaete chrysosporium*, *S. pulverulentum* (*P. chrysosporium* anamorph stage) is a white-rot basidiomycete species able to degrade and metabolize

polysaccharides and lignin found in plant material (Morpeth 1985). *S. pulverulentum* is also able to degrade a broad range of toxic aromatic pollutants, and it is considered a potential bioremediation agent (Gold and Alic 1993). In fact, this fungus is the only known organism which is capable of extensively degrading lignin to CO_2 and H_2O. Indeed, it is able to degrade all major wood polymers: cellulose, hemicellulose, and lignin phenolic complexes (Gold and Alic 1993).

Lignin is a phenylpropanoid polymer synthesized from the phenolic precursors coniferyl, synapyl, and p-coumaryl alcohols (Freudenberg 1968). The condensation of these precursors is initiated by plant cell-wall peroxidases. This results in the formation of a heterogeneous, amorphous, optically inactive, random, and highly branched polymer with at least 12 different linkage types, such as aryl ether and carbon-carbon bonds connecting the aromatic nuclei. These linkages are not degraded by enzymatic hydrolysis. The lignin structure requires depolymerization by extracellular oxidative mechanisms which can be performed by many white-rot basidiomycetes (Crawford 1981; Eriksson et al. 1990). Such ability is due to the lignin and manganese peroxidase activities.

Lignin degradation by *S. pulverulentum* is strongly dependent on the presence of manganese (Leatham 1986). Lignin peroxidase (LiP) and manganese peroxidase (MnP) were described for this fungus in 1983 and 1984, respectively (Glenn et al. 1983; Kuwahara et al. 1984). Since then, these two enzymes have been purified and extensively biochemically characterized. Such studies demonstrated that these enzymes are the major components of the *S. pulverulentum* lignin degradation system (Gold and Alic 1993). These extracellular ligninolytic peroxidases are thought to play a crucial role in lignin degradation and have potential use in industrial processes such as biopulping, biobleaching, and bioremediation (Cameron et al. 2000; Hatakka 2001). Both enzymes catalyze a H_2O_2-dependent oxidation reaction. LiP reacts with a wide variety of nonphenolic lignin model compounds and aromatic pollutants (Buswell and Odier 1987), including synthetic lignin (Hammel and Moen 1991). MnP reacts with lignin (Wariishi et al. 1991), lignin derivatives (Lackner et al. 1991), and also with a variety of phenolic lignin model compounds (Gold et al. 1989). It was recently reported that carbon and nitrogen limitation causes increased expression of LiP and MnP (Wymelenberg et al. 2009). This data, plus the observation that this species expresses proteases along with enzymes which act on lignocellulosic material when grown in cellulose or wood chips, indicates the necessity to scavenge for nitrogen in wood (Sato et al. 2007).

Lignin degradation by *S. pulverulentum* is enhanced by the laccase and cellobiose oxidase activities. Laccase (benzenediol: oxygen oxidoreductase, E.C. 1.10.3.2) is one polyphenol oxidase containing copper atoms in its catalytic center, which is able to catalyze the oxidation of ortho- and paradiphenols, aminophenols, polyphenols, polyamines, lignins, and aryl diamines, as well as some inorganic ions with the simultaneous reduction of molecular dioxygen to water. Laccases have been used in biosensors, biofuel cells, biotransformation, wastewater treatment (Couto and Herrera 2006), prevention of wine decoloration (Lante et al. 1992), paper processing (Reid 1991), enzymatic conversion of chemical intermediates (Agematu et al. 1993), and in the production of useful chemicals from lignin (Sannia et al. 1986).

P. chrysosporium strain RP78 genome revealed the presence of ten lignin peroxidase genes and five manganese peroxidase genes. Interestingly, no conventional laccase gene was reported. Nevertheless, four multicopper oxidases (MCO) may have a role in extracellular oxidation (Martinez et al. 2004).

S. pulverulentum cellobiose-oxidizing enzymes have been extensively characterized: one is a flavocytochrome (*b* type) having a definite oxidase activity and the other one is a simple flavoprotein. Both react with oxygen producing some superoxide radicals, but preferentially use water-soluble quinones or phenoxy radicals resulting from lignin degradation as natural electron acceptors (Canevascini et al. 1991). There are evidences that these CDHs might help in cellulose as well as in lignin degradation (Vallim et al. 1998).

Phanerochaete chrysosporium strain RP78 genome harbors the genetic information to encode more than 240 putative carbohydrate-active enzymes. Cellulases comprehend at least 40 putative endoglucanases, seven exocellobiohydrolases, and at least nine β-glucosidases (Martinez et al. 2004).

28.2.1.15 *Sporotrichum thermophile*

Sporotrichum (*Chrysosporium*, *Myceliophthora*) *thermophile* is the anamorph of the ascomycete *Thielavia heterothallica* (Canevascini et al. 1991). This organism is frequently isolated from soil and self-heating piles of vegetable biomass where it plays an important role by decomposing cell-wall polysaccharides (Bhat and Maheshwari 1987). Also known as *Myceliophthora thermophila*, this fungus is known to produce several thermostable enzymes essential for the hydrolysis of plant biomass, such as cellobiose dehydrogenases (Subramaniam et al. 1999), cellobiohydrolases (Fracheboud and Canevascini 1989), endoglucanases (Roy et al. 1990), laccases (Berka et al. 1997), and xylanases (Katapodis et al. 2003). Unlike, *S. pulverulentum*, this organism is not capable of degrading lignin (Subramaniam et al. 1999). However, *S. thermophile* is capable of producing high levels of xylanases when grown on cheap carbon sources (Katapodis et al. 2003).

S. thermophile growth rate in insoluble cellulose is very similar to the one achieved in glucose. In spite of secreting a low cellulase titer in the culture broth, *S. thermophile* achieved the complete conversion of the cellulosic substrate at a faster rate than *T. reesei* (Bhat and Maheshwari 1987).

Meyer and Canevascini (1981) isolated and characterized two intracellular beta-glucosidases produced by *S. thermophile* upon induction by cellobiose or cellulose as the sole carbon source. Both enzymes showed optimum temperature of 50°C and optimal pH between 5.5 and 6.5. Nonetheless, just one enzyme presented activity towards oligosaccharides such as cellobiose, laminaribiose, and sophorose.

Roy et al. (1990) purified and described the enzymatic properties of an extracellular endoglucanase (1,4-beta-glucanohydrolase, EC 3.2.1.4) produced by the *M. thermophila* strain D-14 (ATCC 48104). This enzyme showed optimum activity at pH 4.8 and at the temperature of 65°C. The purified endoglucanase was quite stable for 60 min at temperatures up to 70°C, but it was denatured above this temperature.

The enzyme displayed the highest activity towards carboxymethyl cellulose and significant inactivation was observed when NH_4^+, Fe_2^+, Cu_2^+ ions were present. Ions such as Hg_2^+, K^+, Na^+, and Ca_2^+ enhanced the enzyme activity, indicating that the enzyme is possibly a metalloprotein and/or that it requires metal ions for its optimum activity.

Subramaniam et al. (1999) described the production of a thermostable cellobiose dehydrogenase by *S. thermophile*. This CDH can be either a monomer or a homodimer (Canevascini et al. 1991), differently from the *S. pulverulentum* CDH which is always a monomer. *S. thermophile* CDH presented a molecular mass of 95 kDa for the monomer. The optimum temperature is 60°C, and the enzyme retained its activity for 3 h when incubated at 70°C.

Vafiadi et al. (2009) reported the purification and characterization of two thermostable *S. thermophile* xylanases. They were named *St*Xyn1 and *St*Xyn2. The first presents a molecular mass of 24 kDa, and the second, 48 kDa. Both enzymes optimum activity is achieved at pH 5 and at 60°C. These enzymes exhibited a half-life of 60 min (*St*Xyn1) and 115 min (*St*Xyn2) at 60°C. *St*Xyn1 was characterized as a member of the endo-1,4-beta-xylanases GH11family, and *St*Xyn2 was characterized as a member of the GH10 family.

S. thermophile also produces a thermostable intracellular beta-xylosidase which is optimally active at pH 7.0 and 50°C. This enzyme is stable between pH 6–8 and temperatures above 50°C. About 58% of its activity is retained after 1 h at 60°C. The enzyme hydrolyzes beta-1,4-linked xylooligosaccharides with chain lengths from 2 to 6 residues, releasing xylose from the nonreducing end, but it is inactive against xylan. The enzyme activity was higher against *p*-nitrophenyl beta-D-xylopyranoside and was inhibited by many alcohols such as butanol, ethanol, methanol, and propanol (Katapodis et al. 2006).

28.2.1.16 *Stilbella thermophila*

S. thermophila was named after its ability to grow at high temperatures: optimum growth occurs in the range of 35–50°C; growth is inhibited at temperatures above 55°C (Fergus 1964). This species was first described by Fergus, in 1964, and it was found in self-heating straw and horse manure compost. Although this fungus was described as a promoter of the mushroom *Agaricus bisporus* mycelial growth on sterilized compost, *S. thermophila* is rarely found on straw or self-heating substrate mixtures (Straatsma et al. 1994a, b).

Due to the paucity of information about this species, to date, there is no data about the enzymatic repertoire of this fungus. However, its habitat strongly indicates that it may be important to degrade organic substrates under high temperatures.

28.2.1.17 *Talaromyces emersonii*

T. emersonii is a moderate thermophilic aerobic ascomycete and a natural saprophyte that inhabits soil and compost heaps (Waters et al. 2010). This filamentous

fungus produces an array of glucan-modifying enzymes, including cellulases (Moloney et al. 1985) and xylanases (Tuohy and Coughlan 1992). *T. emersonii* is able to grow on a wide temperature range (30–90°C), with an optimum range of 30–55°C (Tuohy et al. 2007).

T. emersonii produces a complete thermostable xylan-degrading enzyme system when grown on the appropriate substrate (Tuohy and Coughlan 1992). It also produces a complete cellulase system in which the various components interact synergistically to achieve cellulose hydrolysis (McHale and Coughlan 1980). *T. emersonii* extracellular enzymes are characterized by a remarkable thermostability when compared to other cellulolytic aerobic fungi (McHale and Coughlan 1981; Moloney et al. 1985; Tuohy and Coughlan 1992).

Several *T. emersonii* glycosil hydrolases have been purified and characterized. The enzymes have optimum activity at temperatures between 54 and 85°C, and some of them maintain the activity at temperatures up to 95°C. Most of the components of the cellulolytic and the xylanolytic systems have optimal activity at an acidic pH (Tuohy et al. 2007). *T. emersonii* produces extracellular enzymes in higher amounts than bacteria or yeast, and it is generally regarded as safe (GRAS status), being useful in food processing (Polizeli et al. 2005).

28.2.1.18 *Thermoascus aurantiacus*

Thermoascus aurantiacus is a thermophilic ascomycete commonly found on decaying lignocellulosic material (Carvalho et al. 2006; Brienzo et al. 2008). This organism is characterized by a bright orange color, elliptical ascospores, and does not present asexual cycle (Maheshwari et al. 2000). The spores germinate between 32.5 and 60°C and the hyphal growth occurs between the temperature range of 20–55°C (Cooney and Emerson 1964a, b; Deploey 1995). *T. aurantiacus* grows efficiently on a variety of low-cost carbon sources, such as sugarcane bagasse (Milagres et al. 2004), corn cobs, wheat straw, rice straw, oat bran, and sugar beet pulp (Kalogeris et al. 1998).

T. aurantiacus produces all the cellulolytic enzymes required for the complete conversion of cellulose to glucose. Endo-1,4-beta-D-glucanase is the main cellulase activity produced by this fungus (Himmel et al. 1999). *T. aurantiacus* endoglucanases present high thermostability, a half-life of 98 h at the temperature of 70°C (Gomes et al. 2000), optimum temperature between 65 and 80°C, and optimum pH between 4.0 and 5.5 (Khandke et al. 1989a; Gomes et al. 2000; Parry et al. 2002; Da Silva et al. 2005).

T. aurantiacus beta-glucosidase production is dependent on the culture conditions. Media supplemented with larchwood xylan, blotting paper, bagasse, and pectin enhance the production of beta-glucosidases (Khandke et al. 1989a). These enzymes show high thermostability, half-life at 70°C of 23.5 h (Gomes et al. 2000), optimum temperature between 65 and 80°C, and optimum pH between 4.5 and 6.0 (Parry et al. 2001; Hong et al. 2006).

In *T. aurantiacus*, exoglucanases are produced in lower quantities in comparison with other enzymes from the cellulolytic complex (Khandke et al. 1989a). However, high exoglucanase activity was detected in cultures of *T. aurantiacus* strain 179–5 cultivated in semisolid media containing sugarcane or orange bagasse (Martins et al. 2003).

In general, *T. aurantiacus* exoglucanases present optimum pH between 4.1 and 5.0, optimum temperature at about 65°C, and are stable for 8 h at 50°C (Tong et al. 1980; Khandke et al. 1989a).

This species is also able to degrade hemicellulose and produces all the enzymes that are necessary to achieve this purpose. Xylanase is the main enzyme detected in liquid cultures (Brienzo et al. 2008). Xylanases from *T. aurantiacus* different strains are optimally active at about 70–75°C and around pH 5.0 (Yu et al. 1987; Alam et al. 1994; Gomes et al. 2000).

T. aurantiacus enzymatic inventory is also composed by beta-xylosidase (Kalogeris et al. 1998), alfa-L-arabinofuranosidase (Roche et al. 1994), beta-D-glucuronidase (Khandke et al. 1989b), beta-1,4-Mannanase (Gomes et al. 2000), beta-mannosidase (Guy et al. 1997; Gomes et al. 2007), polygalacturonases, and pectin lyases (Martins et al. 2002) and chitinase (Li et al. 2010).

28.2.1.19 *Thermomyces lanuginosus*

Also known as *Humicola lanuginosa*, *T. lanuginosus* is a thermophilic ascomycete widely distributed in nature, and it is generally found on self-heating masses of organic material (Emerson 1968; Singh et al. 2000). This fungus is unicellular or septated and reproduces asexually by forming aleurioconidia (Hudson 1992). Most *T. lanuginosus* strains grow optimally at the temperature of 50°C and pH 6.5 (Cooney and Emerson 1964a, b).

In spite of not producing cellulases, some *T. lanuginosus* strains are very attractive to industry due to the secretion of large amounts of thermostable cellulase-free xylanases when grown on cheap carbon sources such as corncob, birchwood xylan, and sugarcane bagasse (Purkarthofer et al. 1993; Singh et al. 2003). The production of cellulase-free xylanases is a desirable feature for processes such as pulp bleaching, since even a low cellulase activity may cause serious economic implications in terms of cellulose degradation (Beg et al. 2001). *T. lanuginosus* is also a producer of other hemicellulases (Singh et al. 2003), chitinases (Run-fang et al. 2008), and pectinases (Puchart et al. 1999).

Most *T. lanuginosus* xylanases are optimally active at temperatures above 65°C and at pH near neutral. Some strains are able to produce xylanases that remain stable at a wide pH and temperature range (Damaso et al. 2003; Singh et al. 2003; Li et al. 2005). It has been reported that some *T. lanuginosus* xylanases are capable of hydrolyzing xylan directly to xylose without the production of intermediary xylooligosaccharides (Shrivastava et al. 2011). Such characteristics make *T. lanuginosus* a very interesting organism for industrial processes that requires high quantities of cellulase-free xylanases.

28.2.1.20 *Thielavia terrestris*

Also denominated *Allescheria terrestris* or *Acremonium alabamense* (anamorph), *T. terrestris* is characterized by spherical, non-ostiolate ascomata, with a thin peridium,

and one-celled, darkly pigmented ascospores (Stchigel et al. 2002). This soil organism has optimum growth in the range of 40–45°C (Maheshwari et al. 2000). The highest temperature for cultivation on microcrystalline cellulose is 50–52°C. *T. terrestris* grows well at 62°C on easily metabolizable substrates such as glucose, glycerin, and xylose (Kvesitadze et al. 1999; Stchigel et al. 2002). This ascomycete is also cited for stimulating the white button mushroom *A. bisporus* growth (Straatsma et al. 1994a, b).

This soft-rot fungus presents cellulase, xylanase, and a poor lignolytic activity (Dix and Webster 1995; Rani and Nand 1996). One interesting characteristic of this fungus is the production of distinct enzymatic sets under different temperatures (Haltrich et al. 1996). *T. terrestris* produces higher levels of xylanases when cultivated at 40°C in comparison to 48°C. However, the latter enzymatic set presents higher thermostability than the first (Kvesitadze et al. 1994).

When cultivated at the temperature of 45°C, a twofold reduction of the enzyme activity was observed, but the extract thermostability was augmented by 10°C for both xylanases and cellulases (Kvesitadze et al. 1999). These authors also showed that culture temperature oscillations change the production of the enzymatic set independently of the initial culture conditions. Such changes may play an important role in the microorganism ecology and during the natural degradation of woody materials.

28.2.2 Actinobacteria and Actinomycetes

The phylum *Actinobacteria* is recognized as one of the largest taxonomic units inside the domain *Bacteria* (Ventura and Canchaya 2007). It comprehends a group of Gram-positive bacteria with a high G+C content on their genomes – 51% in some corynebacteria to more than 70% in *Streptomyces* and *Frankia* (Ventura and Canchaya 2007). Organisms from this phylum can be found in a wide variety of environments such as the soil, aquatic habitats, or even extreme environments, like acidic thermal springs or Antarctic regolith, where they play a crucial role in the recycling of refractory biomaterials by decomposition and humus formation, hence participating in carbon cycling (Goodfellow and Williams 1983; Santos and Vieira 2008). *Actinobacteria* comprises numerous important pathogens, nitrogen-fixing symbionts and secondary metabolite-producers, including hydrolytic exoenzymes (Ventura and Canchaya 2007). Plenty of them represent ecologically or economically important organisms. For instance, *Streptomyces* species are important antibiotic producers exploited by the pharmaceutical industry (Berdy 2005). Being a vast phylum as it is, a huge collection of morphologies, physiological and metabolic properties are found.

Actinomycetales comprehends an order of the *Actinobacteria* phylum that is also known as filamentous actinobacteria. This order was first characterized as a group of the fungus kingdom due to the morphological resemblance and pathogenicity properties – the name actinomycetes came from the observation of mycelia. Despite of forming hyphae with true branching, actinomycetes are members of the domain

Bacteria and therefore are imbued with many bacterial characteristics such as sensitivity to Gram-positive antibacterial antibiotics, sensitivity to phages, prokaryosis, and lack of sterols (Lechevalier and Lechevalier 1967).

Numerous actinomycetes are responsible for the decomposition of organic matter and mineralization (Santos and Vieira 2008). For such purpose, many members of this group are capable of synthesizing numerous extracellular hydrolytic enzymes in large amounts. These organisms are of great industrial and scientific interest.

28.2.2.1 *Acidothermus cellulolyticus*

Acidothermus cellulolyticus is a member of the Frankinae family, first isolated by Mohagheghi et al., in 1986, from acidic hot springs at the Yellowstone National Park (Wyoming – USA) and obtained from cellulose agar cultures. This species was found along with some Gram-negative cellulolytic bacteria in a screening for cellulolytic microorganisms resistant to high temperatures and acidic pH. This organism is aerobic, nonpigmented, and acid tolerant, growing between pH 3.5 and 7.0. It is thermophilic, growing between 37 and 70°C; the optimum growth temperature is 55°C. *A cellulolyticus* does not grow below 37°C.

The complete genome sequence of *A. cellulolyticus* 11B was published by Barabote et al. (2009) and revealed an enzymatic profile for biomass degradation larger than expected. *A. cellulolyticus* genome comprises at least 43 genes encoding glycoside hydrolases which seem to be responsible for breaking down structural or storage carbohydrates found in plant and fungal cells, including cellulose, xylan, starch, and chitin. In addition to five previously described cellulase-encoding genes, the genome analysis revealed six new cellulose-degrading enzyme genes, including four endoglucanases and two beta-glucosidases. Moreover, six sequences for hemicellulose decomposition enzymes were identified, including two xylanases, three xylan esterases, and a xylosidase. Most of them are either predicted to be secreted or encode a signal peptide. In this view, this species presents great industrial potential due to the enzymes thermostability and activity at low pH.

A. cellulolyticus genome study also reported that the catalytic domains of two-thirds of the 21 putative secreted biomass-degrading enzymes were fused to one or more carbohydrate-binding modules (CBM), which increase the enzymes specificities.

Genes for the three major classes of cellulases (endoglucanases, cellobiohydrolases, and beta-glucosidases) are present in multiple copies in the *A. cellulolyticus* genome (Barabote et al. 2009). The endo-1,4-beta-glucanase enzyme (E1) presents high thermostability (T_{opt} = 81°C) (Himmel et al. 1994), resistance to acidic pH (4–6), and very high specific activity towards carboxymethyl cellulose (Thomas et al. 1995).

Many studies report the heterologous expression of *A. cellulolyticus* E1 in numerous transgenic plant species. E1 catalytic domain was already expressed in rice (Oraby et al. 2007), tobacco (Ziegelhoffer et al. 2001), *Arabidopsis thaliana* (Ziegler et al. 2000), the duckweed *Lemna minor* 8627 (Sun et al. 2007), corn (Ransom et al. 2007)

and maize seed (Hood et al. 2007). Transgenic plants that overexpress different cellulases represent an alternative to bioreactors in producing inexpensive amounts of cellulases. This is called molecular farming and has been a major goal for other important industrial enzymes and pharmaceutical proteins as well (Owen and Pen 1996). The low enzyme activity at mild temperatures allows the enzyme to accumulate in the cell with minimum effects on the plant growth and development, while its extreme thermostability helps to protect the protein structure and function during extraction and high temperature saccharifications (Himmel et al. 1994).

Recently, Lindenmuth and McDonald (2011) achieved a high production of the synthetic E1 using *P. pastoris* as production host. The heterologous enzyme properties (pH optimum = 5.1 and optimum temperature = 80°C) were in agreement with the values previously determined in other systems. This enzyme production in *P. pastoris* presents many advantages due to its lower growing temperature, tighter expression control, and higher final titers. The production of such thermostable enzyme at high rates is of great commercial and industrial interest.

Using a synthetic sequence, Zhang et al. (2012) produced the recombinant E1 enzyme in transgenic rice seeds in order to evaluate the employment of this cereal as bioreactor for cellulolytic enzyme mass production. Among the different plant tissues, seeds are preferable due to the high protein content and maintenance of the recombinant protein stability during storage. The recombinant enzyme was highly expressed, accumulated in the seeds, and was active in the fermentation process.

28.2.2.2 *Cellulomonas* spp.

Cellulomonas (Cellulosa – cellulose, monas – a unit) are G + C-rich Gram-positive actinomycetes belonging to the coryneform group of bacteria (Chaudhary et al. 1997). *Cellulomonas* species are mesophilic, aerobic bacteria found in the soil and are known to produce an array of thermophilic enzymes involved in the degradation of components of the plant cell wall, mainly cellulases and xylanases (Beg et al. 2001). *Cellulomonas* are capable of using cellulose as their sole carbon energy source (Tomme et al. 1995).

Xylanases (1,4-beta-D-xylan xylanohydrolase E.C. 3.2.1.8) are a family of hydrolytic enzymes that cleave internal linkages on the beta-1,4-xylopyranose backbone (Wong et al. 1988). The hydrolysis of the xylan backbone involves the action of endo-beta-1,4-D-xylanases (E.C. 3.2.1.8) and beta-D-xylosidases (E.C. 3.2.1.37). These enzymes have important applications in pulp and paper industry. They are also frequently used in agricultural and alimentary industries, such as in the extraction of coffee, plant oils, and starch, among others (Beg et al. 2001).

Cellulomonas species are capable of using a variety of carbohydrates, including starch, cellulose, xylan, and even crystalline cellulose. This is due to the production of a complex battery of glucanases in order to degrade substrates possessing extensive microheterogeneity (Chaudhary et al. 1997). Such complex enzymatic inventory induces ultrastructural differences between *Cellulomonas* cells grown in cellulose and cellobiose when compared with cells grown in glucose. It was

described that cells grown in cellulose or cellobiose were covered with multiple protuberant structures resembling cellulosomes, whereas surfaces of glucose-grown cells were nearly devoided of such structures (Vladut-Taylor et al. 1986; Lamed et al. 1987). In addition, *Cellulomonas* glucanases present cross recognition of substrates and are cross inducible (Chaudhary 1995). Therefore, *Cellulomonas* enzymes may be suitable for industrial applications in the food, feed, pulp and paper industries, or in the pretreatment of lignocellulosic biomass required for improving the yields of fermentable sugars for bioethanol production (Amaya-Delgado et al. 2010).

Sánchez-Herrera and collaborators published in 2007 a study demonstrating that the differential expression of cellulases and xylanases by *C. flavigena* is correlated with the presence of diverse carbon sources (glucose, xylan, sugarcane bagasse, and solka-floc). By using liquid chromatography/mass spectrometry, 13 proteins with carboxymethyl cellulase or xylanase activity were identified and sequenced. When grown on sugarcane bagasse or solka-floc supplemented medium, *C. flavigena* presented xylanase, CMCase, and FPase activities. When grown on xylan, only CMCase and xylanase activities were observed. None of these enzyme activities was detected when *C. flavigena* was grown on glucose.

Two thermophilic xylanases (Xyl36 and Xyl53) from *C. flavigena* CDBB-531 were characterized (Santiago-Hernández et al. 2007). Xyl36 and Xyl53 showed optimal activity at pH 6.5 and an optimal temperature at 65 and 55°C, respectively. At the same temperatures, enzyme activity was lost after 1 h. These enzymes exhibited affinity to microcrystalline cellulose and xylan-rich polysaccharides (Santiago-Hernández et al. 2007). Another *C. flavigena* xylanase (Cfl Xyn11A), belonging to glycoside hydrolases family GH11, was reported by Amaya-Delgado et al. (2010). The corresponding gene was isolated from a genomic DNA library and cloned in *E. coli*. The recombinant enzyme, presenting an estimated molecular mass of 31 kDa, showed optimal activity at pH 6.5 and at 55°C. Cfl Xyn11A is thermostable and showed a half-life of 1.06 h at 55°C.

Many studies focusing the molecular cloning and characterization of *C. flavigena* cellulase genes were already reported. Herrera-Herrera et al. (2009) cloned a *C. flavigena* cellobiohydrolase gene named *celA*. Mejia-Castillo et al. (2008) characterized a processive endoglucanase called CBP105, displaying maximum activity towards carboxymethyl cellulose at pH 7.5 and at 60°C. Pérez-Avalos et al. (2008) isolated an enzyme of 49 kDa with a bifunctional cellulase/xylanase activity, presenting the optimal temperature of 50°C, pH optimum of 6.0 for the cellulase activity and 9.0 for the xylanase activity. These data pointed out to separate active sites for each activity. The different pH regulation for each catalytic site provides advantages in numerous industrial processes.

In 2010, Abt and collaborators published the complete genome sequence of *C. flavigena* 134^T, the first genome described for the *Cellulomonas* genus. It comprises a sole circular 4,123,179 long chromosomes with a 74.3% GC content. 98.5% of the genome consists of protein-coding genes. The genome sequence and annotation revealed that almost 10% of the encoded proteins may be related to metabolism and carbohydrate transport. Moreover, the genome sequence revealed 14 genes coding

for putative endo-1,4-beta-xylanases belonging to glycoside hydrolase family 10 and five genes encoding beta-xylosidases. Concerning cellulose metabolism, two genes coding endo-1,4-beta-glucanases (Cfla_0016, Cfla_1897), three genes encoding 1,4-beta-cellobiohydrolases (Cfla_1896, Cfla_2912, Cfla_2913), and three genes coding beta-glucosidases (Cfla_1129, Cfla_3027, Cfla_2913) were identified.

Other *Cellulomonas* species have been studied for their enzymatic properties and their potential use in industrial processes. *C. uda* (Nakamura et al. 1986), *C. biazotea* (Liebl et al. 1992), and particularly, *C. fimi* (Langsford et al. 1984; Din et al. 1995; Sandercock et al. 1996; Mansfield et al. 2002) represent organisms with interesting properties for industrial usage.

28.2.2.3 *Streptomyces* spp.

The genus *Streptomyces* represents a group of microorganisms that is widely distributed in nature (Dastager et al. 2008). With more than 500 recognized species and subspecies, the *Streptomyces* taxon currently harbors the largest number of species in the domain *Bacteria* (Hain et al. 1997). Despite of being a soil genus with mesophilic physiology, many streptomycetes species are described as thermophilic and grow well at 50°C and over the temperature range of 25–55°C (Kim et al. 2000).

Streptomyces species produce a large variety of enzymes and secondary metabolites. *Streptomyces* alone is responsible for the production of more than half of the known biologically active microbial products, including many commercially important antibiotics, immunosuppressive compounds, animal health products, and agrochemicals (George et al. 2010). This vast reservoir of diverse products makes *Streptomyces* one of the most important industrial microbial genera (Herai et al. 2004). *Streptomyces* manage to use a myriad of organic compounds as the sole carbon source, including complex biological materials such as cellulose, hemicellulose, chitin, and lignin and can also utilize an inorganic nitrogen source (Kutzner 1986). Such metabolic versatility requires a huge enzymatic pool.

Chitin (poly-beta-1,4-N-acetylglucosamine) is profusely present in nature as a major structural component of fungi cell walls, arthropod exoskeletons, and crustacean shells. Chitinases (E.C. 3.2.1.14) are enzymes which hydrolyze chitin to chitodextrins (reviewed by Fujii and Miyashita 1993). *Streptomyces* are known as notorious decomposers of chitin. Consequently, media composed by chitin are commonly used for the selective isolation of *Streptomyces* species which produce chitinases (Fujii and Miyashita 1993).

Miyashita et al. (1991) cloned three chitinase genes from *S. lividans*. These genes were expressed in *S. coelicolor* and *E. coli*, and the recombinant enzymes were characterized. The three proteins were named chitinase A, B, and C; their molecular masses were determined as 36, 46, and 65 kDa, respectively. The optimal pH for chitinase A, chitinase B, and chitinase C were 3.0, 5.0, and 6.5, respectively. The production of the three chitinases was induced by chitin and repressed in the presence of readily utilizable carbon sources such as glucose.

Studies on *S. lividans* also revealed that this organism is a potential source of many enzymes required for the degradation of the lignocellulosic biomass. The molecular cloning and sequence characterization of genes encoding for three xylanases (Mondou et al. 1986; Vats-Metha et al. 1990; Shareck et al. 1991; Kluepfel et al. 1992) and for three cellulases (Shareck et al. 1987; Théberge et al. 1992; Wittmann et al. 1994) were reported. These enzymes present interesting characteristics such as optimal pH between 5.0 and 6.5 and optimal temperatures between 50 and 60°C.

Férnandez-Abalos et al. (1992) have cloned and expressed a *S. halstedii* endoglucanase gene (*celA*$_1$) which does not code for a carbohydrate-binding module as confirmed by microcrystalline cellulose-binding experiments. Another endoglucanase (Cel2) is initially produced as a 42-kDa protein (p42) which is later processed at the C-terminus. This protein presents optimal activity against carboxymethyl cellulose and no activity against crystalline cellulose or xylan (Garda et al. 1997). Cel2 has an optimal activity at pH 6 and at 55°C. This study also revealed a protein which has high affinity for microcrystalline cellulose but presented no clear activity against other cellulosic compounds. This protein is encoded by an open reading frame situated 216 bp downstream from p42 and was named p40 (40 kDa). Transcription of the two genes seems to be co-regulated.

Schrempf and Walter (1995) summarized the properties of Cel1, a cellulase produced by *S. reticuli* which is sufficient to degrade crystalline cellulose to cellobiose. This enzyme has a molecular mass of 82 kDa and shows optimal activity at pH 7 and 55°C. George et al. (2010) described a *Streptomyces* species isolated from Indian soil samples with cellulase activity towards more amorphous cellulose.

Thermophilic *Streptomyces* species such as *S. thermocoprophilus* sp. nov. (Kim et al. 2000), *Streptomyces* sp. Ab106 (Techapun et al. 2002), and a new *Streptomyces* sp. isolated by Alani et al. (2008) also produces hydrolytic enzymes of industrial interest.

28.2.2.4 *Thermobifida fusca*

Thermobifida fusca (formerly known as *Thermomonospora fusca*) is a filamentous soil bacterium that belongs to the *Actinobacteria* phylum. *T. fusca* is classified as a moderately thermophilic organism, presenting optimal growth at 55°C. It is a notorious degrader of plant cell walls commonly found in heated organic materials, such as compost heaps, rotting hay, manure piles, or mushroom growth medium (Bachmann and McCarthy 1991). It was first isolated from decaying wood (Bellamy 1977). This species is also known for the generation of allergenic spores which have been associated with a condition called "farmer's lung" (Van den Bogart et al. 1993), an acute inflammatory response which can be seriously dangerous if it reaches the chronic status.

Known as an important agent in carbon cycling, *T. fusca* extracellular glycoside hydrolases are of great interest due to their thermostability, broad pH range (4–10), and high activity, particularly concerning cellulases and xylanases. It degrades all the major plant cell-wall polymers, except pectin and lignin phenolic complexes (Bellamy 1977).

Thermobifida fusca YX complete genome sequence was published (Lykidis 2006). *T. fusca* encodes a total of 45 hydrolytic enzymes predicted to act on polysaccharides. Of these, 36 are glycoside hydrolases, 16 of them are predicted to be secreted (Ventura and Canchaya 2007). In addition, genome analysis revealed 28 putative glycoside hydrolases and enzymes potentially involved in plant cell-wall degradation. This broad enzymatic repertoire comprises endocellulases, exocellulases, cellobiases, xylanases, endochitinases, exochitinases, and some chitin and xylan deacetylases, which suggests that *T. fusca* can utilize these substrates for energy production (Lykidis 2006).

More recently, Adav et al. (2010) published the entire *T. fusca* secretome. The protein profile of *T. fusca* secretome grown in cellulose, lignin, and in a mixture of cellulose and lignin was analyzed. In all three culture conditions, three beta-1,4-endoglucanases (Cel5A, Cel6A and Cel9B), two two-1,4-exoglucanases (Cel6B and Cel48A), one processive endoglucanase (Cel9A), and one beta-glucosidase were detected. Hemicellulases, including endo-1,4-beta-xylanases (family GH-10) and beta-L-arabinofuranosidases (family GH-43) were significantly expressed in cellulose-containing medium. The contents of alfa-L-arabinofuranosidase and acetyl xylan esterase/chitin deacetylase were high when both cellulose and lignin were used in the culture medium in comparison to culture conditions containing either cellulose or lignin. The xylanase and the acetyl xylan esterase were also induced in presence of lignin (Adav et al. 2010).

Huang et al. (2010) cloned and expressed an acetylxylan esterase gene from *T. fusca* NTU22. This enzyme acts in deesterifying acetylxylan and xylooligosaccharides and is essential for the complete xylan degradation. The optimal pH and temperature of the purified esterase were 7.5 and 60°C, respectively. This enzyme acted cooperatively with a xylanase from another transformant and significantly increased the xylooligosaccharides release from oat-spelt xylan. The same was not observed when the lignocellulosic substrate bagasse was used.

Cellulose conversion to higher alcohols, such as 1-propanol and 1-butanol, is desirable due to their potential to substitute gasoline hereafter. The engineered strain B6 expresses a *Clostridium acetobutylicum* bifunctional butyraldehyde/alcohol dehydrogenase. This strain is able to produce directly 1-propanol when grown in various untreated lignocellulosic biomasses. *T. fusca* B6 is the first aerobic organism which presents such characteristics, and this illustrates *T. fusca's* potential utility as a cellulolytic bioprocess organism (Deng and Fong 2011).

28.2.2.5 *Thermomonospora curvata*

Thermomonospora curvata is the dominant bacterial population in a variety of composting materials. It was first isolated in municipal refuse compost samples (Niese 1959; Fergus 1964; Stutzenberger et al. 1970). *T. curvata* grows rapidly at composting temperatures (50–60°C) in cellulose-agar media (Stutzenberger 1972).

T. curvata also produces a wide range of extracellular depolymerizing enzymes, including cellulases and xylanases (Stutzenberger and Busch 1997). Since cellulose

constitutes about one-half of the dry weight of municipal refuse compost (Satriana 1974), the presence of cellulases is essential for the degradation of these residues.

In most bacteria, virtually all hydrolytic enzymes are controlled by catabolite repression. Many *T. curvata* enzymes are not subject to glucose repression. In 1987, Bernier and Stutzenberger observed that this species uptakes cellobiose in preference to glucose when cultivated in the presence of both carbon sources. Furthermore, the production and activity of hydrolytic enzymes such as beta-glucosidase and endoglucanase seem not to be interfered by extracellular glucose. *T. curvata* xylanolytic system is also not subject to glucose repression or inducer exclusion (Busch and Stutzenberger 1997). Once *Thermomonospora* species possess the highest xylanolytic ability amongst the thermophilic actinomycetes (Ball and McCarthy 1989), this feature is particularly relevant for many industrial and solid waste composting processes.

T. curvata cellulases are active against both cotton fibers and carboxymethyl cellulose – C_1 and C_X activities, respectively (Stutzenberger 1972). *T. curvata's* optimum condition for cellulases production is pH 6.0 and 65°C (Stutzenberger 1972).

Some *T. curvata* endoglucanases present interesting characteristics. Enzyme activation by exposure to elevated temperatures is rarely reported. In 1986, Stutzenberger and Lupo discovered a *T. curvata* endoglucanase with such feature. This enzyme activity is increased by a thermal activation step (70°C) at alkaline pH (pH 8) in both crude and purified preparations. However, the activity was depleted when the temperature was increased at acidic pH.

Chertkov et al. (2011) published the genome sequence of *T. curvata* strain $B9^T$, the first complete genome of a member of the *Thermomonosporaceae* family. The genome consists of a 5,639,016-bp-long chromosome with a 71.6% GC content. This strain has 5,061 genes, of which 98.5% are protein-coding genes. Approximately 12% of the genes involve functions such as carbohydrate transport/metabolism and energy production/conversion.

28.3 Future Perspectives and Conclusions

In spite of much information about the enzymes responsible for lignocellulosic substrate breakdown by fungi and actinomycetes, the search for new microorganisms, particularly from extreme environments, such as deserts, thermal pits, polar areas, and isolated tropical areas, must continue in order to increase the repertoire of species which can be employed at the industrial level. Innovative experimental approaches such as secretomics and metabolomics may provide useful information for the optimization of bioprocesses. In this view, the investigation of the molecular basis of enzymes' thermotolerance and thermostability can contribute to the design of more effective catalysts through directed evolution.

In this work, we have performed the most detailed review to date about thermophilic fungi and actinomycetes which are able to convert the vegetal biomass into fermentable sugars and other economically important by-products. Thermotolerant/

thermostable endoglucanases, cellobiohydrolases, beta-glucosidases, xylanases, xylobiases, and ligninases industrial application will certainly increase developing countries economy by generating useful products, particularly biofuels, from wasted agricultural residues. Furthermore, an environmental impact is envisaged, since these residues tend to accumulate and to pollute huge areas where extensive cultures, like sugarcane bagasse, soybean, and corn, are prevalent.

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Chapter 29
Cellulases of Thermophilic Microbes

Linga Venkateswar Rao, Anuj K. Chandel, G. Chandrasekhar, A. Vimala Rodhe, and J. Sridevi

Abstract The renewed interest in cellulase biotechnology is drawing the attention of researchers globally due to their diverse range of applications. The major applications of cellulases (E.C.3.2.1.4) are in textile and detergent industry. Additionally, they are in huge demand in food and feed sector for the improvement in digestibility, in nutritional quality of food/feed material, and in paper industries as de-inking agents. Another most promising application of cellulases is in the bioconversion of renewable lignocellulosic biomass into fermentable sugar constituents that are subsequently used for the production of value-added chemicals after the fermentation reaction with appropriate microorganisms. The success of ethanol-based biorefinery truly depends upon the efficiency of cellulase titers stable at high temperature and their cost at shop floor. Looking at the applications of cellulases, stable and active thermostable cellulases at high pH range would be more advantageous as compared to thermolabile enzymes in terms of time, cost savings, and getting the suitable product with desired yields/productivities. Recent developments on the proteomics, genomics, and fermentation strategies have paved the way for searching more efficient and novel thermostable cellulase titers from thermophilic microorganisms of different habitats.

Keywords Cellulases • Thermophiles • Thermostable cellulase applications • Fermentation

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29.1 Introduction

Enzymes from microorganisms have been the center of attention for researchers and industrialists due to their wide range of physiological, analytical, and industrial applications (Haki and Rakshit 2003; Turner et al. 2007).

Although enzymes required for reaction-based applications are isolated and purified from microbial, animal, and plant sources, microorganisms represent the most common source of enzymes due to their broad biochemical diversity and feasibility at large-scale production by exploiting cheap carbohydrate sources (Demirijan et al. 2001; Rye et al. 2009).

Cellulose is an abundant and readily available renewable energy resource on earth for many sustainable applications. It can be converted into fermentable sugars by the cellulose under appropriate react ion conditions. Microorganisms capable of producing multiple enzymes containing mainly of cellulases to degrade cellulose have been isolated from natural resources, and further for improvement in their production titers, a lot many efforts have been made worldwide (Wilson 2009).

In the current industrial processes, cellulolytic enzymes are employed in the color extraction of juices, in detergents causing color brightening and softening, in the biostoning of jeans, in the pretreatment of biomass that contains cellulose to improve nutritional quality of forage, and in the pretreatment of industrial wastes (Bhat 2000; Nakamura et al. 2001; van Wyk et al. 2001; Zhou et al. 2001).

Looking at the renewed interest in high-temperature-based applications, thermostable enzymes and microorganisms have been the area of interest during the last two decades (Brock 1986; Kristjansson 1989). The potential use of cellulases in various commercial application sectors has been elegantly described by Turner and colleagues (2007).

Among all the cellulase-based applications, their capability of working on cellulose polymers at high temperatures, yielding the sugar monomers is the center of attraction at the moment due to increased demand for lignocellulose-derived fuel ethanol (Wilson 2009; Chandel et al. 2010).

Thermostable cellulases are highly specific with broad range of applications that are well suited for many commercial industrial applications (Margaritis and Merchant 1986; Haki and Rakshit 2003). Thermostable enzymes are stable and active at temperatures which are even higher than the optimum temperatures for the growth of the microorganisms (Saboto et al. 1999).

Thermostable cellulases are isolated primarily from organisms like bacteria, fungi, and yeasts and found to have profound commercial applications (Margaritis and Merchant 1986; Holst et al. 1997; Hough and Danson 1999; Eichler 2001; Demirijan et al. 2001).

The role of thermostable enzymes in many processes is known for a long time. Their existence was associated with the history of ancient Greece where they were using enzymes from microorganisms in baking, brewing, alcohol production, cheese making, etc. (Brock and Freeze 1969). With better knowledge and purification of enzymes, the number of applications has increased by many folds, and with the availability of thermostable enzymes, a number of new possibilities for industrial processes have emerged.

The potential benefits of thermo stable enzymes in the hydrolysis of lignocellulosic substrates include the following: higher specific activity decreasing the amount of enzymes, enhanced stability allowing improved hydrolysis performance, and increased flexibility with respect to process configurations, all leading to improvement of the overall economy of the process (Viikari et al. 2007).

These thermostable enzymes would facilitate development of more efficient and cost-effective forms of the simultaneous saccharification and fermentation process to convert lignocellulosic biomass into biofuels (Rastogi et al. 2010).

This chapter presents an overview of the cellulases produced from thermophiles. Particular emphasis is made on the various kinds of cellulase producing thermophilic microorganisms under different cultivation techniques, strategies developed for their improved production and their catalytic action by adopting latest molecular biology techniques and their applications.

29.2 Structure and Functional Properties of Thermostable Cellulases

Lignocellulosic of plant cell walls are composed of cellulose, hemicellulose, pectin, and lignin (the three former being polysaccharides). Out of the major cell wall ingredients, cellulose is the principle constituent of all plant materials and the most abundant organic source of feed/food, fuel, and chemicals (Spano et al. 1975; Demain et al. 2005) which accounts for around 180 million tons per year (Lynd et al. 2002). It consists of glucose units linked by β-1,4-glycosidic bonds in a linear mode. The highly ordered crystalline form of cellulose makes it more resistant to digestion and hydrolysis (Bayer et al. 1998; Lynd et al. 2002). The enzymes required for the hydrolysis of cellulose include endoglucanases, exoglucanases, and β-glucosidases (Matsui et al. 2000; Bhat 2000; Sukumaran et al. 2005).

A cellulosic enzyme (cellulase) system consists of three major components: endo-β-glucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21). Endo-β-glucanases randomly hydrolyze the internal glycosidic bond to decrease the length of the cellulose chains. Cellobiohydrolases are exo- or endo-processive enzymes that split off cellobiose. Cellobiose is subsequently hydrolyzed by β-glucosidases to glucose (Gray et al. 2006; Wilson 2009). Figure 29.1 shows the mechanism of cellulose conversion into glucose.

Cellulase from *Penicillium citrinum* MTCC 6489 was characterized which revealed two isoforms differing in molecular weight of 90 and 38 kDa with pH optima (6.5). Cellulase of *P. citrinum* was observed to be thermostable in nature. This enzyme may have the potential to be used as an additive in laundry detergents (Dutta et al. 2008).

Morana et al. (2008) isolated a novel cellulase from the thermoacidophilic bacterium *Alicyclobacillus acidocaldarius* ATCC 27009. The enzyme is a glycosylated monomer of 56.2 kDa, relatively thermostable, with optimal pH and temperature of 4.0 and 65°C, respectively.

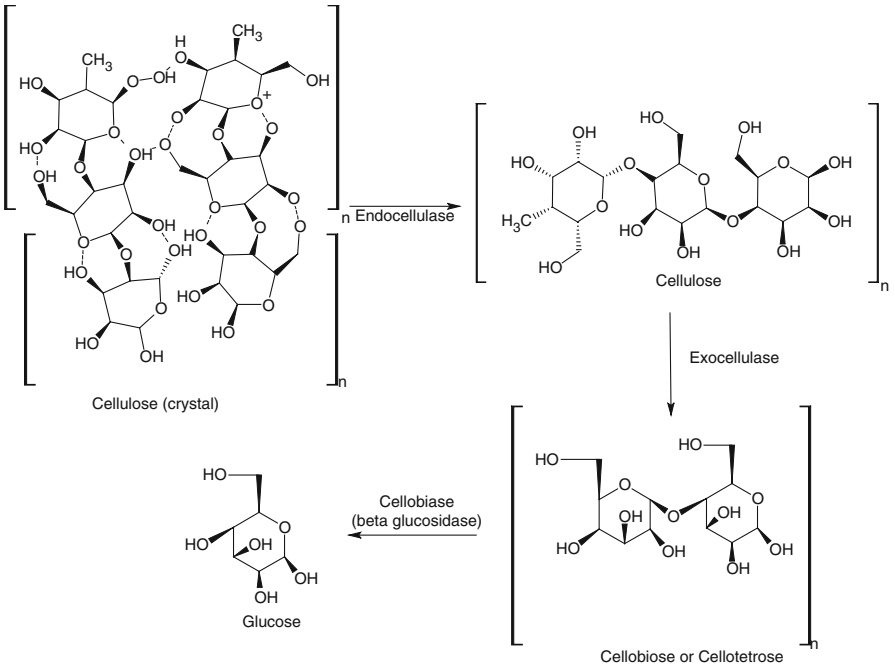

Fig. 29.1 Schematic representation of coordinated action of all three cellulases on cellulose polymer. (**a**) Breakage of the non-covalent interactions present in the crystalline structure of cellulose by endo-cellulase (**b**) Hydrolysis of the individual cellulose fibers to break it into smaller sugars by exo-cellulase (**c**) Hydrolysis of disaccharides and tetrasaccharides into glucose by β-glucosidase

The cellulolytic enzymes are members of a super family of GHs (glycoside hydrolases), with more than 2,200 known protein sequences (Heinzelman et al. 2009). To date, the GHs have been classified into more than 80 different GH families based on their amino acid sequence similarities (Henrissat and Bairoch 1993; Bourne and Henrissat 2001). Cellulases are found in families 5–12, 26, 44, 45, and 48 (Coutinho and Henrissat 1999). Family 12 comprises endoglucanases from mesophilic and bacteria, fungi, and archaea (Crennell et al. 2002; Sandgren et al. 2003). As per the X-ray structures of five enzymes of GH family, 12 do not contain a cellulose-binding module, which explains the low activity of these enzymes on crystalline cellulose (Sandgren et al. 2003; Nurizzo et al. 2002). Figure 29.2 reveals the 3-D structure of cellulase produced by *Humicola grisea* Cel12A. It is a fungus with a growth temperature maximum above 50°C, producing a number of thermostable enzymes including hemicellulases and cellulases (Maheshwari et al. 2000). The secreted product of the *H. grisea* Cel12A gene is identical to that from the GH 12 gene reported for *Humicola insolens* (Dalboge and Heldt-Hansen 1994).

Cellulase from *H. grisea* contains the complete sequence of 224 amino acids. It shares 44% sequence identity (93 residues out of 218) with the *T. reesei* Cel12A enzyme (Sandgren et al. 2003). Equivalent C_α atoms from the two structures can be superimposed with pair wise root-mean-square deviations (RMSDs) in the range of 0.3–0.5 (angstrom). There are no large insertions or deletions in the *H. grisea*

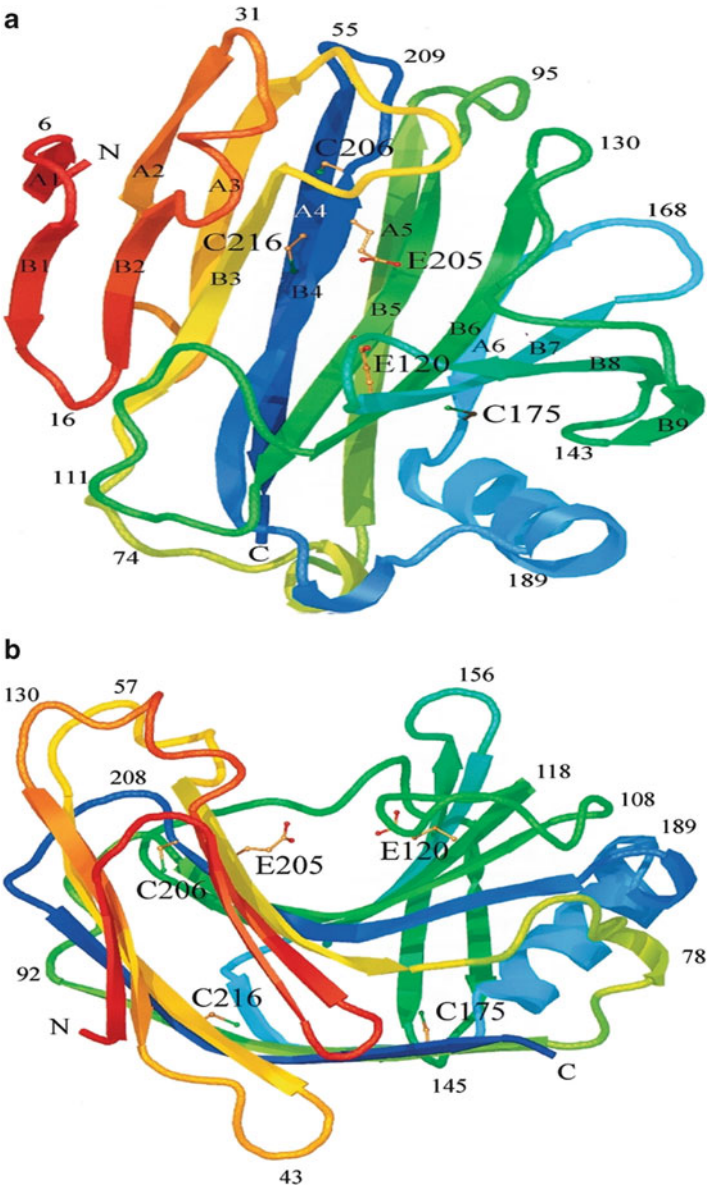

Fig. 29.2 The crystal structure model of cellulase enzyme produced by *H. grisea* Cel12A. Schematic ribbon diagram showing *top* (**a**) and *side views* (**b**) of the *H. grisea* Cel12A crystal structure, color-ramped according to residue number, starting with red at the N terminus and ending with blue at the C terminus of the structure. The structure has the expected fold of a GH family 12 enzyme. It consists mainly of 15 β-strands building up two β-sheets, (**a**) and (**b**), (**a**) consisting of six β-strands and (**b**) of nine β-strands, which stack on top of one another revealing a β-sandwich. The individual β-strands are labeled (A1–A6 and B1–B9) according to their positions in the two β-sheets. The structure has side chains drawn for the three cysteine residues (C175, C206, and C216) and the two catalytic residues (E120 and E205) (Source: Sandgren et al. 2003 with permission and courtesy of "Protein Science")

structure, compared with the *T. reesei* structure. The extra six residues in the *H. grisea* structure are distributed over the molecule as single amino acid insertions. Two of the extra residues are located at the N terminus. There are four loops in the 3-D structure of *H. grisea* Cel12A. These loops correspond to residues 29–32, 40–47, 92–96, and 165–169 and connect β-strands B2 to A2, A2 to A3, A5 to B5, and A6 to B7, respectively.

The 3-D structure of various cellulases varies from one microorganism to the other. For example, cellulase (68 K M*r* Fragment) from *Thermomonospora fusca* consists of barrel fold, family III cellulose-binding domain, and antiparallel β-sandwich fold. It is unusual in nature as it shows the properties of both exo- and endo-cellulases. While acting as exo-cellulase, it cleaves off cellotetraose units. Sakon et al. (1997) reported the crystal structure of cellulase from *T. fusca*. As catalytic residues, two highly conserved glutamic residues have been identified in the cellulases, which are supposed to act as a nucleophile and acid/base catalyst. The genomic sequence of the cellulose degrader *Clostridium thermocellum*, 71 ORFs were identified for the production of cellulosome components (Zverlov et al. 2001). Many of them code for putative or characterized cellulases but also for non-cellulosic polysaccharide hydrolases such as xylanases, glycosidases, a mixed-linkage glucanase, a xyloglucanase, a chitinase, pectinases, and esterases. Most of the latter enzymes are required for separation of cellulose from hemicellulose.

One of the most important microorganisms, *C. thermocellum* ATCC 27405 produces an extracellular cellulase system capable of hydrolyzing crystalline cellulose (Demain et al. 2005). The enzyme system comprises of a multicomponent protein aggregate (the cellulosome) with a total molecular weight in millions, impeding mechanistic studies (Bayer et al. 1998).

29.3 Critical Review

29.3.1 Production of Thermophilic Cellulases: Microorganisms and Their Cultivation

Thermotolerant microorganisms have excelled at producing primary and secondary metabolites from a variety of raw carbohydrates for billions of years under varying cultivation processes (Margaritis and Merchant 1986; Haki and Rakshit 2003). Today, the results of studying the giant “microbial libraries” are currently in vogue for microbial conversion of alternative carbohydrates into value-added products that can also be applied to thermostable cellulase production. Thermophilic fungi have received significant attention in recent years as a source of new thermostable enzymes for use in many biotechnological applications, including biomass degradation. Thermophilic cellulases are the promising enzymes for efficient biomass degradation (Duo-Chuan Li et al. 2011; Muhammad Mohsin Javed et al. 2011).

In addition, timely interventions, such as strain improvement through mutagenesis, gene cloning and expression, and optimization of potential fermentation parameters, can enhance the production of selective metabolites (Margaritis and Merchant 1986; Chandel et al. 2008).

Advances in isolation and screening of thermophilic microorganisms include the isolation of a large number of beneficial microorganisms from different exotic ecological zones of the earth and the subsequent extraction of cellulolytic enzymes from them (Groboillot 1994; Bharat and Hoondal 1998; Bauer et al. 1999; Kohilu et al. 2001).

Haki and Rakshit (2003) reviewed the properties of cellulases (pH and temperature optima) of selected microorganisms derived from natural isolates. Thermostable cellulases of archaeal origin include those isolated from *Pyrococcus furiosus* (Kengen et al. 1993) and *Pyrococcus horikoshii* (Ando et al. 2002) with temperature optima between 102–105 and 97°C, respectively.

For the maximum biomass conversion process, critical factors such as stability of cellulase, substrate and product inhibition, physical factors (temperature, pH, enzyme loadings, and substrate to liquid ratio), and synergism among different cellulases play the key role (Taherzadeh and Karimi 2007).

Thermostable enzymes are generally isolated from thermophiles and hyperthermophiles (Vieille and Zeikus 2001). Due to the rapid growth and high rate of cellulose decomposition, thermotolerant microorganisms like bacteria and fungi are the right source for the cellulases (Bruins et al. 2001; Margaritis and Merchant 1986). Many fungi such as *Chaetomium thermophilum* var. *dissitum*, *C. thermophilum* var. *coprophile*, *Humicola insolens*, *Humicola grisea* var. *thermoidea*, *Myceliophthora thermophila*, *Talaromyces emersonii*, and *Thermoascus aurantiacus* were explored for thermophilic cellulase production (reviewed by Margaritis and Merchant 1986; Maheshwari et al. 2000; Turner et al. 2007).

Often, microorganisms can adapt to a variety of fermentation media. However, multiple essential nutrients along with suitable carbon sources are required for any fermentation reaction (Danson et al. 1992; Stetter 1999). A number of production media were optimized for thermostable cellulase production by different microorganisms. Using the formulated fermentation media for the cultivation of thermophilic microorganisms is an important parameter to get the utmost yield of cellulase titers in either submerged or solid-state cultivations (Margaritis and Merchant 1986; Mathew et al. 2008).

Recently, Rastogi et al. (2009) isolated and characterized the thermostable cellulases from various isolates from compost samples. Eight isolates capable of degrading cellulose, carboxymethyl cellulose (CMC), or ponderosa pine sawdust were identified as belonging to the genera *Geobacillus, Thermobacillus, Cohnella,* and *Thermus*. Two isolates WSUCF1 (*Geobacillus* sp.) and a previously isolated cellulose-degrading deep gold mine strain DUSELR13 (*Bacillus* sp.) were examined for their enzyme properties and kinetics. The optimal pH for carboxymethyl cellulase (CMCase) activity was 5.0 for both isolates. The optimum temperatures for CMCase of WSUCFI and DUSELR13 were 70 and 75°C, respectively.

Li et al. (2009) isolated a novel strain of *E. coli* from soil that produced novel thermoalkalotolerant cellulases showing the titers of CMCase 0.23, FPase 0.08, and β-glucosidase 0.15 U/ml. More than 60% of maximum CMCase activity was retained, for at least 20 min at 50–70°C and 10 min at 80°C, and approximately 50% of its maximal activity was retained after incubating at 90°C for 10 min. Such enzymes could be applied in the bioconversion of lignocellulosic agricultural wastes.

Cultivation of thermophilic microbes at high temperature is technically and economically interesting, as it reduces the risk of contamination and viscosity thus making mixing easier, and leads to a high degree of substrate solubility (Margaritis and Merchant 1986; Turner et al. 2007). However, the biomass production by these organisms is low as compared to mesophilic microorganisms. It affects the enzyme yield considerably if the enzyme is being produced intracellularly. This makes necessary research to be carried out for improving the cell yield (Bhat and Maheshwari 1987). For the large-scale commercial cultivation of thermophiles, some critical factors such as requirement of complex and expensive media (Krahe et al. 1996), low solubility of gas at high temperature, low specific growth rates, and product inhibition (Schiraldi and De Rosa 2002) need to be focused.

Jang and Chang (2005) studied the large-scale production of thermostable cellulase by *Streptomyces* sp. T3-1 when grown in a 50-l fermenter, maximum cellulase production profile (148 IU CMCase/ml, 45 IU Avicelase/ml, and 137 IU β-glucosidase/ml) was recorded after 4th day of incubation when agitation and aeration rate were maintained at 300 rpm and 0.75 vvm, respectively. A comprehensive analysis of various microorganisms, their cultivation conditions, and the enzyme production profile is summarized in Table 29.1.

Thermostable cellulases of archaeal origin include those isolated from *Pyrococcus furiosus* (Kengen et al. 1993) and *Pyrococcus horikoshii* (Ando et al. 2002). While the latter has an optimum temperature of 97°C, the enzyme from *Pyrococcus furiosus* has shown optimal activity at 102–105°C. *Sulfolobus solfataricus* MT4, *S. acidocaldarius,* and *S. shibatae* were also described as producers of β-glucosidases (Grogan 1991). From *Thermotoga maritima* MSB8, optimally active cellulase acting at 95°C and between pH 6.0 and 7.0 was reported (Bronnenmeier et al. 1995).

Reduction in the cost of enzyme production is the fundamental necessity for a breakthrough in large-scale production (Jang and Chang 2005). One of the alternatives to reduce production costs and increase the yield of these processes is to use recombinant technology. Mesophilic organisms such as *Escherichia coli* (Ramchuran et al. 2002), *Bacillus subtilis* (Soutschek-Bauer and Staudenbauer 1987), *Saccharomyces cerevisiae* (Moracci et al. 1992), and *Trichoderma reesei* (Bergquist et al. 2002) have been developed in to thermotolerant microorganisms for the production of thermostable cellulase.

However, differences in codon usage or improper folding of the proteins can result in reduced enzyme activity or low level of expression (Ciaramella et al. 1995; Duffner et al. 2000). Moreover, many complex enzymes, like heterooligomers or those requiring covalently bound cofactors, can be very difficult to produce in mesophilic hosts. This initiated the search of genetic tools for the overexpression of such

Table 29.1 Cellulase production from microorganisms under different cultivation conditions

Microorganisms	Source of isolation	Enzyme activity	Method of cultivation	Carbon source used	References
Aspergillus japonicus URM5620	NA	FPase 953.4, CMCase 191.6, β-glucosidase 88.3 IU/g	SSF (solid-state fermentation)	*Castor bean meal*	Polyanna Nunes Herculano et al. (2011)
Bacillus spp.	Compost	FPase 1.333 IU/ml	SmF (submerged fermentation)	Organic compost	Mayende et al. (2006)
Thermoascus aurantiacus	NA	Endoglucanase 1,572 U/g and β-glucosidase 101.6 U/g	SSF (solid-state fermentation)	Agro-residues	Kalogeris et al. (2003)
Geobacillus sp. 70PC53	Rice straw compost	Acid-swollen Avicel 41.40 , CMC 116.4, β-D-Glucan (barley) 1,267.3, FPase 1.0 (IU/mg)	SmF	Rice straw and rice hull	Ng et al. (2009)
Aspergillus fumigatus	Sugarcane bagasse	Endoglucanase (CMCase) 365 U/l	SmF	Sugarcane bagasse (1%) and corn steep liquor (1.2%)	Grigorevski-Lima et al. (2009)
Aspergillus fumigatus	Sugarcane bagasse	FPase 47 U/g	SSF	Wheat bran and sugarcane bagasse	Grigorevski-Lima et al. (2009)
Bacillus subtilis strain I15	NA	Cellulase activity 2.82 U/ml	SmF	CMC	Yang et al. (2010a, b)
Aspergillus nidulans (Th4)	Farmyard manure	FPase 9.27, CMCase 14.95, cellobiase 134.21, Xylanase 150.16 IU/ml	SSF	Paddy straw and soybean trash	Kumar et al. (2008)
Scytalidium thermophilum (Th5)	Soil	FPase7.49, CMCase 30.36, and cellobiase 69.17 (IU/ml)	SSF	Paddy straw and soybean trash	Kumar et al. (2008)
Humicola sp. (Th10)	Wheat straw compost	FPase11.43, CMCase 15.38, and cellobiase 90.20 (IU/ml)	SSF	Paddy straw and soybean trash	Kumar et al. (2008)
Fusarium chlamydosporum	Soil beneath the rotten wood	FPase 95.2, CMCase 281.8, cellobiohydrolase 182.4, β-glucosidase 135.2 IU/g	SSF	Sugarcane bagasse	Qin et al. (2010)

(continued)

Table 29.1 (continued)

Microorganisms	Source of isolation	Enzyme activity	Method of cultivation	Carbon source used	References
Penicillium citrinum	Soil	Endoglucanase 1.89 ± 0.12, FPase 1.72 ± 0.14 IU/ml	SSF	Wheat bran	Dutta et al. (2008)
P. citrinum	Soil	Endoglucanase 2.04 ± 0.13, FPase 0.64 ± 0.16 IU/ml	SSF	Rice bran	Dutta et al. (2008)
P. citrinum	Soil	Endoglucanase 0.93 ± 0.035, FPase 1.14 ± 0.095 IU/ml	SSF	Rice straw	Dutta et al. (2008)
Geobacillus stearothermophilus	Soil	Avicelase 1.9 IU/ml	SmF	Sugarcane bagasse	Makky (2009)
Acidothermus cellulolyticus	Decaying wood	Endoglucanase 40 IU/ml	SmF	Citrus peel	Kaur et al. (2004)
Bacillus sp. NZ	Degrading lignocellulose	Endoglucanase 1,960–2,280 U/l	SmF	Wheat bran	Nizamudeen and Bajaj (2009)
Anoxybacillus flavithermus EHP1	Egyptian hot spring	CMCase 65.56, Avicelase 25.30, cellobiase 30.12, xylanase 11.5(U/ml)	SmF	Carboxymethyl cellulose	Ibrahim and El-diwany (2007)
Talaromyces emersonii	Compost heap	Endo-cellulase 206, barley β-glucanase 6.1, β-glucosidase 50.5 (IU/g)	SmF	Carob powder	Gilleran et al. (2010)
Anoxybacillus sp. 527	Swine waste	FPU 7 IU/ml	SmF	Avicel cellulose	Liang et al. (2010)
Streptomyces sp. T3-1	Agricultural waste compost	CMCase 33.3, Avicelase 3.2, β-glucosidase 20.5 IU/ml	SmF	CMC	Jang and Chen (2003)
Caldibacillus cellulovorans	Artificial compost	CMCase 44.7 IU/ml	SmF	Paper tissue	Huang and Monk (2004)
Stachybotrys atra BP-A	Rotten rag	CMCase 3.11 U/ml	SmF	CMC or rice straw	Picart et al. (2008)
Brevibacillus sp. JXL	Swine waste	FPU 0.02 IU/ml	SmF	Crystalline cellulose	Liang et al. (2009)

enzymes in host systems. So far, a number of vectors have been developed for expression of proteins in various hosts (reviewed by Turner et al. 2007). Use of the novel expression systems are, however, still at research level, and more work remains to be exploited at large or industrial scale.

29.3.2 Downstream Process for Cellulases from Thermophiles and Their Characterization

Beyond purification, recovering the desired product with stable activity and in pure form after fermentation is one of the tedious tasks for fermentation industries. Downstream processing plays a key role in deciding the economic viability of any fermentative product. Regardless the site-specificity (i.e., intracellular and/or extracellular) of an enzyme, product stability adds another step in the recovery process that can add to the overall cost of the desired product in a fermentation reaction (Torrent et al. 2009). The separation of extracellular enzymes from undesired proteins is less tedious than the separation of enzymes generated within the cell.

Endoglucanases from fungi displayed temperature optima between 50 and 80°C (Maheshwari et al. 2000). A significant amount of work has been carried out on the purification and characterization of endoglucanases from fungi, such as *Chaetomium thermophilum* var. *dissitum*, *C. thermophilum* var. *coprophile*, *Humicola insolens*, *Humicola grisea* var. *thermoidea*, *Myceliophthora thermophila*, *Talaromyces emersonii,* and *Thermoascus aurantiacus* (Torrent et al. 2009).

Nascimento et al. (2010) investigated the effect of several carbon sources on the production of mycelial-bound β-glucosidase by *Humicola grisea* var. *thermoidea* in submerged fermentation followed by purification and characterization. The enzyme was purified by ammonium sulfate precipitation, followed by Sephadex G-200 and DEAE-cellulose chromatography, showing a single band in PAGE and SDS-PAGE. The β-glucosidase had a carbohydrate content of 43% and showed apparent molecular masses of 57 and 60 KDa, as estimated by SDS-PAGE and gel filtration, respectively. The optimal pH and temperature were 6.0 and 50°C, respectively.

An intracellular β-glucosidase from filamentous fungus *Termitomyces clypeatus* was purified to homogeneity by gel filtration, ion exchange chromatography, and HPGPLC from mycelia extract of *T. clypeatus* in the presence of the glycosylation inhibitor 2-deoxy-d-glucose. The enzyme showed around 80% stability up to 60°C, and residual activity was 80–100% between a range of pH 5–8. The enzyme will be useful in synthetic biology to produce complex bioactive glycosides and to avoid chemical hazards (Pal et al. 2010).

β-Glucosidase (BGL) from *Penicillium brasilianum* was purified to homogeneity and was successfully expressed in *Aspergillus oryzae*. A study on enzyme characteristics showed that BGL had excellent stability at elevated temperature of 60°C with no loss of activity even after 24 h of incubation at pH 4–6 (Krogh et al. 2010).

Table 29.2 Engineering methods to develop thermostable proteins

Methodology	Effect of the methodology induced	References
Structure-based changes (rearrangement of oligonucleotides in active site of proteins)	Showing the different specific activity and their diversification	Cicortas et al. (2004)
Structure-based direct evolution	Increasing the temperature range of the proteins and active site region	Hasan et al. (2006), Short (1998)
Site-directed mutagenesis	Reduce the amino acid residue (Gln, Asn, Cys, Met) numbers in protein helix, substitution of surface-exposed amino acid residues to increase the thermostability	Bruins et al. (2001), Vieille and Zeikus (2001)

29.3.3 Stability and Development of Thermostable Cellulases

The application of thermophilic microorganisms and their enzymes is getting more attention than mesophiles and their enzymes due to their stability at higher temperatures (Johri et al. 1999). Particularly, their role in saccharification of cellulosics into fermentable sugars for the production of value-added chemicals is very crucial in renewable chemical-based industries. Thermostable cellulases are those proteins which have high defined unfolding (transition) temperature (T_m) or a long half-life at a selected high temperature (>55°C). Most proteins derived from thermophilic microorganisms are thermostable in nature (Turner et al. 2007). Extracellular proteins generally show high thermostability than intracellular proteins (Santos and da Costa 2002).

Thermostability of mesophilic proteins could be increased by adopting structural and computational biology-based tools and techniques. Apart from the protocols mentioned in Table 29.2, emerging trends in structural biology-based techniques such as stabilization of helix, protein–protein interactions, domain/subunit interactions, and loops and turns have shown interesting results towards the structural changes incorporated for better thermostable reactions (Vieille and Zeikus 2001; Danson and Hough 1998; Yano and Poulos 2003; Crennell et al. 2006; Johannes and Zhao 2006).

Heinzelman and colleagues (2009) demonstrated the genetic stability of cellulase chimeras made by SCHEMA structure-guided recombination, by introducing mutation into *Hypocrea jecorina* and *H. insolens* which improved longtime hydrolysis performance. This mutation also stabilized and improved Avicel hydrolysis by *Phanerochaete chrysosporium* CBH II, which is only 55–56% identical to recombination parent CBH IIs. These results showed that SCHEMA structure-guided recombination enables quantitative prediction of cellulase chimera thermostability and efficient identification of stabilizing mutations.

According to Viikari et al. (2007), fungal thermostable cellulases show better performance relative to their mesophilic counterparts in laboratory scale biomass conversion processes. Enhanced thermostability leads to retention of activity over longer periods of time at both moderate and elevated temperatures.

29.3.4 Applications of Thermostable Cellulases

The global market for enzymes was estimated to be about $2 billion in 2004 in tune with an average annual growth rate of 3.3% (Chandel et al. 2007a). Of the total enzyme market, pharmaceutical enzymes accounted for 41%, food and feed industry 17%, and detergent industry 17% (www.biospectrumindia.com). A recent McKinsey estimate suggests that the total value created by enzymes could be to the tune of $12 billion a year by 2010 globally. In India, the maximum demand of enzymes is in pharmaceutical industries, followed by food/feed and textiles (Chandel et al. 2007a). In all these applications, thermotolerant cellulases play a pivotal role. Therefore, the large-scale production of thermotolerant enzymes showing the broad-substrate ranges and high specificity is essential to meet the requirement for the above applications. Particularly, the role of thermostable cellulases in bioethanol production will be decisive for the commercial success of biorefineries in future.

Currently, thermotolerant cellulolytic enzymes are being employed in the color extractions of juices, in detergents causing color brightening and softening, in the biostoning of jeans, in the pretreatment of biomass that contains cellulose to improve nutritional quality of forage, and in the pretreatment of industrial wastes (Buchert et al. 1997; Niehaus et al. 1999; Bhat 2000; Nakamura et al. 2001; van Wyk et al. 2001; Zhou et al. 2001). In order to attack the native crystalline cellulose, thermostable cellulases which are active at high temperature and high pH are required (Haki and Rakshit 2003).

29.3.5 Recombinant Enzyme Production in Mesophilic Hosts

The recent developments in recombinant DNA technology made it possible to clone and express the thermostable enzymes from thermophiles into mesophiles. In previous studies, the expression of thermophiles into mesophiles has been successfully implemented in mesophilic organisms, such as *Escherichia coli* (Ramchuran et al. 2002), *Bacillus subtilis* (Soutschek-Bauer and Staudenbauer 1987), *Saccharomyces cerevisiae* (Moracci et al. 1992), *Pichia pastoris* (Ramchuran et al. 2006), *Aspergillus oryzae* (Shinohara et al. 2001), *Kluyveromyces lactis* (Walsh et al. 1998), and *Trichoderma reesei* (Bergquist et al. 2002). However, the expression level has been observed low apparently showing poor enzyme yields due to improper folding of proteins.

For the effective degradation of cellulose, Voutilainen et al. (2008) studied the properties of a novel GH-7 family cellobiohydrolases from the fungi *Acremonium thermophilum, Thermoascus aurantiacus, and Chaetomium thermophilum*. The *Trichoderma reesei* Cel7A enzyme was used as a reference in the experiments. As the native *T. aurantiacus* Cel7A has no carbohydrate-binding module (CBM), recombinant proteins having the CBM from either the *C. thermophilum* Cel7A or the *T. reesei* Cel7A were also constructed. All these novel acidic cellobiohydrolases were more thermostable (by 4–10°C) and more active (two- to fourfold)

Table 29.3 Microorganisms showing the sugar utilization profile, temperature, and pH range

Thermophilic microorganisms	Glucose	Xylose	Arabinose	Mannose	Cellulose	Temp. range (°C)	pH range
Clostridium thermocellum	+	−	+	−	+	65	4–8
C. thermohydrosulfuricum	+	+	+	−	−	65	4.7–8
C. thermosaccharolyticum	+	+	+	+	−	60	5–8
C. thermosulfurogenes	+	+	+	+	−	60	4.5–7.5
Thermoanaerobacter ethanolicus	+	+	+	+	−	69	4.4–9.5

in hydrolysis of microcrystalline cellulose (Avicel) at 45°C than *T. reesei* Cel7A. The *C. thermophilum* Cel7A showed the highest specific activity and temperature optimum when measured on soluble substrates. The most effective enzyme for Avicel hydrolysis at 70°C, however, was the 2-module version of the *T. aurantiacus* Cel7A, which was also weakly inhibited by cellobiose.

Yang et al. (2010a, b) recently developed recombinant *E. coli* BL21 (DE3) by the expression of cellulase gene, celI15A extracellularly from novel bacterial strain (*Bacillus subtilis* strain I15) with high cellulase activity (2.82 U/ml). The native thermostable cellulase exhibited the maximum activity at 60°C and pH 6.0. It was very stable since more than 90% of original CMCase activity was maintained at 65°C after incubation for 2 h. The extracellular activity of CelI15 from *E. coli* BL21 was about 6.78 U/ml, and all the other properties were same as that of the wild strain.

Reversely, the expression of mesophilic proteins into thermophiles could be more demanding and useful for the commercial interest due to their direct applications in large-scale reactors. In this direction, a number of shuttle and integrated vectors have been constructed in a variety of hosts (*Thermus thermophilus, Sulfolobus solfataricus*, *Talaromyces* sp. CL240, *Rhodothermus marinus, Pyrococcus abyssi*, *Thermoanaerobacterium saccharolyticum, Sulfolobus acidocaldarius,* and *Pyrococcus furiosus*) for the expression of mesophilic proteins in thermophiles (reviewed by Turner et al. 2007).

Table 29.3 shows the thermophilic bacterium which can be used along with *Clostridia* cultures for the maximum conversion of lignocellulose hydrolysates into ethanol. The pentose-utilizing genes from *P. stipitis*, *C. shehatae,* and *P. tanophillus* can be cloned into the microorganisms mentioned in the table for their best suitability in bioethanol production from lignocellulose hydrolysates.

29.3.6 Recent Developments Seen in Thermostable Cellulase Research

Other endo-cellulases (CelA and CelB) from *Thermotoga neapolitana* were purified and characterized (Bok et al. 1998). Optimum pH for CelA is 6.0 at 95°C, with the ability to hydrolyze microcrystalline cellulose, and the pH range of CelB is broad

(6.0–6.6) at 106°C. Extremely thermostable cellulase was isolated from the bacterium *Anaerocellum thermophilum* (Zverlov et al. 1998), and maximal activity of this enzyme was observed at pH 5.0–6.0 and 85–95°C. A highly thermostable cellobioase (115°C at pH: 6.8–7.8) was also produced from *Thermotoga* sp. FJSS3-B1 (Ruthersmith and Daniel 1991, 1992).

Abdeev et al. (2009) developed a new strategy for the expression of genes from bacteria encoding functional analogues of plant proteins with high specific activity and thermal stability in transgenic plants. They reported the expression of bacterial β-1,4-glucanase which had a significant effect on transgenic tobacco plant metabolism, namely, it affects plant morphology, the thickness of the primary cell wall, phytohormonal status, and the relative sugar content. Jiang et al. (2011) expressed two bacterial thermostable cellulases (E2 and E3) and a E3-E2 fusion form in tobacco. The recombinant cellulases showed good thermostability below 65°C. This is indicative of a useful application for heterologous application of thermostable cellulases in plant for biomass conversion.

Cellulase engineering on natural cellulosic substrates is of importance for emerging biomass-based biorefineries. In this direction, directed enzyme evolution could be a very popular tool for the identification of desired mutants from a large mutant library. Liu et al. (2009) demonstrated a novel selection/screening strategy for finding thermostable β-glucosidase on its natural substrate-cellobiose. The thermostable mutants were identified from a random mutant library of the *Paenibacillus polymyxa* β-glucosidase. The most thermostable mutant A17S showed 11-fold increase in the half-life of thermo-inactivation at 50°C.

Three thermostable cellulases, identified as the most promising enzymes in their categories (cellobiohydrolase, endoglucanase, and beta-glucosidase), were cloned and produced in *Trichoderma reesei* and mixed to compose a novel mixture of thermostable cellulases. The maximum FPU activity of the novel enzyme mixture was about 25% higher at the optimum temperature of 65°C, as compared with the highest activity of the commercial reference enzyme at 60°C (Viikari et al. 2007). Many extremely thermostable enzymes implicated in the deconstruction of lignocellulose can be identified in genome sequences, and many more promising biocatalysts probably remain annotated as "hypothetical proteins" (Blumer et al. 2008).

It is obvious that the successful utilization of cellulose is dependent on the development of economically feasible technologies for the production of cellulase. The bio-polishing process of cotton in the textile industry, for example, requires cellulase stable at high temperature close to 100°C (Ando et al. 2002). Presently used enzymes for this purpose, however, are active only at 50–55°C. Cellulase production is also found to be the most expensive step during ethanol production from cellulosic biomass and accounted for approximately 40% of the total cost (Spano et al. 1975). In the food industry, degradation of cellulose by acids is still unsatisfactory and results in the decomposition of the sugars. Finally, even though many cellulolytic enzymes of thermophilic origin are known, their function under physiological condition remains unclear.

Patents taken for cellulase production from thermostable microorganisms have been shown in Table 29.4.

Table 29.4 Important patents on thermotolerant cellulase production

Patent number	Title	Owner	Issue date
4966850	Production of thermostable xylanase and cellulase	Yu et al.	10/1/1990
5110735	Thermostable purified endoglucanase from bacterium *Acidothermus cellulolyticus*	Tucker et al.	5/1/1992
5275944	Thermostable purified endoglucanas from *Acidothermus cellulolyticus* ATCC 43068	Himmel et al.	1/1/1994
5366884	Thermostable purified endoglucanase II from *Acidothermus cellulolyticus*	Adney et al.	11/1/1994
5432075	Low molecular weight thermostable β-D-glucosidase from *Acidothermus cellulolyticus*	Himmel et al.	7/1/1995
5536655	Gene coding for the E1 endoglucanase	Thomas et al.	7/1/1996
5677151	Thermostable cellulase from a *Thermomonospora* gene	Wilson et al.	10/1/1997
5712142	Method for increasing thermostability in cellulase enzymes	Adney et al.	27/1/1998
6812018	Thermostable cellulase	Wicher et al.	31/11/2004
8137945	Thermostable cellulase having increased enzyme activity	Guo, Rey-ting et al.	20/03/2012

29.4 Analysis

Use of thermostable cellulases, hemicellulases, and ethanol producing microorganisms in the degradation of the lignocellulosic material into sugars and further conversion of sugars into ethanol offers an advantage by minimizing the risk of contamination and could enable a single-step process of enzymatic hydrolysis and ethanol fermentation (Chandel et al. 2007b). In this regard, thermophilic microorganism such as C. *thermocellum* could play a key role in biorefinery due to its following properties (Demain et al. 2005):

- The cellulolytic and ethanogenic nature, allowing saccharification and fermentation in a single step
- The anaerobic nature, negating the need for expensive oxygen transfer
- Low cell growth yield, favoring ethanol conversion
- Facilitates easy ethanol removal and recovery
- Reduction of cooling cost
- Fermentation being less prone to contamination
- Biomass-degrading enzymes enhancing protein stability
- Amenability to coculture with other ethanol-producing and pentose-fermenting microorganisms

The primary advantages of a direct conversion of biomass to ethanol by *Clostridia* include elimination of capital or operating costs for enzyme production, greatly reduced diversion of substrate for enzyme production, and compatible enzyme and fermentation systems. Conventionally, the cost of the cellulase production by *Trichoderma or Aspergillus* and their use in ethanol production by yeast is still high. By adopting direct *Clostridial* coculture process or SSF system (using cellulases and ethanol producing microorganisms together), the overall ethanol production cost could be brought down. As per the observation of Hogsett and colleagues (1992), ethanol production cost could be saved by 50 cents per gallon by adopting the *Clostridial* coculture system as compared to conventional *Trichoderma/Aspergillus*-yeast SSF process, since the former process combines cellulase production, hydrolysis, and fermentation in a single bioreactor.

The conversion of agricultural, forest, and urban resources into ethanol with Clostridial cultures could become an economic substitute for petroleum fuels when oil prices are about US$30 or more per barrel (Demain et al. 2005). Studies conducted for ethanol yield from hardwood hydrolysate using *C. thermocellum* were found 2.5 times higher than with the conventional *Trichoderma* cellulase and *S. cerevisiae* in a continuous cultivation (South et al. 1995).

Lynd (1989) also observed a twofold improvement in ethanol yield using anaerobic bacteria than mesophilic cultures. One of the most famous bioethanol research group, Lynd, and his colleagues further advocated about the adoption of direct Clostridia coculture process for fuel ethanol production. In Clostridial conversion, the use of mixed-culture systems is of great necessity as *C. thermocellum* is capable of utilizing only the hexose sugars and not the pentose sugars generated from lignocelluloses hydrolysis. In order to get the maximum ethanol production, *C. thermocellum* can be cocultivated with other anaerobic bacteria that are capable of utilizing pentose as well as hexose sugars, that is, *C. thermosaccharolyticum* (Saddler and Chan 1982; Wang et al. 1983; Venkateswaran and Demain 1986), *C. thermohydrosulfuricum* (Ng et al. 1981; Germain et al. 1986), *Thermoanaerobacter ethanolicus* (Wiegel and Ljungdahl 1981), and *Thermoanaerobium brockii* (Lamed and Zeikus 1980).

Direct conversion of cellulose into ethanol was also studied by many workers (Venkateswaran and Demain 1986; Wang et al. 1983) at Massachusetts Institute of Technology. They used, dual closely associated, syntrophic and stable cultures for the maximum utilization of pentose and hexose sugars into ethanol (Wiegel 1981).

Cellulose is broken down by the cellulase complex of *C. thermocellum* to cellobiose and cellodextrins, which are then utilized by the organisms to produce ethanol; unfortunately, there are few limitations which make the Clostridial cultures unsuitable for biorefinery. Their inability to utilize the pentose sugars, mainly xylose and xylobiose, formed by the breakdown of hemicelluloses and the production of acetate and lactate during ethanol production make them not a good choice in bioethanol program.

29.5 Future Perspectives

The recent progress in biotechnology of cellulases is truly remarkable and getting worldwide attention. However, development of thermostable cellulase biotechnology has not been rationally discussed. Currently, cellulases are widely used in textile and laundry, biorefinery, food and animal feed, brewery and wine, and pulp and paper industries.

Despite these well studied and set applications, new kind of applications can also be seen in near future with the help of recent developments in biochemistry, genetics, and protein engineering, as well as the structure–function relationships. C. *thermocellum* has been studied extensively for its potential use in research, medicine, and other applied fields of biotechnology.

In today's world, industrial developments and rapid pace of urbanization have called for environmentally sustainable energy and other chemical sources. Harnessing of lignocellulosic biomass for ethanol and other chemicals seems the most appropriate solution to fulfill the future energy demand with the safe and clean environment. Degradation of cellulose polymer by cellulases particularly thermophilic cellulases into simpler sugars at large scale is an important aspect for the successful implementation of biorefinery concept. Thermophilic cellulases can be incorporated in consolidated bio-processing (CBP) of lignocelluloses to ethanol conversion by making the process simpler and less staggered. Thermophilic cellulases are operationally more stable at high temperature thus allowing for, for example, easy mixing, better substrate solubility, high mass transfer rate, and lowered risk of contamination. Thermophiles have often been considered to be important sources of industrially relevant thermostable enzymes.

Looking at the increased applications of thermostable cellulases in future and to realize their potential in biotechnology and research, continued multidisciplinary research on basic and applied aspects is required. These developments together with improved scientific knowledge are expected to pave the way for a remarkable success in the biotechnology of thermophilic cellulases in the twenty-first century.

Now it is clearly understood that thermostable cellulases will decide the fate of success of biorefinery (Fernando et al. 2006; Kamm and Kamm 2004). Future developments include the expression of thermotolerant cellulases in most common hosts for the successful production at industrial scale and their eventual application in biomass saccharification. Development of thermostable cellulases, potent thermotolerant yeast strains, and ethanol production from both pentose and hexose sugars are important for biorefinery.

With the increase in market for thermostable cellulases in biorefinery, textile and detergent, and food and feed sector, leading to production in higher volumes, the cost is, however, predicted to decrease. It can be done by developing recombinant microorganisms for increased cellulase titers production, development of fermentation methods with simpler strategy, and using cheap nutrients and downstream process. Moreover, with a paradigm shift in industry moving from fossils towards renewable resource utilization, the need of microbial catalysts is predicted to increase, and

certainly there will be a continued and increased need of thermostable selective biocatalysts in the future in the form of thermostable cellulases. Characterization of these enzymes will require intensive effort but is likely to generate new opportunities for the use of renewable resources as biofuels (Blumer et al. 2008).

29.6 Conclusions

Cellulases from thermophilic microorganisms gained a great deal of interest due to their various applications in biorefinery and other sectors such as in textile and detergent industry, food and feed, paper industries as de-inking agent, etc. To compare with the mesophilic cellulases, thermophilic cellulases have many important advantages. They can work at high temperature well suited with the direct applications, reducing the cost of temperature maintenance in the biochemical reactions. Particularly the applications in saccharification of cellulosic biomass, textile and paper industries, and thermophilic cellulases play a key role in getting the desired yields of products by saving the costs.

However, due to the scarcity of suitable cellulase producing microorganisms on a large scale, thermostable cellulase production at large scale remains an economical challenge. Recent developments in molecular biology and genetic engineering, structural biochemistry, and fermentation-based formulations/optimizations could provide the necessary push in development of thermostable cellulase-based enzymatic cocktails suitable for the above said applications.

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Chapter 30
Xylanases from Thermophilic Fungi: Classification, Structure, and Case Study of *Melanocarpus albomyces*

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Abstract Xylanases are an important category under the glycoside hydrolase families and together with cellulases constitute nearly 25% market in enzyme sector. Some of the major applications of this enzyme are in bleaching of pulp and paper, food and feed sector, etc. For several of these applications, enzymes from thermophilic sources are preferred. In this chapter, we present information on classification of family 11 xylanases, used in pulp and paper industry. Factors underlying thermostability, such as the length and composition of the N-terminus, Ser/Thr ratio, presence of Arg on enzyme surface, core packing, and hydrophobic interactions, have been described. Based on these principles, protein engineering approaches to achieve thermostability of fungal xylanases are reviewed. Our own work on development of hyper-xylanase-producing mutant and process strategies adopted to enhance production of this enzyme from a thermophilic fungus *Melanocarpus albomyces* is summarized. Role of nitrogen source, pH, temperature, aeration, and agitation is emphasized through this case study whereby productivities of 22,000 IU/L/h have been achieved. Additives, currently in use, to make stable xylanase preparation are also described. Special emphasis is laid on downstream processing, which includes role of carriers and binders in producing the product of desired quality.

Keywords Family 11 xylanases • Thermostability • Protein engineering • *Melanocarpus albomyces* • Process strategies • Spray drying

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30.1 Introduction

Wood, the starting material for preparation of paper, is composed of cellulose, hemicellulose, and lignin. The distribution of cellulose is from 40 to 45%, hemicellulose from 20 to 30%, and lignin from 15 to 25% of dry weight of wood (Sjostrom 1993). Xylan and glucomannan are the two main hemicelluloses with xylan being the most abundant hemicellulose in wood. It varies from 7 to 10% (of the cell-wall content) in softwoods to 15–30% in hardwood and annual plants (<30%) (Singh et al. 2003). Xylan consists of a backbone of β-1,4-glycosidic linked β-D-xylopyranosyl residues. The hydroxyl groups are substituted with acetyl, arabinose, and 4-O-methyl-glucuronose residues. The chemical composition of the substituent group and the frequency of its occurrence depend on the source and the method used during the extraction procedure. Xylan of hardwood is acetyl-4-O-methyl glucuronoxylan with a degree of polymerization of about 200 residues. A small part (10%) of the basic β-D-xylopyranosyl units are substituted at C-2 with a 1,2-linked 4-O-methyl-α-D-glucuronic acid while 70% is acetylated at C-2 or C-3 or both. Hardwoods also contain a small amount of glucomannan in which the ratio of glucose to mannose varies from 1:1 to 1:2 depending on the wood type. Softwood, on the other hand, contains 10–15% xylan as arabino-4-O-glucuronoxylan with a degree of polymerization of more than 120 residues (Kulkarni et al. 1999). It is not acetylated and comprises β-D-xylopyranose, 4-O-methyl-glucuronic acid, and L-arabinose in a ratio of 100:20:13. The 4-O-methyl-α-D-glucuronosyl residues are attached to C-2 and L-arabinofuranosyl residues to C-3 of the basic xylopyranose backbone units. The backbone of softwood glucomannan is made of β-1,4-linked D-glucopyranose and D-mannopyranose units and is partially substituted by α-galactose and acetyl units. The ratio of glucose to mannose is 1:3 to 1:4 (Puls and Schuseil 1993).

Due to the complexity of the xylan structure, several enzymes are required for its complete hydrolysis to sugars. A number of eubacterial and eukaryotic (predominantly filamentous fungal) microbes exist that are the sources for enzymes required for xylan hydrolysis (for review, see Mishra et al. 2008). The most predominant enzyme required for hydrolysis is endo-1,4-β-xylanase (EC 3.2.1.8) or "xylanase" which cleaves the main chain glycosidic bond and β-xylosidase (EC 3.2.1.37) which hydrolyzes the resulting sugar polymeric chains to D-xylose (for review, see Knob et al. 2010). Debranching enzymes such as α-L-arabinofuranosidase (EC 3.2.1.55), α-D-glucuronidase (EC 3.2.1.1), and acetyl esterases (EC 3.1.1.6) are also required for removal of the side-chain residues (Coughlan and Hazlewood 1993).

Xylanases have been applied successfully in the biotechnology sector in the area of animal feed, food processing, and pulp and paper industries. Cellulase-free xylanases, reported to be produced by fungi like novel *Aspergillus sp*. (Sridevi and Charya 2011), *Aspergillus fischeri* (Chandra and Chandra 1995), *Cephalosporium* sp. (Kang et al. 1996), *Fusarium CDC-84* (Sridevi and Charya 2011), *Fusarium oxysporum* strain F3 (Panagiotoy et al. 2003), *Melanocarpus albomyces* (Maheshwari and Kamalam 1985), *Paecilomyces thermophila* J18 (Li et al. 2006), and *Thermomyces lanuginosus*

(Singh et al. 2003) are particularly important as they do not act on the cellulose fiber and impart xylanase treatment on the pulp. In pulp and paper industry, treatment of Kraft pulp (after adjustment of pH and temperature to suit the optimum of the enzyme) with xylanase brings down the requirement of chlorine across a five-stage bleaching sequence to reach the desired brightness (Tolan and Guenette 1997). Due to application of reduced chlorines, absorbable organic halides (AOX) and total organochlorine released in to the effluent also decrease bringing down the color of the effluent. No change in pulp strength but an increase in tear strength is reported due to precipitation of xylan brought about by treatment with xylanases (Tolan et al. 1995). Hence, large-scale pre-treatment has been found to be commercially successful. However, effectiveness of xylanase on the pulp depends on the match between pH and temperature optimum of the xylanase being used and physical condition of the pulp (pH and temperature). Other factors such as dispersion of the enzyme in the pulp and time of interaction between pulp and the enzyme also determine the effectiveness of the process. While most mesophilic xylanases have a pH optimum in the range of 5.5–7.0 and temperature optimum between 50 and 70°C (Polizeli et al. 2005; Ahmed et al. 2009), the stability and the activity of this xylanase are not optimum on the pulp. After chemical pulping, the temperature of the pulp is in the range of 50–60°C depending on the extent of heat removal, and the pH is in the alkaline range (>9.0). Thermophilic producers of high temperature optima xylanases have been reported and would be more suitable for application in the pulp and paper industry.

30.2 Classification of Xylanases

Endo-1,4-β-xylanases (EC 3.2.1.8) hydrolyze the bonds of β-1,4-xylan, the major plant cell-wall polysaccharide component of hemicellulose. On the basis of primary sequence homology and hydrophobic cluster analysis, the xylanases have been grouped into two families of the glycoside hydrolase families, namely, family 10 and family 11, which correspond to former cellulase families F and G, respectively (Henrissat and Davies 1997). A search of the CAZy database (http://afmb.cnrs-mrs.fr/~cazy/CAZY) using the enzyme classification number of xylanase, which is 3.2.1.8, indicated that there exist enzymes with xylanase activities within other families of 5, 7, 8, 16, 26, 43, 52, and 62. A survey of the literature carried out on these enzymes (Collins et al. 2005) showed that only sequences classified in families 5, 7, 8, 10, 11, and 43 contained truly distinct catalytic domains with xylanase activities. Sequences reported in families 16, 52, and 62 appeared to be bifunctional enzymes containing a family 10 or 11 xylanase domain and a second glycosidase domain. Hence, it was suggested that the xylanases belonging to families 5, 7, 8, and 43 should also be considered. For the discussion in this chapter, only members of family 10 and 11 are discussed.

The total number of members placed in family 10 is 1306 with majority belonging to eubacteria (756) and eukaryota (282). A large number (261) still remain unclassified. The structures of 24 members have been determined. Family 10 enzymes

have an $(\alpha/\beta)_8$ barrel structure with approximate molecular mass of 35 kDa. Xylanases from both mesophilic and thermophilic organisms are available in this family. Number of members in family 11 is 664 with almost an equal number of eubacterial (324) and eukaryotic (266) members. A number of unclassified (71) sequences also remain here. Structures of 26 members of this family are available (Last updated: 31.5.2012). In the overall structure of family 11 xylanases, the catalytic domain folds into two β-sheets and one short α-helix which are packed against each other, forming a so-called β-sandwich. While members of both families are important in various industrial applications, the xylanases used in bio-bleaching are usually family 11 xylanases (Shibuya et al. 2000). This is because a low-molecular-mass enzyme is desirable for fiber penetration. In addition, it is desirable that xylanases used in the pulp and paper industry are cellulase-free, stable, and active under alkaline conditions and at high temperatures. There has been considerable amount of research devoted to identifying new xylanases and improving the properties of wild-type xylanases to meet the requirements of the pulp and paper industry.

30.3 Family 11 Xylanases

30.3.1 Comparison of Family 11 Xylanases in Relation to Thermal and pH Stability

Structures of 26 xylanases belonging to family 11 are currently available and are from bacteria as well as fungi. Among the bacterial systems, xylanase from *Dictyoglomus thermophilum* (McCarthy et al. 2000) and *Nonomuraea flexuosa* (Hakulinen et al. 2003) are thermostable. While a number of xylanase crystal structures are available from fungi such as *Aspergillus kawachii* (Fushinobu et al. 1998), *Aspergillus nidulans* (Payan et al. 2004), *Aspergillus niger* (Krengel and Dijkstra 1996; Sansen et al. 2004; Vandermarliere et al. 2008), *Chaetomium thermophilum* (Hakulinen et al. 2003; Janis et al. 2005), *Hypocrea jecorina*, earlier *Trichoderma reesei* (Torronen and Rouvinen 1995), *Neocallimastix patriciarum* (Vardakou et al. 2008), *Paecilomyces variotii* (Kumar et al. 2002a), *Penicillium funiculosum* (Payan et al. 2004), *Scytalidium acidophilum* (Michaux et al. 2010), and *Thermomyces lanuginosus* (Gruber et al. 1998), only three of these, *C. thermophilum*, *P. variotii,* and *T. lanuginosus,* are from thermophilic fungi. A comparison of almost 12 structures including five from thermophilic organisms has been made to understand the structural basis of thermostability (Hakulinen et al. 2003), and, the salient findings are summarized in the following paragraphs.

On the basis of primary sequence comparisons, the sequence identity among the 12 enzymes chosen ranged from 31 to 97%, and they could be placed into four groups. The first group comprises the xylanases from acidophilic group (*A. niger, A. kawachii, T. reesei)*, the second group of alkalophilic xylanase of *Bacillus agaradhaerens* (Sabini et al. 1999) and highly thermostable xylanase (of *D. thermophilum*),

third group of thermophilic *N. flexuosa* and mesophilic *Bacillus circulans* (Wakarchuk et al. 1994; Sidhu et al. 1999) xylanase, and the fourth group of mesophilic (*T. harzianum* and *T. reesei*) and several thermophilic (*C. thermophilum, P. variotii, T. lanuginosus*) xylanases. The classification into these groups was also confirmed by the superimposition of the crystal structures.

Since the crystal structures of mesophilic and thermophilic xylanases did not reveal substantial structural differences, properties such as frequency of amino acids, extent of amino acids present in secondary structures, N-terminus, and its length have been examined as possible factors contributing to thermostability of these enzymes. An increased occurrence of Arg on the protein surface of thermophilic enzymes was observed which was confirmed by large-scale sequence comparison data and also by experimental verification of *T. reesei* xylanase (Turunen et al. 2002). A decrease in Ser frequency accompanied by increase in Thr is also proposed to be responsible in thermophilic xylanases although some exceptions are reported (Kumar et al. 2002b) wherein Arg and Tyr were more frequent. The possible explanation provided (Hakulinen et al. 2003) is that an increase in the Thr/Ser ratio in β-strands improves the β-forming propensities. Since β-structures are known to be more thermostable, the shift to Thr residues could ensure imparting greater thermostability to the enzyme. The frequency of Asn was found to be slightly lowered in thermophilic xylanases (Hakulinen et al. 2003) which matches with the fact that it has lower β-forming tendency. Similarly, lowered glycine content in highly thermostable xylanase of *D. thermophilum* may impart rigidity to the protein structure, although no generalized statement can be made of the same. What appears to be a general principle is an increased network of interactions contributed by hydrogen, ionic (Gruber et al. 1998), and hydrophobic groups; increase in the number of amino acids in the β-strands; and presence of two-layered β-sheets, although a strong evidence for the latter is lacking as in beta proteins, this is a general way of arranging beta strands (Branden and Tooze 1999). An improved internal packing (Hakulinen et al. 2003), increased number of charged surface residues (Turunen et al. 2002), the presence of thermostabilizing domains (Winterhalter et al. 1995; Zverlov et al. 1996; Fonts et al. 1995), and presence of disulfide bridges, in particular, at the N- or C-termini or in the α-helix regions (Kumar et al. 2002a, b; Turunen et al. 2001) all seem to contribute to thermostability of these enzymes. It has also been shown that glycosylation of some xylosidases can contribute to thermal stability of the enzyme (Somera et al. 2009).

The pH dependence of an enzyme depends on the pK_a value of the catalytic residues. This is observed to be, in turn, dependent on the local environments of these groups. Study of a family 11 xylanase showed that residues around the catalytic site that contribute positive charge (such as Lys/Arg) and hydrogen bonds serve to lower the pKa values. The chemical nature of the donor is also important (Joshi et al. 2000), and carboxyl group is one such donor which can either lower or raise the pK_a value. It is significant to note that acidophilic xylanases, belonging to family 11, have an Asp hydrogen bonded to the general acid/base catalyst and that it is replaced by an Asn in the xylanases active under alkaline conditions (Fushinobu et al. 1998; Torronen and Rouvinen 1995; Sapag et al. 2002). Random mutagenesis of *N. patriciarum*

xylanase indicated that an increased negative charge and increased hydrophobicity can increase the pH optimum of this enzyme (Chen et al. 2001). It has also been shown that enzymes that are stable in alkaline conditions have decreased number of acidic residues and increased number of Arg. Alkaliphilic enzymes are indicated to contain more salt bridges than the acidophilic counterparts (Hakulinen et al. 2003).

30.3.2 Engineering of Xylanases for Enhanced Thermal Stability

A number of protein engineering methods have been applied for increasing thermostability of xylanases based on molecular features that are thought to underlie stability. These include modifications of the N-terminus, extending amino acid lengths, stability of the alpha helix and de-novo-designed disulfide bridges at the N-terminus, manipulation of Ser/Thr ratios, and introduction of Arg on enzyme surface. In addition, all thermophilic xylanases have an additional β-strand at the N-terminus. While many such modifications were introduced with success in bacterial xylanases, the data for fungal systems is scant. Few examples available for fungal xylanases are summarized below.

Several of the modifications were introduced at the N-terminus of the xylanases as this location appears to control thermostability. In one study (Sung et al. 1998), extending the N-terminus of *T. reesei* xylanase did increase the stability of the protein. Turunen et al. (2001) introduced a disulfide bridge as well as other minor mutations in *T. reesei* xylanase Xyn II, which increased the thermostability significantly. Introduction of a de-novo-designed N-terminal disulfide bridge was reported (Fenel et al. 2004) to stabilize the *T. reesei* xylanase. Replacement of the Ser/Thr surface of the protein with Arg is also reported to impart thermostability to *T. reesei* Xyn II (Turunen et al. 2002) and *A. niger* xylanase (Sriprang et al. 2006). Introduction of Arg on the surface other than the Ser/Thr side of *T. reesei* Xyn II had no effect on thermostability. In some cases (Turunen et al. 2002), thermostability was accompanied by an increase in enzyme activity provided the substrate was present. In case of *A. niger* xylanase (Sriprang et al. 2006), substitutions of Ser/Thr surface by either 4 or 5 Arg were done with little or slight alterations in the kinetic properties.

Replacement of the N-terminus between mesophilic eukaryotic and thermostable prokaryotic enzymes has also been used successfully for constructing new and improved versions of xylanase enzyme as demonstrated for the *A. niger* enzyme. The N-terminus of this fungal enzyme was substituted with the corresponding region of *Thermomonospora fusca* xylanase A (Sun et al. 2005). The constructed hybrid xylanase was more thermostable and active over a broad pH range (3.0–10.0). Structural analysis of the hybrid enzyme indicated increased aromatic interaction between critical residues leading to stabilization of the N-terminus of the protein. Detailed studies pointed to the involvement of five specific amino acids in this region. Substituting these residues in the *A. niger* enzyme indeed leads to greater thermal stability of this enzyme (Zhang et al. 2010). These mutations seemed to

confer thermostability through both additive and cooperative effects. In a similar fashion, hybrid *T. reesei* xylanases with replaced N-terminus (1–25) by *T. fusca* (1–29) xylanase is also reported to result in increased thermostability (Sung et al. 1998) of the enzyme.

Directed evolution has been lately used to improve the properties of several enzymes. Mimicking Darwinian evolution, it consists of iterative steps of random mutagenesis, recombination, and screening for the desired phenotype (Arnold and Volkov 1999). This approach was used to generate mutant variants of *T. lanuginosus* xylanase (Stephens et al. 2007, 2009); many of which were found to be thermostable. While many of the mutants were compromised for activity, some mutants displayed increased activity and thermostability. In one of the mutants, namely, G41, a total of four mutations were identified, two of which were the changes to Arg, and this was proposed to result in enhanced polar interactions. It also appears that thermostabilization leads to reduced flexibility in the enzyme, and often either thermal stability or catalytic activity is impaired. It has been suggested (Stephens et al. 2007) that both the properties can be improved, although it may be a rare event. Use of directed evolution and site-directed mutagenesis has been made to generate thermostable mutants from anaerobic fungus *N. patriciarum*. Two mutants (G201C and C60A-G201C) were identified that demonstrated increased melting temperatures compared to the parent enzyme by about 10 and 12 degrees, respectively (You et al. 2010). These were mapped in the interior hydrophobic core of the enzyme. Modeling studies performed on the mutant enzymes indicated a potentially stronger hydrophobic interaction between two well-packed cysteines at sites 50 and 201 as a consequence of these mutations. Thus, indirect factors such as close interactions, guided by cysteines, may also contribute to thermostability of the proteins.

While it has been proposed (Arnold et al. 2001) that there is an inherent trade-off between the rigidity necessary for thermostability and the flexibility required for enzyme activity, this may not always be the case as seen for the *N. patriciarum* xylanase. Thus, a combination of several factors is likely to lead to generation of enzymes which exhibit high activity and are also heat stable.

30.4 Assessment, Production, and Stability of Xylanase Produced from *Melanocarpus albomyces*

In our laboratory, we have been working with the xylanases of the thermophilic fungus *M. albomyces*. The cellulase-free xylanases, and also the fact that a number of useful other xylan-debranching enzymes are produced by this fungus, make it a valuable source for production of enzymes suited to application in pulp and paper industry. A consortium of enzyme is thus provided which would be more effective than an individual enzymes. With this in mind, detailed studies were conducted on various aspects of enzyme production in this fungus, and these are summarized below.

30.4.1 Evaluation of Xylanase Produced by M. albomyces

The wild-type *M. albomyces* produces a number of commercially valuable enzymes including xylanases and xylan-debranching enzymes. It has been reported (Maheshwari and Kamalam 1985) to be a non-sporulating fungus with an optimum temperature of growth as 45°C. A xylanase activity of about 170 IU/mL has been reported at shake-flask level from this fungus (Biswas et al. 2010a). The xylanase produced has an optimum temperature of 70°C, and various other isozymes are active within a pH range of 5.5–8.5 (Saraswat and Bisaria 2000). It is also reported to produce thermostable laccase with a neutral pH optimum (Kiiskinen et al. 2002). This laccase binds to softwood and pure microcrystalline cellulose, a property displayed by only a few laccases. A combination of xylanase and laccase produced by this fungus can be used for bio-bleaching in the pulp and paper industry, and this can significantly improve the brightness index of the paper by decreasing the Kappa number. Detailed substrate specificity and properties of various xylanase isozymes (Saraswat and Bisaria 1997) indicate the necessity of using the entire culture filtrate for application to the pulp.

The effectiveness of the xylanases produced by *M. albomyces* has been checked on mixed pulp of eucalyptus and bamboo wood. Different parameters such as Kappa number, pulp filtrate characteristics, viscosity, savings in bleach chemicals, strength and optical properties, and environmental impact have been investigated. A reduction in Kappa number of unbleached Kraft wood pulp (by 1.7 points), a slight gain in pulp brightness, no reduction in viscosity of the treated pulp make it a useful candidate for pulp treatment. Chlorine requirements were also found to be lowered from 41 to 35 $kg.t^{-1}$; alkali usage was lowered to maintain the same brightness index. Strength and optical properties of the untreated and xylanase-treated pulp were found to be almost at par in terms of tensile index (64 and 62 $Nm.g^{-1}$) and burst index (4.4 and 4.2 $kPa.m^2.g^{-1}$). Characterization of the bleach effluent after enzyme treatment showed an improved environmental status in terms of the AOX (reduced by more than 20%), color, and COD (Bisaria et al. 2002). Almost all these properties were at par with many of the commercially available xylanases.

30.4.2 Screening of Hyper-Xylanase-Producing Mutant and Optimization of Xylanase Production

For any commercial application, large-scale availability and high productivity of the enzyme is an essential prerequisite. Two approaches have been taken in our laboratory, namely, the conventional mutagenesis for generating hyper-xylanase-producing mutants and optimization of fermentation strategies for higher yield of xylanases. A number of commercially available media were screened for their ability to induce

sporulation in this fungus, and profuse sporulation was induced on potato-carrot agar medium. The asexual spores remain entangled within the filamentous fibers, and these were successfully removed, without compromising the germination efficiency, by vortexing with glass beads. A hyper-xylanase-producing mutant, namely, IITD3A was isolated (Biswas et al. 2010a) by chemical mutagenesis method (with about ~1% survival rate) followed by screening on remazol brilliant blue (RBB)-xylan agar plates (Biely et al. 1988).

For any fermentation, spore number inoculated and age decides the initial onset of enzyme production. Large number of spores result in faster carbon utilization and hence accelerated growth. The vitality of the inoculum is important, particularly where product formation occurs during exponential growth phase. It is generally accepted that inocula concentrations $>10^5$ spores/mL are required for good growth, although this depends on the strain and is affected by nutrient concentration and fermentation conditions. For pre-culture development of *M. albomyces* IITD3A, a spore count of 3.5×10^5/mL was most suitable and a homogeneously distributed culture of small pellets (size 1–2 mm) was obtained. An activity of 380 IU/mL was obtained with this pre-culture which was nearly 2.5-fold higher than with the wild-type strain. The importance of spore number during fermentation has been shown for *Penicillium chrysogenum* (Smith and Calam 1980) and for citric acid fermentation by *Aspergillus niger* (Papagianni and Mattey 2006).

While both carbon and nitrogen sources are important during fermentation, nitrogen and the carbon to nitrogen ratio are seen to play a significant role, especially if the product of fermentation is a protein or a peptide. It has been reported that addition of complex N source such as peptone, yeast extract is advantageous resulting in higher xylanase activities (Song et al. 2001; Qinnghe et al. 2004) which is largely due to supply of all amino acids which can be absorbed by fungal mycelia and used for protein production. In case of *M. albomyces* IITD3A, complex nitrogen sources lead to an increase in pH to 7.8 at which lower xylanase activities were obtained. Urea supported maximum xylanase activity of 400 IU/mL. Keeping in mind large-scale cultivation of the fungus, preferably in homogeneous aqueous preparation, a cellulose-free soluble alkaline extract media was developed (Sahai et al. 2005) called as soluble alkaline lignocellulosic extract or SALE. It contained primarily xylan (~10/L) and in combination with urea (to maintain a C/N ration of 10–13) lead to xylanase activity of 450 IU/mL (Biswas et al. 2010a). When a large-scale fermentation was carried out at 14 L level, with controlled pH (at 7.8) at 0.05 vvm, a maximum xylanase activity of 415 IU/mL was obtained at 36 h with a total volumetric productivity of ~11,530 IU/L/h which represented an eight-fold increase over the value obtained in shake flask with the wild-type strain. Although this compares well with reported xylanase value of 15,562 IU/L/h for *A. niger* B03 (Dobrev et al. 2007), the enzyme from the latter has a pH and a temperature optimum of 5.0 and 50°C, respectively, making it less suitable for pre-bleaching of pulp.

30.4.3 Process Strategies for Enhanced Production of Xylanase by M. albomyces

While solid substrate fermentation has been used for xylanase production by *M. albomyces* (Narang et al. 2001), production in submerged fermentation mode is preferred due to ease of scale-up and less time taken for product formation. Handling of liquid broth at large scale for partial product recovery is also easier. The production of xylanase in submerged fermentation is governed by several process conditions. Selection of appropriate method of optimization of media components and process parameters can change a less productive fermentation to an enhanced one. In statistical optimization, the widely used response surface methodology accurately predicts the underlying factors in the fermentation which can be controlled to affect the outcome. The parameters that are reported to affect xylanase production are pH, temperature, substrate concentration, cultivation time, aeration, and agitation (reviewed in Biswas et al. 2010b). Cycling of pH is also expected to affect xylanase production as it is a multienzyme system like cellulases, which have been shown to be affected by pH cycling (Mukhopadhyay and Malik 1980). In aerobic fermentation, aeration and agitation are important and affect two underlying physiological parameters. Enhanced oxygen supply and agitation result in availability of high amounts of oxygen for microbial growth and thereby enzyme production and better mixing and increased mass and heat transfer. However, this can also lead to increased energy input associated with higher mechanical stress on fungal biomass. This manifests as morphological change of the filamentous fungus which could lead to variations in growth and product formation. (Amanullah et al. 2000; El-Enshasy et al. 2006; Zmak et al. 2006).

Statistical optimization of fermentation parameters and control of process parameters have been studied for their possible effect on xylanase production in *M. albomyces* IITD3A (Biswas et al. 2010b). Box-Behnken design of response surface methodology was used to analyze the interactive effects of urea concentration, pH, and inoculum size on xylanase production. From the elliptical nature of the 3-D contour plots generated, it was concluded that there was significant interaction among the parameters. The response was found to be particularly sensitive to pH of the production medium. The optimized parameters suggested by the design were concluded to be as follows: a pH of 7.4, an inoculum concentration of 5.0% (v/v), and urea concentration of 1.27 g/L. The predicted value of xylanase activity was 407 IU/mL which matched closely with the observed value of ~400 IU/mL (Biswas et al. 2010b). Since pH cycling has been reported to affect other multi-enzyme systems, its effect was also investigated on xylanase production in *M. albomyces* IITD3A. Cycling of pH between 7.8 and 8.2 leads to a maximum of xylanase production of 415 IU/mL after 24 h with an overall volumetric productivity of 17,291 IU/L/h.

It is well known that filamentous fungi exhibit different morphological forms that vary from pelleted bead-like structures to profuse mycelial mats under the

Table 30.1 Summary of xylanase activities obtained at different stages of optimization by *M. albomyces* IITD3A

Level	Xylanase activity (IU/mL)	Volumetric productivity (IU/L/h)
Shake-flask		
Unoptimized medium	320	4,444
Optimized medium	450	5,555
Fermenter level (14 L)		
Optimized medium	415	11,527
Optimized medium with pH cycling	415	17,291
Aeration control	550	22,000
Agitation control	480	20,000

impact of agitation in a stirred tank reactor. This is accompanied by overall change in the productivity (Papagianni 2004). A high shear rate has a deleterious effect on the productivity by fragmentation of the mycelium. For *M. albomyces* IITD3A, applying an agitation speed of 600 rpm resulted in formation of uniformly dispersed pellets of 1–2 mm (θ) which had a small compact core with a hairy envelope. A significant decrease in the viscosity of the medium, observed on account of pellet formation, may lead to improved mixing and mass-transfer characteristics. Pellets with a compact surface structure display a large diffusional barrier for gases and substrates than pellets with an open peripheral structure, and this transformation thus leads to a maximum xylanase activity of 480 IU/mL after 24 h in *M. albomyces* IITD3A (Biswas et al. 2010b). A combination of active biomass and morphological form is also reported to play an important role in product formation of pectic enzyme and citric acid production in *A. niger* (Kristiansen and Bullock 1988), glucoamylase, and protease production in *A. niger* (Papagianni and Moo-Young 2002; Lin et al. 2010). As observed in our studies with *M. albomyces* IITD3A, the air flow rates also affected xylanase production. An optimum aeration rate is desirable as very high aeration rates give less holding time to air bubbles and create oxygen limitation. Experiments with the use of three different aeration rates (0.1, 0.25, 0.5 vvm) indicated maximum xylanase activity of 550 IU/mL (after 25 h) at an aeration rate of 0.25 vvm with an overall productivity of 22,000 IU/L/h. Table 30.1 summarizes the levels of xylanase activities obtained in this mutant after various stages of optimization. A comparison of xylanase production by this mutant with other fungi reported in literature is given in Table 30.2.

Thus, by employing appropriate strategies (isolation of hyper-xylanase-producing mutant, optimization of carbon, nitrogen source and carbon to nitrogen ratio, development of fermentation strategies), it has been possible to achieve high (~20,000 IU/L/h) productivities of xylanase preparation very suitable for application in pulp and paper industry.

Table 30.2 A comparison of xylanase production by *M. albomyces* IITD3A (in a bioreactor) with other fungal systems

Name of the organism	Substrate used	Mode of operation	Xylanase activity (IU/L)	Productivity (IU/L/h)	References
Aspergillus niger	Xylan (from corncob)	Batch (20 L)	290,000	3,820	Yuan et al. (2005)
Aspergillus oryzae	Spent sulfite liquor	Batch (15 L)	199,000	13,100	Chipeta et al. (2008)
Penicillium oxalicum ZH-30	Wheat bran	Batch (15 L)	16,000	110	Li et al. (2007)
Thermomyces lanuginosus DSM 10635	Oat husk hydrolysate	Batch (2 L)	210,000	4,380	Xiong et al. (2004)
Thermomyces lanuginosus MC 134	Coarse corncob	Batch (5 L)	2,010,000	13,950	Kumar et al. (2009)
Trichoderma reesei Rut C-30	Oat husk hydrolysate	Fed-batch (5 L)	1,350,000	14,060	Xiong et al. (2005)
Melanocarpus albomyces IITD3A	Soluble wheat straw extract	Batch with pH cycling (14 L)	550,000	22,000	Biswas et al. (2010b)

30.4.4 Stability of Xylanase Under Storage and Application Conditions

Post production, storage, and stability are of prime concern in making any enzyme-based technology successful. Various methods have been used for this which include storage in liquid form as well as in solid form. Since enzymes inactivate in dilute solutions, concentrated enzyme preparations, in the presence of specified chemicals, have been used as a method for preservation of enzyme activity. Lyophilization is another method used for long-term storage. Since microbial growth is one of the reasons for loss in enzyme activity, agents that inhibit growth are good preservatives. Effect of common bactericidals was studied on the stability of xylanase produced by *M. albomyces* IITD3A.

Among a number of bactericidals investigated for stability of xylanase at 4 and 30°C, thiomersal was most effective. At 0.01%, it effectively stabilized the enzyme, retaining 87% of original activity even after 50 days (Biswas et al. 2010a). Thiomersal is sodium ethylmercurithiosalicylate and is reported (Magos 2001) to be metabolized or degraded to ethylmercury and thiosalicylate which kill a wide range of microbes. The increased stability of the xylanase produced by the mutant could also be attributed to low extracellular levels of proteases produced by this fungus which was 0.05 IU/mL compared to 0.38 IU/mL produced by the parent strain. No stabilizers were required for preservation of enzyme in the liquid form.

Lyophilization is a method for long-term storage of enzymes in dry form and is useful as transportation over long distances is convenient. Effect of lyophilization of crude culture filtrate of *M. albomyces* IITD3A on xylanase stability was investigated, and it was found that xylanase activity was retained by 100% on lyophilization. Addition of thiomersal increased the shelf life to nearly 2 months with 100% retention of enzyme activity. The complete recovery of xylanase activity from lyophilized powder also suggested that medium components might have acted as lyoprotectants.

Various other methods employing chemical modification, immobilization, and protein engineering have been used to impart stability to commercial enzymes. Chemicals like glutaraldehyde, polyethylene glycol, and dicarboxylic andydrides modify the enzyme by covalent bonds and cross-linking and increase stability of biocatalysts toward organic solvents, extreme pH, and temperature. In immobilization, proteins are immobilized on solid support by covalent attachment, entrapment, or cross-linking, and this often confers considerable stability toward temperature and organic solvents. Through protein engineering, active sites of the enzymes are modulated by rational, combinatorial, and data-driven designs which replace appropriate amino acid residues for desired stability in pH and temperature. With the *M. albomyces* IITD3A enzyme, spray drying was investigated as a process for preparation of dry powder of the enzyme. A large number of binders and carriers were investigated (Gupta et al. 2008), and it was observed that maltose and lactose (at 5%) and maltodextrin (at 7%) acted as good binders. Magnesium sulfate and this salt in combination with mannitol acted as a good carrier for preservation of xylanase activity on spray drying the crude culture filtrate. Table 30.3 gives the data for stability of xylanase with the selected binder and carrier.

Table 30.3 Shelf life of differently prepared spray-dried powders of culture filtrate of *M. albomyces*

Powder	Carrier	Binder	Specific activity of spray-dried powder (IU/mg protein)				Half- life (days)
			Day 0	Day 5	Day 10	Day 15	
A	20% $MgSO_4$	5% malt extract	255	248	240	228	63
B	20% $MgSO_4$	5% lactose	200	198	184	171	75
C	20% $MgSO_4$	7% Maltodextrin	200	182	175	162	49
D	10% $MgSO_4$ + 10% mannitol	5% malt extract	194	190	189	185	96

Stability of the enzyme under application conditions is another requirement for its usage. Both the thermal stability and pH stability of the xylanase in crude cell-free extract of *M. albomyces* IITD3A were investigated, and it was observed to be stable when incubated at pH of 7.5 at 55°C for 2 h, conditions employed for xylanase treatment. The enzyme was also found to be active over a broad range (6.0 to ~9.0) of pH. Similar pH range has also been reported for the parent enzyme (Saraswat and Bisaria 2000). At higher pH (9.0), when incubated at 55°C for 2 h, it was found to retain 50% of its optimum activity (Biswas et al. 2010a). Thus, the general mutation caused in this fungus leads to deregulation and overexpression of xylanases without compromising its activity on the substrate.

30.5 Future Prospects and Conclusions

Enzyme-based technologies are going to gain importance in the coming years as people become concerned about environment. Specificity of enzymatic reactions at ambient temperatures and without any added catalysts makes them ideal for many applications. Xylanases are being considered and used for treatment of pulp as well as other applications. Family 11 xylanases are particularly important in the pulp and paper industry as these are relatively of small molecular weight and can therefore penetrate the substrate more effectively. To match the requirements of the sector, where they are likely to be used, these have been engineered to optimize their pH and make them more thermostable. This is based on a rational approach wherein factors likely to affect pH and thermostability have been studied and applied for enzyme modification. Success has been reported in many cases. New approaches being employed to identify novel thermophilic enzymes (extremophiles, metagenomics, etc.) will further advance our knowledge of factors that govern stability of these enzymes. Techniques such as gene site saturation mutagenesis (Mueller et al. 2000), which completely randomizes successive codons in a gene and evaluates the efficiency of all possible substitutions, have been used in combination with GeneReassembly technology (Solbak et al. 2005) to generate novel types of xylanases (Dumon et al. 2008). Such an experimental approach gives a wider platform to understand the molecular basis of thermostability and other enzymatic properties.

While xylanases are used in the industry in isolation, in nature, they occur as part of a larger biological function, namely, lignocellulose degradation. Hence, an integrated approach whereby production and properties of xylanases are studied and evaluated along with other enzymes is necessary. Basic understanding of how low-molecular-weight proteins are stabilized against denaturation by H^+ ions and temperature will also lead to designing better xylanases.

Our own work with cellulase-free xylanases produced by *M. albomyces* indicates the usefulness of using culture filtrate as an effective starting material. The mixture of enzymes (various xylanase isozymes) produced may also be better for treatment of pulp prepared from different raw materials as well as by different processes. Fermentation strategies, developed for this fungus, can be applied to other fungal sources for enhancing productivities.

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Chapter 31
Thermostable Bacterial Xylanases

Vikash Kumar, Digvijay Verma, A. Archana, and Tulasi Satyanarayana

Abstract Hemicellulose is the second most abundant component in lignocellulosics available in nature. It is a storage polymer occurring in seeds and a prominent structural component of cell walls in plants. Hemicelluloses in agricultural residues constitute up to 40%. Monomers of various hemicelluloses are useful in various biotechnological processes like the production of different antibiotics, alcohols, animal feeds, and biofuels. Xylan is the most abundant of all hemicelluloses. It has a linear backbone of β-1,4-linked D-xylopyranose residues. Immense interest in the enzymatic hydrolysis of xylan has been due to the applications of hydrolysates in feedstocks, production of biochemicals, and paper pulp bleaching. Biodegradation of xylan requires action of several enzymes, among which xylanases play a key role. A wide variety of microorganisms are known to produce xylanases. The interest in thermostable xylanases has markedly increased due to their potential applications in pulping and bleaching processes, in food and feed industry, textile processing, enzymatic saccharification of lignocellulosic materials, and waste treatment. Since elevated temperatures have a significant influence on the bioavailability and solubility of organic compounds, most of these processes are carried out at high temperatures. The elevation in temperature is accompanied by a decrease in viscosity and an increase in the diffusion coefficient of organic compounds, and thus, higher rates of reactions are expected. Thermophilic organisms are of special interest as sources of thermostable xylanases. The development of new analytical techniques and the commercial availability of new matrices have led to the purification and characterization of a large number of xylan-degrading bacterial enzymes. The recombinant DNA technology has permitted selection and overproduction of xylanolytic enzymes that are suitable for industrial applications. The developments in cloning and expression, directed evolution, physicochemical and functional characteristics,

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and biotechnological applications and commercialization of thermostable xylanases of bacterial origin have been reviewed.

Keywords Lignocellulosics • Xylanases • Pulp and paper • Gene cloning • Biotechnological applications • Protein engineering

31.1 Introduction

A fresh look and an adequate management of renewable resources is the need of the hour so as to address the worldwide increasing energy demands. Lignocellulosic biomasses abundantly present on earth are definitely among the most promising candidates to meet the same (Malherbe and Cloete 2002). Lignocellulosics include materials such as agricultural and forestry residues, municipal solid wastes, and other agro-based industrial wastes. The degradation of agro-industrial residues in biotechnology bioprocesses not only provides an alternative substrate for these bioprocesses but also serves to solve some of the pollution problems caused by their accumulation in the environment (Botella et al. 2007). These materials are composed of three main components: cellulose, hemicellulose, and lignin. Hemicellulose is an abundantly available organic biomass on the planet with tremendous applications. It constitutes 20–30% of total plant biomass in association with cellulose (Kulkarni et al. 1999) and is composed of about 26% of hardwood, 22% of softwood, and 30% of various total agricultural residues (Kuhad et al. 1997). Hemicelluloses are complex biopolymers made up of glucuronoxylans, arabinoglucuronoxylans, glucomannans, arabinogalactans, and galactomannans (Haltrich et al. 1996; Sunna and Antranikian 1997; Kulkarni et al. 1999; Subramaniyan and Prema 2002; Collins et al. 2005; Scheller and Ulvskov 2010). Most of the hemicelluloses are built up by β-1,4-linkages between their backbone sugars, except in galactose-based hemicelluloses, which are based on β-1,3-linkages. Xylan is the major component of hemicellulose contributing 15–30% of the total dry weight in angiosperms and 7–12% in gymnosperms (Haltrich et al. 1996; Singh et al. 2003; Izydorczyk and Dexter 2008).

The wood cells from various plant sources are composed of different layers, which mutually differ with respect to their structure and composition. Generally cellulose forms the skeleton, which is surrounded by other materials like hemicellulose as the matrix and lignin as the encrusting material. The side groups arabinose, galactose, and 4-O-methylglucuronic acids are frequently perceived as connecting links to lignin (Fengel and Wegner 1983). It is generally agreed that the hemicellulose molecules are parallel to the cellulosic fibrils (Sharma et al. 2007).

The main raw material for paper and pulp industry is wood that comprises cellulose, hemicellulose, lignin, and other extractives in varying proportions. Removal of hemicelluloses (xylan, mannan, galactan, and arabinan) and consequently of dark-colored lignin from the pulp greatly improves the quality and brightness of thus manufactured paper.

In general, papermaking processes utilize large quantities of chemicals that lead to problems of disposal of hazardous effluents. The need for safer and environmentally sound technologies has become more imperative. Owing to the heterogeneity and complex chemical nature of plant hemicellulose, its complete breakdown requires the action of several hydrolytic enzymes with diverse substrate specificity, namely, β-1,4-endoxylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, and phenolic acid (ferulic and p-coumaric acid) esterase (Singh et al. 2003; Collins et al. 2005). Among hemicellulolytic enzymes, the use of cellulase-free xylanases finds a unique biotechnological application in the pulp and paper industries. They are used primarily as prebleaching agents to reduce the amount of chlorine required to achieve desirable levels of paper brightness (Viikari et al. 1994; Kenealy and Jeffries 2003; Singh et al. 2003; Bajpai 2004; Collins et al. 2005). Ever since Viikari et al. (1986) reported the applicability of xylanases in biobleaching of kraft pulp, the cellulase-free xylanases have emerged as an even better option for this ecofriendly biotechnological application. The dominant chemical pulping process is called kraft pulping which involves cooking wood chips in high concentration of sodium hydroxide and sodium hydrosulfide solution for 2–4 h at 170°C, followed by a washing step that releases most of the lignin in the form of black liquor. The pulp obtained after washing is alkaline in nature and at higher temperature (about 80–90°C), still brown in color due to residual lignin (Gellerstedt 2001).

Since xylan acts as a cementing material between residual lignin and cellulose fibers, therefore thermoalkalistable cellulase-free xylanases are extremely useful in removing this residual lignin in the prebleaching step in papermaking. Most of the naturally occurring xylanases known to date are optimally active at temperatures below 50°C and are active at acidic or neutral pH (Turkiewicz et al. 2000; Ryan et al. 2003). Enzymes active at high temperatures and alkaline pH values have great potential as they can be introduced at different stages of the bleaching process without requiring changes in pH and/or temperature of the reaction mixture (Shoham et al. 1992). Only a handful of xylanases reported till date are active and stable at alkaline pH and high temperature (Rani and Nand 2000; Sharma et al. 2007; Ahlawat et al. 2007; Ko et al. 2010; Azeri et al. 2010; Nagar et al. 2010; Kumar and Satyanarayana 2011).

The xylanases act on xylan by endo-hydrolysis of β-1,4-linkages to generate smaller oligosaccharides, which are further hydrolyzed to D-xylose by the xylosidases. Biodegradation of xylan requires endo-1,4-β-D xylanase, with features like activity and stability at high temperature and high pH for maximum utilization in the paper and pulp industries.

According to some authors, almost all commercially available xylanases can only partially fulfill these requirements, and the optimum temperature for the activity of most xylanases are reported to be 50–60°C with a half-life of about 1 h at 55°C (Jacques et al. 2000). However, some bacterial xylanases have been reported to exhibit higher thermal stability and optimal activity ranging from 80 to 100°C (Saul et al. 1995; Zverlov et al. 1996; Morris et al. 1998; Gupta et al. 2000; Sharma et al. 2007; Kiddinamoorthy et al. 2008; Ko et al. 2010; Kumar and Satyanarayan 2011).

Thermophilic bacteria and fungi are the well-known sources of thermostable enzymes that find a plethora of commercial applications due to their sturdiness and toughness in standing the intense heat generated in various biotechnological industries in which the enzymes are employed. This feature not only supports their industrial exploitation but also proves to be economical as the thermostable enzymes do not have to be replenished from time to time (Demirijan et al. 2001; Techapun et al. 2003). A large number of beneficial thermophilic microorganisms from different exotic ecological zones of earth have been isolated, and enzymes have been extracted from them (Burrows 1973; Antranikian et al. 1987a, b; Groboillot 1994; Bharat and Hoondal 1998; Bauer et al. 1999; Kohilu et al. 2001; Sharma et al. 2007; Ko et al. 2007; Kiddinamoorthy et al. 2008; Nagar et al. 2010; Kumar and Satyanarayana 2011).

A sound recent approach has been to carry out genetic and protein engineering for the maximal and cost-effective commercial production of enzymes with improved stability to high temperatures, extremes of pH, oxidizing agents, and organic solvents. Cloning and expression of relevant genes from extremophiles in a suitable and faster growing mesophilic host has also provided novel possibilities of producing useful thermostable enzymes required for a particular biotransformation process (Blackebrough and Birch 1981; James 1995; Ikeda and Clark 1998; Hough and Danson 1999; Brennan et al. 2004). In this chapter, an attempt has been made to review the developments in finding sources of thermostable xylanases, characteristics of xylanolytic enzymes, the mechanism of thermostability, directed evolution, and important industrial applications.

31.2 Xylan: Occurrence and Structure

The cell wall of land plants contains xylan as the most common hemicellulosic polysaccharide that represents more than 30–35% of their dry weight (Joseleau et al. 1992). Xylan is composed of 1,4-β-D-xylopyranosyl residues (Whistler and Richards 1970). It occurs most commonly as a heteropolysaccharide, containing various substituent groups in the backbone and side chains (Biely 1985; Puls and Poutanen 1989). These are mainly acetyl, arabinosyl, and glucuronosyl residues (Whistler and Richards 1970) (Fig. 31.1). Xylan may also occasionally occur as homoxylan consisting of just xylosyl residues, as found in esparto grass (Chanda et al. 1950), guar seed husk (Montgomery et al. 1956), and tobacco stalk (Eda et al. 1976).

The xylan from hardwood is O-acetyl-4-O-methylglucuronoxylan consisting of at least 70 β-xylopyranose residues [average degree of polymerization (DP) being 150–200], mutually linked by β-1,4-glycosidic bonds (Timell 1964) and is highly acetylated, seen more frequently at C-3 than at the C-2 position, which is responsible for partial solubility of xylan in water. These acetyl groups may readily be removed when xylan is subjected to alkali extraction (Sunna and Antranikian 1997). Every tenth xylose residue carries a 4-O-methylglucuronic acid attached to the second position of xylose (Woodward 1984).

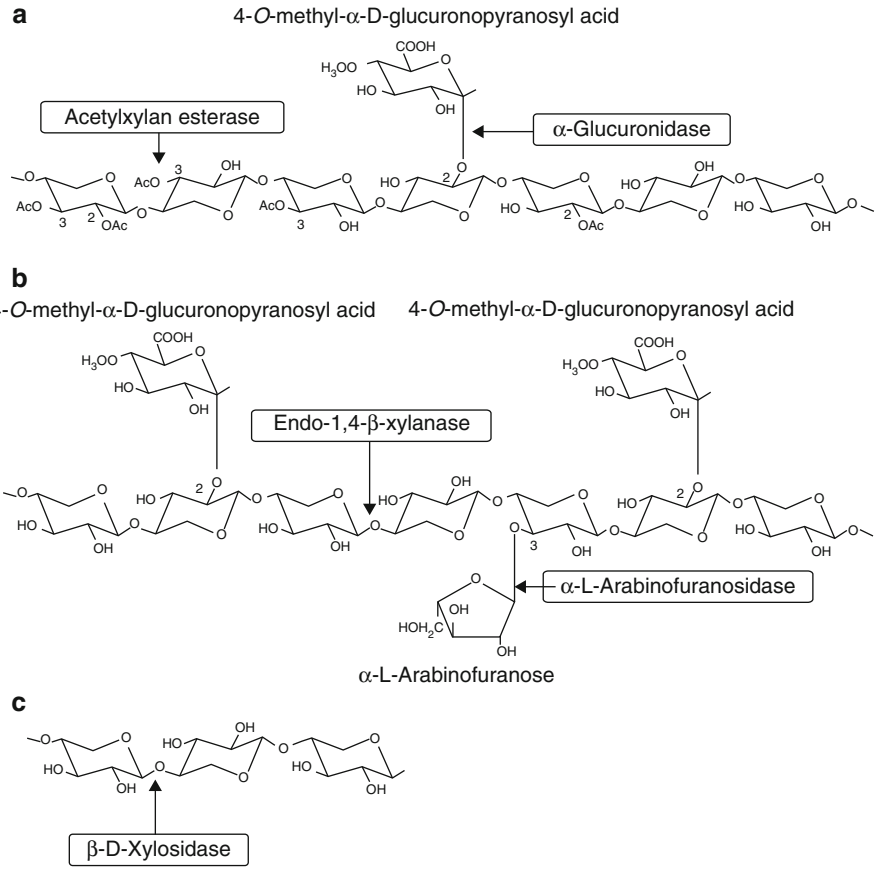

Fig. 31.1 (**a**) Structure of the O-acetyl-4-O-methylglucuronoxylan of hardwood. (**b**) Structure of the arbino-4-O-methylglucuronoxylan of softwood. Xylanolytic enzymes involved in the degradation of the xylan: acetylxylan esterase, α-glucuronosidase, endoxylanase, and α-L-arabinofuranosidase. (**c**) Mode of hydrolysis of β-xylosidase. The numbers indicate carbon atoms to which group substitutions bound. *Ac* Acetyl group

Xylan from softwoods is composed of arabino-4-O-methylglucuroxylans. They have a higher number of 4-O-methylglucuronic acid residues attached to the C-2 position. Instead of being acetylated, softwood xylans possess α-1,3-glycosidic bonds at the C-3 position of xylose (Puls and Schuseil 1993; Izydorczyk and Dexter 2008). They are shorter than hardwood xylans, with a DP between 70 and 130, and are less branched (Sunna and Antranikian 1997). The ratio of β-D-xylopyranose, 4-O-methyl-α-D-glucuronic acid, and L-arabinofuranose is about 100:20:13 in softwoods (Puls and Schuseil 1993).

Xylan from grasses is highly branched and contains large proportions of L-arabinofuranosyl units but possesses a small number of uronic acid residues substituted at C-2 position of xylose. Xylan with higher ratio of xylose:arabinose has been

reported from nodes of wheat and barley (Puls 1992). Xylan from graminaceous plants typically contains O-acetyl groups, along with 1–2% phenolic acid substituents (Bacon et al. 1975). More detailed structural correlation between hemicelluloses from different sources has been cited in the recent literature available (Girio et al. 2010; Scheller and Ulvskov 2010).

31.3 The Xylanolytic Complex

Xylanase represents one of the largest groups of industrial enzymes with increasing market demands due to its applications in prebleaching of kraft pulps, bioconversion of agricultural residues, extraction of coffee and plant oils, improvement of the nutritional properties of agricultural silage, and degumming of plant fibers, such as flax, sun hemp, and ramie (Beg et al. 2001; Subramaniyan and Prema 2002). Agricultural wastes containing hemicelluloses are globally generated. The application of agro-industrial residues in biotechnological bioprocesses not only serves as a suitable substrate but also addresses to solve some of the pollution problems caused by their accumulation (Botella et al. 2007).

Owing to the heterogeneity and complex chemical nature of plant hemicellulose, its complete breakdown requires the action of several hydrolytic enzymes with diverse substrate specificity, namely, β-1,4-endoxylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, and phenolic acid (ferulic and p-coumaric acid) esterase (Bailey et al. 1992; Singh et al. 2003; Collins et al. 2005).

The endoxylanases act on xylan by random hydrolysis of β-1,4-linkages to generate smaller oligosaccharides, which are further hydrolyzed to D-xylose by the action of xylosidases. Besides the two enzymes, the removal of side chains is biocatalyzed by α-L-arabinofuranosidases (EC 3.2.1.55), α-L-glucuronidases (EC 3.2.1.139), acetyl xylan esterases (EC 3.2.1.72), and ferulic acid esterases (EC 3.2.173). All these enzymes constitute the xylanolytic complex as shown in Fig. 31.1.

31.3.1 The "Xylanosome"

Xylanosomes are discrete, multifunctional, multienzyme complexes found on the surface of several microorganisms that play an important role in the degradation of xylan (Sunna and Antranikian 1997). The extracellular xylanosome complex B (CB) from *Butyrivibrio fibrisolvens* H17c exists as a multisubunit protein aggregate (Lin and Thomson 1991). The complex has a molecular weight >669 kilodaltons (kDa) and exhibits 11 protein bands with xylanase activity and 3 bands with endoglucanase activity. *Clostridium papyrosolvens* C 7 possesses a cellulase–xylanase consortium of seven distinct proteins, which is responsible for hydrolysis of both cellulose and xylan hand in hand (Pohlschroder et al. 1994). This multienzyme complex (MEC) ensures efficient bioconversion of agricultural wastes to completion. MECs have

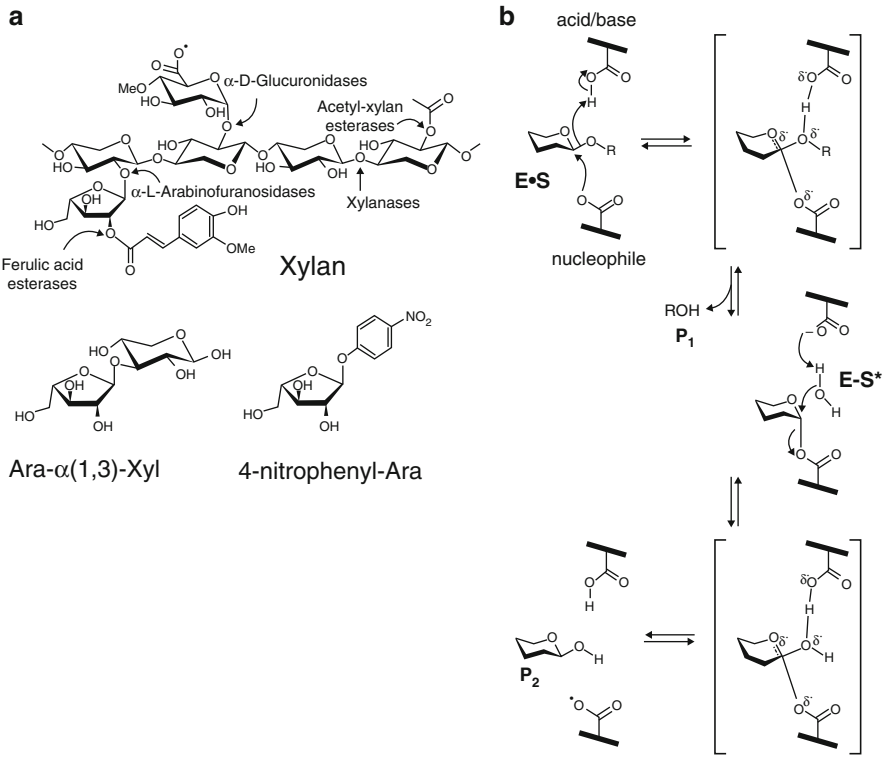

Fig. 31.2 (**a**) Upper, the basic structural components of xylan and the hemicellulases responsible for its degradation; lower, the natural and synthetic substrates of α-L-arabinofuranosidases used in this work: Ara-α(1,3)-Xyl and 4-nitrophenyl-Ara. (**b**) The double-displacement reaction mechanism for retaining glycosidases (Koshland mechanism)

attracted great attention in biofuel technology as they potentially offer a solution to the more effective degradation of complex plant material into fermentable sugars. Recently Van Dyk et al. (2010) have isolated, identified, and characterized an important MEC from *Bacillus licheniformis* SVD1 which has shown seven xylanolytic bands. Two MECs, C1 and C2, having molecular size 371 and 267 kDa, respectively, were purified which consisted of 16 and 18 subunits, five of these are degraded birchwood and oat spelt xylan. The significance of MECs is that they differ structurally from cellulosomes that can hydrolyze substrates with high hemicellulose content (Jones et al. 2012). However, xylanases from various microorganisms have different and unique physicochemical properties, modes of action, and substrate specificity, but a general perception about their action mechanism can be drawn from Koshland mechanism (Koshland 1953; Fig. 31.2). Therefore, characterization of the physicochemical properties of the xylanase being employed and identification of the particular substrate for a particular xylanase are mandatory. Some important bacterial xylanases along with their characteristics were mentioned Table 31.1.

Table 31.1 Some important bacterial xylanases, their mode of production and their characteristics

Microorganism	Substrate	Growth condition	Enzyme (IU/ml)	Optimum enzyme condition		Xylanase stability		Half-life (t1/2)	Reference
				T(°C)	pH	T(°C)	pH		
Bacillus licheniformis A99	1% wheat bran	Solid state fermen tation, 37°C, pH7		60	6–7.5	50–70	6–8	8 h at 60°C pH 7	Archna and Satyanarayana (1997)
Acidobacterium capsulatum		ND		65	5	20–50	3–8		Inagaki et al. (1998)
Bacillus sp. AR009	1% wheat bran	37°C, pH 8,3days, 1, solid-state	621 U/g 12.5 U/g	ND	ND	ND	ND		Gassesse and Mamo (1998)
Bacillus sp. SPS-0	0.5% wheat bran	60°C, pH 8.2, 1day, air 1 vvm in 1.51 fermenter		70	6	60–70	5–8	0.5 h at 70°C, pH 6	Bataillon et al. (1998)
Bacillus sp. Sam3	5% wheat bran	28°C, pH 7,2 days, shake flask	131	60	8	60–70	7–9	2 h at 60°C, pH 8	Shah et al. (1999)
Bacillus sp. BP-23	0.2%birch wood xylan	30°C, pH 6.8, 3 days	1.12	50	5.5	30–60	5–8	1 h at 60°C, pH 7	Blanco et al. (1995)
Bacillus sp.	2% oat spelt xylan	37°C, pH 8, 2 days 180 rpm	9	60	10	30–60	8–10	1 h at 60°C, pH 10	Zheng et al. (2000)
Bacillus sp. NCIM59	5% wheat bran	50°C, pH 10, 2 days (50/150 ml shake flask, 150 rpm)	500	60	7	60–70	7–10	10 min at 70°C, pH 9	Nath and Rao (2000)
Bacillus sp. NCIM2128	3% wheat bran	28°C, pH 9, 2 days	52	60	8	35–70	7–9	5 min at 70°C, pH 8	Balakrishnan et al. (1992)
Bacillus amyloliquefaciencs	1% xylan	45°C, 4 days, air 4 vvm in 3/5 1 fermenter, 200 rpm	10.5	80	7	45–65	4–8	15 min at 70°C, pH 6.8	Breccia et al. (1998)

Bacillus circulans AB16	3% wheat straw	55°C, pH 8, 2 days (100/250 ml shake flask 200 rpm)	50	80	6	55–80	5–9	25 h at 70°C, pH 8	Dhillon et al. (2000)
	0.3% xylan		19	80	6	60–80	5–9		
Bacillus firmus	0.5% birch wood xylan	37°C, pH 10.5, 4 days, shake flask	1.75	37	7	ND	5–9	ND	Tseng et al. (2002)
Bacillus pumilus A-30	0.2% oat spelt xylan	37°C	616	45	6–8	40–50	5–9	ND	Liu et al. (2001)
Bacillus pumilus	1% xylan	45°C, pH 10, 2 days (50/250 ml shake flask, 250 rpm)	328	55–60	8–9	40–50	8–9	2 h at 50°C, pH 8–9	Duarte et al. (1999)
Bacillus subtilis	1% oat spelt xylan		4.9	ND	ND	ND	ND	ND	Khanongnuch et al. (1998)
	1% wheat bran		3.6						
Bacillus subtilis	1% oat spelt xylan	50°C, pH 6, 18 h	12	60	6	50–65	5–8	20 min 65°C, pH 8	Sa-Pereira et al. (2002)
Bacillus stearothermophilus	1% oat spelt xylan	37°C, pH 7, 2 days, shake flask	184	60	7	30–60	5–11	1 h at 70°C, pH 6	Nammori et al. (1990)
Cellulomonas sp. GS2	ND	ND	9.33	ND	ND	ND			Saxena et al. (1991)
Clostridium absonum CFR-702	1% birch wood xylan 75°C, pH 8.5, 4 days (anaerobic condition in 60/100 m serum bottle)		420	75	8.5	78–85	6–9	1 h at 75°C, pH 8.5	Rani and Nand (2000)
	3% rice straw		3.3						
	3% wheat straw		9.9						
	3% corn leaf		6.6						
Dictyoglomus sp. B1	0.15% beech xylan 70°C, pH 8.0, 5 days (anaerobic condition in 750 ml/1 1, fermenter, 40 rpm)		2.3	73	8.0		6–8	ND	Ademsen et al. (1995)
Micrococcus sp. DG10			3.33	ND	ND		ND	ND	Saxena et al. (1991)

(continued)

Table 31.1 (continued)

Microorganism	Substrate	Growth condition	Enzyme (IU/ml)	Optimum enzyme condition		Xylanase stability		Half-life (t1/2)	Reference
				T(°C)	pH	T(°C)	pH		
Rhodothermus marinus IT1376	3% birch wood xylan	61°C, pH 7.5, 4 days (air 1 vvm, in 3.5/51 fermenter, 200 rpm)	7.5	85	5.5–7	65–85	5–8	8.3 h at 85°C, pH 7	Dahlberg et al. (1993)
Staphylococcus sp. SG-13	0.5% oat spelt xylan	37°C, pH 8.0, 1 day (100/250 ml shaking flask, 200 rev/min)	0.8	50	7.5	50	7.5–9.5	ND	Gupta et al. (2000)
	1% cane bagasse		3.9						
	1% wheat bran		4.1						
	1% corn cobs		3.2						
B. thermantarticus	0.5% xylan		N.D.	80	5.6	60–90	5–8	50 min. at 80 pH 5.6	Lama et al. (2004)
B. halodurans S7	N.D.		0.4	70–75	9.5	60–70	6–10	4 h at 60°C, pH 9	Mamo et al. (2006)
Geobacillus thermoleovorans AP07	1% wheat bran		14	80	8.5	70–100	6–11	110 min at 80°C, pH 9	Sharma et al. (2007)
Bacillus sp. GRE7	0.5% xylan, 55°C, pH 7. oat spelt 5		60–80	70	7	70–90	4–11	More than 2.5 h at 60°C	Kiddinamoorthy et al. (2008)
Bacillus pumilus SV-85S	1% wheat bran	37°C, pH 7, Smf, 48 h, 150 rpm	2995.20	50	6	37–75	7–9	1 h at 60°C	Nagar et al. (2010)
Paenibacillus campinasensis BL.11	Rice husk	37°C, pH 7, Smf, 48 h	10.5	50	8	55–65	7–9	4 h at 65°C, pH 9	Ko et al. (2010)
Lysinibacillus sp. Strain P5B1	Corn Straw	35°C, pH7, Smf, 48 h	10.5	60	6	45–60	4.5–10	Not determined	Alvas-prado et al. (2010)
Streptomyces sp. 7b	Wheat bran	50°C, pH 8, Ssf, 72 h	2180	50	6	50	ND	30–45 min at 60°C	Bajaj and Singh (2010)

ND NOt detected

31.4 Regulation of Xylanase Biosynthesis

The regulation of xylanase secretion by microorganisms is not yet completely understood. Since xylan, being bulky, is unable to enter the microbial cell, the induction of xylanase is stimulated by low molecular weight xylan hydrolysis products that are produced in the medium by a small amount of constitutively produced enzyme (Bastawde 1992; Kulkarni et al. 1999). Some positional isomers are also capable of inducing xylanase synthesis in yeast *Trichosporon cutaneum* (Hrmovai et al. 1984).

Xylanases are usually inducible enzymes secreted in media containing pure xylan or xylan-rich residues (Balakrishnan et al. 1997). However, constitutive production of xylanase has also been reported in some cases (Khanna and Gauri 1993; Khasin et al. 1993; Lindner et al. 1994; Segura et al. 1998). Induction is mostly triggered by the substrate (xylan) in *Trametestrogii* (Levin and Forschiassin 1998), *Aspergillus awamori* (Siedenberg et al. 1998), and *Streptomyces* sp. QG-11-3 (Beg et al. 2000a). Xylan was, however, reported to be a poor inducer in *Cellulomonas flavigena* (Avalos et al. 1996). Induction of xylanase has also been reported by several other compounds like L-sorbose, D-maltose, D-glucose, D-arabinose, and various other disaccharides (Sachslehner et al. 1998; Xu et al. 1998; Bocchini et al. 2002) and xylose (Lopez et al. 1998; Liu et al. 1998). Several other reports of xylanase induction by natural lignocelluloses such as wheat bran, rice straw, corncobs, and sugarcane bagasse are available (Kesker 1992; Kuhad et al. 1998; Puchart et al. 1999; Beg et al. 2000a; Gupta et al. 2001). Wheat bran induced xylanase synthesis in polyextremophilic *Bacillus halodurans* TSEV1 (Kumar and Satyanarayana 2011). In some cases, readily metabolizable sugars such as glucose and/or xylose, serve as repressors of xylanase synthesis (Fernandez-Epsinar et al. 1992; Ishihara et al. 1997; Bataillon et al. 1998; Liu et al. 1999; Beg et al. 2000a).

The enhancement of xylanase production in the presence of amino acids has been shown in *Bacillus* sp. No. C-125 (Ikura and Horikoshi 1987), *Bacillus* sp. (NCL-87-6-10) (Balakrishnan et al. 1997), *Trametestrogii* sp. (Levin and Forschiassin 1998), *Staphylococcus* sp. SG-13 (Gupta et al. 1999), and *Streptomyces* sp. QG-11-3 (Beg et al. 2000b). Synthetic calcium-containing zeolite (CaA) at 0.5% has also been reported to enhance xylanase production up to twofold in *Bacillus* sp. NCL 87-6-10 (Balakrishnan et al. 2000). Gupta et al. (2001) reported an improved xylanase production by cultivating *Staphylococcus* sp.

SG-13 in a biphasic medium containing a solid lower layer of agar containing wheat bran and an upper liquid layer, where wheat bran acts as the inducer. Enhancement of xylanase production using DL-β-phenylalanine, niacin, and other surfactants has been recorded in *Bacillus pumilus* MK001 (Kapoor et al. 2008). Vitamin B12 (50 mg) followed by pyridoxine (50 mg) had been shown to enhance enzyme production in *Pseudomonas* sp. 2 (Varalakshmi et al. 2012).

31.5 Multiplicity of Xylanases

The ecological niches of xylanolytic microorganisms are diverse and widespread, but these microbes generally survive in the environments where plant materials accumulate and putrefy. Among xylanolytic enzymes, endo-1,4-β-D-xylanases, commonly referred to as xylanases or endoxylanases, are secreted by microorganisms mostly in the presence of xylan-containing substrates. These enzymes are predominantly found in two discrete sequence families known as GH10 (glycosyl hydrolase family 10) and GH11 (glycosyl hydrolase family 11). Besides, xylanases have also been found in glycosyl hydrolase families 5, 8, and 43 (Collins et al. 2005). An inverse relationship between the pI and molecular mass values has been observed in xylanases belonging to both GH10 and GH11. However, the structure and substrate specificity has been reported to be dissimilar between these two families (Liu et al. 2001; Collins et al. 2005). In addition to the production of debranching enzymes, a number of microorganisms are capable of producing multiple endoxylanases in order to acclimatize to various plant structural polysaccharides. For instance, *Aspergillus niger* produces five types of extracellular xylanases, while *Streptomyces sp*. 3137 also expresses three types of xylanases (Wong et al. 1988). The crude enzyme of *Paenibacillus curdlanolyticus* B-6 also exhibited 12 proteins with xylanase activity (Pason et al. 2006). *Streptomyces* is one of the commonly found bacterium in plant debris (Yang et al. 2001). It typically utilizes hemicellulose with the help of multiple xylanases, although xylanolytic systems of *Streptomyces* were rare to be completely realized. It appears that the presence of multiple xylanases results in enhanced xylan hydrolysis, thereby permitting saprophytes to utilize a wider range of plant debris (Wong et al. 1988; Elegir et al. 1994). There are many reports on the production of multiple forms of xylanases (Ustinov et al. 2008; Sharma et al. 2010a, b). Secretion of multiple forms of xylanases also depends on inducer/repressor present in production medium. Zymography is the most effective way of studying xylanases with different molecular weights (Elegir et al. 1994; Sachslehner et al. 1998). The xylanases of similar size but with different pI values cannot, however, be differentiated from one another on the gel. Immunoblotting has also been used to detect xylanase production (Li et al. 1993), but the antibody preparation necessary for this method of analysis is time-consuming. RT-PCR (reverse transcriptase PCR) is another method for examining xylanase gene expression; the results of RT-PCR, however, do not directly represent the amount of xylanase production (Jun et al. 2003; Calero-Nieto et al. 2007). Recently, 2-DE (two-dimensional electrophoresis) has been widely used for the separation of protein samples on an acrylamide gel according to pI and molecular weight. The amount of expressed protein also can be estimated according to the intensity of the spot on the gel (Oda et al. 2006). However, the catalytic ability of protein is not directly evident on the gel. Therefore, integration of a zymogram with 2-DE will facilitate a more complete examination of xylanase multiplicity. When the xylan-degrading enzymes of *S. thermonitrificans* NTU-88 were resolved, 19 clear spots were observed on zymogram gel after two-dimensional acrylamide gel analysis (Cheng et al. 2009).

31.6 Classification of Xylanases

Xylanases can be classified at least three ways. The first, as suggested by Wong et al. (1988), is based on molecular weight and pI value. They are either high or low molecular weight and have either a high (basic) or low (acidic) isoelectric point. Information for this sort of classification is readily obtained during purification and initial characterization steps. The second is based on the crystal structure of the protein, which may be derived indirectly by a determination of its DNA sequence. According to this, xylanases may belong to family F (now known as glycosidase family 10) or family G (now known as family 11). The third classification system is based on kinetic properties, substrate specificity, and the nature of products. Virtually all xylanases are "endo" acting, as readily determined by chromatography, but the more detailed determination of kinetic properties such as measurement of the relative reaction rates on various substrates and determining the kinetics of intermediate product formation is far less common.

Classifications based on molecular weight and pI is necessarily related to those based on sequence, and sequence analysis can reliably predict crystal structure. Relatively a few studies have been carried out that relate sequence or structural family to action patterns and substrate specificity.

Family 10 xylanases occasionally exhibit endocellulase activity; they generally have a higher molecular weight, and very rarely they possess a cellulose-binding domain. Members of the family 10 act on both PNP-xylobiose and PNP-cellobiose. The overall catalytic efficiency of the enzyme on PNP-xylobioside is, however, about 50 times higher (White et al. 1994). This indicates that the substrate of family 10 xylanases is xylan. Even though all xylanases are endo-acting, they may still differ in their product profiles. Some enzymes produce predominantly xylose and xylobiose, while others predominantly (or exclusively) form xylotriose and other higher oligosaccharides. This distinction may be due to the difference in the number of substrate-binding pockets on the surface of the enzyme molecule. The number of pyranose rings that the enzyme will bind effectively determines the size of the oligosaccharide products.

The family 10 catalytic domain is reported to be a cylindrical α/β barrel resembling a salad bowl with the catalytic site at the narrower end, near the C-terminus of the β-barrel (Derewenda et al. 1994; Harris et al. 1994). There are five xylopyranose binding sites. Catalytic domains of these enzymes belong to a "super family" that includes family A cellulases, β-glucosidase, β-galactosidase, β-(1-3)-glucanases, and β-(1-3,1-4)-glucanases (Jenkins et al. 1995). Xylanases belonging to the family 10 are high molecular weight proteins and tend to yield xylooligosaccharides with a low degree of polymerization (DP).

Family eleven xylanases are considered as true xylanases. They are typically low molecular weight, cellulase-free, and may have either high or low pI values. Family 11 catalytic domains consist principally of β-pleated sheets formed into a two-layered trough that surrounds the catalytic site (Withers and Aebersold 1995) protruding down into the trough, and located towards one side of the protein is a long loop terminating in an isoleucine. Torronen and Rouvinen (1995) have compared the

trough to the palm and fingers and the loop to the thumb of a right hand. The positions of many amino acids are essentially identical in the family 11 xylanases from bacterial (*Bacillus circulans*) and fungal (*Trichoderma harzianum*) origins, suggestive of tremendous conservation of the basic structure of the catalytic site of family 11 xylanases during evolution.

Trichoderma produces two important family 11 xylanases. The first one, Xyn1, has an acidic pI (5.5); possesses a smaller, tighter groove than Xyn2; and has a lower pH optimum (Torronen et al. 1992). It also exhibits a 15-fold higher turnover number (Tenkanen et al. 1992) and a threefold lower K_m than that of Xyn2. The other one, on the contrary, has a basic pI (9.0), a more open structure and a wider pH range for its activity and tends to produce larger oligosaccharides. Both Xyn1 and Xyn2 release xylobiose in β-configuration, indicating that the product is transiently attached to the enzyme surface (Biely et al. 1994). The difference in pI between Xyn1 and Xyn2 is attributable to the presence of more lysine and arginine residues on the sides of the isoleucine "thumb" of the enzyme. The function of these charged groups is not well established, but they could be assisting in binding to acidic side-chain substituents on the xylan backbone. Binding of *Trichoderma* xylanases to polysaccharides is affected by pH and ionic strength (Tenkanen et al. 1992). Enzymes are totally bound to xylan when the pH is below their isoelectric point but become detached at pH values above their pI values.

The pH optimum depends on properties of the acid/base catalyst (Torronen et al. 1992). In Xyn1, this is Glu164; in Xyn2, it is Glu177. In Xyn1, Asp33 makes a strong hydrogen bond (2.9 Å) to Glu164, thereby lowering the pKa. In Xyn2, an asparagine residue (Asn 44) replaces Asp 33; the hydrogen bond is much longer (3.7 Å), and the interaction is weaker. All acidic pI xylanases of family 11 have an aspartic residue in this position, while all the enzymes with basic pI possess an asparagine residue.

Recently a more complete system of classification has been introduced that allows the classification not only of xylanases but of glycosidases too (EC 3.2.1.x), which is now used for classification of these enzymes. This system is based on primary structure comparisons of the catalytic domains only and groups enzymes in families of related sequences (Henrissat 2001). Following this system, 96-glycoside hydrolase families exist (refer to the carbohydrate-active enzyme CAZY server at http://afmb.cnrs-mrs.fr/~cazy/CAZY), with one-third of these families being polyspecific, i.e., having enzymes with diverse substrate specificities. Furthermore, divergent evolution has resulted in some of the families having related three-dimensional structures, and thus the grouping of families in higher hierarchical levels, known as clans, has been introduced (Henrissat and Bourne 2001). Presently, 14 clans have been proposed (GH-A to GH-N), with most clans encompassing two to three families, apart from clan GH-A that has 17 families. Within this classification system, xylanases are normally reported as being confined to families 10 (formerly F) and 11(formerly G) (Wong et al. 1988; Singh et al. 2003; Collins et al. 2005). Interestingly, a search of the appropriate databases using the enzyme classification number EC 3.2.1.8 indicates that enzymes with xylanase activity are also found in families 5, 7, 8, 16, 26, 43, 52, and 62.

31.7 Sources of Thermostable Xylanases

A wide range of living organisms produce xylanases. Among them, microbes are the most important from the commercial viewpoint. Although fungal sources have been significant xylanase producers, they do not qualify the need of certain current biotechnological applications due to lack of stability at high temperature and pH. Many industrial processes employ high temperatures, which have to be lowered in order to use biocatalysts from mesophiles. This can be avoided using enzymes from thermophiles (Hong et al. 2009; Kumar et al. 2009; Zhong et al. 2009; Kumar and Satyanaraayana 2011). Some of the bacterial xylanases from extremophiles are found to be suitable for these industrial requirements owing to their stability at higher temperature (70–80°C) and pH (7–12).

Research into thermophilic microorganisms has demonstrated that thermotolerant proteins are generally more stable than other proteins and retain this property when cloned and expressed in mesophilic bacteria (Techapun et al. 2003; Hayakawa et al. 2010).

31.7.1 Geothermal Sites as Sources of Extremophiles

Most of the naturally occurring xylanases are optimally active at mesophilic temperatures (approximately 40–60°C). Recently xylanases have also been reported that they are not only stable but also active at extremes of pH and temperatures (Collins et al. 2005). In situ temperatures between 80 and 115°C have been found to be conducive biotopes for a number of hyperthermophiles (Huber and Stetter 1998). Some examples are cited in Table 31.1. Although thermophiles require elevated temperatures for optimal growth, this does not necessarily restrict the distribution of thermophiles to very distinct niches where elevated temperatures are provided, such as alkaline hot springs or alkaline (soda) lakes with geothermal or solar heat sources. On the other hand, spore-forming thermophiles including spore-forming polyextremophiles could be found everywhere. One also can speculate that most aerobes are more or less ubiquitous. Extremophiles are also adapted to live at a very low and high pH values (pH 0–3 or 10–12), high salt (5–30%) concentration, at high pressure in the deep sea, and extremely low temperatures in the cold polar regions (Herbert and Sharp 1992). In the last decade, a number of hyperthermophilic archaea, known as the least understood domain of life (Woese et al. 1990), have been isolated, which are able to grow around the boiling point of water (Niehaus et al. 1999). The organisms with the highest growth temperatures (103–110°C) are members of the genera *Pyrobaculum*, *Pyrodictium*, *Pyrococcus*, and *Methanopyrus*. Within the bacteria, *Thermotoga maritima* and *Aquifex pyrophilus* exhibit the highest growth temperatures of 90 and 95°C, respectively (Herbert and Sharp 1992). Prof. Otto Kandler, K.O. Stetter, W. Zillig, and several others have contributed immensely in increasing knowledge about hyperthermophilic microbes which can even withstand autoclaving for 1 h at 121°C, *Pyrolobus fumarii* (Blöchl et al. 1997).

Recently, Ken Takai in Koki Horikoshi's lab was able to isolate a strain of *M. kandleri* that has the ability to withstand high pressure of 200 bar, it even grows up to 122°C and, therefore, reports the highest temperature of growth of a living being (Takai et al. 2008). These properties imply extremely important industrial and biotechnological implications due to the fact that enzymes from such microorganisms may be successfully employed for use in harsh industrial conditions where their specific catalytic activity is retained.

31.7.2 Important Adaptations in Thermophilic Microorganisms

Microorganisms, like all living things, adapt to the condition in which they have to live and survive. Thermophiles are reported to contain proteins that are thermostable and resist denaturation and proteolysis (Kumar and Nussinov 2001). Specialized proteins known as chaperonins are produced by these organisms, which help their proteins to cope with denaturation by refolding to their native form and restore their functions (Everly and Alberto 2000). Saturated fatty acids are major components of the cell membrane of thermophiles. The fatty acids provide a hydrophobic environment for the cell and keeps the cell rigid enough to sustain at elevated temperatures (Herbert and Sharp 1992). The archaea have lipids linked with ether present in the cell wall. The layer formed by this way is much more heat resistant than a membrane formed of fatty acids (De Rosa et al. 1994).

The DNA of thermophiles contains a reverse DNA gyrase that produces positive supercoils in the DNA (Lopez 1999). This raises the melting point of the DNA (the temperature at which the strands of the double helix separate into two single strands) to at least as high a value as the maximum temperature for growth of these microbes. Thermophiles also tolerate high temperature by using a higher degree of the same interactions that non-thermotolerant organisms employ, namely, electrostatic, disulphide bridge, and hydrophobic interactions (Kumar and Nussinov 2001).

Thermostable enzymes are stable and active at temperatures that are even higher than the optimum temperatures for the growth of the microorganisms (Saboto et al. 1999). Very few attempts have been made in this regard and the only available information is that thermostable enzymes are relatively rigid proteins. A clear-cut understanding of this capacity should be possible with new methodologies that clearly indicate changes in protein structure. While determining the conformational entropy of both thermophilic and mesophilic enzymes, the folded state showed a higher structural flexibility in case of the thermostable protein than its non-thermostable counterpart. Thus, it has been assumed that a mechanism characterized by entropic stabilization could be responsible for the higher thermostability of the thermostable enzyme (Fitter et al. 2001; Haki and Rakshit 2003).

31.7.3 Advantages of Using Xylanase from Thermophilic Microorganisms in Biotechnology

Enzymes of thermophilic organisms are gaining wide industrial and biotechnological interest due to the fact that these are better suited for harsh conditions in industrial processes. The bioavailability and solubility of organic compounds is enhanced at higher temperatures and thereby results in more efficient bioconversions (Becker 1997). Elevated process temperatures include higher reaction rates due to a decrease in viscosity and an increase in diffusion coefficient of substrates which results in higher process yield due to increased solubility of substrates and favorable equilibrium displacement in endothermic reactions (Mozhaev 1993; Krahe et al. 1996; Kumar and Swati 2001). One added advantage of conducting biotechnological processes at elevated temperatures is minimizing the risk of contamination by common mesophilic contaminants. Among numerous biotechnological applications of thermostable xylanases, the most demanding ones are in feed industry, textile industry, and prebleaching of paper pulp (Collins et al. 2005). Enzymes from thermophilic microbes may also be used as models for the understanding of thermostability and enzyme activity, which may in turn be conceptually useful for protein engineering.

31.8 Production of Xylanases

Enzymes are proteins and serve as potential biocatalysts for a large number of reactions. Generally, living systems depend on enzymes for the transformation of macromolecules to energy and new materials, besides for growth, repair, and maintenance of cells. Thus, all living beings, like plants, animals, and microorganisms, are sources of enzymes. For commercial applications in industry, microbial enzymes are the most important source of enzymes (Ibrahim 2008). The specificity of thermostable enzymes makes them potent for many industrial applications. The use of such enzymes for industrial utilization may be attributed to economic benefits of being able to degrade plant residues at elevated temperatures (Haki and Rakshit 2003). Although many microorganisms including bacteria (Nakamura et al. 1994; Yang et al. 1995; Dhillon and Khanna 2000; Gupta et al. 2000; Balakrishnan et al. 2002; Choudhury et al. 2006; Kapoor et al. 2008; Ko et al. 2010; Kumar et al. 2012), actinomycetes (Ball and Mccarth 1989; Techapun et al. 2001; Tuncer et al. 2004), and filamentous fungi (Taneja et al. 2002; Angayarkanni et al. 2006; Sudan and Bajaj 2007; Sharma et al. 2008; Maalej et al. 2008; Bajaj et al. 2011; Bajaj and Abbass 2011; Chapla et al. 2010; Pandya and Gupte 2012) have been reported for xylanase production, only a few of them are alkaliphilic or thermophilic. Kraft pulping is carried out under strongly alkaline conditions, and even after multiple washings, alkali continues to leach from the fiber. The activity under alkaline conditions is, therefore, an important characteristic for an enzyme to function in enzymatic prebleaching of paper pulp (Yang et al. 1995). Wild-type *Bacillus* strains capable

of secreting xylanases are devoid of cellulase activity and stable at high temperature and pH and thus, would be an attractive option for such applications.

To obtain maximum yield of an enzyme, it becomes necessary to develop a suitable medium and culture conditions (Narang and Satyanarayana 2001). Hence, the culture medium for the fermentation should be designed in such a way that it is capable of supporting maximal enzyme production, and at the same time, it should be economical and avoids wastage of components. Abundantly available and inexpensive agroresidues such as wheat bran, corncob, corn stover, and wheat straw have been reported to be suitable substrates in achieving higher xylanase yields (Gupta et al. 2000; Damiano et al. 2003; Pason et al. 2006; Kapoor et al. 2008; Ko et al. 2010; Kumar and Satyanarayana 2011; Kumar et al. 2012). A number of microorganisms, including bacteria (Sunna and Antranikian 1997; Archana and Satyanarayana 1997; Gilbert and Hazlewood 1999; Battan et al. 2007; Sharma et al. 2007; Kapoor et al. 2008; Mohana et al. 2008; Nagar et al. 2010; Ko et al. 2010; Kumar and Satyanarayana 2011), fungi (Sunna and Antranikian 1997; Kuhad et al. 1998; Bajaj et al. 2011; Bajaj and Abbass 2011), Actinobacteria (Ball and McCarthy 1989; Kumar et al. 2012), and yeast (Hrmovai et al. 1984; Liu et al. 1998), have been reported for xylanase production in solid-state fermentation (SSF) and submerged fermentation (SmF).

31.8.1 Xylanase Production in Solid-State Fermentation (SSF)

In solid-state fermentation, the culture medium is devoid of free-flowing water in the system (Cannel and Moo-Young 1980). SSF conditions are designed to mimic the natural habitats of microorganisms; therefore, it induces the microorganisms to produce enzymes and metabolites that are either not produced or lesser produced in submerged conditions (SmF) (Jecu 2000). Several reports are available on xylanase production using a bacterial isolate in SSF (Archana and Satyanarayana 1997; Gessesse and Mamo 1998; Subramaniyan and Prema 2001; Sonia et al. 2005; Battan et al. 2007; Sanghi et al. 2008; Dhiman et al. 2008; Mohana et al. 2008; Bajaj and Singh 2010; Garg et al. 2011). In SSF, the solid substrate particles serve as the source of carbon, nitrogen, salts, and growth factors and at the same time, also provide mechanical support and anchorage to the microbial cells.

There are several factors (physical, chemical, and biochemical) which one needs to consider for any SSF (Pandey et al. 2001). The major factors that affect microbial growth and activity in SSF include selection of suitable microorganism and substrate, pretreatment of substrate, particle size (interparticle space and surface area) of the substrate, water content and water activity (a_w) of the substrate, relative humidity, type and size of inoculum control and temperature of the fermenting matter/removal of metabolic heat, period of cultivation, maintenance of uniformity in the environment of SSF, and the gaseous atmosphere (oxygen consumption rate and carbon dioxide evolution rate). There are so many reports about enhancement of bacterial xylanase production by optimizing above parameters (Archana and Satyanarayana

1997; Sanghi et al. 2008; Sindhu et al. 2007; Poorna and Prema 2007; Mohana et al. 2008; Bajaj and Singh 2010; Garg et al. 2011; Nagar et al. 2011).

31.8.2 Xylanase Production in Submerged Fermentation (SmF)

Submerged fermentation involves growth and enzyme production by the producer microorganism in the presence of sufficient free water in which the medium components remain uniformly dissolved, and the microbe derives nutrition from the same. Its physical parameters such as pH, temperature, and dissolved oxygen may be controlled with ease and hence scaling up gives predictable results. The most suitable carbon and nitrogen sources are quite specific for each particular organism. Xylanase production by microbes may be growth associated or growth independent. Dey et al. (1992) reported non-growth-associated xylanase production by *Bacillus sp*., while Esteban et al. (1982) and Nakamura et al. (1993a) demonstrated xylanase production coupled with bacterial exponential phase of growth. Bocchini et al. (2002) described good production of thermostable and cellulase-free xylanase from *Bacillus circulans* through SmF process. There are very few reports on the production of xylanases that are cellulase-free, thermostable, and alkalistable. Sharma et al. (2007) studied thermoalkalistable xylanase in *Geobacillus thermoleovorans* AP7 (syn. *Bacillus thermoleovorans*) using submerged fermentation. Techapun et al. (2002) reported thermostable, alkalitolerant, and cellulase-free xylanase from *Streptomyces sp*. 106 in submerged fermentation. Most of the bacterial xylanases are produced by this process, and the production is optimized by controlling various physical and nutritional parameters.

31.8.2.1 Physical Parameters

The most important physical parameters suitable for the growth and production of industrially important xylanases are temperature, pH, agitation, and dissolved oxygen. Xylanases have been reported from both mesophilic and thermophilic microorganisms. Intensive investigations have been carried out with xylanolytic enzymes from mesophilic bacteria and fungi (Wong et al. 1988; Coughlan and Hazlewood 1993). Most of the xylanases from mesophilic and thermophilic microbes show their growth and optimum activity between 40 and 70°C, but those from extremophiles or hyperthermophiles such as *Dictyoglomus*, *Geobacillus*, and *Thermotoga sp*. have their optima much beyond this temperature range (Archna and Satyanarayana 1998; Qureshy et al. 2002; Sharma et al. 2007; Sanghi et al. 2009; Ko et al. 2010). The pH is well known to have a profound effect on the production as well as stability and activity of the extracellular xylanases. Different organisms have a different optimal pH for the production of enzyme and its activity. Alkaliphiles are reported to frequently produce xylanases with pH optima between 7.0 and 10.0 (Horikoshi and Atsukawa 1973; Okazaki et al. 1984; Tsujibo et al. 1990; Dhillon et al. 2000;

Anuradha et al. 2007; Sharma et al. 2007; Sanghi et al. 2009; Ko et al. 2010; Kumar and Satyanarayana 2011).

Mechanical agitation and aeration are crucial factors for the production of xylanases, since they aid in uniform distribution the nutrients and oxygen supply for the growth of the organisms. An agitation rate of 250–200 rpm supported a higher xylanase production level by various *Bacillus sp*. (Okazaki et al. 1984; Shah et al. 1999; Archana and Satyanarayana 1998; Dhillon et al. 2000; Anuradha et al. 2007; Sharma et al. 2007; Sanghi et al. 2009).

31.8.2.2 Nutritional Parameters

The different sources of carbon as well as their concentrations are important factors for not only the optimal production of enzymes but also their localization (extracellular or cell bound). Xylan and various lignocellulosics like wheat bran, corn straw, and rice straw have been widely reported to support xylanase production (Esteban et al. 1982; Okazaki et al. 1984; Nakanishi et al. 1992; Archna and Satyanarayana 1997; Dhillon et al. 2000; Damiano et al. 2003; Chauhan et al. 2006; Chaudhary et al. 2007; Sharma et al. 2007; Sanghi et al. 2009; Ko et al. 2010, Kumar and Satyanarayan 2011; Kumar et al. 2012).

A suitable nitrogen source in the culture medium is another important factor influencing microbial biomass production as well as metabolite buildup. In general, organic nitrogen sources such as yeast extract, tryptone, peptone, soybean meal, and corn steep solids are routinely used and give better xylanase production. Yeast extract in combination with peptone was reported to enhance xylanase production in *Bacillus sp*. SSP-34 significantly (Subramaniyan et al. 1998; Qinnghe et al. 2004; Kapoor et al. 2008; Sanghi et al. 2009). Similarly, tryptone supported maximum xylanase secretion in *Bacillus sp*. B16 [Subramaniyan et al. 2001; Dhillon et al. 2000; Sharma et al. 2007; Kumar and Satyanarayan (Unpublished work)]. Soybean meal increased the production by 25-fold in *Bacillus sam*-3 (Shah et al. 1999), since soyameal does not cause any catabolite repression, and thus, it is considered as excellent nitrogen source (Nair et al. 2008; Bajaj and Abbass 2011). Corn steep solids supported good production levels of xylanase in *T. reesei* (Lappalainen et al. 2000). The incorporation of trace elements and vitamins affects enzyme production to a great extent, especially for thermoanaerobes (Antranikian et al. 1987a) and some bacilli (Ara et al. 1996). *Thermotoga sp*. FjSS3-B.1 required casamino acid other than the usual carbon and nitrogen sources in the culture medium. Xylanase production by *Streptomyces sp*. QG-11-3 was improved with the addition of amino acids (Beg et al. 2000c). Likewise, a culture medium supplemented with vitamin solution and trace elements fared better with respect to enzyme biosynthesis by *Bacillus sp*. AB16 than with other bacterial sps (Dhillon et al. 2000; Kapoor et al. 2008; Varalakshmi et al. 2012). However, Dey et al. (1992) reported xylanase production in wheat bran and yeast extract medium that did not require additional supplementation of any tonic substance at all.

31.9 Cloning and Expression of Xylanase Genes

Although several xylanases with a great variety of properties have been produced by several bacteria as well as fungi, to fit the bill for industrial exploitation, much needs to be accomplished concerning gene manipulation strategies. Most important prerequisite for the use of xylanases in prebleaching of pulps is their alkalistability and thermostability. Recombinant DNA technology allows cloning of the desired xylanase genes for enhancing the production levels even in the altered physiological conditions. Several reports are available where xylanase genes have been cloned and expressed in a suitable host to overproduce xylanases of interest. Cloning demands the availability of full-length gene of xylanase which can be fished out from any good xylanase-producing microorganism either by PCR approach (Zhang et al. 2007; Guo et al. 2009) or by making genomic libraries (Raha et al. 2006; Hu et al. 2008). Both the approaches have pros and cons. Genomic library construction is a straightforward process to retrieve the full-length genes, but its success rate is quite low till date. Furthermore, this approach is very laborious and time-consuming while PCR approach is much more logical and is based on using primers designed from the conserved regions of the selected xylanases. Isolating the full-length gene in a single attempt is very rare, and therefore, multiple primers are needed to obtain the full stretch of the gene.

As majority of the alkalistable and thermostable xylanases have been reported to be produced by extremophiles, the desired xylanase genes from these bacteria have to be cloned into the genomes of mesophilic bacteria such as *E. coli* or *Bacillus subtilis*. This strategy is logical and energy conserving as well as offers improved features of the enzyme that in turn prove useful in its applications. Several xylanase genes from various bacteria have been cloned by homologous as well as heterologous cloning. Reports are also available on cloning xylanase genes in plant systems (Helbers et al. 1995). Although several bacteria have been reported to produce xylanases, the members of the genera *Bacillus* and *Geobacillus* have more commonly been serving as producers of xylanases. Heterologous cloning of xylanase from the gram-positive bacteria into gram-negative bacteria like *E. coli* is very common in recombinant DNA technology. Improved production of xylanases has been reported from bacteria such as *Bacillus polymyxa* (Yang et al. 1989), *Geobacillus thermoleovorans* (Verma and Satynarayana 2012), *Fibrobacter succinogenes* 135 (Whitehead and Hespell 1989), and *Bacillus circulans* (Sung et al. 1993). Usually such heterologous cloning restricts the localization of enzyme as intracellular and sometimes results in a decline in the production levels too. Such heterologous cloning of a thermostable xylanase gene from *Bacillus coagulans* was successfully attempted in *E. coli* and in *Lactococcus lactis*. A vector pMG36e, a shuttle vector, was used for transferring the genes into *E. coli* (Van de Guchte et al. 1989) and *Lactococcus species* (Raha et al. 2006). Some genes encoding xylanases with interesting properties from *Bacillus stearothermophilus*, *Dictyoglomus thermophilum*, and *Thermotoga maritima* have been cloned and expressed in *E. coli* (Gibbs et al. 1995; Chen et al. 1997; Veoikodvorskaya et al. 1997). The recombinant proteins produced by *Dictyoglomus thermophilum* RT46B.1 showed

a temperature optimum of 90°C with an optimum pH of 5.5 (Gibbs et al. 1995). Likewise, xylanase cloned from *Thermotoga neapolitana* was found to be suitable for bleaching in paper and pulp industries with an optimum temperature at 90°C. Besides, the enzyme possessed a half-life of 2 h at 100°C (Veoikodvorskaya et al. 1997). An unusual xylanase gene was cloned and expressed from *Bacillus polymyxa* NRC 2282. The properties of the recombinant xylanase were entirely different from xylanase produced by the parent bacterium. The pI values were significantly changed from 9.0 to 4.9 in the recombinant xylanase (Yang et al. 1988). A gene encoding a xylanase of low molecular weight (~23 kDa, 213 amino acids) from *Bacillus subtilis* strain R5 has been cloned and expressed in *E. coli*; the activities were further enhanced by metal ions (Jalal et al. 2009). Another recombinant xylanase optimally active at 50°C was cloned from *Bacillus subtilis* (strain B10) in *E. coli* (Huang et al. 2006). The strains of *Bacillus subtilis* and *B. megaterium* are the very common choices as hosts to express the recombinant protein, since they produce low levels of extracellular proteases and possess good secretory system for the release of recombinant proteins (Pariza and Johnson 2001; Radha and Gunasekaran 2009).

The codon usage is the problem in heterologous cloning while homologous cloning reads the same amino acids for the respective codons. An interesting example of homologous cloning was reported in *Bacillus* strain 168, whose xylanase-encoding gene was reintroduced in various strains of *Bacillus subtilis*. Two of these strains BS1 and BS2 produced xylanases similar to the xylanase A of wild-type *Bacillus subtilis* strain 168, while the third strain BS3 produces a xylanase that was different from the wild-type counterpart by two amino acids. Although this xylanase was resistant to xylanase inhibitor present in flour, it still was not suitable for paper and pulp industries due to its instability at 50°C (Zofia Olempska-Beer 2004). A fragment of 9.4 kbp obtained from the genomic library of *B. succinogenes*-encoding xylanase activity was subcloned into pBR322 through a series of subcloning steps, resulting in a trimmed fragment of 3.0 kbp whose translation product actually retained the xylanase activity. However, here the cloning and subcloning manipulations reduced the actual activities ~10-fold as compared to the enzyme activity exhibited from the long 9.2 kbp stretch. This may be attributed to the deletion of a structural or regulatory region from the native stretch (Sipat et al. 1987). A 60-fold increase in enzyme activity was obtained from homologous cloning of xylanase gene from *Streptomyces lividans* 1326 to xyn^- mutant of *Streptomyces lividans*. Therefore, homologous cloning is suitable for codon usage as well as secretion of the desired protein extracellularly in the medium (Iwasaki et al. 1986).

A methylotrophic mesophile *Pichia pastoris* has also been considered as an excellent host system for the expression of the recombinant proteins. It has successfully been utilized for the expression of xylanase genes from several fungi (Chantasingh et al. 2006; Jeya et al. 2009; He et al. 2009; Nan et al. 2011) with a purpose of avoiding the harmful and unwanted mycotoxins produced from these filamentous fungi. In this context, several xylanases from *Thermomyces lanuginosus* and *Trichoderma reesei* have been successfully cloned and expressed in various strains of *Pichia pastoris* (Monica et al. 2003; He et al. 2009; Birijlall et al. 2011; Nan et al. 2011). Besides, bacterial xylanases have also been expressed in this system. An attempt was made to subclone

the xylanase 11A gene from *Thermobifida fusca* NTU22 into pPICZαA followed by its expressed in *Pichia pastoris*. A remarkable 67-fold enhancement of xylanase level, when compared to the native xylanase, is produced by *Thermobifida fusca* NTU22 (Cheng et al. 2005; Wang and Xia 2008). *Pichia pastoris* was also generally compared with *S. lividans* and *E. coli* as a host system and fared better than them. It may also be attributed to the fact that *Pichia pastoris* utilizes its strong promoter AOX1 (alcohol oxidase1) for overexpression of recombinant proteins (Cheng et al. 2005).

Molecular breeding of transgenic plants is considered for the cost-effective production of bacterial xylanases and cellulases. Two different domains of xylanase A gene from *Clostridium thermocellum* were cloned into cultured tobacco BY-2 cells under the control of cauliflower mosaic virus 35S promoter (Kimura et al. 2003). Other attempts were made to produce the endoglucanase I from *Ruminococcus albus* and xylanase B from *Clostridium stercorarium* into tobacco BY2 cells and tobacco plant systems (Kawazu et al. 1999; Sun et al. 1997). Highly thermostable xylanase gene with a broad pH range from hyperthermophile *Thermotoga sp*. strain FJSS3 B.1 was cloned and overexpressed into *E. coli* (Simpson et al. 1991).

Genus *Streptomyces* may be considered as a favorite parent source of a number of xylanase genes into various host systems. Several novel xylanases have been characterized and successfully expressed from *Streptomyces olivaceoviridis* A1 into *E. coli* and *Pichia pastoris*. The amino acid alignment of recombinant xylanase showed ~86% homology with xylanase of *Streptomyces sp*. strain S38, but on the basis of different properties, the xylanase cloned from *Streptomyces olivaceoviridis* A1 was reported as novel (Wang et al. 2007). Another report of novel xylanase from *Streptomyces sp*. S9 shows highest identity (50.8%) with a putative endo-1,4-β-xylanase from *Streptomyces avermitilis* of the glycoside hydrolase family 10. The mature protein was cloned and expressed in *E. coli* BL21 (DE3) although the properties of recombinant xylanase were found to be in close proximity of other xylanases reported from *Streptomyces* sp. (Li et al. 2008). A xylanase gene obtained from the genomic library of *Streptomyces* sp. SP27 has been thought of as novel. The mature xylanase comprises two functional domains, a family 10 glycoside hydrolase and carbohydrate-binding module. Both domains are interconnected by short gly/pro-rich linker region. The CBM-truncated as well CBM linker-truncated versions of the proteins were expressed in *E. coli* BL21 (DE3). The further analysis of these studies reveals that CBM region plays a key role in hydrolysis of insoluble substrate and linker region supports enzyme stability. The recombinant xylanase represent itself a strong tool for producing xylobiose as the main hydrolysis product (Li et al. 2009). The cloning and expression of many xylanase genes from various microorganisms are summarized in Table 31.2.

31.9.1 Rational Protein Design

Gene cloning is a powerful tool that empowers the workers to accomplish the production of thermostable xylanases in mesophilic hosts and also facilitates their

Table 31.2 Cloning and expression of bacterial xylanase

Microorganisms	Host	Vector	Xylanase activity of recombinant (U/mg)	References
Bacillus sp. (NCIM 59)	*Bacillus* sp. (NCIM 59)	PUC8	128	Shendye and Rao (1993)
Bacillus subtilis strain R5	*E. coli*	PTZ57R/T	–	Jalal et al. (2009)
Thermomonospora fusca	*E. coli* JM101	λgtWESλB	28.5	Hu et al. (1991)
Bacillus sp.C125	*E. coli* HB101	pBR322	–	Honda et al. (1985)
Bacillus subtilis B10	*E. coli* JM109	pUC 18	–	Huang et al. (2006)
Cellulomonas sp. NCIM 2353	*E. coli* JM107	pUC18	0.056	Bhalerao et al. (1990)
B. polymyxa NCIB 8158/ATCC 842	*E. coli* C600	pBR322	0.1	Sandhu and Kennedy (1984)
Clostridium stercorarium F9	*E. coli* JM109	PBR322	8.16	Sakka et al. (1990)
C. acedobutylicum P262	*E. coli* JM101	pEcoR 251	4.0	Zappe et al. (1987)
C. thermocellum NRCC	*E. coli*	PUC8	1.5	MacKenzie et al. (1989)
Streptomyces EC3	*S. lividans*	PIJ-702	–	Service et al. (1992)
	S. parvulus	PIJ-702		
Streptomyces halstedii	*S. parvulus*	PIJ-702	–	Arribas et al. (1995)
Bacillus strain 168	*B. subtilis*	pUB110	–	Zofia Olempska-Beer (2004)
Thermobifida fusca NTU22	*Pichia pastoris*	pPICZαA	–	Wang and Xia (2008)
Clostridium thermocellum	Cultured tobacco BY-2 cells	Cauliflower mosaic virus 35 S promoter	–	Kimura et al. (2003)
Streptomyces olivaceoviridis A1	*E. coli*		–	Wang et al. (2007)
	Pichia pastoris			
Streptomyces sp. SP27	*E. coli* BL21			Li et al. (2009)
S. lividans 1326	*S. lividans*	PIJ-702	380	Mondou et al. (1986)
B. pumilus IPO	*Bacillus subtilis* MI 111	PUB110	1.8	Panbangred et al. (1983)
Streptomyces sp.	*S. lividans* TK21	PIJ-702	2839	Iwasaki et al. (1986)

overexpression by coupling their structural genes with their well-known powerful promoters. Despite these concepts, there is practically glaring scarcity in recombinant microbial producers with suitable properties (the most significant being stability at extreme conditions) of xylanases, and only a handful of xylanases have been reported which can accomplish the requisite conditions of industries. Although the enzymes can be stabilized conventionally by various strategies like entropic stabilization, helix capping, and introduction of clusters of aromatic–aromatic interactions such as salt bridges (Matthews et al. 1987; Marshall et al. 2002; Puchkaev et al. 2003), they are not really up to the mark. Industrially useful xylanases often require their sustenance at high temperatures and pH values, especially in papermaking industry. The uses of such enzymes that can tolerate these extremes serve as prebleaching agents by eliminating the dark-colored lignin. This reduces the use of hazardous chemicals in the subsequent chlorine bleaching stage.

The rationale of protein engineering came into existence from studying a large number of enzymes from mesophiles and comparing their amino acids with their counterparts from extremophilic sources. This alignment and comparison of their amino acid sequences in catalytic domains, thermal stabilizing domains, or substrate-binding domains led to a conclusion that some amino acids are truly crucial in imparting their significant properties. Conversely stated, these amino acid residues could be targeted for alteration in the pursuit of enhancing the desirable properties of the protein being manipulated (Sterner and Liebl 2001; Vieille and Zeikus 2001; Hakulinen et al. 2003). Therefore, protein engineering has now come to occupy a very significant niche if one wishes to alter the protein of their interest with respect to some specific features. The basic information of protein such as active sites, catalytic domains, and structure–function relationship provide tools to make a logical design for protein engineering.

31.9.2 Protein Engineering for Thermal Stabilization

A comparative study was conducted among twelve members of family 11 xylanases, including mesophilic and thermophilic proteins, by using molecular dynamics (MD). It elucidated that thermostable xylanases endured heat better than their counterparts obtained from mesophiles and also indicates the unstable regions that are hotspots for mutations with respect to thermostability (Purmonen et al. 2007; Joo et al. 2010; Uzuner et al. 2011; Noorbatcha and Hamzah 2011).

Enhancement of protein stability towards extreme temperatures may be accredited to various fundamental structural features such as disulfide bridges (Wakarchuk et al. 1994; Xiong et al. 2004; Yang et al. 2007), more surface-charged residues (Turunen et al. 2002) and a higher degree of hydrophobic packing in protein core (Xie et al. 2006). A number of xylanase genes have been engineered to stabilize the xylanases at higher temperatures.

Based on the 3-D structure of the enzyme, an enhancement has been achieved for thermal stabilization of xylanase from *Bacillus circulans*. Introduction of inter- and

intramolecular disulfide bridges by means of site-directed mutagenesis improved the thermal stability of the mutants, although there was no enhancement in xylanase activity of the mutants (Wakarchuk et al. 1994). Structure–function analysis of xylanases has already revealed that N-terminal, C-terminal, and α-helix regions have significant contributions in thermostability owing to their labile structures. A mutant was engineered by substitution of N-terminus of *Streptomyces olivaceoviridis* xylanase XYNB with the corresponding region of *Thermomonospora fusca* xylanase TfxA. A significant enhancement in thermostability was observed where optimum temperature was shifted to 70°C at pH 6 and a sixfold enhancement in thermostability was accomplished at 80°C for 3 min (Yang et al. 2006). The thermostability of this mutant was further improved by incorporation of disulphide bridge (C_{109}–C_{153}) and expressed in *Pichia pastoris*, wherein an approximately 12.4-fold enhancement was observed compared to XYNB at 70°C, pH 6 for 20 min. The synergistic effect of N-terminus and disulfide bridge remarkably extended the half-life of XYNB from 3 min to a great 150 min, coupled with a better resistance in acidic conditions (Yang et al. 2007).

Local and long-range interaction determines the stability of protein in extreme conditions. Sometimes stepwise successive substitutions with a small quantum effect each also adds up to a desired thermostability at extreme temperatures. Another popular strategy for thermal stabilization comes through comparative analysis of respective proteins from mesophiles with their counterparts from extremophiles with the goal of identifying amino acid (s) responsible for the thermal stabilization. Furthermore, correlation among some amino acids really noteworthy for the thermal stabilization, such as more proline coupled with less asparagine and glutamate residues and more arginines and tyrosines plus less cysteine and serine residues, increased numbers of salt bridges and side-chain–side-chain hydrogen bonds. Likewise, increased numbers of arginines in combination with reduced numbers of proline, cysteine, and histidine residues in α-helices of the protein are also reported to stabilize the enzyme at high temperatures (Sriprapundh et al. 2000).

31.9.3 Protein Engineering for Other Properties

The pH optimum was changed from 8.5 to 9.5 for xylanase J of *Bacillus sp*. 41 M-1 by introducing a salt bridge in the catalytic cleft and incorporating excess arginine residues (Umemoto et al. 2009). Another remarkable example to improve the properties of xylanase by the aid of protein engineering is enhancing the hydrolyzing activity towards insoluble xylan. Random mutations were done in xylan-binding domain (XBD) of xylanase J gene, and the repertoire was cloned for phage display. The substitution of Thr_{317} by isoleucine improved its xylan-binding activity (Sakata et al. 2006).

Besides substitution of various amino acids at specified position, protein engineering has also been used for integrating two distinct xylanolytic enzymes. Two chimeric enzymes, xyn–ara and xyn–xylo, were constructed by linking the catalytic

portion of a xylanase (xyn) to that of either an arabinofuranosidase (ara) or a xylosidase (xylo) with the help of a flexible peptide linker. Although their optimum pH shifted from 8.0 to 6.0, both not only retained their activities but also demonstrated much better hydrolytic activities than earlier (Fan et al. 2008).

31.9.4 Directed Evolution

Protein engineering demands mandatory information regarding structure and catalytic mechanism of the proteins to be engineered, which make it even tougher to implement, particularly in case of protein thermostabilization, where the mechanism is feebly understood (Adams and Kelly 1998; Farinas et al. 2001). In addition, very few examples are available to enhance the stability of xylanase in alkaline conditions. Therefore, it may become tough at times to achieve the specific amino acid residue substitution to get the desired results using this approach. An alternate strategy of protein engineering, therefore, has been attempted to improve the properties of xylanase and make it suitable for the factual conditions of industries. Significant work has been done to improve the properties of xylanase by implementing the directed evolution without compromising upon its catalytic action. A noteworthy enhancement has been achieved in thermal stabilization by attempting the combinatorial approach. When gene site-saturation mutagenesis (GSSM) was attempted along with gene-reassembly technology, a total of nine significant mutations were identified for the thermal stabilization. Therefore, all 512 possible combinatorial variants of nine mutations were screened that gave 11 variants whose denaturation temperature transition midpoints were improved from 61 to 96°C (Palackal et al. 2004). Due to less information on the catalytic mechanism of xylanase A of *Thermobifida fusca*, directed evolution was tried to stabilize the xylanase in alkaline conditions. Two rounds of DNA shuffling resulted in an efficient mutant with improved alkali stability. In addition, a 12-fold enhancement was also achieved in K_{cat}/K_m along with a fivefold decrease in K_m (Wang and Xia 2008). Another success was achieved by implementing three rounds of DNA shuffling. An improved mutant was obtained from xylanase B of *Streptomyces lividans* by substituting eight different amino acids whose individual effect was also investigated by site-directed mutagenesis. All of the eight amino acids were positioned at N-terminal region of the protein, which reflected a significant role of N-terminal region towards pH as well as thermal stabilization (Xia and Wang 2009). It was noticed that a single shot of directed evolution does not suffice to improve the properties of the proteins, but such repeated attempts are mostly successful to get the desired results. For instance, thermostability of an endoxylanase from *Thermomyces lanuginosus* could be enhanced by two rounds of random mutagenesis. In the first round, four mutants were selected having slightly better stability at 80°C as compared to the wild-type strain. All these four clones were further subjected to mutagenesis. Of these, three potent mutants were obtained with much enhanced thermostability. Among these, one mutant was the best, having a single mutation (Y58F) in β-sheet, and could retain 71% of its activity at 80°C for 1 h, with a half-life of 215 min at 70°C

(Stephens et al. 2007). The thermostability of a xylanase belonging to family 11 from *Bacillus subtilis* was improved by merging random point mutation along with saturation mutagenesis and DNA shuffling. A variant obtained with three unique mutations (Q7H, N8F, and S179C) exhibited an enhancement in temperature, leading to half inactivation from 58 to 68°C for 10 min duration. In this case, the directed evolution revealed that a bulky and hydrophobic cavity was the weak point cavity (Miyazaki et al. 2006). Although divalent ions play a significant role in thermal stabilization, their precipitation has deleterious effects on various downstream processing, particularly calcium-ion precipitation for beer industries (Haki and Rakshit 2003). Therefore, an attempt was made to obtain a calcium-ion-independent xylanase functional at extremely high temperatures. Thermostability of a xylanase from *Cellvibrio japonicus* has been enhanced by forced protein evolution. A mutant (D262N/A80T/R347C) was identified after three rounds of error-prone PCR, which was more thermostable in absence of calcium ions as compared to wild type. The cumulative effect of all three mutations resulted in an extremely useful mutant strain for industrial usage (Andrews et al. 2004).

31.9.5 Sequence Homology

Xylanases belong to glycosyl hydrolase families and categorized into families 5, 7, 8, 10, 11, and 43. Among all the families, 10 and 11 are more conserved and true xylanases. There is no stringent rule that restricts the particular class of xylanase to a specific microorganism; rather it diversifies the xylanases to a plethora of microbes including bacteria, fungi, algae, protozoa, and lower invertebrates such as arthropods and gastropods (Prade 1995). This is suggestive of horizontal as well as lateral gene transfer of xylanase genes. As mentioned earlier in this chapter, xylanase families F and G are now known as families 10 and 11, respectively (Henrissat and Bairoch 1993; Collins et al. 2005). Usually xylanases have been classified on the basis of amino acid sequences, but the contribution of hydrophobic cluster analysis is also noteworthy. The catalytic domain of XynA from *Glaciecola mesophila* KMM 241 shares a wide homology with family 10 xylanases on the basis of hydrophobic cluster analysis (Guo et al. 2009). The two different genes of xylanase from *B. firmus* have been cloned and sequenced. The proteins Xyl 10A and 11A encoded by the gene xyl10 A from *Bacillus firmus* gene showed sequence homology with alkaliphilic xylanase A of *Bacillus halodurans* (Chang et al. 2004). Likewise, two other xylanases isolated from *Paenibacillus sp*. were compared with preexisting available xylanases. Deduced amino acid sequence of XylA from *Paenibacillus sp*. indeed showed the expected homology with other bacterial xylanases. This family 11 xylanase showed maximum identity (83%) with XylA of *Aeromonas caviae*, about 80% identity with other *Bacillus* species and 57% with *Cellulomonas pachnodae* Xyn11A. The other xylanase from *Paenibacillus sp*. was recognized as XylB, which does not show any homology with XylA of *Paenibacillus sp*., while it shared maximum proximity (51%) with *Rhodothermus marinus*, 42% with

Pseudomonas fluorescens xynB, and about 14–30% sequence homology with corresponding amino acid sequences of *Streptomyces thermoviolaceus*, *Cellulomonas fimi*, *B. stearothermophilus*, and *C. pachnodae* (Lee et al. 2000). In another report, three different genes were cloned and sequenced from *Streptomyces lividans*. These genes were categorized into two groups: stx-I and stx-II, and stx-III. The amino acid sequence of stx-I and stx-II showed homology with the families F and G, while stx-III was not found similar to the expected xylanase family. Rather, it exhibited homology with acetyl xylan esterase and chitin deacetylase of *S. lividans* and *Mucor rouxii*, respectively. Their catalytic domains were highly conserved with their respective families. In addition, amino acid homology of stx-II and stx-III showed extensive homology (~98%) with each other at C termini, which is a highly conserved region for CBD II of xylanases, chitinases, and cellulases. Tsujibo et al. (1997) and Shareck et al. (1991) reported multiple genes of xylanases from *Streptomyces lividans*. The three genes xylA, xylB, and xylC belong to family F and G. The deduced amino acid of xylA showed homology with xylZ of *Clostridium thermocellum*; BACxyl of *Bacillus sp*. C-125; cex, a xylanase from *Cellulomonas fimi*; and xyl B of *Pseudomonas fluorescens* subsp. There was no homology of Xyl B/C with XylA, while noteworthy homology was observed between Xyl B and C. The amino acid alignments of xyl B and C shared maximum homology with bacterial xylanases of *S. lividans* sp. No. 36A, *Bacillus pumilus*, and *B. subtilis* as well as with fungal xylanases of *Trichoderma harzianium* and *Schizophyllum commune* (Shareck et al. 1991). Another gene of xylanase cloned from *Streptomyces sp*. strain EC3 belongs to glycanase C family and showed homology with other xylanases (Mazy-Servais et al. 1996). The cluster of xylanase genes (Xyl 10D, Xyl 10E, and Xyl 10B) was identified from *Fibrobacter succinogenes* S85. All the three genes were cloned and characterized for the amino acid homology. Analysis of sequence homology revealed that all of them belong to Xyl 10 family that has conserved N-terminal catalytic and C-terminal carbohydrate-binding domains (Jun 2003). A xylan-binding xylanase, Xyl 30, from *Streptomyces avermitilis* showed 83% homology with xylA of *Streptomyces coelicolor* A3(2) and *S. lividans*, and 79% with STX-I of *Streptomyces thermoviolaceus*. The deduced amino acids showed close proximity with family F/10 having highly conserved region from 165 to 170 and 271 to 281 amino acids. Besides, these three highly conserved histidine residues (His_{173}, His_{178}, and His_{249}) were also recognized from the multiple sequence alignment of the amino acids (Hernandez et al. 2008). A single domain xyl C was obtained from *Cellvibrio mixtus*. Its protein sequencing revealed a maximum homology of its amino acids (43%) with xyl A of *Bacteroides ovatus*, 39% with the enzyme of *P. bryantii* and 36% with xyl X of *Aeromonas caviae*. All these homologues are non-modular xylanases and belong to a subset of family 10 enzymes. Another xylanase (xyl G), which was used as a probe for the detection of xyl C of *Cellvibrio mixtus*, has also been recognized as a single domain xylanase of family 10 (Fontes et al. 2000). Amino acid sequence of xylanase A of *Bacillus sp*. 23 indicated it to be a member of family F or family 10. It has maximum resemblance with xylanase A of *Pseudomonas fluorescens*. In addition, the initial 40 amino acids showed a 48% identity with N-terminal sequence of Xyl B from *Pseudomonas fluorescens*. Other

than this, the xylA of *Bacillus sp*. 23 showed very poor homology with other xylanases of family F, namely, XynA of *Caldocellum saccharolyticum,* xylanase of *Bacillus* sp. strain C-125, and Xyn of *Cryptococcus albidus* (Blanco et al. 1995).

The property of multiplicity is very common among xylanases of both bacterial as well as fungal origin. *Streptomyces sp*. B-12-2, *Aspergillus niger*, and *Trichoderma viride* possess 5, 15, and 13 forms of xylanases, respectively (Biely et al. 1985; Elegir et al. 1994). A xylanase was obtained from *Bacillus halodurans* S7 with some unique and interesting properties. Although the enzyme belongs to family 10 xylanases that is not supposed to reflect the extreme properties of xylanases at high pH and temperatures, this enzyme was different, which made it a suitable candidate for industrial usage for prebleaching. ClustalW alignments showed its substantial amino acid homology with other family 10 xylanases, namely, Xyn10C from *C. japonicus*, Xyn-T6 of *G. stearothermophilus*, and XynA2 of *G. stearothermophilus* (Mamo et al. 2009). Another cluster of xylanases has been reported from *Caldicellulosiruptor sp.* strain Rt69B.1. Three family 10 xylanase (XynA, Xlnb, and XynC) and one family 11 xylanase (XynD) genes were obtained by genome-walking PCR. The full-length genes of XlnB and XlnC are the largest reported modular xylanases (5Kb) till now. The consensus stretch of XlnA, XlnB, and XlnC were 64–71% identical with each other. All these three genes shared 98% identity with *Caldicellulosiruptor sp.* isolate Rt69B.1 (Morris et al. 1999). An acidic xylanase reported from *Aspergillus kawachii* shared 40–50% identity with bacterial xylanases such as *Bacillus pumilus, Bacillus circulans*, and *C. acetobutylicum* while possessing quite less or negligible homology with fungal xylanases (Iwashita et al. 1998). An exceptional xylanase was reported from *Clostridium stercorarium* which has high molecular weight (~56 kDa) even it belongs to family 11 xylanase as it showed homology with other reported xylanases from family 11 which further validates the lateral transfer of genes of xylanases (Sakka et al. 1993).

Xylanases from *Streptomyces* are mainly endo-acting, which have been reported in *Streptomyces lividans*, *Streptomyces flavogriseus*, *Streptomyces olivaceoviridis*, *Streptomyces thermonitrificans*, and *Streptomyces cyaneus* (Srivastava et al. 1991; Wang and Xia 2007; Ninawe et al. 2008). The recombinant xylanase showed ~75.9% identity to the xylanase obtained from *Streptomyces* spzxy19. The xylanases of Streptomyces *olivaceoviridis* A1and *Streptomyces sp*. S27 possess a similar optimum temperature for their enzyme activity in acidic conditions (Li et al. 2009). Some fungal xylanases have amazingly been reported to show more sequence homology with bacterial xylanases as compared to the fungal ones, e.g., the enzyme of *Trichoderma reesei* was observed to be more homologous with bacterial family 11 xylanases in various aspects (Oku et al. 1993).

31.10 Industrial Applications of Xylanases

Microbial xylanases represent one of the largest groups of industrial enzymes, and they have attracted a great deal of attention during the past few decades. Their potential biotechnological applications in various industries include the food, feed, fuel, textile,

detergents, and paper and pulp industries as also in waste treatment (Beg et al. 2001; Nath and Rao 2000; Collins et al. 2005; Dhiman et al. 2008; Satyanarayana et al. 2012). Xylanases constitute the major commercial proportion of hemicellulases and have a worldwide market of around 200 million dollars (Katapodis et al. 2007). The sale trends are expected to increase owing to the fact that these enzymes have attracted increasing attention for their potential use in several new applications such as coffee extraction, preparation of soluble coffee, protoplasting of plant cells, and production of alkyl glycosides for use as surfactants and washing of precision devices and semiconductors. Some of the important biotechnological applications are discussed as follows.

The use of xylanases in pulp and paper industry has increased significantly with the discovery of Viikari et al. (1986). Since then, the researchers worldwide have focused their attention towards newer extremophilic microbial isolates, the xylanases from which can be used in the pulp and paper industries. The scientific interest in this field is reflected by the number of research papers published during recent years describing numerous xylanases from newer sources, as well as pulp bleaching experiments and protocols reported using various natural hemicellulases. The xylanases for various biotechnological applications have been reported mainly from bacteria (Gilbert and Hazlewood 1993; Ragauskas et al. 1994; Sunna and Antranikian 1997; Sharma et al. 2007; Ko et al. 2010; Kumar and Satyanarayana 2011), fungi (Sunna and Antranikian 1997; Chapla et al. 2011), actinomycetes (Ball and Mccarth 1989; Beg et al. 2000a; Nair et al. 2008; Kumar et al. 2012), and yeast (Hrmovai et al. 1984; Liu et al. 1998, 1999; Adsul et al. 2009). Over the years the number of possible applications of xylanases in the pulp and paper industry has increased steadily, and several have become commercially useful candidates. Currently, the most effective application of xylanase is in prebleaching of kraft pulp to minimize the use of harsh chemicals in the subsequent treatment stages of kraft pulp. While many applications of enzymes in paper industries are still in the research and developmental stage, several applications have found their way into the mills in an unprecedented short period of time in the last decade (Bajpai 1999).

31.10.1 Prebleaching of Kraft Pulps

In the paper and pulp industries, cellulase-free xylanases are extremely useful for prebleaching of kraft pulp (Bajpai 1999). In conventional papermaking processes, manufacturers use more quantity of chemicals, which has resulted in hazardous effluent disposal problems (Ayyachamy and Vatsala 2007). Treatment with xylanases facilitates the chemical extraction of lignin from pulp and helps in significant reduction of consumption of hazardous chemicals such as chlorine to achieve comparable levels of paper brightness while simultaneously preserving the important paper characteristics such as tensile strength, brightness, fibrillation, and drainage. For biobleaching applications, the candidate xylanase should be thermostable, alkalitolerant, and stable on kraft pulp. Its properties such as effective molecular weight, net ionic properties, and specific action pattern must suit the process requirements. Moreover, to

avoid damage to cellulose pulp, enzyme preparations should essentially be free from cellulase activity (Subramaniyan and Prema 2002; Damiano et al. 2003; Ayyachamy and Vatsala 2007; Sharma et al. 2007; Sudan and Bajaj 2007; Dhiman et al. 2008). Cellulase-free xylanases are also useful in yielding cellulose from dissolved pulps for rayon production and biobleaching of wood pulps (Bajpai et al. 1994; Viikari et al. 1992; Srinivasan and Rele 1999; Mamo et al. 2006). In recent years there have been so many published works where chlorine consumption was reduced up to 12–30% by using microbial xylanases without compromising with paper quality (Beg et al. 2001; Polizeli et al. 2005; Dhiman et al. 2008; Ko et al. 2010; Garg et al. 2011). For effective biobleaching some authors have suggested the use of other hemicellulases along with xylanases (Ahlawat et al. 2007; Dhiman et al. 2009; Kaur et al. 2010).

31.10.2 Use of Xylanases in Poultry

Depressions in weight gain and feed conversion efficiency in rye-fed broiler chicks was shown to be associated with its intestinal viscosity. Incorporation of xylanase into a rye-based diet of broiler chickens could result in reduced intestinal viscosity, thus improving both their body weight as well as feed conversion efficiency (Bedford and Classen 1992; Silversides et al. 2006; Bayram et al. 2008). Xylanase supplementation has alleviated the impairment of intestinal mucosal barrier induced by *C. perfringens* challenge (Liu et al. 2012).

31.10.3 Use of Xylanases in Bakery

The efficiency of xylanases in improving the quality of bread has been demonstrated along with an increase in specific bread volume. This is further enhanced when amylase is used in combination with xylanase (Maat et al. 1992; Beg et al. 2001; Courtin et al. 2006; Jiang et al. 2005; Shah et al. 2006; Butt et al. 2008). In situ enrichment of bread with arabinoxylan-oligosaccharides (AXOS) through enzymic degradation of wheat flour arabinoxylan (AX) by the hyperthermophilic xylanase B from *Thermotoga maritima* (rXTMB) during bread making was studied by Dornez et al. (2011). An enhanced production of AXOS along with increased loaf volume was recorded when the dough was supplemented with xylanases from *Bacillus subtilis* or *Pseudoalteromonas haloplanktis*.

31.10.4 Saccharification and Xylooligosaccharide Production

Xylan is present in large amounts in wastes from agricultural and food industries. Hence, xylanases are used for conversion of xylan into xylose from agricultural

waste. The development of an efficient process of enzymatic hydrolysis offers new prospects for treating hemicellulosic wastes (Biely 1985; Rani and Nand 1996; Heck et al. 2006; Mohana et al. 2008). There are several reports on the application of partially purified/purified β-xylosidase-free xylanase in releasing xylooligosaccharides from xylan-containing agroresidues (Adsul et al. 2009; Kumar and Satyanarayana 2011; Chapla et al. 2011; Verma and Satyanarayana 2012).

31.10.5 Xylanases in Agriculture

Xylanase treatment of plant cells can induce glycosylation and fatty acylation of phytosterols. Treatment of tobacco suspension cells (*Nicotiana tabacum* CV. KY 14) with a purified endoxylanase from *Trichoderma viride* caused a 13-fold increase in the levels of acylated sterol glycosides and elicited the synthesis of phytoalexins (Moreau et al. 1994). Some xylanases may be used to improve cell wall maceration for the production of plant protoplasts (Wong et al. 1988). A recent application of a truncated bacterial xylanase gene from *Clostridium thermocellum* has been demonstrated in rhizosecretion in transgenic tobacco plants (Borisjuk et al. 1999). Besides, forage crops may be pretreated to improve the digestibility of ruminant feeds and to facilitate composting (Gilbert and Hazlewood 1993).

31.10.6 In Breweries and Distilleries

Xylanases are used in conjunction with cellulases and pectinases for clarifying musts and juices and for uniform liquefaction of fruits and vegetables (Biely 1985). O-L-arabinofuranosidase and O-D-glucopyranosidase have been employed in food processing for aromatizing musts, wines, and fruit juices (Spagna et al. 1998).

31.10.7 Xylanases for Producing Effective Surfactants

Alkyl glycosides are one of the most promising candidates as new age surfactants. Commercially they are produced from monomeric sugars such as D-glucose and a fatty alcohol. But the direct glycosylation of polysaccharides is feasible for their industrial production, because by using this approach, hydrolysis of polysaccharides and subsequent steps may be omitted. Xylanases incorporated in this mixture enhance this process. Recently, xylanase from *Aureobasidium pullulans* has been used for direct transglycosylation of xylan, 1-octanal, and 2-ethyl hexanol into octyl-O-D-xylobioside, xyloside, and 2-ethylhexyl-O-D-xylobioside, respectively (Matsumara et al. 1999).

31.10.8 Biofuels

Xylanases in synergism with several other enzymes, such as mannanases, ligninases, xylosidases, glucanases, and glucosidases, can be used for the generation of biofuels, such as ethanol and xylitol, from lignocellulosic biomass (Kuhad and Singh 1993; Olsson and Hahn-Hagerdal 1996; Dominguez 1998). The biological process of ethanol fuel production requires delignification of lignocellulosics to liberate cellulose and hemicellulose from their complex with lignin, followed by depolymerization of the carbohydrate polymers (cellulose and hemicellulose) to produce free sugars, and finally fermentation of mixed pentose and hexose sugars to produce ethanol (Lee 1997).

31.10.9 In Degumming and Debarking

A potential application of the xylanolytic enzyme system in conjunction with the pectinolytic enzyme system is in the degumming of bast fibers such as flax, hemp, jute, and ramie (Puchart et al. 1999; Sharma 1987). A xylanase–pectinase combination is also used in the debarking process, which is the first step in wood processing (Wong and Saddler 1992; Bajpai 1999). The fiber liberation from plants is affected by retting, i.e., enzyme can be used for removal of binding material present in plant tissues using enzymes. Pectinases are believed to play the major role in this process, but xylanases may also be involved (Sharma 1987). Replacement of slow natural retting by treatment with artificial mixtures of enzymes holds good promise as the new fiber liberation technology in the near future (Bajpai 1999).

Very few bacterial strains are reported for the commercial production of xylanases like *Bacillus subtilis* (Khanongnuch et al. 1998) and *Streptomyces lividans* (Senior et al. 1992; Ragauska et al. 1994). Table 31.3 gives the comprehensive view of several commercially produced microbial xylanases (mostly fungal) finding potential applications in several industries along with their characteristics.

31.11 Future Perspectives and Conclusions

The global research efforts on microbial xylanases have suggested that it is not an easy task to get thermoalkalistable xylanases from mesophilic microbes for pulp bleaching. To get xylanases of requisite properties, isolation of xylanaolytic microbes from polyextremophilic conditions will be a better approach. Despite the fact that a good beginning has been made to improve the properties of xylanase by protein engineering, it is far from the target. There is a long way to go before it can be called a sure short tool for developing tailor-made enzymes to suit industrial applications. Another Pandora's box has just been opened that deals with cloning genes from the environmental DNA encoding the desirable enzymes. This approach

Table 31.3 Some of the commercially available xylanases and their characteristics along with potential application in biotechnology

Commercial name	Distributors	Microorganism	Fermentation	Optimal pH	Optimal temperature (°C)	Application
Allzym PT	Alltech	*Aspergillus niger*	SbmF	5.3	65	Animal feed improvement
Ammano 90	Amano Pharmaceutical		SSF wheat raw	4.5	50	Pharmaceutica analysis, food industry
Bio-Feed Plus	Novo Nordisk	*Humicola Insolens*	SbmF	n.c.	n.c.	Animal feed
Resinase	A/S	n.c.	n.c.	n.c.		Cellulose and paper industry
Bleachzyme	Biocon, India	n.c.	n.c.	6.5–7.0	40–50	
Cartazyme	Clariant, UK	*Thermomospora fuca*	n.c.	5.0	45–55	
EcopulpX-200	Primalco	*Trichoderma*	SbmF	5.0–6.0	50–55	Cellulose pulp bleaching
Ecosane	Biotec	*reesei*	sbmF	n.c.	n.c.	Animal feed
Ecozyme	Thomas Swan, UK	n.c.	n.c.	7.0	50	Cellulose and paper industry
Grindazym GP e GV	Danisco Ingredients	*A. niger*	SbmF	n.c.	n.c.	Bird and pig feed
Irgazyme 40	Nalco-Genencor, Ciba, -Geigy	*Trichoderma longibrachiatum*	SbmF	n.c.	n.c.	Paper industry and animal feed
Multifect XL	Genencor	*T. longibrachiatum*	SbmF	5.0–5.5	55–60	Food industry
Pulpzyme	Novozymes, Denmark	*Bacillus* sp.		9.5	50	Cellulose and paper industry
Solvay pentonase	Solvay Enzymes	*T. reesei*	SbmF	5.3–5.5	55	Starch and bread-making industries
Stremzym HC 46	SternEnzym		SSF	n.c.	n.c.	Bread-making
Sumizyme X	ShinNihon	*Trichoderma koningii*	SSF wheat raw	5.0	55	Manufacture of mushrooms and vegetables extracts, bread-making, enzymatic peeling of cereals, animal feed

(continued)

Table 31.3 (continued)

Commercial name	Distributors	Microorganism	Fermentation	Optimal pH	Optimal temperature (°C)	Application
Xylanase	Seikagaku	*Trichoderma* sp.	SbmF	n.c.	n.c.	Carbohydrate structural studies
Xylanase	Granotec do Brasil	n.c.	n.c.	n.c.	n.c.	Weight decreasing in Cream-Crackers, better texture and taste, Wafer's uniformity improvement
Xylanase GS35	Iogen	*T. reesei*	SbmF	4.5	40	Cellulose pulp bleaching, animal feed
Biobrite		n.c.	n.c.	5.0–6.0	55	Cellulose and paper industry

n.c. stands for Not checked

opens the door for obtaining novel genes from the inaccessible 99% of the unculturable microorganisms present in the environment. Very few xylanases have been recovered by the metagenomic approaches, and therefore, further research efforts are needed in this direction.

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Chapter 32
Thermostable Proteases

Rajeshwari Sinha and Sunil K. Khare

Abstract Proteases are one of the largest selling enzymes in the world. This is rationalised by their extensive usage in the detergent, food, pharmaceutical, leather and textile industries. Thermostability in industrial enzymes remains a desirable attributes for (1) achieving faster conversion rates, (2) greater catalytic efficiencies and (3) protection from microbial contamination while operating at higher temperatures. Proteases endowed with such characteristics are all the more needed for baking and textile processing. In general, most of the industrial proteases are sourced from *Bacillus* sp. Thermostability in protease is accorded by protein engineering or appropriate immobilisation methods. Proteases from hyperthermophiles and thermophiles are natural choice for exploring the inherent heat stability. Few classical thermostable proteases especially those from *Pyrococcus* and *Thermococcus* have generated considerable interest. Heat stability in these cases has been attributed to large proportion of hydrophobic residue, extensive hydrogen bonding and increased share of disulphide bonds. Extensive screening of large range of unexplored thermophiles is well called for. Understanding their protein architecture may enable rationale design for heat-stable proteases in future.

This chapter highlights the enzymatic characteristics and novel properties of known thermostable proteases and focuses on their structure–function relationship. Recent developments and future perspectives in screening new proteases from hyperthermophiles/thermophiles, metagenomic studies, directed evolution, site-directed mutagenesis, modern immobilisation methods such as CLEC, CLEA and PCMC and immobilisation on nanoparticles are comprehensively covered.

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32.1 Introduction

Life on the earth exists in various ecological niches spanning conditions ranging from extremes of temperature, pH, salinity, pressure and toxicants to various other factors. Microbial life has shown an immense ability to adapt to such environments, well beyond the familiar mesophilic envelope. Such organisms have been termed 'extremophiles'. The understanding about extremophilic diversity has improved considerably over the past two decades.

Temperature is an important physical parameter that sets a line of demarcation between mesophiles and those grouped under extremophiles. Microorganisms that thrive in environments ranging from moderate to very hot terrestrial habitats such as geysers, hot springs, volcanic eruptions or in short 'love' heat are further subgrouped as 'thermophiles'. They require elevated temperatures (>50°C) for growth and sustenance (Gottschal and Prins 1991).

For inhabiting such high temperatures, these organisms suitably adapt a thermostable matrix within which important biochemical reactions, cellular activities and physiological functions take place. Existence of such heat-stable cells raises questions pertaining to the inherent thermal stability of their biomolecules, their enzymes in particular and their evolutionary origin. It is suggested that the intricate strategies for thermoprotection adapted by thermophilic cells have originated during course of microbial evolution which eventually becomes dependent on the extreme living conditions in which they survive (Daniel and Cowan 2000). The molecular basis of heat resistance is still not very clear. Though the thermal profiles of major cell components have been thoroughly investigated over a period of time, yet smaller molecules, metabolites or coenzymes have evaded the attention.

32.2 Thermozymes

Enzymes which have been found from thermophilic or hyperthermophilic microbes have been termed as 'thermozymes'. With the discovery of life existing at higher temperatures, way back in 1960s (Brock 1967, 1985), the study of thermophiles and related enzymes has become a major domain for research. Thermozymes are unique in their ability to retain optimal biocatalytic activity at very high temperatures (ranging from 60°C to about 120°C). They also exhibit increased resistance to irreversible denaturation by chemical reagents, detergents, chaotropic agents, organic solvents and extreme pH values (Bruins et al. 2001). However, it is known that their catalytic mechanisms do not differ much from their mesophilic counterparts. Structural

comparisons between mesophilic and thermophilic enzymes have indicated numerous modes of protein stabilisation in the latter. A greater conformational stability clubbed with stabilisation strategies involving hydrophobic interactions, ionic interactions and higher packing efficiency impart compatibility with the existing high temperature environments in these enzymes.

Thermophiles and hyperthermophiles thus present an obvious source of robust thermostable catalysts capable of functioning under high temperatures. Several important enzymes have been isolated from hyperthermophilic sources like *Pyrococcus furiosus* and *Thermotoga* sp. (Adams et al. 1995; Fischer et al. 1996; Adams and Kelly 1998) and thermophilic ones like *Bacillus* sp., *Clostridium* sp. and *Thermus* sp. (Zentgraf 1992).

Other than sourcing heat-stable enzymes from thermophilic/hyperthermophilic microorganisms, several approaches have been adopted to impart stability to enzymes from mesophilic origin. It is relevant to briefly discuss some commonly employed techniques which include chemical modification, immobilisation, protein engineering and directed evolution.

- Immobilisation of enzymes onto solid supports is one of the most widely accepted modes of enzyme stabilisation. It has been stated that apart from stabilisation, immobilisation also renders a better product separation, lesser chances of product contamination and efficient recovery and reusability of enzymes. It also enhances protein thermostability, storage stability and stability against denaturation. Immobilisation has been extensively used to produce stable proteases (Guisán et al. 1991, 1997; Filippova and Lysogorskaia 2003; Lei et al. 2004). Encapsulation, entrapment and covalent coupling are some of the immobilisation methods reported to have dramatically increased the thermal stability of the enzyme post their immobilisation.
- Cross-linked enzyme crystals (CLEC) are reportedly novel biocatalyst preparations designed by enzyme crystallisation followed by molecular cross-linking with glutaraldehyde (Häring and Schreier 1999; Abraham et al. 2004). This imparts extreme stability to protein structure with respect to temperature, organic solvents, resistance to proteolytic action and higher enzyme activity over free enzyme. CLEC of subtilisin have been reported to exhibit increased thermostability and have been used advantageously in organic synthesis (Noritomi et al. 1998; Fernandes et al. 2005).
- The process of enzyme crystallisation involved in CLEC formation is laborious and cost-intensive. Hence, newer approaches towards development of cross-linked enzyme aggregates (CLEA) are being considered over CLEC (Gupta and Raghava 2011). CLEAs are produced by simple precipitation of the enzyme from the aqueous solution upon addition of salt or organic solvents followed by cross-linking (López-Serrano et al. 2002; Shah et al. 2006; Gaur et al. 2006). Throughout the process the enzyme activity is retained simultaneously. They are thus inexpensive, exhibit improved stability, better enzymatic performance and may be reused when required. Protease CLEAs are predicted to be promising tools for peptide synthesis in future.

- Over the last decade, the application of nanoparticles for immobilisation of enzymes has gained significant attention. Gold and titanium dioxide nanoparticles in association with proteins, peptides, enzymes, antibodies and nucleic acids are being harnessed as biocatalysts in biochemical processes. The assembly of TiO_2 nanoparticles as templates for immobilisation of protease enzyme has been reported very recently (Sadjadi et al. 2009). The immobilised enzyme showed significant increase in thermal and pH stability. Conjugation of trypsin on magnetic nanoparticles (MNP) of Fe_3O_4 was carried out, and the enzyme was assessed in terms of activity, kinetics, thermal stability and reusability (Li et al. 2010). Its potential application in proteomics and miniaturised analytical devices is suggested to be promising. *B. subtilis* keratinase immobilised onto PEG-supported MNP resulted in fourfold increase in activity and significantly enhanced thermal stability, storage stability and recyclability (Konwarh et al. 2009).
- Protein-coated microcrystals (PCMC) have proved to be another very recent and efficient mode of immobilisation. Such microcrystals are formed by simultaneous precipitation and immobilisation on the microcrystal surface (Kreiner and Parker 2005). Protein-coated microcrystals of *Pseudomonas aeruginosa* lipase have been prepared by immobilisation of the protein (Gaur et al. 2008). Applications of proteins stabilised by PCMC for biocatalysis in organic solvents have been reported (Shah et al. 2008).
- Directed evolution or random mutagenesis is coming up as a powerful approach for generating a large library of variants of thermally stable enzymes. Abrahmsen et al. (1991) have reported engineering of the protease subtilisin and its substrates conveniently for peptide bond formation in aqueous solution. The organic solvent stability of the PST-01 protease from *P. aeruginosa* was enhanced by the introduction of a disulphide bond between two cysteine residues (Ogino et al. 2001). Random mutagenesis to suitably improve the thermal stability of subtilisin has also been reported (Adamczak and Hari Krishna 2004; Takagi et al. 2000).

32.3 Industrial Relevance of Thermozymes

Thermophilic enzymes demand more attention in current scenario because they serve as appropriate tools for development of commercial biotechnology, as well as provide model systems to study protein stability. The major advantages of performing industrial processes at elevated temperatures include reduced risk of microbial contamination, faster reaction rates, lowered viscosity and increased substrate solubility. Pathogenic bacteria are most likely to succumb under temperatures higher than 65°C, thereby reducing the chances of contamination in industrial food processes (Haki and Rakshit 2003). Further, a lowered viscosity under high temperature implies reduced costs of pumping, filtration and centrifugation and reduced usage of water levels. Also, an increased temperature promotes increase in substrate solubility, which, in turn shifts the equilibrium for a higher yield of products (Zeikus 1996).

Enzymatic processes at higher temperatures have their own share of drawbacks. Occurrence of side reactions, by-product formation, substrate or product instability and loss of selectivity are some such factors which cannot be ruled out (Peek et al. 1992). Nevertheless, the numerous advantages surmount such drawbacks arising only in few cases.

Heat-stable proteases are required for industrial processes involving high temperatures above 50°C. For enzymatic processes involving higher operational temperature, the thermal stability of the biocatalyst becomes the core issue of biotechnology. The prime reason behind the choice of thermophilic enzymes in high temperature processes is their heat stability. The heat-stable DNA polymerase from *Thermus aquaticus*, a thermophilic bacterium (Brock and Freeze 1969), popularly known as *Taq* has gained prominence for materialising the powerful technique of PCR. Some more polymerases like those from *Thermococcus litoralis* and other thermophiles are now dominating the market, and their usage are envisaged to increase in the future. Thermozymes are also expected to play an important role in paper, pulp, pharmaceutical, food, chemical and waste treatment industries. It is believed that the discovery of new thermophilic enzymes will subsequently replace their mesophilic counterparts in the industrial arena.

32.4 Thermostable Proteolytic Enzymes

The global market of industrial enzymes was assessed to be around two billion US$ in 2004 which might have increased many fold by now. Hydrolytic enzymes like proteases, lipases, amylases and xylanases comprise about 52% of the world market. The quantum of proteolytic enzymes produced on commercial scale is larger than any other industrial enzyme. Biotechnological applications of proteases have been extensively renewed from time to time (Gupta et al. 2002; Kumar and Takagi 1999).

Proteases are essential for all forms of life on earth including prokaryotes, fungi, plants and animals. They catalyse hydrolysis of proteins into smaller peptides or free amino acids. They have been categorised as serine, cysteine, aspartic or metalloproteases. Microbial proteases are of greater interest because of their versatile characteristics and diverse applications in food, feed, dairy, textile, biomedical and pharmaceutical industries, waste management in tannery, silver recovery and detergent formulation. The success of microbial proteases may be owed to their vast occurrence among diverse microorganisms, ease of genetic manipulation and improvised methods of enzyme production, purification and characterisation.

32.5 Sources of Thermostable Proteases

Endo (1962) was the first to characterise a thermostable protease, thermolysin, from *Bacillus thermoproteolyticus*. Since then, varieties of thermoactive proteolytic enzymes have been identified from different classes of microorganisms.

Some interesting proteases have been identified from thermophilic archaea, belonging to the genera *Pyrococcus*, *Thermococcus*, *Staphylothermus*, *Desulfurococcus*, *Pyrobaculum* and *Sulfolobus*. Proteases from archaea have been extremely stable and their optimum activities varied in the range of 90 and 110°C. Records of extremely thermostable serine proteases, produced by the hyperthermophilic archaeon *Desulfurococcus* strain (Hanzawa et al. 1996), are also available.

Thermophilic fungi namely *Achaetomium*, *Chaetomium*, *Penicillium*, *Rhizopus*, *Sporotrichum*, *Torula* and *Rhizomucor* have also been reported to produce novel thermostable protease. Some of these are characterised by higher thermal stability and higher reaction rates. One of the most thermostable fungal acid proteases was detected in a *Penicillium duponti* K1014, isolated from compost (Emi et al. 1976; Hashimoto et al.. 1973). On the contrary, alkaline thermostable proteases are reported from *Malbranchea pulchella* var. *sulfurea* and *Humicola lanuginosa* (Ong and Gaucher 1973, 1976).

Among bacteria, *Bacillus* sp. has been a major source of heat-stable proteases. The earliest isolate belonged to *Bacillus stearothermophilus* (Salleh et al. 1977). Proteolytic enzymes from different strains of *Bacillus stearothermophilus* differ in their heat stability, e.g. protease from one of the *Bacillus stearothermophilus* sp. was found to be optimally active at 85°C (Rahman et al. 1994) while that from *Bacillus stearothermophilus* TP26 was active at 75°C (Gey and Unger 1995). Alkaliphilic *Bacillus* sp. JB-99 produced thermostable alkaline proteases (Johnevelsy and Naik 2001). An elaborate list about more proteases from above-described domain of archaea, fungi and bacteria with details about thermal stability and other properties is discussed later in this chapter.

32.6 Purification and Characterisation of Thermostable Proteases

It is quite evident that most thermostable proteases, intracellular or secreted extracellularly, are of the serine type. Heat-stable serine proteases have been reported from hyperthermophilic archaeon *Desulfurococcus* strain SY, having a half-life of 4.3 h at 95°C (Hanzawa et al. 1996), *Desulfurococcus* strain Tok12S1 (Cowan et al. 1987), thermoacidophilic *S. solfataricus* (Burlini et al. 1992), *S. acidocaldarius* (Fusek et al. 1990; Lin and Tang 1990), *Staphylothermus marinus* (Mayer et al. 1996) and *P. furiosus* (Eggen et al. 1990). Extracellular serine proteases from a number of *Thermococcus* species have been studied (Klingeberg et al. 1991). The proteinase from *T. stetteri* is characterised by unique resistance to chemical denaturation and retains 70% activity in the presence of 1% sodium dodecylsulphate (Klingeberg et al. 1995). A gene encoding a subtilisin-like serine protease, named aereolysin, has been cloned from *Pyrobaculum aerophilum* (VolkI et al. 1995).

In addition to the serine proteases, other types of enzymes have been identified as thiol proteases from *Pyrococcus* sp. KOD1 (Morikawa et al. 1994), a propyl

peptidase and a new type of protease from hyperthermophilic archaeon *P. furiosus* (Halio et al. 1996; Harwood et al. 1997). The purification methods and characteristic properties of some of the interesting thermophilic proteases are summarised in Table 32.1.

For the purification of thermostable proteases, ion-exchange chromatography followed by gel permeation chromatography has been most commonly used. Simple chromatography using anionic exchanger DEAE-cellulose followed by gel permeation chromatography on a range of matrices like Sephadex G75, G100, G150 and G200 or Sephacryl S200 and Sephacryl S-100HR has successfully eluted out the purified protease. In addition to these, affinity chromatography using bacitracin–Sepharose and HgPhNH-Sepharose has been a method of choice for efficient purification of protease (Manikandan et al. 2009; Zhu et al. 2007; Emi et al. 1976, Shenolikar and Stevenson 1982).

Purification using advanced methods such as FPLC and HPLC has also been attempted lately. The purification of a thermostable thiol protease from *Pyrococcus* sp. KOD1 using gel permeation chromatography followed by FPLC and hydroxyapatite chromatography has been reported (Morikawa et al. 1994). Likewise, three thermophilic proteases of *B. stearothermophilus* TSL33 have been purified from each other using lysine-affinity chromatography, anion-exchange (Q Hyper D matrix) and gel permeation (Ultrogel AcA44) chromatography (Sookkheo et al. 2000).

The optimum temperature of the purified thermostable proteases lies in the range of 50–85°C. However, an exception to the above generic trend has been observed in case of proteases from *Pyrococcus* sp. origin. The protease from *Pyrococcus abyssi* strain st 549 shows a temperature optimum of 95°C, while that from *Pyrococcus* sp. KOD1 has an optimum temperature of 110°C (Dib et al. 1998; Morikawa et al. 1994).

The optimum pH of most of the thermophilic proteases is found to be in the range of 6.0–12.0. The protease from *Thermoascus aurantiacus* shows pH optimum of about 5.5 (Merheb et al. 2007). A pH optimum of 2.5 has been reported in case of protease from *Penicillium duponti* K1014 (Emi et al. 1976). Interestingly, another protease from *Sulfolobus acidocaldarius* has lower pH optimum of 2.0, while its temperature optimum goes as high as 90°C (Lin and Tang 1990).

The molecular weights of the purified proteases vary in the range of 45–80 kDa. Surprisingly, thermostable proteases derived from *Bacillus* sp. have lower molecular weights in the range of 20–30 kDa. One of the three proteases purified from *B. stearothermophilus* TSL33 is reported to have a molecular weight of 36 kDa (Sookkheo et al. 2000). The protease from *B. subtilis* PE-11 is documented to have a molecular weight as low as 15 kDa (Adinarayana et al. 2003). Proteases from *Bacillus polymyxa* B-17, *Bacillus pumilus* MK6-5 and *B. licheniformis* LHSB-05 have similar observations (Matta and Punj 1998; Kumar et al. 1999; Olajuyigbe and Kolawole 2011). Thermophilic proteases, with higher molecular mass, are also encountered. For example, protease with a molecular mass of 86 kDa from *Halogeometricum borinquense* strain TSS101 and thermostable keratinase with

Table 32.1 Purification strategies and enzymatic properties of some novel thermostable proteases

Source	Type of protease	Purification procedure	Enzyme properties			References
			Temp. optima (°C)	pH optima	Molecular weight (kDa)	
Talaromyces duponti	Aminopeptidase	Sephadex G-150, DEAE Sephadex A-50 gel permeation chromatography	60	6.9		Chapuis and Zuber (1970)
Malbranchea pulchella var. *sulfurea*	Alkaline	DEAE Sephadex affinity chromatography using Sepharose-4 phenyl butyl amine	45	8.5		Ong and Gaucher (1973)
Torula thermophila	Alkaline	Sephadex G-100 gel permeation chromatography	70	8.0		Karavaeva et al. (1975)
Penicillium duponti K1014	Acid	Ion-exchange using DEAE Sephadex A-50 and gel permeation chromatography	30	2.5		Emi et al. (1976)
Sporotrichum pulverulentum		DEAE Sephadex A-50 and gel permeation chromatography	30	5.0		Erikson and Petterson (1982)
Humicola lanuginose	Thiol	Affinity chromatography on HgPhNH-Sepharose, Sephadex G-150 gel permeation chromatography	30	8.0		Shenolikar and Stevenson (1982)
Thermoactinomyces thalpophilus		DEAE-cellulose ion-exchange chromatography and Sephadex G-150 gel permeation chromatography	70	6.0	55.0	Odibo and Obi (1988)
Sulfolobus acidocaldarius	Acid	DEAE–Sepharose CL-GB, phenyl-Sepharose CL-4B, Sephadex G-100, monoQ on FPLC and gel permeation chromatography on HPLC	90	2.0	45.0	Lin and Tang (1990)
Thermococcus litoralis	Serine		85	8.5		Klingeberg et al. (1991)
Thermus aquaticus YT1		Ion-exchange and affinity chromatography	80	10.0	28.0	Green et al. (1993)

Bacillus stearothermophilus			85			Rahman et al. (1994)
Chloroflexus aurantiacus J-10-fl	Metalloprotease	Butyl-Toyopearl 650 M chromatography		8.0	66.0	Watanabe et al. (1993)
Pyrococcus sp. KOD1	Thiol	Ammonium sulphate fractionation, gel filtration chromatography on TSKgel Toyopearl HW-55F column, FPLC with Superose 12, and hydroxyapatite chromatography	110	7.0	44.0	Morikawa et al. (1994)
Bacillus stearothermophilus TP26			75			Gey and Unger (1995)
Staphylothermus marinus	Serine			9.0		Mayer et al. (1996)
Thermomyces lanuginosus (2 proteases)			70	5.0 and 9.0		Li et al. (1997)
Bacillus brevis			60	10.5		Banerjee et al. (1999)
Pyrococcus abyssi, strain st 549	Serine	DEAE and SP Trisacryl M resin on FPLC	95	9.0	60.0	Dib et al. (1998)
Bacillus licheniformis			70	9.0		Manchini and Foretina (1998)
Bacillus polymyxa B-17		Ammonium sulphate fractionation, Sephadex G-100 gel permeation chromatography	50	7.5	30.0	Matta and Punj (1998)
Bacillus pumilus MK6-5		Ammonium sulphate fractionation, ion-exchange and gel filtration chromatography	55–60	11.5	28.0	Kumar et al. (1999)

(continued)

Table 32.1 (continued)

Source	Type of protease	Purification procedure	Enzyme properties			References
			Temp. optima (°C)	pH optima	Molecular weight (kDa)	
Bacillus stearo thermophilus TLS33 (3 proteases: S,N,B)		Lysine-affinity chromatography, anion-exchange Q HyperD chromatography, and Ultrogel AcA44 gel permeation chromatography	70, 85, and 90 for S, N, B, respectively	8.5, 7.5, and 7.0	36.0, 53.0, and 71.0	Sookkheo et al. (2000)
Bacillus sp. JB-99			80	6.0–12.0		Johnevelsy and Naik (2001)
Bacillus subtilis PE-11		Ammonium sulphate fractionation and Sephadex G-200 gel permeation chromatography	60	10.0	15.0	Adinarayana et al. (2003)
Thermophilic bacillus strain HS08		Ammonium sulphate fractionation, DEAE–Sepharose chromatography and Sephacryl S-100HR gel permeation chromatography	65	7.5	30.9	Guangrong et al. (2006)
Halogeometricum borinquense strain TSS101		Ethanol precipitation, Sephacryl-S and Sephadex G-75 gel permeation chromatography	60	10.0	86.0	Vidyasagar et al. (2006)
Chaetomium thermophilum (2 proteases)		Ammonium sulphate fractionation, DEAE–Sepharose ion-exchange chromatography and phenyl-Sepharose hydrophobic interaction chromatography	65	10.0 and 5.0	33.0 and 63.0	Li et al. (2007)
Thermoascus aurantiacus			60	5.5		Merheb et al. (2007)
Geobacillus sp. YMTC 1049		DEAE Sephadex-A50, Sephadex G-75 gel permeation chromatography	85	7.5	56.5	Zhu et al. (2007)

Bacillus sp. HUTBS71		Ammonium sulphate fractionation, Sephadex G-100 gel permeation chromatography and DEAE-cellulose ion-exchange chromatography	65	7.8	49.0	Akel et al. (2009)
Haloferax lucentensis VKMM 007		Bacitracin–Sepharose affinity chromatography and Sephadex G-100 gel permeation chromatography	60	8.0	57.8	Manikandan et al. (2009)
Bacillus licheniformis Lbbl-11			60	8.0		Olajuyigbe and Ajele (2008)
Streptomyces fungicidicus MML1614	Alkaline		40	9.0		Ramesh et al. (2009)
Bacillus Subtilis (KHS-1)		Ammonium sulphate fractionation and Sephadex G-200 gel permeation chromatography	60	10.5	20.5	Ramakrishna et al. (2010)
Bacillus halodurans JB99	Serine	Ammonium sulphate fractionation, CM cellulose chromatography and Sephadex G-100 gel permeation chromatography	70	11.0	29	Shrinivas and Naik (2011)
Lactobacillus brevis		Ammonium sulphate fractionation and Sephadex G-150 gel permeation chromatography	60	9.0	33.2	Femi-Ola and Oladokun (2012)

molecular mass of 76 kDa from *Meiothermus* sp. I40 have been reported (Vidyasagar et al. 2006; Kuo et al. 2012).

Proteases from mesophilic sources start losing activity beyond 45–50°C in general. The stability in thermostable proteases starts from 50°C onward, the average being 60°C. The thermal stability of some typical proteases along with pH influence is summarised in Table 32.2.

Apparently, the thermostable proteases from *Bacillus* sp. are generally stable up to 60°C. The proteases belonging to the actinomycetes *Streptomyces* sp. are more stable, reaching to the temperature of 70°C. *Thermococcus* spp. touch the range of 80–85°C (Klingeberg et al. 1991). The exceptional stabilities of hyperthermophilic archaea are worth highlighting, for instance, a *Pyrococcus* sp. KOD1 isolated by Morikawa et al. (1994) from Kodakara Island, Japan, exhibited stability well beyond 90°C, with a half-life of 1 h at 100°C. Extracellular protease secreted from psychotrophic bacteria *Pseudomonas fluorescens* BJ-10 showed heat stability. It was reported to retain more than 94% activity even after treatment at 100°C for 3 min (Zhang and Lv 2012). The basis of thermal stability in thermophilic bacteria such as *Bacillus stearothernophilus* and others is generally attributed to extensive hydrogen bonds, ionic bonds, hydrophobic bonds, and disulphide bonds to some extent. This aspect is further elaborated in next section.

32.7 Structural Basis of Thermal Stability

Over the decades, there has been a considerable amount of research towards understanding the stability of thermophilic enzymes. However, an established concept as to how this stability is achieved and maintained still remains elusive.

Combinations of various structural parameters play a pivotal role in imparting thermal stability to an enzyme. Some of the basic mechanisms include improved packing efficiency, increased ionic interactions, decrease in amount of available hydrophobic surface area, loop/helix stabilisation, alterations in amino acid sequences of proteins and hydrogen bonds, reduction in conformational strain, extended secondary structure and greater rigidity in subunit assembly or oligomerisation. The following section discusses the generic adaptive features observed in thermostable enzymes, including those in proteases.

32.7.1 Hydrophobic Interactions

Hydrophobic interactions are major driving forces in molecular folding and thermostability of proteins (Goodenough and Jenkins 1991). Total hydrophobicity of a protein is directly correlated with thermostability in some cases. The concept of entropy–enthalpy compensation has been aptly used to describe the role of increased hydrophobicity in thermostabilisation (Daniel et al. 2008).

Table 32.2 pH and temperature stability of some thermostable proteases

Source	Thermal stability	pH stability	Reference
Pyrococcus sp. KOD1	Significant thermal stability up to 90°C Half-life (100°C): 1 h		Morikawa et al. (1994)
Thermomyces lanuginosus	Fully stable at 50°C Half-life (60°C): 160 min (70°C): 60 min	Stable over a broad pH range of 4.0–11.0	Li et al. (1997)
Bacillus polymyxa B-17	Retained 35% activity at 70°C for 10 min	Active over a wide pH range of 5.5–10.0	Matta and Punj (1998)
Bacillus brevis	Stable at 25°C for 288 h Half-life (50°C):60 h (60°C):7 h	Stable in pH range 10.0–12.0	Banerjee et al. (1999)
Alkalophilic *Bacillus* spp.	Stable up to 50°C Half-life (50°C):50 and 40 min for proteases AP1 and AP2	Stable in the pH range of 6.0–12.0	Kumar et al. (1999)
Bacillus sp.JB-99	Stable up to 80°C	Active in the pH range 8.0–12.0	Johnevelsy and Naik (2001)
Streptomyces tendae	Stable at 55°C, retained 70% activity after 30 min at 60°C	Stable in the pH range 4.0–9.0	Seong et al. (2004)
Thermoascus aurantiacus	Quite stable at 60°C. Activity lost within 1 h at 60°C.	Stable in pH range 3.0–9.5	Merheb et al. (2007)
Geobacillus sp. YMTC 1049	Stable up to 65°C. No loss in activity up to 10 h at 65°C	High activity at pH range 6.0–9.0	Zhu et al.(2007)
Streptomyces fungicidicus MML1614	Stable up to 60°C	Stable up to pH 11.0	Ramesh et al. (2009)
Streptomyces clavuligerus strain Mit-1	Temperature optimum: 70°C thermally stable at 60–80°C	Stable in pH range 8.5–11.0	Thumar and Singh (2007)
Haloferax lucentensis VKMM007	Stable over a temperature range of 20–70°C with maximum stability at 60°C	Stable in pH range 7.0–10.0	Manikandan et al. (2009)

(continued)

Table 32.2 (continued)

Source	Thermal stability	pH stability	Reference
Aeromonas veronii PG01	Stable up to 60°C, while only about 53% of maximum activity was observed at 70°C	Stable in pH range 6.0–10.0	Divakar et al. (2010)
Streptomyces sp. strain AB1	Stable up to 60°C	Stable in pH range 6.0–12.0	Jaouadi et al. (2010)
Oceanobacillus theyensis O. M. A_l8	Stable in the range of 60–90°C for 24 h	–	Purohit and Singh (2011)
Haloalkaliphilic bacterium O. M. E_l2	Retained 50% of activity after 3 h at 60°C		
Meiothermus sp. I40	Retained 96.7% and 71.3% activity when incubated at 65°C and 70°C, respectively, for 12 h	Demonstrates more than 60 % of maximum activity in the pH range of 5.0–9.0	Kuo et al. (2012)

32.7.2 Packing Density

The strength of hydrophobic interactions which are known to stabilise thermophilic proteins originates from van der Waals' interactions between hydrophobic groups buried within the protein core. It may therefore be presumed that increased packing of a protein with hydrophobic amino acids may lead to better van der Waals' interactions and enhanced thermal stability. It is also suggested that increased amount of alanine, proline or isoleucine is functional in providing tighter packing in hydrophobic interiors (Russell et al. 1997). The compactness of thermophilic proteins may be further enhanced by shortening of loops, optimised packing of side chains in the interior and elimination of unnecessary cavities (Corazza et al. 2006).

32.7.3 Increased Stabilising Interactions

While disulphide bridges are reported to stabilise the protein by enhancing conformational stability, hydrogen bonds at the same time stabilise by forming intramolecular interactions (Nosoh and Sekiguchi 1990).

Tanner et al. (1996) observed that these H-bonds were favoured over salt bridges. The stabilising effect of disulphide bridges is confirmed by the many mutagenesis studies involving the introduction of disulphide bonds in enzymes (Li et al. 2005). The role of optimised electrostatic interactions by increasing of the number of salt bridges has also been demonstrated to enhance thermostability in case of hyperthermophilic microorganisms (Karshikoff and Ladenstein 2001).

Liang et al. (2005) have reported that the amounts of Glu, Lys and Arg are higher in thermophilic proteins than in their mesophilic counterparts. This increase in charged residues suggests that there are more salt bridges in thermophile proteins as compared to corresponding mesophile proteins (Das and Gerstein 2000). Ion pairs are organised in large networks on protein surfaces. Salt bridges and ionic interactions thus assume prime importance towards enhancement of stability of proteins.

32.7.4 Increased Rigidity and Lesser Flexibility

Thermozymes are generally more rigid than mesozymes. Overall enzyme rigidity increases through multiple factors as α-helix stabilisation, electrostatic interaction optimisation, conformational strain reduction etc. This increased rigidity is essential for preserving their catalytically active structure at elevated temperature. Enhanced rigidity is demonstrated by lower susceptibility to proteolytic degradation and chemical denaturants and reduced thermally induced unfolding (Bruins et al. 2001).

32.7.5 α-Helix Stabilisation

Enhanced helix stabilisation also contributes to thermostability in many ways. α-Helix stabilisation is achieved by substituting low helical propensity residues with high helical propensity residues. A comparison of the X-ray structures of thirteen thermophilic proteins with their mesophilic homologues revealed that the β-branched residues (Val, Ileu, Thr) were conspicuously lacking in α-helix of thermostable proteins.

32.7.6 Amino Acid Exchanges

Sequence comparison between thermophilic and mesophilic enzymes has revealed many significant substitutions in thermophilic enzymes, viz. Asp to Glu, Lys to Arg, Ser to Ala, Gly to Ala, Ser to Thr and Val to Ileu (Argos et al. 1979). These amino acid substitutions increase the internal hydrophobicity. The decrease in protein flexibility and the increased hydrophobicity by means of amino acid substitutions is further confirmed to be the major driving force in achieving thermal stability. Another advantage of the amino acid exchanges could be the facilitation of more hydrogen bonds and salt link formation.

Interestingly, comparison of the thermodynamic and kinetic parameters of protein folding among mesophiles, thermophiles and hyperthermophiles also provides significant insight into thermophilic protein stabilisation strategies. Three thermodynamic models have been proposed to explain the higher stability of thermostable proteins (Nojima et al. 1977; Jaenicke and Bohm 1998).

According to the first model, the thermostable protein would be more thermodynamically stable throughout the temperature range as compared to mesophilic proteins.

The second model proposes that the free-energy profile of the thermostable protein would be horizontally displaced to higher temperatures. The maximum value for ΔG_U would be equal for both thermophilic and mesophilic proteins, but the maxima would appear at different temperatures. At high temperatures, the thermostable protein would be more stable, while at lower temperatures, stability of the mesophilic protein predominates.

A flattening of the free-energy profile for the thermostable protein in comparison to that of the protein from the mesophile becomes evident in the third model. Support for all three models, and combinations thereof, has been reported for different thermostable proteins (Razvi and Scholtz 2006; Kumar et al. 2001).

Recent understandings of thermostability of proteins from thermophilic sources reveal that besides structural and thermodynamic parameters which contribute to stability, increased charge dipole on helices, cation-π interactions also serve as important factors in imparting stability (Yano and Poulos 2003). Thermophiles have significantly greater number of cation-π interactions over corresponding mesophiles,

and abundance of the lysine–tyrosine cation-π interactions is of prime importance (Gromiha et al. 2002; Chakravarty and Varadarajan 2002).

32.8 Applications of Thermostable Proteases

The spectrum of protease application ranges from hydrolysis of peptide bonds to peptide synthesis. The applications discussed here will be restricted to the processes wherein thermal stability could be useful.

32.8.1 Peptide Synthesis

Lately, proteases are being used for peptide synthesis in low water/nonaqueous environments. This could be possible because of discovery of some proteases showing compatibility with organic solvents (Wilson et al. 1994a, b). More specifically, thermolysin is in use for the synthesis of the dipeptide N-CBZ-L-Asp-L-Phe methyl ester, which is the precursor of the sweetener aspartame (Isowa et al. 1979). In these cases, the compatibility of the enzyme to organic solvents is of critical significance (Nakanishi et al. 1990; Gupta 1992). Lately, a protease from a solvent tolerant *Pseudomonas aeruginosa* was found to retain stability in organic solvents (Gupta and Khare 2006).

32.8.2 Detergent Formulations

Proteases are extensively used as additives in domestic laundry detergents to remove proteinaceous stains. Thermostable nature of these proteases should be advantageous in washing at elevated temperatures. Another larger application might be the use of thermophilic enzymes in dishwashing detergent. A thermostable protease would easily be able to withstand the alkaline pH and temperatures above 60°C. Kanchana et al. (2012) have reported a thermostable extracellular protease from *Enterobacter* sp. with potential application as additive in detergent formulation and removal of blood stains.

32.8.3 Molecular Biology Reagent

A thermostable protease derived from *Thermus* sp. (Pretaq) is being increasingly advocated for cleanup of DNA in the polymerase chain reaction (PCR) prior to amplification.

32.8.4 Leather Processing

The leather industries need proteolytic enzymes endowed with high keratinolytic and elastolytic activities for soaking (Niehaus et al. 1999). A keratin degrading thermophilic bacterium *Fervidobacterium pennavorans*, isolated from hot springs of the Azores Island, was successfully used in complete conversion of chicken feather to amino acids and peptides at 80°C. Thermostable keratinolytic protease from Bacillus *halodurans* JB99 successfully dehaired buffalo and goat hide with causing any damage to the collagen layer (Shrinivas and Naik 2011). Thermophilic proteases can also be better catalysts in bating processes under acidic and alkaline conditions (Friedrich and Antranikian 1996).

32.8.5 Meat Tenderisation

Applications of proteases in meat tenderising are known for long. Since the process is carried out at 40–60°C, a thermostable protease could be better applied for the purpose. Advantages of using bacterial proteolytic thermophile enzymes E A.1 protease (from *Bacillus* strain E A.1) and 4-1.A protease (from *Thermus* strain Rt4-1.A) in meat tenderisation have been discussed (Wilson et al. 1992).

32.9 Future Perspectives

- It is desirable to screen new thermophilic microorganisms for obtaining novel enzymes.
- A precise mechanism of their inherent stability needs to be clearly established.
- Cloning and expression of thermostable enzymes in mesophilic hosts will ease their production and usage. Protease genes from *Pyrobaculum aerophilum* and *B. stearothermophilum* (VolkI et al. 1995; Kubo and Imanaka 1988) have been expressed in *E. coli*. Very recently, a keratinase from *Bacillus pumilus* KS12 was cloned and functionally overexpressed in *E. coli* (Rajput et al. 2011). The keratinase was highly thermostable with $t_{1/2}$ of more than 4 h at 70°C, 2 h at 80°C and 30 min at 90°C.
- It is envisaged that overexpression of thermotolerant enzymes in the *E. coli* or *S. cerevisiae* may allow increased enzyme production as well as easier purification by heat treatment. Thermal treatment is likely to denature and precipitate out the host's mesophilic proteins, while the necessary thermostable enzyme may be retained in solution.
- Usage of thermophilic protease may be explored for difficult biotransformation processes.
- The structure function relationship in thermophilic protease may provide a clue to engineer the stability of mesophilic proteases.

- Metagenomic studies of the soil profile near volcanic eruptions, thermal vents and hot springs are yet to be initiated for obtaining unculturable thermophiles and their novel proteases.

32.10 Conclusions

The discovery of thermophiles has encouraged serious research into their enzymes with a view to envisage the molecular basis of protein stabilisation and their potential applications. Their unique enzyme catalytic activities still further add to the interest of large-scale biotechnological applications.

Thermostability appears to be naturally implemented in thermophilic organisms by an amalgamation of various strategies. The upper limits for activity of enzymes seem to be governed by the extent of protein stability. Thermal stability of the enzyme plays a major role in deciding the maximum temperature limit for life under extreme conditions. This chapter also highlights the tremendous potential of thermostable proteases in modern and advanced biotechnological processes and also the valuable genetic resource they represent. Nevertheless, the extent of such developments will depend largely on the economic viability of the processes. Utilisation of thermostable proteases may however be limited by the higher cost involved in making the commercially pure enzyme available.

Microbial communities with novel and unique enzymatic properties continue to colonise environments once assumed to be too harsh and hostile for existence. With the development of newer methods of culturing, exploring the biodiversity of extreme environments should serve as means of yet undiscovered sources of thermophilic enzymes, more specifically proteases.

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Chapter 33
Microbial Keratinases: Diversity and Applications

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Abstract Keratinases are unique proteases that are capable of degrading recalcitrant, "hard-to-degrade" keratin residues. Diverse microorganisms that belong to Eukarya, bacteria, and Archaea produce these enzymes. A large number of Bacilli, Actinobacteria, and fungi are reported to produce keratinases. Microbial keratinases present great diversity in their biochemical and biophysical properties. They are robust enzymes with wide temperature and pH activity range. They are optimally active at neutral to alkaline and 40–60°C, but examples of microbial keratinolysis at alkalophilic and thermophilic conditions have been well documented. Studies with specific substrates and inhibitors indicated that keratinases preferentially act on hydrophobic and aromatic residues at P1 position. Keratinases have several current and potential applications in agro-industrial, pharmaceutical, and biomedical fields. These enzymes are useful in processes related with the bioconversion of keratin waste into feed and fertilizers. Other promising applications are enzymatic dehairing for leather and cosmetic industry, detergent industry, and development of biopolymers from keratin fibers. The use of keratinases to enhance drug delivery in some tissues and hydrolysis of prion proteins arises as novel outstanding applications. Their use in biomass conversion into biofuels may address the increasing concern on energy conservation and recycling. Looking into their biotechnological impetus, they are being cloned and expressed in a variety of heterologous hosts.

Keywords Keratin • Keratin waste • Keratinolysis • Keratinase • *Bacillus* • Prion proteins

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Keratin is insoluble structural protein known for its recalcitrant nature and high stability (Bradbury 1973). It is abundant protein in nature in the form of animal skin, horn hair, wool, feather, nail, hooves, scales, etc. (Onifade et al. 1998). On the basis of secondary structure, keratins have been classified into α-helix (hair and wool) and β-sheets (feather and silk fibroin) (Voet and Voet 1995; Akhtar and Edwards 1997; Parry and North 1998). In α and β form of keratin, fibrils are twisted parallel or antiparallel to form stable fibers (Kreplak et al. 2004; Zerdani et al. 2004). The keratin protein is intensively cross-linked with disulfide bridges which hinder its degradation by common proteases like trypsin, pepsin, and papain (Papadopolous 1985).

Despite being recalcitrant, keratin wastes are efficiently degraded by a large number of bacteria, actinomycetes, and fungi continuously in nature. These microorganisms are known to produce special proteolytic enzymes, "The keratinases" (Gupta and Ramnani 2006). Keratinases are special class of proteases, since they target insoluble keratin substrate (Onifade et al. 1998; Gupta and Ramnani 2006). They show high affinity toward hard-to-degrade proteins and have broad substrate specificity in comparison to conventional proteases. They are generally alkaline and thermostable in nature (Gupta and Ramnani 2006; Brandelli et al. 2010). Thus, they have not only gained importance in various conventional biotechnological sectors of detergent, feed, fertilizers, etc. (Gupta and Ramnani 2006; Brandelli 2008) but also find application in environmental cleanup of feather keratin for converting it into feather meal for multiple applications (Thanikaivelan et al. 2004; Karthikeyan et al. 2007). They are also considered as dehairing enzymes and better proteases for leather industry and pharmaceutical preparations as ungual enhancers, etc. (Bertsch and Coello 2005; Mohorčič et al. 2007).

Till date a large number of microorganisms have been reported which produce keratinases (Onifade et al. 1998; Gupta and Ramnani 2006; Brandelli et al. 2010). These enzymes have been purified and characterized. Lately, with the realization that these enzymes target β-sheet rich keratin protein and can be good catalyst for degrading even proteinase K-resistant prion protein, they have gained a lot of biotechnological impetus. Therefore, a lot of literature has accumulated during the last decade. The subject has been reviewed several times with focus on the biodiversity, production, downstream processing, purification, biochemical characterization, and biotechnological applications (Gupta and Ramnani 2006; Brandelli 2008; Brandelli et al. 2010).

Here, a comprehensive updated review of literature on various aspects of microbial keratinases has been presented.

33.1 Sources of Microbial Keratinases

Microbial keratinases are elaborated by a compendium of microorganisms like bacteria, actinomycetes, and fungi. Keratinase producers are mainly isolated from keratinous wastes or keratin-rich areas, but they are widely distributed in nature and have been isolated from variety of habitats, viz., antarctic soils, soybean wastes,

Mediterranean Sea, solfataric muds, polluted rivers, and hot springs. Diverse source of isolation like Amazon Basin has also been explored for keratinolytic microbes by Giongo et al. 2007; Correa et al. 2010; Daroit et al. 2009.

In the laboratory, keratinase producers are by and large isolated by enrichment method, using chicken feather as carbon and nitrogen substrate. Feather degraders are selected as potential keratinase producers and keratinases are purified from extracellular broth. Hence, microorganisms capable of degrading keratin substrates like chicken feather, hair, wool, feather meal, hooves and horns, and shrimp shell powder are considered as source of keratinase. In this respect, till date a lot of literature on diversity of keratinolytic microorganisms comprising of bacteria, fungi, and actinomycetes has been accumulated. Among these, keratinolytic fungi by and large fall in the category of dermatophytic microorganisms as they attack α-keratin and are not regarded as safe (Gupta and Ramnani 2006). Therefore, here only diversity of keratinolytic bacteria and actinomycetes of biotechnological relevance is comprehended (Table 33.1).

From the literature, it can be observed that among bacteria, strains of *Bacillus* species are the most prominent feather degraders and, hence, keratinase producers (Gupta and Ramnani 2006; Zhang et al. 2009; Brandelli et al. 2010; Prasad et al. 2010). Diverse strains of *Bacillus licheniformis* and *Bacillus subtilis* have been described for feather degradation and keratinase production (Brutt and Ichida 1999; Evans et al. 2000; Suh and Lee 2001; Macedo et al. 2005; Suntornsuk et al. 2005; Hossain et al. 2007; Kumar et al. 2008; Cai and Zheng 2009; Fakhfakh et al. 2009; Matikeviciene et al. 2009; Rai et al. 2009; Kumar et al. 2010; Desai et al. 2010; Lin and Yin 2010; Mazotto et al. 2010). Other *Bacillus* sp., like *B. pumilus*, *B. cereus*, *B. amyloliquefaciens*, *B. halodurans*, *B. thuringiensis*, *B. megaterium*, and *B. pseudofirmus*, have also been reported for keratinase production (Kojima et al. 2006; Adigüzel et al. 2009; Cortezi et al. 2008; Park and Son 2009; Fakhfakh-Zauari et al. 2010; Infante et al. 2010; Lateef et al. 2010; Nagal and Jain 2010). First thermostable keratinase was produced from *Bacillus licheniformis* PWD-1 which was later on commercialized under the trade name of "Versazyme" by Shih and coworker at Bioresources International, Inc. (USA). Some of the thermophilic and alkaliphilic strains of *B. pseudofirmus* AL-89, *B. pseudofirmus* FA30-01, and *B. halodurans* AH-101 have also been reported for keratinase production (Gessesse et al. 2003; Takami et al. 1999; Kojima et al. 2006).

Besides these *Bacillus* sp., many other bacteria like *Thermoanaerobic* sp. (Riessen and Antranikian 2001; Kublanov et al. 2009), *Lysobacter* (Allpress et al. 2002), *Kocuria* (Vidal et al. 2000; Bernal et al. 2003), *Microbacterium* (Thys et al. 2004), *Nesterenkonia* (Bakhtiar et al. 2005), *Serratia* (Khardenavis et al. 2009), *Chryseobacterium* (Wang et al. 2008; Silveira et al. 2010), *Stenotrophomonas* (Yamamura et al. 2002; Jeong et al. 2010), *Pseudomonas* (Tork et al. 2010), and *Vibrio* (Sangali and Brandelli 2000) have also been reported for feather degradation and keratinase production.

Among actinomycetes, mainly *Streptomyces* spp. have been reported as a keratinolytic microbes. These have been largely isolated from poultry wastes, and few have also been reported from various habitats like antarctic soil, Mediterranean Sea, and Lonar Lake (Chitte et al. 1999; Szabo et al. 2000; Gushterova et al. 2005; Esway

Table 33.1 Microbial keratinases: Sources and production conditions

Microorganism	Source of isolation	Production conditions (Temperature (°C)/pH/ Agitation (rpm)/Time)	Additional carbon/nitrogen	References
Bacteria				
Bacillus licheniformis PWD1	Poultry wastes	50/7.5/120/30 h	None	Williams et al. (1990), Lin et al. 1992
Bacillus licheniformis RG-1	Compost	37/7/250/72 h	Peptone/glucose	Ramnani and Gupta (2004)
Bacillus licheniformis RG1	Compost	37/7/200/60 h	Glucose/peptone	Ramnani and Gupta (2006)
Bacillus licheniformis RPk	Polluted river	37/–/200/48 h	Yeast extract	Fakhfakh et al. (2009)
Bacillus licheniformis 511	Poultry waste water	37/7.4/200/24 h	None	Matikeviciene et al. (2009)
Bacillus licheniformis KI 8102	Poultry soil	37/7/120/48 h	None	Desai et al. (2010)
Bacillus licheniformis YJ4	Decomposed feather	37/7.5/150/72 h	Rice husk, yeast extract	Lin and Yin (2010)
Bacillus licheniformis	Poultry waste	40/7/150/7 days	Nutrient broth	Vigneshwaran et al. (2010)
Bacillus subtilis RM-01	Soil	50/8/SSF/72 h	Maltose/sodium nitrate	Rai et al. (2009)
Bacillus subtilis [a]	Horn meal	37/7/static/48 h	Wheat husks	Kumar et al. (2010)
Bacillus subtilis KI 8101	Poultry soil	37/7/120/48 h	None	Desai et al. (2010)
Bacillus pumilus	Avian plumage	–/–/–/48–60 h	Yeast extract	Brutt and Ichida (1999)
Bacillus pumilus A1	Slaughter house poultry water	37/7/200/24 h	None	Fakhfakh-Zauari et al. (2010)
Bacillus cereus KB043	Feather compost	37/7/150/7 days	None	Nagal and Jain (2010)
Bacillus cereus LAU08	Keratin disposal site	37/7.5/100/120 h	Yeast extract	Lateef et al. (2010)
Bacillus amyloliquefaciens	Poultry wastes	40/8/150/3 days	NH_4Cl	Cortezi et al. (2008)
Bacillus megaterium F7-1	Feather	30/7.5/200/5 days	NH4Cl/yeast extract	Park and Son (2009)
Bacillus megaterium L4 [b]	Raw wool	30/7/–/48 h	None	Infante et al. (2010)
Bacillus thuringiensis L11				
Bacillus halodurans PPKS-2	Rice mill effluent	37/11/180/48 h	Soy bean flour/peptone /sodium sulfite	Prakash et al. (2010)
Bacillus halodurans JB 99	Sugarcane molasses	55/10/220/48 h	Yeast extract/peptone	Shrinivas and Nayak (2011)

Bacillus sp. 50-3	Calotes versicolor feces	37/7/150/36 h	None	Zhang et al. (2008)
Kocuria rosea LBP-3	Soil	40/7.5/75/36 h	Yeast extract	Vidal et al. (2000), Bernal et al. (2003)
Nesterenkonia sp. Al20	–	37/10/–/148 h	None	Bakhtiar et al. (2005)
Chryseobacterium sp. strain kr6	Poultry wastes	30/8/70/48 h	None	Silveira et al. (2010)
Chryseobacterium L99 sp. nov.	Wool	30/7.5/200/30 h	None	Long-Xian et al. (2010)
Fervidobacterium islandicum AW-1	Geothermal hot spring	70/7/static/48 h	Yeast extract	Nam et al. (2002)
Thermoanaerobacter keratinophilus sp. nov.	Geothermal hot spring	70/6.8/static/96 h	Yeast extract/tryptone	Riessen and Antranikian (2001)
Thermoanaerobic sp.[c]	Thermal spring	40–70/8.1–9.6/–/–	Sucrose/yeast extract/peptone	Kublanov et al. (2009)
Stenotrophomonas sp. D1	Soil containing deer fur	20/7/130/4 days	None	Yamamura et al. (2002)
Stenotrophomonas maltophilia	Soil and roots of plants	30/7/200/24 h	Glucose/polypeptone	Jeong et al. (2010)
Vibrio sp. kr2	Industrial poultry	30/6/180/32 h	None	Sangali and Brandelli (2000)
Lysobacter NCIMB 9497	Culture collection	25/–/120/29 days	None	Allpress et al. (2002)
Xanthomonas maltophilia POA-1	Poultry waste	30/7/200/72 h		De Toni et al. (2002)
Microbacterium arborescens kr 10	Industrial poultry wastes	30/7/180/36 h	None	Thys et al. (2004)
Clostridium sporogenes bv. Pennavorans	Solfataric muds	42/7/–/7 days	None	Ionata et al. (2006)
Serratia sp. HPC 1383	Waste water of tannery	30/7.5/150/72 h	Yeast extract	Khardenavis et al. (2009)
Pseudomonas sp.	Poultry wastes	37/7.2/static/24 h	None	Tork et al. (2010)
Actinomycetes				
Thermoactinomyces[d] *candidus*	Soil	65/7.2/–/5 days	None	Ignatova et al. (1999)

(continued)

Table 33.1 (continued)

Microorganism	Source of isolation	Production conditions (Temperature (°C)/pH/ Agitation (rpm)/Time)	Additional carbon/nitrogen	References
Streptomyces graminofaciens	Soil from poultry wastes	40/7.6/250/–	Glucose/peptone	Szabo et al. (2000)
Thermoactinomyces sp.[d]	Soil	55/7.2/280/–	None	Gousterova et al. (2005)
Actinomycete[e]	Antarctic soil	55/7.2/280/5 days	None	Gousterova et al. (2005)
Streptomyces sp. 594	Soil	30/7.5/200/4 days	Casitone-molasses	De Azaredo et al. (2006)
Streptomyces	Poultry soil	30/8.5/–/4–5 days	Glycerol/starch/ polypeptone/gelatine	Ya-Peng et al. (2006)
Streptomyces sp. 7	Poultry house	45/11/300/5 days	None	Tatineni et al. (2007)
Streptomyces albus [e]	Mediterranean sea	30/9/SSF/5 days	None	Esway (2007)
Streptomyces	Poultry waste water	40/8/160/48 h	None	Tapia and Contiero (2008)
Streptomyces gulbargensis	Soil	30/7.5/250/7 days	Starch	Syed et al. (2009)
Streptomyces	Poultry grange soil	30/7.2/120/5 days	None	Kansoh et al. (2009)
Streptomyces sp. strain AB1	Soil	30/9/200/5 days	None	Jaouadi et al. (2010)
Streptomyces sp. strain [f] 16	–	30/ 8/200/4 days	Soluble starch/yeast extract	Xie et al. (2010)

Substrate used *a* = horn meal, *b* = wool, *c* = pig hair, *d* = sheep wool, *e* = wheat flour, *f* = native human foot hair

2007; Jaoudai et al. 2010). Highly keratinolytic actinomycetes strains, *Streptomyces flavis* 2BG and *Microspora aerate* IMBAS-11A, were isolated from antarctic soil (Gushterova et al. 2005). Thermophilic sp. like *Streptomyces gulbargensis* (Syed et al. 2009), *Streptomyces thermoviolaceus* (Chitte et al. 1999), and *Streptomyces thermonitrificans* (Mohamedin 1999) were isolated from soil. Few mesophilic streptomyces like *Streptomyces pactum* DSM 40530 (Bockle et al. 1995), *Streptomyces graminofaciens* (Szabo et al. 2000), and *Streptomyces albidoflavus* $K_{1\text{-}02}$ (Bressollier et al. 1999) were also characterized for keratinase production.

Thermophilic and alkalophilic conditions facilitate keratin degradation, so thermophilic and alkalophilic microorganisms have also gained importance for feather degradation and keratinase production. In this respect, *Fervidobacterium pennavorans* (Friedrich and Antranikian 1996), *Fervidobacterium islandicum* (Nam et al. 2002), *Clostridium sporogenes* (Ionata et al. 2008), and *Thermoanaerobacter* (Riessen and Antranikian 2001; Kublanov et al. 2009) were isolated from extreme environments like hot springs, geothermal vents, solfataric muds, and volcanic areas. Alkaliphilic *Nesterenkonia* and *Nocardiopsis* TOA-1 have also been used for keratinase production (Bakhtiar et al. 2005; Mitsuki et al. 2004).

It can, thus, be put forth that keratinolytic microorganisms are widespread to a variety of environment and habitat; however, generally they are selected on the basis of their capability to degrade keratin in the form of chicken feather, wool, horn, or hoove among them chicken feather is the most preferred substrate. Till date, strains of *Bacillus* species are predominant among bacteria and *Streptomyces* sp. among actinobacteria.

33.2 Mechanism of Keratin Degradation

Keratin hydrolysis by microorganisms is supposed to be assisted by keratinases and requires additional involvement of reducing agent for breakdown of disulfide bonds. Release of thiol groups has been often observed during the microbial growth on keratinous substrate which supports the essential role of reduction of disulfide bonds during keratin hydrolysis (Cao et al. 2008; Mabrouk 2008; Daroit et al. 2009). High mechanical stability and resistance to proteolytic attack of keratinase are due to cross-linking of keratin chains by disulfide bonds (Böckle and Müller 1997). It is evident that cleavage of disulfide bonds may play a significant role in keratin degradation (Yamamura et al. 2002b; Ramnani et al. 2005). The definitive mechanism(s) and exact nature of keratinolysis are highly complex and not yet fully understood. Production of intracellular and/or extracellular disulfide reductase (Yamamura et al. 2002; Ramnani et al. 2005; Ghosh et al. 2008; Kumar et al. 2008; Prakash et al. 2010), sulfite and thiosulfate release (Ramnani et al. 2005), and cell-bound redox system (Bockle and Muller 1997; Ramnani et al. 2005; Ramnani and Gupta 2007) also leads to sulfitolysis. In case of fungi, mechanical pressure occurs at keratinous substrate during mycelia penetration (Mitola et al. 2002; Moreira et al. 2007). Thus, it can be inferred that sulfitolysis and proteolysis may be involved in keratin degradation.

Till date, none of the purified keratinase could degrade keratin completely. Keratin hydrolysis using purified keratinase was observed only in the presence of reducing agents, which promote sulfitolysis (Takami et al. 1992a; Gradisar et al. 2005; Cai et al. 2008; Cao et al. 2008). Hydrolysis of disulfide bonds changes the confirmation of keratin and exposes more sites for keratinase action leading to keratin degradation (Vignardet et al. 1999).

The extent of keratin hydrolysis varies from substrate to substrate. Stratum corneum an α-keratin is more susceptible to keratinases in comparison to hair and wool (Friedrich and Kern 2003; Brandelli 2005; Gradisar et al. 2005; Xie et al. 2010). β-keratin mainly feather are hydrolyzed more than α-keratin, i.e., hair and wool (Brandelli 2005; Cao et al. 2009; Moreira-Gasparin et al. 2009). Wool and hair contains high cysteine concentration (Barone et al. 2005; Gousterova et al. 2005; Poole et al. 2009) in comparison to feather (Szabo et al. 2000; Takahashi et al. 2004; Bertsch and Coello 2005) which results in compact structure of hair and wool, thus limiting the accessibility to keratinases in comparison to β-keratin (Coward-Kelly et al. 2006).

In some cases, keratinases from *Doratomyces microsporus* and *Paecilomyces marquandii* hydrolyzed α-keratin more efficiently in comparison to β-keratin (Friedrich and Kern 2003; Gradisar et al. 2005) and vice versa (Kojima et al. 2006; Pillai and Archana 2008; Rai et al. 2009). Few keratinases from *Bacillus subtilis* and *Stenotrophomonas* are reported to hydrolyze both α- and β-keratins with similar potential (Cai et al. 2008; Cao et al. 2009). Other than disulfide bond, fibril structure and porosity (Cowerd-Kelly et al. 2006) may also be responsible for differential hydrolysis of keratin substrates.

Thus, it can be concluded that multiplicity of catalytic mechanisms are observed involving a variety of microbial keratinases, i.e., serine, thiol, or metalloproteases. However, keratin hydrolysis in nature is still a result of synergistic action of keratinolytic microbes and their enzymes and metabolites which hydrolyze recalcitrant protein in comparison to pure enzyme (Ichida et al. 2001).

33.3 Biochemical Properties of Keratinases

Keratinases from various microorganisms have been characterized biochemically. The biochemical and physiochemical properties of keratinases from several microorganisms have been extensively studied. The important properties, viz., pH and temperature optima, pI, molecular weight, and substrate specificity are presented.

33.3.1 pH and Temperature Kinetics

Keratinases from most bacteria, actinomycetes, and fungi show activity in the pH range neutral to alkaline pH 7–9 (Gupta and Ramnani 2006; Brandelli et al. 2010).

Among various *Bacillus* sp. mainly *B. licheniformis*, *B. subtilis,* and *B. pumilus* showed optimum activity at neutral to alkaline pH ranging from 7 to 9. Other *Bacillus* sp. like *Bacillus thuringiensis* (Infante et al. 2010) and *Bacillus cereus* (Lateef et al. 2010) were active at neutral pH, while few *Bacillus* strains *B. pseudofirmus* (Gessesse et al. 2003; Kojima et al. 2006) and *B. halodurans* (Prakash et al. 2010; Shrinivas et al. 2011) were active at highly alkaline condition pH 8.9–11. Keratinases from *Bacillus halodurans* AH101 possess extreme alkalophilic optima of pH >12.0 (Takami et al. 1999; Mitsuiki et al. 2004); however, keratinase from *Bacillus subtilis* MTCC 9102 was found to be active in slightly acidic pH (Balaji et al. 2008).

Apart from *Bacillus* sp., keratinases from *Fervidobacterium*, *Stenotrophomonas*, *Chryseobacterium*, *Thermoanaerobacter*, *Nesterenkonia*, *Lysobacter*, *Xanthomonas, Kocuria*, *Clostridium,* and *Serratia* were optimally active in alkaline pH ranged from pH 8 to 11. Keratinase from various *Streptomyces* sp. was also active in alkaline pH range pH 7.5–11, while highly alkaline keratinase with optima at pH >12.5 was reported in case of *Nocardiopsis* sp. TOA-1 (Mitsuiki et al. 2004).

Most of the keratinases from strains of *Bacillus*, *Nesterenkonia*, *Chryseobacterium indologenes* TKU014, *Stenotrophomonas*, *Streptomyces pactum,* and *Streptomyces* AB1 exhibited stability over a wide range of pH from 5 to 12. Noteworthy is the remarkable stability of keratinase from *Nocardiopsis* which showed stability over extreme pH range from 1.5 to 12.0 for 24 h at 30°C (Mitsuki et al. 2002). Recently, keratinase from *Bacillus licheniformis* MKU3 showed increased pH stability after expression in *Pichia pastoris* X33 (Radha and Gunasekaran 2009). Keratinase from *Streptomyces pactum* DSM40530 showed stability at pH 4; however, keratinase from *B. halodurans* AH 101 was stable at highest pH 13 (Bockle et al. 1995; Takami et al. 1999).

Temperature optima of keratinases vary according to the source and origin of the isolate. Generally the temperature optima of keratinases from different *Bacillus* species, i.e., *B. licheniformis, B. subtilis, B. pumilus, B. halodurans, B. thurengiensis, and B. cereus* ranges between 40–70°C. Keratinase from other bacteria like *Lysobacter* (Allpress et al. 2002), *Xanthomonas* (De Toni et al. 2002), *Stenotrophomonas* (Yamamura et al. 2002; Cao et al. 2009), *Thermoanaerobacter* (Kublanov et al. 2009), *Chryseobacterium* (Wang et al. 2008; Long-Xian et al. 2010), *Serratia* (Khardenavis et al. 2009), *Clostridium* (Ionata et al. 2008), *Microbacterium* (Thys et al. 2004), *Kocuria* (Bernal et al. 2003), *Nesterenkonia* (Gessesse et al. 2003), and *Vibrio* (Sangali and Brandelli 2000) was also optimally active at 40–70°C. Thermophilic organisms *Fervidobacterium pennavorans* (Friedrich and Antranikian 1996) and *Thermoanaerobacter keratinophilus* (Riessen and Antranikian 2001) had optima at 80 and 85°C, respectively, while highest activity optima of 100°C was observed for keratinase from *Fervidobacterium islandicum* AW-1 (Nam et al. 2002). Keratinase from *Chrysosporium keratinophilum* (Dozie et al. 1994) showed high-temperature optima of 90°C. Keratinase from *Streptomyces* showed optimal activity in the temperature range of 40–70°C (Böckle et al. 1995; Bressollier et al. 1999; Chitte et al. 1999; Ignatova et al. 1999; Korkmaz et al. 2003; Esway 2007; Tapia and Contiero 2008; Tatineni et al. 2008; Xie et al. 2010).

Keratinases exhibit varied thermostability which may range from 10 min to 36 h at various temperatures. Keratinases from *Bacillus* were observed to be stable in the temperature ranging from 10 to 70°C. *B. licheniformis* PWD1 keratinase was stable at 20–25°C for 4–5 days while for 1 h at 30°C (Lin et al. 1992; Cheng et al. 1995). *Bacillus subtilis* KS-1 and RM-01 keratinase showed stability of 24 h at 25°C and 30 min at 60°C (Suh and Lee 2001; Rai et al. 2009). Keratinase from *Bacillus* sp. P7 was stable at 45 and 50°C for 173 and 53 min, respectively (Corrêa et al. 2009), and from *Bacillus pumilus* A1 was stable for 2 h at 50°C (Fakhfakh-Zauari et al. 2010). Keratinase of *Bacillus halodurans* showed 3–4 h stability at 70°C (Prakash et al. 2010; Shrinivas and Nayak 2011). Exceptionally keratinase from *Bacillus thuringiensis* L11 was stable at −20°C for 2 weeks (Infante et al. 2010). *Fervidobacterium islandicum* AW-1 keratinase was stable at 100°C for 90 min (Nam et al. 2002), and *Thermoanaerobacter keratinophilus* was stable for 6 h at 80°C (Riessen and Antranikian 2001). Keratinase from *Kocuria* was stable for 12 h at room temperature and for 1 h at 60°C (Bernal et al. 2003), *Clostridium* for 36 h at 55°C (Ionata et al. 2008), and *Serratia* for 10 min at 60°C (Khardenavis et al. 2009). *Streptomyces* keratinase was also observed to be stable at 35–65°C. Keratinase from *Streptomyces pactum* DSM40530 was stable for 5 h at 35°C (Böckle et al. 1995), *S. thermoviolaceus* SD8 was 30 min at 65°C (Chitte et al. 1999), *Streptomyces* sp. for 1 h at 50°C (Tatineni et al. 2008), and *Streptomyces* sp. strain 16 for 1 h at 40–60°C (Xie et al. 2010). Exceptionally, keratinase from *Streptomyces* BA7 was stable for >30 min at 90°C (Korkmaz et al. 2003).

Thermostability of enzyme was also increased in the presence of divalent cations like calcium and magnesium (Dozie et al. 1994; Bressollier et al. 1999; Chitte et al. 1999; Ignatova et al. 1999; Riessen and Antranikian 2001; Nam et al. 2002; Gessesse et al. 2003; Riffel et al. 2003; Farag and Hassan 2004; Kublanov et al. 2009; Jaouadi et al. 2010). Thermostability of keratinase from *Thermoanaerobacter* increased from 10 min to 13 h at 94°C by addition of calcium (Kublanov et al. 2009). Thermostability of keratinase from *Streptomyces* ap. AB1 was increased in the presence of 5 mM $MgSO_4$ up to 240, 160, and 60 min from 180, 110, and 30 min at 60, 70, and 80°C, respectively (Jaouadi et al. 2010).

33.3.2 Molecular Weight

Molecular weight of keratinases has been reported in the range between 18 and 200 kDa. In case of *Bacillus* sp., size of keratinase varied in the range of 20–69 kDa. The lowest molecular mass is 18 kDa for *Streptomyces albidoflavus* (Bressollier et al. 1999) and highest 240 kDa for *K. rosea* (Bernal et al. 2003b). Most of the reported keratinase are monomeric; however, dimeric keratinase of 58 kDa from *Bacillus licheniformis* ER-15 (Tiwary and Gupta 2010a) and multimeric keratinases are reported by Nam et al. (2002) and Xie et al. (2010) from *Fervidobacterium islandicum* AW-1 and *Streptomyces* sp., respectively. High molecular mass of keratinases from thermophiles, i.e., *Fervidobacterium pennavorans* (130 kDa) (Friedrich

and Antranikian 1996), *Thermoanaerobacter keratinophilus* (135 kDa) (Riessen and Antranikian 2001), *Thermoanaerobacter* (150 kDa) (Kublanov et al. 2009), or metalloprotease *Lysobacter* (148 kDa) (Allpress et al. 2002) have been reported. Isoelectric point of the keratinases is reported in range of pI 6.5–8.5, but lower pI 5.9 and >10.5 was also reported in case of *Bacillus pseudofirmus* and *Nocardiopsis* TOA-1, respectively (Mitsuiki et al. 2004; Kojima et al. 2006).

33.3.3 Substrate Specificity

Keratinases show broad substrate specificity and are reported to be active against both soluble and insoluble proteinaceous substrates. They have the unique ability to cleave complex, insoluble substrates such as feather keratin, collagen, elastin, fibrin, and nail which are otherwise resistant to degradation by conventional proteases (Evans et al. 2000). Keratinases efficiently hydrolyze soluble protein substrates like casein, azocasein, bovine serum albumin, gelatin, and insoluble proteins like azokeratin, collagen, elastin, feather, horn, hair, nail, silk, stratum corneum, wool, and skin. Study on synthetic *p*-nitroanilide substrates indicated that keratinase preferably cleaves at hydrophobic and aromatic amino acids at P1 position (Bressollier et al. 1999; Evans et al. 2000; Gradisar et al. 2005; Brandelli et al. 2010).

33.3.4 Effect of Inhibitors, Metal Ions, Solvents, Nonionic Detergents, and Reducing Agents

Keratinases mostly belong to serine or metalloproteases irrespective of the microorganism. Therefore, PMSF, EDTA, and 1,10-*o*-phenanthroline (Dozie et al. 1994; Riffel et al. 2003; Gupta and Ramnani 2006) are potential inhibitors of these enzymes. Recently, few cysteine proteases have also been reported (Rai et al. 2009; Lin and Yin 2010). In some cases, EDTA causes partial inhibition indicating the importance of cations as stabilizing agents (Böckle et al. 1995; Bressollier et al. 1999; Ignatova et al. 1999; Riessen and Antranikian 2001; Nam et al. 2002).

The effect of metal ions on keratinolytic activity has also been studied, and a general trend was observed that Ca^{2+}, Mg^{2+}, and Mn^{2+} often stimulated keratinases (Riessen and Antranikian 2001; Nam et al. 2002; Riffel et al. 2003; Suntornsuk et al. 2005; Werlang and Brandelli 2005; Kojima et al. 2006; Tatineni et al. 2008; Cao et al. 2009; Brandelli et al. 2010). Some keratinases are activated by Co^{2+} (Nam et al. 2002; Werlang and Brandelli 2005; Tatineni et al. 2008; Kainoor and Naik 2010), Al^{3+} (Suntornsuk et al. 2005), Zn^{2+} (Balaji et al. 2008; Long-Xian et al. 2010), Fe^{2+} (Khardenavis et al. 2009; Rai et al. 2009), Cu^{2+} and Ba^{2+} (Cao et al. 2009). Metals might help in maintaining the active enzyme conformation and thus stabilize the enzyme substrate complex (Farag and Hassan 2004; Suntornsuk et al. 2005;

Cao et al. 2009). In subtilisin type keratinase, interaction of calcium with specific Ca^{2+} binding sites or autolysis sites may result in the improved activity and stability of the enzyme in the presence of such metals (Fakhfakh et al. 2009).

Keratinase activity is generally inhibited by transition and heavy metal ions Ag^{+} (Nam et al. 2002), Ba^{2+}, Co^{+} (Dozie et al. 1994), Cu^{2+} (Nam et al. 2002; Riffel et al. 2003; Thys et al. 2004), Hg^{2+} (Riffel et al. 2003; Thys et al. 2004; Werlang and Brandelli 2005; Kojima et al. 2006), Pb^{+} (Farag and Hassan 2004), and Zn^{2+} (Dozie et al. 1994; Thys et al. 2004). Enzyme activity of *Bacillus pseudofirmus* FA 30-01 was inhibited by Fe^{2+} (Kojima et al. 2006), while keratinase of *Chrysosporium keratinophilum* was activated by $Fe^{2+.}$ (Lin et al. 1992; Dozie et al. 1994). A thiol keratinase from *Bacillus licheniformis* K508 was completely inhibited by Hg^{2+} (Rozs et al. 2001).

It can be inferred from the literature that keratinases are inhibited, stimulated, or stable in the presence of nonionic detergents and solvents. Activation in enzyme activity by SDS was reported by Bressollier et al. (1999), while a decrease was reported by Böckle et al. (1995) and Mitsuiki et al. (2004). Many keratinases were reported to be stable in the presence of SDS (Riffel et al. 2003). Keratinases are generally stable in the presence of organic solvents (Nam et al. 2002; Riffel et al. 2003; Mitsuiki et al. 2004), DMSO (Böckle et al. 1995; Mitsuiki et al. 2004), and Triton X100 (Riffel et al. 2003; Mitsuiki et al. 2004; Kojima et al. 2006).

Keratinases are thiol activated as it has been largely observed that reducing agents like cysteine, glutathione, DTT, mercaptoethanol, sodium sulfite, and thioglycolate enhance keratinolytic activity (Onifade et al. 1998; Werlang and Brandelli 2005). In fact, it is indicated that degradation by purified keratinases in vitro is only accomplished by addition of reducing agents which help in sulfitolysis by breaking disulfide bonds as discussed in the section on mechanism of keratinolysis. Purified keratinases are generally unable to degrade keratin (10% degradation reported in most cases) in the absence of reducing agents (Böckle et al. 1995; Santos et al. 1996; Bressollier et al. 1999; Ignatova et al. 1999; Suh and Lee 2001; Yamamura et al. 2002; Riffel et al. 2003).

Thus, microbial keratinases are largely serine or metalloproteases marked by thiol activation. They are highly robust enzymes as they are active over a wide range of pH and temperature, possess a broad substrate range, and are stable in the presence of detergents, solvents, and metals.

33.4 Cloning and Expression of Keratinases

Till date, many keratinases from bacteria such as *Bacillus licheniformis* PWD-1 *kerA*, *Bacillus licheniformis* OWU 1411T (Acc no. AF282893), *Bacillus licheniformis* RG1 (Acc no. AY590140), *Bacillus licheniformis* RG2 (Acc no. AY817143), *Bacillus licheniformis* MKU2 (Acc no. DQ071569), *Bacillus licheniformis* MKU3 (Acc no. DQ071570), *Bacillus subtilis aprA* (Zaghloul 1998), *Bacillus mojavensis* (Acc no. AY665611), *Bacillus pumilus* (ACM477351), *Bacillus pumilus* KS12

(HM219183), *Bacillus* sp. JB99 (ADD644651), *Bacillus licheniformis* RPk (ACA979911), *Bacillus cereus* (AAR192201), *Fervidobacterium pennivorans* (Kluskens et al. 2002), actinomycetes *Nocardiopsis* sp. TOA-1 NAPase (Mitsuiki et al. 2004) and *Streptomyces fradiae* (Li et al. 2007), and *Pseudomonas aeruginosa* (Lin et al. 2009; Sharma and Gupta 2010) have been cloned and sequenced. Among these, keratinase from *Bacillus licheniformis* PWD-1 – *kerA* has been extensively studied and has been expressed many times in various systems by Shih and coworkers. Similarly, keratinase from *Bacillus licheniformis* MKU3 (Radha and Gunasekarn 2009) and *Pseudomonas aeruginosa* (Lin et al. 2009; Sharma and Gupta 2010a) was also expressed many times. Keratinases from *Fervidobacterium pennavorans* (Kluskens et al. 2002) and actinomycetes *Nocardiopsis* sp. TOA-1 NAPase (Mitsuiki et al. 2004) and *Streptomyces fradiae* (Li et al. 2007) have also been expressed.

33.4.1 Heterologous Expression in E. coli System

The *kerA* gene has been cloned and expressed in *E. coli* BL21 using pET vector system using T7 promoter (Lin et al. 1995). Inhibitory effect of soluble protein was observed in expression of Ker A (Lin et al. 1995). Level of keratinase was high in *E. coli* (Wang et al. 2003) and protein expressed as inclusion bodies which required in vitro refolding of pro-keratinase and resulted in limited enzyme yields. Along with this, plasmid-based expression was unstable when keratinase was expressed in *E. coli*. Further, keratinase from *Bacillus licheniformis* MKU3 was cloned and expressed in *E. coli* BL21 using pET30b vector which resulted lower expression of keratinases. Keratinase from *Pseudomonas aeruginosa* was also expressed in *E. coli* AD494 (DE3) pLys S using pET43b(+) expression vector. In all the cases, yield of keratinase expression was very low, and high expression became lethal for expression host or resulted in inactive keratinase as insoluble inclusion bodies.

To overcome this, keratinases from *Bacillus licheniformis* and *Pseudomonas aeruginosa* were expressed as extracellular protein in *E. coli* using pEZZ18 vector (Tiwary and Gupta 2010b; Sharma and Gupta 2010a).

33.4.2 Expression in Bacillus System

Expression of keratinases in *E. coli* was not feasible since most of the protein was accumulated as inclusion bodies. So, attempts were made to express keratinase in *Bacillus* system. In this respect, *kerA* gene has been cloned using pUB 18/18-P43/10-P43-Pker vector system and expressed in *Bacillus subtilis* DB104 and *Bacillus subtilis* WB600, respectively (Lin et al. 1995; Wang et al. 2003). The vector system was constructed by addition of vegetative growth promoter P43 to the pUB18 vectors. This vector system resulted in high expression of keratinase in *Bacillus subtilis* DB104 but plasmid was unstable. Ker A expression yield has been increased by

additional promoter in plasmid pUB in *Bacillus subtilis* DB104 from *Bacillus licheniformis* PWD1 and *Bacillus subtilis*. Keratinase expression level was increased using extrachromosomal plasmid in *Bacillus* and scaled up to 150 L, but this was also unstable due to segregational instability (Wang and Shih 1999). Further, stable and improved keratinase yield was obtained by chromosomal integration of keratinase gene in *Bacillus subtilis* DB104 and *Bacillus licheniformis* T399D (Wang et al. 2004). An increase of 4–6 folds in keratinase expression was observed in comparison to wild strain which was achieved by integration of 3–5 copy of gene. Translational capacity, posttranslational processing, and secretion were constrained to the strain which resulted in low enzyme yield even randomly amplified copy number of enzyme was present (Wu et al. 1991). Bio-immobilization of keratinases has been developed by fusion of two genes KerA-encoding keratinase and *stp*-encoding streptavidin that binds biotin (Wang et al. 2003). This construct secreted keratinase-streptavidin (KE-STP) into the medium which can be separated from medium directly by biotinylated beads. Further, a new asporogenic strain of *Bacillus licheniformis* PWD1 has been developed in strain development techniques using the splicing by overlap extension PCR method. However, in case of *Bacillus subtilis* as expression host, plasmid stability was observed to be less so *Bacillus megaterium* was used as an expression host.

In this respect, keratinase from *Bacillus licheniformis* MKU3 was cloned and expressed in *Bacillus megaterium.* Keratinase gene was subcloned into *E. coli-Bacillus* shuttle vector pWH1520, and extracellular expression was obtained under the *xyl A* promoter using expression host *Bacillus megaterium* ATCC14945 containing pWHK3 plasmid (Radha and Gunasekaran 2007). Because of unstability of the plasmid in *Bacillus megaterium* ATCC14945, a new strain of *Bacillus megaterium* MS941 was used for expression studies. Inducible expression using xylose promoter (*pXyl* A), constitutive expression using α-amylase promoter (*pAmy*L), and xylose and amylase promoter (pXyl A-pAmyL) resulted in increased expression level of keratinase (Radha and Gunasekaran 2008) in *Bacillus megaterium* MS941. A sustained expression was obtained for about 60 generation in *Bacillus megaterium* MS941 in comparison to *Bacillus megaterium* 14945 (Radha and Gunasekaran 2008).

33.4.3 Expression in Pichia pastoris System

Though, *Bacillus* system was better as expression host and keratinase expression was increased in this system by medium optimization, but the yield of keratinase was not sufficient for industrial applications.

In this respect, methylotrophic yeasts *Pichia pastoris* have been used for expression of heterologous proteins. Process of expression and production is simple in comparison of *Bacillus* system so keratinase expression was attempted into *Pichia* system. They produced high yields of extracellular protein in simple and inexpensive medium mediated by inducible alcohol oxidase (AOX1) promoter. Keratinase

from *Bacillus licheniformis* PWD1 was expressed into *Pichia pastoris* using pPICZαA and pGAPZαA, and higher expression level was achieved after 144 h of induction (Porres et al. 2002). Keratinase from *Bacillus licheniformis* MKU3 was expressed into *Pichia pastoris* X33 using plasmid pPICZαA containing broker gene (pPZK3) which resulted in threefold-enhanced expression of keratinase in comparison to wild strain (Radha and Gunasekaran 2009). Recombinant keratinase expressed in *Pichia* was glycosylated which resulted increase in size of keratinases.

Keratinase from *Pseudomonas aeruginosa* was also successfully expressed in *Pichia pastoris* pPICZαC expression vector, and high yields of keratinase were obtained in glycosylated form (Lin et al. 2009).

Apart from these, keratinase SFP2 from *Streptomyces fradiae* var k11 was also expressed in *Pichia pastoris* SMD1168 using expression vector pPIC9 (Li et al. 2007). However, keratinase from *Nocardiopsis* sp. TOA-1 was successfully expressed in *Streptomyces lividans* using plasmid pUC702 (Mitsuiki et al. 2004).

Thermophilic keratinase, fervidolysin from *Fervidobacterium pennivorans,* was cloned and expressed as inactive protein in *E. coli* and *Bacillus subtilis* (Kluskens et al. 2002). This was the first keratinase which was crystallized (Kim et al. 2004). Crystal structure suggests that fervidolysin is closely related fibronectin like domain of human promatrix metalloprotease which degrades fibrous polymeric substrate (Kim et al. 2004).

33.5 Applications of Keratinases

Keratinases from microorganisms have attracted a great deal of attention in the recent decade as special proteases with their extended substrate spectrum as they act on insoluble structural proteins. Today, due to their wide substrate specificity, they are considered as better catalyst for their application in conventional protease sectors like feed, fertilizer, detergent, leather, and pharmaceutical/biomedical industries. Apart from these, they are indispensible for their application in keratin waste management especially chicken feather wastes. Keratinases are special proteases which act on structural proteins.

Most of the applications of keratinases are common to proteases, but here we are describing only specific keratinases.

33.5.1 Hydrolysis of Hard-to-Degrade Proteins

Insoluble, structural, and hard-to-degrade proteins are ubiquitously present in animal body. A lot of these proteins are generated as industrial waste from meat, poultry, and leather industries. Currently, disposal of these wastes is preferred by incineration or by landfilling (Suzuki et al. 2006). Incineration produced a large amount of carbon dioxide, while landfilling needed large area where naturally degradation is

very slow. In this respect, keratinases gained focus due to their unique ability to degrade these resistant proteins.

These hard-to-degrade proteins are collagen, elastin, keratins, bone, horn, nail, hooves, hair, wool, etc. generated mainly from poultry and leather industries. Apart from these, prion proteins are also resistant to common proteases. Among these proteins, feather is most widely used for production of feather meal which has multifold application in various areas. In the following section, production and application of feather meal has been discussed.

33.5.1.1 Production of Feather Meal from Feather

Feathers, waste product of poultry processing industries, are being generated in billion of tons every year (Onifade et al. 1998; Gupta and Ramnani 2006; Vasileva-Tonkova et al. 2009; Brandelli et al. 2010). These feathers are generally landfilled or burnt which cause environmental problems (Vasileva-Tonkova et al. 2009). Feathers are made up of >90% protein and rich in hydrophobic amino acids and essential amino acids like cysteine, arginine, and threonine (Coward-Kelly et al. 2006). Nowadays, feathers are converted into feather meal and used as poultry feed, cattle feed, fish feed, etc. (Brandelli et al. 2010). Most popular method of conversion of feathers is by hydrothermal process where feather is cooked under high pressure at high temperature (Onifade et al. 1998; Gousterova et al. 2003; Gupta and Ramnani 2006). This hydrothermal treatments result in the destruction of essential amino acids like methionine, lysine, tyrosine, and tryptophan and produced feather meal that has poor digestibility and low nutritional value (Papadopolous et al. 1986; Wang and Parsons 1997). In the last decade, bioconversion of feather into feather meal using feather degraders has gained importance. These microbes offer considerable opportunities for bioconversion of poultry feathers from a potent pollutant to a nutritionally upgraded protein-rich feedstuff for livestock (Onifade et al. 1998).

Bioconversion of feather into feather meal and improvement as poultry feed has been studied extensively (Onifade et al. 1998; Grazziotin et al. 2006). Biodegradation of feather can be achieved either by crude culture filtrate containing keratinases or by cultivation of keratin-degrading microorganism (Gupta and Ramnani 2006).

Feather degradation was demonstrated mainly during fermentation (Brandelli et al. 2010), and a variety of cultures have been used. Among them, *Bacillus* sp. were mainly used for feather degradation (Deivasigamani and Alagappan 2008; Vigneshwaran et al. 2010), while mixed thermophilic actinomycetes (Vasileva-Tonkova et al. 2009), *Vibrio* (Grazziotin et al. 2007), and *Streptomyces* AB1 (Jaouadi et al. 2010) were also used. Recombinant *Bacillus* species were also used for feather degradation (Zaghloul et al. 2004; Zaghloul et al. 2011). Complete feather degradation was achieved only in fermentation method (Deivasigamani and Alagappan 2008; Zaghloul et al. 2011). Crude culture supernatant from *Bacillus* sp. (Korkmaz et al. 2004; Hossain et al. 2007; Fakhfakh-Zauari et al. 2010), *Streptomyces* (Korkmaz et al. 2003), and *Pseudomonas* (Sharma and Gupta 2010b) has also used for feather degradation. Immobilized cells of *Bacillus halodurans* PPKS were also used for feather degradation where 60–80 % degradation was achieved in 48 h in the

presence of reducing agents like sodium sulfite, dithiothreitol, SDS, and triton X-100 (Prakash et al. 2010). Feather degradation using enzymes was performed in the presence of reducing agents like β-mercaptoethanol at higher temperature (Liang et al. 2010). Culture-mediated feather degradation demonstrated at 30–55°C for 24 h–7 days.

33.5.2 Keratinase as Feed Supplement

Keratinases can be directly used as additive in animal feed and improve the digestibility and nutritional value of feed proteins. Use of crude keratinases in feed increased the amino acid digestibility of raw feather and commercial feather meal (Lee et al. 1991; Odetallah et al. 2003). Keratinases supplemented in chicken's diet improved the growth performance of broiler chickens at different ages and also the meat yield (Odetallah et al. 2003, 2005; Wang et al. 2006).

Keratinase from *Bacillus licheniformis* PWD-1 is a well-studied feed enzyme and named as "Versazyme" which is approved keratinase-based feed additive (Stark et al. 2009). The addition of "Versazyme" in mashed and pelleted diets showed beneficial effect on early growth and feed utilization of broilers (Stark et al. 2009). Versazyme increased the body weight of broilers in pelleted diet (Stark et al. 2009).

33.5.3 Keratinase for Production of Nitrogen Fertilizers

Recently, in organic farming, use of keratinolytic microorganisms has been shown to be an alternative for the enrichment through keratin waste. In addition to this, a keratinolytic strain with biocontrolling property is inferred to show the dual purpose (Raaijmakers et al. 2002). In this respect, a keratinolytic strain of *Stenotrophomonas maltophilia* R13 was used as a bioinoculated in the agricultural environments. This strain possessed antifungal activity and inhibited mycelial growth of some pathogenic fungi and produced keratinases which exhibited plant growth-promoting activity (Jeong et al. 2010).

33.5.4 Keratinase in Biomedical/Pharmaceutical Industry

33.5.4.1 Degradation of Prion Proteins

Prions are infective agents of fatal neurodegenerative diseases called transmissible spongiform encephalopathies (TSE). This includes bovine spongiform encephalopathy in cattle, scrapie in sheep and goat, and Creutzfeldt-Jakob disease in human. Infection of prion is accompanied by conversion of harmless PrP^{c} to infectious PrP^{sc}. Conformational changes in the α-helices polypeptide of PrP^{c} to β-sheets resulted

the infective conformation of PrP^{sc}. These PrP^{sc} are resistant to proteinase K. Degradation of prion or prion-like protein has been demonstrated by microbial keratinases (Langveld et al. 2003; Chen et al. 2005; Wang et al. 2005; Yoshioka et al. 2007; Sharma and Gupta 2010b).

Shih and coworker at BRI reported degradation of prion from brain tissue of bovine spongiform encephalopathy (BSE) and scrapie-infected animals in the presence of detergent and heat treatment (Langveld et al. 2003). Keratinase from *Nocardiopsis* sp. TOA-1, NAPase, efficiently degraded PrP^{sc} at pH 10.0 and 60°C in the absence of any chemical treatment (Mitsuiki et al. 2006). Thermally denatured prions were hydrolyzed by anaerobic thermophiles *Thermoanaerobacter* subsp. S290, *Thermosipho* subsp. VC15, and *Thermococcus* subsp. VC13 (Tsiroulnikov et al. 2004). Keratinase from *Streptomyces galbus* achromogenes 695–206 also acted on β-keratin-like prion plaques (Tsiroulnikov et al. 2004). Pretreatment of prion with SDS made the protein accessible to the bacterial keratinase from *Bacillus licheniformis* MSK103 (Yoshioka et al. 2007). A thermophilic keratinase from *Bacillus* sp. WF146 disintegrates prion protein at 80°C (Liang et al. 2010). These enzymatic methods have also been used in the decontamination of precision instruments that are susceptible to prion contamination (Yoshioka et al. 2007).

33.5.4.2 Keratinase in Enhanced Drug Delivery, Dermatological Treatment, and Cosmetics

Keratinases have also been utilized in the elimination of keratin in acne and psoriasis, elimination of human callus and degradation of keratinized skin, depilation, preparation of vaccine for dermatophytosis therapy and increased ungual drug delivery (Vignerdet et al. 2001; Friedrich et al. 2005; Gradisar et al. 2005; Mohorčič et al. 2007).

Keratinases are gaining importance as ungual enhancer to increase drug delivery through nail for the treatment of onychomycosis and psoriasis, common fungal infections of nails. Current oral treatments increase the complication and side effects such as pain and systemic adverse events. Topical applications of medicine are limited due to poor permeability of nail plates. Keratinases can act on nail plates (Friedrich and Kern 2003; Gradisar et al. 2005) to increase its porosity which may facilitate drug diffusion through nail plates (Mohorčič et al. 2007).

In addition to this, keratinases accelerate healing processes and also be used in the medicine of trauma. It might act to remove scar and regenerate epithelia (Chao et al. 2007). Bioactive peptides generated after degradation of keratin may also be explored for pharmaceutical applications (Riessen and Antranikian 2001; Matsui et al. 2009).

33.5.5 Keratinase in Leather Industry

Keratinolytic enzymes are extensively used in leather industry mainly for dehairing process. Utility of sulfide for tanning process can be replaced by the use of keratinases

(Friedrich et al. 2005; Anbu et al. 2006; Macedo et al. 2005; Wang et al. 2007). In this respect, many keratinases from *Bacillus halodurans* PPKS2 (Prakash et al. 2010), *Bacillus halodurans* JB99 (Shrinivas and Nayak 2011), and *Bacillus cereus* MCM-B-326 (Nilegaonkar et al. 2007) were demonstrated for dehairing of goat and buffalo skin.

Use of mixture of enzymes, i.e., proteases, lipases, and carbohydrates are well exploited for various tannery stages (Saravanabhavan et al. 2004; Thanikaivelan et al. 2004) in the bioprocessing of leather. Non-collagenolytic and mild elastolytic keratinases are increasingly being explored for dehairing processes. Enzyme would help in the selective breakdown of keratin tissues in the follicle thereby pulling out intact hair without affecting the tensile strength of leather (Macedo et al. 2005).

Keratinases are successfully used as depiling agents (Letourneau et al. 1998; Bressollier et al. 1999; Allpress et al. 2002; Friedrich and Kern 2003). Keratinase from *Bacillus subtilis* S14 (Macedo et al. 2005) completely eliminated the use of sodium sulfide during the tanning processes.

33.5.6 Other Applications

Apart from above-mentioned uses, keratinases can also be used to modify the functional properties of proteins, such as solubility, emulsification, and gelation in the food industry. Keratinases might be useful for the preparation of peptides by hydrolysis of casein and keratin (Wang et al. 2003; Casarin et al. 2008).

Keratinases also find application in detergent industry. Keratinases exhibited significant activity and stability in alkaline pH, low-to-moderate temperature, surfactant, compatible to detergents, and stable in bleach which makes them potential additive in detergent formulation (Rai et al. 2009; Zhang et al. 2009; Prakash et al. 2009; Xie et al. 2010). Keratinases show great ability to hydrolyze solid substrates. This property can be used to clean proteins attached to hard and solid surfaces. They can encounter keratinous soil attached to the collar of shirts where generally proteases fail to act (Gessesse et al. 2003). Keratinases could also be used in waste water treatment to remove clogs in bathroom drain pipes and to clear obstructions in sewage system (Takami et al. 1992; Chitte et al. 1999; Tapia and Contiero 2008).

Keratinases show potential hydrolysis of structural proteins which can be exploited for degumming of silk and finishing textile fibers like wool. They finish the fibers and increase smoothening, shining, and dying capacity (Riessen and Antranikian 2001; Onar and Saruşik 2005; Cao et al. 2009).

Nowadays, use of biodegradable films, coatings, and glues is gaining importance. In this respect, processed keratinous wastes have been used for the formation of biodegradable products (Schrooyen et al. 2001; Schrooyen and Radulf 2004; Gupta and Ramnani 2006). Keratinous wastes are also used in the medium for production of mosquitocidal toxins (Poopathi and Abidha 2008).

33.6 Conclusions

Keratinases are valuable enzymes owing to their unique ability to act on recalcitrant proteins. These enzymes are largely produced in the presence of keratinous substrates like hair, feather, wool, horn, and native human foot skin. A vast diversity of keratinolytic microorganisms has been added to the literature in the last two decades. Keratinases are robust enzymes with wide pH and temperature activity range and are largely serine and metalloproteases.

Keratinases play an important role in environmental cleanup and convert keratinous waste into various biotechnological products as protein supplements for feed and as nitrogen fertilizer. They outstand from conventional proteases with their potential applications in the pharmaceutical sectors as ungual enhancers, prion degrader, treatment of dermatophytosis, and removal of scar and acne. However, still their utility in these upcoming sectors is under exploited due to limited availability of efficient keratinases with versatile substrate specificity and better catalytic efficiency. To overcome this, the advances in keratinase research should be directed toward improving the characteristics of existing keratinases or searching for novel keratinases with broader substrate specificity.

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Chapter 34
Biocatalysis Through Thermostable Lipases: Adding Flavor to Chemistry

Rohit Sharma, Vishal Thakur, Monika Sharma, and Nils-Kåre Birkeland

Abstract In this technology-oriented world, when every phase is going green, enzymes have found tremendous applications. Thousands of enzymes have been identified and are being used commercially. However, microbial origin enzymes have gained more relevance than those from other sources. Among others, lipases are spectacular enzymes known for their unique attributes and significant industrial potential. They are one of the most important biocatalysts known for their applications in the biotechnology industry. Since these enzymes find massive applications, with the passage of time, the trend has shifted towards the identification of thermostable lipases which could be used in the industries which require harsh conditions to work in. Thermostable lipases have found applications in various areas such as in pharmaceuticals, food, and chemical industries. The following article talks about the thermophilic lipases derived from various microorganisms and their applications.

Keywords Biocatalysts • Lipases • Thermophile/s • Thermostable • Triacylglycerols

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34.1 Introduction

Today's technologies survive mostly on the basis of the products derived from various life forms, be it a product obtained from a plant, animal, or microorganisms. Pharmaceutical, agrochemical, biotechnology, and other such related industries, all make use of the biologically derived products. Enzymes derived from microorganisms possess a wide variety of applications. These biomolecules produced by living organisms are important to carry out various biochemical reactions to support life (de Carvalho 2011). Out of the huge lot of enzymes, lipases form the most viable and versatile enzyme. Lipases derived from different microorganism have been reported for their massive applications, but with the development, the trend has shifted from mesophilic to thermophilic lipases because of their unique attributes. In the present scenario, microorganisms from extreme environmental conditions have been drawing attention because of their adaptability and huge biotechnological applications (Morozkina et al. 2010). Extremozymes from extremophiles possess increased stability not only for one condition but for multiple conditions, for example, enzyme that stays active in organic solvent and at high temperature (Sharma et al. 2002a, b; Baharum et al. 2003; Satyanarayana et al. 2005; Borkar et al. 2009).

Most of the industrial processes are carried out at high temperature where biocatalysts from mesophilic origin suffer denaturation and are therefore not suitable for biocatalysis. However, biocatalyst from thermophilic microorganisms due to their adaptability not only catalyzes biochemical transformation but also maintains their biocatalytic efficiency and reproducibility. Biocatalysis at elevated temperature offers certain advantages like increased solubility of compound in organic solvents, reducing viscosity, increased product yield, and lower contamination chances, and all these attributes offered by biocatalyst are of significance (Haki and Rakshit 2003; Torres and Guillermo 2004; Hasan et al. 2006).

Lipases are known as one of the most imperative biocatalysts in today's epoch and carry out novel reactions both in aqueous and nonaqueous environment as they possess *regio-*, *enantio-,* and *chemo*-selectivity. Generally lipases are known as glycerol ester or serine hydrolases and true lipases as triacylglycerol ester hydrolases and are known to be distributed in the five kingdoms of life (Jaeger et al. 1999; Chakravorty et al. 2011). Structurally they contain α/β hydrolases fold (Schrag and Cygler 1997). The fold contains eight parallel β strands with second one antiparallel, connected by α helices packing on either side of the β strands. Lipases possess a three-dimensional structure with a catalytically active site which comprise of three residues, namely, a serine residue (nucleophilic in nature) in a Gly-X-Ser-X-Gly motif, an acidic residue (glutamic acid or aspartic acid), and a histidine residue (Jaeger and Reetz 1998). All the three residues act in cooperation with each other so as to bring about the hydrolysis of the esters. Depending on various biological properties and amino acid sequences comparisons, these enzymes have been classified into eight diverse families (I–VIII) (Royter et al. 2009).

These enzymes are found to be highly active at the water-lipid interface, but in the presence of the monomeric substrate, there is a massive decrease in their activity, which increases dramatically as soon as the substrate emulsion is formed, that is, the

lipases activity increases beyond the solubility limit. This attribute of lipases has led to the development of a phenomenon known as the "interfacial activation" that can be traced to unique structural characteristic of this class of enzyme (Mohanty et al. 2001; Reetz 2002; Salameh and Wiegel 2007). Lipases hydrolytic activity is enhanced during the availability of the water-lipid interface. These activities are reversed in the presence of the nonaqueous environment, during which the active site of these enzymes is covered by the hydrophobic lid. It is proved by both computational and experimental approaches that interfacial activation, a hallmark for lipase action mechanisms, is because of flap or lid movements where lid opens only when it recognize oil–water interface or otherwise remained closed (Balashev et al. 2001; Wang et al. 2010).

Lipases are one of the most versatile, relevant, and important enzyme in the field of biotechnology and find massive applications in the food (ripening of cheese, flavor development), detergent, wastewater treatment, fine chemistry, diary, paper, textile, agrochemical (pesticides, insecticides), oleochemical (biosurfactant synthesis, fat and oil hydrolysis), and pharmaceutical (ibuprofen, naproxen) industries (Arvindan et al. 2001; Saxena et al. 1999; Sharma et al. 2001; Jaeger and Eggert 2002; Reetz 2002; Cammarota and Freire 2006). During the last decade, lipases have gained a massive preference over other industrially important enzymes such as proteases and amylases especially in the field of organic synthesis. The most favorable characters make it a gracious enzyme of choice especially for chiral drug resolutions, cocoa butter substituents synthesis, fat modification, biofuels, personal care products, and in the synthesis of flavor enhancers. Thus, these characteristics of lipases make it an enzyme of choice for microbiologists, biotechnologists, pharmacists, biochemical and process engineers, biophysicists, organic chemists, and biochemists (Gaur et al. 2008; Saxena et al. 1999). Microbial lipases find extensive applications in the industries comparative to the lipases derived from plants and animals, since microbes can be easily cultured in the laboratories. Lipases derived from microorganisms take part in the catalysis of wide spectrum of synthetic and hydrolytic reactions either in short chain fatty acids (esterases) or in long-chain triglycerides (lipases) (Arpigny and Jaeger 1999; Gunasekran and Das 2005).

34.2 Global Enzyme Market

Industries continue to demand more selective and efficient catalysts and processes for the manufacture of fine chemicals. The use of biocatalysis for industrial synthetic chemistry is on the way of significant growth. The process of biocatalysis has been commercially employed since long time by mankind. After 1894 when Emil Fischer elaborated the essence of enzyme catalysis, this discipline gained more momentum. The main reason for using enzyme for biotransformation is because of their high selectivity, precision to allow process simplification, and waste stream reduction to meet the set principle of green chemistry. Cost of the process can further be reduced if improvements are made to reach high space time yields using biological and engineering principles. As the use of biocatalysis for industrial chemical synthesis becomes easier, several chemical companies have begun to increase significantly the number and sophistication of the biocatalytic processes used in their synthesis operations

(Schmid et al. 2001). Enzyme market of industrial importance is continuously increasing and further likely to grow in future. It is manifested from data that enzyme market was about 5.1 billion US dollar in 2009 (Sanchez and Demain 2010).

Key factors driving market growth include new enzyme technologies endeavoring to enhance cost efficiencies and productivity and growing interest among consumers in substituting petrochemical products with other organic compounds such as enzymes. Other factor propelling market growth includes surging demand from textile manufacturers, animal feed producers, detergent manufacturers, pharmaceutical companies, and cosmetics vendors.

According to market survey that is recently published by CHEManager in 2008, the expected growth of chemistry market is likely to be 3,235 US dollar by 2015 and 4,000 US dollar by 2020 (www.chemanager-online.com/en). However, the industrial biotechnological market is representing about 50 billion US dollar. From 2001 to 2010, the share of biotechnologically manufactured product increases from 8 to 60%. It is also speculated that by 2020 approximately 20% of fine chemical are likely to be produced only through biotechnological route (Ghisalba et al. 2010).

Similarly BCC (2011) reported that global market for industrial enzyme is likely to grow by 4.4$ billion by 2015. Global Industry Analysts, Inc. USA, released a comprehensive global report on industrial enzymes market which is likely to reach $ 3.74 billion dollar by 2015 (GIA 2012). According to report published in 2012, Europe represents the largest region for industrial enzymes worldwide. However, United States trails Europe in terms of sales of industrial enzymes. United States and Europe collectively still command a major share of the world industrial enzymes market. On the other hand, Asia Pacific is poised to register the fastest compounded annual growth rate of more than 8.0% over the analysis period.

From the above statistical data, one can elaborate the importance of biocatalysis in chemical industries. Presently more than 75% of industrial enzymes belong to hydrolases family. Lipases are one of the most important groups of biocatalysts among this family for biotechnological applications that account for approximately 5% of the market (www.freedoniagroup.com/World-enzymes.html). Therefore, need for biocatalyst that can carry out biotransformation of industrially important compounds without losing their biocatalytic prowess is the main focus. Extremophile and their biomolecules can serve this purpose successfully owning to their adaptation to extreme conditions. For processes requiring high temperature to operate, biocatalysts from thermophilic origin can solve the problem. Also their stability in alkaline pH and organic solvent for long time is mostly sought for industrial processes.

34.3 Thermostable Lipases: A Versatile Tool in Biocatalysis

Lipases (EC 3.1.1.3) have appeared as one of the most extensively used enzymes in the field of organic chemistry and biotechnology applications (Aly et al. 2012).

Lipases derived from mesophiles act in wide range of pH but are unstable at higher temperatures (generally beyond 70°C). The temperature optima for bacterial

lipases generally lie between 30 and 65°C. A large number of thermostable lipases have been isolated from thermophiles recovered from diverse of environments (Akanbi et al. 2010). Their thermostability particularly in the absence of sufficient amount of water has made them more attractive (Nawani et al. 1998). Certain thermostable lipases have been isolated from moderate thermophiles, especially from the representatives of the genus *Bacillus* (Sugihara et al. 1991; Wang et al. 1995; Fakhreddine et al. 1998; Sharma et al. 2002a, b). Thermostable lipases have been reported from *Bacillus thermoleovorans* ID-1 (Lee et al. 1999), *Bacillus* sp. J33 (Nawani and Kaur 2000), *B. stearothermophilus* (Kim et al. 1998, 2000; Sinchaikul et al. 2002), *Lactobacillus* (Lopes et al. 2002), and *Pseudomonas* sp. (Sugihara et al. 1992; Lee and Rhee 1993; Ahn et al. 1999).

The inherent thermostability of proteins from thermophiles is brought about by an increased number of stabilizing molecular interactions including extended ionic networks of hydrophobic interactions, hydrogen bonding, and disulfide bonds, as well as shorter surface loops and capped N- and C-terminal compared to the mesophilic counterparts. In addition to this, thermophilic enzymes possess a more rigid and packed conformational structure, causing low catalytic activity at low and moderate temperatures acquire low activity, which is a reason of dilemma since a number of fine chemistry compounds are stable at these temperatures only (Iyer and Ananthanarayan 2008). The main problem associated with these biocatalysts is the instability which they acquire in the presence of adverse conditions such as the presence of organic solvents, extremes of pH, temperature, pressure, and ionic strength which makes the processes involved economically unfeasible.

Immobilization can be used in certain cases to improve the stability of the enzymes. The use of immobilized enzymes over soluble enzymes provides certain advantages in industrial processes such as easy recovery of product and biocatalyst, reduction of denaturant effects, continuous processing, inhibition of aggregate formation in organic media, and modification of physical and chemical properties. Enzyme immobilization requires a rational and carefully planned strategy since random immobilization can lead to decreased enzyme rigidity and decreased enzyme stability, caused by unpredicted interactions of the support with the enzyme (Mateo et al. 2007).

Immobilization of lipases and esterases has been brought about by various techniques such as adsorption by covalent and ionic bonds as well as occlusion and physical adsorption (Fernandez-Lafuente et al. 1995; Palomo et al. 2003; Palomo et al. 2004; Wang and Hsieh 2008; Bolivar et al. 2009). Some researchers reported the immobilization of lipases derived from thermophilic microorganisms *Geobacillus thermoleovorans* on propylene support which shows increased thermostability after immobilization (Sánchez-Otero et al. 2008). Nawani *et al*. (2006) studied lipase derived from *Bacillus* sp. which showed optimum activity at pH of 8.5 and at 60°C temperature. Immobilization of lipases derived from various bacterial and fungal sources like those derived from *Bacillus thermoleovorans* CCR11*, B. coagulans, B. stearothermophilus, B. thermocatenulatus, Rhizopus oryzae, Thermus thermophilus, T. aquaticus* on different matrices like silica, DEAE cellulose matrix have shown an increase in the thermostability of the enzyme (Cowan and Fernandez-Lafuente 2011).

For commercialization of important biocatalysts like lipases, thermostability is a major factor and an important enviable characteristic. As the temperature increases, there is an increase in the rate of the reaction; therefore, relatively higher temperatures are feasible for an increase in the reaction productivity (Sharma et al. 2001). The most precious advantage associated with reactions at higher temperatures is that it reduces the contamination by common mesophiles at higher temperatures (Becker 1997). Since most of the industrial processes which make use of lipases work at a temperature beyond 45°C, hence it becomes important that the optimum temperature exhibited by the enzymes should be at least in access of 50°C (Sharma et al. 2002a, b).

34.3.1 Factors Affecting the Thermostability of Lipases

To determine the mystery behind the thermostability of the proteins at elevated temperatures, a significant amount of research has been carried out (Yano and Poulos 2003; Trivedi et al. 2006). Increased protein hydrophobicity (Haney et al. 1997; Sadeghi et al. 2006) and protein rigidity is due to the presence of proline residue (Bogin et al. 1998), compact protein structure (Russell et al. 1997), increased hydrogen bonding (Vogt and Argos 1997; Gromiha 2001; Sadeghi et al. 2006), and decreased thermolabile residues occurrence (Russell et al. 1997) which are mainly responsible for the thermostability of the proteins (Vieille and Zeikus 2001; Sharma et al. 2011; Chakravorty et al. 2011).

Knowing physical bridge between sequence difference in mesophilic and thermophilic enzymes and their adaptability for higher temperature is the prerequisite to understand the robust nature of life. Quest for understanding the thermostable nature of protein is forced by two factors: first to find answer for evolutionary trends since thermostable protein are believed to be originated from their mesophilic ancestors, and second is by developing successful design model for such proteins, would have numerous applications for industrial processes (Vogt et al. 1997).

In thermophilic organisms membrane plays an important role as it protects the cellular components from denaturation. Saturated fatty acids on the membrane provide a hydrophobic environment and maintain the cell rigidity at elevated temperature. In case of hyperthermophiles, which belong to the Archaea domain, lipid linkage on to the cell wall accounts for their resistance to heat denaturation (De Rosa et al. 1994). Thermostable lipases can be obtained from various thermophilic microorganisms. However, they can usually not be used directly because of their low yield. The alternative way of obtaining thermostable lipases is to screen thermophilic microorganisms for thermostable lipases and compare the sequence and structure with mesostable counterparts so as to floor the way for the in vitro evolution of enzymes from mesophilic organism with enhanced thermostability.

The major factors responsible for the thermostability of the proteins.

34.3.1.1 Role of Metal Ions

One of the area known as the most potential habitat for thermophiles is Indonesia. A number of thermophilic bacteria have been isolated from various geothermal locations in Indonesia, especially from West Java. A thermophilic bacterium was isolated from Manuk hot spring for its lipases, and it was found that the interaction of lipases with metal ions such as Ca^{2+} was responsible for its structural thermostability (Widhiastuty et al. 2011).

Ca^{2+} and Zn^{2+} found tightly linked to lipases. Ca^{2+} restricts the conformational flexibility of certain helices and loops and brings about the stabilization of His residue through hydrogen bonding and thus leads to lipases thermostability (Chakravorty et al. 2011). The same has been studied in *Pseudomonas* and *Burkholderia* sp. derived lipases. Removal of Ca^{2+} leads to unfolding and aggregation of proteins (Invernizzi et al. 2009). Ca^{2+} ion acts as an activator of lipase and plays a vital role in protein stabilization. Li et al. (2011) reported a twofold increase in lipase activity from *S. mercescens* ECU1010, when calcium ions were added in the buffer to refold the lipase. Since Ca^{2+} ion manages the stability of both thermostable and mesostable lipases, therefore it is not the sole criteria to govern thermostability of the protein. It was found that Zn^{2+} ions in lipases were solely playing their structural role in bringing about the stabilization of catalytic domain of lipases and not being involved in the catalysis. Apart from this it has been found that the Zn^{2+} ion coordinating residues are not only found in thermostable lipases of *Bacillus* and *Staphylococcus* but also in mesostable *Staphylococcus epidermis* lipase and occupying same position. Thus, it clearly depicts that Zn^{2+} ion, although being responsible for an important characteristic, is solely not responsible for thermostability of lipases, but at the same time if both Ca^{2+} and Zn^{2+} ions are found together in lipases, their coordination helps in stabilizing the lipases structure and hence lead to the stability of lipases at elevated temperatures (Chakravorty et al. 2011).

34.3.1.2 Role of Amino Acids

The variation in the amino acids composition is known to be a reason for difference between thermostable and mesostable proteins. There is a difference in the amino acids composition in meso- and thermostable lipases. The majority of thermophilic proteins in comparison to their mesophilic counterparts show increased number of salt bridges and side chain hydrogen bonds that significantly contribute to lower the enthalpy of folded state of protein. High resolution structures for many hyperthermophilic proteins provide significant information about the role that side chains of amino acids played in ionic networks (Chisti 1999; Ladenstein 2008).

Thermostable bacterial and fungal lipases (TBFL) have lesser Cys percentage (0.87%) than in mesostable bacterial lipases (MBL) which is 0.97%. Thermostable fungal lipases (TFL) have more Cys content than the thermostable bacterial lipases (TBL), that is, 1.99% than 0.48%. TBL possess more number of charged amino acids residues like Glu, Arg, and His (3.94%) than those present in MBL which is

just 3.23%. Another difference is in the composition of Asp and Lys residues which is 4.85% in TBFL and 5.33% in mesostable lipases (ML), whereas the amount of Asp in TFL is 6.18% which is far more than those for TBL (5.67%) and ML (5.92%). Aromatic and proline residues are in higher amounts in TBFL (3.26% and 4.65%, respectively) than in ML (2.95% and 4.24%, respectively). ML have higher Gly residues, that is, 9.98% than 9.91% in TBFL; however, TBL have 10.55% of Gly residues which is higher than TBFL. Thermostable lipases have labile amino acid residues like Gln and Met in lesser amount (3.32%) than those in ML (3.73%). B-branched residues like Val, Leu, and Ile are comparatively less in TBFL (6.63%) than in ML (7.37%). Val and Ile show an increment in mesostable lipases, whereas hydrophobic residues demonstrate varying trend in TFL, TBL, and ML (Chakravorty et al. 2011).

The presence of more charged amino acids residues leads to proteins thermostability as they are responsible for electrostatic interactions which play an important role in stabilizing the secondary structures of proteins. Cys residues are sensitive to oxidation at higher temperatures, and thus when present in disulphide linkages, they enhance the thermostability, and when present in disulphide linkages, they enhance the thermostability, and when present as free residues, they decrease the thermostability (Chakravorty et al. 2011). Apart from this, understanding about thermostability is not only required only from a theoretical point of view about physiochemical principles responsible for protein folding at high temperature but also for rational design of a new and efficient biocatalyst (Kumar et al. 2000).

34.3.1.3 Role of Organic Solvents

Water serves as a molecular lubricant for enzyme. However, these smart biomolecules remember their history in organic solvent as they are kinetically trapped in their previous conformations (Rupley and Careri 1991). Lipases are the most preferred enzymes from an industrial point of view because of their solvent tolerance (Khare et al. 2000; Tsai et al. 2006). Polar organic solvents are not pernicious than nonpolar solvents as they place enzymes in rigid conformation by stripping off essential water layer surrounding the biomolecule. From industrial point of view, solvent tolerance is an added advantage for a potential biomolecules. Due to rigid structure acquired by enzymes in dry media, their resistances increased for thermal vibrations and ultimately enhance their industrial values (Hernandez-Rodriguez et al. 2009).

34.4 Sources of Thermostable Lipases

The widespread flora and fauna of our mother earth is a native place where lipases are found in abundance. Lipases are most abundantly found in bacteria, yeast, and fungi and are generally extracellular (Abd Rahman et al. 2009; Contesini et al. 2010; Wu et al. 1996). Microbial lipases are commercially more significant as these pos-

sess greater stability, have low production costs, and are more easily available than lipases from plant and animal (Joseph et al. 2007; Pandey et al. 1999). However, to meet global demand for thermostable, lipases screening of more thermostable lipase producers from diverse locations like hydrothermal vent and hot spring is required. A number of *Bacillu*s sp. have been reported as the main source of lipases (Ahmed et al. 2010; Kim et al. 1994; Schmidt et al. 1994; Luisa et al. 1997: Sharma et al. 2002a, b). The microorganisms are isolated from diverse extreme conditions like hot spring and marine environmental conditions (Nawani et al. 1998; Sharma et al. 2002a, b; Annamalai et al. 2011).

Most of these enzymes are generally stable and active at a temperature of about 60°C and pH around 7.0, but the lipases derived from *Bacillus thermoleovorans* and a thermophilic *Rhizopus oryzae* work extremely well at extreme of temperatures and pH values (Dong-Woo et al. 1999; Abel et al. 2000). A lipase reported from *Bacillus thermoleovorans* AAD30278 (NCBI accession number) retained 50 and 30% of initial activity after 1 h of incubation at 60 and 70°C, respectively (Cho et al. 2000). Lipase *Bacillus licheniformis* was also found to be quite stable at high temperature. The temperature stability study showed 100% retention in enzyme activity at 60°C and 65% activity at 80°C, respectively, after 1 h of incubation (Annamalai et al. 2011). Akanbi *et al.* (2010) isolated thermostable lipase producer *Bacillus* from Selayang hot spring in the western part of Peninsular Malaysia that shows 98% homology with *Bacillus cereus* strain. This lipase retained 77% of its original activity after exposed to 80°C for 30 min. Lipase producer *Bacillus sp.* RSJ-1 isolated from hot spring of Manikaran, India, was found to be quite stable at 50°C for 1 h and retain more than 90% of its original activity after 2 h of exposure to same temperature (Sharma et al. 2002a, b).

Many *Geobacillus* species, for example, *G. thermoleovorans, G. stearothermophilus*, *G. thermocatenulatus*, *G. toebli* sp., *G. thermodenitrificans*, are known for their ability to survive in the mesophilic to thermophilic temperature range (Balan et al. 2010; Sunna et al. 1997; Nazina et al. 2001; Sung et al. 2002). Rahman et al. (2007) isolated and screened 29 potent lipase producers from oil palm mills from Malaysia. One of the isolated selected on the basis of maximum lipase production was identified using physiological and biochemical tests, DNA/DNA hybridization, RiboPrint analysis; the length of lipase gene and protein pattern allowed genotypic and phenotypic differentiation as *Geobacillus zalihae* sp. nov. Crude lipase studied from *G. zalihae* sp. shows optimum temperature of 70°C and was also stable up to 60°C after 30 min of incubation without significant loss of enzyme activity (Table 34.1).

34.5 Production of Lipases

The production of thermostable lipases has been reported from different microorganisms under different conditions. Wang et al. (1995) reported a highly thermostable alkaline lipase from a thermophilic *Bacillus* strain A30–1 (ATCC 53841). Similarly a thermostable lipase produced by a thermophile growing optimally at

Table 34.1 Catalytic activity of some thermostable lipases at respective optimum temperatures and pH

Microorganism	Enzyme optimum pH	Enzyme optimum temperature (°C)	References
Bacillus sp. RSJ-1	8.0–9.0	50	Sharma et al. (2002a, b)
Bacillus acidocaldarius	–	70	Manco et al. (1998)
Bacillus strain J33	8.0	60	Nawani et al. (1998)
Bacillus thermocatenulatus	8.0–9.0	60–70	Klibanov (1983)
Bacillus stearothermophilus	–	68	Gupta et al. (1999)
Geobacillus sp.	9.0	70	Abdel-Fattah (2002)
Pseudomonas sp.	11.0	90	Rathi et al. (2000)
Pseudomonas sp.	9.6	65	Kulkurani and Gadre (1999)
Pyrobaculum calidifontis	–	90	Hotta et al. (2002)
Pyrococcus furiosus	–	100	Ikeda and Clark (1998)
Pyrococcus horikoshii	7.0	95	Yan et al. (2000)
Pyrococcus horikoshii	5.6	97	Ando et al. (2002)
Staphylococcus aureus	5.0–12.0	55	Horchani et al.(2008)
Rhizopus chinensis	6.0–9.0	30–50	Sun et al.(2009)
Thermoanaerobacter thermohydrosulfuricus	8.0	75–90	Royter et al.(2009)
Staphylococcus xylosus	4–11	60–70	Khoramnia et al. (2010)
Bacillus sp.	6–10	55–80	Shariff et al. (2011)
Bacillus pumilus RK31	6.0	40–70	Kumar et al. (2012)

50°C and showing the high activity and stability in temperatures above 50°C has been reported by Sharma et al. (2002b). *Bacillus megaterium* AKG-1-derived lipases under submerged fermentation conditions have been reported for its thermostable attribute (Sekhon et al. 2006). A mesophilic bacterium producing thermostable lipases was isolated from the food oil waste in Malaysia. The bacterium was identified as *Staphylococcus xylosus.* Enzyme was characterized biochemically and claimed as the first report about a mesophilic *Staphylococci* bacterium with a high thermostable lipase which is able to retain 50% of its activity at 70°C after 90 min and at 60°C after 120 min (Khoramnia et al. 2010). Lipases derived from *Staphylococcus* are extracellular in nature, and like other lipases, they are also influenced by various environmental parameters like temperature, pH, and oxygen solubility (Ebrahimpour et al. 2008; Talon and Montel 1997).

Lipases derived from *Bacillus* sp. L2 are known to produce extracellular thermophilic lipases which possess maximum activity in the temperature range of 55–70°C, with a lipolytic activity of more than 70% and maximum activity at a temperature of 70°C (Shariff et al. 2011). Other *Bacillus* sp. that has been isolated

from Malaysian hot springs produces extracellular lipase for one of the isolate (SY7) which shows thermostability and retains 77% of its original activity at 80°C after 30 min. The modern technique 16s RNA analysis of the thermostable lipase producer shows 98% gene sequence homology with *Bacillus cereus* J-1 (Akanbi et al. 2010).

34.6 Applications of Thermostable Lipases

The use of chemical catalysts not only possesses many disadvantages associated with it but also gives rise to many undesired by-products along with noxious effluents, whereas on the other hand, substrate specificity, biodegradability, and many such advantages associated with biocatalysts give them an edge over the use of chemical catalysts in the industries. Thermostable enzymes are able to brave high temperature, thus endow longer half-life to the biocatalyst. These enzymes can also endure higher concentrations of substrates and are also resistant to chemical denaturants. Their ability to conduct various reactions at a higher process rate because of increase in substrate diffusion coefficient and reduced viscosity at higher temperatures makes them a preferred choice over mesophilic sources (Ebrahimpour et al. 2011). The biotechnological potentials associated with lipases make them an appropriate biocatalyst in the arena of many biotechnology industries.

34.6.1 In Food Industry

Lipases encompass a substantial significance in the food industry wherein they are used for the production and modification of oils and fats (since they form an important component of food) so as to produce healthier food. The modification of the lipids is brought by lipases by bringing about the alteration in the position of the fatty acid chains in various glycerides (Sharma et al. 2001). The specificity associated with lipases makes them an ideal choice for oleochemical industries for the production of numerous high added value products like human milk fat substitute, cocoa butter equivalents, and other specific-structured lipids (Xu 2000; Fernandez-Lafuente 2010). Since no chemical method presents such specificities, this specific attribute of lipases can be targeted for commercial and industrial developments.

Lipases from *Thermomyces lanuginosus* have been known to bring about various esterification and transesterification reactions. Lipases from *T. lanuginosus* have been known to carry out the formation of glycerides so as to produce healthier margarines (Zhang et al. 2004, 2006; Fernandez-Lafuente 2010). Lipases have also been involved in the development of various flavoring agents. Thermostable lipases from many microorganisms have been used to bring about the modification of oils, production of human milk fat-resembling lipids, formulation of margarines using glycerides, etc. (Fernandez-Lafuente 2010).

34.6.2 In Detergent Industry

The detergent industry is one of the largest markets for the enzymes, and a massive development is taking place in order to bring in the new enzymes like lipases, proteases, and amylases with higher and better potential. One of the most commercially important applications of lipases is in the detergent industry wherein it is added to the detergent for enhancing the action of the latter (Sharma et al. 2002b). Enzyme sales in 1995 have been estimated to be $30 million, with detergent enzymes making up 30%. Approximately 1,000 t of lipases are added to the approximately 13 billion tons of detergents produced each year. One of the major issues in the laundries is the removal of the adsorbed lipids from the fabrics, which are generally comprised of the long chain, water insoluble triacylglycerols, whose removal can be accomplished by employing the use of detergents containing lipolytic enzymes which can bring about the degradation of these triacylglycerols into free fatty acids, mono- and di-acylglycerols (Snabe et al. 2005; Thirunavukarasu et al. 2008).

The first commercialized lipase to be used in detergents was Lipolase™ which was introduced by Novo Nordisk in 1994. Lipolase™ is a fungal lipase as it was derived from fungus *Thermomyces lanuginosus*. Since the production was low commercially, therefore it was expressed later in *Aspergillus oryzae*. In 1995, two bacterial lipases were introduced—Lumafast™ from *Pseudomonas mendocina* and Lipomax™ from *Pseudomonas alcaligenes*, both produced by Genencor International (Jaeger and Reetz 1998; Romdhane et al. 2010).

The presence of lipases as a component of the detergent improves the washing capability of the detergent, helps in removal of stringent stains, and prevents scaling (Hasan et al. 2006). Lipases derived from *Pseudomonas* sp. and *Talaromyces thermophilus* are active at alkaline pH and are thermostable; therefore, they are promising candidates for the detergent industry (Hemachander and Puvanakrishnan 2000; Romdhane et al. 2010).

34.6.3 In Drug and Drug Intermediates

Lipases have also been reported for their ability to bring about the resolution of numerous racemic mixtures of alcohols and acids (Parve et al. 1993; Schmidt et al. 1996). Thermostable lipase from *Thermomyces lanuginosus* (TLL) has been described as one of the best enzymes for bringing about the kinetic resolutions of synthetically important chiral building block, (Z)-4-triphenylmethoxy-2,3 epoxybutan-1-ol, making use of vinyl acetate as acyl donor. Another instance shows the double enantioselective hydrolysis by TLL-derived lipases of (1S,2R,3R,5S,6R)-2-bromo-3-butanoyloxy-6-hexanoyloxybicyclo [3.2.0] heptane, so as to obtain optically highly pure compounds like (−)-bromodiol and (−)-epoxyalcohol (Parvwe et al.1993; Fernandez-Lafuente 2010).

Lipozyme *T. lanuginosus* IM has been successfully used for the regioselective synthesis of 5′-O-acyl 5-fluorouridines, which is a strong antitumor agent compared to the parent molecule 5-flourouridine (Fernandez-Lafuente 2010).

Patel et al. (1994) reported the stereoselective enzymic hydrolysis of cis-3-(acetyloxy)-4-phenyl-2-azetidinone to the corresponding (S)-(−)-alcohol was carried out lipase PS-30 (*Pseudomonas cepacia*) and BMS (Bristol-Myers Squibb) lipase New Jersy, USA (*Pseudomonas* sp. SC13856). Reaction results in the hydrolysis, resulting in (S)-(−)-alcohol and the desired (R)-(+)-acetate. Reaction yields of >96% and optical purities of >99.5% were obtained. The chiral intermediate formed using enzymatic method is a key for the semisynthesis of paclitaxel (taxol), an anticancer compound. A thermostable lipase derived from *P. aeruginosa* was capable of carrying out the enantioselective hydrolysis of methoxyphenyl glycidic acid methyl ester [(±)-MPGM], which is an intermediate essential for the synthesis of diltiazem, a cardiovascular drug (Sharma et al. 2003; Singh and Banerjee 2005).

34.6.4 Other Applications of Thermostable Lipases

The degradation of polymers present in the environment has always been a trouble, but with the isolation of thermostable lipases, this problem has been overcome to a degree. The degradation of side chain of poly (vinyl acetate) was performed at 60°C by different lipases in the presence of toluene. The longer side chains were hydrolyzed in the order as hog-pancreas lipase > Novozyme 435 > TLL > *Candida rugosa* lipase; on the other hand, the hydrolysis of short side chains was brought about in the reverse order. The degradation of many other polymers such as polycaprolactone and poly (*bis*phenol) has been brought at different temperatures ranging between 26 and 70°C using different lipases such as lipases from *Candida rugosa*, hog-pancreas, TLL, and Novozyme 435 in the presence of various solvents. The optimum temperatures reported for hog-pancreas lipases and others, respectively, were around 50 and 60°C, whereas the degradability potential/lipase activity was in the array of TLL > *Candida rugosa* > Novozyme 435 > hog-pancreas (Fernandez-Lafuente 2010).

Zheng et al. (2011) showed that the fungi *Thermomyces lanuginosus* is one of the best eukaryotic sources of thermostable lipases. Lipase derived from the said source was found to be stable within a temperature range of 40–70°C, compared to thermostable lipases from other eukaryotic sources which are found to be stable till 50°C. The highly stable lipase from *Thermomyces lanuginosus* is thought to be highly suitable for cosmetic industry.

Because of abundance of enthralling characters like biodegradability, permeability, and biocompatibility associated with poly ε-caprolactone (PCL), it has gained massive attention in the field of biomedicines (Srivastava, and Albertsson 2006). As of today many esterases and lipases have been reported and used for their ability to bring about the ε-caprolactone ring opening polymerization, such as *Pseudomonas cepacia* lipase, *Humicola insolens cutinase* (HIC), porcine pancreatic lipase, and *Candida Antarctica* lipase B (CALB) (Henderson et al. 1996; Namekawa et al. 1999; Kumar and Gross 2000). But due to instability associated with mesophilic enzymes in the production of PCL at industrial level, wherein harsh conditions such

as high temperatures and exposure to organic solvents is concerned, these enzymes are not preferred. The instability associated with these enzymes inhibits the integration of the enzymatic and chemical polymerization as a single step for the synthesis of novel polymerases (Duxbury et al. 2005). Novel thermostable lipases from anaerobic bacterium *Fervidobacterium nodosum* were isolated which are capable of growing at a temperature of 60°C with the enzyme optima is 80°C. The thermophilic lipase so isolated leads to effective esterases hydrolysis, cleavage of short triglycerides to mild acyl chain and olive oil (Quanshun et al. 2010).

34.7 Purification and Kinetic Characterization

A significant degree of purity in biocatalyst enables their successful use. Purification of enzyme facilitates the determination of primary and three-dimensional structures (Saxena et al. 2003). Lipases have been identified, purified, and characterized from different sources. They have been characterized on the basis of their stability profile and activity depending on various parameters such as temperature, pH, presence of chelating agents, and metal ions. Lipases have mostly been purified with the help of techniques such as gel filtration hydrophobic interaction chromatography, ion exchange chromatography, and precipitation. Some cases have employed the use of affinity chromatography as to reduce the number of steps involved in enzyme purification (Woolley and Peterson 1994).

Sharma et al. 2002a have reported purification and characterization of a thermostable alkaline lipase from a new thermophilic *Bacillus* sp. RSJ-1. The enzyme was purified to homogeneity through Q-sepharose followed by Sephacryl s-200 HR. Yet another lipase purified from *Pseudomonas* species showed enhanced thermostability. Also their stability in organic solvent further enhances their thermostability due to rigidity conferred by solvent on to the enzymes. These attribute makes lipase from *Pseudomonas* species most sought biocatalyst for industrial applications. A thermostable lipase was isolated from *Pseudomonas aeruginosa* MB5001 by Chartrain et al. (1993). The enzyme was isolated using a three-step procedure wherein the enzyme was concentrated using ultrafiltration followed by the ion exchange chromatography and gel filtration. The molecular mass of the enzyme determined by SDS-PAGE was 29 kDa, and the maximum activity for the enzyme was reported at a temperature of 55°C and pH optima of 8.0. Other thermostable extracellular lipase isolated from *Pseudomonas sp*. was stable at temperature less than 60°C and within a pH range of 6–12 (Dong et al. 1999).

Borkar et al. (2009) reported thermostable and solvent-tolerant lipase from *Pseudomonas aeruginosa* SRT 9. Enzyme was reported to be purified using ammonium sulfate precipitation followed by phenyl Sepharose CL- 4B and Mono Q HR 5/5 column chromatography steps. The purification process resulted in 98-fold purification factor and a final recovery (yield) of 7.53% of the enzyme with specific activity of 12307.81 U/mg. Molecular weight was determined as 29 kDa by isoelectric point of 4.5. The enzyme was quite stable at 55°C for 120 min and also retains

78% of its initial activity at 65°C after 60 min of incubation. A partially purified solvent-tolerant lipase from *Pseudomonas fluorescence* P21 was found to stable for 2.5 h at 60°C retaining 93.8% of its residual activity. At higher temperature (70°C) lipase retained only 72% of its initial activity after 2 h of incubation. SDS-PAGE and zymogram analysis were exhibited two lipases with a molecular weight 15 and 38 KDa (Cadrici and Yasa 2010).

Thermostable lipase has been also reported from fungus like *Penicillium and Aspergillus*.

Gutarria et al. (2009) reported production of thermostable and acidic lipase from mesophilic origin fungus *P. simplicissimum*. Lipase shows enhanced thermostability with half-life of 300 min at 50°C. However, lipase characterized through whole cell biocatalyst from *Aspergillus niger* displayed on *Saccharomyces cerevisiae,* using a-agglutinin as an anchor protein, shows almost 80% of the full activity after incubation at 60°C for 1 h and >80% of the full activity at 50°C for 6 h. These results were in agreement with purified enzyme from wild strain of *A. niger* FO44 (Liu et al. 2010). Results show that there is huge variation in thermostability of enzyme that varies from species to species.

Like other enzymes lipases also obey Michaelis-Menten kinetic equation (Guit et al. 1991; Malcata et al. 1992). The two main characteristics of Michaelis-Menten equation are K_m (measure of affinity of an enzyme for particular substrate) and Vmax (maximum rate of reaction). Low value of K_m refers to high affinity, and the range of K_m for industrially important enzymes varies between 10^{-1} and 10^{-5} M (Fullbrook 1996).

34.8 Cloning and Sequencing

Alberghina et al. (1991) discussed the initial work on cloning and sequencing of lipases, and since then the same has attracted more attention. Lipases from different sources have been cloned till date and the process still continuing.

A proficient expression system was developed for the weakly expressed thermophilic lipase BTL-2 (*Bacillus thermocatenulatus* lipase II). The latter was overexpressed in *E. coli*. The gene was then subcloned downstream of the temperature-inducible lambda promoter PL in the pCVT-EXP1 (pT1) expression vector. A comparable lipase expression was seen for the expression vectors pT1-BTL2 and pT1-pre BTL 2 in the levels of 7,000–9,000 Ug^{-1} cells. The production level of soluble lipase using expression vector pT1-Omp ABTL2 was between 30,000 and 660,000 Ug^{-1} cells, depending on the specific *E. coli* strain which was used to express the gene (Rua et al. 1998).

Cho et al. (2000) cloned and sequenced thermophilic lipase of *Bacillus thermoleovorans.* Like other lipases derived from *Bacillus* sp., this lipase also contained a conserved pentapeptide sequence Ala-X-Ser-X-Gly, also the gene coded for 416 amino acid residues. The lipase gene was cloned along with a strong T7 promoter in a vector, namely, pET-22b (+) for expression in *E. coli*, and it was found that the activity of the enzyme was 1.4-folds more than that of the native promoter.

Lipase gene derived from thermophilic *Geobacillus thermoleovorans* was subcloned in pET-15b vector by bringing about the digestion of PCR II cloning vector which had the lipase insert with NdeI/BamHI. This was followed by bringing about the ligation of same in the expression vector *E. coli*. The pET-15b vector is known to have His tag and strong T7 promoter (IPTG induced) which facilitates easy purification steps afterwards (Abdel-Fattah and Gaballa 2008).

Recombinant lipase generated by inserting lipase gene fragment from thermophilic bacteria *Geobacillus* sp. SBS-4S, in pET-21a expression vector utilizing NdeI and HindIII restriction enzymes, showed optimum activity at 60°C, with the half-life of 20 min. The enzyme was found to be stable within a range of 45–70°C (Tayyab et al. 2011).

Sabri et al. (2009) cloned the thermostable lipase from *Bacillus sp.* L2 in *Picha pastoris* expression system. Recombinant thermostable lipase was secreted into production medium driven by the *Saccharomyces cerevisiae* α-factor signal sequence. Purified lipase was 44.5KDa and showing maximum activity at 70°C and pH 8.0.

In a study conducted by Sharma et al. (2011), wild type (Wt) lipase gene (jkP01) cloned initially from metagenomic DNA, extracted and purified from hot spring, has been modified using error-prone PCR to generate a thermostable mutant lipase (lip M1), with mutation N355K in the mature polypeptide. The lip mutant lipase (M1), that is, the mutant lipase verified 144 folds improved thermostability over the Wt (wild type) enzyme when assayed at 60°C. Also, both the enzymes, that is, Wt and lip M1 have been characterized in details using various biophysical, biochemical, and computational approaches (Sharma et al. 2011).

34.9 Directed Evolution: A Tool to Create Designer Biocatalysts

Biocatalysis is used increasingly in industrial synthetic chemistry, especially in the cases where implementation of chemical routes is difficult. Presently over 500 products covering 50 applications ranging from beer making to detergent industries are employing enzymes produced by microorganisms for large-scale fermentation processes (Cherry and Fidantsef 2003). Biocatalysts offer various important advantages over the synthetic chemical catalysts, like they function under moderately mild temperatures, pressures, and pH; are biodegradable; possess exquisite chemo-selectivity, *stereo*selectivity, and *regio*selectivity; and thus fabricate the production of enantiomerically pure compound (Fujii et al. 2005). However, a functional fissure separates the natural wild type enzyme from what we desire from an enzyme to function under different conditions in different industrial processes. Various characteristics of the enzymes like their selectivity, activity, stability, pH optima, and solubility are required to be optimized for any given process. Different processes like rational protein design and directed evolution have been used to tailor the enzymes as per the requirement of the specific industrial process. Unlike rational protein design, where the structural

and functional information of the protein is used in order to enhance the specific properties of the enzymes, directed evolution is simply based on the powerful Darwinian principles of selection and mutations (Johannes and Zhao 2006). Directed evolution is used to improve the enzyme characteristics like resistance to thermal denaturation, increased specificity, and solvent tolerance (Ahmed et al. 2008; Arnold and Volkov 1999; Kaur and Sharma 2006; Petrounia and Arnold 2000). The success rate of directed evolution in laboratory to mimic natural molecular evolution depends upon three key factors, namely, mutagenesis, selection of mutants, and further selection of mutants for mutations (Arnold and Bloom 2009). A library of mutant genes is created starting from a set of target genes or a single gene using either recombination of the gene or random mutagenesis. The mutant genes library so generated is then inserted into the desired microorganism for the expression of the proteins with the aid of the expression vector, and this is followed by careful selection of desired mutants using high throughput screening which is then used as a template for the following rounds of improvement. Therefore, this technique mimics the natural evolution process where first step is to generate molecular diversity followed by selection of suitable variants (Leibeton et al. 2000; Arnold et al. 2001).

Lipases are known as the versatile catalysts that carry the transfer of the acyl group and are involved in bringing the interconversion of various alcohols, esters, and carboxylic acids; besides this, they are also employed for the production of many fine chemicals and optically active pharmaceuticals. The improvement in the amide-hydrolyzing activity (Fuji et al. 2005; Johannes and Zhao 2006) enhanced activity for carrying out the hydrolysis of diethyl 3-(30, 40-dichlorophenyl) glutarate (Suen et al. 2004; Johannes and Zhao 2006), and enantioselectivity (Qian and Lutz 2005; Johannes and Zhao 2006) of lipases has been brought about by different mutagenesis approaches like DNA and DNA family shuffling and epPCR and saturation mutagenesis. At higher temperatures, many enzymes become inactivated and partly unfolded as a result of which they are unable to perform their desired actions. These instabilities can be improved with the aid of various techniques of directed evolution. The enantioselectivity of lipases from *Pseudomonas aeruginosa* has been improved towards specific substrates by carrying out consecutive rounds of error-prone PCR along with DNA shuffling (Zha et al. 2001). Kuhrana et al. (2011) reported enhanced thermal stability of lipase from *Bacillus* sp. using directed evolution. Enzyme mutated for isoleucine in place of threonine was screened and shows twofold enhancement in specific activity with $T_{1/2}$ of 21min at 50°C in comparison to wild type.

Similarly epPCR and DNA shuffling methods (tools for directed evolution) were used to enhance the lipase activity from *Proteus vulgaris* T6. Most desired mutant selected shows mutation near catalytic side (V102), where distance between Serine at 79 and V102 is only 0.1Å that leads to forming hydrogen bonding that might be responsible for higher specific activity under alkaline conditions in PVL than its wild type enzyme (Fang et al. 2009). Acharya et al. (2004) employ directed evolution to enhance the thermostability of *Bacillus subtilis* lipase. Mutants were generated by epPCR followed by their screening. Three hundredfold increases in half-life for denaturation was observed for lipase.

The thermostability of lipases and other enzymes has been targeted using directed evolution so as to bring an increase in the melting temperature of the enzymes by 1–2°C (Kuchner and Arnold 1997).

It is therefore clear that directed evolution is an effective strategy for optimizing the enzymes as per the requirements of the industrial processes. It has relatively made a great impact on the applied biocatalysis. Various other enzymes like proteases, amylases, laccase, and chitinases have been subjected to targeted improvements over the period of time with the help of directed evolution (Mollania et al. 2011). The list continues to expand, and it has now become feasible to incorporate the improvement in activity, stability, selectivity, and in various other such properties of enzymes with directed evolution.

34.10 Future Perspectives

Lipases are incredible biocatalysts for its high value applications in the pharmaceuticals, oleo-chemicals, and biotechnology industries. These enzymes are capable of carrying out novel reactions both in aqueous and nonaqueous environments as well as biotransformations with highly specified *enantio*-, *regio*-, and *stereo*selectivity. Hence, lipases are a prospective and an efficient tool for the bioeconomy-based industries. The enhancement in the applications of the lipases derived from microorganisms in biotechnology has paved the way for further research in the isolation and development of thermostable lipases from novel sources with potential to utilize a broad range of substrates, has enhanced stability, and can tolerate other extreme reaction conditions. The growing interest and demand of lipases has given rise to a new trend which focuses on improving the characteristic properties of the lipases which already exist, tailoring the enzymes as per the requirement of the industrial applications and finally looking for novel sources of thermostable enzymes. Using present knowledge we can engineer biomolecule and incorporate many attribute like higher selectivity, enhanced thermostability, and solvent tolerance. However, designing such a multitasking biomolecule is huge task as it requires evolution of each and every parameter we altered. Therefore, designing such enzyme molecules is the necessity of industrial processes, trends, and enhancement in the technologies.

34.11 Conclusions

As the world is targeting new heights everyday and with the advancement in technologies, the enzymes derived from thermophiles have gained massive applications and are a source of attraction for many industries. Thermophilic lipases have gained gigantic recognition in the past few years and are one of the most important biocatalysts because of their extraordinary ability to carry out novel interfacial reactions and

capability to carry out transformations. Because of their competence to endure harsh conditions, they have achieved colossal applications in the field of biotechnology, microbiology, and pharmaceuticals. Keeping up with all the advanced and unique features of thermophilic lipases, it is now important to look up for new microbial sources for these enzymes and thoroughly search for their applications in new areas. Apart from this, techniques like molecular dynamics and advancement methodology for the free energy calculation help us to get an insight into the factors responsible for thermal resistance. These studies will enhance our understanding of protein flexibility and to design new and robust biocatalyst for industrial processes.

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