Sanjay Sahay *Editor*

Extremophilic Fungi

Ecology, Physiology and Applications

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Editor
Sanjay Sahay
Sarojini Naidu Government Postgraduate
Girl's (Autonomous) College
Bhopal, Madhya Pradesh, India

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To my wife Alka Sahay and son Amogh Sahay

Preface

Physico-chemical extremities provide opportunities for life forms to evolve mechanisms and biomolecules, helping them to thrive under such conditions. Fungi among eukaryotes constitute dominant flora in extreme habitats. They can thrive in extremes of temperature, pH, salinity, pressure, water activity, etc. Although fungi have common and important ecological functions as decomposers, symbionts, and parasites, to play to maintain the abiotic and biotic components of the Earth's ecosystem, their survival in extreme conditions is key to the maintenance of these extreme parts of the Earth's ecosystem. The current pace of climate change has affected all ecosystems and life forms including the bare proportion of explored and largely unexplored proportion of fungi of extreme climates. Therefore, the last few decades have witnessed intensive efforts to explore fungi from various extreme climates and develop techniques for the study and maintenance of extremophilic fungi.

The biological mechanisms behind the adaptabilities of diverse extreme climatic groups of fungi are also very attractive and interesting subjects of study. The results unraveled several exciting physiological pathways and mechanisms, many of them being novel ones. The novel pathways are also supplemented with novel biomolecules that support the adaptability of extremophilic fungi. Coincidently, these biomolecules also prove to be important for various human uses.

The objective of the book is to present updated information about various types of extremophilic fungi for young graduates and researchers. It is organized into three parts: (1) Basic information comprising the techniques for their isolation, identification, and maintenance; (2) Ecology and physiology; and (3) Biotechnological applications.

Bhopal, India — Sanjay Sahay

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Editor and Contributors

About the Editor

Sanjay Sahay, Professor of Botany, is currently serving Sarojini Naidu Government Girls Postgraduate College, Bhopal (India). He obtained his PhD in fungal genetics (Patna University, Patna, India), and postdoctoral research experience in yeast molecular biology (Indian Institute of Science, Bangalore, India). He has teaching experience of 27 years, guided 18 PhD students, published more than 30 research papers, contributed three book chapters, and filed two patent applications in lignocellulosic ethanol, one of which has been awarded. His areas of research include psychrophilic fungi, cold-active enzymes, and lignocellulosic ethanol. He has been awarded Biotechnology National Associateship by the Department of Science and Technology, Government of India, and Research Award by University Grants Commission, India.

Contributors

Roheena Abdullah Department of Biotechnology, Lahore College for Women University, Lahore, Pakistan

Prakruthi Acharya School of Basic and Applied Sciences, Dayananda Sagar University, Bangalore, Karnataka, India

Farooq Ahmad Ahanger Government Science and Commerce College, Bhopal, Madhya Pradesh, India

Rajkumar Ahirwar Department of Biotechnology, Barkatullah University, Bhopal, Madhya Pradesh, India

Ali Akbar Faculty of Life Sciences, University of Balochistan, Quetta, Pakistan

S. Akshay School of Basic and Applied Sciences, Dayananda Sagar University, Bengaluru, Karnataka, India

Imran Ali School of Life Science and Engineering, Southwest University of Science and Technology, Mianyang, Sichuan, China
Faculty of Life Sciences, University of Balochistan, Quetta, Pakistan
Plant Biomass Utilization Research Unit, Botany Department, Chulalongkorn University, Bangkok, Thailand

Abdel Rahman Al-Tawaha Department of Biological Sciences, Al-Hussein Bin Talal University, Maan, Jordon

K. S. Anantharaju Department of Chemistry, Dayananda Sagar College of Engineering, Bangalore, Karnataka, India

J. Angelin School of Biosciences and Technology, Vellore Institute of Technology, Vellore, Tamil Nadu, India

Anam Asghar Department of Zoology, Lahore College for Women University, Lahore, Pakistan

Rintu Banerjee Agricultural and Food Engineering Department, Indian Institute of Technology, Kharagpur, West Bengal, India

Subhodeep Banerjee Advanced Technology Development Centre, Indian Institute of Technology, Kharagpur, West Bengal, India

Sunil Bhapkar Department of Microbiology, Savitribai Phule Pune University, Pune, Maharashtra, India

Sourav Bhattacharya Department of Microbiology, School of Sciences, JAIN (Deemed-to-be University), Bangalore, Karnataka, India

Ragini Bodade Department of Microbiology, Savitribai Phule Pune University, Pune, Maharashtra, India

Ercan Bursal Department of Biochemistry, Mus Alparslan University, Muş, Turkey

Swapnil Chaturvedi Department of Biotechnology, Jaypee Institute of Information Technology, Noida, Uttar Pradesh, India

Xiaoming Chen School of Life Science and Engineering, Southwest University of Science and Technology, Mianyang, Sichuan, China

Kanak Choudhary Department of Biotechnology, Barkatullah University, Bhopal, Madhya Pradesh, India

Sangita Chouhan Department of Biotechnology, Barkatullah University, Bhopal, Madhya Pradesh, India

Vinod Chourse Department of Biotechnology, Barkatullah University, Bhopal, Madhya Pradesh, India

Annada Das Department of Livestock Products Technology, Faculty of Veterinary and Animal Sciences, West Bengal University of Animal and Fishery Sciences, Kolkata, West Bengal, India

Arijit Das Department of Microbiology, School of Sciences, JAIN (Deemed-to-be University), Bangalore, Karnataka, India

Arun Kumar Das Eastern Regional Station, ICAR-Indian Veterinary Research Institute, Kolkata, West Bengal, India

Mohan Das Agricultural and Food Engineering Department, Indian Institute of Technology, Kharagpur, West Bengal, India

Regina Sharmila Dass Fungal Genetics and Mycotoxicology Laboratory, Department of Microbiology, School of Life Sciences, Pondicherry University, Pondicherry, India

Tuyelee Das Department of Life Science, Presidency University, Kolkata, West Bengal, India

Vishal Dawkar MITCON Biotechnology and Pharmaceutical Technology Business Incubator Center, Pune, Maharashtra, India

Abhijit Dey Department of Life Sciences, Presidency University, Kolkata, West Bengal, India

Subhara Dey P. K. Sinha Centre for Bioenergy and Renewables, Indian Institute of Technology, Kharagpur, West Bengal, India

Joy Elvin Dhinakar Department of Studies in Biochemistry, University of Mysore, Mysore, Karnataka, India

Aneesa Fasim School of Basic and Applied Sciences, Dayananda Sagar University, Bengaluru, Karnataka, India

Mayukhmita Ghose Fungal Genetics and Mycotoxicology Laboratory, Department of Microbiology, School of Life Sciences, Pondicherry University, Pondicherry, India

Irfana Iqbal Department of Zoology, Lahore College for Women University, Lahore, Pakistan

Mehwish Iqtedar Department of Biotechnology, Lahore College for Women University, Lahore, Pakistan

Umesh U. Jadhav Department of Microbiology, Savitribai Phule Pune University, Pune, Maharashtra, India

Afshan Kaleem Department of Biotechnology, Lahore College for Women University, Lahore, Pakistan

M. Kavitha School of Biosciences and Technology, Vellore Institute of Technology, Vellore, Tamil Nadu, India

Abdul Majid Khan Department of Biotechnology, Barkatullah University, Bhopal, Madhya Pradesh, India

Vijay Kumar Department of Biotechnology, Lovely Faculty of Technology and Sciences, Lovely Professional University, Phagwara, Punjab, India

Krutika Lonkar Department of Microbiology, Savitribai Phule Pune University, Pune, Maharashtra, India

Jing Luhuai School of Life Science and Engineering, Southwest University of Science and Technology, Mianyang, Sichuan, China

Irena Maček Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia Faculty of Mathematics, Natural Sciences and Information Technologies (FAMNIT), University of Primorska, Koper, Slovenia

Ashiq Magrey Department of Biotechnology, Barkatullah University, Bhopal, Madhya Pradesh, India

Suhaib Mohamad Malik Government Science and Commerce College, Bhopal, Madhya Pradesh, India

H. K. Manjushree School of Basic and Applied Sciences, Dayananda Sagar University, Bengaluru, Karnataka, India

Imran Mohsin Turku Bioscience Centre, University of Turku and Åbo Akademi University, Turku, Finland

Sunil S. More School of Basic and Applied Sciences, Dayananda Sagar University, Bengaluru, Karnataka, India

Veena S. More Department of Biotechnology, Sapthagiri College of Engineering, Bangalore, Karnataka, India

Anuradha Mukherjee MMHS, South 24 Parganas, West Bengal, India

Ajay Nair School of Basic and Applied Sciences, Dayananda Sagar University, Bangalore, Karnataka, India

Samapika Nandy Department of Life Science, Presidency University, Kolkata, West Bengal, India

K. Nivetha School of Basic and Applied Sciences, Dayananda Sagar University, Bangalore, Karnataka, India

Potshangbam Nongdam Department of Biotechnology, Manipur University, Imphal, Manipur, India

Anupama Pable Department of Microbiology, Savitribai Phule Pune University, Pune, Maharashtra, India

Ananya Pal P. K. Sinha Centre for Bioenergy and Renewables, Indian Institute of Technology, Kharagpur, West Bengal, India

Devendra Kumar Pandey Department of Biotechnology, Lovely Faculty of Technology and Sciences, Lovely Professional University, Phagwara, Punjab, India

Anastassios C. Papageorgiou Turku Bioscience Centre, University of Turku and Åbo Akademi University, Turku, Finland

Tejpal Singh Parmar Department of Biotechnology, Barkatullah University, Bhopal, Madhya Pradesh, India

Deeksha Patil Department of Microbiology, Savitribai Phule Pune University, Pune, Maharashtra, India

Rushikesh Pol Department of Microbiology, Savitribai Phule Pune University, Pune, Maharashtra, India

K. Poornima School of Basic and Applied Sciences, Dayananda Sagar University, Bengaluru, Karnataka, India

A. Prakruti School of Basic and Applied Sciences, Dayananda Sagar University, Bengaluru, Karnataka, India

Nitish Rai Department of Biotechnology, Mohanlal Sukhadia University, Udaipur, Rajasthan, India

Archana S. Rao School of Basic and Applied Sciences, Dayananda Sagar University, Bangalore, Karnataka, India

S. Rashmi School of Basic and Applied Sciences, Dayananda Sagar University, Bengaluru, Karnataka, India

Puja Ray Department of Life Sciences, Presidency University, Kolkata, West Bengal, India

Sanjay Sahay Department of Botany, SN Government PG College, Bhopal, Madhya Pradesh, India

Indira P. Sarethy Department of Biotechnology, Jaypee Institute of Information Technology, Noida, Uttar Pradesh, India

Beenish Sarfaraz Department of Biology, Lahore Garrison University, Lahore, Pakistan

Sanhita Sarkar Department of Biotechnology, Mohanlal Sukhadia University, Udaipur, Rajasthan, India

Kaushik Satyaprakash Department of Veterinary Public Health and Epidemiology, Faculty of Veterinary and Animal Sciences, Banaras Hindu University, Mirzapur, Uttar Pradesh, India

Mahipal S. Shekhawat Biotechnology Unit, Kanchi Mamunivar Government Institute for Postgraduate Studies and Research, Puducherry, India

Harshita Shukla Department of Biotechnology, Sri Guru Tegh Bahadur Khalsa College, Jabalpur, Madhya Pradesh, India

Shyamji Shukla Department of Biotechnology, Mata Gujri Mahila Mahavidyalaya (Autonomous), Jabalpur, Madhya Pradesh, India

V. Sindhuja School of Basic and Applied Sciences, Dayananda Sagar University, Bengaluru, Karnataka, India

Namita Ashish Singh Department of Microbiology, Mohanlal Sukhadia University, Udaipur, Rajasthan, India

Angeline Jessika Suresh Fungal Genetics and Mycotoxicology Laboratory, Department of Microbiology, School of Life Sciences, Pondicherry University, Pondicherry, India

Ayesha Tahir Department of Zoology, Lahore College for Women University, Lahore, Pakistan

Kalhoro Muhammad Talib School of Life Science and Engineering, Southwest University of Science and Technology, Mianyang, Sichuan, China

Akriti Tirkey Fungal Genetics and Mycotoxicology Laboratory, Department of Microbiology, School of Life Sciences, Pondicherry University, Pondicherry, India

Najeeb Hussain Wani Government Science and Commerce College, Bhopal, Madhya Pradesh, India

Part I

Basic Information

Isolation, Culture, and Maintenance of Extremophilic Fungi

1

Kalhoro Muhammad Talib, Jing Luhuai, Xiaoming Chen, Ali Akbar, Ayesha Tahir, Irfana Iqbal, and Imran Ali

Abstract

Extremophilic fungi are the ones that can survive in extreme available habitats, which either can be natural or artificial. They can be divided mainly into thermophilic, psychrophilic, acidophilic, alkaliphilic, halophilic, and barophilic fungi. This chapter covers two major parts including (a) general isolation techniques, sample collection, preparation of isolation media, strain separation, cell maintenance, morphological, and molecular identifications and (b) maintenance of species, preparation of maintenance media, and preservation of extremophilic fungi. Apart from general operation procedures, the isolation and maintenance of extremophilic fungi needs special adjustments according to the obligate and tolerant needs of their handlings. This chapter is expected to be a consultative protocol resource for the students, academicians, and for industries working of extremophilic fungi.

K. M. Talib · J. Luhuai · X. Chen
School of Life Science and Engineering, Southwest University of Science and Technology, Mianyang, Sichuan, China

A. Akbar
Faculty of Life Sciences, University of Balochistan, Quetta, Pakistan

A. Tahir · I. Iqbal
Department of Zoology, Lahore College for Women University, Lahore, Pakistan

I. Ali (✉)
School of Life Science and Engineering, Southwest University of Science and Technology, Mianyang, Sichuan, China

Faculty of Life Sciences, University of Balochistan, Quetta, Pakistan

Plant Biomass Utilization Research Unit, Botany Department, Chulalongkorn University, Bangkok, Thailand

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Keywords

Fungal protocols · Fungal isolations · Culture media · Fungal preservations

1.1 Introduction

It is a miracle of nature that many kinds of microorganisms can survive in all habitats even in hostile climatic conditions where virtually all life cannot exist (Sharma et al. 2019). These extremely harsh environments are high or low temperature, high saline, high acid, high alkali, high pressure, and high radiation intensity (Yegin 2017). Besides this, some exceptional environments, such as deserts barren areas, oil fields, mining places, subversive anaerobic atmosphere, and high halogen environment are also extreme environments. With the development of science and technology, we find some microorganisms that can survive in these environments (Sekova et al. 2015).

Thermophilic microbes were isolated in 1860, which is the first extremophile to be discovered. With the extensive development of thermophilic microbes research, new strains have been found (Yegin 2017). Recently, there is an increasing trend in the research of fungal species that inhabit extreme environments for reasons that vary from gaining insight into the origin of life to explore new biotechnological applications (Álvarez-Pérez et al. 2011).

Thermophile fungi have many biotechnological, industrial, pharmaceutical and medical applications. Under heavy metal stress, some extremophile fungi have been found to be better than bacteria in degradation and release of heavy metals, hence recovering heavy metal polluted biomass (Jing et al. 2020). Due to safety, sensitivity and cost-effectiveness, natural antimicrobial agents isolated from extremophile fungi have been found to be better for the safety and preservation of food as compared with synthetic antimicrobials (Akbar et al. 2019a, b). Industrial processes with severe conditions (high salt concentrations, high temperatures, and high alkalinity levels) can utilize enzymes produced by extremophile strains that pose no hazard to humans and can be used in medical and food industries (Ali et al. 2015; Prasongsuk et al. 2018).

This chapter is intended to cover the skimmed introduction of extremophilic fungi accompanied by the general lab techniques for the isolation and maintenance of extremophilic fungi. It is expected that this chapter will be helpful for the academicians and students for the consultation of protocols for handling extremophilic fungi.

1.2 Classification of Extremophilic Fungi

Extremophiles mostly refer to microbes isolated from extreme environments. However, there are few reports on extreme fungi, but they have great potential value (Khan et al. 2020a). Exploration of the globe intended for the discoveries of life in

the biosphere led to the finding of the organisms in the environments, which were hitherto considered as non-habitable; consequently, life can persist and even flourish under extreme environmental conditions. In recent decades, extremophiles have aroused great interest and gradually become one of the research hotspots in life science (Ali et al. 2016b). According to the characteristics of the environment, we divide the extremophilic fungi into thermophilic, psychrophilic, acidophilic, alkalophilic, halophilic, barophilic, radio resistant, extreme anaerobic fungi, and so on (Hujslová et al. 2019). These extremophilic fungi define the boundary of biosphere, revealing the origin of life, and enrich the biodiversity of nature (Khan et al. 2020a, b). They are the result of life's adaptation to the environment and contain rich information in the evolution of life (Dumorne et al. 2017). Extremophilic fungi can not only tolerate these extreme natural conditions but also depend on these extreme factors for their growth. Therefore, the revelation of their diverse adaptive mechanisms will provide a new breakthrough for the development of life science (Ali et al. 2014b).

1.2.1 Thermophilic Fungi

Temperature is an exceptionally significant environmental variable, which plays a key role in the existence, development, dissemination, and biodiversity of microbes on the globe. Thermophilic fungi are adapted to grow at higher temperature at or around 60 °C and require nominal nutrients for metabolism and growth as compared with mesophilic fungi. Quite a few research about evolution, growth pattern, respiration, substrate exploitation, nutrition interest, and protein metabolism rate can provide basic info about thermophilic fungi (Maheshwari et al. 2000).

1.2.2 Psychrophilic Fungi

Psychrophilic fungi can thrive at a temperature lower than 15 °C, the highest growth temperature is lower than 20 °C, and the lowest growth temperature is below 0 °C (Singh et al. 2006). Psychrophilic fungi are mainly distributed in Arctic and Antarctic polar regions, deep sea, high mountains, ice cellars, glaciers, cold storage, and frozen soil areas (Sayed et al. 2020). The predominant extreme situation in Antarctic is extra low temperature, unavailability of water (frozen desert) and rainfall, frequent freeze–thaw sequences, heavy winds and high altitudes, vaporization, and UV radioactivity (Durán et al. 2019). Cold thriving fungi have adapted and developed distinct characteristics in evolution process, for example, enzymes, alteration in membrane permeability, and several cell mechanisms, which permit these fungi to develop at ultra-low temperatures as compared with mesophilic species, which survive at adequate temperature (Robinson 2001; Hassan et al. 2016).

1.2.3 Acidophilic Fungi

Basically fungi surviving in acidic environments ought to be considered as acid tolerant fairly instead of being acidophilic (Saroj et al. 2020), for the reason that they can also grow in neutral or sometimes alkaline habitat, yet no obligate acidophilic fungus is reported (Hujslová and Gryndler 2019). Normally, fungi prefer a wide range of pH 1.0–11.0 (Chan et al. 2016) and mostly isolated from acidic habitats such as volcanos, springs, mining areas, or acidic industrial liquid wastes (Hassan et al. 2019).

1.2.4 Alkaliphilic Fungi

In alkaline lakes and some alkaline environments, even in some neutral environments, alkalophilic fungi can be isolated (Horikoshi 2016). Biodiversity of filamentous fungal species, which often grow beyond high ambient pH ranges, i.e., 8–11, still remain mainly unexplored (Grum-Grzhimaylo et al. 2016). Alkaliphiles constituted of two important physiological groups of fungi, alkaliphilic and haloalkaliphilic, first one requires an alkaline pH of 9 or above for the growth and devise an optimum growth pH of near 10; however, haloalkaliphilic fungi require both an alkaline pH 9 and extraordinary saline environment up to 33% w/v NaCl (Bondarenko et al. 2018).

1.2.5 Halophilic Fungi

Naturally high concentration of NaCl is often considered as hypersaline sites regarded solar salterns or thalassohaline (Ali et al. 2018) enriched by different ions and high ultraviolet radiation and sometimes extremes in pH value (Plemenitaš et al. 2014). Their habitats entail several cellular reactions in response of high saline tolerance (Khan et al. 2020a, b). Fungal species, which endure extreme concentrations of NaCl, represents standard and typical physiological systems that enable them to tolerate osmotic and salinity strain and maintain intracellular absorptions (Bano et al. 2018).

1.2.6 Barophilic/Deep Sea Fungi

Bottom of the sea is considered the most enigmatic and uncharted extreme environment acquiring potential and attention for evaluation of fungal biodiversity and future direction for deep-sea fungal exploration (Nagano and Nagahama 2012). Extremophiles generally live in the deepest seafloor. They live in an environment with a pressure of more than 1000 atmospheres, but they cannot survive under normal pressure (Straub et al. 2017).

1.3 Isolation of Extremophilic Fungi

For the isolation of extremophilic fungi, we usually choose to isolate extreme fungi by creating an extreme culture environment. Because extremophiles can grow in conditions different from ordinary microorganisms, we can use these characteristics to isolate extremophiles (Samiullah et al. 2017). For example, ordinary fungi grow well at 28 °C, so when we change the temperature to 60 °C or higher, we can isolate thermophilic fungi (Merino et al. 2019). Similarly the halophilic fungi are isolated in brine solutions (Khan et al. 2017).

1.3.1 Sample Collection

In addition to the general scientific method of isolating fungi from the environment, we also need to pay attention to the selective sampling according to the extreme environment that the extreme fungi adapt to, which can reduce the workload of the later separation (Yanwisetpakdee et al. 2014). For example, when separating thermophilic fungi, it is necessary to collect samples in places with high ambient temperature, such as undersea fire vents or hot spring accessories (Straub et al. 2017); for samples separated for psychrophilic fungi are generally collected from the north and south poles or high mountains (Fariha 2016); the samples of acidophilic fungi are generally collected from the waste water of acid mines or sulfur-containing hot springs (Hujslová et al. 2019); the alkaline fungi are generally from the water with high pH (Ali et al. 2016b). The samples of halophilic fungi come from a wide range of sources. Seawater, salt fields, and salted products can be used as samples for halophilic fungi isolation (Ali et al. 2016b); deep sea samples are generally collected for the isolation of barophilic fungi; Specimens preserved under aseptic conditions until transported to the laboratory in thermocole box (Akbar et al. 2016). At the same time, the collected samples should be kept in a proper way. For example, the samples isolated from psychrophilic fungi should be kept at a lower temperature (0–4 °C) (Wang et al. 2017).

1.3.2 Culture Medium

Potato dextrose agar media (PDA) is mostly used for isolation of various extreme fungi. The formula is: potato 200 g, glucose 20 g, agar 16 g, water 1000 mL, and pH natural (Ali et al. 2016a). Yeast–mold (YM) media is used for isolation of psychrophilic fungi (Paola et al. 2019). The formula is: 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 2% glucose, 2% agar, and pH 6.2 ± 2. Sabouraud agar media is used for isolation of acidophilic fungi (Ali et al. 2015). The formula is: peptone 10 g, agar 20 g, glucose 40 g, and adding distilled water to volume to 1 L (Akbar et al. 2019b). Czapek Yeast Autolysate agar media is made of: agar 15.0 g, yeast extract 5.0 g, $NaNO_3$ 3.0 g, K_2HPO_4 1.0 g, KCL 0.5 g, $MgSO_4$ $7H_2O$ 0.5 g, $FeSO_4$ $7H_2O$ 0.01 g, sucrose solution 100.0 mL, and pH 7.3 ± 0.2 at 25 °C. Malt

extract agar media is composed of: malt extract 30 g, mycological peptone 5 g, agar 15 g, water 1000 mL, and pH 5.4 ± 0.2 (Szulc et al. 2017).

It is not easy to isolate acidophilic fungi. When the pH is very low, especially at 1, the required agar concentration needs to be very high to ensure the solidification of the separation medium. But using silica gel instead of gel can increase the number of culturable microorganisms (Hujslová et al. 2019). For the isolation of extreme fungi, the material of culture medium also needs special attention. In general, some changes may occur in the material of culture medium under extreme conditions, resulting in the failure of the separation work. This is also one of the main problems we need to solve in the future (Ali et al. 2014c).

1.3.3 Strain Separation

Fungi can be isolated by serial dilution method. The sample can be diluted to 10^{-1}–10^{-6} with sterile water, and 0.3 mL is drawn and coated on the sterile PDA medium plate (Makhathini-Zincume 2017). In the isolation of different extreme fungi, different selection conditions are set according to their types. For example, when isolating thermophilic fungi, the culture temperature should be set be at 50 °C (Ali et al. 2014a); when isolating psychrophiles fungi, the temperature should be set at 4–15 °C (Tapia-Vázquez et al. 2020), but the growth time must more than 30 days; when separating acidophiles fungi, the pH should be adjusted to 2.5 (Yu et al. 2014); when isolating alkalophilic fungi, the pH should more then 8.0 (Hujslová et al. 2019), usually most of them live pH is 9–10 (Merino et al. 2019); when isolating halophilic fungi, the salt concentration in the medium should reach 2.5–5.2 mol/L; for the isolation of barophilic fungi, the whole process needs to be carried out in a special pressure vessel (Ali et al. 2014d).

In general, the extremophilic fungi need to be cultured in a constant temperature incubator for 3–10 days, observed at any time during the cultivation process. When colonies grow on the plate, they are transferred to a new medium and continue to culture under the corresponding conditions until pure culture is obtained (Ali et al. 2013). Rifampicin (100 μg mL^{-1}) or chloramphenicol (100 μg mL^{-1}) can be added to prevent any bacteria growth (Gray et al. 2019).

1.3.4 Cell Culture

For details its recommended to consult Durán et al. (2019). Briefly, laying a layer of sterile cellophane (5 cm in diameter) on the medium plate and transferring the mycelial block with least common multiple (LCM) diameter on the cellophane, and cultivating according to the growth rate of different strains. When the cellophane is full of hyphae, scrape off the mycelium with sterilized scalpel, and place it in a 1.5 mL centrifuge tube for DNA extraction or cryopreservation (Verma et al. 2017).

1.4 Maintenance of Extremophilic Fungi

Naturally extremophilic fungi do not take place in pure culture; bacteria free cultures can be maintained by growing fungi in media under ultraviolet radiation, which may inhibit the development of bacteria with minimum or no side effect on the fungi (Bovio et al. 2019). Pure culture techniques allow mycologists for detection, isolation, identification, and quantification of records and several types of fungi from various environments and define nutrition, chemistry, and the environmental requirements for their growth and metabolism (Goers et al. 2014). Pure cultural studies not only develop understanding of any natural habitat in the infected host or environment but also help to determine the molecular structure and exploration of new activities/compounds. Pure cultural methodology is the foundation of basic and applied research and commercial utilization of a fungus (Jong and Birmingham 2001; Sharma et al. 2019). Understanding the basic requirements of a growth media is a limiting factor in culturing various extremophilic fungi. Expansion of knowledge will require wide research in this field to achieve inexpensive culture of the noncultivable extremophiles (Tiquia-Arashiro and Grube 2019).

1.4.1 Medium for Maintenance

Extremophilic fungi pose a versatile biodiversity and several metabolic pathways, so there is no specific and standard culture medium for their growth. Keeping in view the basic physical conditions that influence their growth and better understanding of nutritional requirements will provide an intelligent approach to formulate a media and culture conditions best for their growth (Hauser 1986; Dighton 2016). Mostly culture medium can be very common and simple, formulated with easily available ingredients, but sometimes require some particular and proficient recipe to formulate (Waheeda and Shyam 2017). Essential materials necessary for fungal growth and development comprise simple sugars, like dextrose and sucrose, inorganic salts, air, and water (Smith and Onions 1994). Mostly fungi prefer ammonium nitrate as basic source of nitrogen, whereas others oblige organic forms of nitrogen like asparagine or simple amino acids (Hesami et al. 2014). Several species are capable to grow and yield spores on a simple agar medium prepared with tap water, whereas specific fungi with definite growth features need essential amino acids, fatty acids, trace elements, vitamins (such as pyridoxine, nicotinic acid, thiamin, biotin, inositol, and pantothenic acid), and even sometimes blood components (Wang et al. 2014). In some cases, combinations of different growth factors are formulated. Amino acids are easily available by acid hydrolysis of casein and extracts of yeast cells, which are also considered as common sources of vitamins (de Souza et al. 2015).

Culture media are classified as natural, semisynthetic, or synthetic based on their composition and ingredients. Natural medium is created from natural materials, like V-8 vegetable juice, lima beans, carrots, potatoes, cornmeal, onions, prunes, dung, or soil. These ingredients are typically utilized as extracts, infusions, or decoctions. Semisynthetic media comprise together natural components and distinct synthetic

ingredients, such as potato dextrose agar, yeast extract dextrose agar, and peptone glucose agar (Basu et al. 2015).

Synthetic media contain identified components, and constituents in concentration are appropriately measured. These media are basically used for biological analysis and enzyme activity or specific biochemical research (Kahraman and Gurdal 2002). Culture medium can be solid and liquid form. Solidified media are either natural materials, such as root pieces, potato or carrot slices, or filament of beans, or nutrient solutions coagulated by the adding agar or gelatinous materials (Figueira and Hirooka 2000). Agar is a multifaceted polysaccharide mostly extracted from several kinds of red aquatic algae. It is practically and exclusively used at a concentration of 1.5%, although for specifically acidic medium, 2% or more is prerequisite. Commonly, agar medium do not liquefy up to the temperature beyond 95 °C and cannot resolidify till the temperature drops lower than 40 °C, whereas gelatin liquefies simply when temperatures exceeds 30 °C (Abdollahi et al. 2016). Agar media are usually used basically in experiments conducted for isolation, identification, maintenance of cultures, and study of sporulation parameters. Liquid media are ideal for biochemical research, predominantly during investigation of microbial analyses, metabolic by-products, and metabolic insufficiencies (Hauser 1986). Constituents are typically mixed in a specific medium and sterilized in autoclave. If agar is used in media for solidification, then it should be heated gradually, generally up to boiling, for melting the agar. To avoid interaction of media constituents, such as metals, that may precipitate, the ingredients are arranged and sterilized individually before mixture (Valente et al. 2016). The pH of medium is generally adjusted before to sterilization, although sometimes sterile acid or alkali needs to be mixed after sterilization to maintain the pH. When temperature sensitive combinations are contained within media preparations, then different sterilization technique, such as membrane filtration, is preferred (Rezali et al. 2017).

1.4.2 Culture Conditions

Extremophilic fungi are extensively vulnerable to variations in temperature, light, pH, and aeration that affect their growth (Ali et al. 2019). Normally, most of fungi grow well at room temperatures (Ametrano et al. 2019). However, the optimum temperature may differ among species of a genus and even for strains of same species. Some fungi, which can infect vegetation in frost areas or make food spoilage in refrigerator, can tolerate low temperatures and even grow better at or below 0 °C (Hassan et al. 2016). Heat loving fungi grow adequately at preeminent temperatures beyond 45–55 °C. Agar medium where thermophiles are cultured be likely to dehydrate quickly, and attention must be given to confirm extra humidity during incubation. The degree of temperature for sporulation is typically narrower than for mycelial growth (Joshi and Chettri 2019). Fungi show embellishment to some extent in acid media. Even though mostly fungi prefer pH range from 3 to 7, however for sporulation its limited range (Pommerville and Alcamo 2012). Light effects equally on growth and reproduction of fungi; however, they grow similarly well in light or

dark. Sometimes mycelial growth gets slow by incessant acquaintance to light and also provides stimulatory or an inhibitory effect on sporulation (Jong et al. 1996).

1.4.3 Nutritional Requirements

Fungi are virtually ubiquitous, assorted, and obligatory to human life; for detailed research and exploration of fungi, suitable culture media preparation is one of the prerequisites. Variety of microorganisms grow and inhabit at different environments and have diverse growth requirements, like temperature, nutrients, osmotic conditions, and pH. Because of versatile culture requirements and media, availability of the favorable in vitro environment, almost 99% of all extremophilic fungi, is still not cultivable. As per limitations of fungal growth in laboratory, preparation of novel media is a much desirable push for current microbiology (Tregoning et al. 2015).

Extremophilic culture media may be of diversified subject to the nutritive requirements of different fungi, which require about 10 macro-elements namely C, H, N, O, P, S, K, Ca, Fe, and Mg, which are required for synthesis of carbohydrates, lipids, proteins, and nucleic acids and play a vital role in metabolism. Besides these elements they require several microelements like (Mn, Zn, Co, Mo, Ni, and Cu) for synthesis of enzymes and cofactors. These fungi also need carbon-based compounds, which are essential growth factors (Basu et al. 2015).

1.5 Challenges in Culturing

It is important to learn the phenomena of stoutness of extremophilic fungal applications in biotechnology, evolution, and most fascinatingly their life in extreme environments (Tiquia-Arashiro and Grube 2019). Despite conservative culture methods sometimes flop in sustenance of extremophilic fungi at high temperature and pressure circumstances, fluctuation in pH occurs. Cultivation of thermophiles on cellulose and agar plates containing Luria-Bertani broth or nutrient broth is not convenient because these are unstable above 70 °C for longer time (Tsudome et al. 2009). Solid culture media having spongy solid plate in nonfibrous cellulose is used currently that may keep its reliability up to 260 °C at 25 MPa. This technique can be used to growth most of extremophilic fungi including acidophiles, alkaliphiles, thermophiles, acidothermophiles, and alkalithermophiles in extreme physiochemical environments in lab (Tsudome et al. 2009). Extremotolerant fungi can exhibit differences in tolerance to the medium that simulates the conditions of their natural habitation (Álvarez-Pérez et al. 2011).

1.6 Culture Media

1.6.1 Synthetic Media

1.6.1.1 Czapek's Solution Agar

The medium contains sucrose as the main source of carbon, whereas sodium nitrate as sole inorganic source of nitrogen, potassium chloride as essential ions, magnesium glycerophosphate, ferrous sulfate as cations, and potassium sulfate as buffering agent and agar for solidification (Sun et al. 2020). Czapek's agar medium is recommended for the general cultivation of fungi especially from water samples (Polyak et al. 2020). The acidity of the medium may be increased for the cultivation of acidophilic fungi (Baird et al. 2017).

1.6.2 Saborauds Medium

Sabouraud agar or Sabouraud Dextrose agar (SDA) is a type of growth medium containing digests of animal tissues (peptones), which provide a nutritious source of amino acids and nitrogenous compounds for the growth of fungi and yeasts. Dextrose is added as the energy and carbon source. Mostly it is used to cultivate dermatophytes and some other types of fungi. The acidic pH of this medium (pH about 5.0) inhibits the growth of bacteria but permits the growth of yeasts and most filamentous fungi (Seirafinia et al. 2019). Additionally, chloramphenicol and/or tetracycline may be added as broad spectrum antimicrobials to inhibit the growth of a wide range of gram-positive and gram-negative bacteria (Widyana et al. 2019).

1.6.3 Semisynthetic Media

1.6.3.1 Potato Dextrose Agar (PDA)

Potato dextrose agar is considered as general purpose basal medium and first choice of mycologists for culture, identification, enumeration, and maintaining stock cultures of various type of fungi (Dutta et al. 2008; Devi and Kanwar 2017). It is composed of dehydrated potato infusion and dextrose that stimulate and encourage luxuriant fungal growth (Nonaka et al. 2014). Agar is added as the solidifying agent and sometimes added sterile tartaric acid (10%) to lower the pH of this medium to 3.5 +/− 0.1, inhibiting bacterial growth (Bano et al. 2018). Chloramphenicol is added to inhibit bacterial overgrowth of competing microorganisms from mixed specimens, while permitting the selective isolation of fungi.

1.6.3.2 Modified Leonian's Agar

Different fungi devise a complex of concealed potentials, which may not be observed unless exposed to the appropriate environmental stimulations. This is especially favorable culture medium formulated that mitosporic fungi produce

pycnidia readily on it (Leonian 1924). Mostly, it is used to promote sporulation in ascomycetes and hyphomycetes. This media contains malt extract, which provides carbon, protein, and nutrient sources, where maltose is chief carbohydrate source, peptone for nitrogen supply, dihydrogen potassium phosphate, and magnesium sulfate serving as sources for the essential inorganic salts. Agar is used for solidification of media, which is melted in a separate lot of water before it is added to the nutrient portion (Hearle 2018).

1.6.3.3 Martin's Rose Bengal Agar

Rose Bengal agar is recommended for the selective isolation and enumeration of extremophilic fungi isolated from air, soil, lakes, ponds, rivers, streams, wastewaters, and well waters (Ottow and Glathe 1968), which can initiate growth over a wide pH range and temperature (Kanazawa and Kunito 1996). Rose Bengal agar contains Papaic Digest of soybean meal, which provides the carbon and nitrogen sources required for good growth of a wide variety of fungi (Halili et al. 2016). Dextrose is an energy source, monopotassium phosphate provides buffering capability, and magnesium sulfate provides necessary trace elements (Gawai and Mangnalikar 2018).

1.6.3.4 Yeast Extract-Peptone-Dextrose Agar (YEPD Agar)

YEPD consists of yeast extract, peptone, and glucose or dextrose for use in the cultivation of aquatic fungi (Deka and Jha 2018). Mostly, fungi grow well on a minimal medium containing only dextrose and salts, but addition of protein and yeast cell extract hydrolysates allows faster growth during exponential or log-phase growth (Kunert et al. 2019). Yeast extract supplies B-complex vitamins and it contains all the amino acids necessary for growth (Babcock et al. 2019). Peptone acts as the source of nitrogen, vitamins, and minerals. Dextrose serves as the carbon source. This medium supports the growth of most heterotrophic microorganisms, but due to their simple composition, they have been adopted as the basal media for the routine cultivation of fungi (Vanderwolf et al. 2019).

1.6.4 Natural Media

1.6.4.1 V-8 Agar

V8 juice medium has traditionally been recommended for sporulation and the examination of various fungal characteristics throughout the life cycle (Basu et al. 2015). This medium contains V8 juice supplemented with calcium carbonate used to induce sexual development in many kinds of fungi. Yeast extract provides essential growth nutrients, and L-asparagine serves as the amino acid source and glucose as the carbohydrate source for the growth of fungi. V-8 juice is a blend of eight vegetable juices, which supplies the trace ingredients to stimulate the growth of fungi (Kent et al. 2008). The acidic pH of the medium favors fungal growth and suppresses bacterial growth (Bhattacharjee and Dey 2014).

1.6.4.2 Weitzman and Silva-Hutner's Agar

Weitzman and Silva-Hutner's agar is selective medium and formulated to augment sporulation in most common and important fungi (Scott et al. 1993a), also used for molds as well. It is a good alternative for V-8 agar and used in routine culture in the labs (Benny 2008). Most of the fungi grow better on both Weitzman and Silva-Hutner's and Leonian's agar and some fail in sporulation on one or the other media (Weitzman and Silva-Hutner 1967). This medium is enriched with organic carbon for energy, a source of nitrogen for protein and vitamin synthesis, and several minerals (Scott et al. 1993b). It contains alphacel cellulose powder, pablum baby oatmeal, hunt's tomato paste, potassium dihydrogen phosphate, magnesium sulfate, sodium nitrate, and agar (Nugent et al. 2006).

1.6.4.3 Corn Meal Agar

Corn meal agar is a well-established, general mycological purpose medium recommended for use in the cultivation, maintenance of fungal stock cultures, and for the inducement of chlamydospore formation (Bharathi 2018). This is a very simple formulation containing only cornmeal infusion and agar; however this infusion has enough nutrients to enhance the growth of fungi (Mehta and Anupama 2016). Polysorbate 80 is a mixture of oleic esters, which, when added to the corn meal infusion, stimulate production of chlamydospores (Saeed et al. 2018). Dextrose provides an energy source to enhance fungal growth and chromogenesis (Kovács and Pusztahelyi 2018).

1.7 Preservation Extremophilic Fungi

Understanding the metabolic and evolutionary patterns of microorganisms has played a pivotal role in the development of agriculture, industry, and health sectors (Kannojia et al. 2020). Therefore, for the ex situ conservation of the microbial diversity, microbial culture collections also known as Biobanks or Microbial Resource Centers remain the most important scientific infrastructure (Karaduman et al. 2012; Paul et al. 2015; Sharma et al. 2017). The primary methods of culture preservation are continuous growth, drying, and freezing (Paul et al. 2015). Recently, research relating to biodiversity, classification, epidemiology, biotechnology, biosafety, and biosecurity of extremophilic fungi has attracted the attention of researchers. Conserved cultures of fungal species are applied by the investors related with agricultural science, pharmacological, winery, and manufacturing for emerging new technologies and produces for consumption and welfare of humanity (Sharma et al. 2017). Numerous preservation techniques for fungi are given in this chapter with special reference to extremophilic fungi, which are mostly used, easy, simple, and cost-effective.

1.7.1 Short-Term Preservation

Short-term preservation implicates conserving the culture for a period less than 1 year. Mostly fungus culture can be sustained throughout this retro by periodic transfer. Continual growing approaches, where cultures are grown on agar, usually kept for short time storing (Boundy-Mills et al. 2016). The method is modest, low in price, and extensively used (Hu et al. 2014). Though it is time-consuming and laborious, systematic serial transfer is a better choice for the continued use of local trivial collections for a short period of time (up to 1 year) (Iqbal et al. 2017). However, this method also has some disadvantages. The culture must be often examined for drying and contamination by small insects or other microorganisms (Cui et al. 2018). However, with the time interval, morphology and physiology of cultured fungi may change. Particularly, after frequent transfers, spores or the ability to infect the host may be diminished (Boundy-Mills et al. 2016). Due to these shortcomings, this technique is usually not suitable for long-term (over a year) preservation of culture (Fong et al. 2000). Most filamentous fungi can endure about 1–2 years at 4 °C (Caleza et al. 2017). Viable spore cultures can also be sealed tightly and stored at a temperature −20 °C, or stored at −70 °C to improve survival and increase the transfer required interval (Al-Bedak et al. 2019).

1.7.1.1 Culture Maintenance and Periodic Transfer

There is no comprehensive method for storing extremophilic fungal cultures; the prime objective of maintenance is to be a sanctuary of viability without contamination, genomic variant, or deterioration. Particularly, the procedure targets either to diminish the possibility of variations or exclude regular transfer by prolonging the time period between subculture and attempts to carry cellular activity to a cessation (Ilyas and Soeka 2019).

Most extremophilic fungi may be well-preserved by periodic serial transfer to fresh culture media. Time interval between these transfers differs according to physiology of fungi, medium used, and the environmental circumstances. Generally, nominal medium is ideal for subculturing because basal metabolic rate of the fungi and consequently extend the time within transfers. Fungal growth is often much more rapid in nutrient rich medium, with the absorption of metabolic contents (Iqbal et al. 2017).

Such substances may change environmental factors such as the pH and gas process and reduce the transfer rate. Some fungi need diverse media for growth, or they require the addition of organic molecules to the medium to retain specific physiochemical activities. Perspective must evaluate the subculturing duration for those organisms (Sharma et al. 2017).

At room temperature, the time span may differ from days to months. Transferring the culture after every 3 months interval is adequate for most of fungi, though certain type of fungi can persist viable for years (Caleza et al. 2017). Certain fungal species in their dormant state can persist up to 10 years deprived of a nutrient resource before becoming inviable (Richter et al. 2016). The fungi, which cannot produce particular

propagative cells, enormous fragments of mycelium from young, vigorously developing fringe areas may be transferred (Kumari and Naraian 2016; Singh 2017).

While preserving extremophilic fungi by periodic serial transfer, it should be considered to use the tubes that can be properly sealed. Glass and plastic test tubes and 10 mL bottles with plastic screw lids are extensively used for this purpose. Fungi can be cultured with faintly loose caps up to sufficient growth is attained and then tighten up to avoid desiccation (Marvanová 2020).

Solid medium is desired to broth to watch contaminants which can be easily visible. Replication of tubes should be carried out as a provision contrary to any damage, at least till the subculturing is obtained. Pure culture should be confirmed after each transfer, and curtailed description of traits must be guaranteed and tested from time to time (Singh et al. 2018). Preservation in a fridge is the ideal technique for sub cultures. Many fungi can be stored for 3–5 months interval for transfers if appropriate precautionary measures are taken to avoid desiccation of the culture media (Kannojia et al. 2020).

In fact, preservation of cultures by periodical transfer is risky, laborious, and little bit expensive. Consequences of recurrent culture transfer can be contamination, mislabeling, and assortment of strain variation. Short-term conservation and preservation processes are not suggested for industrial strains because lot of glitches may happen in these methods (Akhtar et al. 2016). The main risks are contaminants, species changes in assortment causing loss of genetic and biological properties, and the expenses of maintaining cultures under these conditions**.**

1.7.1.2 Disposable Screw-Cap Plastic Tubes

This method for conservation and maintenance of fungal cultures, widely used in gelatinous media, has been successfully applied to the storage of mycelial forms of fungi and has been fully described and discussed by Fennell (Fennell 1960). The periodic transfer method, in an appropriate substrate, is commonly used and such transfers are carried out at time intervals determined by the rate of dehydration and by will of the researcher maintaining the collection (de Capriles et al. 1989). Medium can dry out hastily even at 25 °C and may require transfer after 3 months (Thakur and Sandhu 2010). But reducing the storage temperature to 5 °C will reduce the rate of evaporation from the media (O'Donnell and Peterson 2013), thereby lengthening the period of transfer to once every year. Keeping in view the periodic transfer as tedious, lavish, and time lapsing, this method can be practiced diminishing these disadvantages.

These cultures are maintained at required temperatures, with the cap lightly fastened until a fungal growth has ensued (Onions 1971). Then the caps are strongly secured, and the cultures moved to a storing temperature of 5 °C. This method is advantageous as plastic tubes are low-cost, resilient, and hygienic (Humber 2012); the tubes are easy to use, and the cultures are easy to spot, and they remain standard deprived of extreme drying for more than 2 years at 5 °C; this technique is swift, and the tubes occupy minimum space in storage, minimum risk of mite invasion (Onions) and contaminants of the cultures by other fungi during storage (Smith 1971)**.**

1.7.2 Long-Term Preservation

1.7.2.1 Sclerotization

Fungi have multifarious regulated phenomenon for rapid adaptation of biochemical response to produce sclerotia to compete with fungal predators, growth competitors, and environmental stress (Calvo and Cary 2015). Some fungal species produce squeezed mass of hardened mycelium or long time persistent resting spores, stored with reserve food material are called sclerotia; naturally or in culture medium, conserving these survival bodies (Wang et al. 2019), generally at 3–5 °C, is a decent technique for preservation of fungal strains (Gutiérrez-Barranquero et al. 2012). Some higher fungi becomes separated and remains dormant until a favorable opportunity for growth is germinated successfully after years of storage (Frawley et al. 2020).

Mostly, soil borne fungi produce sclerotia or micro-sclerotic bodies, which may persist and viable up to many years. Protocols for induction and development of sclerotia, spherules, and microsclerotia can be reviewed (Daniel and Baldwin 1964; Nakasone et al. 2004). Sclerotization can also be induced by selective media (Frisvad et al. 2014) exposing the culture to white daylight (Gutiérrez-Barranquero et al. 2012) induced by oxidative stress (Georgiou et al. 2006), fungal antioxidant response (Huarte-Bonnet et al. 2019), secondary metabolites (Calvo and Cary 2015), and reactive oxygen (Liu et al. 2018). Rice straw or toothpicks can be used in culture medium as substratum for induced production of sclerotia. A simple method to induce production sclerotia in fungal species is described by Ainsworth and Sussman (2013). Briefly, cut a piece of sterilized cellophane into the size of a petri dish and place it on a disk containing 1% water agar. The rapidly growing plasmodium is then transferred to cellophane and allowed to expand at night. The cellophane is then removed from the agar, placed in a sterile, dry petri dish, covered, and allowed to dry for 24 h. Then, covering the petri dish to keep the sclerotia air-dried until they are hard. The cellophane is cut into small pieces, and each is stored in its own screw cap vial. The nasal tube is removed from the cellophane and is stored in a vial as a substitute. Glycerol can considerably induce formation of sclerotia on nutrition supply in sawdust; carbon source and extreme temperatures may induce sclerotium formation (Cheng et al. 2006).

1.7.2.2 Mineral Oil Overlay

The mineral oil is a colorless and odorless petroleum by-product as alkane with 0.8 g/cm^3 density commonly known as paraffin or white oil (Kannojia et al. 2020). It is a cost-effective and easy to handle method for culture preservation grown on agar plates (Tariq et al. 2015). In this technique, extremophilic fungal cultures may retain its viability for more than a few years or, in extraordinary case, more than 30 years at room temperature or 15–20 °C (Abd-Elsalam et al. 2010). This technique is suitable in mycelial or fungi that fail to produce spores or cultures that are not acquiescent to freezing temperatures (Okolo et al. 2017). Another advantage of this method is that oil decreases mite invasion and contamination (Karabıçak et al. 2016). Even though several basidiomycetes could be preserved by this technique, but the growing level

of culture slows down as with increase in storage time (Kannojia et al. 2020). Main drawback of the oil overlay method is that the fungal culture remain growing and making mixture of mutants that may develop during adverse environments (Lohnoo et al. 2018), and fungi may lack capability of sporulation and decreased virulence (Al-Bedak et al. 2019). Good quality mineral oil or liquefied paraffin is extensively sterilized for this purpose (Goel et al. 2020).

Fungal culture fledged on agar plates are enclosed with 10 mm of high quality mineral oil and free from any toxic substance. The complete agar superficial layer and fungal culture must be sunken entirely in the oil (Goel et al. 2020). Test tubes should be retained in vertical position at room temperature. Oil content in test tubes or ampules should be checked from time to time, and additional oil may be added, if needed (Tariq et al. 2015). Care should be taken that medium should not get dry and float in the tube. For reclamation of a culture from oil overlay, a slight quantity of the fungus colony should be placed on suitable medium keeping oil separated from culture (Ajello et al. 1951). Repeated culture can help to get rid of oil from the fungus sample (Okolo et al. 2017).

1.7.2.3 Soil or Sand

Extremophilic fungi can be conveniently and effectively preserved for long period in dried and sterilized soil or sand (Nakasone et al. 2004). This method is used since 1918, and advantages of this technique comprises augmented prolonged viability of the culture, decreased or exclusion of morphological variations, and the accessibility of identical inoculum for several years (Ryan et al. 2000). This easy, economic, and low-cost technique is suitable for extremophilic fungi (Kannojia et al. 2020). Some fungi have been reported having morphological and physiological changes due to dormancy and dryness for elongated period in preservation (Windels et al. 1988). Sandy or loamy soil (20% moisture content) in 60 ml glass flasks filled to two-thirds volume and then autoclaved at 120 °C for 20 min. Then the flasks should be cooled down and repeatedly sterilized. Sterilized, deionized distilled water DDH_2O is added to the culture, and fungus colony superficial layer is scratched lightly to produce 5 mL of spore or mycelial suspension. One milliliter of the suspension is added to each flask of soil or sand (Kannojia et al. 2020). After few days of colony growth 20–25 °C temperature, the flasks are topped lightly and kept in the fridge at 4 °C (Nakasone et al. 2004). For retrieval of culture, some grains of soil are scattered in to agar medium (Jong and Birmingham 2001). Test tubes, vials, or screw cap bottles can be used in place of glass flasks to save space (Tariq et al. 2015).

1.7.2.4 Silica Gel

Preservation in silica gel technique is widely practiced to reserve sporulating fungal species when freeze drying or liquid nitrogen facilities are unavailable (Perkins 1977). This method was initially suggested by Perkins (Perkins 1962) who found that sporulating fungi preserved in skimmed milk and kept on silica gel were viable for 4–5 years (Karabıçak et al. 2016). Generally, viability of fungal strains preserved on silica gel depends on the growth medium and fungal species (Trollope 1975). Main advantage of silica gel storage is that all metabolic and growth process is

prevented. Sometimes mycologists use glass beads as an alternative to silica gel (Humber 1997). Retrieval of culture from silica gel is very convenient; just few silica gel crystals are sprinkled on an agar plate (Okolo et al. 2017). The storage container can be reused again for sequential samples.

Screw cap plastic tubes filled partly by 6–22 mesh size already sterilized silica should be stored in strongly airtight containers (Perkins 1962). Fungal spores should be in suspension with 10% dry powder of skim milk in DDH_2O, already chilled at 4 °C. Silica gel should be chilled to about 4 °C and kept in an ice water. Suspension of spores then mixed in silica gel about 3/4 of the gel 0.5 mL/4 g and left in ice water bath for half hour. Plastic tubes then kept with the loose caps at 20–25 °C for one to two weeks. Viability of spores should be checked by quaking some crystals onto appropriate medium (Deshmukh 2003). If the culture is viable, then caps must be tighten up, and the tubes are stored in a strongly sealed vessel at 4 °C (Jong and Birmingham 2001). For nonsporulating species of fungi glass test tubes with cotton plug can be used to preserve mycelium and vegetative parts of fungi following the process discussed above (Perkins 1962).

1.7.2.5 Immersion in Distilled Water

Use of distilled water for preservation of extremophilic fungi is an economical, cost-effective, and easy method commonly used by mycologists (Tariq et al. 2015). Actually, the water quashes morphological changes in many fungi (Lohnoo et al. 2018). The technique applied in casing fungal culture on agar medium in oil overlay can also be possible if overlayed with sterilized deionized distilled water (Escobar et al. 2020). On the other hand, sterile stubbles or Pasteur pipettes can be applied for cutting disks from margins of growing culture. The culture disks are the shuffled to screw cap plastic tubes already sterilized and containing some water (Karabıçak et al. 2016). Small vials of 1.8 mL can also be used instead of tubes to save space (Lohnoo et al. 2018). These plastic tubes can be kept at room temperature, and screw caps should not be fully tightened; after few days, caps should be tightened, sealed, and stored at 4 °C. Culture disks can be transferred carefully to fresh agar medium to retrieve cultures avoiding contamination (Sakr 2018). For sporulating fungi, same technique can be used for spore preservation with a little modification as discussed earlier in oil overlay method (Caleza et al. 2017). A novel technique for fungal species isolated from marine environments preserved in sterile marine water is appraised where marine-oriented fungi can be preserved up to 5 years and retrieved rate is 100%. This method is adapted as castellany method by using marine water compromises the cheap and alternate approach of preservation of marine fungi (Reddy and Vijaya 2020).

1.7.2.6 Organic Substratum

Since long time, scientists have established useful, reliable, and creative approaches for preservation of fungal species on several organic substances such as wooden chips, cereal grains, straws, filter paper, and insects and plant tissues. Mostly, these methods were used for pathogenic or some particular fungi and carefully/extensively examined on wide range of fungal species (Siwarungson et al. 2013).

1.7.2.7 Preservation in Wood

Wood inhabiting fungal species are easily and effectively preserved on wooden chips or toothpicks as far as the culture is growing robustly. Some fungi are reported to be preserved on wooden chips and found viable till 10 years (Nakasone et al. 2004). Although some fungi do not strongly grow on wooden chips, then the method can be failed. Slight fragments of crude beech wood are dipped in 2% malt-extract broth and autoclaved at 125 °C for 20 min and resterilized after 24 h (Delatour 1991). Wooden chips then smashed with fungus culture in petri dish and sealed to get colonization on wood. Fifteen days later, colonized wooden chips should be moved to sterilized test tubes partially filled with 2% malt agar and plugged with cotton and wrapped with aluminum foil and kept for 7 days at 4 °C. A piece of wood can be inoculated in to fresh medium for retrieval of culture (Jong and Birmingham 2001).

1.7.2.8 Preservation on Cereal Grains

Some fungal species have been reported to be preserved for more than 10 years on grains of wheat, barley, sorghum, rye, millet, and oats (Singleton et al. 1992). Seeds are sodden in water having chloramphenicol (250 g/mL) solved and kept for overnight (Jong and Birmingham 2001). Next day, water is drained. Tubes with screw cap filled with grains and sterilized in autoclaved for 1 h at 120 °C are repeated for 2 days. The grains should be inoculated with fresh fungus culture and kept for 10 days, then screw caps should be stiffened and enfolded with parafilm and stored room temperature (Nakasone et al. 2004).

1.7.2.9 Use of Agar Strips for Preservation

Some fungi can be preserved on dried agar strips, which can be viable for 18 months to 3–4 years. Fungi grown on suitable culture medium in petri dishes, being cut from the growing margin of the colony, are kept in sterilized petri dishes (Jong and Birmingham 2001). One week later at room temperature, the fragments of dry agar can be moved in sterile vials, sealed, and dried in vacuum. Agar strips can be inoculated on fresh medium for retrieval of culture (Nakasone et al. 2004).

1.7.2.10 Insect or Plant Tissue

Some pathogenic fungi can be preserved on host plant tissues used as a substratum, which is used to preserve the culture (Jong and Birmingham 2001). Sometimes viable conidia can be preserved on frozen, infested aphid cadavers (Nakasone et al. 2004).

1.7.2.11 Freezing

Common practice to preserve fungal cultures is freezing at temperatures below −20 °C to −80 °C in electrical freezers (Simione et al. 1991). Isolates have been reported viable, pure, and morphologically stable when frozen over a year, and this technique proved as safe, easy to handle, cost-effective, and reliable (Sakr 2018). Frozen cultures in liquefied nitrogen are explained in detail in the subsequent section. Screw cap tubes or cotton ploughed test tubes containing cultures grown

on agar medium can be kept in deep freezers. Cultures can be kept in 10% glycerol below −80 °C temperature. PDA petri dishes inoculated with fungus cultures attaining appropriate growth should be frozen at 16 °C temperature for 1 year, and then these cultures can be defrosted at 4 °C for 24 h. Cultures can be retrieved periodically for viability test. To minimalize the chances of variant strains in culture circumstances, fungal isolates should be subcultured on variable culture media (Álvarez-Pérez et al. 2011). Cultures colonized on different organic substratum, just like cereal grains, agar strips, plant parts, and filter paper, are dried and then frozen. Particularly, fungi growing vigorous can survive more in freezing method and better than less vigorous species (Atlas 2010). Recurrent freezing and liquefying should be avoided, which may significantly diminish viability of the cultures (Imathiu et al. 2014). The cost of electricity for equipment, repairs, and power outage are restraining element.

1.7.2.12 Lyophilization/Freeze-Drying and Desiccation

Presence of moisture in preserved samples often deteriorate the morphology and viability and removal of water from sample by heating can also be lethal to the samples (Tapia et al. 2020). Lyophilization is a procedure of desiccation of samples, while freezing water is detached in dual phases: i.e., sublimation and followed by desorption. Since freeze drying is executed at ultra-low temperatures, intended for reduced metabolic activity, stabilization and elongate shelf life of thermos-labile fungi and those strains or else unstable in liquid state are necessary to be dried. The principle behind this is sublimation of water at temperature and pressure below its tripartite, i.e., 611 Pascal and 0.0098 °C. Water separation from a sample is frozen by annealing through conduction or radiation (Bissoyi et al. 2016). Lyophilization, or freeze-drying, is a cost-effective method of long-term preservation and applicable to selective strains of fungi. Over increasing reputation of this method has achieved a global attention in this research (Wei et al. 2012). However, the method was used mainly for fungi that form frequent, comparatively minute spores. However, mycelium fragments of basidiomycetes were lyophilized successfully in the existence of trehalose by Croan (2000). Frozen dried propagules of dictyostelids are preserved effectively over 30 years by Raper (2014). The process of preservation technique suits to several spore-bearing fungal species, which produce minute spores. Bigger spores have a tendency to breakdown during the lyophilic procedure, if morphological destruction affected cannot be revocable during retrieval (Berny and Hennebert 1991). Many spores of proper size may also be physically broken and destroyed by lyophilization during the establishment of ice crystals (Nireesha et al. 2013). For this reason, the ampoules in the beginning need a lot of sustainable spores. Quick freezing and adding of a menstruum, which thaws ice crystals, can reduce the progression of ice crystals (Wang 2000). There are two widely used solvents as menstruum, i.e., skimmed milk powder and bovine serum, while other proteinic substances can be used too (Gaidhani et al. 2015).

Material necessary for lyophilization include good quality automated vacuum pump; vacuum gauge; vacuum manifold with stand; cold trap; hoses to connect pump, trap, and manifold; insulated bath; oxygen-gas torch; oxygen supply; 10 cm

length of 6 mm soft glass tubes with one end heat sealed, or lyophilization ampoules; cotton for plugging tubes; Pasteur pipettes; mechanical or electrical pipetting aid; sterile menstruum; and permanent ink suitable for writing on glass (Nakasone et al. 2004).

Agar slants with a suitable medium, which enables vigorous growth and induce sporulation, should be inoculated with the fungus and allowed to develop up to a resting stage. Lyophilized fungal isolates from cultures before or after resting phase frequently show much lower viability after lyophilization (Ellis and Roberson 1968). Five or some extra lyophilization vials should be sterilized and tagged for instant use. Approximately, 1.5–2.0 mL sterilized menstruum should be mixed in agar slant; Pasteur pipette should be scraped on agar surface to make suspension of spores in menstruum. If spores are not enough, then another agar slant can be scraped for more dense suspension. About 200 μL spore suspension is poured in every of several freeze drying tubes. Tubes must be gently closed with cotton plug, one open end of the glass tube is greased by castor oil, and then tubes should be kept on a vacuum manifold. The manifold is let down till the freeze drying tubes are kept dipped in the dry ice and ethylene glycol bath at −40 °C to −50 °C temperatures up to filling of every tube frozen. The system should be vacuumed for half hour, while the bath reached a temperature about 0 °C for annealing. The manifold is then elevated to take out the tubes from bath. Lyophilization materials then dried at room temperature at a pressure of the system becomes 30 millitorrs. Cooling by condensation allows the samples freeze during the drying course (Rey 2016). Then the tubes are sealed under vacuum using a gas-oxygen torch. After lyophilization, tubes are kept in sequenced plastic cases or wrapped in plastic bags at 4 °C temperature (Nakasone et al. 2004). Viability of lyophilized cultures should be checked after a week. When the culture is to be retrieved, ampules are opened, and the contents of sample are transferred to sterilized deionized distilled water in a test tube using sterile pipette; let the spores to rehydrate for an hour, before transferring to freshly make suitable agar medium. Media and culture conditions specified for fungal strains should be maintained as recommended and incubated the culture at suitable temperature. The remnants of the suspension should be kept in refrigerator, letting for extra retrieval effort if the first was failed. Given proper cure and conditions, mostly culture may propagate in few days. However, some fungi may exhibit an extended lag period and should be given more incubation time before being discarded as nonviable.

As we discussed above, the protocol of lyophilization is a classical technique, but recently advanced technology is available for quick freeze drying of samples with the help of automated equipment (Pisano 2019), which are more reliable, economical, and easy to use (Shanley 2017). In latest lyophilization technique, samples are prepared in glass vials or ampules, sealed with rubbers stoppers and metal caps and then freeze dried in an automated freeze dryer (Boundy-Mills et al. 2020).

1.7.2.13 Liquid Nitrogen/Cryopreservation

Storing with liquid nitrogen is an efficient method for preserving several extremophilic fungi (Zucconi et al. 2012) and the fungi which are not suitable for lyophilization (Pegg 2015). It is a little bit expensive as compared with

lyophilization, for this reason that liquid nitrogen requires to be refilled timely (Silber 2018). Liquid-nitrogen storage is recommended for the preservation of extremophilic fungi (Duarte et al. 2018), except thermophiles (Iqbal et al. 2017). Since ratio of mutation in most cultured fungal strains is likely to resemble to those of mitotic division and high metabolism, this technique is best suitable for such fungi because it completely renders the metabolic activity and cell division (Paul et al. 2015). This accomplishes due to temperature below extreme levels when water crystals cannot develop and metabolic rates are about to cease that affect cellular life (Nannou et al. 2016). Most of the fungal species can be preserved by freezing, but this technique usually is reticent to fungi, which do not produce spores in culture or have big fruiting structures unable to persist in lyophilization, genome banks, and hazardous human pathogens. American type-culture collection (ATCC) stock the seed reserves in liquid nitrogen because if supply of stock is in shortage, then materials used for renewal may be genetically close to the novel deposit and feasible (Boundy-Mills et al. 2020).

Keeping in view the susceptibility of living cells to freezing and melting, commonly two kinds of cryoprotectants are used: penetrating agents such as glycerol and dimethyl sulfoxide (DMSO), which freely penetrate through cell membrane and protect cells internally and externally (Homolka 2013). Glycerol and DMSO have shown efficacy for nonpenetrating agents such as sucrose, lactose, glucose, mannitol, sorbitol, dextran, polyvinyl-pyrrolidone, and hydroxyethyl starch, which exert their defensive effect externally to the cell membrane (Paul et al. 2015), even though another penetrating agent polyethylene glycol, can be used too (Ohmasa et al. 1996).

Key advantages of liquid nitrogen preservation comprises prevention for chances of genetic variation of samples; streamlined, convenient; no frequent tests for pathogenicity, less probabilities of contamination; and better assertion for accessibility of cultures (Humber 2012). The main drawbacks of this method is that liquid nitrogen is expensive and should be replenish after 2 days and require more space, and continuous observation is needed. Long-term availability of liquid nitrogen and shipping cost is also increased (Fahy and Wowk 2015).

Fungi, which do not sporulate in culture or grow mycelium deep into the agar, sterilized 2 mL screw cap polypropylene ampoules are filled with 0.5–1.0 mL sterile 10% glycerol. Plugs of 4 mm diameter are cut from robustly growing cultures using a sterilized plastic straw. Quite a few plugs are placed in the vial, the cap is tighten up, and the tube is retained directly into the vapor phase of a liquid nitrogen tank at −170 °C temperature. Each vial should be labeled with a cryoresistant ink pen or printed onto paper sticker, and then stuck to the vial, or it should be printed onto a special cryoresistant adhesive label, which is easily available from biotechnology supply agencies. The location of storage in the freezer must be indexed for rapid retrieval. Frozen preparations are retrieved by removing the vials from the freezer and rapidly thawing them in a 37 °C water bath. The thawed agar plugs are placed on appropriate agar plates. Viability of the cultures should be checked from 2 to 7 days after storage (Prakash et al. 2013).

For suspensions of spores or mycelial fragments from cultures growing on the surface of agar slants or plates, the colony surface is flooded with 10% glycerol or

5% DMSO and gently scraped with a pipette. Kept in liquid nitrogen as discussed before (Escobar et al. 2020).

The mycelium of a fungus growing in liquid culture can be infused before it can be pipetted into vials. The broth culture is fragmented for a few seconds in a sterile mini blender and mixed with equal parts of 20% glycerol or 10% DMSO to give a final concentration of 10% glycerol or 5% DMSO, respectively; vials are then transferred instantaneously to liquid nitrogen vapor at −150 °C to −180 °C (Nakasone et al. 2004; Lohnoo et al. 2018).

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Modern Tools for the Identification of Fungi, Including Yeasts

2

Ayesha Tahir, Irfana Iqbal, Kalhoro Muhammad Talib, Jing Luhuai, Xiaoming Chen, Ali Akbar, Anam Asghar, and Imran Ali

Abstract

Fungi is a group of eukaryotic and multicellular heterotrophs with a wide range of diversity of phenotypic characters. Therefore, the traditional practice of identifying fungi solely based on morphological characters is incomplete and outdated. With the advancement of molecular biology and bioinformatics, molecular data surge, i.e., DNA and proteins based on which fungal species can be identified. This chapter discusses DNA barcoding techniques using ITS, nuclear ribosomal subunits, protein-coding genes, secondary DNA markers, and DNA taxonomy to identify fungal species and their placement across different taxonomic levels. In addition to the list of curated molecular databases, this chapter provides a step-wise procedure for identifying extremophilic fungi.

Keywords

DNA barcoding · DNA taxonomy · Identification of extremophilic fungi

A. Tahir · I. Iqbal · A. Asghar
Department of Zoology, Lahore College for Women University, Lahore, Pakistan

K. M. Talib · J. Luhuai · X. Chen
School of Life Science and Engineering, Southwest University of Science and Technology, Mianyang, Sichuan, China

A. Akbar
Faculty of Life Sciences, University of Balochistan, Quetta, Pakistan

I. Ali (✉)
School of Life Science and Engineering, Southwest University of Science and Technology, Mianyang, Sichuan, China

Faculty of Life Sciences, University of Balochistan, Quetta, Pakistan

Plant Biomass Utilization Research Unit, Botany Department, Chulalongkorn University, Bangkok, Thailand

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2.1 Introduction

Identification of fungi primarily depends on phenotypic characteristics such as mode of reproduction (sexual/asexual) to form spore-producing structures, mycelial growth pattern, and morphology of hyphae (Chethana et al. 2021). Using morphology, fungal identification is crucial for understanding fungal evolution and ontological studies at higher taxonomic levels, i.e., order and family levels (Aguilar-Trigueros et al. 2015). Still, it provides insufficient knowledge at lower classification, i.e., species level (Branco 2019). Morphological characterization of fungi is problematic even for trained mycologists due to plasticity of characters (sporulation, asexual structures, shape/size of spores) (Raja et al. 2017b), cryptic speciation (Lücking et al. 2014; Sepúlveda et al. 2017), convergent evolution (Shang et al. 2016) and species hybridization (Brasier 2000). The International Code of Nomenclature does not accept this practice as trustworthy in fungal taxonomy (Hibbett and Taylor 2013). As a result, there is much progress in using DNA sequence-based methods, i.e., DNA barcoding, to identify the much-diversified group of fungi (Hibbett et al. 2011; Insumran et al. 2021).

For sequence-based identification of fungus, two techniques are in use: DNA barcoding and DNA taxonomies. DNA barcoding was developed to identify animals at the species stage by using a short-standardized region of DNA (400–800 bp long) (Kress and Erickson 2012). The concept of DNA barcoding states that variance between species (interspecific differences) would be higher than variations among the species (intraspecific variation). The difference between interspecific and intraspecific variation is called the barcode gap, which is the actual measure for fungal identification. DNA barcoding amplifies the ITS region of unknown fungi and compares it to standard sequence databases to identify species based on similarity. The European Nucleotide Sequencing Archive of the European Molecular Biology Laboratory (EMBL) and DNA Data Bank of Japan (DDBJ); International Sequence Database (INSD); GenBank at the National Center for Biotechnology Information GenBank, NCBI, and UNITE (User-friendly Nordic ITS Ectomycorrhiza Database are the main reference for sequences used to identify species (Batista et al. 2020).

DNA taxonomy studies the sequence alignments among two or more genes and assesses the phylogenetic relationships by estimating the evolutionary relationship between homologous sequences. However, the rapid evolution of DNA barcodes has resulted in high divergence at lower taxonomic levels (species and genus); hence could not be used in phylogenetic reconstruction at higher levels (familial and ordinal) (Raja et al. 2017b).

2.2 Morphological Identification of Extremophilic Fungi

The isolated strains of extremophilic fungi are inoculated on medium plates, and the colony culture characteristics are observed under the corresponding extreme environment (Akbar et al. 2014). The slides can be cultured according to the method of

(Chung et al. 2019). The specific operation method is as follows: firstly, the colony edge of the sterilized perforator is cut. The portion of the fungi can be placed on the glass slide with a diameter of 50 mL on the glass slide. It is cultured in the corresponding extreme environment for 4–7 days (Ahirwar et al. 2017). A sterile absorbent cotton ball can be placed in the centrifuge tube to prevent the strain block from losing water too fast. After the mycelium grows well, another clean slide can be taken, dropping a drop of sterile water, taking off the cover glass of the mycelium, and covering it. Then observing, photographing, and measuring the morphological characteristics of the mycelium, conidia, and conidia under the microscope and consulting the relevant data for identification. If one wants to make a permanent slide, the emulsion phenol oil or the lactic acid phenol cotton blue dye can be used as the floating carrier.

The morphology of yeast cells is observed after culture grown in YM and YMA media and incubated at 30 °C for 3 days (Kreger-van Rij 2013). Morphological analysis cannot be ruled out from modern systematics, although fungal systematics has stepped into an era of phylogenetic analysis. For preliminary identification of pathogenic yeasts, their growth on CHROMagar medium is also examined. Prepared CHROMagar Candida medium is used to prepare semisolid medium, and colonies are patched on the medium. Various pathogenic yeasts showed the same characteristics color of colonies after 5 days (Kurtzman et al. 2011; Nadeem et al. 2010).

2.3 Biochemical/Physiological Characterization

2.3.1 Physiological Characterization

According to Bridge (1985), growth and colonies morphology are investigated in the influence of 0.032% sodium selenite, 0.005 or 0.001% crystal violet, or 0.05 or 0.001% copper sulfate. The tests were developed for sporulation in a liquid medium containing glucose, citric acid, ethanol, ammonium oxalate, lactic acid, or ammonium tartrate as the only source of carbon, as well as aesculin and gelatin hydrolysis. After 30 days, growth at 4 °C is assessed in malt extracted agar (MEA), and heat tolerance of conidial aggregates is determined after a 5-min incubation at 75 °C (Bridge 1985).

Physiological characterization is essential for yeast identification. Yeasts are mainly cultivated in a lower limit medium supplemented with a carbon or nitrogen component as the only carbon or nitrogen resources to determine their capacity to metabolize the spectrum of carbon or nitrogen substances. The growth of ascomycetous yeasts in the influence of cycloheximide is also investigated (Hutzler et al. 2015; Kurtzman et al. 2011). Yeasts are also examined for their ability to use certain carbon compounds anaerobically. This test is performed in an especially designed durham tube and using the basic medium containing yeast extract −0.5% (w/v) and test sugar—50 mM to the final concentration. Inoculated tubes are incubated at 25 °C on a rotary shaker at 150 rpm for 10 days. The formation of an air column in

the inner tube indicates the ability of yeast cells to utilize the carbon compound anaerobically. For Basidiomycetous yeasts, positive response in starch formation, urea hydrolysis, and DBB (Diazonium blue B) tests are considered important diagnostic characters and are performed in small capped vials (Barnett et al. 1990; Lloyd 2000).

2.3.1.1 Sporulation Test

All the six yeast isolates were tested for their ability to form spore on yeast malt extract (YM) agar, PDA, 2% malt agar, 5% malt agar, corn meal agar, and McClary's acetate agar media, the incubation conditions being temperature 20 °C and 25 °C and time 1 month. The inoculum used was cells from single (pure) or all six (mixed) cultures. Since all the six isolates turned out to be the same based on morphological and physiological data, one isolate, HG112, was finally selected for further analysis.

2.4 Biochemical Characterization

Potato dextrose agar (PDA) is utilized to culture all single-spore isolates for 3 days at 25 °C, mostly in darkness (Chung et al. 2019). Agar media blocks are taken from the colonies' borders and in 250-mL Erlenmeyer flasks, employed as inocula of GYM medium (Mugnai and Bridge 1989) at 25 °C, without shaking, collected during 9 days, cleaned with sterile water, and centrifuged at 2000 rpm 3 g for 10 min at 4 °C. The resulting mycelial mass is clasped and grinded with a pestle before having shaken for 5 min with 0.45-mm-diameter ballotini glass particles in a cold potter containing 1.5 mL of sterile water. Light microscopy is performed to examine cell disruption. The cell homogenates were moved to microcentrifuge tubes employing a Pasteur pipette and centrifuged at 13,000 3 g for 2 min at 4 °C. 0.45-mm-pore-size The resulting precipitate has been filtered utilizing a Sterivex H-A filter (Millipore, Bedford, Mass.) and saved in micro aliquots at 20 °C awaiting isozyme investigation. Materials for electrophoresis were developed at a maximum concentration of 100 mg protein (Bradford 1976) via suspension 30 ml of mycelial isolate throughout 10 ml of 0.5 M Tris-HCl, pH 6.8, incorporating 1.2 mL of 0.5% bromophenol blue as identifying dye in glycerol (1:4 dilution). Enzymes are isolated using the technique outlined. Procedures were applied to synthesize enzyme staining mixtures (Paterson and Bridge 1994) and Shaw and Prasad (Shaw and Prasad 1970). Isoenzyme patterns are determined for alkaline phosphatase (ALP) (EC 3.1.3.1), catalase (CAT) (EC 1.11.1.6), acid phosphatase (ACP) (EC 3.1.3.2), malate dehydrogenase (MDH) (EC 1.1.1.37), alcohol esterase (EST) (EC 3.1.1.1) and dehydrogenase (ADH) (EC 1.1.1.1) activities. Cellulase (1,4-(1,3:1,4)-b-D-glucan-4-glucanohydrolase) (EC 3.2.1.4) is detectable via developing an overlying gel of 1% agarose in 0.05 M acetic acid-acetate buffer, pH 5.2, over a gel bond membrane, afterward staining using Congo red according to Mateos et al. Isoenzymes are implemented to determine well-resolved bands across all single-spore isolated for yeast's identification (Mateos et al. 1992).

2.5 Molecular Tools for Fungal Identification

2.5.1 Ribosomal Genes and ITS Region

Innis et al. (2012) described the fungal nuclear ribosomal operon primers two decades ago after which the practice of using molecular data for the identification of fungi flourished. These primers amplified three fungal DNA sequences; nuclear ribosomal large subunit (nrLSU-26S or 28S), nuclear ribosomal smaller subunit (nrSSU-18S), and the whole internal transcribed spacer region (ITS1, 5.8S, ITS2; ca. 0.45–0.80 kb) that are being used in molecular phylogenetic sequence identification of fungi (Bruns et al. 1991; Seifert et al. 1995). Various levels of evolution have produced the slowest development in the SSU area and the quickest growth in the ITS domain (Mitchell and Zuccaro 2006). As a result, the SSU region may be transcribed and sequenced utilizing primers NS1 and NS4 for phylogenetic placing at high taxonomic categories, such as family, order, class, and phylum (Innis et al. 2012). The LSU area may be amplified and sequencing employing the specific primers such as LROR and LR6 for identifying and placing at intermediary taxonomic stages, i.e., family and genus (Rehner and Samuels 1995; Vilgalys and Hester 1990). The hypervariable regions of LSU areas D1 and D2 individually (Liu et al. 2012) or combined with the ITS section are incredibly effective in fungal classification (Porras-Alfaro et al. 2014; Raja et al. 2017a; Schoch et al. 2009; Seifert 2009). Due to the fastest evolving portion of the rRNA cistron, the ITS region is suitable for fungal identification at the species level. ITS region is the official barcode for fungi due to its widespread use, easy amplification, and large barcode gap (Kõljalg et al. 2013). For species-level identification, the ITS region can also be used in combination with protein-coding genes.

2.6 Protein-Coding Genes in Conjunction with ITS Region

Because of the existence of intron sections, protein-coding genes are used to determine species through bar codes, particularly, when compared to ITS, occasionally progresses at a quicker pace (Tkacz and Lange 2004), and were utilized phylogenetically because of to having an excellent resolution at higher taxonomic rates compared to rRNA genes (Schoch et al. 2009). Additionally, these genes are supposed to survive as a separate copy in fungi, are far less varying in lengths, leading for easy identification of homology and convergence, as they accrue minimal mutations within their exons, and are simple and easy to align than rRNA genes since they contain little ambiguity related to codon regulations (Berbee and Taylor 2001; Einax and Voigt 2003). Protein-coding genes detected in fungal systematics had also been widely utilized in the formation of molecular phylogenetic analysis to identify a range and categorization of species, due mainly to the National Science Foundation's seminal research in systematic and taxonomy mycology, such as Assembling the Fungal Tree of Life (Blackwell et al. 2006; Hibbett et al. 2007; James et al. 2006; Lutzoni et al. 2004). Such attempts by fungal systematists

contribute to our understanding of developmental interactions between fungi (McLaughlin et al. 2009) and pave the way for a more reliable categorization of such fungal kingdoms (Hibbett et al. 2007). Among the protein-coding markers (Liu and Hall 2004; Matheny et al. 2002; Reeb et al. 2004; Stiller and Hall 1997), beta-tubulin (tub2/BenA) (Glass and Donaldson 1995; O'Donnell and Cigelnik 1997) and transforming growth factor 1-alpha (tef1), the highest (RPB1) and second-highest (RPB2) RNA polymerase subunits would be most commonly implemented to establish phylogenetic connections within fungi (James et al. 2006). Moreover, the mini-chromosome management protein (MCM7) shows potential as a newly developed marker, implying higher and lower-level phylogenetic interactions (Gillot et al. 2015; Hustad and Miller 2015; Morgenstern et al. 2012; Raja et al. 2011; Schmitt and Barker 2009). Compared to several extensively adopted protein-coding markers, the MCM7 area provides a preferable gene for phylogenetic analysis (Aguileta et al. 2008). Furthermore, it functions effectively when combined alongside the large subunit (LSU) gene, as displayed by Ascomycota members (Raja et al. 2011).

2.7 Secondary Barcode Marker

To obtain a more effective determination at the species stage (e.g., Aspergillus, Penicillium, and Trichoderma), this could be required for the users to sequence one or more single-copy protein-coding genes for specific fungal genera and/or lineages as compared to utilizing the Interior transcriptional spacer (ITS) marker only for identifying, which may not be sufficient in such fungal clades. Because of the limitations of a single-marker barcoding technique in fungi, another community of mycologists recently completed an experiment evaluating >1500 Dikarya (Ascomycota and Basidiomycota) species (1931 strains or specimens) with various ribosomal and single-copy protein-coding markers (Stielow et al. 2015). The study concluded that an innovative, high-fidelity primer couple for tef-1 (EF1–1018F GAYTTCATCAAGAACATGAT and EF1–1620R GACGTTGAADCCRACRTTGTC) must have the most potent ability to facilitate as a supplementary DNA barcode, providing superlative resolution to ITS (Stielow et al. 2015), that has been already extensively employed as a phylogenetic marker in mycology (Rehner and Buckley 2005).

The genetic sections tub2/BenA, RPB1, RPB2, and incomplete calmodulin (CaM) are particularly favorable enabling species detection in specific lineages of fungus such as Eurotiales, including Penicillium and Aspergillus, among of the highly abundant genera of fungi for additional compounds, that contain several therapeutic activities and industrial applications valuable species (Houbraken et al. 2011; Houbraken and Samson 2011; Samson et al. 2011, 2014). Tub2/BenA has been suggested as a supplementary barcoding marker for *Penicillium sp.* Barcoding (Samson et al. 2014). For phylogenetic studies of Penicillium sp., RPB2 or CaM genes are suggested. They are generally simpler to align than tub2/BenA, which would be challenging to align primarily to the existence of many ambiguous areas on Penicillium (Samson et al. 2014). The CaM gene is proposed for an additional

barcoding marker, whereas the tub2/BenA and RPB2 genes seem strongly suggested for Aspergillus (Samson et al. 2014). The user is guided to Samson et al. for the priming sequences with advance and reversal primers of genes utilized in segments for Aspergillus and Penicillium for PCR procedures (Samson et al. 2014).

Although *Trichoderma* sp. having significant consequences in living creature and wildlife health, industries, agriculture, and the ecosystem (Mukherjee et al. 2013; Schuster and Schmoll 2010), recognizing Trichoderma is vital to natural products investigation, particularly when applying for a position on peptaibols (Neumann et al. 2015). Therefore, Trichoderma determination employing the ITS area could be problematic because it lacks sufficient diversity to differentiate between species. The ITS region can be used for BLAST search for this genus using the TrichoKeys database, which is hosted on the ISHT webpage (Table 2.1) but provides information only on species clusters. Hence, for species-level identification, the tef-1 region has been widely used in the systematics and taxonomy of this genus (Degenkolb et al. 2008; Druzhinina et al. 2005; Lu et al. 2004; Montoya et al. 2016). However, using tef-1 intron four in conjunction with intron five is extremely helpful for species verification (Jaklitsch and Voglmayr 2015; Nagy et al. 2007). Furthermore, for Trichoderma identification, a 1200 bp portion of RPB2 may be transcribed, and sequencing via primer pairing RPB2–5f and RPB2–7cR (Liu et al. 1999) is indicating advantages of applying phylogenetic techniques.

2.8 D1/D2 Domain and Identification of Yeasts

In the past, two gene regions have been used for the identification of *C. membranifaciens* (Yamadazyma) phylogenetic clade species, namely the D1/D2 domains of the large subunit (LSU) and the nearly complete small subunit (SSU) nrRNA gene (Sung-Oui et al. 2005). Recently Kurtzman et al. (2011) described the genus Yamadazyma phylogenetically, employing the D1/D2 and almost entire SSU sections and introducing an unexpected 11 CoQ-9-forming Candida species as a component of this teleomorphic clade. Yamadazyma had also been classified as a member of the Debaryomycetaceae family, according to Kurtzman et al. (2011). A total of 5 samples extracted from plants in French Guiana and Thailand (four and one strains, respectively) were described for this research based on physiological, morphological, and phylogenetic characteristics and determined to categorize three novel varieties of Yamadazyma. Including isolated, the ITS and D1/D2 domains of the 26S nrDNA have indeed been identified. Because the ITS region was still only publically available for a couple of the type strains of previously investigated Yamadazyma species, additional strains were sequenced to evaluate the variance across the ITS and the D1/D2 regions between the related species.

Except for CBS 8535T, the novel species' ITS and D1/D2 sequences do not match any known yeast species in GenBank or the CBS yeast sequence database. Their sequences matched those of an unidentified Candida species (CBS 9930). S.H. identified a variant from the CBS yeast resource from Scheffleraoctophylla leaves and placed it in the CBS stock cultures in 2004. Yang. The combination of

Table 2.1 List of curated molecular databases

Name of Database	URL	Molecular markers	Scope
Barcode of life database, BOLD	http://www.boldsystems.org/index.php/IDS_OpenIdEngine	ITS	
CBS-KNAW	www.cbs.knaw.nl	ITS	Aspergillus and PenicilliumDermatophytes*Fusarium*indoor fungimedical fungi*Phaeoacremonium**Pseudallescheria/Scedosporium*ResupinateRussulales*Russula*yeasts
FUSARIUM-ID	http://isolate.fusariumdb.org	ITS, *tef1*, *RPB1*, *RPB2*, *tub2*	*Fusarium*
Fungal barcoding	http://www.fungalbarcoding.org	ITS	
Fungal MLST database Q-Bank	http://www.q-bank.eu/Fungi/	Partial actin, *tub2*, *RPB1*. *RPB2*, *tef1* among others	Quarantine organisms
ISHAM	http://its.mycologylab.org	ITS	Human and animal pathogens
Naïve Bayesian Classifier	http://rdp.cme.msu.edu/classifier/classifier.jsp	28S, ITS	
RefSeq Target Loci (RTL)	http://www.ncbi.nlm.nih.gov/refseq/targetedloci/	ITS, 18S, 28S	Mainly sequences from type material, re-annotated from INSDC
International Subcommision on Hypocrea and Trichoderma (ISHT) TrichoKey and TrichoBLAST (Trichoderma)	http://www.isth.info/tools/blast/	ITS and *tef1*, *RPB2*	
UNITE, user-friendly Nordic ITS Ectomycorrhiza database	https://unite.ut.ee/	ITS	Wide taxonomic range, re-annotated from INSDC

Adapted from the work of Robert et al. (2015) and Rebecca et al. (2016)

D1/D2 and ITS sequence alignments (placed in TreeBASE) that included 40 types such as the group and out-group sequence used to have a length of 1120 characters, of which these 130 were phylogenetically unspecific, 704 seemed stable, and 286 have been relevant (Groenewald et al. 2011).

Even though sequence analysis gave a more significant percentage of identification than both phenotypic procedures for the D1/D2 or ITS2 regions, each of these techniques had limitations. Sequence analysis of the ITS2 and D1/D2 sections detected just 79 percent and 87 percent of the isolated species, respectively. The identification rates for both the ITS and D1/D2 areas were lower than previously reported for human diagnostic isolates (Hall et al. 2003; Leaw et al. 2006; Pryce et al. 2003; Putignani et al. 2008).

2.9 Curated Molecular Databases for Fungal Sequences

Various curated molecular databases for numerous fungal groupings had been developed to identify ITS segments, ribosome subunits, and protein-coding sequences (Paterson and Lima 2015). Table 2.1 provides a comprehensive overview of selected molecular libraries, their URLs, molecular markers, and fungal sequencing reportage. It is based on the research of Robert et al. (2015).

2.10 DNA Taxonomic Studies for Phylogenetic Trees Construction from DNA Sequences

DNA taxonomy, like DNA barcoding, is founded on the concept of categorizing taxa by utilizing genetic diversity contained in sequences from different people. The distinction between these two approaches is that an uncertain is identified depending on a phylogenetic assumption (Tautz et al. 2003), promoting an evolutionary viewpoint for detecting fungal species and employing a predicted strategy (Tkacz and Lange 2004). Considering a specified fungus group, DNA classification could depend on one or more protein-coding segments of rDNA. It may be obtained by combining phylogenetic techniques with any gene region (Dissanayake et al. 2020). Although DNA-based categorization relying on the phylogenetic concept can definitely assist via putting an alien species into a phylogenetic clade or group, thereby giving a possible species identity, this could not consistently help identify a specific genus of a fungus. Schmitt and Barker recently published a technique and software description that helped do studies that inferred or used phylogenetic trees (Schmitt and Barker 2009). Using sequences from known species with shared ancestors (homologous sequences) might anticipate species characteristics like ecological and metabolic (Tkacz and Lange 2004). Taylor and Fisher (2003) developed the Genealogical Concordance Phylogenetic Species Recognition (GCPSR) concept to define the boundaries of sexual species utilizing shared housekeeping genes based

on the phylogenetic concordance of numerous unlinked genomic loci. Considering it possesses more discriminating strength over other species ideas, like morphological and biological species categories, the GCPSR concept is considerably quite helpful in detecting fungus (Harrington and Rizzo 1999). A single gene marker can also be exploited to enable broad species confirmation, while GCPSR needs two or even more genetic loci to identify species (Tkacz and Lange 2004). Once the ribosomal genes have supported a taxonomic category, including a genus or family, nuclear protein-coding genes may be applied through phylogenetic experiments and/or BLAST searches to determine further convincing species.

2.11 Protocol in Brief

2.11.1 DNA Extraction

DNA barcoding and DNA categorization are based on the concept of utilizing intrinsic genetic diversity between distinct individual sequences to assess classifications (Chung et al. 2019). A specific group of fungi's DNA taxonomy could be defined on protein-coding one or even other rDNA sections. It may be produced via plant genetic techniques by adopting any gene section alone or combination (Schoch et al. 2012). Whereas DNA-based categorization derived from plant genetics hypothesis does not always facilitate accurately identify species of fungus, it does help place undetectable species in plant genetics or groups, allowing a specific species verification (Raja et al. 2017b). Mycelial powdered was utilized, which is produced via crushing a tiny part of the fungal colony in liquid nitrogen utilizing a pestle and mortar. The Doyle & Doyle technique may be used to isolate DNA (Spadoni et al. 2019). Specific steps are: taking 10–50 mg of the scraped fresh mycelium into a 1.5 mL centrifuge tube, adding sterilized quartz sand and 0.01 g PVP, and first adding 100 μL Cetyltrimethylammonium Bromide (CTAB) buffer solution of 65 °C is grounded into homogenate with a sterilized ground glass rod, and then 500 μL of the same CTAB buffer solution is added for several times to make it evenly mixed (Worasilchai et al. 2018). The mixture is kept in a 65 °C water bath for 0.5–1 h after cooling; 600 μL saturated phenol: chloroform: isoamyl alcohol (25:24:1) is added to the extraction solution: centrifugation at 12000 rpm for 10 min (Gonzalez-Franco et al. 2017). The supernatant is centrifuged at 12000 rpm for 10 min again. The supernatant is added with an equal volume of isopropanol and mixed evenly. The supernatant is placed at room temperature for 10–20 min, centrifuged (12,000 rpm, 15 min), and the supernatant is removed. 100 μL alcohol (75%) is washed two times, and then 75% ethanol is poured. After drying, 100 μL TE buffer is added. The product is mixed with a 6× Loading Buffer with agarose gel of 0.8% for electrophoresis detection and stored at 20 °C (Sandona et al. 2019). Isolates can be identified with the services of National Center of Biotechnology Information (NCBI) web by means of BLAST for the matches amongst the sequences.

2.11.2 PCR Amplification

Metagenomic technologies are reliable tackles that can provide a more wide-ranging assessment of fungal communities. The amplification of rDNA followed by next-generation sequencing has given good results in studies aimed particularly at extreme environments (Baeza et al. 2017). This technique is proved very useful for easy and swift identification and classification of all the fungal species, rDNA primers are effectively used for fungal detection and identification in samples (Mohammad et al. 2017).

1. Primer synthesis: primers ITS1 and ITS4 for amplification and sequencing are generally synthesized such as following (1–3) (Guo et al. 2020).
   ITS1:5′ — TCCGTAGGTGAACCTGCGG — 3′
   ITS4:5′ — TCCTCCGCTTATTGATATG — 3′
2. Amplification reaction system: 40 ul system, adding the following reagents in turn:

dd H_20	30.4 μl
Buffer (10×)	4.0 μl
ITSl (10 pmol/μl)	1.2 μl
ITS2 (10 pmol/μl)	1.2 μl
dNTP (2.5 mM)	2.0 μl
Taq (5 U/μl)	0.4 μl
DNA	0.8 μl

3. The amplification conditions are as follows (3–5) (Hashimoto and Kunieda 2017).
   After 94 °C for 4 min, Enter the following 30 consecutive cycles:

55 °C	30 S
72 °C	1.5 min
94 °C	40 S

   72 °C 2 min, the last cycle is extended at 72 °C for 10 min.
4. Purification of PCR products: 1% amplification of agarose gel is used to detect the amplified products. The products show a single band with enough concentration and are further purified.
5. Sequencing and Sequence Analysis
   The purified PCR products are sequenced. The primer is used to determine the positive direction of the fragment reverse primer retest. The reverse sequence is reverse complementary by bio edit software and connected with the forward sequence to form a complete sequence, and compared with the related sequence in Gene Bank (Nicolaus et al. 2016).

2.11.2.1 Phylogenetic Analyses

To evaluate protein-coding genes, the individual dataset is aligned employing MAFFT's electronic edition employing the E-INS-I option (Katoh et al. 2002). Mesquite (version 2.74) is used to analyze aligned segments manually, and adjustments are made (Morgenstern et al. 2012). The original alignment has been modified to remove gap-rich regions with weak similarity within sequences, locations represented by just one sequence, and the C- and N-terminal ends have been trimmed. Protesters use the Akaike Information Criterion (AIC) to find the most incredible amino acid replacement models independent of the number of unchanging domains (Abascal et al. 2005). Most probable experiments are carried out using RAxML 7.2.6, adopting both the quick bootstrapping and fast maximum likelihood alternatives (Stamatakis 2006). It seems that every dataset's branching stability is calculated using 100 bootstrap repetitions. Sequences derived during the research must be uploaded in GenBank and issued accession numbers.

The phylogeny is rebuilt by maximum likelihood by analyzing the ITS region using MEGA 5.0 (Tamura et al. 2011). Along with Model Test, the Kimura two-parameter nucleotide-substitution model is run with uniform rates and partial deletion (95%) parameters (Posada and Crandall 1998).

2.12 Proteomics of Extremophilic Fungi

Proteomic studies of extremophilic fungal species had extended insignificance hastily from when the biology of such creatures exposed their extraordinary confrontation to severe environmental and ecological situations (Tesei et al. 2019). Proteomics techniques demonstrated the strength of an investigative attempt to discover extremophilic fungi and deliver adequate information, predominantly about quantitative dimensions of their cell biology. To identify the fungal proteome, two primary proteomic techniques are used: lotion separating procedure combined using mass spectrometry (MS) and shotgun (gel-free) methodology (Wasinger et al. 2013). A proteome comprehends the full information related to proteins that may be produced, their profusion, disparities, alterations, interactions, and linkages. Proteomic quantities are accomplished through a grouping of extraordinarily profound equipment and the application of potent computational procedures to provide interpretation of cellular pathways and procedures in a given cell (Pérez-Llano et al. 2020). On this principle, proteomics of extremophilic and extremotolerant fungi allows recognizing them by the changes in structure and stability of proteins, biologically dynamic during extreme conditions (Moreno et al. 2018).

Virtual proteomic describing approaches such as standard, Two-Dimensional Difference Gel Electrophoresis (2D-E and 2D-DIGE) to label proteins and the more latest shotgun proteomics methods are helpful to investigate response mechanisms to various stress like temperatures, salinity, pH, dryness, Mars-like environments (Blachowicz et al. 2019), and host–pathogen interaction (Seyedmousavi et al. 2013). The combined use of diverse proteomics processes and bioinformatics investigations provides advanced knowledge about protein

characteristics, functionalities, and cellular pathways during the survival of such organisms (Gómez-Silva et al. 2019). The accessibility of whole-genome sequences and annotation play a vital role in the exploration of mechanisms of adaptation. Comparative genomics has demonstrated that extremophiles overall own a fantastic set of genes and proteins that allow them with the natural ability to thrive in extreme situations (Kumar et al. 2018).

Matrix-assisted laser desorption/ionization (MALDI) time of flight (MALDI-TOF) is the latest proteomic technique used to identify microbes (Blättel et al. 2013; Moothoo-Padayachie et al. 2013). In this mass spectrometric technique, the ion source is matrix-assisted laser desorption/ionization (MALDI), and the mass analyzer is a time-of-flight (TOF) analyzer. The Sabouraud dextrose agar' inoculated strains for 24 h (48 h if the colonies are not present or when the purity cannot be verified after 24 h) at 30 °C are transferred into 1.5- mL screws cap tubes and mixed methodically in 0.3 mL of double-distilled ultrapure water. Pure ethanol (0.9 mL) is added to the tubes, and they are centrifuged at 13,000 × g for 2 min after vortexing. The supernatant is castoff, and the pellet is mixed thoroughly with 50 μL of 70% aqueous formic acid. The mixture is centrifuged at 13,000 × g for 2 min after adding 50 μL of acetonitrile. A polished steel MALDI target plate (Bruker Daltonics) is used to place one microliter of the microorganism extract supernatant in duplicate and allowed it to dry at room temperature. Two microliters of a matrix solution are used to overlay each sample consisting of saturated α-cyano-4-hydroxycinnamic acid in 50% acetonitrile–2.5% trifluoroacetic acid, and the plate is air-dried at room temperature. The analysis is performed after the plate is loaded into MALDI-TOF mass spectrometer. The spectra was automatically recorded in the linear positive ion mode within a mass range from 4000 to 10,000 Da with delayed extraction at a laser frequency of 20 Hz. Spectra are suitable for more analysis when the peaks have a resolution better than 400 intensity in arbitrary units. Each run includes a bacterial test standard with a characteristic protein and peptide profile, Bruker's for calibration, a negative extraction control, and the reference QC strains. The same methods were repeated using new colonies for failures (Yaman et al. 2012).

2.12.1 Identification of Extremophilic Fungi Using Molecular Tools

Extremophilic fungi show diversity in ecological, metabolism, morphological and phylogenetic characteristics, especially their bioactive molecules that catch the interests of scientists to discover novel chemicals for pharmaceutical, agricultural and industrial applications (Zhang 2016). Despite the significance of extremophilic fungi, their identification still vestiges an intimidating challenge for chemists, particularly without coordination of mycologists (Chamekh et al. 2019). Currently, there are numerous problems concerning identifying fungus just by morphology at the species and strain levels; therefore, morphological and molecular data are utilized for this objective. Three nuclear ribosomal genes are commonly utilized in fungal identification. The potential benefits and drawbacks of the internal transcribed spacer (ITS) region, the authorized DNA barcoding marker for species classification

of fungus. The most current method is the use of NCBI-BLAST search for DNA barcoding, with a cautionary note about its limitations; many systematized molecular databases containing fungal sequences; several protein-coding genes used to improve or replace ITS in species-level identification of specific fungus; and technologies employed to build phylogenetic trees from DNA sequences to allow identifying (Raja et al. 2017b).

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Part II

Eco-physiology

Major Habitats and Diversity of Thermophilic Fungi

3

Swapnil Chaturvedi and Indira P. Sarethy

Abstract

More than 80% of the earth surface has extreme environmental conditions, not conducive for normal life, as assessed on anthropogenic parameters. Along with the discovery of novel bacteria and archaea from various extreme environments, it has been found that fungi also are capable of colonizing such areas. These fungi have been shown to have diverse phylogenetic characteristics, belonging to many genera. Conditions of extremes of pH, temperature, pressure, and radiation necessitate special strategies for survival, involving genetic changes, which can result in novel natural products. Thermophilic fungi occurrence is generally in soil or in habitats where decomposition of plant material such as grains, compost, husk, municipal refuse, and other organic material takes place, under humid and aerobic environment conditions. In these habitats, thermophiles occur as either resting propagules or as active mycelia depending on the nutrients and environmental condition. The occurrence of thermophilic fungi is due to the dissemination of propagules from masses of organic material. Thermophilic fungi belong to various genera such as *Zygomycetes*, *Ascomycetes*, and *Deuteromycetes*. Metabolites from these organisms, not found in normal habitats, can be of good value. Based on the biological activity and structure of compounds isolated from such extremophilic fungi, more than 150 compounds have been documented from thermophilic species of *Penicillium*, *Aspergillus*, and others. The current chapter focuses on the major habitats of thermophilic fungi, their ecology, physiology, and molecular biology, elicitation of specialized metabolites from them, and their various documented activities.

S. Chaturvedi · I. P. Sarethy (✉)
Department of Biotechnology, Jaypee Institute of Information Technology, Noida, Uttar Pradesh, India
e-mail: indirap.sarethy@jiit.ac.in

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Keywords

Thermophilic fungi · Extremophilic fungi · Metabolites · Metagenomics

3.1 Introduction

Thermophilic fungi are eukaryotic organisms, which generally grow at temperatures of 60–62 °C. According to Cooney and Emerson (1964), thermophiles are divided into three categories: thermophiles grow at above 45 °C, extreme thermophiles 66–79 °C, and hyperthermophiles 80 °C (Maria et al. 2016). Thermophilic fungi grow at above 45 °C and above, and the minimum temperature for growth is 20 °C (Oliveria et al. 2015). They occur in soil or in habitats where decomposing plant materials such as grains, compost, husk, municipal refuse, and other organic material are present. In these habitats, thermophiles occur as either resting propagules or as active mycelia depend on the nutrients and the environmental conditions. These fungi are spread by the dissemination of propagules from masses of organic material. They belong to genera such as *Zygomycetes*, *Ascomycetes*, and *Deuteromycetes.* They have a role in the production of thermostable enzymes and are used as components of recombinant organisms for the production of proteases, pectinases, and cellulases (Surrough et al. 2012). Thermophilic fungi can be cultivated in the laboratory on different media such as yeast-starch agar (YpSs), oatmeal agar (OA), and Czapek's agar (C3) as expounded by Cooney and Emerson (1964). *Themomyces stellatus* can grow at above 45 °C (Morgenstern et al. 2012). These fungi contain saturated fatty acids (Maheshwari et al. 2000) and can survive in stress conditions such as high water pressure and desiccation (Mahajan et al. 1986). Thermophilic fungi have an important role to play in environment as they can degrade organic matter, act as biodeteriorants by producing intracellular and extracellular enzymes, phenolic compounds, polysaccharides, antibiotics, and can also serve as single-cell protein (SCP). Single-cell proteins can act as bioconversion agents, for instance such as in preparation of mushroom compost. *Chaetomium* and *Pulverulentum* produce SCP from lignocellulosic wastes. *Chaetomium thermophile* and *Humicola lanuginose* compost are rich in different minerals such as sodium, potassium, and phosphorus. Thermophilic activities of microbes are associated with the thermostability of enzymes and proteins. Advantages of using these thermostable enzymes and protein are reduction in contamination risk of mesophilic microbes in industrial processes and products; as the viscosity of culture medium decreases, solubility of organic compounds increases and as the coefficient of diffusion of reactant and product increases, the rate of reaction becomes higher.

3.2 Types

Thermophilic fungi grow at temperatures between 25 and 80 °C, which is associated with biological conditions. According to Cooney and Emerson (1964), the maximum temperature for their growth is above 50 °C and minimum 25 °C.

Based on various studies, some terms are given to thermophilic fungi to further categorize them:

1. Thermophilic: Fungi that grow at or above 45 °C (Maheshwari et al. 2000).
2. Thermotolerant: Those that grow best at a maximum of 50 °C and minimum of 20 °C (Mouchacca 2000).
3. Thermophilius fungi: They include both thermophilic and thermotolerant fungi (Apinis 1963).
4. Thermoduric fungi: Fungi whose reproductive structures can resist temperature of 80 °C or above, but normal growth is at 22–25 °C (Apinis and Pugh 1967).
5. Transitional thermophile*:* Fungi that grow below 20 °C but can survive at temperature up to 40 °C (Apinis and Pugh 1967).
6. Stenothermal: Those that grow at a narrow temperature range mostly found in a habitat with a constant temperature range (Brock and Fred 1982).
7. Eurythermal: Fungi that grow at a wider temperature range mostly found in the habitat where temperature fluctuates (Brock and Fred 1982).

3.3 History

Earlier studies on thermophilic fungi were more focused on their occurrence in various habitats. The first thermophilic hyphomycetes, *Thermomyces lanuginosus*, was reported from potato and described by Tsiklinskaya (1899). Four thermophilic fungi, *Thermoidium sulphureum*, *Mucor pusillus*, *Thermoascus aurantiacus*, and *Thermomyces lanuginosus*, are based on the growth of thermophilic fungi to the thermogenesis of agriculture stock by Miehe (1907). Later he also studied the thermophilic and mesophilic fungi to check the maximum temperature for their growth. Led Kurt Noack (1920) isolated thermophilic fungi from different natural substrates and observed that thermophilic fungi are present in habitats with varying temperatures. Allen and Emerson (1949) isolated many thermophilic fungi, which were able to grow at 60 °C. They found that thermophilic microflora in retting guayule decreased the amount of resin in the extract of crude rubber, which resulted in improved physical properties. In 1950, La Touche discovered new cellulolytic ascomycetes, which had industrial applications. Notable publications on thermophilic fungi during the sixties and seventies included ones by Apinis (1963), Eggins and Malik (1969) for temperate climate soil, and Hedger (1974) and Gochenaur (1975) for tropical regions. Morphology of thermophilic fungi is based on their biological activities, which help in the discovery of industrial application. Approximately 75 types of thermophilic fungi species have been discovered till now,

comprising around 0.1% of the total fungal species, from compost, soil, desert soil, wood husk, organic material and coal, and many more.

3.4 Habitats

Several extreme conditions are found in the environment such as large or small temperature ranges, acidity, radiation, and drought conditions; few organisms are able to survive in these conditions, with a vast majority unable to adapt to such variations. Thermophilic fungi prefer composts, snuff, municipal waste, wood husk, grains, and other such organic material where the preferred humidity and aerobic environmental conditions are present for their growth and development. Figure 3.1 shows various habitats from where thermophilic fungi have been isolated. Table 3.1 shows that the earliest studies of thermophilic fungi, which have shown these to be

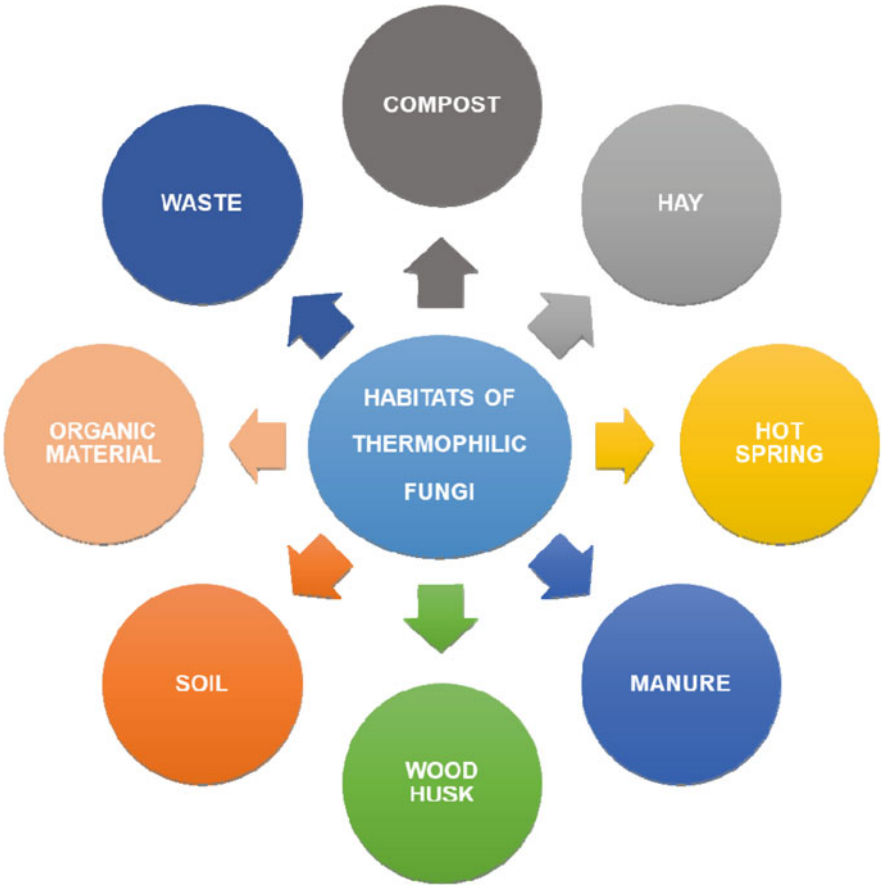

Fig. 3.1 Habitats from where thermophilic fungi have been isolated

Table 3.1 Origin of thermophilic fungi and their habitats

Class	Organism	Habitat	Reference
Ascomycetes	*Canariomyces thermophile*	Soil	von Arx et al. (1988)
	Chaetomium britannicum	Mushroom compost, soil	Ames (1963), Chen and Chen (1996)
	Chaetomidium pingtungium	Sugarcane field Decomposing leaves	Ames (1963)
	Chaetomium virginicum	Plant remains, seeds of *Capsicum annuum*, soil	Ames (1963)
	Chaetomium senegalensis	Soil, decomposing wheat straw	Ames (1963)
	Corynascus sepedonium	Soil, pasture soil, hay, coal spoil tips, compost	von Arx (1975)
	Coonemeria crustacean	Coal spoil tips, bagasse, soil	von Arx (1975)
	Thielavia australiensis	Nesting material of malleefowl	Tansey and Jack (1975)
	Talaromyces byssochlamydoides	Forest soil	Guarro et al. (1996)
Zygomycetes	*Rhizomucor pusillus*	Municipal wastes, horse dung, composted wheat straw, guayule, hay, seeds of cacao, barley, maize and wheat, groundnuts	Schipper (1978)
	Rhizomucor miehei	Soil, sand, coal mines, hay, stored barley, compost	Cooney and Emerson (1964)
Eurotiomycetes	*Paecilomyces themophila*	Wheat straw	Yang et al. (2006)
	Malbranchea cinnamomea	Compost, soil	Maijala et al. (2012)
	Sporotrichum thermophile	Soil	Sadaf and Khare (2014)
Deuteromycetes	*Scytalidium thermophilum*	Compost, soil	Narain et al. (1983)
	Scytalidium thermophilum	Compost	Robledo et al. (2015)
	Acremonium thermophilum	Sugarcane bagasse	Van Oorschot (1977)
	Chrysosporium tropicum	Dung, soil, air	Gams and Lacey (1972), Carmichael (1962)
	Acremonium alabamense	Alluvial soil	Malloch and Cain (1973)

widely present in decomposing organic matter or hot springs, are still the habitats from where these continue to be isolated. In these habitats, thermophilic fungi may occur as resting or active mycelia depending upon the favorable conditions of the environment.

In 1939, Waksman and his team isolated thermophilic fungi from soil. As seen from Table 3.1, thermophilic fungi have been isolated from a wide variety of materials. Thermophilic fungi grow easily in temperate countries but not in tropical countries. Their occurrence is due to self-heating masses of organic material (Maheshwari et al. 1987). A thermophilic fungus was found in Australia in temperate soil region (Ellis and Keane 1981). Thirty-two thermophilic and thermotolerant fungi were found in coal soil with well-organized colonies (Evans 1971). Pine hardwood pine scrub (Ward Jr and Cowley 1972) were found to be good sources too. Approximately 75 types of thermophilic fungi species have been discovered till now, comprising around 0.1% of the total fungal species, from compost, soil, desert soil, wood husk, and organic material land coal.

3.4.1 Natural Habitats

In these habitats, where high temperature conditions prevail round the year, thermophilic fungi have been obtained using culture-dependent techniques. Some of the characteristics of these habitats and the fungi isolated are discussed in this section.

3.4.1.1 Hot Springs

This is a type of thermal spring generated from geothermally heated water. Water flows from hot spring and is heated either by geothermal process or when water flows from the hot rock surface. On the other hand, in volcanic areas, water gets heated on coming in contact with magma. Boiling water, which builds up steam pressure, which comes out in the form of jet on the earth surface, is called a geyser. Countries from which fungi have been documented from such hot springs are Canada, New Zealand, Japan, Chile, Hungary, Israel, India, and Fiji. Hot spring water contains many minerals, which provide the growth of microbiota. These hot spring thermal areas are niches with some unique qualities, which can be explored for biotechnological purposes. Isolation and characterization of thermophilic fungi from hot spring thermal regions have been done by several researchers in the past decades (Sharma et al. 2012). Thermophilic fungi are present in a larger population in the hot spring region of Indonesia, but all of them are not able to grow under the hot spring volcanic conditions and is restricted to some species. Five types of thermophilic fungi were isolated from WU-Rai of northern Taiwan: *Humicola insolens*, *Rhizoctonia*, *Aspergillus fumigatus*, *Penicillium dupontii*, and *Thermomyces lanuginosus*, all of which could grow between 55 and 65 °C (Chen et al. 2000). A research of the mycoflora in Yangmingshan National Park, northern Taiwan, from August 1999 to June 2000, of thermophilic and thermotolerant fungi inhabiting sulfurous hot spring soils, identified 12 taxa (Chen et al. 2003). Further four thermophilic fungi species were reported from Xiaoyoukeng sulfurous area:

Sporotrichum sp., *Chrysosporium* sp., *Scytalidium thermophilum*, and *Papulaspora thermophile*. Pan et al. (2010) isolated thermophilic fungi from geothermal sites with alkalescent hot spring in Tengchong Rehai National Park, China and utilized the ITS region Internal transcribed spacer) sequencing system to classify fungi. More than a hundred fungal strains were isolated such as *Talaromyces byssochlamydoides*, *Rhizomucor miehei*, *Thermoascus aurantiacus*, *Talaromyces thermophiles*, *Thermomyces lanuginosus*, *Scytalidium thermophilum*, and *Coprinopsis species*.

3.4.1.2 Soil

Soil is the upper land surface on earth, which is the mixture of all the organic material present with minerals and other components, which provide support for the growth of the organism. In soil, various microorganisms provide nutritious condition to plant and animal species. Thermophilic fungi grow in soil; however, it depends on the nature of the soil. According to researchers, such fungi are present in the upper layer of the soil or the debris of the plant on the soil; Rajasekaran and Maheshwari (1993) estimated the respiratory rates of thermophilic and mesophilic fungi. They discovered that the respiratory rate of thermophilic organisms was especially receptive to changes in temperature; however, that of mesophilic fungi was generally free of such changes, suggesting that in thermally fluctuating conditions thermophilic fungi might be at a physiological setback in contrast to mesophilic organisms. Their work shows that thermophilic fungi are inactive components of soil microflora. Different types of species were isolated from south-central Indiana soil, a sun-heated soil: *Myriococcum albomyces*, *Aspergillus fumigatus*, *Talaromyces thermophilus*, *Humicola lanuginosa*, *Allescheria terrestris*, *Malbranchea pulchella var. sulfurea*, *Thielavia heterothallica*, *Mucor pusillus*, *Chaetomium thermophile var. dissitum*, *Thielavia minor*, *Thermoascus aurantiacus*, *Mucor miehei*, *Torula thermophila*, *Humicola stellata*, *Acrophialophora nainana*, *Thielavia sepedonium*, *Dactylomyces thermophilus*, *Talaromyces emersonii* (Tansey and Jack 1976), *Malbranchea cinnamomea* (Maijala et al. 2012), and *Sporotrichum thermophile,* (Lu et al. 2013).

Ten species of thermophilic fungi was identified from different zones of Darjeeling in eastern Himalayan soil. It was observed that there was a decline in the pervasiveness of thermophiles with expanding altitudes (Sandhu and Singh 1981). Later, thermophilic and thermotolerant fungi were isolated from 77 regions of Iraq. Out of these, six were true species of thermophilic fungi, while the rest were thermotolerant. *Aspergillus terreus*, *A. fumigatus*, *and A. niger* were available with frequencies of the event of 70%, 68%, and 60%, individually, and thus the investigation revealed that thermophilic and thermotolerant fungi are widely present mycoflora of Iraq soils (Abdullah and Al-Bader 1990).

3.4.1.3 Desert Soil

Desert soil has the amount of precipitation that falls based on temperature or due to geographical location. Around one-third land surface of the earth is arid and semi-arid. Researchers have isolated thermophilic fungi from the desert where the temperature is up to 55 °C or above. However, water is required for the growth, even

when spores are present in dry soil. Abdel-Hafez (1982) studied the desert soil of Saudi Arabia for thermophilic fungi and isolated 48 species of thermophilic fungi from 24 genera on different laboratory media such as Czapek's agar media and containing cellulose or glucose. The organisms isolated were *A. fumigatus*, *Humicola grisea var*, *Aspergillus nidulans*, and *C. thermophile var. copropile.* Further, it was reported that 16 species were reported: *Mucor pusillus*, *Talaromyces*, *Thielavia*, *Myxococcus*, *Stilbella thermophile*, *A. fumigatus*, *C. thermophile var. copropile*, *C. thermophile var. dissitum*, *Chaetomium virginicum*, *Torula thermophile*, *Malbranchia pulchella var. sulfurea*, *Malbranchia pulchella var. sulfurea*, *Sporotrichum pulverulentum*, *Talaromyces thermophilus*, *Myriococcum albomyces*, *Allescheria terrestris*, *Papulaspora thermophile*, *Sporotrichum pulverulentum*, and *Humicola lanuginose.* In Middle East region (Egypt, Iraq, Syria, and Kuwait) thermophilic and thermotolerant fungi from desert soil were isolated from which *Malbranchea cinnamomea*, *Scytalidium thermophilum*, *Myceliophthora thermophile*, and *Thermomyces lanuginosus* were obtained by Mouchacca (1995). Thermophilic fungi from the Thar desert of India were isolated such as *Aspergillus flavus*, *A. niger*, *A. terreus*, *A.versicolor*, *Chaetomium*, *Emericella*, *Emericellanidulans*, *Fusarium*, *Chlamydosporum*, *Penicillium*, *Scytalidium*, *Thanatephorus cucumeris*, *Cunninghamella*, *Eurotium*, *Mucor*, *Chrysogenum*, *Talaromyces*, *Alternaria alternata*, and *Rhizopus stolonifer* (Sharma et al. 2010). Eight thermophilic fungi were identified from desert area of Yard province: *Ulocladium*, *Fusarium*, *Penicillium*, *Aspergillus*, *Alternaria*, *Rhizopus*, *Stemphylium*, and *Paecilomyces* (Rafiei and Banihashemi 2019).

3.4.1.4 Coal Mine Soil

Thermophilic fungi were isolated from coal mine soil of Chandameta, Parasia, from Chhindwara District of Madhya Pradesh, India. The normal temperature and precipitation rate of this region is 21 °C and 64 cm. A total of 14 fungi species were isolated: *Achaetomium macrosporum*, *Emericella nidulans*, *Rhizopus rhizopodiformis*, *Absidia corymbifera*, *Thermomyces lanuginosus*, *Thielavia minor*, *Humicola grisea*, *Aspergillus fumigatus*, *Thermoascus aurantiacus*, *Torula thermophile*, *Rhizopus microsporus*, *Penicillium sp*., *Sporotrichum sp*., *and Aspergillus fumigatus* (Johri and Thakre 1975). Coal mine near Hazaribagh Jharkhand exhibited the presence of thermophilic and thermotolerant fungi such as *Chrysosporium tropicum*, *Melanocarpus albomyces*, *Chaetomium piluliferum*, *Chaetomium thermophile*, *Penicillium chrysogenum*, *Aspergillus fumigatus*, *Curvularia lunata*, and *Cladosporium* spp. (Tulsiyan et al. 2017).

3.4.1.5 Coal Soil Tips

Coal soil tip is waste accumulated together in one place. Thermophilic fungi can grow on coal soil tip easily isolated thermophilic fungi from coal soil tip and observed that thermophilic fungi grow in these habitats due to its high temperature, organic material waste, and lack of soil crumbs. More than 30 thermophilic fungi species from different genera were isolated: *Aspergillus, Penicillium, Rhizopus, Mucor, Chrysosporium, Acrophialophora, Aspergillus, Calcarisporium,*

Chrysosporium, Geotrichum, Penicillium, Scolecobasidium, and *Talaromyces* (Evans 1971).

3.4.2 Man-Made Habitats

Man-made habitats are largely those created and lived in by human beings. These types of habitats having effluents, thermal insulation system, stored grains, and compost piles provide a suitable environment for growth of thermophilic fungi. Heating in these environments can be by natural means (such as the heat generated during decomposition) or provided artificially by solar heating or other self-heating systems in which temperature can rise to 70 °C.

3.4.2.1 Manure

It is an organic material that is obtained from human, animal, and plant residues. They contain nutrients in organic material form (Larney and Hao 2007). Agricultural processes require a high demand for manure, but as manure has directly come in contact with agricultural land, it may cause many adverse effects on soil, water, and nutrient leaching. Manure compost provides better nourishment to the product as they are rich in nutritious value. Holman et al. (2016) have isolated thermophilic fungi such as *Thermomyces lanuginosus* and *Remersonia thermophile.*

3.4.2.2 Municipal Waste

Municipal waste is the waste material that has lignocellulosic material and some inorganic waste material. According to a study by Kaiser et al. (1968), municipal corporations generate more than 50% paper waste. Stutzenberger and their group (1970) studied the microorganisms that could degrade lignocellulosic materials. In municipal waste, the temperature can rise to 60 °C, suitable for growth of thermophilic fungi. Kane and Mullins (1973) isolated *Torula thermophile*, *Humicola lanuginosa*, *Mucor pusillus*, *Aspergillus fumigatus*, *Chaetomium thermophilum*, and *Thermoascus aurantiacus*. Sen et al. (1979) studied thermophilic fungi such as *Mucor* and *Humicole*, which were rich in nutrients such as nitrogen, potassium, and phosphorus. From different parts of India, various representatives such as *Myceliophthora thermophile* and *Thermo mucor sp.* have been isolated. Thermotolerant fungi such as *Cladosporium* and *Absidial* spp. were isolated from municipal waste in Iran (Ghazifard et al. 2001).

3.4.2.3 Wood Chip Piles

Wood chip piles are strong pieces of wood made by cutting or chipping parts of freshly harvested wood and are commonly utilized for the production of wood mash and as a crude material for specialized wood handling. Brown et al. (1994) found that these wood chip pile consists more than 50% weight of water due to which bacteria can grow easily by a fermentation process. Tansey (1971) studied that as the temperature rises in these stored wood chip piles, an ignition process starts due to which growth of thermophilic fungi takes place, and this causes deterioration in

wood quality. He also isolated thermophilic fungi from wood chip piles obtained from paper factory: *Humicola lanuginose, Chaetomium thermophile var. dissitum, Talaromyces emersonii, Chaetomium thermophile var. coprophile*, *Talaromyces thermophiles*, and *Sporotrichum thermophile.*

3.4.2.4 Hay

Hay is related to grass, vegetables, or different herbaceous plants that have been cut, dried, and put away as bundles for later use as grain, especially for nibbling creatures. The hay bundles are stacked one over another, and the moisture present in these lead to the growth of microorganisms. Miehe (1907) isolated *Thermoascus aurantiacus*, *Rhizomucor pusillus*, and *Thermomyces lanuginosus* from a self-heating haystack. After inoculating hay and other organic material with a pure culture of fungi, he observed that sterilized hay does not produce heat, whereas the inoculated fungus does generate heat. Gregory and Lacey (1963) found that a different type of microflora develops in haystack, which has some moisture content. They demonstrated that hay having a 16% moisture content heated up moderately, while that having 25% moisture heated up to 45 °C, allowing the development of thermotolerant molds. But the temperature in wet bales with 45% moisture soared to 60–65 °C, and a range of thermophilic fungi, including *Aspergillus fumigatus, Mucor pusillus, and Humicola lanuginose*, and some actinomycetes were also recovered. Thermophilic fungal strains are capable of growing at 50–60 °C, at pH 2.0. More than 70 fungal strains belonging to *Ascomyota* were isolated from decaying plant matter and compost (Thanh et al. 2019).

3.4.2.5 Stored Grains

Stored grains have been used for centuries to fulfill the requirement for food necessity. Tansey and Brock (1978) studied that thermophilic fungi, though present on grains, do not damage grains prior to storage. Stored grains are a good substrate for the colonization of thermophilic fungi. Clark (1967), Clarke (1969), and Lacey (1971) discovered various thermophilic fungi from stored grains such as *Aspergillus candidus*, *Absidia corymbifera*, *Humicola lanuginosa*, and *Mucor pusillus*. Mulinge and Apinis (1969) isolated *Eurotium amstelodami*, *Absidia spp*., *Monascus spp*., *Aspergillus fumigatus*, *Aspergillus terreus*, and *Dactylomyces crustaceus* from stored moist barley grains. Hansen and Welty (1970) isolated thermophilic fungi from cocoa beans. Wareing (1997) isolated the thermophilic and thermotolerant *Aspergillus flavus*, *Paecilomyces variotii*, *Rhizomucor pusillus*, *Thermomyces lanuginosus*, and *Thermoascus crustaceous* from stored maize grain in sub-Saharan Africa. *Thermomyces*, *Fumigatus*, *Dupontii*, *Thermomyces lanuginosces*, *Rhizomucor*, *and Thermoascus crustaceus* were obtained from corn grain at 52 °C (Sandona et al. 2019).

3.5 Biodiversity of Thermophilic Fungi

3.5.1 Zygomycetes

1. *Rhizomucor miehei* form colonies with sporangia at 40–45 °C. Initially colonies are white and later turn into gray–brown (Schipper 1978). Spherical sporangia are 30–60 μm in diameter. The zygospore is subspherical and initially reddish-brown, later turning a black color with a diameter of 30–50 μm. They are found on soil, coal mines, hay, stored barley, and compost. They have been studied and geographically found in the United States, India, Ghana, the United Kingdom, and Saudi Arabia (Salar and Aneja 2007).
2. *Rhizomucor nainitalensis* produces colonies with sporangiospores and grows at 48 °C and is very similar to *R. miehei*. Sporangiospores are ellipsoidal, dumb bell-shaped and 3–6 μm in diameter (Joshi 1982). They grow predominantly on decomposed oak log and mainly have been reported in tropical places such as India (Salar and Aneja 2007).
3. *Rhizopus rhizopodiformis* produces colonies and grows well at 45 °C. Initially colonies are white and later turn black (Thakre and Johri 1976). They have a hyphae structure 3–7 μm in diameter; sporangia are spherical with a smooth, black surface and 76–175 μm in diameter and are found in coal mine soils, nesting material of birds, bread, wooden slats, soil, seeds of *Lycopersicon esculentum*, and oil palm effluents. They are found in India, the United Kingdom, South Africa, China, Hong Kong, Indonesia, Malaysia, and Japan (Salar and Aneja 2007).

3.5.2 Ascomycetes

1. *Canariomyces thermophila* colonies grow at 45 °C under laboratory conditions (Von arx et al. 1988). Ascospores are dark colored and irregularly shaped with spore size 14.0–18.0 × 7.5–10.0 μm in diameter. They have been largely found in Africa (Salar and Aneja 2007).
2. *Chaetomidium pingtungium* have colonies that grow at 45–50 °C. They produce dark globose cleistothecia with thick hairy walls and are 2–5 μm wide and 350 μm long. Asci are cylindrical in shape with a diameter of 40–52 × 7–9 μm and have an 8-spored structure (Chen and Chen 1996). Ascospores are uniseriate, dark brown, and are thick-walled structures 8.5–10.0 × 6.5–8.5 μm diameter. They have been reported from sugarcane fields of Taiwan (Salar and Aneja 2007).
3. *Chaetomium britannicum* produces colonies that grow at 45–50 °C under laboratory conditions (Ames 1963). Ascomata are cylindrical in shape with a hairy surface. Ascospores are brown in color and irregularly shaped (19–24 × 11–14 μm diameter). They have been reported from mushroom compost and soil of the United Kingdom (Salar and Aneja 2007).
4. *Chaetomium mesopotamicum* has colonies that grow at 30–50 °C. They are different from other species (Abdullah and Zora 1993). It produces long and

highly branched structures and has been isolated from date palm in Iraq (Salar and Aneja 2007).

5. *Coonemeria aegyptiaca* produces colonies growing at 25–55 °C under laboratory conditions. Ascocarps are a crusty mass with red brown color during the initial growth phase and have a coiled hyphae structure. Ascospores are single celled and appear yellowish to pale reddish orange, are thick-walled and smooth. Conidiophores are produced on aerial hyphae, with a smooth-walled surface, and diameter of 50–300 × 5–7 μm with apical parts irregularly branched. Phialides are solitary or irregularly verticillate and cylindric structure, with diameter size 12–30 × 3–6 μm. They are usually found in soil and have been reported from Egypt and Iraq (Ueda and Udagawa) Mouchacca 1997; Salar and Aneja 2007).
6. *Melanocarpus thermophilus* has eight-spored asci (Guarro et al. 1996), with ascospores being ovoid, dark brown, 7.5–9.0 × 6.0–7.5 μm, and each provided with a single germ pore. This isolate has been reported from forest soil of Iraq (Salar and Aneja 2007).

3.5.3 Deuteromycetes

1. *Arthrinium pterospermum* colonies have been shown to grow at 37 °C. They have hyphae like structure 2 μm in diameter (Apinis 1963). Conidiogenous cells are colorless during the initial phase of growth and later become dark brown with a diameter size of 7.6 × 5.3 μm. These have been described from hay and soil in the USA (Salar and Aneja 2007).
2. *Myceliophthora thermophile* produces colonies at 45 °C. Colonies have floccose surface or granular (Van Oorschot 1977). Initially, they have white colored colonies, which subsequently turn brown with a diameter of 2 μm. These have been reported to grow in soil from various countries with diverse climatic conditions such as Canada, India, Japan, and Australia (Salar and Aneja 2007),
3. *Papulospora thermophila* colonies have two types of mycelia: those with an arrow diameter (1.5 μm) and those with a wider diameter (5–7 μm) and with highly branched structures (Fergus 1971). Bulbils are present on hyphae in the form of globes to subglobose with diameter of 105 × 90 μm. These hyphae have an irregular shape, and initially, they are yellow later orange in color. These have been reported from mushroom compost and soil in India and Japan (Salar and Aneja 2007).
4. *Scytalidium indonesicum* colonies grow at 45 ° C on laboratory media. Conidia are brown with barrel-shaped to ellipsoid shape; they have a thick wall surface with a diameter size 25–12 μm (Hedger et al. 1982). Once conidia are fully grown they form irregular shaped brown structures with thick wall. These have been reported from Indonesia (Salar and Aneja 2007).

3.6 Metagenomic Analyses of Thermophilic Fungi

Metagenomics-based analysis has considerable potential in understanding industrial applications of thermophiles. The general strategy of metagenomics utilizes both functional- and sequence-based techniques for a comprehensive understanding (Rodriguez et al. 2018). Thermophiles surviving above 55 °C have high potential for biotechnological applications for production of biocatalysts, which can be beneficial in reducing contaminations, especially in food industry. Thermoenzymes are efficient under high pH, temperature, pressure, and high concentration of substrate and hence are used in production of paper, textile, food, and pharmaceuticals (DeCastro et al. 2016). Table 3.2 shows some cellulases obtained from thermophilic fungi.

3.7 Metagenome-Based Classification

The classification of fungi in metagenomics studies is based on the Internal Transcribed Spacer (ITS) sequence obtained from next-generation sequencing data (Oliveria et al. 2015; Thanh et al. 2019). The so-obtained sequence data must be deposited in a public repository such as the National Centre for Biotechnology Information (NCBI). Table 3.3 shows the study of thermophilic fungi based on taxonomy and diversity. Different repositories and tools (http://www.indexfungorum.org/, http://www.mycobank.org/) are available where the description of each taxon is available based on their nomenclature and classification database. Many of the sequences obtained from such studies have been identified/tallied with appropriate MycoBank numbers, some of them mapped with fungi which had been identified considerably earlier. An example is *A. fumigatus*, identified in a study by Latge (1999), which has been mapped with an isolate identified in 1863.

3.8 Phylogenetic Analysis: Case Study

A case study of *Thermomyces lanuginosus* (Fig. 3.2) shows that many isolates of this species have been obtained, sequenced, and submitted in GenBank. A phylogenetic analysis using Mega X (Kumar et al. 2018) shows that the isolates map close to each other though many clades and subclades have formed. This indicates that considerable differences also exist among the isolates. This directly indicates possible genetic diversity among produced compounds such as enzymes and other metabolites too.

Table 3.2 Some cellulases obtained from thermophilic fungi

Enzyme	Optimum temperature (°C)	Optimum pH	Source	Reference
AtCel7A	60	5.0	*Acremonium thermophilum*	Voutilainen et al. (2008)
CBHI	55	3.0	*Aspergillus aculeatus*	Takada et al. (1998)
CtCel7A	65	4.0	*Chaetomium thermophilum*	Voutilainen et al. (2008)
CBH3	65	5.0	*Chaetomium thermophilum*	Li et al. (2006)
CBH1	55	5.0	*Humicola grisea* var. *thermoidea*	Takashima et al. (1998)
TeCel7A	55	4.0	*Talaromyces emersonii*	Voutilainen et al. (2010)
Cel7A	65	3.0	*Penicillium funiculosum*	Texier et al. (2012)
TaCel7A	55	5.0	*Thermoascus aurantiacus*	Voutilainen et al. (2008)
CBHI	65	6.0	*Trichoderma viride*	Song et al. (2010)
ThCBHI	50	5.0	*Trichoderma harzianum*	Colussi et al. (2011)
Bgl	60	5.0	*Fusarium oxysporum*	Zhao et al. (2013)
Bgl4	55	6.0	*Humicola grisea* var. *thermoidea* IFO9854	Takashima et al. (1999)
Bgl1	55	5.5	*Orpinomyces* sp. PC-2	Li et al. (2004)
Bgl1G5	50	6.0	*Phialophora* sp. G5	Li et al. (2013)
Cel3A	50–60	5.0	*Amnesia atro brunnea*	Colabardini et al. (2016)
Cel3B	50–60	5.0	*Amnesia atro brunnea*	Colabardini et al. (2016)
BglB	52	5.5	*Aspergillus nidulans*	Calza et al. (1985)
Bgl	50	5.0	*Aspergillus oryzae*	Tang et al. (2014).
MoCel3A	50	5.5	*Magnaporthe oryzae*	Takahashi et al. (2011)
MoCel3B	50	5.5	*Magnaporthe oryzae*	Takahashi et al. (2011)
NfBGL1	80	5.0	*Neosartorya fischeri*	Yang et al. (2014)
PtBglu3	75	6.0	*Paecilomyces thermophile*	Yan et al. (2012)
RmBglu3B	50	5.0	*Rhizomucor miehei*	Guo et al. (2015)
β-Glucosidase	75	4.5	*Talaromyces aculeatus*	Lee et al. (2013)
CBH1	55	5.5	*Fusarium lini*	Mishra et al. (1983)
CBH11	55	5.5	*Fusarium lini*	Mishra et al. (1983)

(continued)

Table 3.2 (continued)

Enzyme	Optimum temperature (°C)	Optimum pH	Source	Reference
S1	50	4.0	*Irpex lactus*	Kanda et al. (1980)
En1	50	5.0	*Irpex lactus*	Kanda et al. (1980)
E2A	60	5.0	*Irpex lactus*	Kubo and Nisizawa (1983)
E2B	60	5.0	*Irpex lactus*	Kubo and Nisizawa (1983)
GB1(E)	60	5.5	*Pyricularia oryzae*	Hirayama et al. (1978)
GB2(E)	45	5.0	*Pyricularia oryzae*	Hiramaya and Nagamaya (1979)

Table 3.3 Taxonomy of thermophilic fungi

Taxon name	MycoBank number	Reference
Aspergillus fumigatus	MB#211776	Latge (1999)
Chaetomium thermophilum	MB#807382	Amlacher et al. (2011)
Myceliophthora fergusii	MB#317954	van den Brink et al. (2012)
Myceliophthora heterothallica	MB#519877	Houbraken et al. (2011)
Myceliophthora guttulata	MB#802335	Zhang et al. (2014)
Rhizomucor miehei (=Mucor miehei)	MB#322483	Schipper (1978)
Rasamsonia byssochlamydoides	MB#519877	Houbraken et al. (2011)
Thermomyces dupontii	MB#805186	Houbraken et al. (2014)
Thermothelomyces heterothallica(=Myceliophthora heterothallica)	MB#809491	Stchigel et al. (2015)
Thermothelomyces thermophila (=Myceliophthora thermophila)	MB#809493	Stchigel et al. (2015)
Thermomyces lanuginosus (=Humicola lanuginosa)	MB#239786	Singh (2003)

3.9 Metabolites

Thermophilic and thermotolerant fungi are considered as good sources of thermostable enzymes, which can be useful in industrial processes that function at higher temperatures. A variety of thermostable enzymes have been obtained from thermophilic fungi such as cellulases, lipases amylases, and proteases (Thanh et al. 2019).

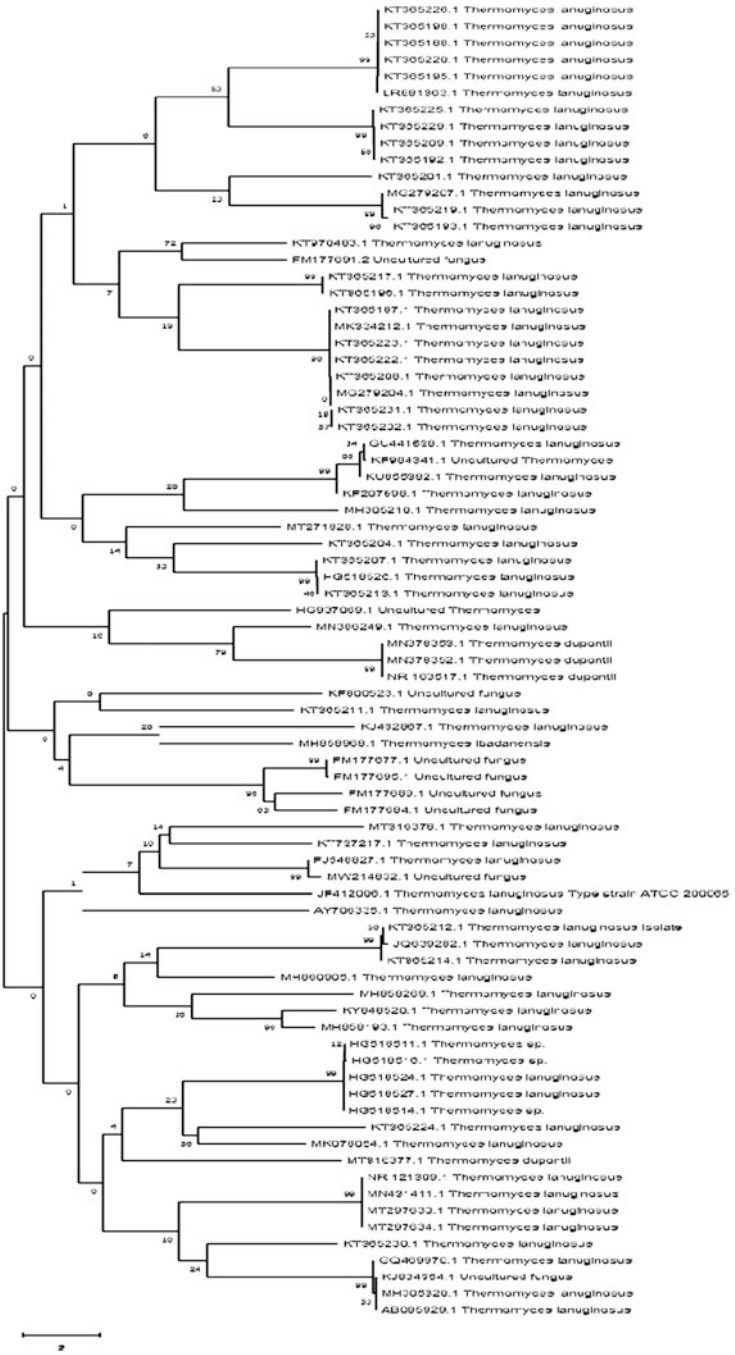

Fig. 3.2 Phylogenetic tree of 95 GenBank nucleotide sequences from isolates of *T. lanuginosus*. Branch lengths are in the same units as those of the evolutionary distances, computed using the Maximum Composite Likelihood method

Hence, the information provided in Sect. 3.8 can be relevant in further purifying and better understanding of more potentially novel thermo-stable enzymes, considering that many of the isolates of *T. lanuginosus* fall into distinct clades.

Many carbohydrate-active enzymes (CAZymes) have been found in thermophilic fungi. For example, four polysaccharide lyases (PLs), 28 carbohydrate esterases (CEs), and more than 50 enzymes with auxiliary activities (AAs) have been described. The genome of *Thielavia terrestris* has been shown to encode nearly 500 CAZymes, including 212 glycoside hydrolases (GHs), 91 glycosyl transferases (GTs), and 80 carbohydrate-binding modules (CBMs). Studies have shown that common strategies for thermal adaptation include a reduced genome size and increased frequency of the amino acids such as Ile, Val, Tyr, Trp, Arg, Glu, and Leu (IVYWREL) in proteins (Thanh et al. 2019), contributing to thermostability. *T. lanuginosus* has encouraging potential in production of xylanase too, as seen from its genetic sequence (Oliveria et al. 2015).

Orfali and Perveen (2019) isolated two new compounds - 3-(furan 12-carboxylic acid)-6-(methoxycarbonyl)-4-hydroxy-4-methyl-4 and 5-dihydro-2H-pyran 1 3-α-methyl-7-hydroxy-5-carboxylic acid methyl ester-1-indanone - from a thermophilic *Penicillium* species that was initially isolated from Ghamiqa hot spring sediments (Saudi Arabia). Austinol, emodin, and 2-methyl-penicinoline, already documented, were also isolated. Emodin showed cytotoxicity against HTB-176 cell line, while austinol exhibited antibacterial activity against *Pseudomonas aeruginosa.* It is interesting that new bioactive molecules are elicited from thermophilic fungi and need further studies.

Metabolite profiling of *T. lanuginosus* and *Scytalidium thermophilum* (Yang et al. 2020) showed the presence of 23 metabolites from *T. lanuginosus*. Among these, there was also a new metabolite, therlanubutanolide, and 15 known compounds. In addition, seven known compounds were obtained from *S. thermophilum*. Also, a polyketide synthase pathway-derived metabolite, three 3,4-dihydronaphthalen-1 (2H)-ones, was obtained from both fungi. This metabolite has been shown to possess antimicrobial activity and is known to be a phytotoxin in plant pathogenic fungi.

Nematicidal compounds have been described from a thermophilic fungus, belonging to a class of PKS-NRPS hybrid metabolites (Guo et al. 2012). These compounds from *Talaromyces thermophilus* were found to have a 13-membered lactam-bearing macrolactone, namely, thermolides A–F, as per NMR spectra. Two compounds showed nematicidal activity against three destructive nematodes: *Meloidogyne incognita, Bursaphelenches siyophilus*, and *Panagrellus redivevus.*

It is interesting to see that thermophilic fungi can not only synthesize bioactive metabolites but also facilitate biotransformation. Sreelatha et al. (2018) have shown that *T. lanuginosus* can convert spironolactone to the minor mammalian metabolites 7α-thiospironolactone (M1) canrenone (M2), 7α-thiomethylspironolactone (M3), and 6β-OH-7α-thiomethylspironolactone (M4). Hence, possessing similar mammalian enzyme systems, their potential in biotransformation, and production of important metabolites warrants exploration.

These thermophilic fungi can also be utilized for metabolic engineering as evidenced by the work of Li et al. (2020). They used the cellulolytic thermophilic

filamentous fungus *Myceliophthora thermophile* in an attempt to produce ethanol from glucose and cellobiose. They introduced the ScADH1 gene into the wild-type strain and found that ethanol production was increased when glucose was used as substrate. However, overexpression of a glucose transporter or cellodextrin transport system from *N. crassa* resulted in increased ethanol production. Transcriptomic analysis showed downregulation of genes involved in oxidation–reduction reactions and stress response but upregulation of protein synthesis related genes.

3.10 Conclusion

It is obvious from this chapter that thermophilic fungi hold great potential for future prospects in industry. While many such fungi have been isolated, identified, and sequenced, whole-genome sequences and the mining of information from their genes are yet to be comprehensively elucidated. Next-generation sequencing technologies are set to play a vital role in better understanding this unique group of fungi. Considering that metagenome analysis of thermophilic fungi, though very less, has shown matches with fungi already documented, this can ease out the process of information mining. It is expected that in the next few years, a wealth of information repository would be developed focusing on thermophilic fungi.

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Thermophilic Fungi: Habitats and Morpho-Molecular Adaptations

4

Regina Sharmila Dass, Joy Elvin Dhinakar, Akriti Tirkey, Mayukhmita Ghose, and Angeline Jessika Suresh

Abstract

The study of extremophilic fungi has received manifold global attention during the past decade. Among the members belonging to the monophyletic fungal kingdoms, very few species have the capacity to survive and proliferate between the temperature range of 45–55 °C. These temperatures are considered as high temperatures while studying thermophilic and thermotolerant fungi. Earlier classification and studies were arbitrarily carried out based on their cardinal temperatures. The temperature endured by the fungi are not as high as those witnessed in bacteria and archaebacteria, adding to the very many reasons for not receiving due publicity in the past. However, drastic improvements in the methods employed for molecular fungal phylogeny and DNA-based studies has eliminated such hassles and paved the way for the elucidation of thermophily as an interesting phenomenon in fungi. Such fungal candidates have lent themselves as tools and excellent laboratory material for classical, genetic, and applied research. Their morphological, physiological, and molecular adaptations, characteristics, diversity and their role in different habitats such as soils, compost heaps, agricultural and forest debris, etc. have been reviewed and presented.

Keywords

Diversity · Fungi · Habitats · Molecular studies · Thermophilic · Thermotolerant

R. S. Dass (✉) · A. Tirkey · M. Ghose · A. J. Suresh
Fungal Genetics and Mycotoxicology Laboratory, Department of Microbiology, School of Life Sciences, Pondicherry University, Pondicherry, India

J. E. Dhinakar
Department of Studies in Biochemistry, University of Mysore, Mysore, India

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4.1 Introduction

Fungi are widely distributed eukaryotes present in almost all the known habitats of microorganisms. Their presence in diverse environments is owed to their structure and ability to produce several exoenzymes to degrade and solubilize complex, insoluble macromolecules. Fungi generally prefer dark and moist or humid environments. Geared with an extremely diverse metabolic activity, fungi have taken over several ecological habitats (Naranjo-Ortiz and Gabaldon 2019). Being saprobes, fungi are abundantly present in soil rich in organic matter and play a major role as decomposers of dead matter or recyclers of nutrients in the soil. They can easily grow on and in between the soil aggregates. The presence of fungi in soil is affected by several factors such as soil pH, moisture, temperature, salinity, structure, and the presence of other organisms (Rouphael et al. 2015). Most of the fungi have temperature optima between 20 and 25 °C and prefer acidic conditions. Several basidiomycetes have been reported to lose their tolerance above pH, 7 but many other taxa of fungi have a wide pH tolerance range (Dangles and Chauvet 2003). Fungi are found in varying types of soils ranging from grasslands, forest, and agricultural soils to extreme environments like tundra regions and soils of the desert. In grasslands, the fungi are important in terms of their nutrient recycling activity, production of enzymes, and organic compounds due to interaction with plants, further resulting in the inhibition of grassland biodiversity (Huhe et al. 2017). Basidiomycetes are reported as the dominant taxon in grasslands, while ascomycetes are the major decomposers of organic matter in agricultural soils (Deacon et al. 2006).

The ability to withstand and thrive in the extremes of temperature is found throughout the domains of life. However, the extent of tolerance to high temperatures varies, with Eukarya showing a more restricted nature of thermophily. The taxonomically diverse group of thermophilic fungi is perhaps one of the most enigmatic life forms on Earth, despite them occupying only a fraction of Kingdom Fungi (Salar and Aneja 2007). There are numerous classification systems to define thermophilic fungi based on minimum, optimum, and maximum temperatures of growth starting from Cooney and Emerson (1964) classification (Oliveira et al. 2015). While on the other hand, Maheshwari et al. (2000) defines thermophilic fungi with a more straightforward definition as those fungi whose optimal growth temperature lays between 40 and 45 °C or greater. Among multiple definitions for thermophilic fungi, a recent one defines them as the fungi which demonstrate their optimum growth between 40 and 50 °C (Oliveira et al. 2015).

4.2 Terrestrial Thermophilic Fungi

The fungi are the most fascinating creatures of the eukaryotic microbial world. Of the abundant fungal forms, a specific few species possesses the unique attribute to survive, grow, and multiply between 45 and 55 °C. These are referred to as thermophilic or thermotolerant fungi, which are classified purely on their cardinal

temperature requirements (Cooney and Emerson 1964; Crisan 1973). Unlike in bacteria, thermophily in fungi does not necessarily exhibit growth at temperatures close to 100 °C as seen in archaebacteria or eubacteria isolated from solfatara habitats, hydrothermal vents, and thermal springs (Brock 1995; Blochl et al. 1997). Ever since the discovery of thermophilic fungi, their identification had always been a challenging and tedious task for mycologists due to the lack of availability of sequences of reference strains in public/genetic databases. However, taxonomic studies, advancement in sequencing technology leading to the discovery of rapid DNA-based methodologies, chemo- and numerical taxonomic methods and, new age sequencing (next-generation sequencing) have made their identification much easier and faster (Oliveira et al. 2015; Raja et al. 2017).

4.3 Aquatic Thermophilic Fungi

Fungi are widespread in aquatic habitats. But most of them are still unknown to us, since many locations across the globe are yet to be explored. This is due to the challenges associated with aquatic sampling, especially the marine habitat. However, understanding the beneficiary role of aquatic fungi to the environment and in turn to mankind has led scientists to undertake various fungal biodiversity studies in aquatic habitats. Studies on such ecosystems report the prevalence of fungi of the phyla *Ascomycetes* and *Chytridiomycetes* in aquatic habitats, while *Basidiomycetes* have found be occurring the least. Moreover, species similarities were commonly found between freshwater habitat and terrestrial habitats, while the marine habitat shows uniqueness in its fungal biodiversity (Shearer et al. 2009) and therapeutic potential.

Aquatic fungi are mostly found to be proliferating on submerged organic substrates of either plant or animal origin. Some of these fungi are well adapted to grow in freshwater habitats and hence are referred to as "resident" aquatic fungi, while "transient" aquatic fungi are those which comprise spores from the adjoining terrestrial habitat. Studying freshwater fungi is tedious because of their tight adherence to their substrate through their elongated hyphae. Freshwater fungi mostly thrive on dead coarse particulate organic matter, especially on plant litter, using them as primary energy source and nutrient source. In turn, these fungi function in the decomposition of plant litter in freshwater ecosystem, thereby transferring energy and nutrients to higher trophic level, such as invertebrates (Gulis et al. 2009). The importance of aquatic fungi in the ecosystem has been extensively studied (Krauss et al. 2011). They can also serve as potential agents for pollution study, as climatic change has a direct impact on them. Aquatic fungi play a major role in recycling organic matter in aquatic environment and in maintaining the aquatic food web.

Brackish water is another aquatic habitat that has been investigated at a large scale. Such water bodies have a higher salinity as compared with freshwater, thereby favoring the growth of fungi of well-known marine taxa such of *Halosphaeriaceae* and Lulworthiaceae family. Studies carried out in lakes (such as Lake Fuxian of China, Lake of Italy and many more), containing brackish water, have reported

similar occurrences (Grasso and La Feria 1985; Cai et al. 2002) and were initially referred to as the "saltwater fungi." Besides, fungi show a great biodiversity in brackish waters based on its climatic condition. As such, *Ascomycetes* supersede mitosporic fungal community in temperate climate, while the tropical region reports the contrary (Hyde and Goh 1998; Cai et al. 2002).

Marine habitat is the largest aquatic habitat in the world that offers an enormous range of biodiversity. Of these, extremophiles and thermophilic fungi have been widely exploited as a potential source of novel bioactive compounds in last 15 years. In order to grow in such hypersaline environments alongside other stress-inducing factors such as high pressure and extreme temperature, these extremophiles produce special enzymes, which are of medicinal and industrial importance (Dalmaso et al. 2015).

4.4 Morpho-Molecular Adaptations

4.4.1 Morphological Adaptation

Records suggest that Lindt was the first scientist to isolate a thermophilic fungus namely *Mucor pusillus*from bread. He was the pioneer for the world of thermophilic fungi (Lindt 1886). *Mucor pusillus* is now rechristened as *Rhizomucor pusillus*. In 1899, another scientist named Tsiklinskaya studied yet another thermophilic fungus growing on potatoes. The fungus was then identified as *Thermomyces lanuginosus* (Tsiklinsky 1899). However, there was no information available at that time, regarding nutritional requirements and natural habitats that favored growth of thermophilic fungi. In the early twentieth century, Kurt Noack worked with thermophilic fungi and isolated them from a vast range of natural substrates (Noack 1920). He used respiration as a probe to determine if the metabolic rate of the thermophilic fungi had any effect on their characteristic nature. Following him, another independent research scientist Hugo Miehe intensively researched on thermophilic fungi and accumulated substantial information regarding their primary habitat. He isolated four species of thermophilic fungi, namely, *Mucor pusillus*, *Thermomyces lanuginosus*, *Thermoidium sulfureum*, and *Thermoascus aurantiacus* (Miehe 1930), that set the stage for future research. A compilation, such as this one, would rather be incomplete without acknowledging their pioneering work.

Thermophilic fungi are found across the fungal phyla. They are especially abundant in the phylum *Ascomycota* followed by the "traditional" phylum *Zygomycota* (Salar and Aneja 2007). These unique fungi are often found ubiquitously in soils or in impermanent microhabitats consisting of degrading plant biomass such as composting bins, lake sediments, and birds' nests (Oliveira et al. 2015). Much is yet to be discovered of the ecology of thermophilic fungi, but the growth of these transient communities seems to be fostered by high temperatures and precipitation (van Noort et al. 2013). Certain thermophiles can be pathogenic to human beings, livestock, or plants while others are known to be saprophytic in nature (Di Piazza et al. 2020). Taxonomic classification of thermophilic fungi has

undergone major revision over the past few decades due to clashes in nomenclature, misidentification of thermotolerant species as thermophilic species, and the recovery of new fungal sequences from environmental samples (Oliveira et al. 2015). It is suggested by many that the ancestors of the thermophilic fungi might have been mesophilic, which eventually developed thermal tolerance. Some also believe that this was a result of adaptation of fungi to seasonal changes and higher temperatures during daytime and not specifically to occupy new thermophilic habitats (Powell et al. 2012). Since then, several researchers have proposed cardinal temperature points for the classification of thermophilic fungi based on their optimal growth temperatures. This led to a divergence of these fungi into two groups: thermophilic and thermotolerant. It was not before the monograph published by Cooney and Emerson (1964) on thermophilic fungi that this group of fungi drew focused attention of the mycologists across the world. Thermophilic fungi were seen to play a key role in resin utilization in crude rubber, thereby making the extraction of rubber from its shrub much feasible. Eventually Allen and Emerson's work provided evidence for the same. According to them, these thermophilic fungi could thrive at temperature as high as 60 °C (Allen and Emerson 1949). Later, the term "thermophiles" was used by Apinis to describe fungi having optimal growth at 35–40 °C (Apinis 1953), who's descriptions recorded lower temperatures compared with other researchers (Allen and Emerson 1949).

The fungal cells are encapsulated with a polysaccharide rich wall, which is a pivotal entity for the preservation of cellular integrity, which in turn aid in their protection from external invaders, namely, the environmental agents and host infections (Geoghegan et al. 2017). The fungal cell wall is more than merely being an external shield. It harnesses an exquisite architecture in form and its composition, controlling the morphological structure and recognition of an extensive range of external environmental stimuli. The cell wall of the fungi is thought to have a stratified structure while under observation using an electron microscope; the electrons are profound and densely present in the outer layer (Osumi 1998). The composition in the inner layer of the fungal cell wall is majorly composed of the chitin–glucan matrix that is cross-linked; mannosylated proteins are observed to be rich in the outer layer. The substantial increase in the electrons observed in the inner segment of the cell wall is because of the presence of melanin. Occasionally, the fungal cell wall exhibits itself as a granular form if not as a distinct individual electron rich layer (Latge 2007). In contrast to the genome reduction, duplication of genes for hyphal melanization in thermophiles, which provides resistance against desiccation, high temperature, and UV radiation, may provide newer insights into their evolutionary process (van Noort et al. 2013). Due to climate change and global warming, it is predicted that as the average global temperature rises, more thermotolerant and thermophilic fungi will come into picture (Paterson and Lima 2017).

The fungal cell wall (FCW) composition by mass is composed of β-1, 3 glucans being the most frequently occurring (approximately about 80 per cent) in the hyphal cell wall of the rice blast fungus *Magnaporthe oryzae* (Samalova et al. 2016). Chitin and of β-1, 6 glucan each comprise less than 10% of the entire polysaccharide,

whereas in a couple of species β-1, 6 glucan occurs as a single residue branch points off β-1, 3 glucan chains rather than a polymer instead (Free 2013; Samalova et al. 2016; Geoghegan et al. 2017). Chitin turns out to be a straight polymer of β- 1,4 -*N*-acetylglucosamine. The polymers with individual chains undergo hydrogen bonding in the interchain region, giving rise to the genesis of microfibrils along with a massively rigid structure. In comparison, the primary constituents of the fungal isolates associated with the glucan portions of the cell wall are noncrystalline. The β-1, 3 linked glucan chains form a branched structure, which is amorphous. Every β-1,3 linked glucan chain is variegated with branch points, alongside β-1,6 linkages (Borchani et al. 2016). Both β-1,3 and β-1,6 linked glucans can be interlinked to chitin. The polysaccharide constituents present in the cell wall form an extremely interwoven and interlinked matrix that imparts great strength. The protein constituents present in the cell wall are usually a small cell wall component. They incorporate the proteins possessing structural roles like sensor proteins, which are membrane-bound and enzymes required for synthesis and rearrangement. The proteins are mostly *N*- or *O*-glycosylated, with side chains comprising of mannose-rich residues. The aforementioned proteins can be intensely attached to the glucan network (Borchani et al. 2016). This in turn provides solid attachment to the fungal cell wall matrix, with most of the cell wall proteins being soluble and non-covalently bonded to the fungal wall with polysaccharide-binding modules (Osumi 1998). The fungal cell wall is a balanced blend of mannoproteins, chitin, alpha, and beta-glucans; they play a couple of roles, namely, rigidity, shape, metabolism, ion exchange, and interactions with the host defense mechanisms (Pan et al. 2010; Lord and Vyas 2019). There is a variation in the cell wall compositions of fungi according to their species. Out of all, the major constituent of most fungal cell wall is β-1,3 glucans. The fungal cell wall contains proteins that play a role in tight adhesion or binding of the fungi to the substratum/substrate. This is very useful for pathogenic fungi. The cell wall of fungi is essential for its viability, considering the mechanical viewpoint; the cell wall facilitates tolerance of turgor pressure, which aids in the prevention of cell lysis.

In order to survive, the thermophilic fungi must also find means to protect themselves against oscillating temperatures experienced by their habitats by mounting adequate responses. One such response is the heat shock response. A team of researchers (Ianutsevich et al. 2016) probed into the molecular basis of heat shock responses in thermophilic fungi, through studying membrane lipid composition and the production of soluble carbohydrates. The group demonstrated an increase in phosphatidic acids (PA) and sterols in thermophilic species *Rhizomucor tauricus* and *Myceliophthora thermophila* while simultaneously observing a decrease in phosphatidylcholines (PC) and phosphatidylethanolamines (PE). While a significant alteration of membrane composition was reported, more insight about the specific regulatory and functional roles of certain lipids in thermal adaptation is yet to be elucidated. Interestingly, the group also reported that in the response to prolonged heat shock, there was virtually no change in the levels of unsaturation, leading the group to believe unsaturation does not play a role in heat shock responses of thermophilic fungi. Apart from elevated temperatures, thermophilic fungi also

need to withstand other environmental stresses such as cold shock (Gunde-Cimerman et al. 2014), osmotic stress, and oxidative stress. In a study done by Ianutsevich et al. (2020), thermophilic fungi *Rhizomucor miehei* was found to have an increased degree of membrane polar lipid unsaturation and decreased ergosterol concentration during cold shock. In the same report, osmotic stress was found to trigger the production of compatible solutes such as glycerol and arabitol in the thermophilic fungus. The role of disaccharide trehalose remains unclear in thermophilic eukaryotic organisms, although certain evidence suggests it may be required for thermophilia (Zhou et al. 2014). However, the levels of trehalose were significantly decreased under both heat shock and cold shock (Ianutsevich et al. 2016, 2020).

An extremely popular model that has been studied over the years to derive maximum scientific information on the fungal cell wall is *Saccharomyces cerevisiae*. This yeast from the baker's armamentarium has been considered as the darling of biochemical and genetic research, because of the manner in which it has lent itself for cell biology studies too, apart from being credited as the first eukaryote, whose genome was sequenced.

Individually the cells of fungi utilize a sequence of stress sensors, which crossways with the plasma membrane of the cell to constantly monitor the cell wall integrity. The structure of these sensors is quite indistinguishable with a short C terminal cytoplasmic domain. As we venture to understand further, these sensors have single transmembrane domains and periplasmic amino terminus, which are rich in serine and threonine residues. These amino-terminal portions are necessary for the process of endocytosis. The polarized dissemination of the wall stress component Wsc1 is regulated endocytotically. This leads to polarized accumulation of the protein in the fungal cell wall (Piao et al. 2006; Lord and Vyas 2019).

4.4.2 Molecular and Enzymatic Adaptations

Thermophilic fungi are the ones that live in high temperature conditions, while thermotolerant fungi usually live at mesophilic temperatures but can also survive at higher temperatures. One example of a thermotolerant fungus is *Aspergillus fumigatus*, which also can grow at above 50 °C (Mouchacca 2000a). Many fungi are found in very dry ecosystems like dry and hot deserts or in regions where water is present in the bound form, thus drastically reducing the availability of water. Temperature greatly affects the structure and function of biological molecules, thereby serving as one of the main regulators of growth and activity in thermophilic fungi. These fungi do not seem to have a specific organelle or developmental pattern different from that of mesophiles, but survival at elevated temperature requires specific modifications of the already existing structures. In order to grow actively at such high temperatures, these fungi have evolved themselves to adapt their DNA, proteins, and cytoplasmic membrane to thermo-resistant mechanisms (Oliveira and Rodrigues 2019).

The role of mycologists and taxonomists studying fungal phylogeny who have followed painstaking, time-consuming, and elaborate procedures has been well-documented ever since fungi were classified using the five-kingdom classification (Whittaker 1959, 1969) in a number of studies, up to the present-day classification (Alexopoulos et al. 2010). Traditional classification systems have been based almost entirely on morphological characteristics, for instance, nature of the asexual spore formed as a result of mitosis, structure of spore-bearing structures, nature and arrangement of sexual spores, which are a resultant of a meiotic process between opposite mating types, etc. The nomenclature and taxonomic classification of fungi with emphasis to thermophilic fungi is muddled, often leading to ambiguity and confusion (Mouchacca 1997, 2000b). Natural classification systems, which have combined the use of molecular markers, have eliminated this chaos to a great extent. The concept of fungal barcoding is extremely prevalent in the current times and has been used as a resort by most mycologists during elaborate morphological identification procedures, while following lengthy protocols. The evolution of fungal classification using purely morphological methods to the present-day phylogenetic approaches based on DNA-based and chemotaxonomic approaches is well outlined in general (Raja et al. 2017) and particularly in thermophilic fungi (Pan et al. 2010). The genomes of very few thermophilic fungi have been sequenced; several taxa and their barcode sequences are unavailable in genetic databases. The dearth of such genetic information certainly limits the identification and potential use of thermophilic fungi, thus impeding the way for the assessment of newer technologies for the description of a thermophilic species. The paradigm shifts in fungal taxonomy were witnessed, due to the advent and use of computers that led to numerical taxonomic methods. While the role of conventional techniques routinely used in identification of fungi using phenotypic characteristics is still important, chemotaxonomic methods have played an enormous role, leading to the formation of three monophyletic kingdoms of fungi. When chemotaxonomic approaches are being used, cell wall composition is a major parameter used in fungal phylogeny.

Of the six million fungal species that exist (as suggested by high-throughput sequencing techniques), nearly over 1.5 lakh species of fungi that have been isolated and identified till date, which constitute the most intriguing creatures of the microbial world; both mycelial and unicellular forms are bound with the fungal cell wall. This structure is not just vital to fungal growth and survival but also crucial in the morphogenesis (e.g., cell compartmentalization and septal development) of a particular species. Adequate studies on the mutational analysis have evidenced the role of the cell wall as a shielding barrier against a multitude of environmental parameters such as high and low temperatures, desiccation, osmotic stress, and nutrition stress. It is not surprising to note that, today, thermophily in kingdom fungi is believed to have arisen as an adaptive mechanism to seasonal changes and high temperatures prevailing and probably did not emerge due to occupying high-temperature ecological niches. The recent modifications announced by the International Code of Nomenclature for algae, fungi and plants could lead to simplifications in thermophilic fungal taxonomy, making the use of thermophilic fungi in biotechnological, industrial, and research purposes more feasible. The survival of thermophilic fungi

can mainly be attributed to the thermostability of its proteins, particularly its enzymes such as lipases, esterases, amylases, peptidases, and cellulolytic enzymes. Certain features of thermophilic adaptation have been observed across the domains of life. For example, the substitution of lysine by arginine (known as amino acid bias) has been observed on several occasions in thermophilic organisms. A research team (van Noort et al. 2013) reported the increased levels of arginine and tryptophan and decreased amounts of aspartic acid, lysine, and glycine in thermophilic species *Chaetomium thermophilum*, *Thielavia terrestris*, and *Thielavia heterothallica* of the order *Sordariomycetes*. The proteins of *C. thermophilum* were also found to contain large amounts of cysteine, which, as hypothesized by the authors, increases the thermostability of the proteins.

One of the major characteristics of thermophilic fungi is homeoviscous adaptation (Sinensky 1974). The fungi have devised a cellular framework to overcome and circumvent the challenges of experiencing varied temperatures by altering the fatty acid composition of the phospholipids that are present in their cell membranes. This is achieved by maintaining their fluidity of the membrane to keep it intact, for the flawless functioning of the enzymes and transporters localized in the membrane. The fungi recruit a high number of saturated fatty acids upon the exposure to high temperatures; at lower temperatures, it produces adequate number of unsaturated fatty acids. When there is a shift from low to high temperature, an enzyme called fatty acid saturase comes into play. This enzyme converts fatty acid linoleate to oleate in fungal cell membrane. In other words, oleic acid is the most dominant fatty acid of fungal cell membrane at higher temperature (Kates and Baxter 1962; Mumma et al. 1970; Sekura and Fergus 1971). Also, most microbes alter their fatty acid composition of their cell membrane phospholipids, as one of their primary functions in response to the temperatures prevailing in the growth environments. Consequently, there is an increase in the saturated fatty acid content with the increase in temperature. On the contrary, at lower temperatures, there is an increase in the unsaturated fatty acid content. The adaptive phenomenon is referred to as "homeoviscous adaptation." Although, this is true with most microscopic creatures, Wright et al. (1983) reported an exception with the fungal species *Talaromyces thermophilus*, wherein a shift from a higher temperature (50 °C) to a lower (30 °C) growth temperature failed to result in the increase in the degree of unsaturation. The fatty acid composition remained virtually unaltered. There was, however, a difference when *T. lanuginosus* was studied and examined. When mycelia were grown in 50 °C, the phospholipid unsaturated fatty acid composition was 0.88 in contrast to mycelia grown at 30 °C, which was equivalent to 1.00 (Rajasekaran and Maheshwari 1990). *Chaetomium thermophile* also showed a decrease in the degree of unsaturated fatty acids, when a heat shock was applied (Oberson et al. 1999). These examples point toward a certain inability to adjust membrane fluidity and could be the reason for their high minimum growth temperature.

A comparative study on the lipid composition of psychrophilic, mesophilic, thermophilic, and thermotolerant species of *Mucor* and *Rhizopus* by Sumner and his colleagues gave enough evidence to support the aforementioned property of the fungal cell wall (Evans 1969; Sumner and Morgan 1969). Their experiments

revealed that a high content of saturated fatty acids in thermophilic fungi is at high and low growth temperature, while fatty acid composition of thermotolerant fungi matched with that of psychrophiles and mesophiles at low temperature and to thermophilic fungi at higher temperature. Additional and interesting facts that emerged from their study showed that spores contain low amount of lipid but predominating in saturated fatty acid as compared with fungal mycelia. They also understood that molecular oxygen was crucial and played a pivotal role in fatty acid desaturation (Sumner and Morgan 1969). In contrast, reduced oxygen tension at high temperature activates saturases (Harris and James 1969). A study involving *Saccharomyces cerevisiae* subjected to both high (40 °C) and low (26 °C) temperatures, revealed that a high amount of saturated fatty acids and negligible amount of unsaturated fatty acids being present in their cell membrane at 40 °C (Chang and Matson 1972). Mumma and his coworkers elucidated the fact that thermophilic fungi contain higher level of lipid content as compared with mesophilic fungal isolates of same genera (Mumma et al. 1970; Sekura and Fergus 1971). Sterols are present in significant amounts in cell membranes of thermophilic fungi in addition to saturated fatty acids and propose a significant role in providing thermal stability. Thermophilic fungi usually grow under aerobic conditions (Kane and Mullins 1973). However, the thermophilic fungus, *Humicolainsolens*, is an exception and usually prefers anaerobic or microaerophilic environment for its growth.

Complete genome sequencing of thermophilic fungi has provided mycologists with the inner workings of thermophilia in eukaryotic organisms. A research group (van Noort et al. 2013) reported that thermophilic fungi tend to have reduced genome sizes when compared with their mesophilic relatives. The team presented evidence showing a large portion of genes found in the closely related mesophilic species *Chaetomium globosum* and *Neurospora crassa* but not in thermophilic fungi. *C. thermophilum*, *T. terrestris*, and *T. heterothallica* were related to transposable elements. Based on their findings, the group suggested that the phenomenon of transposition is not preferred at elevated temperatures. van Noort et al. (2013) also noted that genes coding for certain enzymes (e.g., oxygenases) are present in fewer copies in comparison with mesophilic species.

Over many years, fungi have evolved and acquired thermostability and thermotolerance to overcome stressful and drastic temperatures. Some fungi have been briefly exposed to sublethal temperatures and have adapted tolerance to be exposed to lethal temperatures. Some of the mesophilic fungi synthesize a set of heat shock proteins (HSPs) following a sudden exposure to higher temperatures. The synthesis or the formation of heat shock proteins turns out to be an adaptive response to encounter stressful conditions and thermotolerance (Trent et al. 1994). Based on the research gone by, scientist groups have inferred that conidia of the fungal species *T. lanuginosus* germinates at 50 °C, while it is heat treated at 55 °C for about 60 min prior to the brief exposure of 58 °C. These conidia priorly exposed to such heat shock, exhibited prolonged survival compared with that of non-treated conidia (Maheshwari et al. 2000). Therefore, based on this observation, the heat shock proteins come into play in order to enhance the thermotolerance in fungi. If in case the protein synthesis is blocked or inhibited during the heat shock period or heat

shock interval using cycloheximide, it either deteriorated or eliminated the thermotolerance in fungi. Pulse labeling of proteins during the heat shock interval or the heat shock period led by the separation through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), revealed an increase in the genesis of the heat shock proteins. A transitory heat shock protein was also observed in *Chaetomium thermophile* var. *thermophile* being specific a HSP60 (heat shock protein) that was abundantly expressed in this species; this posed to be an important member of thermophily (Oberson et al. 1999). The heat shock proteins (HSPs) prompt fungal genera to withstand or overcome lethal temperatures. The enzymes present in the fungi also enable them to survive in adverse conditions, thus making them resilient to strive all odds in the environment. The fungi emerge out to become more competent over a period of time after the exposure to all environmental fluxes. As mentioned earlier, there are a set of enzymes that are present in thermophilic fungi that aid in sustaining lethal and adverse conditions. These enzymes are called the secretory enzymes and have found wide applications in industrial bioprocesses (Maheshwari et al. 2000), like various food business organizations like fermentation sectors, breweries, food and beverage industries, etc.

Just like prokaryotic extremophiles, several fungal species also make up the eukaryotic extremophiles. AM (arbuscular mycorrhizal) fungi (Macek et al. 2011) and soil yeast *Occultifur mephitis* sp. nov. (Sibanc et al. 2018) have been reported from the mofette sites (natural CO_2 springs) of Slovenia, which are ambient temperature areas, rich in gas vents of pure geological CO_2 rising to the surface from the deep mantle. The elevated levels of CO_2 lead to reduction in O_2 concentration making the soil hypoxic and high carbonic acid in the soil results in the lowering of pH. Such fields are common in volcanically or tectonically active sites (Macek et al. 2016).

Apart from thermophily, several psychrophilic fungi have been isolated in large numbers from the sub-glacial ice of polythermal glaciers (Gunde-Cimerman et al. 2003). Populations of *Penicillium crustosum* have been reported from the glaciers of Svalbard, Norway (Arctic), which have unique features like they are capable of producing andrastin A, a secondary metabolite or are unable to use creatine as their sole source of carbon (Sonjak et al. 2007, 2009). Two novel endemic genotypes *Thelebolus globosus* and *T. ellipsoideus* have been isolated from the extreme cold environments of Antarctica (De Hoog et al. 2005). Halophilic fungal populations have been abundantly and consistently reported from naturally hypersaline ecosystems (Gunde-Cimerman et al. 2000), which have unique molecular adaptations to survive at high ion concentrations and low water activity (Plemenitas et al. 2008). *Hortaea werneckii*, a halotolerant fungus, is capable of growing in almost saturated solutions of sodium chloride and also without it (Gunde-Cimerman et al. 2000). Acidophilic and acid-tolerant fungi have been reported from acidic environments like acid mine drainage, volcanic springs, and industrial wastewaters (Gross and Robbins 2000). *Aspergillus* sp. and *Geotrichum* sp. have been isolated from copper mine (Orandi et al. 2007). Eurotiomyces and Dothideomycetes are found in highly acidic environments of acid mine drainage at Richmond Iron Mountain where the pH was 0.8 (Baker et al. 2004, 2009).

Ascomycetous, filamentous fungus *Xeromyces bisporus* is one of the most xerophilic organisms, which can survive in extreme dry conditions and has a water activity optimum at 0.85 (Grant 2004). *Aspergillus penicillioides* is a polyextremophilic fungus as it demonstrates features of being a halophile (can grow at high-salt concentrations), xerophile (has very low water activity), osmophile (can withstand high sugar concentrations), and a psychrophile (can grow at near 0 °C) and can also grow anaerobically (Chin et al. 2010; Zhang et al. 2013; Nazareth and Gonsalves 2014; Stevenson et al. 2015).

Thermophilic fungi are cosmopolitan. In both natural and man-made environments, they have been observed to be present as propagules of active mycelia (Oliveira and Rodrigues 2019). The dissemination of propagules or spores (sexual or asexual) by air may be the cause behind their ubiquitous distribution (Le Goff et al. 2010). Forest soils are covered by plant debris and litter, which make a very favorable environment for diverse groups of fungi. The terrestrial ecosystems, which accumulate lignocellulosic biomass, are broken down by lignolytic or cellulolytic fungi by the production of extracellular lignocellulases. Such enzymes also be useful in a multitude of industrial and bioremediative processes. Thermophilic fungi are also potential candidates for the production of thermostable enzymes. The use of fungal isolates for the industrial production of such bioenzymes may greatly reduce the costs involved in synthetic production (Banerjee et al. 2010). Their use in the production of industrial enzymes has also increased economic feasibility of industrial processes. Additionally, such utilization has minimized problems caused in the environment as a result of its disposal (Reddy et al. 2003; Sanchez 2009). Among the microbes cultivated using these biological wastes, filamentous fungi have topped the list, especially by adopting solid state fermentation techniques (Leite et al. 2008; Alves-Prado et al. 2010; Moretti et al. 2012). In a focused study by de Cassia Pereira and team (2015) who aimed at obtaining new cellulases and xylanases from thermophilic fungi, they reported that *Myceliophthora thermophila* JCP1-4 was the best producer of endoglucanase, β- glucosidase, and xylanase. The enzymes produced remained active at 55–70 °C and stable between 30 and 60 °C. Many countries in the world whose businesses run on agro-industries accumulate millions of tons of lignocellulosic wastes. Such accumulated bio-wastes serve as culture medium for thermophilic fungi or microbes in general. Maheshwari and his research colleagues (2000) have documented such thermophilic mycelial forms and their role in industrial processes, involving the production of enzymes, in large bio-reactors. These enzymes are active and stable at higher temperatures, which serves as an important feature in such industrial processes.

Self-heating environments like composting systems have high temperatures due to the exothermic metabolic reactions of the microorganisms and therefore are one of the most suitable habitats for thermophilic fungi. These fungi mostly occur as propagules and are generally dispersed by the agency of wind (Rajasekaran and Maheshwari 1993), especially from composting piles while turning the piles for aeration and facilitate adequate degradative processes of biomass used. Thermophilic fungi produce and secrete a wide range of extracellular enzymes in their environment with substrate specificity and in large quantities, which have shown

to act in a synergistic way in the decomposition process (Johri and Rajani 1999; Dashtban et al. 2009). When these enzymes were compared with the enzymes of mesophilic fungal species, slight differences were found in their structure, sequence, function, and thermodynamic features (Niehaus et al. 1999; Oliveira et al. 2018).

Thermophilic fungi possess a large potential for the commercial market (Singh et al. 2016). They contain numerous genes coding for a secretion of various industrially important enzymes such as proteases, amylases, xylanases, and other lignocellulolytic enzymes (Maheshwari et al. 2000). These enzymes are often thermostable and confer several advantages to an industrial process such as less contamination by mesophilic organisms and reduced reaction times, therefore minimizing overall energy costs (Oliveira et al. 2015). *Thermomyces lanuginosus*, *Myriococcum thermophilum*, *Thermoascus aurantiacus*, *Myriococcum thermophilum*, and *Thermoascus aurantiacus* are a few examples of thermophilic fungi that have been investigated for industrial use. The biofuel sector especially may greatly benefit from the use of thermophilic fungi, in which extracted thermostable enzymes (e.g., glycoside hydrolases) are used for the bio-conversation of plant biomass into fermentable sugars for the production of second-generation biofuels such as bioethanol. Thermophilic fungi can also be useful for scientific purposes (e.g., in structural studies or biochemical studies), the paper industry, and in food biotechnology (van Noort et al. 2013; Zhou et al. 2014).

The rate of growth of thermophilic fungi is much slower than that of mesophilic fungi (Brock 1967). Moreover, thermophilic fungi undergo slower metabolism than their mesophilic counterparts (Prasad et al. 1979). In case of thermotolerant fungi, certain nutritional supplements favors increase in their growth under conditions of high temperature (Loginova et al. 1962). This is evident in the fungus, *Coprinus fimetarius* (Fries 1953). In a particular research study by Morgenstern and co-workers (2012), the growth performances of 30 fungal strains were studied whose identity was established by previous studies as thermotolerant and thermophilic fungi (Maheshwari et al. 2000; Mouchacca 2000a). Mesophilic strains were also used for comparative studies. Few to name are *Calcarisporiella thermophila*, *Chaetomium thermophilum*, *Paecilomyces*, *Remersonia thermophila*, *Rhizomucor miehei*, *R. pusillus*, *Talaromyces thermophilus*, and *Thermomyces ibadanensis*. Mesophilic fungal strains included *Acremonium alcalophilum*, *Amorphotheca resinae*, *Aureobasidium pullulans*, *Chaetomium globosum*, *Gloeophyllum trabeum*, *Lentinula edodes*, *Pleurotus ostreatus*, and *Tramates versicolor*. The classification of thermophiles only with respect to their growth requirements has been a debatable topic ever since the study of thermophilic fungi has been initiated. The upper and lower limit temperatures set as per Cooney and Emerson (1964) requires further investigations in the current age due to evolving nature of the fungi and classification methodologies. Some species like *Thielavia australiensis*, which grows above 50 °C as they also do below 20 °C, are not covered under traditional or conventional definitions (Morgenstern et al. 2012) of thermophilic fungi.

A modification of the previous definitions was presented by a team of researchers (Morgenstern et al. 2012) who redefined thermophilic species as those that could exhibit faster growth at 45 °C than at a mesophilic temperature of 34 °C, in spite of

growth being observed at 20 °C. This has led to a reclassification of *Calcarisporiella thermophila*, *Remersonia thermophila*, and *Thielavia australiensis* as thermophiles, though they were earlier classified as thermotolerants by different research groups (Seifert et al. 1997; Mouchacca 2000b).

A reduction in the genome size of the thermophilic fungi has also been noticed due to the loss of transposable elements, intergenic regions, protein-coding genes, and shorter introns in comparison with their nearest mesophilic species (van Noort et al. 2013). Upon comparison with phylogenetically related mesophilic fungi, significant reduction in the number of peptidase coding genes in thermophilic fungi has been reported (Oliveira et al. 2018).

An examination of the peptidases from thermophilic fungi revealed the presence of Glu, Pro, Ala, Gly, Val, and Arg residues in larger proportion and Cys, Ile, His, Met, Gln, Thr, Asn, Trp, and Ser in lesser proportion in comparison with mesophilic peptidases. Also, the number of internal cavities in thermophilic peptidases was reduced. According to the researcher, two possible scenarios for these modifications are (1) loss of peptidases with large number of internal cavities and retention of only compactly folded peptidases and (2) enzyme optimization to contain fewer cavities (Oliveira et al. 2018).

4.5 Conclusions

The taxonomically diverse group of thermophilic fungi is perhaps one of the most enigmatic life forms on Earth, despite them occupying only a fraction of kingdom fungi. The genetic constitution of an organism is the key for it to be classified as thermophilic, which is true even in case of thermophilic fungi. Thermophilic fungi serve as a great source of heat stable enzymes, which are of industrial importance. Once scientists became well aware of their remarkable potency in enzyme production, this group of fungi will continue to draw much attention. Research in the last five decades has undoubtedly increased our knowledge and understanding of the biology and molecular mechanisms in thermophilic fungi, as they have educed interests in the field of pharmaceuticals and enzymes. There will be more additions to the list of thermophilic fungi, an understanding to their biology, lifestyle, etc., as routine and advanced techniques to study them evolve. Both conventional and non-conventional methodologies have been adopted in the current era, which have contributed to the advancement of this area of research inquiries.

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Modulation of Physiological and Molecular Switches in Thermophilic Fungi: A Brief Outlook

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Tuyelee Das, Samapika Nandy, Abdel Rahman Al-Tawaha, Potshangbam Nongdam, Ercan Bursal, Mahipal S. Shekhawat, and Abhijit Dey

Abstract

Thermophiles are significantly important microorganisms utilize in biotechnological research and industrial purposes. Thermophiles can grow at a temperature ranges between 45 and 80 °C. The survival of thermophilic fungi at such conditions induced by genomic evolution and natural selection that in turn gives thermophiles the thermotolerant abilities. Like other thermophilic organisms, thermophilic fungi get inclusive attention due to their potential to produce thermostable enzymes. Research studies mostly demonstrated physiological aspects of thermophilic fungi based on their nutrition requirement, metabolism, growth, and their interaction with the environmental factors. Furthermore, investigating molecular features such as genome size, protein, and nucleotides screening is likewise significant. Moreover, the physiological and molecular study gives us a concise idea about thermophilic fungi adaptation strategies and parameter to sustain in an extreme environmental condition. Therefore, in this chapter, we endeavor to convey thermophilic fungi physiological and molecular highlights about adaptation to elevated temperature.

T. Das · S. Nandy · A. Dey (✉)
Department of Life Science, Presidency University, Kolkata, West Bengal, India
e-mail: abhijit.dbs@presiuniv.ac.in

A. R. Al-Tawaha
Department of Biological Sciences, Al-Hussein Bin Talal University, Maan, Jordon

P. Nongdam
Department of Biotechnology, Manipur University, Imphal, Manipur, India

E. Bursal
Department of Biochemistry, Mus Alparslan University, Muş, Turkey

M. S. Shekhawat
Biotechnology Unit, Kanchi Mamunivar Government Institute for Postgraduate Studies and Research, Puducherry, India

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Thermophilic fungi · Adaptations · Temperature · Molecular biology

5.1 Introduction

Thermophilic means heat-loving microorganisms that can be demarcated as hyperthermophile (>80 °C), thermophilic (45–80 °C), and mesophilic (<45 °C). Most hyperthermophiles are confined to the Archaea domain, whereas thermophilic organisms belong to the Bacteria, Archaea, and Eukarya domain. A small number of fungi can sporulate at 45–55 °C. Thermophilic fungi cannot grow in as extreme as bacteria and archaea can grow, excepts only a few species can grow at 113 °C (Blochl et al. 1997). Consequently, it gets important to see how thermophilic fungi can grow in such extreme temperatures, while most living creature can't endure. Thermophily in fungi isn't just about as outrageous as in eubacteria or archaea, a few types of which can develop close or above 100 °C in solfatara fields, hydrothermal vents, or thermal springs (Brock 1995; Blochl et al. 1997). Definition for thermophilic organisms (bacteria and archaea) cannot embrace the same for thermophilic fungi because temperature tolerance for them differs. According to Maheshwari et al. (2000), fungi can grow optimally at or above 45 °C called thermophilic fungi (Maheshwari et al. 2000). Cooney and Emerson (1964) characterize fungi growing between ≥50 °C and ≥20 °C temperature as considered thermophilic (Cooney and Emerson 1964). Thermophilic fungi have an immense role in degrading organic matter, production industrial production in extracellular and intracellular enzyme, antibiotics, and organic acids. Maybe due to their moderate level of thermophily and on the grounds that their habitats are not fascinating, thermophilic fungi have not gotten a lot of exposure and attention. Additionally, research on this category of extremophiles has been ignored. Moreover, uncertainty in taxonomic affiliation and misidentification leading thermophilic fungi places them in a state of confusion (Madigan and Orient 1999; Stetter et al. 1990).

For the industrial production of enzymes, one of the problems is the maintenance of temperature during the entire farming procedure at an optimal level. Hence, the utilization of thermophilic strains of fungi can be a real solution, so the study of their thermostability related to genome or molecular level functioning is important. The study of genetic engineering is an exceeding recent development. In different environment conditions, thermophilic fungi act differently, but the research related to this topic is scant. If we can understand the physiology of thermophilic fungi, we undoubtedly use them for industrial production. Therefore, this chapter aims to compile data from the past on whatever little information is available on the physiology of thermophilic fungi that will assist with improving industrial production. Figure 5.1 represents physiological and molecular adaptive features of thermophilic fungi. This chapter also focuses on the understanding of the genome structure, size, and thermostable genes concerning thermostability.

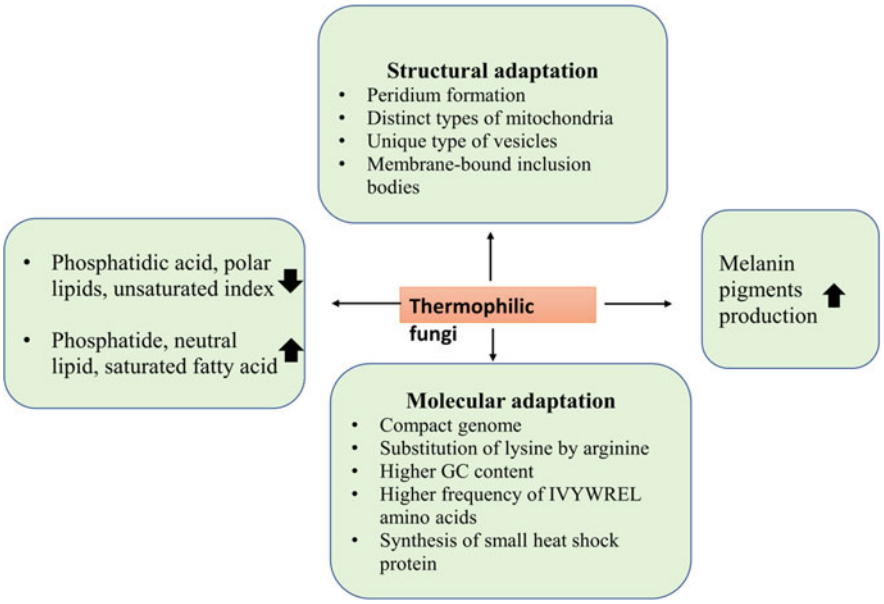

Fig. 5.1 Physiological and molecular adaptative features of thermophilic fungi

5.2 Physiology of Thermophilic Fungi

5.2.1 Nutrition Requirements

Thermophilic fungi do not need any special nutrition for their growth. They can grow in media composed of basic micronutrients and macronutrients. For the growth, reproduction and maintenance of basic biosynthetic pathway, thermophilic fungi need a constant supply of nutrients. Earlier, it was assumed that thermophilic fungi require complex nutrition, but now, it is clear that they need simple media for their growth contains carbon, nitrogen, and salts (Maheshwari et al. 2000). Additionally, it is known that they are autotrophic fungi.

Many thermophilic fungi observed to utilize polysaccharides (cellulose, hemicellulose, starch, pectin, and lignin) as a carbon source to grow efficiently (Basu 1980; Deploey 1976a, b; Satyanarayana and Johri 1984). Chauhan et al. (1985) revealed *Torula thermophila*, *Sporotrichum thermophile*, *Thermomucor indicae-seudaticae*, and *Thermoascus aurantiacus* can grow on methanol and formate (Chauhan et al. 1985). *T. aurantiacus* observed to grow densely on dulcitol, mannitol, oxalic acid, and citric acid; however, *Scytalidium thermophilum* reported deprived growth on oxalic and citric acids, with no growth on dulcitol (Subrahmanyam 1977). Thermophilic fungi *Thermomyces lanuginosus* and *Penicillium duponti* utilized a mixed source (combination of glucose and sucrose) of nutrition at 50 °C. The growth rate of these two fungi is more in mixed culture rather than single carbon source culture,

indicating that the two sugars proportionally affected their use in the combination. The two sugars were likewise used simultaneously at 30 °C but at almost indistinguishable rates. Combination of glucose and sucrose was picked on the grounds that concentration of glucose and sucrose can easily determine in the medium utilizing commercially available enzymes (Maheshwari and Balasubramanyam 1988). Thermophilic fungi utilize amino acids as a nitrogen source was investigated. Subrahmanyam (1980) reported poor growth and sporulation of *T. aurantiacus* in the presence of magnesium nitrate, glycine, peptone, thiourea, L-serine, DL-phenylalanine, DL-leucine, DL-alanine, and ammonium acetate (Subrahmanyam 1980).

Satyanarayana and Johri (1984) revealed that nitrates of Na and K are a better source of nitrogen than ammonium nitrate and sulfate (Satyanarayana and Johri 1984). Rosenberg in 1975 examined 21 species of thermophilic and thermotolerant fungi growth in a glucose-containing mineral medium complemented with 0.01% yeast extract. He reported that eight species required 0.01% yeast extract for growth on a solid complex medium (Rosenberg 1975). Wali et al. (1978) reported organic acid succinate needed in a concentration-dependent manner for the growth of thermophilic fungi *Humicola lanuginosa, P. duponti, S. thermophile,* and *Mucor pusillus* in addition to glucose media. The growth of these fungi stimulated because of their buffering action. They also observed glucose utilization increase in the presence of succinate (Wali et al. 1978). In contrast to Wali et al. (1978), Gupta and Maheshwari (1985) reported that for the growth of *T. lanuginosus*, succinate is not essential in addition to the need for organic acid that could be deleted if the pH of the medium can properly maintain (Gupta and Maheshwari 1985). Oxygen withdrawal from the growing medium of thermophilic fungus severely affected their growth. Emerson 1968 reported that *Humicola insolens* grow better under anaerobic conditions at elevated temperatures (Emerson 1968). Cooney and Emerson (1964) detected that thermophilic *Talaromyces duponti* initiated conidial stage in aerobic cultures, whereas the sexual stage was formed in agar culture only (Cooney and Emerson 1964).

5.2.2 Growth and Metabolism

The growth of filamentous thermophilic fungi is complex and unpredictable. Aerial hyphae from filamentous fungi get nutrition when mycelium interacts with the medium. For that, nutrient transport needs more time to cover the distance from mycelium to aerial hyphae. Therefore, continuous supply of nutrient source needs to be maintained for the rapid growth of thermophilic fungi, mostly essential for industrial purposes. *T. aurantiacus* grown at 45 °C in minimal medium utilized 55% of sugars for the synthesis of growth and biomass production and 45% for metabolism. Polysaccharide is utilized by thermophilic fungi so that they can produce cellulose and hemicellulose. The fungi *Chaetomium thermophilum* and *H. insolens* were recorded to grow better when they utilized xylan (Chang 1967). However, later Bhat and Maheshwari (1987) informed that *S. thermophile* fungus

can grow on both cellulose and glucose identically (Bhat and Maheshwari 1987). The growth rate of the fungus on cellulose was indistinguishable from that on glucose uncovering the remarkable capacity of growth of this fungus to utilize cellulose as efficiently as glucose (Gaikwad and Maheshwari 1994). Thermophilic fungi growth rates increase in shake flasks than in static cultures (Prasad and Maheshwari 1978). Similarly, *Chaetomium cellulolyticum* boomed in broth containing 15% glucose with a growth rate of 0.14 per hour (Chahal and Hawksworth 1976). Additionally, in 1978, Chahal and Wang displayed that *C. cellulolyticum* can synthesized protein with 1% cellulose concentration in 0.09 per hour, where additional 1% cellulose in the medium help to synthesized protein rapidly in just 0.3 per hour (Chahal and Wang 1978).

5.2.3 Lipids and Fatty Acids Composition

Thermophilic fungi grow at such temperatures at which most of the fungi cannot live the required course of thermostability and permeability of the membranes. The lipid components are mainly responsible for membrane stability at high temperature as most of the cellular events are membrane linked. In low temperature, thermophiles cannot produce unsaturated fatty acids so they are unable to grow. A low amount of phosphatidic acid was reported in *Malbranchea pulchella* var. *sulfurea* and *Absidia ramose*, while a very high level of phosphatide was observed in *H. lanuginosa* and *M. pulchella* var. *sulfurea*. All the mentioned fungi contained fatty acids, monoglycerides, diglycerides, and triglycerides, whereas sterols are detected in *A. ramose* (Raju et al. 1976). A low amount of phosphatidic acid also is found in *Acremonium alabamensis* and *T. indicae-seudaticae*. The lipid content of dry mycelium of *A. alabamensis* ranged from 2.6% to 7.3% and of *T. indicae-seudaticae* ranged from 8.5% to 13.0%. During growth, neutral lipid increased, whereasphospholipids and polar lipids decreased (Satyanarayana et al. 1987). Mumma et al. (1971a, b) compared neutral lipids and the fatty acid profile between thermophilic and mesophilic fungi and reported thermophiles produced more saturated neutral lipids than mesophiles (Mumma et al. 1971a). Mumma et al. (1970) reported the same that the fatty acids of thermophilic fungi *M. pusillus* are more saturated than mesophilic *Mucor globosus*. They also reported the presence of palmitic, oleic, and linolenic in both species with considerable and no amount of linolenic acid in mesophile and thermophile, respectively (Mumma et al. 1970). The polar lipids of the thermophilic fungus *Humicola grisea* var. *thermoidea* consisted of 38.4–42.3%. Polar lipids consist of sterol glycosides and neutral lipids consist of tri-glycerides, free fatty acids, sterols, sterol esters, and diglycerides (Mumma et al. 1971b). The unsaturated index in *T. thermophila* decreased as the temperature of culture increased (Bruszewski et al. 1972). Wright et al. (1983) examined and observed regulation of inability in membrane fluidity at high minimum temperature. They reported shifting of growth temperature of *Talaromyces thermophilus* from 50 to 33 °C was unable to increase the degree of unsaturation of fatty acids. Addition of palmitic acids, oleic acids, stearic acids, and ergosterol induced fatty acid

synthesis, which was before inhibited by the antibiotic cerulenin (Wright et al. 1983). However, Rajasekaran et al. noticed that growth of *T. lanuginosus* was different in high (50 °C) and low (30 °C) temperature. Linoleic acid synthesis increased at low temperature than at high temperature (Rajasekaran and Maheshwari 1990).

5.2.4 Ultrastructure

Thermophilic fungi reported to developed special dense vesicles for storage. A unique papillate germ pore and pustulate surface ornamentation in *C. thermophile* fungus are reported to be a notable ultrastructural feature compared with other species of *Chaetomium*. *Chaetomium semen-citrulli* ascospores contained vacuoles, many nuclei, mitochondria, and glycogen-like particles (Millner et al. 1977). Another species *Chaetomium brasiliense* reported forming a typical ascus vesicle close to the plasma membrane of asci (Rosing 1982).

In *T. aurantiacus*, subcellular events of ascosporogenesis take place. Events like ascogenous hyphae formation, ascospores delimination, cell-wall formation between ascospore-delimiting membranes, electron-transparent endospore, and an electron-dense epispore were observed (Ellis 1981a). Perithecia are reported to be a remarkable ultrastructural feature of *Chaetomiun thermophile*. It was revealed that two-layered peridium formation may be a structural adaptation of *Ascomycotina* fungi that defends the ascocarp centrum from desiccation (Ellis 1981c). Similarly, two-layered peridium is also found in *T. aurantiacus*. Ascocarps of *T. aurantiacus* was also reported to consist of well-defined cleistothecia (Ellis 1981b).

Furthermore, ultrastructural features of lipid bodies have been observed in *T. thermophilus* (Bokhary et al. 1988). Goh and Hanlin (1998) reported in *Melanospora zamiae* cell two morphologically distinct types of mitochondria such as D-type and T-type that were present in different ascomal development stages. A unique type of vesicles is found in *M. zamiae* cells at the distal end of the elongated ascus (Goh and Hanlin 1998). Similarly, two different spore types such as type 1 conidia and type 2 chlamydospores reported in thermophilic *H. insolens* (Ellis 1982). Three species of hyphomycete *T. lanuginosus*, *T. stellatus*, *and T. ibadanensis* reported developed thick-walled and terminal holoblastic conidia. The presence of numerous dense body vacuoles and the formation of holoblastic conidia are also reported. Vacuoles are observed only when *T. lanuginosus*, *T. stellatus*, *and T. ibadanensis* grew in not only high temperature (52 °C) but also low temperature (40 °C) (Ellis 1981d). Similarly, *Thermomyces stellatus*, *T. lanuginosa*, *Humicola insolans*, and *H. grisea* var. *thermoidea* have been reported to contain membrane-bound inclusion bodies in the cytoplasm when grown at high temperatures (Garrison et al. 1975).

5.2.5 Pigments

The pigment formation gives the protection of several organisms under heat stress condition. The pigmentation range differs depending upon temperature and age. Some thermophilic fungi, *T. duponti*, *T. aurantiacus*, *Malbranchea pulchella var. sulfurea*, *T. lanuginosus*, and *H. grisea var. thermoidea*, produce melanin pigments in cell walls of ascocarp, ascospores, peridia, and conidia. By spectrometric studies, it was reported that pigments were found to be similar to aphins (Somasundaram et al. 1986). The pigment formation is completely sensitive to elevated temperature. *Mucor miehei* sporulated at 35 °C but produced a brown pigment after longer incubation at 35 °C (Lasure and Ingle 1976).

5.2.6 Abiotic Factors and Growth of Thermophilic Fungi

Thermophilic fungi, which grow at or above 50 °C, are significant for the decomposition of organic matter (Cooney and Emerson 1964) and mushroom compost (Cooney and Emerson 1964; Fergus 1964; Renard and Cailleux 1972). Some excellent sources are *Talaromyces thermophilus*, *H. insolens*, *H. grisea*, *S. thermophile*, *M. miehei*, and *Papulaspora* for the investigation of the effects of temperature (Chapman 1974). At 40 and 45 °C, fungi grew fastest, whereas at 30, 35, 50, and 55 °C, slower rates of fungi occurred. All of the species of fungi formed spores with different temperature range (Chapman 1974). *M. miehei* produced zygospores after incubation for 51 h at 35 °C, but not at 46 °C in liquid medium (Lasure and Ingle 1976). *Chaetomium thermophile* var. *dissectum* and one strain of *C. thermophile* var. *coprophile* have shown a more restricted temperature range for sexual and asexual reproduction; vegetative growth of thermophilic fungi takes place in a wider temperature range (Tansey 1972). The mitochondria of *Talaromyces* sp. contained higher amounts of oleic acid (39%) and palmitic acid (30%) but considerably less amount of linoleic acid (10%) when grown at high temperature (50 °C). The higher amount of saturated fatty acids and lower unsaturated fatty acids production is correlated with higher temperature. Mitochondrial membranes of mesophilic and thermophilic fungi possess significant difference (Asundi et al. 1974). The respiratory rates of thermophile *T. lanuginosus* reported to be highly sensitive to temperature changes, but not in mesophile *A. niger*, where respiration rates were insensitive to temperature changes (Prasad et al. 1979). Complex nutrients contained factors cannot synthesize at the low temperatures. *Humicola*, *Thermoascus*, and *Aspergillus* when incubated in liquid medium included complex supplements at 20 °C after ten days; they showed good growth, but they did not grow below 30 °C lacking any complex supplements (Maheshwari et al. 1987). Wright et al. (1983) supported the observation of growth of *T. thermophilus* fungi at 33 °C, which was helpful by supplemented culture medium with ergosterol (Wright et al. 1983). Most of the thermophilic fungi survive exposure to 60 °C for 1–48 h observed by Satyanarayana and Johri (1984). Besides temperature, pH range is another abiotic factor that influences the growth of thermophilic fungi. Rosenberg

(1975) studied pH optima of 21 fungi and reported that the pH optima of *T. lanuginosus*, *Malbranchea cinnamomea*, and *T. thermophilus* was found to be near neutral (pH 7), whereas *Talaromyces emersonii* and *Allescheria terrestris* optimally grew in an acidic environment (pH 3.4–6.0). Additionally, it observed that between pH and temperature, no correlation was found (Rosenberg 1975). In contrast, the temperature change is directly correlated with water availability. As temperature increases, water availability also decreases. *Humicola* is a xerophilic strain of thermophilic fungus found in the Thar Desert reported to produce higher proline and sterol to overcome desiccation stress (Mahajan et al. 1986). For the optimum growth of thermophilic fungi, sufficient humidity required. *C. thermophile* var. *coprophile* ascospores survive longer in a dry state and stored at low relative humidity with high temperatures than in water or stored dry at high relative humidity (Celerin and Fergus 1971).

5.3 Acquired Thermotolerance

Acquired thermotolerance in fungi refers to the enhanced survival in lethal temperatures subsequent exposure to sublethal high temperatures. Acquired thermotolerance related with synthesis of heat-shock proteins (HSPs) under temperature stress. Normal growth condition HSPs act as a molecular chaperone. All organisms included fungi have grew in such a way that they developed a variety of strategies for surviving in elevated temperature, and among these strategies, HSP synthesis is one of them. Bacteria, Archaea, and Eucarya have been shown to develop acquired thermotolerance by synthesizing HSPs after heat shock. Trentet al. observed *T. lanuginosus* germinated at 50 °C and heat shocked at 55 °C for 60 min showed enhanced survival at 58 °C. The synthesis of small HSPs ranging from 31 to 33 kDa notably dominated in the heat-shock temperatures. Thermotolerance in *T. lanuginosus* was removed by the effects of cycloheximide when added during the heat shock period that subsequently inhibit protein synthesis (Trent et al. 1994). Oberson et al. (1999) detected a transient HSP synthesis lasting about 60 min in *C. thermophile*. Translation of HSPs stopped beyond 56 °C (Oberson et al. 1999).

5.4 Molecular Features

The molecular biology of thermophilic fungi has gained attention to molecular structure and functioning of cell linked adaptation to extreme conditions. Molecular genetic functioning has been effectively studied in thermophilic bacteria and archaea, and little information is available on the thermophilic fungi.

5.4.1 Reduction in Genome Size

Thermophilic fungi genome size is small as compared with other fungi. Reduction of genome size is one of the strategies to sustain in high temperature. The compact genome is an interesting feature, as it helps in fast reproduction and reduces energy consumption. Thermophiles living at a temperature of >60 °C reported having smaller genomes (4 Mb) than those species which grow at temperatures of <45 °C with larger genomes 6 Mb (Van Noort et al. 2013). Van Noort et al. (2013) revealed some common strategies of thermophilic fungi *Chaetomium thermophilum*, *Thielavia terrestris*, and *Thielavia heterothallica*. They found that a certain amino acid residue for a specific protein contribute to thermostability. The steadiest pattern showed the substitution of lysine by arginine (Van Noort et al. 2013). They also compared the genome of those three thermophilic fungi to mesophilic fungi *Chaetomium globosum* and *Neurospora crassa* and observed that the genome size of mesophilic fungi is smaller than that of mentioned three thermophilic fungi. Reduced genome size is due to fewer protein-coding genes and shorter introns and intergenic regions (Van Noort et al. 2013). Besides those strategies, horizontal gene transfer could be another evolutional strategy of thermophilic fungi to sustain in such an extreme environment (Averhoff and Müller 2010).

5.4.2 GC Content

Thermophiles have a much more stable genomic structure than mesophiles. Higher genome stability is correlated with higher guanine (G) and cytosine (C) content that related to an adaptation to high temperature of the protein-coding genes of thermophile. The genome sequence study of thermophilic *M. thermophila* and *T. terrestris* when compared with the mesophile *C. globosum* and *T. reesei* showed higher GC contents in thermophilic fungi coding regions in the genomes (Berkeley and Cancer 2011). Zygomycete fungus *Rhizomucor miehei* CAU432 exhibited a genome size of 27.6 Mb with a 43.8% GC content of fungal whole genome (Zhou et al. 2014). Other thermophilic fungi *T. lanuginosus, T. terrestris*, and *M. thermophila* content are 52.14%, 54.7%, and 51.4% of GC, respectively, which reported to much higher than other zygomycetes mesophilic fungi (Berkeley and Cancer 2011; Mchunu et al. 2013; Van Noort et al. 2013). The GC content of the whole genome of *T. lanuginosus* Strain SSBP was 52.14% with higher GC content in the coding region 55.6%. Thermal adaptation of *T. lanuginosus* fungus was associated with DNA-related pathways. Activation of the ubiquitin degradation pathway in *T. lanuginosus* is related to several stresses (Mchunu et al. 2013). On the other hand, Zeldovich et al. (2007) revealed that the GC content in genomes does not exhibit a significant correlation with optimum growth temperature. However, the content of AG in the coding region of DNA is significantly correlated with optimum growth temperature due to IVYWREL (Ile, Val, Tyr, Trp, Arg, Glu, and Leu) amino acids (Zeldovich et al. 2007).

5.4.3 Proteome

The amino acid arrangement of proteins of thermophilic organisms is related to their adaptations to extreme environments. Proteome studies of thermophilic *C. thermophilum*, *T. terrestris*, and *T. heterothallica* fungi were to provide new paths to protein engineering related to stability. They reported that an enhanced level of cysteines in *C. thermophilum* might be related to thermophily by forming disulfide bridges and metal binding. A higher frequency of IVYWREL amino acids was observed in *C. thermophilum* than in its mesophilic counterpart. IVYWREL is observed to be related to optimum growth temperature and is also related to the survival at higher temperatures (Van Noort et al. 2013). Lowering glycine is correlated with acquiring thermostability. Thermophilic *C. thermophilum* lowers glycine content, whereas mesophilic *C. globosum* has changed in the reverse direction (Van Noort et al. 2013). A higher amount of IVYWREL, depletion of glycine, and elevation of arginine and alanine likewise are observed in *T. lanuginosus* and *T. thermophilus* when compared with the same subclass mesophilic fungi *Aspergillus fumigatus* and *Emericella nidulans* (Van Noort et al. 2013). During translation, cysteine, lysine, and all the amino acids of IVYWREL are involved with tRNA by class I aminoacyl tRNA synthetases and thus associated with thermostable proteins synthesis (Eriani et al. 1990).

5.4.4 Genes Responsible for Thermostability

Until now, several numbers of genes have been isolated and screened from different thermophilic fungi. β-Glucosidase is an important cellulose hydrolyzing enzyme that has been widely utilized in several industrial fields. Zhao et al. (2015) expressed mtbgl3b β-glucosidase of glycoside hydrolase (GH family 3) gene from *Myceliophthora thermophila* in *Pichia pastoris* and reported properties of mtbgl3b genes. Recombinant MtBgl3b was reported to show thermostability after incubated at 60 and 65 °C (Zhao et al. 2015). β-Glucosidase from *T. aurantiacus* fungus also showed thermostability on semisolid fermentation medium (De Palma-Fernandez et al. 2002). β-Glucosidase (cel3a) genes from *Talalaromyces emersonii* was reported to show thermostability at 71.5 °C after expressed in *Trichoderma reesei* with a strong cbh1 promoter (Murray et al. 2004). *Tachit1* and *Ctchit1* chitinase genes have been isolated from *T. aurantiacus* var. levisporus and *C. thermophilum*, respectively, and expressed in *P. pastoris*. Single domain protein TaCHIT1 of *Tachit1* gene showed lower thermostability than CtCHIT1 of *Ctchit1* gene, which makes CtCHIT1 protein potentially suitable for chitin hydrolysis at high temperatures (Li et al. 2010).

5.5 Conclusion

The biology of thermophiles has acquired consideration and anticipated the comprehension of adaptation at the molecular level. Thermophilic fungi grown at high temperature have greater saturated lipids, palmitic, oleic and linolenic palmitic, oleic and linolenic acid, and a low amount of phosphatidic acid. They can grow in basic media containing amino acids, organic acids, etc. Sometimes, they grow in a concentration-dependent manner. Ultrastructure study uncovered that thermophilic fungi produced well-defined epispore, special dense vesicles, cleistothecia, and membrane-bound inclusion bodies. The molecular biology of thermophiles has recently gained attention both in terms of adaptation to temperature and the development of manipulative genetic tools. Their genetic study discloses reduced high G/C content, genomic size, shorter introns, enhanced level of cysteines, and IVYWREL amino acids that exhibit adaptations. Future research on genetic engineering and biotechnology related to thermophilic fungi is essential insight into thermophilic fungi role in biology, ecology, and in economic importance, in addition to understanding their ability to grow at high temperatures.

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Psychrotrophic Microfungi: Major Habitats, Diversity and Living Strategies

6

Kanak Choudhary, Najeeb Hussain Wani, Farooq Ahmad Ahanger, Suhaib Mohamad Malik, Vinod Chourse, Abdul Majid Khan, and Sanjay Sahay

Abstract

Ecology of extreme cold areas with subzero temperatures at least in some part of the year is the subject of interest. Microfungi from these areas show special morphological and physiological adaptations to avoid cold stresses. Some of them are endemic, but majority are cosmopolitan in distribution. Except for a few, mostly fungi from these areas show a wide range of growth temperatures.

Keywords

Cold-active microfungi · Psychrotrophic fungal diversity · Cold adaptations

6.1 Introduction

Cold climates are very low-temperature regions and known for their low water and poor nutrient availability. Despite this, many fungal species are found here that can grow and survive near-zero temperatures. Fungi can grow on various types of substrate and can thrive or survive under many extreme environments. Hence their abundance and diversity spread throughout the world. This category of microorganisms was relatively neglected by the 1950s. These organisms possess potential commercial value due to their cold-active enzymes. Many cold-active fungi

K. Choudhary · V. Chourse · A. M. Khan
Department of Biotechnology, Barkatullah University, Bhopal, Madhya Pradesh, India

N. H. Wani · F. A. Ahanger · S. M. Malik
Government Science and Commerce College, Bhopal, Madhya Pradesh, India

S. Sahay (✉)
Sarojini Naidu Government Postgraduate Girl's (Autonomous) College, Bhopal, Madhya Pradesh, India

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are known as the active agent of spoilage of frozen foods, so it is important to study these fungi as well (Margesin and Schinner 1994). These cold-active fungi are also of interest to study due to their biogeographical and ecological importance. Cold active organisms are those that are adapted to a climate characterized by very low temperatures below 20 °C. Around 85% of Earth including polar and alpine regions and deep-sea exhibits this harsh condition. The cold-active organisms may be psychrophilic or psychrotolerant, depending on their inability or ability to thrive above a threshold lower temperature, respectively, and their threshold temperature may be 15 or 20 °C (Morita 1975; Robinson 2001; Cavicchioli et al. 2002).

It is well known that the rate of reaction decreases by half when the temperature reduces by 10 °C. This slow rate of reaction interferes with many biological processes of fungi. Extremely low temperature, low water availability, and high UV radiations are some general cold stress associated with the cold climate. High UV radiation affects the cell by damaging the membrane lipoproteins, cellular proteins, and DNA. Microorganisms protect themselves from the high UV radiation by producing pigments such as melanin and mycosporines. Some fungi form micro-niche in rocks or stones in cold ecosystems to protect them from high UV radiation. Intracellular accumulation of osmoprotectants or compatible solutes protects the cells from water loss. Low-temperature interferes with the membrane integrity and enzyme activity of an organism. Fungi cope with this problem by altering the membrane lipids and acquire flexibility in the membrane. Microfungi comprise a group of filamentous fungi belonging to all major classes; the common characteristic they exhibit is the inability to produce visible melanized fruiting bodies that can be seen by naked eyes as found in most *Ascomycetes* and *Basidiomycetes*. The most common microfungi are molds (bread molds, slime molds, and water molds), powdery mildew, and rusts. This group includes both saprophytic and pathogenic (for plants and animals) fungal species that comprise 4468 genera and 55,989 species. For a detailed classification of microfungi, readers are suggested to visit the dedicated website (https://www.microfungi.org/).

Cold-active microfungi have been reported from almost all the cold areas exist on the earth (Hassan et al. 2016). Their extraordinary capacity enables them to occupy diverse types of niches such as the interior of living plants (endophytes), over the surface of plants (epiphytes), on the decaying plant materials (saprobes), on or inside the insects (entomopathogen), associated with roots (ectomycorrhiza), deep sea animals (such as sponges) or sediments, soil, water, etc. in the cold environments. They are expected to have faced diverse types of challenges enabling them to evolve varied types of bio-molecules to respond to them. These bio-molecules are what we are concerned with to feed our industries to get newer products or processes. The most attractive features of all applications are the lower energy consumption either in the bio-production (at low temperatures) of biomolecules or application of these bio-molecules (in the process at low temperatures) or both and subsequent reduction in CO_2 release. The chapter will unfold these aspects in detail.

6.2 Habitats

Cold-active fungi exist in extensively diverse types of cold habitats (Hassan et al. 2016). Almost 85% of the Earth's biosphere exhibits cold temperature (<5 °C) for a longer period in the year. Specifically, this cold area includes deep ocean (about 90% of the volume of the ocean shows temperature < 5 °C), snow-covered land (35% of land surface), permafrost (24% of land surface), sea ice (13% of the land surface), glaciers (10% of land surface), cold water lakes, cold soils, and cold deserts. Major cold land surfaces are present in the Arctic, Antarctic, and high-mountains (Margesin and Miteva 2011; Hassan et al. 2016).

6.3 Terrestrial Cold Environments

6.3.1 Arctic Soil

Low nutrients availability, freeze-thaw cycles, low soil moisture, extreme cold, and low annual precipitation are several limiting factors that reduce microbial activity in arctic soils. Microbial communities in Finnish Lapland (Männistö and Häggblom 2006; Männistö et al. 2007) are dominated by a wide variety of these microorganisms.

6.3.2 Alpine Soils

The term "alpine" implies a high altitude belt that lacks trees and is located above continuous forests on mountains. Alpine soils are subjected to large temperature fluctuations, a high number of frost and ice days, moisture fluctuations, regular freeze-thaw events, and high precipitation. Microbial communities may vary seasonally (Lipson 2007).

6.3.3 Antarctic Soils

Antarctic terrestrial ecosystems differ from those in the Arctic. Antarctic terrestrial environment is colder (subzero temperatures down to –60 °C), drier (moisture content of 1–10%), lower availability of nutrients, and often alkaline. Due to the extreme aridity, soils accumulate salts through precipitation and weathering. Microbial diversity abundance in the terrestrial Antarctic region has been reviewed recently. *Cryptococcus* and *Mrakia* are the dominating fungal species of Antarctic soil (Aislabie et al. 2006; Smith et al. 2006; Babalola et al. 2009; Bej et al. 2009).

6.3.3.1 Historic Sites

Many historic sites present in the Antarctic environment have become a matter of concern for their long preservation (Blanchette et al. 2002). Historic huts and pristine

environment is a habitat of many diverse fungi that utilize wood as their nutrition (source of carbon and nitrogen). They show temperature-dependent growth and produce a significant amount of extracellular enzyme especially endo-1,4-β-glucanase for wood degradation. Much cold-active fungal microbiota such as *Cadophora* species (*C. malorum, C. luteo-olivace, and C. cladosporioides*) and *Geomyces* sp. decompose the wood and organic matter of many artifacts and historic huts located in the Antarctic pristine region (Held et al. 2003; Farrell et al. 2004). These fungal species play an important role in nutrient cycling in a cold climate. Soft rot are associated with the degradation of woody historic huts. The species of *Cadophora* are known to be native saprophytes of the Antarctic region as they are abundantly found in the environment with limited human interference. *Cadophora* and *Geomyces* are the two most common fungal genera found in the historic hut located on the Ross Island and are also found associated with many historic wooden structures from various cold habitats and represent the fungal diversity in a cold climate (Farrell et al. 2011).

6.3.3.2 Cryptoendolithic and Rock

Studies revealed that the total fungal diversity present in the Antarctic region is composed of water molds (0.6%) and true fungi (99.4%). The cryptoendolithic community especially lichen-dominated communities found in the deep zones of porous sandstones of Antarctica. Filamentous fungi form cryptoendolithic lichen association with chlorophycean algae. This fungal community is endemic as they evolved with the harsh environments of Antarctica. Microscopic black melanized fungi form microcolonies in rocks. Some filamentous hyphomycetes also found associated with rocks. Melanized cell wall of these fungi enables them to survive under cold stress (Ruisi et al. 2007).

6.4 Aquatic Cold Environments

6.4.1 Atmosphere and Clouds

The stratosphere and mesosphere layers are inhabited by many bacterial and fungal cells as they associate with the aerosols present in the atmosphere and remain viable in these layers. These microbes can stay here for a long time depending on the capacity and environmental condition. Some of them can stay for only a week while others can even stay for years. High UV radiation, desiccation, poor nutrients availability, and oxidative stress in this ecosystem affect the survival of microbes in this cold environment. However, the cloud is a more suitable environment for cold fungi due to the presence of liquid water. Many bacterial and fungal communities form a surface where water vapors condense and thus help in cloud formation and ice nucleation. Thus atmosphere and clouds are considered as the ecosystem for diverse microorganisms (Wainwright et al. 2004; Griffin 2008; Pearce et al. 2009).

6.4.2 Permafrost

Permafrost is the land surfaces such as soil, rock, or sediments usually at high altitudes that are covered with ice for at least two consecutive years. Permafrost regularly faces subfreezing temperatures. The mean temperature value for Arctic permafrost is −10 °C, while it ranges from −18.5 °C to −27 °C for Antarctic permafrost. Bacteria and fungi of these permafrost exhibit growth and metabolic activity up to a temperature of −39 °C. Several viable Basidiomycetous yeasts were found in the permafrost soil of Siberia. Genus *Penicillium* was found as dominating fungus in Arctic permafrost (Margesin and Miteva 2011).

6.4.3 Snow

Snow is frozen atmospheric water and makes it a part of the cryosphere. Thirty-five percent of the land surface of Earth is permanently or seasonally covered with snow. Seasonal temperature variation, aerobic conditions, high light, and high UV radiation are the characteristics of this ecosystem (Jones 1999; Cockell and Cordoba-Jabonero 2004). Atmospheric microbes together with the dust particles reach the snow and form a niche. Microorganisms inhabiting snow play important role in nutrient cycling in the cold environment through exchanging of reactive oxygen species. The microbial community present in snow also varies with changing latitude and altitude (Hodson et al. 2008; Pearce 2009; Pearce et al. 2010). Many archaea, bacteria, cyanobacteria, and fungi are isolated from this ecosystem. Many of them are known psychrophilic and psychrotolerant species (Segawa et al. 2005; Amato et al. 2007).

6.4.4 Polar and Alpine Lakes

Polar and high-altitude alpine lakes are the general cold lakes present on Earth that possess significant limnological diversity. Due to different dominating temperatures, these cold lakes show high diversity ranging from freshwater to hypersaline, from highly oxygenated to anoxic, from highly acidic to alkaline, and permanently ice-covered to ice-free (Vincent et al. 2008). The number of lakes in the Arctic region (1432) exceeds the Antarctic region (174), and thus the two groups of lakes exhibit different origin, geography, and environmental conditions (Ryanzhin et al. 2010).

6.4.5 Deep Sea and Sea Ice

Oceans cover around 71% of the Earth's surface, and more than half of this area is over 3000 m deep. The deep sea is defined as the lowest layer in the ocean at a depth of more than 1000 fathoms (1 fathom = 1.8288 m). The deep sea is characterized by

poor light (dark), cold, high pressure, and low nutritional availability. Microorganisms living in the deep sea adopt several unique features that allow them to thrive in this extreme environment (Abe 2004; Deming 2009). Most isolated strains from the deep sea are psychropiezophilic as they possess both psychrophilic and piezophilic (optimal growth at pressures >0.1 MPa) properties and cannot be cultured at temperatures higher than 20 °C (Nogi 2008).

6.4.6 Glaciers

Glacier is a dynamic ecosystem as they possess high fluctuating conditions and subfreezing temperatures. Glacial ice serves as a habitat for many fungal communities. *Penicillium* species are abundantly found in the glacial ice of Antarctica and source of various new bioactive secondary metabolites. The microbes inhabit liquid veins or ice crystals in the glacial due to the presence of few amount of liquid water. Glaciers are the important cold habitat for the genetic study of these cold-active microbes as the ancient DNA of these microbes remains preserved in this cold condition (Margesin and Miteva 2011).

6.4.7 Cryoconite Holes

Cryoconite holes are water-filled tiny cavities formed by atmospheric debris and dust particles in the snow-free glaciers. These holes can be located at the upper surface or subsurface in the glacier. The dust and other particles that form a cryoconite hole absorb solar radiation at a high rate to melt the ice near the hole and thus forms a micro-ecosystem for various microbes. *Rhodotorula glacialis* and *Mrakiella cryoconiti* yeast are isolated from cryoconite holes (Margesin et al. 2007; Margesin and Miteva 2011).

6.5 Artificial Ecosystem

Refrigerators, cold storage room, and refrigerated foods, where temperatures are usually below 0 °C, are man-made environment from many of the cold-adapted fungi have also been isolated (Altunatmaz et al. 2012).

6.6 Fungal Investigations in Cold Environments

Fungi present in glaciers obtain its nutrient from archaea, bacteria, virus, and plants and their metabolic products. However, embedded decaying vegetations and atmospheric debris also provide nutrients to fungi in glaciers. This interactive ecology enables the survival of various microbes in the glacial environment. Several fungi

from high Arctic glaciers are known to produce acid phosphatase to solubilize the inorganic phosphate and make it available for other species (Hassan et al. 2016).

6.7 Key Drivers of Fungal Abundance

During the fungal survey in Antarctica, the key drivers for fungal presence were studied, and the percent composition of carbon and nitrogen in the soil was found to be important in determining the fungal abundance (Arenz and Blanchette 2011). Other factors such as soil moisture, pH, and salinity have hardly any correlation with fungal abundance. The most frequently isolated fungi were the species of *Geomyces* and *Cadophora*.

6.8 Diversity

The saying that "everything is everywhere but the environment selects" has served as a motivational principle for surveying microbes from various ecosystems and geographical regions (Beijerinck 1913; Baas-Becking 1934). Thus there is the possibility of occurrence of similar phytotypes in similar habitats in different geographical locations (Finlay and Clarke 1999). There are two more working parameters, viz., environmental heterogeneity and spatial limitations that decide the biodiversity of various geographical regions. Thus a geographically isolated ecosystem may be dominated by endemic species (Papke et al. 2003; Souza et al. 2008; Whitaker et al. 2003). Advancement in molecular isolation techniques such as "metagenomic one" has expedited the isolation of hitherto unknown species and helped studying biogeographical distribution, dispersal, disseminating vectors, speciation, and the survival mechanisms and also verification of principles underlying therein (Margesin and Miteva 2011).

Psychrophilic fungal communities have essential roles in that ecosystem including nutrient cycle, water cycle, and energy flow and maintain an ecological balance and ensuring the release of nutrient sources for other organisms (Treseder 2005; Watling 2005).

Many psychrophilic fungi are cosmopolitan in distribution, e.g., *Phoma herbarum* (Domsch et al. 1980; Singh et al. 2006). Again many of the psychrophilic fungi exhibit broad growth temperature ranges, e.g., *Epicoccum purpurascens* can grow in the temperature range of −3 to −4 °C to 45 °C with the optimum temperature being 23 to 28 °C (Domsch et al. 1980; Hassan et al. 2016). Hardly a few psychrophilic fungi exhibit a narrow growth temperature range (Table 6.1).

Fungi isolated from various cold areas are as follows:

Table 6.1 Psychrotrophic fungi growing in narrow range of temperatures (Source: Wang et al. 2017)

Taxa	Growth temperature	Location/substrate/host	Reference
Mucor strictus	OGT below 10 °C	Alpine soils	Schipper (1967)
Coprinus psychromorbidus	OGT between −8 and −3 °C	Plant pathogen infecting cereals (winter wheat, oats), grass, and conifers	Traquair and Smith (1982)
Typhula ishikariensis	OGT at 10 °C		Hoshino et al. (1998)
Typhula incarnate	OGT between 5 and 10 °C		Nakajima and Abe (1994)
Microdochium nivale	OGT between 15 and 18 °C		
Sclerotinia borealis	OGT between 4 and 10 °C		Hoshino et al. (2010)
Rhodotorula himalayensis	–	Soils of Himalayan mountain ranges	Shivaji et al. (2008)
Phoma herbarum	OGT at 4 °C	Endophytic fungi of trees	Moghaddam and Soltani (2014)
Humicola marvinii	OGT below 15 °C, MGT at 20 ° C	Soils in the maritime Antarctic	Weinstein et al. (1997)
Pseudogymnoascus destructans	OGT at 4 °C	Hibernant bats	Gargas et al. (2009)
Pseudogymnoascus pannorum	OGT at 5 °C	Soil and litter	Flanagan and Scarborough (1974)
Thelebolus microsporus	–	Pangong Lake, Himalayan region	Anupama et al. (2011)
Mrakia robertii		Antarctic and alpine soils	
Mrakia blollopis	MGT below 20 °C		Thomas-Hall et al. (2010)
Mrakiella niccombsiis			
Mrakiella cryoconiti	MGT below 20 °C	Northern Siberian glacier sediment	Margesin and Fell (2008)
Mrakia psychrophila	OGT 10, MGT18	Antarctic soils	Xin and Zhou (2007)
Rhodotorula psychrophila	OGT below 15 °C	Alpine soils	Margesin et al. (2007)
Tetracladim ellipsoideum	OGT below 15 °C	Glacier soils in Qinghai–Tibet plateau	Wang et al. (2015)
Tetracladim globosum	OGT below 15 °C		
Tetracladium psychrophilum	OGT below 15 °C, MGT below 20 °C		

OGT optimum growth temperature, *MGT* maximum growth temperature

6.8.1 Antarctica

6.8.1.1 Soil

Cadophora sp., *C. fastigiata*, *C. luteo-olivacea*, *C. malorum*, *Chaetomium* sp., *Fusarium* sp., *Geomyces* sp., *Gliocladium* sp., *Hormonema* sp., *Hormonema dematioides*, *Mortierella* sp., *Penicillium* sp., *P. echinulatum*, *P. expansum*, *P. mali*, *P. roqueforti*, *Pestalotiopsis* sp., *Petriella* sp., *Pseudogymnoascus* sp., *Rhizopus* sp., *Talaromyces* sp., *Tetracladium* sp., *Thelebolus* sp.

6.8.1.2 Lake

Acremonium sp., *Cadophora luteo-olivacea*, *C. malorum*, *Davidiella tassiana*, *Fontanospora* sp., *Geomyces pannorum*, *Mortierella* sp., *Penicillium commune*, *P. dipodomyicola*, *Thelebolus globosus*, *Th. Ellipsoideus*, *Th. Microspores*, *Trichoderma atroviride.*

6.8.1.3 Endoliths

Cryomyces minteri, *C. antarcticus*, *Friedmanniomyces* s*implex*, *F. endolithic*, *Fusarium proliferatum*, *Geomyces* sp., *Penicillium* sp., and some more from unidentified members of *Sporomiaceae*.

6.8.2 Arctic

6.8.2.1 Soil

Aspergillus spp., *A. aculeatus, A. nidulans, Cladophora finlandica, Cenococcum geophilum, Geomyces* spp., *Mortierella* spp., *Myrothecium* spp., *Penicillium* spp., *Phialocephala fortinii, Phialophora* spp., *Preussia* spp.

6.8.2.2 Moss

Botrytis verrucosa, Corynespora sp., *Fusarium oxysporum, Geomyces pannorum*, *Microdochium* sp., *Mortierella* sp., *M. alpine*, *M. schmucker*, *M. simplex*, *Mucor* sp., *Mucor hiemalis*, *Penicillium* sp., *P. citrinum*, *P. frequentans*, *P. rugulosum*, *Phialophora* sp., *Pithomyces chartarum*.

6.8.2.3 Lichen

Acremonium sp., *Alternaria* sp., *Antarctomyces* sp., *Arthrinium* sp., *Atradidymella muscivora*, *Cadophora* sp., *C. thielavia*, *Cenococcum* sp., *Cosmospora* sp., *Elasticomyces elasticus*, *Fusarium* sp., *Herpotrichia* sp., *Lecanicillium* sp. (insect pathogen), *Monodictys* sp., *Mortierella* sp., *Oidiodendron* sp., *Penicillium* sp., *Phaeosphaeria* sp., *Phialocephala* sp., *Polyblastia terrestris*, *Preussia* sp., *Rhizoscyphus ericae.*

6.8.3 Deep Sea

Acremonium sp., *Alternaria* sp., *Aspergillus* sp., *A. terreus*, *A. ustus*, *A. versicolour*, *Beauveria* sp., *Cadophora* sp., *Curvularia* sp., *Cylindrocarpon* sp., *Emericella* sp., *Emericellopsis* sp., *Eurotium* sp., *Fusarium* sp., *Galactomyces candidum*, *Geomyces* sp., *Graphium* sp., *Gymnascella* sp., *Leptosphaeria* sp., *Mortierella* sp., *Paecilomyces* sp. (insect pathogen), *Penicillium* sp., *P. lagena*, *Pestalotiopsis* sp., *Petriella* sp., *Phoma* sp., *Pseudogymnoascus* sp., *Rhizoscyphus* sp., *Sagenomella* sp., *Spicellum* sp., *Trichoderma* sp., *Xeromyces* sp.

6.8.4 Alpine

Aspergillus sp., *Beauveria* sp., *Cadophora* sp., *Curvularia* sp., *Fontanospora* sp., *Geomyces* sp., *Leotiomycetes* sp., *Mortierella* sp., *M. alpine*, *Mucor* sp., *Mucor hiemalis*, *Penicillium* sp., *P. canesense*, *P. chrysogenum*, *Periconia* sp., *Phoma* sp., *Phoma sclerotioides*, *Pseudogymnoascus pannorum*, *Ps. roseus*, *Pseudeurotium* sp., *Scopulariopsis* sp., *Thelebolus microspores*, *Trichoderma* sp., *Truncatella angustata*, *Umbelopsis isabellina.*

6.8.5 Refrigerator

Acremonium sp., *Aspergillus* sp., *Aspergillus ochraceus*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus Alternaria alternate*, *Botrytis* sp., *B. cinerea*, *Mucor* sp., *M. racemosus, M. plumbeus*, *Penicillium* sp., *Penicillium italicum.*

6.9 Living Strategies

Psychrotrophic fungi carry out ecological functions under extreme climatic conditions through various forms such as symbionts, saprobes, parasites (plant and animal), and pathogens and adopting various living strategies, some of which are species-specific too.

6.9.1 Freeze Thaw and Desiccation

Freeze-thaw cycles and desiccation are two very hostile conditions impacting fungal survival in a cold environment. The Arctic and Antarctic environments are characterized by fluctuations in temperature near 0 °C as an annual cycle. For example in Arctic polar semidesert soil during winter, 30 freeze-thaw cycles have been found at the depth of 3 cm (Coulson et al. 1995). Studies have shown that filamentous fungi, e.g., spores of hyphomycete can tolerate lower temperatures (even freezing ones), but they are not much tolerant to regular freeze-thaw cycles (Vishniac

1996). In an experiments on an Antarctic yeast, *Nadsoniella nigra* var. *Hesuelica*, it has been found that 33% and 10% of cells remain alive after one and ten cycles of freeze (−13 °C) thaw cycle, respectively (Lyakh et al. 1984; Wynn-Williams 1990).

Desiccation is another very important factor impacting fungal survival in low temperatures. Availability of free water around fungal cells at very low temperature is only rarely or intermittently found in extremely cold areas (cold deserts), e.g., Antarctica (McRae and Seppelt 1999) that hampers fungal survival. Accumulation of polyols makes the cell desiccation tolerant.

6.9.2 Molecular Strategies

A higher proportion of polyunsaturated and branched fatty acids maintain the fluid condition of the membrane and ensure the transportability of nutrients under cold conditions as in *Rhodosporidium diobovatum* (Turk et al. 2011) and *Geomyces pannorum* (Weinstein et al. 2000). This is a very common strategy to avoid transport constraints under freezing temperature. Accumulation of glycerol as cryoprotectant as in *Mrakia psychrophile* (Su et al. 2016), compatible solutes such as polyols, melanin, mycosporines, trehalose, and betaine (Ruisi et al. 2007) is another important strategy. Reactive oxygen scavenging systems have also been observed in some cold-active fungi such as *Penicillium* sp. p14 and *Penicillium* sp. m12 (Gocheva et al. 2006) and Arctic fungi *Keissleriella* sp. YS 4108 and *Aspergillus versicolor* (Sun et al. 2004).

6.10 Saprobes

Saprobes carry out a very important function of decomposition of dead plant/animal or their detached parts with secreted hydrolases in the soil. The psychrotrophic *Cadophora* and *Geomyces* spp. are found associated with the degradation of wooden structures with secreted cellulases (historic huts and artifacts) in Antarctica (Arenz et al. 2006; Arenz and Blanchette 2009; Farrell et al. 2011).

Cryptoendolithic fungi thrive in cold employing various morphological and physiological adaptations. Important morphological adaptations include pigmentation and meristematic growth; the latter keeps the volume/surface ratio optimal, minimizing the impact of external stressors. Important physiological adaptations include an increased intracellular concentration of trehalose and polyol, secretion of antifreeze proteins, and psychrophilic enzymes. *Arthrobotrys ferox* an Antarctica fungus exhibits higher resistance to UV radiations because of the abundant pigments its cell wall contains (Zucconi et al. 2002). Some fungi even produce extracellular polymeric substances during biofilm formation that help them in tolerating stresses arising out from desiccation, freeze-thaw cycles, etc. (De Los Ríos et al. 2002; Selbmann et al. 2005).

6.11 Plant Mutualists

Lichen and mycorrhiza comprise the common types of fungal mutualism in cold areas. Mycorrhizae are fungi associated with the plant root, the association benefitting both the partners. Fungi get carbohydrate from the plant while the plant is helped in water and mineral absorption by fungi. The Arctic plants are deemed to obtain 86% of N_2 with the help of mycorrhizae (Hobbie and Hobbie 2006; Bjorbaekmo et al. 2010; Deslippe et al. 2011; Geml et al. 2012; Timling and Taylor 2012).

Lichens are another mutualistic association wherein algae can do photosynthesis at temperature −20 °C. They can absorb water vapor from the snow cover and clouds and tolerate extreme desiccation conditions. All the three kinds of lichens, viz., Crustose, Foliose (e.g., *Xanthoria elegans*), and Fruticose (e.g., *Usnea antarctica*) exist in Antarctica. They can tolerate salinity and pigments such as melanin, parietin, and usnic acid that are present in them and protect them from UV radiation (Little 2009; Zhang et al. 2015).

6.12 Endophytes

Cold-active endophytic fungi studied in the plants belonging to *Cupressaceae* are found to contain bioactive compounds with antibacterial, antifungal, and antiproliferative activity conferring upon the plants' tolerance to abiotic and biotic stress. The endophytic fungi *Phoma* and *Dothideomycetes* species show optimal growth at 4 °C (Moghaddam and Soltani 2014). Cold-active endophytic fungi isolated from bryophytes, e.g., *Barbilophozia hatchery, Chorisodontium aciphyllum*, and *Sanionia uncinata* in maritime Antarctica have been found to be indispensable for the survival of the hosts (Zhang et al. 2013). Psychrotrophic endophytic fungi from high altitude Baima Snow Mountain, Southwest China belonging to genera *Cephalosporium, Discula, Dothiorella, Phoma, Seimatosporium,* and *Sirococcus* exhibit optimal growth at 15 °C (Li et al. 2012).

6.13 Parasites

Fungal pathogens in the cold area do develop survival strategies, for example, Snow molds ≤0 °C pathogenizes several plants covered with snow in the polar regions and thrive in that climate through spores and sclerotia. Some important diseases are typhula blight caused by *Typhula incarnate, Typhula idahoensis*, and *Typhula ishikariensis*; snow rots caused by *Pythium iwayami* and *Pythium okanoganense*; snow scald caused by *Myriosclerotinia borealis*; and pink snow mold such as *Microdochium nivale* associated diseases (Matsumoto 2009). Rust fungi *Melampsora* causes mortality of willows and smuts (Ustilaginales) cause disease in plants belongs to family *Cyperaceae* in the Arctic region (Parmelee 1989; Scholler et al. 2003; Smith et al. 2004).

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Physiology and Molecular Biology of Psychrotrophic Fungi: An Insight

7

Tuyelee Das, Samapika Nandy, Devendra Kumar Pandey, Abdel Rahman Al-Tawaha, Potshangbam Nongdam, Ercan Bursal, Mahipal S. Shekhawat, and Abhijit Dey

Abstract

Among other environmental parameters, the temperature is an important abiotic factor in which the diversity of spices reduces at the extremes, and moreover, only a few microorganisms become the main flora of that region. These extremophiles represented bacteria, archaea, fungi, and algae that are found in both aquatic and terrestrial cold and hot environments. Cold-tolerated microorganisms known as psychrotrophic or psychrotolerant found in the snow, ice, and rocks. They have a significant role in the ecology of that particular environment. Like other extremophilic fungi, psychrotrophic fungi get broad attention due to their potential to produce cold-adapted enzymes, which have importance in pharmaceutical and biotechnological fields. However, research data on psychrotrophic fungi are lacking. Physiological studies of psychrotrophic fungi based on nutrition, growth medium, pH requirement, and the interaction of fungi with environmental factors are significantly important for fungus to grow optimally on a larger scale. The

T. Das · S. Nandy · A. Dey (✉)
Department of Life Science, Presidency University, Kolkata, West Bengal, India
e-mail: abhijit.dbs@presiuniv.ac.in

D. K. Pandey
Department of Biotechnology, Lovely Professional University, Phagwara, Punjab, India

A. R. Al-Tawaha
Department of Biological Sciences, Al-Hussein Bin Talal University, Maan, Jordon

P. Nongdam
Department of Biotechnology, Manipur University, Imphal, Manipur, India

E. Bursal
Department of Biochemistry, Mus Alparslan University, Muş, Turkey

M. S. Shekhawat
Biotechnology Unit, Kanchi Mamunivar Government Institute for Postgraduate Studies and Research, Puducherry, India

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molecular basis of psychrotrophic or psychrotolerant fungi is reviewed in terms of biochemical mechanisms in this chapter. Moreover, the physiological and molecular study gives us a brief idea about psychrotroph adaptation strategies to sustain in a low environmental condition. Therefore, in this chapter, we attempt to deliver psychrotroph physiological and molecular features in relationship with adaptation to low temperature.

Keywords

Psychrotolerant · Psychrotrophic fungi · Cold tolerant · Low temperature

7.1 Introduction

Psychrotrophs are organisms that have the ability to grow at low temperatures (0 °C), but their optimal growth occurs at 15 °C, and maximal growth occurs above 20 °C (Ingram 1965; Morita 1975; Maheswari 2005). Psychrotrophs are also known as psychrotolerant. The psychrotrophic term is more preferable by dairy microbiologists, whereas the psychrotolerant term is utilized by environmental microbiologists (Moyer et al. 2017). Cold places on earth range from deep-sea ocean to cold desert, from frozen ground to terrestrial environment, and from high mountain to glaciers. Cold environments considered as extreme environments when rigid conditions like low water, low nutrient, and high pressure create hard conditions. Microorganisms that flourish in the cold atmospheres have evolved different adaptations strategies to live beneath such condition, which includes production of cold-active enzymes that hydrolyze the compounds, which they can utilize as nutrients (Buzzini and Margesin 2014; Alcaíno et al. 2015; Baeza et al. 2017). Microorganisms such as psychrophiles, mesophiles, and thermophiles have been defined traditionally depends on basic temperature for growth of psychrotrophic microorganisms that are found in cold environments in both terrestrial aquatic regions. Psychrotrophic microorganisms can be isolated from meat, milk, vegetables, cider, and fruit kept in the refrigerator. Psychrotrophs maintain the ecology of cold regions, which cover a significant portion (80%) of the planet (Hoshino and Matsumoto 2012). However, research related to psychrotrophs has not been studied more intensively. In addition, psychrotrophs also could be used in different biotechnological processes. Psychrotrophs grow in low temperature determined by membrane fluidity, nutrients transport, enzymes, and temperature-dependent interaction with DNA, RNA, ribosome, and enzyme (Fukunaga and Russell 1990; Russell 1990; Hamamoto 1994). The psychrotrophic microorganisms possess the ability to grow at low temperature, but not at moderate temperature owing to modification in their membrane proteins and lipids. Membrane protein changes are genotypic, whereas lipids changes are genotypic or phenotypic, which are significant for alternating membrane fluidity and permeability. The capacity to acclimate solute uptake through membrane lipid alternation might differentiate psychrotrophs from psychrophiles. If we can understand the physiology of

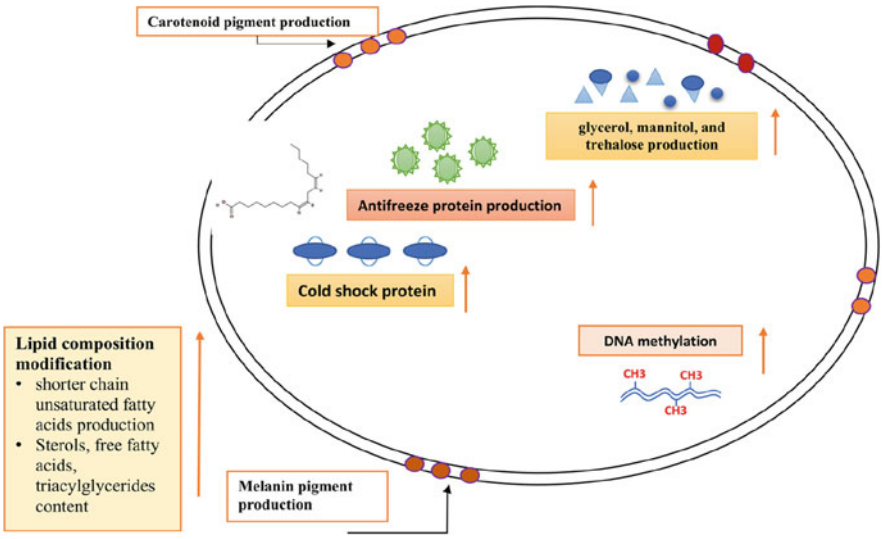

Fig. 7.1 Physiological and molecular adaptative features of psychrotrophic fungi

psychrotrophy, then we can easily utilize them for industrial cold-adapted enzyme production. Figure 7.1 represents physiological and molecular adaptative features of psychrotrophic fungi. Therefore, this chapter aims to compile data from the past on whatever little information is available on the physiology of psychrotrophy that will help to improve industrial production.

7.2 Nutritional Necessities

One cold-tolerant Himalaya strain of *Penicillium pinophilum* MCC 1049 is supplemented with carbon, nitrogen sources, vitamins, and antibiotics and found to produce lactase at 25∘C. Fructose, potassium nitrate, chloramphenicol, and folic acid were found to be the top accompaniments for laccase production (Dhakar et al. 2014). *Mortierella minutissima* 01 fungi from arctic soils grew on agar plates with limonene vapor in medium containing 0.8% substrate (Trytek and Fiedurek 2005). Psychrotrophic micro-fungi (*Aspergillus* sp., *Fusarium* sp., *Mucor* sp., *Rhizopus* sp., and *Penicillium* sp.) grew best on different agar medium (CzapekDox Agar, Malt extract agar Sabouraud dextrose agar, and Potato dextrose agar) (Maharana and Ray 2014). Cho et al. 2007 showed *M. minutissima* 01 improved perillyl alcohol production to 0.3% by the addition of methanol at 15 °C with 6.0 pH (Trytek and Skowronek 2015). *Penicillium chrysogenum* 9 showed to produce the maximum lipase at pH 6.0 but did not produce lipase at pH 3.0–5.0. (Cho et al. 2007). *P. chrysogenum* 9 enzymes showed maximum activity at 30 °C with pH 5.0 for triacylglycerols substrate and with at pH 7.0 for natural substrates like oils (Cho et al. 2007).

7.3 Pigments

The pigment formation gives the protection of fungal spores from stress. Thick pigmented walls protected fungi from extreme cold conditions. A cold-tolerant fungus *Thelebolus microsporus* produces carotenoid pigments detected by high-performance liquid chromatography studies (Singh et al. 2014). Psychrotrophic Antarctic sea fungus *Arthrobacter agilis* accumulates C-50 carotenoid bacterioruberin and its glycosylated derivatives, regulating cellular membrane fluidity (Fong et al. 2001). In cold weather, UV rays are present throughout the year. Therefore, fungi from those places developed some strategies that protect against UV light (Gorbushina 2003; Butler and Day 1998). Antarctic fungal taxa *Alternaria alternata*, *Stachybotrys chartarum* and *Ulocladium consortiale* have melanized strains that resist UV radiation (Hughes et al. 2003). Hughes et al. (2003) observed that *Phoma herbarum*, isolated from Antarctica, was able to produce a brown pigment, probably melanin, within 24 h of disclosure to high radiation of UV-B. Psychrotolerant yeasts from the Antarctic region *Cryptococcus gastricus*, *Dioszegia* sp., *Leuconeurospora* sp. (T27Cd2), *Rhodotorula mucilaginosa*, and *Rhodotorula laryngis* showed the maximum UV-C radiation tolerance. *Dioszegia* sp., *C. gastricus*, and *R. mucilaginosa* showed production of the highest percentage of carotenoids (torulene, 2-gamma carotene, gamma carotene, and lycopene) (Villarreal et al. 2016). *Penicillium cordubense* produced yellow pigment at 25 °C optimal temperature (Pandey et al. 2016). The formation of green conidia has importance in UV radiation tolerance (Braga et al. 2006). Greenish conidia production observed in *Trichoderma velutinum* ACR-P1 fungus isolated from Shiwalik hills, Jammu, and Kashmir (Sharma et al. 2016).

7.4 Antifreeze Proteins

Antifreeze proteins are structurally diverse polypeptides that attach to ice nucleators and adsorb to ice surface that let down the freezing temperature without changing the melting point (Urrutia et al. 1992). This phenomenon is known as thermal hysteresis, in which the ranges vary from organism to organism. Antifreeze protein is a key strategy of fungi in a low-temperature atmosphere (Duman and Olsen 1993). Antifreeze proteins alter the ice crystals shape led to inhibition of recrystallization (Knight et al. 1984). Antifreeze protein from *Antarctomycespsychrotrophicus* was identified as an extracellular protein that generated bipyramidal ice crystals and showed thermal hysteresis phenomenon under alkaline conditions (Xiao et al. 2010). The psychrophilic or psychotropic snow molds are plant pathogen, which also acts as a plant pathogen of grasses and cereals. Antifreeze proteins of *Coprinus psychromorbidus* and *Typhula ishikariensis* present in extracellular space and represent 22 and 23 kDa molecular masses, respectively, which suppress the freezing of the extracellular environment. Antifreeze proteins from *T. ishikariensis* do not form proper hexagonal ice crystals instead bind with ice crystals to inhibit their growth

(Hoshino et al. 2003). Antifreeze protein binds to the various stratum of ice crystal that suggested by observing ice crystal patterns linked with snow mold.

7.5 Compatible Solutes

Compatible solute accumulation results in a decrease in freezing point, which linked to cytoplasmic macromolecules stability. The most extensive cryoprotective substances are glycerol, mannitol, and trehalose that synthesized by induction of low temperatures. Changes in polyol concentration led to typical physiological adaptation for maintaining turgor pressure by fungi (Weinstein et al. 2000). Disaccharide trehalose (a-D-glucopyranosyl(1–1)-aD-glucopyranoside) extensively found in bacteria, yeasts, and fungi in different growing stages. Trehalose maintains fungi survival in environmental stress conditions. Trehalose not only acts as a reserve carbohydrate but also plays a role as an effective protectant that enhanced protection against adverse conditions (Chi et al. 2003). Trehalose plays a significant role in thermotolerance but the cold tolerance of the yeast. An Antarctic psychrotolerant yeast *Guehomyces pullulans* 17-1 grows the best at 15 °C. *G. pullulans* 17-1 cells was grown at 10, 15, and 25 °C and resulted in expression of trehalose-6-phosphate synthase (*Tps1*) gene activity higher at 25 °C than those grown at 10 and 15 °C. Trehalose synthesized by *G. pullulans* 17-1 linked with adaption to high temperature (Zhang et al. 2013). These observations suggest that psychrotolerant *G. pullulans* 17-1 are more delicate to elevated temperature than to low temperature. Therefore, the production of more trehalose needs to be synthesized in cell to protect the cells grown at high temperature (Zhang et al. 2013). Glycogen and trehalose production is detected in *Penicillium olsonii* p14 after 4–6 h of cold treatment. In this psychrotolerant strain accumulation of glycogen and trehalose content increases up to 2.0–2.5 times and 2.5-fold, respectively (Gocheva et al. 2009).

7.6 Lipid Composition

Membrane lipid composition changes in response to a different temperature. Psychrophiles and psychrotrophs contain more short-chain branched unsaturated and polyunsaturated fatty acids. In psychrophile, polyunsaturated fatty acid level increased by decreasing the temperature. In low temperature, acyl chain length in the phospholipids also decreased. Psychrophiles are capable of maintaining the higher proportion of hexadecenoic (16:1) and octadecenoic (18:1) acids than are mesophiles (Hamamoto et al. 1995). *Geomyces pannorum* in static liquid cultures reported producing higher degrees of sterols, sterol esters, free fatty acids, triacylglycerides, and mono−/diacylglycerides. Unsaturated fatty acids and triacylglycerides present more in *Pseudogymnoascus destructans* as compared with *G. pannorum* (Pannkuk et al. 2014). The common fatty acids found in *G. pannorum* and *Geomyces vinaceus* were tetradecanoic acid, 12-octadecadienoic acid, exadecanoic acid, octadecanoic acid, cis 9-octadecenoic acid, cis

9-exadecenoic acid, cis 9, and eicosanoic acid. *G. vinaceus* and *C. merdarium* fungi showed a different unsaturation level at different temperatures (Finotti et al. 1993). Two strains of *G. pannorum,* VKM FW-224 and VKM F-3808, showed an improved level of linoleic acid (C 18:2) in a medium containing NaCl at 20 °C (Konova et al. 2009). Gram-negative psychrotolerant bacterial species were also shown to contain branched-chain fatty acids (Fukunaga and Russell 1990). At low temperatures, shorter chain unsaturated fatty acid production increases in both bacteria and fungi that lead to membrane fluidity increase (Weete 1974; Tearle and Richard 1987). *T. microsporus* produced myristic acid, palmitic acid, stearic acid, heptadecanoic acid, linolenic acid, and linoleic acid, which detected by gas chromatography. Presence of these fatty acids suggested that fungus growing at low temperature modified membrane fluidity by altering membrane lipids (Singh et al. 2014).

7.7 DNA Methylation

Gene expression alternation in the response to cold stress has been studied in prokaryotes and plants (Fan et al. 2013; Dai et al. 2015). Turchetti et al. (2020) studied DNA methylation in psychrophilic (*Naganishia antarctica*) and psychrotolerant (*Naganishia albida*) yeast species in different temperature. Both the species showed a different level of DNA methylation. In *N. antarctica* total methylated fragments did not change. In contrast, psychrotolerant *N. albida* showed a nonsignificant increase of methylation when incubated at 4 °C and also during the regaining stage (Turchetti et al. 2020). DNA methylation is a significant epigenetic modification. Numerous studies have associated with DNA methylation of gene; however, those studies are quite controversial. DNA methylation studies in some yeast displayed this modification, while some other confirmed the presence of DNA methylation (Hattman et al. 1978; Bulkowska et al. 2007; Baum and Carbon 2011; Tang et al. 2012; Capuano et al. 2014).

7.8 Enzymes

Psychrotrophic microorganisms utilized different enzymes for survival at a lower temperature. Their survival strategies include reduced ionic pairs number, hydrophobic interactions, inter-subunit interactions, and increased solvent interaction, nonpolar residues in the solvent, the decline in cofactor binding, and glycine (D'Amico et al. 2006). Cold-tolerant microorganisms produce lipases that raise their tolerance to survive in an extremely cold environment (Kour et al. 2019). Various cold-adapted lipases have been studied from psychrotrophic and psychrophilic microorganisms (Kavitha 2016). *P. chrysogenum* 9, *Penicillium canesense*, and *Pseudogymnoascus roseus* were found to produced lipase (Bancerz et al. 2005; Sahay and Chouhan 2018). The psychrotolerant *Penicillium* isolates (GBPI_P98, GBPI_P228, and GBPI_P150) exhibited lipase production at 25 °C. Mostly lipase

production increases at low temperature. All the isolates were shown to possess tolerance from a temperature between 4 and 35 °C (Pandey et al. 2016).

7.9 Cold Shock Proteins and Mycosporine

Cold shock (CS) proteins are multifunctional RNA/DNA binding proteins, enable translation through RNA secondary structure destabilization. Fang and St. Leger (2010) identified glycine-rich RNA binding proteins (GRPs) homologues in various fungi. Nevertheless, *Metarhizium anisopliae* and *Aspergillus clavatus* possessed CS domains. *Crp1* and *Crp2* displayed a high resemblance to CS proteins. CRP1 (cold response protein 1) also protected *M. anisopliae* from oxidative stress and maintain metabolism (Fang and St. Leger 2010). Psychrotolarent *Leuco neurospora creatinivora* can produce mycosporines (Villarreal et al. 2016). The synthesis of mycosporine mechanisms can reduce cell damage due to UV radiation in organisms growing at high altitude (Margesin et al. 2007).

7.10 Temperature Effect

Psychrotrophic microorganisms are found in equally aquatic and terrestrial cold environments. Psychrophilic and psychrotrophic microorganisms play a significant role for maintain ecological balance, but, shockingly, research based on their physiology has not yet been conducted intensely. *Rhodotorula aurantiaca* psychrophilic Antarctic strain A19 cannot grow beyond 20 °C, whereas Belgium strain 31,345 staring higher growth-limiting temperature was 32 °C. These two yeast produced maximum cell at 0 °C and by increasing temperature cell production decreased. Glucose uptake was increased at temperatures above l0 and 17 °C for the psychrophilic strain and psychrotrophic strain, respectively. The difference in growth rate and substrate affinity was associated with the adaptation strategy of *R. aurantiaca* strains to environmental conditions (Sabri et al. 2000). Snow mold fungus *Typhula incarnata* formed small-sized sclerotia, which reduced by elevating temperature but large-sized sclerotia formation increased by raising the temperature (Hoshino et al. 2004). Gocheva et al. (2009) displayed that psychrotolerant strain *P. olsonii* p14 from Antarctica can tolerate temperature downshift. Temperature changes from 15 to 6 °C directed cells to oxidative response, which linked to a drop of biomass production and rise in the levels of superoxide dismutase (SOD) and catalase (CAT) enzymes, storage carbohydrates, and oxidative damaged proteins (Gocheva et al. 2009). Gray investigated Antarctic fungus growing in various temperature was not found any psychrophilic species but two Antarctic psychrotolerant nematophagous fungi *Monacrosporium ellipsosporum* and *Monacrosporium cionapagum*, which grew between 4 and 15 °C and adapted to cold habitats (Gray 1982). Kerry (1990) investigated growth rates of fungi isolated from the Antarctic Continent at temperatures ranging from 4 to 35 °C. They specified that those fungi that grew at 4 °C were classified as psychrotrophs (Kerry 1990).

Lipase production in different isolates of *Penicillium* spp. depends upon temperatures. Maximum production of lipase was reported at 15 and 25 °C temperatures, while minimum production recorded at 5 and 35 °C (Pandey et al. 2016). At temperature ranges from 4 to 50 °C soil ascomycete fungus *T. velutinum* ACR-P1 can grow and formed compact and hyphae with greenish conidia on solid media (Sharma et al. 2016).

7.11 Conclusion

From the past few decades, the thermal features of psychrotrophs have been established accurately; however, we are still understanding how psychrotrophs have the ability to grow at such low temperatures. Psychrotrophs are naturally exposed to very low temperature, high UV-B radiations, and low water and nutrient available environment. Psychrotrophs survival is not possible without a proper nutrient cycle in a functional ecological niche. The presence of Psychrotrophic fungus in cold habitats like glaciers is quite interesting. The physiology of psychrotrophs has gained attention and predicted the understanding of adaptation at the molecular level. Psychrotrophs grown at low temperature have low acyl chain length phospholipids, synthesis of melanin and mycosporine, production of cold-active enzymes, and presence of compatible solutes. However, all the physiological and molecular adaptive mechanisms still need more investigation. It is a fact that psychrotrophic fungi show potential in biotechnological and pharmaceutical fields. Future research on cold-active enzymes production, pharmaceutical metabolites, and biotechnology related to psychotrophy is essential for understanding the role of psychrotrophic or psychrotolerant fungi.

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Ecology, Physiology, and Diversity of Piezophilic Fungi

8

Shyamji Shukla and Harshita Shukla

Abstract

Deep-sea hydrothermal ecosystems (>1000 m below sea level) are just like fertile spot of life in oceans and symbolize one of the most extreme ocean environments. Many indigenous species of Bacteria, Archaea, and other organisms, like annelids and crabs, are reported since the late 1970s when the development of these ecosystems took place. The deep-sea possesses great variety of microbial diversity, in spite of the extreme environmental conditions viz., low temperatures, high hydrostatic pressure, high salinity concentrations, absence of sunshine, etc. Considerable information has been gathered about the variety of microorganisms in deep-sea ecosystems, but the diversity of fungi has not been examined up to now. These deep-sea fungi are called piezophilic fungi. Fungi are recognized as important organisms in terrestrial ecosystems because of their environmental functions and predominantly their capability to break down organic matter. The paucity of information about fungal diversity in the deep-sea has generally established a notion that they are terrestrial organisms. For many years, the majority of the facts about deep-sea fungal diversity were revealed via culture-dependent techniques. In recent times, with the advancements in efficient sequencing techniques, there has been a speedy growth in the research of deep-sea fungal flora. This chapter highlights the studies related to the variety, ecology, and physiology of piezophilic fungi. The aim of this chapter is also to focus on the evolution of the techniques to evaluate the diversity and ecological role of the piezophilic fungi which could stimulate new prospecting for biomolecules from these fungi.

S. Shukla (✉)
Department of Biotechnology, Mata Gujri Mahila Mahavidyalaya (Autonomous), Jabalpur, India

H. Shukla
Department of Biotechnology, Sri Guru Tegh Bhadur Khalsa College, Jabalpur, India

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8.1 Introduction

The deep-sea has great potential and is one of the most inexplicable and unknown extreme environments. In spite of widespread studies on prokaryotes of deep-sea, very little is known about the rich biodiversity of fungi which are ecologically very important groups of eukaryotic microorganisms. Though, the occurrence of fungi in these deep-sea ecosystems is now being acknowledged. Piezophiles dwell deep-sea sediments (>3000 m depth) under high hydrostatic pressure and also inhabit the guts of bottom-dwelling animals. Numerous fungi similar to terrestrial species have been isolated by culture-dependent methods from various deep-sea environments. On the other hand, these methods have also revealed the presence of various novel fungal phylotypes viz., Cryptomycota, which do not possess the characteristic chitin-rich fungal cell walls. Even though exact information about the diversity of fungi and its function in deep-sea ecosystem remains not very clear, the purpose of this chapter is to evaluate recent understanding of the ecology, physiology and variety of fungi in these ecosystems recommending future path for investigating deep-sea fungal flora.

Fungi are a vital constituent of all ecosystems and are omnipresent in environment because of their extremely adaptable physiological character. Deep-sea fungi inhabit sediments of deep-sea at a depth of quite 1000 m below the surface of the ocean. The deep-sea is that part of the ocean which is around 1000 m deep, and covers 60% of the Earth's surface. It therefore makes the planet's largest homogeneous ecological formation called biome. The geological, geochemical and physical conditions of the seabed and water column define varied habitats, sheltering specific biological communities. The bottom of the ocean consists of a variety of different environments, viz., continental margins, abyssal plains, oceanic trenches, mid-ocean ridges and seamounts. Around 90% of the deep-seabed contains fine sediments composed of particles of different origin viz., biogenic (produced by living organisms), terrigenous, volcanic and authigenic (originating from the rock where it's found). The seabed is however rockier on mid-ocean ridges and seamounts, furthermore as in some isolated areas of continental slopes, into which submarine canyons with abrupt cliffs is also cut. The marine environment is globally characterized by an absence of sunlight, high, low, and comparatively constant temperatures, low currents and oxygen content generally sufficient for animal life to develop.

The deep-sea environment is among the most intense environments for living organisms. Conditions like lack of sunlight, mostly lesser temperature (some places like hydrothermal vents have a particularly warmth up to 400 °C), excessive pH, and high hydrostatic pressure (up to 110 MPa) create extreme environment in deep-sea (Damare et al. 2006a; Burgaud et al. 2010). These environmental factors are known to affect the abundance, diversity, activity, and distribution of fungi within the

natural habitats. Piezophilic fungi are able to survive in such extreme environmental conditions and are found in large numbers in deep-sea environments (Gadanho and Sampaio 2005; Wang et al. 2015). It is supposed that these fungi evolved from terrestrial environments, and acquainted well to the deep-sea environmen. A current estimation indicates that overall fungal species are approximately 2.2–3.8 million, out of which only 120,000 species are explained by taxonomists, while some species are identified from the deep-sea environments.

The biodiversity of deep-sea-derived fungal species and their applications in biotechnology are not completely described, which raises the need of further research to discover their potential. However some bioactive molecules having antibacterial, antiviral, antidiabetics, anti-inflammatory, antitumor, and enzymatic potential are isolated from deep-sea fungal communities (Wang et al. 2015). The aim of this chapter is to provide an overview about Piezophilic fungal diversity, including their ecology and physiology. This chapter also discusses the tools and techniques employed in the collection, isolation, and identification of the Piezophilic fungi.

8.2 Exploring Piezophilic Fungal Diversity

8.2.1 Collection of Sample

Collection of fungi from deep-sea waters can be performed using routine oceanographic samplers such as Niskin or Nansen bottles or a Rossette or ZoBell sampler. In-situ hydrostatic pressure is not maintained in these samplers. First water sampler, having potential to sustain deep-sea pressure to collect water sample for isolation of piezophilic bacteria was reported by Jannasch et al. (1973). Usually deep-sea fungi procure their nutrients from organic matter present in the sediments and generate hyphae, which extend and develop amid the sediment particles. It is more suitable to search for deep-sea fungi in the sediments as they are not well adapted as free living forms. Several types of sampling devices for collection of sediment samples have been used successfully by mycologists.

A deep-sea workspace was created at JAMSTEC, Japan under the Deep-Star program for collecting and culturing microorganisms from deep-sea conditions. This system has a pressure-retaining sediment sampler, which is able to maintain hydrostatic pressure and low temperature of the sediment samples after collecting from the bottom of deep-sea (Yanagibayashi et al. 1999). Whereas regular sampling of deep-sea sediments for isolation of microbiological flora is done by techniques like multiple corers, long gravity corers or box corers.

Box corers are more commonly used for sampling of flat oceanic floors. It is made to penetrate the underside by lowering on a ship's trawl wire. Since the corer is taken out of the seabed, the top and bottom of the sample box are closed. The benefit of a box corer is that it accumulates samples which are usually $\geq$20 cm long including most of the deep-sea organisms. Research submarines can also be employed for deep-sea sediments collection. An enquiry submarine is comparatively larger in size

and can carry a pilot and one or two scientists in a pressure sphere of about 2 m in diameter. Around the sphere is a basket carrying equipments for support, propulsion, ascent and descent, and those for scientific purposes like manipulator arms, cameras and specialized payload in a very carrying basket.

Another method for sampling includes self propelled instrument packages viz., remotely operated vehicles. Some operate a two-way communications link at the top of a cable that has power and hosts, while others are untethered, carrying their own power, recording images and data. Instrument package consists of Propulsion unit, sensors (particularly television), and manipulator arms. Their design enable them either to fly or crawl, e.g., the Remote Underwater Manipulator over bed surface and are further appropriate for sampling from deep-sea sediments.

8.2.2 Studying Microbial Communities

Microbial communities play essential role in diverse biogeochemical cycles of different habitats. The major problem in correct estimation of microbes at a particular habitat is sampling and analyzing comparatively very small proportion of total area. In addition, due to spatial heterogeneity of the species or the habitat, diversity analysis of the microbes may be underestimated. Other factor for inability of correct estimation of microbial communities at a particular habitat is their low culture ability. It has been observed that approx. 99% of bacteria analyzed under a microscope are not cultured by common laboratory techniques. The majority of the fungal species also escape laboratory culturing techniques. Diversity of species includes species richness, the overall number of species present, species constancy, and the allocation of species. Some of the techniques used for the measurement of cultured microbial diversity are as following:

8.2.3 Community Level Physiological Profiling

Applying this technique the culturable microorganisms may be recognized on the basis of a range of carbon source utilization patterns. To perform this technique designed Biolog plates containing all the well-known carbon sources are commercially available. For examination, samples containing microbes are inoculated and monitored over time for their ability to utilize substrates. Thereafter the speed at which these substrates are utilized was also monitored. With the application of various statistical techniques to the information, relative differences are assessed.

8.2.4 Fatty Acid Methyl Ester (FAME) Analysis

Fatty acids build up a comparatively invariable fraction of the cell biomass. Some characteristic fatty acids exist that can distinguish chief taxonomic groups of a community. Therefore, a modification in the fatty acid profile signifies a

transformation in the microbial population. Hence fatty acid profiles can be exploited to analyze composition and population changes of a microbial community. Further researches on bacteria have, revealed that fatty acid compositions affect the phenotypic characteristics of organisms based on DNA and rRNA homology.

Every bacterial species and strain depicts a particular fatty acid profile and thereby can be identified on this basis. Yeast identification can also be performed by using this method reported differential expression of fatty acids by different phyla of fungi which can be in turn employed as a biomarker for their identification. For example, fungi belonging to Oomycota express 20:5 fatty acids, Zygomycota produce 18:3 fatty acids which are higher in number as compared to Dikaryotic forms i.e., Ascomycota and Basidiomycota. On the other hand sterile forms of fungi produce 22:0 fatty acid, which is not found in any other taxon.

8.2.5 Molecular Based Techniques

Polymerase Chain Reaction (PCR) targeting the 16S rDNA has been used comprehensively to examine the identification and diversity of prokaryotes. In case of eukaryotes, particularly fungal species, 18S and internal transcribed spacer (ITS) regions of SSU rDNA are increasingly used for study. Moreover, the D1/D2 domain as well as different regions of Large Sub Unit (LSU) of 28S rDNA of eukaryotes can also be employed for identification and analysis of diversity (Gadanho and Sampaio 2005; Burgaud et al. 2010). Besides this diversity analysis can also be done by applying Restriction Fragment Length Polymorphism (RFLP) for the above amplified products on the basis of DNA polymorphisms. RFLP is the dissimilarity in banding patterns of DNA fragments obtained after electrophoresis of restriction digests of DNA from different individuals of a species. The cause for the unlike banding patterns is due to the occurrence of a restriction enzyme cleavage site at one place in the genome in one individual and the lack of that specific site in another individual. Therefore, strains can be identified and compared on the basis of these different and specific banding patterns after cleavage with a particular restriction enzyme obtained in electrophoresis of samples from different strains.

8.2.6 Culture-Dependent Approach

Recently some reports have described presence of fungi in deep-sea habitats using culture-dependent approaches. This approach has been used for the isolation of several fungi from various marine habitats. Besides it has also been known for the isolation of few terrestrial fungi viz., *Acremonium, Aspergillus, Penicillium*, and *Trichoderma* (Raghukumar et al. 1992). A wide range of culturable marine fungi has been reported from deep-sea hydrothermal vents using this technique (Burgaud et al. 2009).

Their physiological characterization proved them to be more or less adapted to deep-sea conditions. Burgaud et al. (2010), isolated yeast species belonging to

genera, *Rhodotorula, Rhodosporidium, Candida, Debaryomyces, and Cryptococcus* from hydrothermal vent animals and identified them by analysis of 26 rRNA gene sequences. Fungi were isolated in the earlier reports from the deep-sea sediments of the Central Indian Basin (CIB) from different sites with geographical locations of ~10–16°S and 73–77°E (Raghukumar et al. 2004; Damare et al. 2006a). Earlier studies have reported the identification of fungi only on the basis of classical taxonomic method, which may give biased results about actual fungal diversity in this region (Raghukumar et al. 2004; Damare et al. 2006a).

8.3 Ecological Distribution of Piezophilic Fungi

Around 65% area of the whole earth surface is covered with deep-sea. The main characteristic features of deep-sea environment include intense conditions of temperature, hydrostatic pressure and nutrient conditions. There is an increase in the hydrostatic pressure with depth at a rate of 0.1 MPa/10 m (0.1 MPa is reminiscent of 1 bar hydrostatic pressure). Therefore, the hydrostatic pressure is ~50 MPa at 5000 m depth. On the other hand, there is a decrease in temperature with increase in depth. It is around ~2–3 °C at 5000 m depth. This environment is furthermore described by low nutrient conditions and is intended to be physically stable.

Deep-sea fungi were primarily reported from the shells collected from deep-sea waters of 4610 m depth (Höhnk 1969). Further reports suggests isolation of fungi from subtropical ocean water samples having depth of 4500 m using sterile van Dorn bags or Niskin samplers (Roth et al. 1964). Deep-sea fungi have also been isolated by submerging wooden panels at a depth of 1615–5315 m directly (Kohlmeyer 1977). However, four fungi out of these were found growing on wooden panels while one on chitin of a hydrozoan. Poulicek et al. 1986 depicted mycelial fungi growing inside shells of mollusks at 4830 m depth within the Atlantic. Numerous filamentous fungi were isolated using 1/5 diluted malt extract medium prepared with seawater from surface sterilized calcareous fragments at a depth of 300–860 m within the Bay of Bengal (Raghukumar et al. 1992). The high-pressure and low-temperature tolerance assay can indicate whether the isolated fungal species are native deep-sea forms or not (Kohlmeyer and Kohlmeyer 1979). In an another study, it was demonstrated that conidia of *Aspergillus ustus* (Bainier) and *Graphium sp.* isolated from the calcareous sediments germinated at 10 and 20 MPa pressure in Czapek-Dox medium and on shells suspended in seawater (Raghukumar and Raghukumar 1998). In a similar study culturable fungi, *Penicillium lagena* (Delitsch) Stolk & Samson and *Rhodotorula mucilaginosa* (A. Jörgensen) F. C. Harrison, were obtained from a depth of 10,500 m sediment samples from the Mariana Trench within the ocean.

In marine ecosystem, fungi holds a significant position as they are present everywhere and perform decomposition and mineralization of organic matter (Kohlmeyer and Kohlmeyer 1979). The Deep-sea fungi were first observed in shells collected from deep-sea waters at a depth of 4610 m. Presence of fungi inside molluskan shells was reported by Poulicek et al. (1986). Isolation of cultures of

many marine yeasts viz., *Debaryomyces sp., Rhodotorula sp., and Rhodosporidium sp.* over a range of temperature and hydrostatic pressure conditions have also been recorded. Reports are also available on the presence of fungi in Mariana trench within the Pacific at a depth of ~11,000 m.

A variety of filamentous fungi isolated from calcareous sediments show germination of spores at elevated hydrostatic pressure (Raghukumar and Raghukumar 1998). Fluorescent brightner calcofluor under epifluorescence microscope depicted the presence of fungal filaments in calcareous fragments (Raghukumar and Raghukumar 1998). The fungal filament isolated from the deep-sea sediments of Chagos trench in the Indian Ocean is thought to be oldest in age (Raghukumar et al. 2004). More than 200 fungal isolates were procured in culture form from deep-sea sediments of the CIB by the application of different techniques like dilution plating, particle plating and pressure enrichment techniques (Damare et al. 2006a). Immunofluorescence technique revealed that one of these isolates was a native of deep-sea sediment.

8.4 Diversity of Piezophilic Fungi

Damare et al. (2006a) identified *Aspergillus* sp. by applying classical morphology based taxonomy as the most dominant form from the CIB. Sixteen filamentous fungi and 12 yeast species from CIB were reported by Singh et al. (2010) as culturable fungi using ITS and 18S sequences of SSU rDNA. Occurrence of *Sagenomella* sp., *Exophiala* sp., *Capronia coronata* Samuels and *Tilletiopsis* sp. from deep-sea sediments were reported for the first time by using this approach**.** It was observed that the majority of the culturable filamentous fungi belonged to ascomycetes whereas most of the yeast isolates belonged to basidiomycetes. Sixty-two filamentous fungi associated with animals from a range of deep-sea hydrothermal vent sites mostly belonging to ascomycetes were isolated by Burgaud et al. (2009). Inspite of so many fungal species isolated from deep-sea, exploration of Piezophilic fungi associated with deep-sea dwelling macrofauna and zooplankton should be undertaken further to unravel their diversity and ecological role. A novel genus of deep-sea ascomycetes, *Alisea longicola* including one new species was reported by Dupont et al. (2009) via analyses of 18S and 28S rDNA sequences and morphological characters. Its isolation was performed from sunken wood of Pacific Ocean at Vanuatu Islands.

In a similar study Connell et al. (2009) isolated eight yeasts and yeast-like fungal species from cold hydrothermal environment and basalt rock surface from an active deep-sea volcano, Vailulu'u Seamount, Samoa. Recently, 32 isolates of yeasts belonging to phyla Ascomycota and Basidiomycota were obtained by Burgaud et al. (2010). These fungal species were associated with deep-sea fauna from hydrothermal vents. On the other hand techniques like PCR amplification of SSU rRNA genes and sequence analyses have revealed the occurrence and diversity of microbial eukaryotes from a deep-sea in the aphotic zone at 250–3000 m depth in the Antarctic polar front (Lopez-Garcia et al. 2001). With the advent of these reports

which showed fungi as one of the eukaryotic groups in these sediments, techniques employing amplification of sediment DNA with fungal specific primers to study culture-independent diversity attracted most of the scientific attention.

Investigation of culture-independent fungal diversity by examination of the small-subunit rRNA gene sequences amplified by PCR using DNA extracts from hydrothermal vent samples was performed by Le Calvez et al. (2009). Three fungal phyla viz., Chytridiomycota, Ascomycota, and Basidiomycota were reported with new species by them. On the other hand, particularly lesser variety of filamentous fungi was reported by Bass et al. (2007). Only about 18 fungal 18S-types from 11 deep-sea sediment samples were obtained. It can therefore be concluded that combination of culturing techniques with phylogenetic analysis gives much better range of fungi within the deep-sea environment. Further, several researchers have reported the diversity of fungi in deep-sea sediments isolated by culture-independent methods and the challenges accompanied by this method (Lai et al. 2007; Takishita et al. 2007; Le Calvez et al. 2009; Nagano et al. 2010; Biddle et al. 2008; Edgcomb et al. 2010). Most of the fungi isolated so far from the deep-sea are like terrestrial fungi. Though, Alker et al. (2001) and Zuccaro et al. (2004) reported some terrestrial fungi from marine habitats, which have evolved into marine forms.

It is a matter of research that if these fungi are physiologically different from their terrestrial counterparts in their nutritional requirements and enzyme activities. These fungi are probably associated with deep-sea dwelling marine fauna. However some of the novel fungi isolated from various deep-sea locations are true piezophiles as described by several research groups Lopez-Garcia et al. 2001; Gadanho and Sampaio 2005, Lai et al. 2007; Le Calvez et al. 2009; Nagano et al. 2010). But still more techniques need to be developed for culturing fungi which are yet not cultured. Moreover the host population and diversity in the deep-sea environment are significantly affected by the association of many eukaryotic phylotypes with parasitic organisms (Brown et al. 2009).

The Ocean Drilling Program has aided to a great extent in the examination of microbial diversity from deep marine seafloor sediments in spite of the extreme environment (Parkes et al. 2005). Ascomycetous fungi belonging to the genera *Cladosporium, Penicillium, and Acremonium* spp. were recovered from sediment core collected at 200 mbsf (meter below sea floor) of the Peru margin by direct plating and enrichment culturing technique (Biddle et al. 2005). Identification of these cultured fungi was performed by ITS sequencing. In a similar study Edgcomb et al. (2010) obtained fungal sequences from the deep-sea sediment isolated fungal trains. These fungi were isolated from the depth 37 m below the seafloor of the Peru Margin and Peru Trench and were mostly found to be belonging to Basidiomycetous phyla.

Besides this, fungal species belonging to different phyla has also been reported from a few more extreme environments. *Cryptococcus curvatus* (Diddens & Lodder) Golubev, a basidiomycetous yeast was found to be the main fungal phylotype in oxygen-depleted sediments from deep-sea methane seeps (Takishita et al. 2006, 2007). These areas are particularly rich in methane and hydrogen sulfide concentrations. On the other hand, gas-hydrate-bearing sediments containing

ice-like minerals that have crystallized under low temperature, elevated hydrostatic pressure and methane concentrations are also known to possess rich fungal diversity whose sequences have been recovered from there (Cao 2010). Gas hydrates are in gas hydrates, where methane is the major hydrocarbon in the gas mixture held in water molecules. Several fungal sequences in such methane hydrate-bearing deep-sea sediments have been recovered that were not associated to any known fungi or fungal sequences Lai et al. (2007). Besides, fungal clones showing similar characteristics to *Phoma, Cylindrocarpon, Hortaea, Cladosporium, Emericella, Aspergillus, Malassezia, Cryptococcus, Lodderomyces, Candida,* and *Pichia* were also procured.

Several studies have confirmed the presence of diverse fungal forms from the Indian Ocean (Raghukumar and Raghukumar 1998; Damare et al. 2006a). Identification of these fungal species was carried out by classical taxonomic methods. But the disadvantage of the classical identification method is that it cannot identify the non-sporulating forms of fungi on the basis of morphological features. In recent studies identification of fungal cultures is done by employing amplification and sequencing of their 18S rDNA and ITS regions (Burgaud et al. 2009). With the aid of these molecular techniques the identification of both sporulating as well as non-sporulating forms of fungal cultures upto generic or species level is possible.

Culture-independent approach also has reported isolation of a large variety of fungal species from different regions throughout the world using. In an another study, Bass et al. (2007) revealed that yeast are among the most dominant fungal forms found in the deep ocean. Fungal specific primers for SSU rDNA region were used for this analysis. A similar study indicated the presence of fungal strains in hydrothermal sediments of the mid-Atlantic ridge by analysis of 18S rDNA sequences amplified with eukaryote-specific primers (Lopez-Garcia et al. 2003). Likewise recovery of fungal sequences from different deep-sea habitats viz., methane hydrate bearing deep-sea sediments (Lai et al. 2007), hydrothermal vents, anoxic sites etc. have been reported by several researchers.

Hardly any research work has confirmed the identification of fungi using molecular methods from the CIB. In the previous researches classical morphological methods have been used for the identification of fungal diversity however these studies were not able to justify presence of unidentified non-sporulating forms with classical methods. Consequently, in order to observe the exact diversity of fungi in the deep-sea sediments of the CIB, both culture-dependent as well as culture-independent approaches were applied for the study in addition to the classical methods. Further culture identification and environmental gene libraries construction was performed by targeting the 18S and ITS regions of SSU rDNA. Thereafter the results obtained were compared with earlier reports on fungal diversity from the same sampling area of the Indian Ocean.

Even though the deep-sea environment acts as a habitat for a huge number of microbial species, but, the origin, diversity, and distribution of deep-sea fungal community remain mostly unexplored. The first report on deep-sea fungi was proposed by Höhnk (1969) when he isolated fungi from 4610 m depth below the sea. Similarly, Roth et al. (1964) isolated fungi from the subtropical Atlantic Ocean

at a depth of 4500 m. However, these fungal isolates were not obtained in culture form under laboratory conditions. In an another study numerous filamentous fungal strains were primarily isolated from calcareous fragments of the Bay of Bengal at a depth of 300–860 m using culture-dependent method Raghukumar et al. (1992). Thereafter, several fungal species were isolated and identified from different deep-sea environmental samples, such as from the Mariana Trench at a depth of 11,500 m, the Chagos Trench 5500 m (Raghukumar et al. 2004), the CIB 5000 m (Singh et al. 2011), Gulf of Mexico sediment 2400 m, South China Sea 2400–4000 m, East India Ocean 4000 m, Canterbury Basin (New Zealand) 4–1884 mbsf (below the seafloor), Okinawa 1190–1589 m, and the pacific ocean off the Shimokita Peninsula, Japan, 1289–2466 mbsf (Liu et al. 2017).

Around 120 deep-sea fungal species have been isolated and identified by several researchers using culture-dependent techniques (Gadanho and Sampaio 2005). While some unknown fungi have also been isolated by the application of culture-independent techniques. It has been reported that Ascomycota comprises about 78% of the total species in deep-sea environment followed by Basidiomycota (17.3%), Zygomycota (1.5%), and Chytridiomycota (0.8%). On the other hand unidentified fungal isolates forms 2.4% of the total deep-sea microbial flora. *Aspergillus* sp., *Penicillium* sp., and *Simplicillium obclavatum* are the most diverse and common fungal species. Whereas, *Alternaria alternata*, *Aureobasidium pullulans*, *Cryptococcus liquefaciens*, *Exophiala dermatitidis*, *Epicoccum nigrum*, and *Neosetophoma samarorum* were reported to be the rarest fungal species found in the deep-sea environments. Lately, 69 fungal isolates belonging to 27 species were isolated by Liu et al. (2017) from deep coal-associated sediment samples collected at depths ranging from 1289 to 2457 mbsf in the Pacific Ocean off the Shimokita Peninsula, Japan. Around 88% of the isolated strains belonged to the phyla Ascomycota dominated by *Penicillium* and *Aspergillus*, while only 12% of the strains belong to Basidiomycota. Besides these a number of new fungal species were also reported.

In one such study, isolated six new phylotypes of genera *Ajellomyces*, *Podosordaria*, *Torula*, and *Xylaria* from the sediment of the South China Sea at a depth of 2400–4000 m. Similarly, one novel fungal phylotype, DSF-Group1, was explored in deep-sea sediments at depths ranging from 1200 to 10,000 m by using three fungal specific primer sets, targeting the ITS1–5.8S–ITS2-28S rRNA regions (Nagano et al. 2010). In another study, environmental clade KML11, a group of the parasitic genus *Rozella* in *Cryptomycota*, was also identified by using eukaryotic-specific primers EK-82F and EK-1492R. Likewise, fungus-specific primers viz., nu-SSU- 081 and nu-SSU-1536-39 were used for the isolation and identification of a novel fungal strain the BCGI clade. As majority of deep-sea fungal species exhibit similar morphological characteristics with the terrestrial fungi, molecular phylogenetic analysis played vital role in the detection and identification of the unknown fungi.

8.5 Role of in Deep-Sea Ecosystem

Deep-sea fungi play significant roles in the marine environments viz., parasitic, symbiotic or pathogenic and is found in various associations. A marine basidiomycete fungal species, *Mycaureola dilseae*, has been identified to parasitize the subtidal alga, *Dilsea carnosa*. This marine macroalga is infected by two widespread, zoosporic fungal pathogens viz., *Eurychasma dicksonii* and *Chytridium polysiphoniae* (Sparrow 1960). Moreover some fungal species are known to have symbiotic association with marine sponges which secrete useful antimicrobial compounds. While some other fungi viz., *Phoma sp.* isolated from marine and estuarine environment are reported to be the parasites of seaweeds, sea-grasses, molluscs, and sponges. Likewise few marine yeast species namely, *Rhodotorula, and Rhodosporidium* are identified to be potential mycoparasites and phytopathogens. These host-parasite interactions are facilitated by specific cellular structures referred to as colacosomes present in the fungal species. Similarly, a black yeast isolated from mussel and other animals residing in hydrothermal vent sites is reported to be pathogenic (Moreira and López-García 2003). On the other hand a number of potential pathogenic forms of *Aspergillus sp.* were isolated from deep-sea sediments of the CIB (Damare et al. 2006a).

8.5.1 Aggregate Formation and Carbon Contribution

It is well-known fact that transformation of organic matter in terrestrial soil is one of the major functions of bacteria and fungi. On the contrary, major studies on bacterial flora of deep-sea sediments have revealed their lesser important role in organic matter degradation (Turley and Dixon 2002). Since deep-sea fungi remain embedded in water and cannot be often noticed, their role in the deep-sea sediments has remained neglected (Damare and Raghukumar 2008). However, recent reports have suggested the use of chelating agent, like EDTA which increases the visibility of fungi in the deep-sea sediments by effectively solubilizing polysaccharides (Liu et al. 2002). Recent studies also reveal the function of deep-sea fungi in humic aggregate formation via mechanisms very similar to those in terrestrial sediments. The humus formed is basically composed of some by-products of microbial metabolism viz., reducing sugars and amino acids, which form brown-colored products by undergoing non-enzymatic polymerization (Tisdall and Oades 1982).

Microaggregates are formed by the combination of humic material with soil particles. Fine particles of soil are trapped with the fungal hyphae further which act as binding agents to form macro-aggregates (Kandeler et al. 1999). Therefore fungi or bacteria stay sheltered and safe in some specific particle size classes (Suberkropp and Meyers 1996). Cations such as Si^{4+}, Fe^{3+}, Al^{3+} and Ca^{2+} acts as bridges between particles in terrestrial microaggregates (Bronick and Lal 2005). Reports suggest more effective aggregate stabilization by deep-sea fungi as compared to the other soil microflora. These aggregates are primarily formed through hyphal entanglement of soil particles (Molope et al. 1987). Formations of micro

aggregates by fungi grown in sediment have been well described by Damare and Raghukumar (2008) on extract medium under 20 MPa pressure. Similar to previous reports on fungi from terrestrial environments, it is found that the microaggregates formed by deep-sea fungi stain positively for humic substances indicating that the fungal activities are responsible for the formation of humus possibly in deep-sea sediments (Stevenson 1994). These findings therefore indicate that deep-sea fungi play a significant role in nutrient cycle by acting upon from inside these microaggregates. Aggregation is a key mechanism for accumulation and maintenance of organic matter in soil thereby providing protection to the soil organic matter (Beare et al. 1994). This is chiefly a useful characteristic in natural environment as it inhibits the extracellular enzymes from diffusing away from the cells secreting it.

Therefore, the whole nutrient dynamics of the sediments is known to be largely affected by the humic-enzyme complexes (Burns 1978). According to recent reports fungi secrete a large quantity of exo-polysaccaharides (Selbmann et al. 2003), which in turn leads to enrichment of the aggregates. Hyphal sheaths act as a connecting link responsible for the attachment of fungal mycelium to surfaces and particle entrapment (Hyde et al. 1986).

Fungal biomass forms a huge fraction of the probably mineralizable organic matter present in the forest and grassland soils (Chiu et al. 2006). Damare and Raghukumar (2008) evaluated fungal biomass in deep-sea sediments supported fungal bio-volume. Conversion of this bio-volume into Carbon was reported at a rate of 1 pg of C per μm^3 (Van Veen and Paul 1979). Similarly, the fungal organic carbon contribution within the deep-sea sediments of CIB was reported to be 2.3 to 6.3 $\mu g\ g^{-1}$ of dry sediment. Whereas the carbon contribution by bacterial species for the same site was reported to be about1 to 4 $mg\ g^{-1}$ of dry sediment (Raghukumar et al. 2001).

Around 10–150 $pg\ g^{-1}$ sediment of fungal carbon contribution within the coastal waters off Goa has been reported (Jebraj and Raghukumar 2009). On the contrary, about 453–3375 $\mu g\ g^{-1}$ of dry sediment of fungal carbon contribution was reported in soil from grassland and forests (Chiu et al. 2006). The results of fungal biomass estimation obtained by hyphal length method are more accurate in comparison to other biochemical methods viz., phospholipids (Balser et al. 2005), hexosamine (Gessner and Newell 2001) or ergosterol (Mille-Lindblom et al. 2004) content. Since the components of fungal cell wall viz., melanin and chitin, easily degradable, as compared to phospholipids of bacterial cell walls thus carbon accumulation by fungi is estimated to be more persistent in contrast to that stored by bacterial species (Bailey et al. 2002). Therefore a comparative quantitative analysis using pure cultures of deep-sea bacteria and fungi with ^{14}C-labeled sugars and amino acids for growth, uptake kinetics and carbon sequestration under simulated deep-sea conditions would help us in understanding their individual role to a particular extent.

8.5.2 Extracellular Enzymes

A number of enzymes are recognized to be involved in the cycling of nutrients in the deep-sea and hence can be employed as possible indicators of nutrient cycling processes. Among many such enzymes alkaline phosphatase activity (APA) in aquatic ecosystems like that of the deep-sea, plays a significant role in the regeneration of inorganic phosphate via catalysis of organic esters (Chróst 1991). Organic P content as measured during one such study varied from 0.007 to 011% and alkaline phosphatase activity was in the range of 0.06 to 7.8 U which is equivalent to μmol of phosphate phosphorus released h^{-1} g^{-1} dry sediment (Raghukumar et al. 2006).

During laboratory microcosm experiments Damare (2007) tried to clear the difference between the contribution of APA in deep-sea sediments by fungi and bacteria under simulated deep-sea conditions.

APA was particularly very low in the plain sediment without any additional nutrients. But as detritus was added the APA increased 20-fold, signifying sharp rise in microbial activity. It was further observed that APA was elevated in the presence of antibacterial agents, which is indicative of the fact that major share of APA is coming from fungi. This indicates that fungi have a high degradative activity toward detritic material might similar to that in the terrestrial environments (Newell 1996). In an another study the piezophilic fungal isolate *A. terreus* (#A4634), showed a very high APA in the presence of antibacterial agents as compared to APA exhibited by deep-sea bacteria in the presence of a fungicide under the same experimental conditions. These results clearly depict the significant role of fungi in the P cycle within the deep sea.

Besides their key role in the biogeochemical cycles, piezophilic microorganisms growing under intense conditions are a potential source of some novel useful enzymes with unusual properties (Synnes 2007; Dang et al. 2009). Out of various extracellular enzymes being produced by the fungi, protease plays a significant role in their nutritional requirements. Damare et al. (2006b) confirmed that entire fungal flora isolated from CIB deep-sea sediments exhibited active protease production at low temperatures. On the other hand these fungi when grown under elevated pressure synthesized extracellular protease in good amounts. Enzyme production and fungal growth are directly related therefore reduced growth at 5 °C leads to low levels of enzyme production. Whereas the enzyme production was increased in the presence of several commercial detergents and 0.5 M NaCl, equivalent to 29 PSU (Practical Salinity Unit) of seawater salinity. These factors are advantageous for enzyme to be used as additive in detergents at industrial of enzymes level (Raghukumar et al. 2009). Thus deep-sea fungal proteases are efficient detergent enzymes.

Fungal polygalacturonases are yet another class of useful enzymes used for clarification of fruit juices in the food industry. From the supernatant of deep-sea yeast (strain N6) culture two novel endopolygalacturonases having activity at 0–10 °C were purified. This deep-sea yeast (strain N6) was isolated from the Japan Trench at a depth of 4500–6500 m (Miura et al. 2001; Abe et al. 2006). The

hydrolytic activity of these enzymes was found to be unaffected even at a hydrostatic pressure of 100 MPa at 24 °C (Abe et al. 2006).

According to another study strain of *Cryptococcus* was found to be tolerant to $CuSO_4$ up to a concentration of 50 mM. Moreover this strain also exhibited high activity of superoxide dismutase, an enzyme responsible for scavenging superoxide radicals (Abe et al. 2001). It was also observed that high pressure shifted the required temperature for any chemical reaction toward lower temperature (Daniel et al. 2006). Therefore, in such reactions a mesophilic enzyme could be used in place of thermophilic enzyme.

8.6 A Novel Source of Extremozymes

Nearly three fourth of the Earth's area is covered by ocean, having a depth of 3800 m, indicating that the majority of our planet consists of deep-sea environments. However, the deep-sea is one of the most inexplicable and unexplored environments on the earth. It inhabits a vast diversity of microbial communities that play vital functions in biogeochemical cycles (Sogin et al. 2006). In addition the deep-sea is acknowledged as an extreme environment, because it comprises certain intense environmental conditions such as the lack of sunlight, the presence of mostly low temperatures and high hydrostatic pressures. But sometimes environmental conditions become even more harsh particularly in the habitats, like deep-sea hydrothermal vents with their extremely high temperatures (approx. >400 °C), deep hyper saline anoxic basins with their extremely high salinities and abysses of up to 11 km depth with their extremely high pressures.

Deep-sea extremophiles includes organisms that survive and grow in deep-sea environments having extreme environmental conditions viz., pressure, temperature, pH, salinity and redox potential etc. that are usually lethal to other organisms. The majority of deep-sea extremophiles are prokaryotic microorganisms particularly including the domains of Archaea and Bacteria (Horikoshi and Bull 2011; Harrison et al. 2013). There is a huge diversity of extremophilic microorganisms inhabiting the deep-sea. They are functionally diverse and cosmopolitan in taxonomy and are classified into thermophiles (55–121 °C), psychrophiles (−2 °C to 20 °C), halophiles (2–5 M NaCl or KCl), piezophiles (>500 atmospheres), alkalophiles (pH > 8), acidophiles (pH < 4), and metalophiles (high concentrations of metals, e.g., copper, zinc, cadmium and arsenic). Mostly deep-sea extremophiles tolerate more than one extreme condition and therefore are called polyextremophiles (Cavicchioli et al. 2011). These extreme conditions are normally injurious to the majority of organisms, but extremophilic microorganisms are capable to stay alive and flourish in them. It is only because of their highly flexible metabolisms and therefore the unique structural characteristics of their bio-macromolecules (Nath and Bharathi 2011; Dalmaso et al. 2015).

Since past few years, piezophilic microorganisms have attracted the attention of researchers who are constantly searching for newer bioactive compounds like enzymes that may be employed in the industries worldwide (Zhang and Kim

2010). The wide-ranging temperatures, salinities, pHs and pressures occurring naturally in extreme deep-sea environments serve as promising sites to explore enzymes having industrial potential (Samuel et al. 2012). Several studies have demonstrated that the extremozymes secreted by deep-sea extremophilic microorganisms have a number of industrial applications as a result of their high activities and great stabilities under extreme conditions. Therefore the stability and variety of activities of extremozymes make them important alternatives to ordinary biotechnological processes. These enzymes possess considerable economic potential within the agricultural, feed, food, beverage, pharmaceutical, detergent, leather, textile, pulp, and biomining industries (Raddadi et al. 2015).

Even though till date many enzymes have been isolated and identified around the world, the majority of which have been examined for industrial applications, the commercial enzyme market remains insufficient in fulfilling industrial demands (Van Den Burg 2003). The reason being that most of these enzymes are unable to tolerate industrial conditions (Irwin and Baird 2004). The industrial process requires biocatalysts that can tolerate a variety of intense conditions, viz., temperature, pH, salinity, and pressure, while exhibiting high conversion rates and reproducibility's (Haki and Rakshit 2003). Besides this, ecological compatibility is also significant for industrial enzymes used in technologies (Gao et al. 2019). Though only a few extremozymes are currently being produced and used at the industrial level, the improvement of novel industrial processes based on these enzymes is being promoted by development in deep-sea extremophile and extremozyme research. The rising demand for novel biocatalysts in industries, could be achieved by development in deep-sea sampling techniques, rapid expansion of new molecular and omics technologies, such as metagenomics, proteomics, protein engineering, gene-directed evolution and synthetic biology (Ferrer et al. 2007). Thus, the priority in enzyme research is the discovery of enzymes with unusual enzymatic activities and enhanced stability (Raddadi et al. 2015).

8.7 Strategies to Explore Extremozymes

The conventional method employed for the discovery of novel extremozymes from deep-sea microorganisms is that the cultivation of microorganisms followed by screening for the particular enzymes. Although 99.9% of these deep-sea microorganisms cannot be cultivated by using conventional laboratory techniques but a number of extremozymes with great potential for industrial applications are isolated from deep-sea environments using this method (Amann et al. 1990). To overcome the need for isolation or cultivation of microorganisms metagenomic technologies are developed as a tool for isolation and identification of novel genes and enzymes directly from uncultured microorganisms (Madhavan et al. 2017). Several reports suggest the application of metagenomes for exploring extremozymes from deep-sea environments, thereby surpassing the disadvantages accompanied with the conventional culturing methods of extremophiles (López-López et al. 2014).

Metagenomic analyses involves the direct isolation of genomic DNA from deep-sea environmental samples by either sequence-based (isolation of putative enzymes based on conserved sequences) or function-based methods (isolation of functional enzymes on the basis of expressed features like a selected enzyme activity) (Popovic et al. 2015). The sequence-based technique utilizes the colony hybridization approach for screening metagenomic clones. It is performed by using an oligonucleotide primer or probes for the target gene, which is further amplified by polymerase chain reaction (PCR) and consequently cloned into appropriate expression vectors.

Besides, this by application of metagenomics data followed by suitable bioinformatic annotations particular gene sequences can also be isolated directly. This sequence-based technique therefore leads to the discovery of novel sequences which in turn increases the chances of identifying enzymes efficiently (Lee et al. 2010). Though, the potential of screening specific enzymes by employing this method is based on classical bioinformatic analyses, several novel or unknown activities may not be recognized.

8.8 Physiology of Piezophilic Fungi

Piezophilic fungi residing the deep-sea atmosphere possess some potential physiological characteristics according to the prevailing extreme conditions. The deep-sea environment is identified by certain characteristic conditions viz., high hydrostatic pressure (1 bar/10 m), low temperatures (2–4 °C) or extremely high (>400 °C) (Nagano et al. 2010) high salinity concentrations (240 ppt) up to 500 ppt (Gladfelter et al. 2019; Barone et al. 2019), and also the absence of sunlight (Nagano et al. 2010; Wei et al. 2014). Out of all these conditions fungi has remarkable capacity to tackle hydrostatic pressures. But most of the initial studies have not assessed the barotolerance capacity of deep-sea fungi. In one such study it was observed that *Aspergillus ustus and Graphium sp.,* isolated from the Indian Ocean showed potential barotolerance, and also exhibited conidia germination at a pressure of 100 bar. Other studies reported that *Aspergillus sydowii* can grow and sporulate at 500 bar (50 MPa) (Damare et al. 2006a; Raghukumar et al. 2004). Recent research works have depicted that Piezophilic fungi are actually facultative peizophile and are therefore able to modify the composition of the deep-sea environment. These fungi are also capable of changing the fluidity of their semipermeable membrane in order to tolerate the pressure (Simonato et al. 2006).

The confirmation of these physiological adaptations has been made by the recognition of elevated levels of transcripts involved in the biosynthesis of ergosterol, signifying that marine fungi undertake alterations in their membrane composition to bear high hydrostatic pressures (Pachiadaki et al. 2016). Up regulation of the OLE1 gene involved in the synthesis of fatty acids was observed in case of *S. cerevisiae* grown under a high hydrostatic pressure of 2000 bar (200 MPa) (Simonato et al. 2006). In addition, another important feature of deep-sea fungi is their ability to grow under high salt concentrations which in turn makes their survival and growth easy in the deep-sea environments. Even though deep-sea fungi have

capacity to tolerate high salt concentrations, they're not usually halophilic fungi (Gladfelter et al. 2019). This ability of marine fungi to grow in presence of high salt concentrations although salt is not one of the requirements for growth is referred to as halotolerant or halophytic. On the contrary, halophilic fungi require a specific salt concentration (from low to high concentrations) for their optimal growth (Burgaud et al. 2010; Gunde-Cimerman et al. 2009). Some halotolerant species from the genera *Penicillium, Cladosporium,* and *Aspergillus* growing at 5700 mbsl and are able to sporulate in an extremely high salt concentration of 1.7 to 34 ppt, have been reported.

Another halophilic species isolated from the genera *Candida*, dwelling in a deep-sea hydrothermal vent, was able to grow only under a salinity concentration of 30 ppt (Burgaud et al. 2010). In another research work it was demonstrated that deep-sea hypersaline basins (salinity between 70 and 172 ppt) located in the Eastern Mediterranean Sea harbored Ascomycota and Basidiomycota (Bernhard et al. 2014). During a metatranscriptomic analysis of these fungal species it was observed that the majority of the detected rRNA signatures were related to the genera *Aspergillus and Penicillium* (Edgcomb et al. 2016).

The most commonly occurring genus in the deep-sea environments is *Hortaea* (Lai et al. 2007; Singh et al. 2012; Singh et al. 2011; Burgaud et al. 2010; Li et al. 2019; Wei et al. 2019). Majority of its species particularly the *H. werneckii* species, which is black yeast has been classified as halotolerant in Mediterranean deep-sea waters (Romeo et al. 2020) and as halophilic in deep-sea hydrothermal vents (Burgaud et al. 2010). Various potential bioactive secondary metabolites have been isolated by marine-derived *Penicillium* and *Aspergillus* species (Lee et al. 2013; Liu et al. 2017). *Penicillium* and *Aspergillus* species isolated from the deep-sea are reported to produce around 106 novel bioactive compounds (Zain Ul Arifeen et al. 2019). Out of these some compounds were found to have potential cytotoxicity activity on different cancer cell lines (Zain Ul Arifeen et al. 2019; Sun et al. 2012).

In a similar study few other deep-sea fungal species belonging to the *Simplicillium*, *Acaromyces*, and *Engyodontium* genera have been recognized as an attractive source of secondary metabolites with cytotoxicity (Deshmukh et al. 2018). Zain Ul Arifeen et al. (2019) isolated six novel alkaloid-bioactive compounds from *Aspergillus* species and demonstrated efficient antimicrobial activity against some fungal and bacterial pathogens like *Monilia albicans*, *Staphylococcus aureus, Pseudomonas aeruginosa, and Klebsiella pneumoniae*. Therefore from different studies it can be inferred that the vast diversity of fungal species within the deep-sea are an efficient source of different bioactive compounds including anticancer, antibacterial, antiviral, antifungal, antioxidant, and nontoxic antifouling compounds. Moreover, the wide range of potential osmotic techniques can be applied to explore more novel compounds from the deep-sea fungal flora (Barone et al. 2019; Zain Ul Arifeen et al. 2019; Wang et al. 2015; Zhang et al. 2014a, b).

8.8.1 Effect of Elevated Pressure and Low Temperature

Different pressures and temperatures magnitudes produce different effects on organisms. Usually, high hydrostatic pressure equivalent to several dozen MPa, have been proved to be nonlethal, but may exert adverse affects on organisms inhabiting the environments with normal atmospheric pressures (Abe 2004). For instance it has been demonstrated that the growth of mesophilic microorganisms gets inhibited at a high pressure of 40–50 MPa. Along with inhibition of growth some morphological modifications including formation of filaments in *Escherichia coli* and cell chains or pseudomycelia in the marine yeast *Rhodosporidium sphaerocarpum* have also been observed (Lorenz and Molitoris 1992). Application of higher pressures, ranging from 100 MPa may also be used for sterilization purposes. The growth inhibitory effects not only depends on the amount but also on the duration of pressure applied in combination with temperature, pH, oxygen supply and composition of culture media (Abe 2007). Initial steps of translation are inhibited by both low temperature and elevated pressure. The cold-shock response has been recommended to be an adaptive response to assist the gene expression during initiation of translation.

Studies also suggest that conditions involving elevated pressures, results in the controlled synthesis of proteins indicating decreased translation capacity. Lastly, conditions involving low temperature and elevated pressure reduce membrane fluidity, which affects a variety of membrane associated processes, including transport of membrane ions, nutrient flux, and DNA replication.

E. coli, *Bacillus subtilis*, and the budding yeast *S. cerevisiae* have been selected as model organisms to study the effect of increased hydrostatic pressure and low temperature since their complete genomes have been sequenced, and are used as powerful genetic tools by several workers. These microbes can be classified either as piezophilic i.e., high-pressure loving or piezotolerant i.e., capable of tolerating elevated pressure but showing better growth at atmospheric pressure. In 1979, the first pure culture of a piezophilic bacterial isolate, strain CNPT-3 was reported by Yayanos (1979). At an elevated pressure of about 50 MPa this spirillum-like bacterium depicted efficient doubling rate, growth could be was not observed at atmospheric pressure conditions even after incubation for several weeks. Isolation and characterization of various piezophilic and piezotolerant bacteria have been performed from deep-sea sediments of Japan at depths ranging from 2500–11,000 m. Among these isolates majority of the strains were observed to be piezophilic as well as psychrophilic. Such strains exhibited optimum growth at low temperatures and almost no growth at temperatures above 20 °C. Additionally, studies in *E. coli* confirmed high-pressure induction of heat-shock proteins (Grob et al. 1994). Induction of heat-shock proteins has also been demonstrated in piezophilic microorganisms upon decompression. In another research work subsequent to shift to atmospheric pressure resulted in induction of stress protein similar to heat- shock proteins in the deep-sea piezophilic hyperthermophilic bacteria *Thermococcus barophilus*. As it is well known that high pressure and low temperature produce similar effects on protein synthesis and membrane structure,

the initiation of both pressure-shock and cold-shock proteins may correspond to an effort by a few bacterial isolates to improve the destructive effects of high pressure on membrane structure, translation processes, and the macromolecules stability.

8.8.2 Response of Spores and Mycelia to Low Temperature and Elevated Hydrostatic Pressure

Different environmental conditions of sea water viz., temperature, elevated hydrostatic pressure and low nutrient concentrations exert mycostatic effect on fungal spore germination within the deep-sea (Kirk Jr. 1980). A characteristic feature of spores is their capacity to develop into a complete individual without fusing with other cell. The most important step during this process is spore germination, followed by growth of germ tube producing a mycelium by elongation, septum formation, and branching.

Comparative study on the effect of subsequent environmental conditions on the germination of conidia of the deep-sea *Aspergillus* isolates, and their viability was conducted by Damare et al. (2008). Germination of spores of three deep-sea and terrestrial *Aspergillus* isolates and a Piezophilic *Cladosporium* isolate in sediment extracts of various dilutions was found to be better at 20 MPa pressure and 30 °C temperature but no germination was obtained at 5 °C. On the other hand, percentage germination lowered steadily at high pressure. According to Damare (2007) the spores did not germinate at a temperature of 5 °C, even at 0.1 MPa pressure even after 20 days.

Even though all fungal isolates were able to produce biomass at elevated hydrostatic pressure and became vasoconstrictive when began with mycelial inoculums, spores did not germinate under these conditions. Therefore it seems that vasoconstriction and not high hydrostatic pressure may be a limiting factor for spore germination and further biomass production within the deep-sea. Dormancy of spores in three *Aspergillus* species was broken by temperature shock of 15 min at 50 °C resulting to their germination at 0.1 MPa at 5 °C but not at 20 MPa/5 °C. It can be further inferred that fungal mycelial fragments which seem to be more active metabolically are able to tolerate high hydrostatic pressure and temperature as compared to the dormant fungal spores. Hence, mycelial fragments have greater probability of proliferation under deep-sea conditions than the spores. As reported by Ivanova and Marfenina (2001), the continued existence of fungal species in tense extreme environments is largely influenced by the size of mycelial fragments.

8.8.3 Pressure Effects on Yeasts

The effect of air mass on growth, viability and cellular responses in living cells is termed as piezophysiology (Abe 2004). It has been observed that growth and cellular activity in *S. cerevisiae* is not affected at pressures below 20–30 MPa. Elevated pressures exert several stress response via pressure inducible genes and proteins.

Piezotolerance is dependent on the period of air mass application. Yeast cells in stationary phase are more resistant to pressure than actively growing cells (Abe 2004; Fernandes 2005).

Another study revealed various morphological changes in wild-type and trehalose-6-phosphate synthase (tsp1) mutant cells of *S. cerevisiae* caused by hydrostatic pressure of 200 MPa for 30 min (Fernandes et al. 2001). These mutant cells are not able to gather an eminent tissue layer protectant called trehalose, which is a disaccharide, during unfavorable environmental conditions. When both wild and mutant cells were subjected to preheat treatment at 40 °C temperature for 60 min before subjecting to the pressure treatment, both the types of cells gained resistance to the pressure treatment. Similar induced pressure tolerance in *S. cerevisiae* via heat shock was earlier reported by Iwahashi et al. (1991). Besides this many researchers have studied the effect of lethal pressures on yeast cells (Iwahashi et al. 1991, 2003; Fernandes et al. 2004).

Iwahashi et al. (2003) examined the DNA microarrays of *S. cerevisiae* and evaluated expression levels of approx. 6000 genes. The gene expression profiles depicted that pressure of about 180 MPa at 4 °C for 2 min resulted in damage to cellular organelles just similar to that caused by detergents, oils, freezing and thawing. Likewise Fernandes et al. (2004) applied whole genome microarray hybridization and reported some characteristic modifications in retort to hydrostatic pressure of 200 MPa for 30 min in *S. cerevisiae*.

It was confirmed by further researches that most of the upregulated genes played a key role in stress defense and carbohydrate metabolism while, a variety of downregulated genes were involved in processes like cell cycle progression and protein synthesis. It was also noticed that the effects of growth inhibiting pressures in *S. cerevisiae* were different from those caused by lethal pressures (Abe 2004). According to Iwahashi et al. (2005) *S. cerevisiae* grown under 30 MPa pressures exhibited growth inhibiting genome-wide mRNA expression profile. They reported upregulation of genes membrane metabolism which produced fundamentally diverse response to pressures.

8.8.4 Pressure Effects on Filamentous Fungi

At high pressure microbial activity is inhibited as compared to at 0.1 MPa. Elevated pressures affect features like growth, respiration, and specific biochemical processes (Abe and Horikoshi 1995; Fernandes et al. 2004; Daniel et al. 2006; Abe 2007). Marine bacteria possess pressure inducible genes, which aid in pressure adjustment and withstand huge vertical changes within the water column (Bartlett 1991). On one hand in the case of bacteria, the effects of pressure on organic phenomenon, membranes, membrane proteins, DNA structure and organic process, are studied thoroughly, on the other hand in the case of fungi the studies have been directed within the deep-sea sediments toward growth under elevated pressure and production of extracellular enzymes under elevated hydrostatic pressure (Raghukumar and Damare 2008).

Prokaryotic and eukaryotic cells were found to secrete selected classes of proteins under physiological stress conditions. This phenomenon is called as "stress response" and also the newly formed transitory proteins are termed as "stress proteins." These proteins play a chief role in adaptation of cells and aid them in defense against a number of stress conditions (Lindquist and Craig 1988). Since specific kinds of proteins provide adaptation to different stresses or shock conditions viz., heat-shock, cold-shock or antifreeze proteins. Peizophiles exhibit therefore some kinds of proteins available for helping in adaptation toward different hydrostatic pressures.

Some fungal species isolated from deep-sea of CIB exhibited abnormal morphology (Damare et al. 2006a). They possessed extremely long conidiophores with vesicles being covered by long hyphae, rather than phialides of metulae or conidia, similar to that of the *Aspergillus* species. Majority of these abnormal features vanished after subculturing for 4–6 times. It was observed in case of many deep-sea fungi that initially they remain non-sporulating, but ultimately begins producing spores after repeated (~ 6–8 times) subculturing at 0.1 MPa pressure. Many such fungi when grown under elevated hydrostatic pressure presented discrete swellings.

The morphology is believed to be greatly affected by the type of nutrients used for culturing. In one such study the Piezophilic fungi produced fungal hyphae with several swellings when grown in malt extract seawater broth, however when grown in sediment extract medium the fungal hyphae produced were absolutely normal (Damare and Raghukumar 2008). At a pressure of 10 MPa two deep-sea fungi showed microcyclic conidiation during growth (Raghukumar and Raghukumar 1998). Immediate conidiation was observed after conidial germination without the extension of vegetative mycelium. The studies suggest that under nutrient-limiting conditions fungi produce microcyclic conidiation which in turn help them to complete their life cycles in a very shorter time. Arrest of apical growth followed by lateral differentiation of conidium producing cells might be the reason for this occurrence.

8.8.5 Effect of Deep-Sea Conditions on Growth and Protein Patterns

As compared to bacteria, very fewer studies have been made on the effect of high hydrostatic pressure and low temperature conditions on fungal growth patterns. Raghukumar et al. (2004), observed germination and growth of spores of deep-sea fungus *A. Sydowii*, isolated from deep-sea sediments of Chagos Trench under a high hydrostatic pressure and low temperature conditions. In another research work, mycelial inocula of fungi isolated from deep-sea sediments of the CIB were found to produce considerable growth at both 30 °C and 5 °C under elevated hydrostatic pressure (Damare et al. 2006a). *S. cerevisiae* is a facultative anaerobe and therefore is used in a variety of studies for evaluating the effects of elevated hydrostatic pressure and low temperature. Because of its facultative anaerobic nature,

S. cerevisiae has been selected as most appropriate organism to survive under closed pressure vessel condition in very low oxygen concentration (Abe 2007).

Approximately 6000 genes are encoded by the genome of this yeast out of which more than 4800 are non essential ones. Recent studies have demonstrated the presence of unexpected genes and metabolic pathways that are responsible for environmental stresses tolerance. Moreover this yeast exhibited no growth at elevated pressure and low temperature conditions when examined via genome-wide gene expression profiles. However, high levels of upregulated genes involved in energy metabolism transaction, protein and cell defense metabolism were recovered from the stress conditions (Iwahashi et al. 2003). It was also observed that *S. cerevisiae* survived more efficiently at elevated pressures after prior heat-shock treatment (Iwahashi et al. 1991). The heat-shock treatments lead to increased production of heat-shock proteins (Hsps) and trehalose metabolism in this yeast.

Out of all the heat-shock proteins (Hsp), Hsp104 plays a vital role in developing tolerance by carrying out ATP- dependent unfolding of denatured intracellular proteins. Additionally, synthesis rate of various proteins viz., ubiquitin, few glycolytic enzymes, and a plasma membrane protein in *S. cerevisiae*, was found to be enhanced greatly upon exposure to stress conditions. It was further observed that a plasmid carrying the *TAT2* gene, encoding a high affinity tryptophan permease, supported *S. cerevisiae* growth at a pressure ranging between 15 and 25 MPa. Moreover, cells producing high levels of the Tat2 protein became capable to grow under low-temperature (10–15 °C) as well as at high-pressure conditions. They also reported that vacuoles in yeast cells were more acidified at hydrostatic pressures of 40–60 MPa and that the growth of yeast cells was highly affected by expression of the tryptophan permease i.e., *TAT2* gene.

Besides *S. cerevisiae*, very few other fungal isolates are utilized as model organisms to study the effect of high hydrostatic pressure and cold stress conditions. By application of advance techniques these stress conditions were applied for determining their effects on filamentous and unicellular marine fungi. The marine ecosystem is the world's biggest ecosystem spreading over three-fourths of the Earth's surface. The average depth of the marine biome is 3800 m, and is subjected to a pressure of 38 megapascals (MPa). Several factors affect the biodiversity prevailing in oceans, but hydrostatic pressure is the most significant parameter out of all.

Deep-sea microorganisms are categorized on the basis of their cardinal growth pressures into different classes' viz., piezosensitive, piezotolerant, piezophiles, or hyperpiezophiles. Recent studies suggest that *Pyrococcus yayanosii*, an obligate piezophilic hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent, exhibited potential to grow even at high hydrostatic pressure ranging from 20 to 120 MPa. These results demonstrate the important role of hydrostatic pressure in the life cycle of prokaryotic organisms within the deep-sea (Burgaud et al. 2015).

On the other hand numerous DNA-based studies have reported the presence of a number of fungal communities in the deep-sea ecosystems along with prokaryotic organisms (Abe 2007). These fungal communities live in deep sediments hydrothermal vents, sunken woods, cold seeps and even deep hypersaline anoxic basins

(Le Calvez et al. 2009). The sequencing techniques based on rRNA and mRNA approaches have disclosed some important informations about these communities viz., their metabolically functional components, their imperative ecological roles in biogeochemical cycles or their interactions with prokaryotes and antibiotic defense mechanisms (Takishita et al. 2007). The fungal communities thus recovered were mainly belonging to a subkingdom Dikarya and two phyla Ascomycota and Basidiomycota. In addition few species belonging to the basal phyla Chytridiomycota and Cryptomycota were also obtained. Nevertheless culture-based methods will always be preferred to isolate deep-sea fungal strains for assessing their ability to tolerate hydrostatic pressure. This can develop our awareness about the deep-sea fungal flora and their ecological roles in such stressful environments.

Different effects of high pressure on biological processes include fluidity modification of pressure-sensitive lipids, membrane permeability, multimeric associations and stability of pressure-sensitive proteins, thereby inducing motility and organic process and replication and transcription processes by pressure-stabilized DNA hydrogen bonds. In a similar study the impact of high hydrostatic pressure on cell physiology has been comprehensively demonstrated within the yeast cells. From these studies it was indicated that the tryptophan auxotrophy and also the availability of this organic compound affects the power of *S. cerevisiae* cells to grow at moderate pressure, between 15 and 25 MPa. It was further observed that *S. cerevisiae* cells were killed at pressures ranging from 100 to 200 MPa as a result of disruption of microtubule ultrastructures, actin filaments or nuclear membranes.

A number of research works have especially paid attention on the development of marine fungi growing under hydrostatic pressure. On the basis of these studies most of the fungal species isolated were from the *Aspergillus* genus, while others were marine yeasts namely *Rhodotorula rubra*, *Debaryomyces hansenii,* and *Rhodosporidium sphaerocarpum*. However, almost all isolated fungal species were considered as piezosensitive, since their growth was found to be much better at gas pressure (0.1 MPa) in comparison to high hydrostatic pressures. Another study highlighted that the three marine yeasts cultivated under high hydrostatic pressure were able to grow at a pressure of 20 MPa, whereas only the basidiomycetous species *R. rubra* and *R. sphaerocarpum* were observed to grow at 40 MPa, indicating better piezotolerance of basidiomycetes compared to ascomycetes. Yeasts recovered in these studies seems to be well adapted.

8.9 Conclusion

As a result of several research works it could be inferred that deep-sea sediments emerge as one of the potential habitat for Piezophilic fungi. Therefore a technical review of oceans of the world is generally suggested for determining the culture-dependent and culture-independent fungi. Novel techniques and media compositions to separate and culture them need to be designed. Moreover their function in macro aggregate formation is very important in biogeochemical cycling of nutrients and

carbon fixation within the deep-sea. They are also an efficient source of bioactive secondary metabolites and enzymes with new characteristics and applications (Pettit 2010). But still Piezophilic fungi are an unexplored part of the deep-sea environment. Inspite of their physiological adaptability to vasoconstrictive, and elevated hydro pressure, and imperative roles within the ecosystem, the deep-sea fungal flora have not been studied much. The diversity of deep-sea fungi currently known is just like a drop from the ocean and a huge amount of the unidentified fungal community is yet to be explored. Deep-sea fungi have shown great potential as a source of interesting enzymes, exclusive metabolics, and novel secondary metabolites. Numerous biotechnologically significant enzymes are produced by deep-sea fungal community isolated from a range of marine habitats.

Piezophilic fungal diversity possesses genetic diversity and adaptive nature to numerous extreme environmental conditions makes them an attractive candidate for research works. For the investigation of deep-sea fungal diversity both cultural-dependent and cultural-independent techniques are used. However, cultural-independent techniques have been demonstrated to be more effective for the discovery of unknown fungal species inhabiting the deep-sea, which are not able to grow via standard culture-dependent techniques. These fungi are a promising source of important products like extracellular bioactive compounds including polysaccharides, enzymes, and other secondary metabolites. Enhanced coordinated research work is necessary to expand our current knowledge about the diversity, activity, and capability of deep-sea fungi to adapt to the extreme environmental conditions of the marine ecosystem.

Future marine mycological research should focus particularly on the "unidentified" or "unclassified" sequences within the deep-sea fungal datasets, which not only are of great biotechnological potential, but also represent key fungal species for unravelling the origin of marine fungi. Besides more research works on marine fungal biology are needed to disclose attractive biochemical and physiological features of the deep-sea fungal flora. With the advent of recent scientific techniques to assess the physiology and biochemistry of uncultured and unusual marine-derived fungal flora would definitely pave the path for exploring potential of piezophilic fungi.

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9 Halophilic, Acidophilic, Alkaliphilic, Metallophilic, and Radioresistant Fungi: Habitats and Their Living Strategies

Tuyelee Das, Abdel Rahman Al-Tawaha, Devendra Kumar Pandey, Potshangbam Nongdam, Mahipal S. Shekhawat, Abhijit Dey, Kanak Choudhary, and Sanjay Sahay

Abstract

The magnificent stress-resistant mechanism, capacity to transform extreme abiotic factors as triggers for genetic modulation and physiological evolution, synced speciation in response to altered environment, and highly innovative succession cum resource management skill have crowned the microorganisms as the "specialist messenger of life" that thrive under extreme conditions. However, in recent decade, the ubiquitous fungi have gathered attention after archaea and bacteria for their versatile ecological adaptation, morphological resilience, and biochemical flexibility that allowed them to sustain and flourish under nature's deadliest environmental conditions. The inhospitable temperature, pressure, radiation, desiccation, salinity, and pH (both acidic and basic)-induced

T. Das · A. Dey (✉)
Department of Life Science, Presidency University, Kolkata, West Bengal, India
e-mail: abhijit.dbs@presiuniv.ac.in

A. R. Al-Tawaha
Department of Biological Sciences, Al-Hussein Bin Talal University, Maan, Jordan

D. K. Pandey
Department of Biotechnology, Lovely Professional University, Phagwara, Punjab, India

P. Nongdam
Department of Biotechnology, Manipur University, Imphal, Manipur, India

M. S. Shekhawat
Biotechnology Unit, Kanchi Mamunivar Government Institute for Postgraduate Studies and Research, Puducherry, India

K. Choudhary
Department of Biotechnology, Barkarullah University, Bhopal, Madhya Pradesh, India

S. Sahay
Sarojini Naidu Government Postgraduate Girl's (Autonomous) College, Bhopal, Madhya Pradesh, India

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stress has capacitated a large number of extremophilic fungi with better sustainability factors. The "extraterrestrial" type of existence has been reported from hostile and lethal niches like frozen world of Antarctic and Arctic, deep sea ice and hydrothermal vents, hot springs, areas of high salt concentration, barren desert with extreme climate, toxic heavy metal and organic matter polluted regions, ocean trenches with high pressure, radiation contaminated zones, etc. The phylogenetic diversity of extremophilic fungi is highly complex exactly as their multidimensional mechanism of primary and secondary resource management, niche utilization, and physiological metabolism. From the bed of life-enriched rainforests to barren worlds full of toxic materials and fluctuating climate, this eukaryotic group has manifested great evolutionary plasticity and molecular strategies that are the center of interdisciplinary research that connects evolutionary biology, astrobiology, biochemistry, molecular biology, ecology, and many related fields of science. The modification of genetic make-up and introduction of specialized survival technique controlled via manipulation of metabolic pathways are not only associated with successful colonization of these fungal members but also important in terms of exploration of natural products from unexplored sources.

Keywords

Halophiles · Acidophiles · Alkaliphiles · Living strategy

9.1 Introduction

Until microbiologist exposed that the extreme environment of earth is truly occupied by a various range of microorganisms, humans assumed that in such extreme parameters no organism can live. Nonetheless, lately, a diverse variety of extremophiles has been discovered across a wide range of environment like hydrothermal vents, hot springs, polar regions, acid mine drainage sites, deserts, acidic lake, saline–alkaline lakes, sodic lakes, etc. (Gunde-Cimerman et al. 2003; Gunde-Cimerman and Zalar 2014; Plemenitaš et al. 2014; Selbmann et al. 2013). Extremophiles (eukaryotes, bacteria, and archaea) are microbes that have been found at extremes of pressures of up to 110 MPa, pH (0–12.5), temperature (122 °C − 20 °C), salinity (>1.0 M NaCl), and UV radiations. Archaea is the most flourish group of extremophiles. Alternatively, fungi are the most adaptable, ubiquitous, and effective ecological group having progressed gradually toward a wide range of ecological niches. Accordingly, they need to utilize prime sources for the establishment and production of essential enzymes. These fungi are additionally impacted upon by main abiotic factors like pH, salinity, temperature, and water availability and accessibility. Therefore, species of fungi occupy a respective niche due to their unique kind of survival mechanism based on particular ecological abiotic factors. Fungi apply diverse strategies to survive in different and extreme environmental conditions. These strategies are mainly C-selected (combative), S-selected (stress), and R-selected (ruderal) (Cooke and Whipps 1993). In this chapter, we are

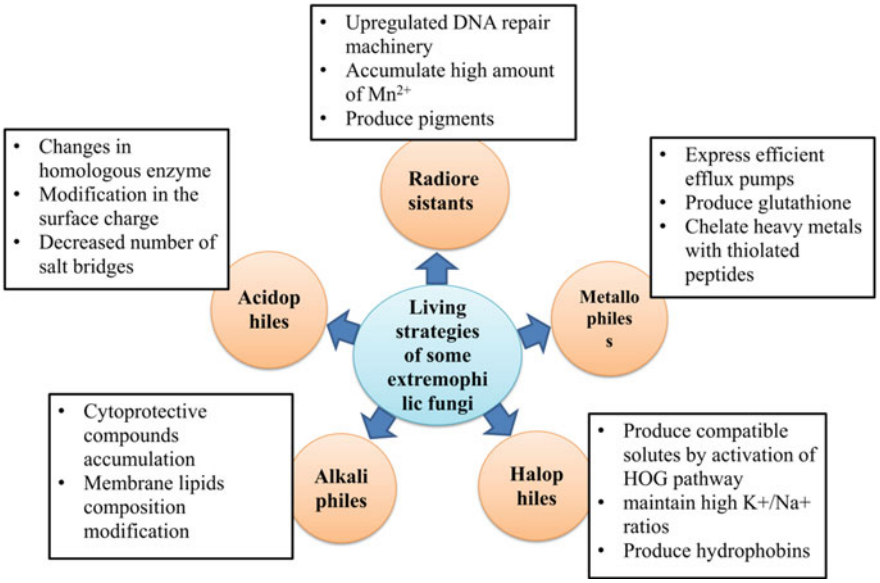

Fig. 9.1 Representative extremophilic fungi and their adaptive strategies to survive in extreme environmental conditions

specifically concerned with extremophilic fungi, which may use S-selected strategies for growth and survival in a range of so-called extreme environments. Extremophilic fungi are gaining ecological importance as well as biotechnical interest due to their ability to produce different kinds of bioactive compounds, enzymes, and proteins with prospective application in the industrial fields. Extremophilic fungi have some unique feature that were evolved based on extreme environmental conditions. Types of extremophilic fungi and its adaptative strategies to survive in extreme environment conditions are presented in Fig. 9.1. Many of the biomolecules, viz., enzymes and proteins produced by these fungi, are attributed to some defense strategies for their survival in the extreme environment. Apart from industrial benefits, these fungi possess unique genes that promote the growth of plant when applied as biofertilizers in sustainable agriculture (Yadav 2017). Thus, this chapter focuses on the strategies adopted by the other extremophilic fungi (halophiles, acidophiles, and alkaliophiles) to grow in harsh environments linked to some genes' expressions and the production of natural products as a response, which lead to an ecological impact on the environment.

9.2 Halophiles

Halophilic fungi require more than 0.2 M salt for their growth and are divided into (1) slight halophiles (0.2–0.85 M; 2–5%), (2) moderate halophiles (0.85–3.4 M; 5–20%), and (3) extreme halophiles (3.4–5.1 M; 20–30%) (El Hidri et al. 2013; Guesmi et al. 2013).

9.2.1 Habitats

Halophilic fungi have been reported from various habitats including the following.

9.2.1.1 Saline Soil

A saline soil is soil with high but variable sodium concentration.

9.2.1.2 Saline Water

Saline water is water with salinity 3% or above (De-Dekker 1983). It includes brackish water, marine water, and water from salt lakes and salterns. The saline water is broadly divided into two types, viz., NaCl-rich thalassohaline and $MgCl_2$- and $CaCl_2$-rich athalassohaline. Of these, thalassohaline water is an important habitat of halophilic life including fungi. Some typical thalassohaline habitats are the Dead Sea, Grate Salt Lake of USA, and Natrun Valley of Egypt. The Dead Sea is about 320 m in depth and a salt concentration of 78% NaCl. It has slightly acidic pH and important ions such as Na+, Cl-, and Mg2+ (Javor 1989). The Great Salt Lake, USA, has slightly alkaline pH and salinity of 33% NaCl (Javor 1989). The Solar Lake, Egypt, may have salinity of 20% NaCl in the summer. Lakes at Natrun Valley (Wadi El Natrun), Egypt, have salinity in the range of 3.1–8.6% NaCl,

9.2.1.3 Solar Salterns

These are manmade series shallow ponds for making salt. The ponds are fed by sea water or other saline water bodies, the last in the series is crystallizer having salt above 30% (Antón et al. 2000). Inland saltern of La Mala, Spain, has salinity of 18% NaCl and other ions like Mg2+, Ca2+, and K+.

9.2.2 Halophilic and Halotolerant Fungi

The fungi isolated from various saline habitats are mostly halotolerant rather than halophilic. They can grow in growth medium supplemented with or without salt. They have been isolated from saline and nonsaline habitats (Plemenitaš et al. 2008) including from food as food contaminants. The orders Capnodiales, Eurotiales, and Dothideales of Ascomycota and the genus Wallemia of Basidiomycota have been reported to comprise halophilic or halotolerant species (Al-Abri 2011). They include meristematic melanized yeast-like fungi, the so-called black yeasts such as *Hortaea werneckii* (Zalar et al. 1999b), *Phaeotheca triangularis* (Zalar et al. 1999b, c), *Aureobasidium pullulans* (Zalar et al. 1999b), and a new species *Trimmatostroma salinum* (Zalar et al. 1999a), different related species of the genus *Cladosporium* (Gunde-Cimerman et al. 2000; Zalar et al. 2007; Butinar et al. 2005a), non-melanized yeasts *Pichia guilliermondii*, *Debaryomyces hansenii*, *Yarrowia lipolytica*, *Candida parapsilosis*, *Rhodosporidium sphaerocarpum*, *R. babjevae*, *Rhodotorula laryngis*, *Trichosporon mucoides*, *Metschnikowia bicuspidata*, *Candida atmosphaerica*-like and *Pichia philogaea*-like (Butinar et al. 2005b), the filamentous genera *Wallemia*, *Scopulariopsis* and *Alternaria* (Zalar et al. 2005;

Gunde-Cimerman et al. 2005), and different species of the anamorphic genera *Aspergillus* and *Penicillium*, including some of their teleomorphic genera *Eurotium* and *Emericella* (Butinar et al. 2005, 2011). Of all, *Wallemia ichthyophaga* (Basidiomycetes) is the most well-known and in true sense halophilic fungus that requires a minimum of 10% NaCl for its growth (Zalar et al. 2005; Zajc et al. 2014).

9.2.3 Living Strategies

9.2.3.1 Lower Water Activity

Halotolerants are adapted to lower water activity (a_w) and can thrive in the presence of lower concentration of available water.

9.2.3.2 Compatible Solute

Fungi face in hypersaline environment two stresses, viz., osmotic and ionic ones. Fungi adapted to life at a_w do this by accumulating compatible solutes to counter the impact of lowering turgor pressure in the presence of hypersaline environment. They apply same strategy to counter salinity-related osmotic stress. The halophilic *W. ichthyophaga* and halotolerants *A. pullulans*, *H. werneckii,* and other halotolerant fungi accumulate primarily glycerol as compatible solute. In addition, *W. ichthyophaga* does accumulate little amount of arabitol and traces of mannitol to supplement glycerol (Zajc et al. 2013a, b). The black yeast *H. werneckii,* on the other hand, at lower salinities produces mycosporine–glutaminol–glucoside (prime function of mycosporine being involved in fungal sporulation and UV protection) (Oren and Gunde-Cimerman 2007), and at higher salinities produces other polyols (e.g., erythritol, arabitol, and mannitol) to supplement glycerol (Kogej et al. 2004, 2006). In case of salt-tolerant yeasts *Debaryomyces hansenii*, *Candida versatilis*, *Rhodotorula mucilaginosa*, or *Pichia guilliermondii* trehalose and other polyols supplement glycerol (Andre et al. 1988; Prista et al. 1997; Almagro et al. 2000).

9.2.3.3 Ion Homeostasis

There are at least three physiological strategies halotolerant fungi apply to overcome ion stress. The halotolerant *H. werneckii* is said to use the two salt-responsive P-type (ENA-like) ATPases (Gorjan and Plemenitas 2006) to extrude Na + at higher concentration of NaCl as supported by genomic data (Lenassi et al. 2013). The halophilic *Wallemia ichthyophaga,* which lacks most cation transporters, seems to use avoidance strategy by preventing entry of excess Na + with its extremely thickened cell walls (Kralj Kuncic et al. 2010, 2013; Zajc et al. 2013a, b).

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9.2.3.4 Cell Wall Structure and Pigmentation

At the differential level of melanin on outer cell wall of *H. werneckii* in the presence of different salt concentrations (e.g., thin layer of melanin when there is no NaCl, but thick layer of melanin at optimal salt concentration) (Kogej et al. 2004, 2006), the melanin seemingly gives mechanical support to counter higher turgor pressure

(Kogej et al. 2004, 2006). The meristematic growth of *Wallemia ichthyophaga* forming bigger (fourfold) and compact multicellular clumps and thickened (threefold) at higher salinity (cf. growth phenotype at lower salt concentration) is considered as an important adaptation to tolerate extreme salinity (Kralj Kuncic et al. 2010, 2013).

9.2.3.5 Plasma Membrane Fluidity

It is generally seen that eukaryotic cells that accumulate glycerol as a compatible solute, its back outflow has to be stopped by using active transport system (energetically costly) or by reducing fluidity of membrane through enhancing sterol content (Oren 1999). In case of *H. werneckii*, it has been shown that membrane remains fluid over a wide range of salinities (Turk et al. 2004, 2007) and its sterol content remains largely unchanged (Turk et al. 2004), suggesting that its hypermelanized cell wall also helps maintain glycerol at higher concentrations in the cells even in the presence of highly fluid membrane (Gostincar et al. 2009).

9.2.3.6 Molecular Basis

Halophilic and halotolerant fungi developed a novel molecular mechanism so that they can maintain their growth in high salt condition. Halophilic fungi possess a few features for osmotolerance via utilizing compatible solutes by activation of the HOG pathway. The HOG pathway produces glycerol that reestablishes the osmotic balance in the cell (Gostinčar et al. 2011; Zajc et al. 2012; Hohmann 2009). Plemenitaš et al. (2014) observed that halophilic *W. ichthyophaga* produced compatible solutes (glycerol) by HOG pathway activation implicated to their survival in a high osmolar environment. *W. ichthyophaga* also maintains high K^+/Na^+ ratios since in a high saline environment toxic Na^+ ions are over K^+ ions. Thus, halophilic fungi developed some mechanisms that can maintain high K^+/Na^+ ratios (Plemenitaš et al. 2014). Hydrophobin is a type of protein that contains a high number of acidic amino acids. These acidic amino acids are exposed to the protein surface and bind with salt and reduced salt-induced changes (Siglioccolo et al. 2011). Hydrophobins were found to be present in both *W. ichthyophaga* and *W. sebi* (Zajc et al. 2013a, b). Hydrophobins also induced microconidial chain formation in *W. ichthyophaga,* which might involve the accumulation of cells for the formation of the cluster. Production of haloadaptation is primarily attributed to the response against salt stress (Fuchs et al. 2004; Gostincar et al. 2010). Hydrophobins can also maintain cell wall rigidity so that halophilic fungi take advantage of osmolarity changes in stress (Wosten 2001; Bayry et al. 2012). *H. werneckii* contains acidic proteins that are involved in the accumulation of K^+ ions besides glycerol in response to hypersalinity (Kogej et al. 2005).

9.3 Alkaliphiles

Biochemical processes can occur at different hydrogen ion concentrations. However, biochemical events function better close to neutral pH. Very high or low pH harms the activity of biochemical events mostly via damaging the protein structure. Alkaliphiles have been defined as organisms that grow optimally at pH above 9. Alkaliphiles are further divided into obligate alkaliphiles (incapable of growing at or below pH 7.0) and facultative alkaliphiles (capable of growing at pH 7.0) (Padan et al. 2005; Slonczewski et al. 2009).

9.3.1 Habitats

Alkaline habitats have been classified into

1. High Ca^{2+} environments (groundwaters bearing high CaOH). Various locations of this type have been reported in California, Oman, the former Yugoslavia, Cyprus, Jordan, and Turkey (Barnes et al. 1982; Jones et al. 1994).
2. Low Ca^{2+} environments (e.g., soda lakes, soda soil, and deserts with major salt being sodium carbonate) (Grant and Horikoshi 1989, 1992). These are stable environments with soda lakes being a productive system because of the presence of favorable temperatures (30–45 °C), high sunlight intensities, and abundance of HCO3 for photosynthesis (Ulukanli and Diurak 2002). The soda lakes are characterized by higher pH (11–12) and around of 5–30% salinity ($NaCO_3$ and NaCl in almost equal proportion) conditions (Duckworth et al. 1996).

Alkaliphiles are also found in a few insect guts and littoral soils (Hicks et al. 2010).

9.3.2 Alkaliphilic Fungi

Alkaliphilic fungi are very rare and reported sporadically from soda soil, soda lake, and limestone cave (Nagai et al. 1995, 1998; Grum-Grzhimaylo et al. 2013a). Alkalitolerant fungi *Fusarium oxysporum*, *F. bullatum,* and *Penicillium variabile* capable of growing at pH have been isolated in 1923 (Johnson 1923). Okada et al. (1993) isolated alkaliphilic fungus *Acremonium alcalophilum* growing optimally at pH 9.0. Most of the fungi thus isolated were alkalitolerants that can grow at alkaline pH of 10. For example, *Acremonium alternatum*, *A. furcatum*, *Acremonium* sp. 6, *Gliocladium cibotii* (YBLF 575), *Phialophora geniculata*, *Stachylidium icolor,* and *Stilbella annulata* isolated from soil *Acremonium* sp. 6 were said to be alkalophile (Nagai et al. 1995). Likewise out of six *Acremonium* and *Chrysosporium* species from limestone caves (stalactite caves) in Japan capable of growing at alkaline pH, one species each of *Acremonium* sp. and *Chrysosporium* sp. were alkalophiles (Nagai et al. 1998). Then eight species of alkaliphilic and alkalitolerant

soil fungi from Argentina have been reported belonging to families *Bionectriaceae*, *Trichocomaceae*, *Sporormiaceae*, *Ceratostomataceae,* and *Sordariaceae* (Elíades et al. 2006). Generally, the alkaliphilic fungi are anamorphic without forming any sexual structure, for example, *Acremonium* or *Verticillium* species (Okada et al. 1993; Kladwang et al. 2003). An alkaliphilic fungus *Sodiomyces* alkalinus showing optimal growth at alkaline pH, however, is able to form cleistothecium (Grum-Grzhimaylo et al. 2013b). Another novel alkaliphilic fungus *Emericellopsis alkalina* sp. nov. (grow at pH 4–11.2, but optimally at 10–10.2) besides several alkalitolerant isolates of *Acremonium* has been reported (Grum-Grzhimaylo et al. 2013b).

9.3.3 Living Strategy

The fungi found in soda soil/water face at least three stresses, namely, high osmotic pressures, low water potentials, and elevated ambient pH (>9) (Grum-Grzhimaylo et al. 2013b).

Alkylophilic fungi regulate their internal pH near neutral through active and passive regulation mechanisms. Passive regulation involves the low membrane permeability and cytoplasmic pools of polyamines (PA). Active regulation mechanism of homeostasis involves the sodium ion channels (Sharma et al. 2017). Cell wall components are very different in alkaliphiles. Many acidic polymers are present on the cell wall that reduces the pH. Altered membrane lipids and presence of cytoprotectant molecules enable them to survive at alkaline pH (Masato et al. 2010). Na^+/H^+ and K^+/H^+ type of antiporters are used to produce acid to reduce the internal pH and thus regulate the proton motive force (Charlesworth and Burns 2016). They employ different adaptation mechanisms against stress via accumulation of cytoprotective compounds (carbohydrate osmolytes) and modification of the composition of their membrane lipids. *Sodiomyces alkalinus* (Plectosphaerellaceae, Sordariomycetes, Ascomycota) is an alkalophilic fungus that accumulates cytosol carbohydrate trehalose, mannitol, phosphatidylcholines (PC), and PA in the mycelium of the fungus. Fruit bodies of this fungus were detected with high amounts of trehalose, triacylglycerols (TAG), PC, and sterols (Kozlova et al. 2019a). Bondarenko et al. (2018) observed trehalose, mannitol, and arabitol accumulation in two obligate alkaliphilic fungi *Sodiomyces magadii* (Plectosphaerellaceae, Sordariomycetes, Ascomycota) and *S. alkaline* (Plectosphaerellaceae, Sordariomycetes, Ascomycota) with almost double proportion of PA and lower proportions of PC and St (Bondarenko et al. 2018). Kozlova et al. (2019b) demonstrated unique features of Ascomycete *S. alkalinus*, which in the early lysis of cell walls of asci releases immature ascospores inside the fruit body whereas pseudoparenchymal and peridium cells degradation occur long before the ascospores maturation at extremely high pH of soda lakes. After maturity, these ascospores are forcefully released due to higher turgor pressure by cracking the fruit body. It was assumed that these features could develop to cope with the high pH (Kozlova et al. 2019b).

The fungi *Fusarium oxysporum,* was found to respond to hypersaline conditions by the expression of gene *ena1* encoding P-type Na^+A-ATPase. This gene is also upregulated when the pH of growth environment is increased (Caracuel et al. 2003). This coincidence suggests commonality of alkalitolerance and halotolerance mechanisms.

9.4 Acidophiles

Acidophiles are organisms that grow optimally at pH < 4.0. Another criterion to differentiate acidotolerant and acidophilious is the growth curve; the former exhibits bimodal growth while the latter shows unimodal growth (Cavicchioli and Torsten 2000; Gimmler et al. 2001). Fungi are mostly found to be acidotolerants.

9.4.1 Habitats

The acidophilic fungi may be isolated from neutral or acidic habitats (pH < 3) such as acidic soil, lake, swamp, and peat bogs (Middelhoven et al. 1992). Some of the highly studied sites are solfatara soil studied in the USA, Japan, Russia, Italy, Iceland, New Zealand, acid rock drainage of São Domingos (Portugal) and Rio Tinto (Spain), etc.

9.4.2 Acidophilic Fungi

Acidophilic fungi are rarely found; generally fungi growing at lower pH can also grow at neutral to slightly alkaline pH and thus mostly they are acidotolerant. Fungal biodiversity study in highly acidic Tinto river (Spain) revealed species of *Scytalidium*, *Bahusakala*, *Phoma*, *Heteroonium*, *Lecythophora*, *Acremonium,* and *Mortierella* (López-Archilla et al. 2004).

Three highly acidotolerant fungi *Acidothrix acidophila* (Amplistromataceae, Sordariomycetes, Ascomycota), *Acidea extrema,* and *Soosiella minima* (Helotiales, Leotiomycetes, Ascomycota) have been isolated from highly acidic soils in the Czech Republic and a coastal site in the Antarctic Peninsula (Hujslová et al. 2014) while another anamorphic brown mold fungus *Scytalidium acidophilum* was isolated from acidic soil and acidic solutions in an industrial plant and a uranium mine that show optimum growth at acidic pH (Sigler and Camichaeil 1974).

Acidophilous fungi have been explored from Iberian Pyrite Belt (IPB), and acid rock drainage in two localities São Domingos (Portugal) and Rio Tinto (Spain). The most acid-tolerant found was yeast Cryptococcus spp. 5 followed by *Cryptococcus* spp. 3 and Lecytophora spp. Moderately tolerant species were *Candida fluviatilis*, *Rhodosporidium toruloides*, *Williopsis californica*, and three unidentified yeasts belonging to *Rhodotorula* and *Cryptococcus* (Gadanho et al. 2006).

A novel acidophilic fungus *Teratosphaeria* (Capnodiales, Dothideomycetes) was reported from biofilms collected from an extremely acidic and hot spring. It is a ascomycetous teleomorphic fungus belonging to ascomyetes; phylogenetically close to *Acidomyces acidophilus* and *Bispora* spp., earlier reported acidophilic anamorphic fungi (Yamazaki et al. 2010).

From various studies, the domination of dematiaceous fungal species has been found in various acidic habitats (Amaral Zettler et al. 2002, 2003; Baker et al. 2004, 2009; Hujslová et al. 2010, 2013; López-Archilla et al. 2004). Of these, the three fungi *Acidomyces acidophilus* (Selbmann et al. 2008), *Hortaea acidophila* (Hölker et al. 2004), and *Acidomyces acidothermus* (Yamazaki et al. 2010; Hujslová et al. 2013) have been considered as acidophilic ones. All these plus the acidotolerant fungus *Acidiella bohemica* (Hujslová et al. 2013) belong to the family Teratosphaeriaceae (Capnodiales, Dothideomycetes, Ascomycota). Moreover, the three fungal species *A. acidophilus*, *A. acidothermus,* and *H. acidophila* along with two unidentified fungal isolates *Paecilomyces* spp. and *Penicillium* sp. 4 can grow at pH 1 (Gimmler et al. 2001; Hölker et al. 2004; Hujslová et al. 2010; Yamazaki et al. 2010).

9.4.3 Living Strategy

Fungi being eukaryotes face four main challenges: very high H^+ concentration, higher concentration of toxic metals, oligotrophic conditions, and extreme temperatures (Whitton 1970; Brock 1978; Brake and Hasiotis 2010). Extremely low pH irreversibly destroys primary and secondary structures of proteins (Kapfer 1998; Nixdorf and Kapfer 1998).

The acidotolerants employ twin mechanisms to tolerate hyperacidic environments; extrusion of protons out of the cell and maintaining low proton membrane permeability (Nikolay et al. 2018). Fungi by virtue of these internal pH regulation mechanisms exist commonly in acidic environments (Gross and Robbins 2000).

Acidophiles maintain the intracellular pH by preventing proton influx, buffering of intracellular protons, and efflux of protons. Although a number of protein transporter systems are located on the cell membrane to regulate the cytosolic pH levels (Gupta et al. 2014; Sharma et al. 2017; Christel 2018).

Acidophiles have highly impermeable cell membrane or reduced size of membrane pore to reduce entry of protons into the cytoplasm and maintain the pH homeostasis (Mirete et al. 2017) or have efficient proton pumps, which maintain the proton gradient across the cytoplasm and its pH at or near neutral pH (Mirete et al. 2017). They cope with the heavy metals by rapid efflux of these metals, inactivate them, or convert them into less toxic compounds (Charlesworth and Burns 2016; Christel 2018) and manage their oxidative stress by regulating the reacting oxygen species (ROS). They possess some antioxidants such as glutathione to inactivate these ROS or possess some enzymatic machinery such as superoxidase mutase or peroxidase to neutralize or inactivate the ROS (Christel 2018). They have

highly expressed chaperons that help them in rapid repair of the damaged proteins. The protein protects the DNA and other proteins from damage caused by the low pH (Mirete et al. 2017). An acid-tolerant strain of *Penicillium funiculosum* growing actively at pH 1.0 possesses a major facilitator superfamily transporter (PfMFS) involved in the acid resistance and intracellular pH homeostasis (Xu et al. 2014).

Acidophilic microorganisms are ecologically and economically important extremophiles found in solfataric fields, hydrogen sulfide (H_2S) emissions, active or abandoned mines, acidic copper mine wastes, and geysers (Sharma et al. 2012). Although a few acidophiles have been studied up to now, those data are not yet sufficient to clearly understand the adaptive features of acidophilic fungi. Determination of endo-1,4-b-xylanase crystal structure from *Scytalidium acidophilum* (Chaetomiaceae, Leotiomycetes, Ascomycota), XYL1 acidophilic fungi adds understandings of low pH adaptation. This study revealed the changes in the homologous enzyme to maintain stability in an acidic environment. Alterations include modification in the surface charge, decreased number of salt bridges, changes like the conserved residue of the active site, etc., at low pH (Michaux et al. 2010). Bacteria control internal low pH through increasing ATPase pump efficiency, which rapidly pumps out protons from the cells to raise the internal pH of the cell. Bacterial adaptation in such an environment (low pH) includes alternation of the cell membrane and controlling of flagella. This kind of observation is lacking in fungi and needs to be elaborated to enable a better understanding of fungi present in such ecological niches.

9.5 Metallophiles

Metallophiles are the organisms that thrive under metal-rich condition or environment with high metallic concentration. They are able to tolerate and detoxify high concentration of heavy metals. Most of the metallophiles are acidophiles, thus enhancing their survival 1000-fold than mesophiles and efficiently tolerate the high level of heavy metals (Anahid et al. 2011; Gupta et al. 2014).

9.5.1 Habitat

Naturally metal-rich environment such as water bodies and land around mining areas are the main habitats of metallophiles. Apart from these metal-contaminated areas around industries are also habitats of such metallophiles.

9.5.2 Metallophilic Fungi

Penicillium verrucosum KNU3 is metallophilic as it shows increased growth in the presence of Cr^{3+}, Cu^{2+}, and Pb^{2+} at 1 mM concentration (Joo and Hussein 2012). Similarly, *Penicillium simplicissimum* shows higher growth in the presence of heavy

metals at concentration up to 8000 ppm (Anahid et al. 2011). Other fungi *Aspergillus niger*, *Aspergillus foetidus* and *P. simplicissimum* showing high tolerance to molybdenum and vanadium have been reported. Of these, *P. simplicissimum* and *A. foetidus* are adapted to high concentration of heavy metals and show enhanced growth in the presence of heavy metals up to concentration of 2000 ppm (Valix et al. 2001).

Fungi that are tolerant to various metals have also been reported. For example, chromium- and nickel-resistant *Aspergillus* sp. tolerating chromium toxicity up to 10,000 mg/L chromium have been reported (Congeevaram et al. 2007). Ectomycorrhizal fungi *Hymenogaster* sp., *Scleroderma* sp., and *Pisolithus tinctorius* show higher tolerance against increased concentration of Al, Fe, Cu, and Zn (Tam 1995). Heavy metal biosorption analysis revealed that *Aspergillus* sp.1 accumulated 1.20 mg Cr and 2.72 mg Cd, *Aspergillus* sp. 2 accumulated 1.56 mg Cr and 2.91 mg Cd while *Rhizopus* sp. accumulated 4.33 mg Cr and 2.72 mg Cd per gram of biomass (Zafar et al. 2007). *Saccharomyces cerevisiae* and *Rhizopus nigricans* accumulate zinc (Sprocati et al. 2006). *Fusarium solani* shows tolerance to Ag (I) up to 1100 mg/L concentration (El Sayed and El-Sayed 2020). Another strain of fungus *A. niger* tolerates high concentration of heavy metal (Acosta-Rodríguez et al. 2018). *Fomitopsis meliae*, *Trichoderma ghanense*, and *Rhizopus microsporus* are some other metalloresistant filamentous fungi isolated from gold and gemstone mine sites that can tolerate various heavy metals such as Cu, Pb, and Fe (Oladipo et al. 2018).

9.5.3 Living Strategies

Presence of heavy metals such as Zn, Cd, Hg, Pb, Ag, Co, and Cr makes the environment very toxic. Generally high metal concentration inhibits the growth and functioning of microbes, but metallophiles develop the strategies to function optimally under these conditions. Some metallophiles possess efficient efflux pumps for the rapid removal of toxic metals while others associate these metals by binding them with protein molecules (Gupta et al. 2014). Ascomycete fungi such as *S. cerevisiae*, *Schizosaccharomyces pombe,* and *Candida albicans* have been studied for their adaptations to cope with high concentration of heavy metals. Some fungi chelate these heavy metals with thiolated peptides and make a complex that is either accumulated in the vacuole or extruded out of the cell. Some produce an antioxidant glutathione in high amount that prevents the oxidative stress. *S. cerevisiae* transports the heavy metals into external environment through a plasma membrane transporter Pca1 (Otohinoyi and Omodele 2015). They exhibit two general mechanisms: extracellular and intracellular, to fight with the high concentration of heavy metals. Extracellular mechanism involves the chelating and cell wall binding (biosorption) of heavy metals to restrict the entry of heavy metals into the cell while intracellular mechanism involves the binding of heavy metals to proteins to reduce the concentration of heavy metals inside the cell and prevent itself from damage (Anahid et al. 2011).

9.6 Radioresistants

Radioresistants or radiophiles are the extremophiles that are highly resistant to high level of ionizing and ultraviolet radiation. Radioresistant organisms tolerate extreme radiations for longer period of time while radiotolerant organisms tolerate extreme radiations for only a short period of time. Ionizing radiation such as gamma radiation and nonionizing radiation such as ultraviolet radiation are the two major radiations that cause lethal effect on an organism. Radiophiles are polyextremophiles as they can tolerate extreme cold, dehydration, vacuum, and high acidic concentration (Coker 2019).

9.6.1 Adaptations of Radiophiles

Gamma radiations causes double-stranded breaks in the DNA of an organism and produce reacting oxygen species that interfere with the metabolic processes leading to cell death. They also damage proteins and lipids and produce persistent oxidative stress. UV radiations cause more destruction by DNA damage through formation of thymine dimer and pyrimidine radio tolerant photoproducts. Radiophiles protect them from gamma radiation by adapting efficient DNA repair mechanism that rapidly repairs the damaged DNA, production of antioxidants, enzymatic defense system (increased production of enzyme such as catalase to inactivate free radicals and reactive oxygen species), and condensed nucleoid. UV-resistant radiophiles protect them from radiation through multiple mechanisms. Their genome is composed of very small number of bipyrimidine sequences. They possess gene duplication phenomenon causing polyploidy. Carotenoids, superoxide dismutase, and hydroperoxidases reduce the stress developed by radiation (Coker 2019). Radiophiles possess the capability to survive under starvation and high oxidative stress condition. They can even survive in condition with high amount of DNA damage. Ionizing radiations induce changes in upregulation of cell repair system and genetic component of an organism. Some UV radiation-resistant radiophiles protect their DNA from lethal radiation by the presence of UV-absorbing pigments such as scytonemin in sheath around the cell while some radiophiles accumulate UV-absorbing pigments such as mycosporine like amino acids in the cytoplasm of the cell (Dighton et al. 2008; Kazak et al. 2010).

Fungi are resistant to chronic ionizing radiations evolved from various radiation sources such as radioactive waste and nuclear disaster. The main strategy adopted by the radiation-resistant fungi against high radiation stress is to scavenge reactive oxygen species. They accumulate high amount of Mn^{2+} metabolite antioxidant complex for scavenging reactive oxygen species induced by the ionizing radiations as Mn^{2+} complexes with other compounds to inactivate the reactive oxygen species. Low concentration of iron ions and high concentration of manganese ions protect the cell from oxidative stress. Radiotolerant fungi possess high Mn^{2+}/Fe^{2+} ratio (Dadachova and Casadevall 2008; Dighton et al. 2008; Matusiak 2016). Melanin and some other pigments play an important role for the development of resistance to

radiations. A complex polymer melanin is important in energy transduction and shielding as they possess the capability to absorb various kinds of electromagnetic radiations. Radiation exposure causes fungal melanin pigment to alter the shape and induce them to form a thick layer of melanin. Some fungi, especially melanized fungi, harvest energy from the radiation with the help of melanin pigment and utilize this energy for their growth and development (Dadachova and Casadevall 2008; Dighton et al. 2008).

Ascomycota yeast possess resistance to chronic ionizing radiation is correlated with Cr^{+3} while resistance of Basidiomycete yeast to chronic ionizing radiation is correlated with the highest temperature that allows the growth (Shuryak et al. 2019). Biofilms of radioresistant fungi are adapted to high mutation rate and are more resistant to ionizing radiation than other radioresistants (Ragon et al. 2011). *Cryptococcus neoformans* is a radioresistant fungi that generally can be found in high radiation environment. Genome-wide radiation resistance analysis of this fungus explains the upregulation of DNA repair machinery for reducing the radiation stress. Rad53 protein kinase regulates the transcription factor Bdr1 and controls the transcription (Jung et al. 2016).

9.7 Fungi in Exoplanet-like Environment

For the study of life outside of our planet, extremophilic organisms are considered the best suitable model. As we already discussed, these organisms can survive in extreme acidic, alkaline, heat, cold, salt, and pressure. The real challenges to grow extremophilic fungi in exoplanet-like environment are space vacuum, solar, galactic and ionizing radiation, and extreme cold and heat. The precondition for Mars would be water availability. Fungi-producing melanin pigment are mostly colonized in the Antarctic to the Arctic to high-altitude terrains. For growing in such regions, extremophilic fungi have to deal with UV radiations, dry, and cold. So, melanized fungi could be a suitable model for studies in Mars-like habitat. Microcolonial fungus *Cryomyces antarcticus* (incertaesedis), Dothideomycetes, Ascomycota) can live in Mars-like habitat in a good way. *C. antarcticus* in Mars-like habitat for 24 h showed a decrease in protein number, but after 4- and 7-day treatment protein number was increased again and protein patterns matched to normalcy. This result indicated that *C. antarcticus* needs 1 week for recovery of its metabolic activity in a Mars-like condition (Zakharova et al. 2014). Another melanin-forming fungi *Cryomyces minteri* (incertaesedis, Dothideomycetes, Ascomycota) and known *C. antarcticus* exposed in Mars-like habitat for 18 months resulted in 10% of the sample being able to form colonies. Additionally, high stability in DNA is also observed in the hostile conditions of space (Onofri et al. 2015). Onofri et al. (2018) isolated *C. antarcticus* and *C. minteri* from cryptoendolithic microbial communities in Antarctica. After the screening of their DNA, it was observed that *C. antarcticus* displayed higher resistance than *C. minteri*. They concluded that the apparent presence of thicker melanized cell wall of *C. antarcticus* could be a reason for higher resistance (Onofri et al. 2018). Pacelli et al. (2019) experimented with black

fungus *C. antarcticus* with a simulated space vacuum or Mars-like condition and found that this black fungus can tolerate such a condition with high integrity of DNA even after the treatments (Pacelli et al. 2019) So the theory that in space biological material can be preserved is somehow true as we cited that fungi DNA remains undamaged in space. However, exact space condition cannot be created in the laboratory.

9.7.1 Genes and/or Secondary Metabolites

The EhHOG gene has an important role in the osmoregulatory pathway. EhHOG gene, isolated from *Eurotium herbariorum* from the dead sea, where salinity is the utmost on earth, showed resistance against salt, water, and low- and high-temperature stress. EhHOG genes encode mitogen-activated protein kinase (MAPK), which is a homolog of the HOG gene from *Aspergillus nidulans, Saccharomyces cerevisiae, Schizosaccharomyces pombe,* and many other eukaryotes. In the hog1 mutant gene of *S. cerevisiae*, when supplemented by the EhHOG gene, growth of the fungi is restored in high salt stress condition. Additionally, glycerol content also increased (Jin et al. 2005). Halophilic fungus *Aspergillus glaucus* contains RPL44 (ribosomal protein L44), a conserved protein related to salt resistance (Liu et al. 2014). Same kind of result was found in aquaglyceroporins (GlpFs), 60S protease subunit, and AgRPS3aE, ribosomal subunit from *A. glaucus*. Aquaglyceroporins transport glycerol and water, which are related to osmoregulation (Liang et al. 2015; Liu et al. 2015). Altogether these genes are highly conserved; they can support transgenic plants or cells surviving under high salt and heat stress conditions. Analysis of these genes may further support genetic engineering tools and crop improvement under high salt, water, and temperature stress. Extremophilic fungi develop exclusive defenses to survive in extreme conditions like temperature, salinity, pH, pressure, and desiccation, which leads to the production of diverse secondary metabolites. Secondary metabolites have no direct role in the adaptation process of extremophilic fungi. However, they have an indirect role by inhibiting the different microorganisms (viruses, pathogenic fungi, and pathogenic bacteria) in a competition to survive in an environment with limited nutrients (Table 9.1).

9.8 Conclusion

Extremophilic features are great parts of evolution, and scientists would get a better understanding of the effect of different proteins, genes, or metabolites responsible for survival in extreme environments. The presence of several harsh environmental conditions can lead to weighty challenges for living, resulting in unique survival strategies. Fungi are one of the most adaptable organisms for their splendid environmental and structural flexibility. They are physiologically changed for vigorous growth under extreme temperature, salt, pressure, pH, and minimal water availability through employing biochemical pathways, which are responsible for synthesizing

Table 9.1 List of antimicrobial activity of metabolites from extremophilic fungi

Category	Species	Collected from	Compound	Antimicrobial activity against	Reference
Acidophiles	*Penicilliumpurpurogenum* JS03–21	Red soil	Purpurides B, purpurides C berkedrimane B	*Candida albicans, Enterobacter aerogenes, Pseudomonas aeruginosa*	Wang et al. (2013)
Halophiles	*Aspergillus flavus, aspergillus gracilis, aspergillus penicillioides*	Solar saltern	Not mentioned	Not mentioned	Ali et al. (2014)
Psychrophiles	*Penicillium chrysogenum*	Benthisenviornment	Rugulosin, skyrin	*Staphylococcus aureus, Escherichia coli, Candida albicans*	Brunati et al. (2009)
Piezophiles	*Aspergillus* sp. SCSIO Ind09F01	Deep sea	Liotoxin, 12,13-dihydroxy-fumitremorgin C, helvolic acid	*Mycobacterium tuberculosis*	Luo et al. (2017)
	Aspergillus versicolor	Deep sea	Anthraquinone	Methicillin-resistant *Staphylococcus aureus*	Wang et al. (2018)
	Neosartorya fennelliae KUFA 0811	Marine sponges	Dihydrochromone dimer	*Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212	Kumla et al. (2017)
	Oidiodendron griseum UBOCC-A-114129	Deep sea sediment	Dihydrosecofuscin/ secofuscin	*Enterococcus faecalis*	Navarri et al. (2017)
Thermophiles	*Elaphocordyceps ophioglossoides*	Soil	Ophiosetin	Not mentioned	Putri et al. (2010)
Xerophilies	*Aspergillus felis*	Atacama Desert	Cytochalasins	*Paracoccidioides brasiliensis Pb18*	Mendes et al. (2016)

compounds (organic compounds, glycerol, trehalose, mannitol, arabitol, erythritol, etc.). In future, investigations on the extremophilic fungal genomes can be helpful to reveal the alteration in their cellular response in response to the extreme environment. Extremophiles that can survive in a wide range of harsh environments can further be used in a range of industrially important bioprocesses and in astrobiology studies.

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Ecology and Diversity of Microaerophilic Fungi Including Endophytes

10

Deeksha Patil, Vishal Dawkar, and Umesh Jadhav

Abstract

This chapter explores the extremophilic microaerophilic fungi along with endophytes. The relation between microaerophilic fungi and endophytes with the environment which decide the diversity is discussed here. From the overall population of one to five million fungal species, 1.5–1.6 million species are predominating. These are widely spread in extreme conditions like deep sea sediments, ionizing radiations along with areas where high salt concentrations prevail. Another perspective is of endophytic fungi. They are classified majorly as The Clavicipitaceae Fungal Endophyte (1) and the Non-Clavicipitaceae Fungal Endophyte (2). The microaerophilic fungi in extreme conditions are not explored much. There is a huge scope for the study of these microaerophilic fungi and endophytes that can thrive in adverse conditions. These extreme environments pose several stresses on fungi. The fungi use various mechanisms to adapt to these adverse conditions.

Keywords

Microaerophilic fungi · Extremophiles · Fungal endophytes · Biotic stress · Abiotic stress · Adaptations

D. Patil · U. Jadhav (✉)
Department of Microbiology, Savitribai Phule Pune University, Pune, Maharashtra, India
e-mail: ujadhav@unipune.ac.in

V. Dawkar
MITCON Biotechnology and Pharmaceutical Technology Business Incubator Center, Pune, Maharashtra, India

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10.1 Introduction

Human beings have a general tendency to look around the world as per their perspective which tends to be normal for the Earth. But that is not the case; there are things that thrive in extreme conditions that don't seem to be normal for human beings. MacElroy first introduced the term extremophiles (Macelroy 1974). Extremophiles can live and thrive under harsh physical conditions like pressure, radiation, and temperature and also in geochemical extremes like desiccation, oxygen levels, pH, and salinity. These harsh conditions threaten the general functions of life (Shrestha et al. 2018). All the three domains of life can survive in extreme conditions, but they are dominated by prokaryotes. The growth limiting factors for some organisms are listed in Table 10.1.

Talking about Endophyte, it means "in the plant," where *endon-* within and *phyton-* plant (Schulz and Boyle 2005). It was coined by the German scientist Heinrich Anton De Bary. Endophytes are organisms living inside the plant tissue (Vega et al. 2008). It colonizes the internal tissue of the plant without harming the host (Sikora et al. 2007; Backman and Sikora 2008; Hardoim et al. 2015; Puri et al. 2016). This definition holds true for cultivable fungi and it doesn't consider the unculturable fungi (Lugtenberg et al. 2016). Many variations are witnessed in the endophytic partners along with their relationships with each other (Schulz and Boyle 2005). They are ubiquitous in nature. They are accompanied with various organisms (Hartley and Gange 2009). They colonize various body parts of host plant (Saikkonen et al. 2006). They protect pants from herbivores. The grasses are infected with endophytic infection artificially and this infected grasses act as biocontrol agent against insects' pests of grasses (Clay 1989; Carroll 1995; Breen 1994; Saikkonen et al. 1998). Some of the fungal endophytes shield host plants from pests, nematodes attack and contribute to enhanced plant growth (Elmi et al. 2000; Sikora et al. 2007; Backman and Sikora 2008; Ownley et al. 2008; Reddy et al. 2009; Akello and Sikora 2012; Biswas et al. 2012; Jaber and Enkerli 2016; Jaber and Araj 2017). On the other hand, "Oecologie" or Ecology was the term first coined by Ernst Haeckel from Latin words Oikos (home) and logos (study of) (Haeckel 1866). Taylor defined ecology as, a science of all relations of all organisms to their entire environment (Taylor 1936). It can also be said in other words, that ecology deals with the organisms and

Table 10.1 Organisms and their known limits for growth (Coker, 2019)

Organism	Growth limiting factors
Psychromoas ingrahamii	Minus 12 °C
Geogemma barossii	129 °C
Picrophilus torridus	pH less than 0
Plectonemanos tocorum and Hydrogenophaga sp.	pH 13
Shewanella benthica	100 Mpa pressure
Haloferax volcanii	High concentration of NaCl and KCl
Halobacterium sp. NRC-1 and Deinococcus radiodurans	Ultraviolet (UV) and gamma radiation

environment and is associated with the study of factors that control organism's abundance and distribution and clearly describes the characteristics needed to thrive in natural environment (Winkelmann 2007).

10.2 Types of Microaerophilic Fungi with Endophytes

The Latin word *fungus* originated from the Greek word *sphongos* that meant having morphology of mushroom (Ainsworth 1976; Simpson 1979). The five-kingdom classification of Whittaker replaced the traditional classification of fungi as plants and gave rise to three different kingdoms that are- animal, fungi, and plantae (Baldauf and Palmer 1993).

These three multicellular eukaryotic kingdoms of life are believed to be ramified from each other some billion years ago (Bruns 2006). Fungi are considered as key players in terrestrial environment, where they perform essential functions like-decomposers, operating nutrient cycles, transforming elements other than carbon and behave as parasites and symbionts (Fig. 10.1) (Wainwright 1981; Wainwright 1988; James et al. 2006; Richards et al. 2012). Like other organisms' fungi also perform basic metabolic function for which they require energy supply along with carbon supply for the biosynthesis of cellular constituents. Basically, when fungi grow aerobically and as heterotroph, to supply energy they generally use available

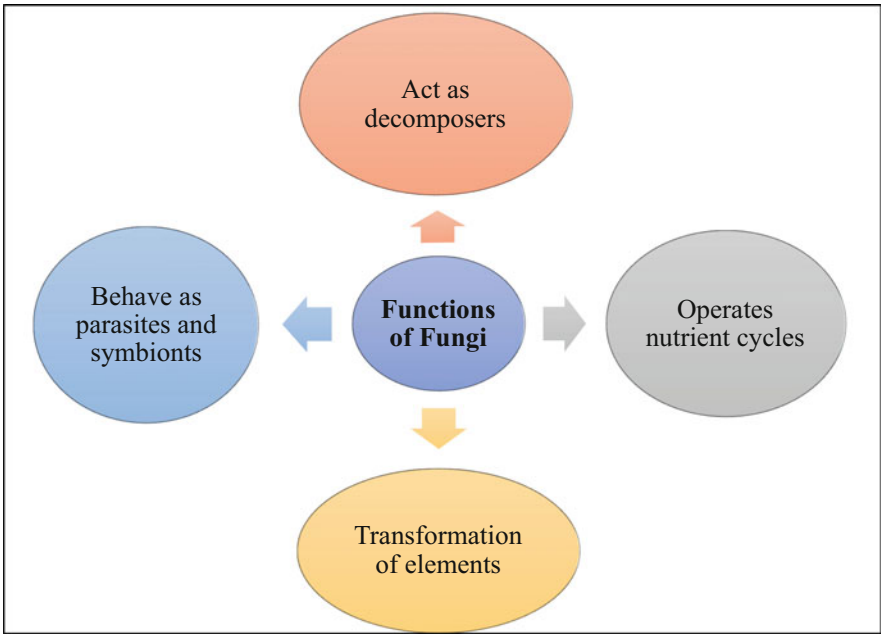

Fig. 10.1 Basic metabolic functions performed by fungi

organic substrate. Here, oxygen plays an important role as terminal electron acceptor for oxidation of substrate to CO_2 (Wainwright 1988; Grahl et al. 2012).

But it was seen that some rumen microorganisms that were recognized as zoospores of anaerobic phycomycetous fungi that are now considered as normal microbiota (Teunissen et al. 1991; Durrant 1996). Some of the scientists studied that, there are few filamentous fungi along with yeasts that can grow in low oxygen condition or in microaerophilic conditions (Waid 1962; Tabak and Cooke 1968). Basidiomycete specie, *Trichocladium canadense, Geotrichum sp.,* and *Fusarium sp*. are said to require low oxygen concentrations for their growth and enzyme productions (Pavarina and Durrant 2002). Fungi like *Blastocladiella prings/ zeimiana, Penicillium roquefortii*, along with several yeasts are found to have somatic growth at low oxygen levels (Durrant et al. 1995). Scientific classification for some of them is given in Table 10.2. Fungal endophytes occur naturally in many host plants (Saikkonen et al. 1998). They showed presence in plants like wheat, bananas, tomatoes (Vega et al. 2008). Various species are present in solitary vegetal part like root, stem, leaf (Rodriguez et al. 2009; Fürnkranz et al. 2012). Higher vascular plants exhibit symbiotic relationship with plant and endophytic fungi, where the endophytes provide benefits to host as indirect defense (Vega et al. 2008) in exchange of nutrition from plants (Kim et al. 2008; Quesada-Moraga et al. 2009; Lugtenberg et al. 2016). Endosymbiont derived from non-vascular plants, ferns, conifers, monocots and dicots are associated with foliage and compromises of ascomycetous fungi (Arnold and Lewis 2005). Many naturally occurring insect pathogenic fungi like *Beauveria bassiana, Clonostachys rosea, Isaria farinosa,* and *Acremonium sp.* have been isolated from symptomless body parts (Cherry et al. 1999; Pimentel et al. 2006; Vega et al. 2008; Orole and Adejumo 2009; Quesada-Moraga et al. 2009). The host plants were artificial inoculated with *Metarhizium anisopliae*, *B. bassiana*, *Fusarium oxysporum, Hypocrea lixii, Gibberella moniliformis,* and *Trichoderma asperellum* and they were re-isolated (Bing and Lewis 1991; Akello and Sikora 2012; Akello and Sikora 2012; Akutse et al. 2013). Colonization of endophytic entomopathogenic fungi using artificial methods of inoculation was successful in *Triticum aestivum* (Gurulingappa et al. 2010), *Phaseolus vulgaris* (Akutse et al. 2013), *Zea mays* (Bing and Lewis 1991), *Lycopersicon esculentum* (Ownley et al. 2008), *Glycine max* (Russo et al. 2015), Coffea spp. (Posada et al. 2007), *Papaver somniferum* (Quesada-Moraga et al. 2009), *Manihot esculenta* (Greenfield et al. 2016), *Sorghum bicolor* (Tefera and Vidal 2009), *Gossypium hirsutum* (Ownley et al. 2008). Petrini (1991) propound that some endophytic fungi exist in latent or inactive form. They become active due to changes in environmental/host/pathogen conditions (Petrini 1991).

10.2.1 Classification of Fungal Endophytes

Although sundry endophytic species are present, but characterization of only few of them has been done till now (Hawksworth 1991, 2001). Fossil study suggested that the plants were associated with endophytes from ancient time (Krings et al. 2007).

Table 10.2 Classification of microaerophilic fungi

Kingdom	Fungi	Fungi	Fungi	Fungi	Fungi
Division	Ascomycota	Ascomycota	Blastocladiomycota	Ascomycota	Ascomycota
Sub division	Pezizomycotina				
Class	Sordariomycetes	Eurotiomycetes	Blastocladiomycetes	Saccharomycetes	Sordariomycetes
Order	Sordariales	Eurotiales	Blastocladiales	Saccharomycetales	Hypocreales
Family	Chaetomiaceae	Trichocomaceae	Blastocladiaceae	Dipodascaceae	Nectriaceae
Genus	*Trichocladium*	*Penicillium*	*Blastocladiella*	*Geotrichum*	*Fusarium*
Species		*P. Roqueforti*			

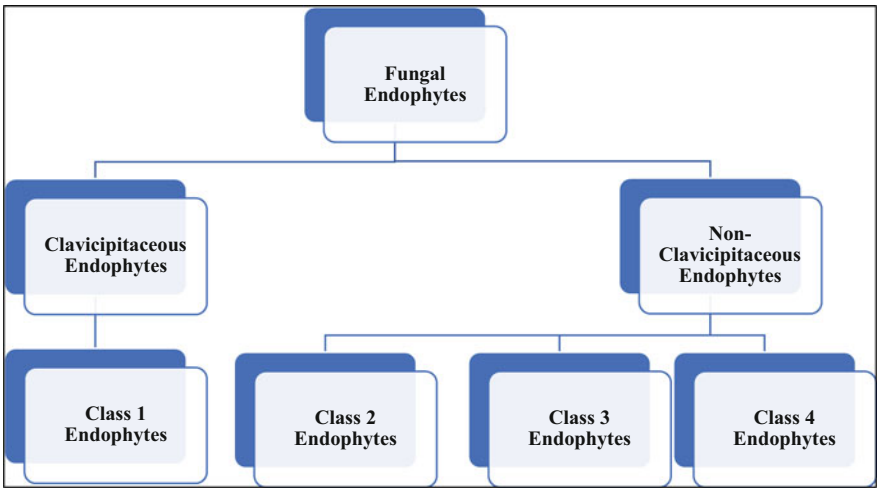

Fig. 10.2 Sorting of fungal endophytes

Fungal endophytes are categorized in two groups (Fig. 10.2) the clavicipitaceous endophytes—they infect some grasses; and the nonclavicipitaceous endophytes. They can be recovered from symptomless tissues of various vegetations (Lugtenberg et al. 2016).

10.2.1.1 Clavicipitaceous Endophytes (C-Endophytes)

They are known as Class 1 endophytes. These were first noted in late nineteenth Century by a group of European investigators in seeds of *Lolium temulentum, Lolium arvense, Lolium linicolum,* and *Lolium remotum* (Rodriguez et al. 2009). It was found that clavicipitaceous grow along with host and both of them benefited (Clay 1988; Bacon and White, 2000). However, they are small in number. They require particular media for their growth i.e., fastidious in nature. They are limited to seasonal grass family like poaceae and sometimes in cyperacae (Rodriguez et al. 2009). Class 1 types are host specific. They cause systemic infections in plant shoots. The family is said to be derived within the Hypocreales (order of fungi within the class Sordariomycetes) (Spatafora and Blackwell, 1993; Rehner and Samuels 1995; Spatafora et al. 2007). They involve plant pathogens, saprotrophs, and endophytes. Some of them are capable of production of bioactive compounds. They are said to be derived from insects' pathogens (Rodriguez et al. 2009). Transmission takes place vertically (Saikkonen et al. 2002). Three types of clavicipitaceous endophytes are observed (Clay and Schardl, 2002). They are said to add up in plant biomass. They help to tolerate water shortage. They protect host by producing compounds that are harmful to herbivores (Cheplick and Clay 1988; Rodriguez et al. 2009). Epichloë species of endophytes has stromata production that helps in sexual reproduction (White and Bultman 1987). They are found in Northern hemisphere. Whereas, C-endophytes that are found in Southern hemisphere do not produce stromata and

reproduces asexually (Clay and Schardl 2002). They are referred as Type III endophytes and classified as Neotyphodium species (White 1988; Moon et al. 1999; Leuchtmann et al. 2000).

These types of endophytes confer several beneficial effects to the host plant. First being herbivore deterrence. They retard insect attack using several mechanisms. They cause reduction in insect developmental rate. The insect growth is inhibited through feeding deterrence. Some class 1 endophytes are found to show anti-nematode activity. Mammal herbivory deterrence is also witnessed in USA, where *Achnatherum robustum* also known as sleepy grass is associated with endophyte. The domestic animals avoid consumption of such grasses. In South America there are grasses that are harmful to mammals. These grasses are also getting infected by *Neotyphodium tembladerae*. *Achnatherum inebrians* in Asia infects *Neotyphodium gansuense* and is resistant to mammal herbivory. C-endophytes are also involved in disease resistance. *Epichloë festucae* endophyte that infects turf grasses produces derivatives like a sesquiterpene, and a diacetamide are resistant over leaf spot pathogens and red thread disease. Ecophysiology of host plants is also enhanced by C-endophytes. Abiotic stress like drought, metal contamination is countered by infection of endophytes (Rodriguez et al. 2008).

10.2.1.2 Nonclavicipitaceous Endophytes (NC Endophytes)-

NC endophytes are varied and represent ascomycetous molds primarily. NC endophytes are recovered from all terrestrial ecosystems (Arnold and Lutzoni 2007). Several properties of these endophytes attracted attention of scientists. The diversity, ability to play an ecological role, and their endophytic and free-living lifestyles are some of these properties (Vasiliauskas et al. 2007; Selosse et al. 2008; Rodriguez et al. 2009). NC endophytes have presence of three functional classes—Class 2, Class 3, and Class 4.

Class 2 Endophytes

They are diverse as compared to class 1 endophytes and they comprise of species such as pezizomycotina (ascomycota), agaricomycotina, and pucciniomycotina (basidiomycota) (Rodriguez et al. 2009; Lugtenberg et al. 2016). Diversity in individual host plant is limited (Rodriguez et al. 2008). Roots, stem, leaves or sometimes an entire plant is colonized by them i.e., extensive colonization of host is possible. Colonization takes place through appressoria or hyphae. Transmission can be vertical or horizontal. They are seen to enhance the fitness of their host to enable the host to sustain habitat-specific stresses (Rodriguez et al. 2008). They are found in both underground and above-ground tissues. C-2 endophytes are essential for normal growth of plants. They are also seen to help plants to sustain in adverse conditions. Not much study is done on ecology of class 2 endophytes and hence limited information is available for distribution and abundance of same in rhizosphere. Species like *Phoma sp.* and *Arthrobotrys spp.* are present in high number in soil, whereas *Fusarium culmorum, Colletotrichum magna, and Curvularia protuberata* are in low number (Rodriguez et al. 2008).

As stated above, endophytes increase root and shoot biomass. The possible reason can be increased production of plant hormones by the host or their biosynthesis by fungi (Tudzynski and Sharon 2002). Some endophytes are witnessed to avoid stress by practicing plant symbiosis. *Fusarium culmorum* grow along all non-embryonic tissues of coastal dunegrass. When they are grown individually, they do not tolerate high salt concentration and growth is retarded. But if grown symbiotically can tolerate salt concentration of 300–500 mM NaCl (Rodriguez et al. 2008). Class 2 endophytes provide protects plants in various ways against fungal pathogens. It includes production of secondary metabolites, fungal parasitism, and induction of systemic resistance. Resistance against virulent pathogen is seen in Barley, as endophytic isolate *Fusarium oxysporum* had increased amount of phenolic metabolite. Plants like *Colletotrichum spp.* that grows in agricultural fields, *Fusarium culmorum* growing in coastal beaches or *Curvularia protuberata* that grows in geothermal soils has the ability to infect plants asymptomatically (Redman et al. 2001,2002).

Class 3 Endophytes

Class 3 endophytes are members of the Dikaryomycota (Ascomycota or Basidiomycota), Pezizomycotina, Saccharomycotina. They grow in the tissues that are above ground in localized manner. Their diversity is very high in individual plant or tissue. Around>20 species may be present on single tropical leaf (Rodriguez et al. 2009). Variety of species associated with above-ground plant tissues are seen in Class 3 endophytes. They are observed in photosynthetic and herbaceous tissues; flowers, fruits, asymptomatic wood and inner bark. In Central Panama tropical forest, it is seen that endophytes are diverse and individual leaves harbor around one isolate/2mm^2 of leaf tissue. Class 3 endophytes exists in temperate and boreal communities as well. These communities were recovered from Arctic endophytes. Reproduction takes place by sexual or asexual way.

Ecological functions of class 3 endophytes are not explored yet. The infection of plants with class 3 endophytes has insignificant effect on growth rate. Bark endophytes protect trees against Dutch elm disease. Even if they are parasitic or pathogenic, they may become mutualistic. It is also witnessed that class 3 endophytes have negative effect on host.

Class 4 Endophytes

They are mostly observed in above-ground root and colonization is same as class 2 (Rodriguez et al. 2009). Transmission is horizontal. They are characterized based on presence of darkly melanized septa. They are ascomycetous fungi, conidial or sterile and referred as “dark septa endophytes” (DSE). Host or habitat specificity is very less. They are associated with non-mycorrhizal plants from all types of ecosystems. They are also found in boreal and temperate forests associated with fine roots of trees and shrubs. It is witnessed that they are prevalent in highly adverse conditions, ubiquitous and abundant in different ecosystems. DSE when studied across North and South Pole was seen to be associated with 320 genera, 587 plant species. Root colonization can be intercellular or intracellular.

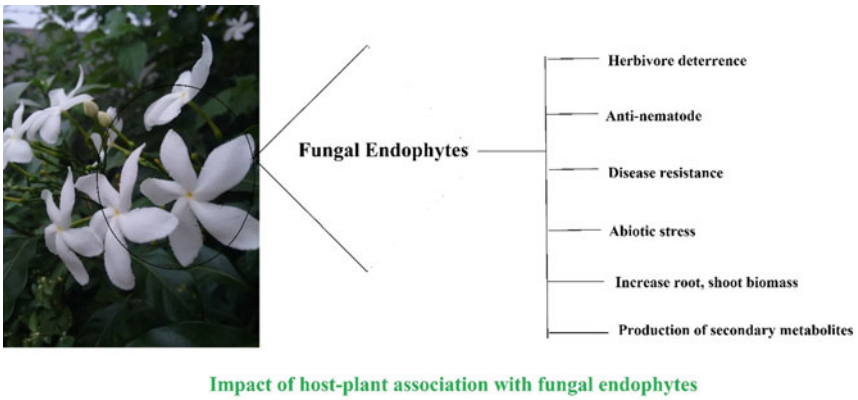

Fig. 10.3 Summary of impact of host-plant association with fungal endophytes

Like other endophytes, ecological role of DSE is not well defined. But it is seen that DSE have symbiotic association like mycorhizza. They perform many roles. It is also proposed that DSE acquisition can also play role in deterring pathogens. This can be achieved by controlling carbon availability in rhizosphere. Secondary metabolites production can be toxic to herbivores. Figure 10.3 shows interaction of endophytes with host plant.

10.3 Major Habitats

Biodiversity means the biological variation on Earth (Hawksworth, 1991). It is estimated to be around one to five million for fungi, where 1.5–1.6 million species are predominating (Hawksworth 1991,2001; Richards et al. 2012; Hawksworth and Lucking 2016). They are distributed worldwide and in extreme conditions like deep sea sediments (Raghukumar and Raghukumar 1998), ionizing radiations (Dadachova et al. 2007) along with areas where high salt concentrations prevail (Vaupotic et al. 2008). The distribution studies of endophytic fungi showed their presence in various ecosystems like terrestrial, semiaquatic, freshwater and marine habitats.

10.3.1 Terrestrial Ecosystem

Relationship of growths with earthbound plant species has gotten significant consideration contrasted with sea-going territories (Sandberg et al. 2014). Various geographic areas were assessed to study the occurrence of endophytic fungi like tropical, subtropical, and temperate regions of forest trees (Sun and Guo 2012).

10.3.1.1 Trees and Shrubs

In this part we will discuss about the interrelation of endophytic fungi with trees and shrubs. Diverse endophytic fungi are found on tropical trees and their leaves (Arnold and Lutzoni 2007). Some endophytic fungi like *Colletotrichum, Pestalotiopsis, Phomopsis,* and *Xylaria* are dominant irrespective of habitat. In India, *Tectona grandis* host of *Phomopsis sp. and Plumeria rubra* in *Colletotrichum sp., Phyllosticta sp.* are dominant taxa in tropical trees, where as in Brazil, *Tairira guianensis* host in *Phomopsis sp., Euterpe oleraceae* in *Xylaria cubensis, Spondias mombin* in *Phyllosticta sp*. and *Phomopsis sp., Malus domestica* in *Colletotrichum spp.* are dominant (Suryanarayanan 2011). *Phoma spp.* that infects *Calluna vulgaris* was analyzed of Mediterranean plants stated that they are root endophytes and confers fitness benefits to plants (Rayner 1915). Sun and Guo (2012) measured endophytic fungi in *Betula platyphylla, Quercus liaotungensis*, and *Ulmus macrocarpa* tree species and tissues from China's woodland habitats. Overall, 48.5 to 65.6% of twigs were colonized. The leaves showed slightly more colonization compared to twigs. In case of, *Tectona grandis* seasonal and geographical variation of endophytes was observed (Singh et al. 2017). About 5089 isolates that were recovered and assigned to 45 distinct morphotypes. Out of this, 43 morphotypes were assigned to ascomycotina and 02 to basidiomycotina. In case of DSE, Zubek et al. (2011) reported the co-occurrence of DSE with Arbuscular mycorrhizal (AM) in the ten plant species of Pamir-Alay Mountains of Central Asia. Bagyalakshmi et al. (2010) studied mycorrhizal types and DSE fungal associations in Western Ghats region.

10.3.1.2 Medicinal Plants

The fungal endophytes are known for the production of a broad variety of secondary metabolites (Kusari et al. 2014). They can be used for discovery of new medicinal agents in health care (Fig.10.4) (Kharwar et al. 2011).

Xanthomonas oryzae bacterial disease of rice was targeted using several secondary metabolites produced by endophytic fungus *Phomopsis longicolla.* Antitumor medicines like paclitaxel (Taxol), camptothecin, podophyllotoxin, vinblastine, and vincristine are identified from fungal endophytes (Kharwar et al. 2011). Taxol is produced by various endophytes associated with Japanese yew tree (Kumaran et al. 2010). Several endophytic fungi were isolated from liverwort *Scapania verrucosa* that belonged to the Xylariaceae family. They were classified into seven genera and showed antioxidant activities for DPPH radicals and hydroxyl radicals and proved to be novel antioxidant compounds (Zeng et al. 2011). From these around fifty endophtytic fungi were isolated from medicinal plants *Alpinia calcarata, Bixa orellana, Calophyllum inophyllum, and Catharanthus roseus*. They were screened for different enzymatic activities (Sunitha et al. 2013). Another study reported isolation of several species of endophytic fungi from various plant parts of five medicinal plant species present in the Western Ghats of India (Raviraja 2005).

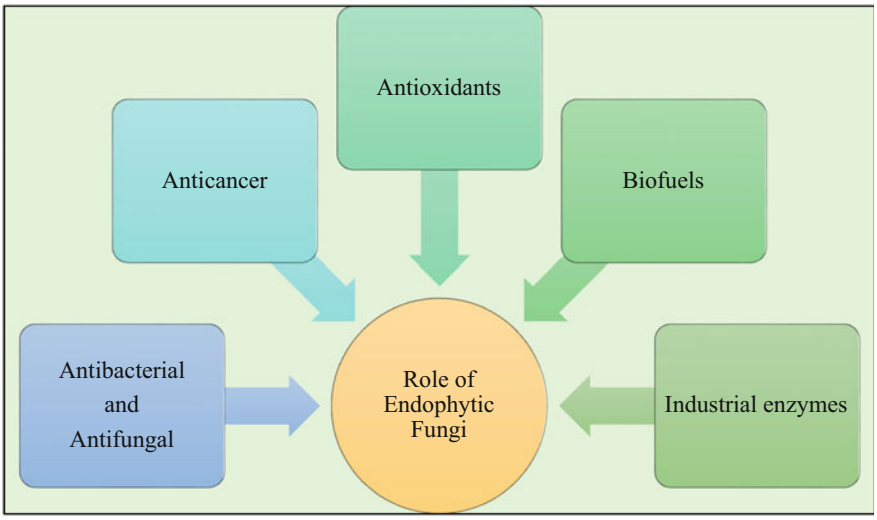

Fig. 10.4 Role of endophytic fungi in different medicinal fields

10.3.1.3 Ferns

In the tropical and subtropical ecosystems several thousand species of epiphytic ferns are reported that are associated with pteridophyte (Jones 1947). Raviraja et al. (1996) studied the roots of four riparian ferns. They observed colonization of pteridophytes by aquatic hyphomycetes. Kumaresan et al. (2013) screened five species of Pteridophytes, *Adiantum sp., Gleichenia linearis, Lygodium flexuosum, Pteris sp.,* and *Selaginella sp*. Out of the 40 species of fungal endophytes recorded, *Colletotrichum sp.* 1 occurred in all the Pteridophyte species.

10.3.1.4 Orchids

The presence of endophytic and Rhizoctonia-like fungi in Aegean and Mediterranean regions was studied. *Fusarium, Papulaspora,* and *Rhizoctonia* were isolated (Gezgin and Eltem 2009). Ascomycetes—Helotiales, Hypocreales, and Xylariales were found associated with five orchids from the tropical jungle of southern Ecuador (Herrera et al. 2010). Ten orchid species have a place with the genera Dendrobium in China which yielded high variety of endophytic organisms. The endophytes showed varied degree of host specificity (Chen et al. 2011). Orchid *Anoectochilus setaceus* which is found in Sri Lanka is associated with endophytic fungi *Xylaria sp*. (Ratnaweera et al. 2014). Several isolates were obtained the roots of two orchids (Yu et al. 2015).

10.3.2 Aquatic Ecosystem

10.3.2.1 Freshwater Habitats

The freshwater environments are less explored for the presence of endophytic fungi. In such environments macrophytes act as host (Bärlocher 1992; Rajagopal et al. 2018). Endophytes from macrophytes uncovered low disengagement recurrence yet contrasted in species extravagance, variety, and local area structure with closeness with general earthbound networks (Sandberg et al. 2014). Aquatic medicinal fern was found associated with hyphomycetes, Coelomycetes, and endophytic fungus (Udayaprakash et al. 2018). Endophytic parasites were observed in riparian tree species in two altitudinal reaches of the Western Ghats (Ghate and Sridhar 2017). The variety of amphibian hyphomycetes was higher in mid elevation than high-height (Raviraja et al. 1998). Several endophytic growths were recuperated with predominance of *Cladosporium, Fusarium,* and *Geotrichum* from the three oceanic and two riparian plant species (Li et al. 2010).

10.3.2.2 Marine Habitats

Mangroves

The plant species occurring in river mouths give possible living spaces for the colonization of endophytic fungi. Suryanarayanan and Kumaresan (2000) isolated four halophytes *Acanthus ilicifolius* (Acanthaceae), *Arthrocnemum indicum*, *Suaeda maritima* (Chenopodiaceae), and *Sesuvium portulacastrum* (Aizoaceae) from the mangrove plants. All of them harbored fungal endophytes in which *Acremonium, Phomopsis, Phyllosticta*, and *Sporormiella* minima were common foliar endophytes. Cheng et al. (2008) isolated endophytic fungus from mangrove *Kandelia candel*. Using molecular and morphological evidence they identified the endophyte as *Diaporthe phaseolorum* var. *sojae*. A wild legume *Sesbania bispinosa* was evaluated for presence of endophytes. Several endophytic fungi with six dominant taxa were identified (Shreelalitha and Sridhar 2015). In some cases, there is single species dominance in endophytic fungi (Suryanarayanan and Kumaresan 2000; Suryanarayanan et al. 1998; Kumaresan and Suryanarayanan 2001). The multispecies domination was also witnessed (Ananda and Sridhar 2002; Kumaresan and Suryanarayanan 2001).

Coastal Sand Dunes

The roots of three plant species—*Ipomoea pes-caprae, Launaea sarmentosa, and Polycarpaea corymbosa* were studied for the presence of fugal endophytes. About 31 species were isolated—19 Deuteromycetes, 6 Ascomycetes, and 6 sterile fungi (Beena et al. 2000). *Chaetomium globosum* was the most prevailing organism. It plays an important role in plant protection. Just a single marine parasite was endophytic. Wild vegetable of seaside sand and mangroves showed presence of six endophytes (Shreelalitha and Sridhar 2015).

Seaweeds

Ocean growth of wide geographic areas has endophytic parasites (Raghukumar 2008; Schulz et al. 2008; Ariffin et al. 2011; Flewelling et al. 2013; Singh et al. 2018). The endophyte *Mycophycias ascophylli* is related to the existence of certain seaweeds—*Ascophyllum nodosum* and *Pelvetia canaliculate*. Macroalgae showed presence of different types of endophytes than terrestrial plant species. The assessment of seaweeds in coastal region showed presence of several endophytes (Sridhar 2019). In the seaweeds of Atlantic coast of Canada 79 endophytic fungi were associated with red, brown, and green algae (Flewelling et al. 2013).

Seagrass

Seagrass has been studied as a possible niche for endophytic fungi colonization. The east coast of India's Halophila ovalis leaf blade, petiole, and rhizome showed lower presence of endophytic fungi (Devarajan and Suryanarayanan 2002). Also, a culture-based analysis, showed lower presence of endophytic fungi in coastal seagrass species compared to terrestrial plant species (Venkatachalam et al. 2015). Around 26 endophytes are known from 3 seagrass (Sridhar 2019). Three endophytes—*Fusarium, Penicillium,* and *Nigrospora*) were predominant. Strangely, *Nigrospora sp.* showed antifungal property against the dermatophyte. An intensive study on the endophytic growths in southern Thailand resulted in identification of four seagrass species—*Cymodocea serrulata, Enhalus acoroides, Halophila ovalis,* and *Thalassia hemprichii* (Supaphon et al. 2017).

10.4 Adaptations to Biotic and Abiotic Stress

Diverse environmental conditions are present on earth (Fig. 10.5). Drought, high and low temperatures, scarcity of water, and salinity are few extreme environmental conditions.

These conditions may influence the structure of types of plants present in a particular region and thereby the types of fungal endophytes present. To survive such extreme conditions the fungal endophytes adapt themselves in a variety of ways. A study explored the endophytic fungal biota associated with plants adapted to arid habitats. They reported diverse number of fungal endophytes. The dominant genera among these were *Aspergillus, Alternaria, Penicillium, Nigrospora, Chaetomium* and *Curvularia* (Sangamesh et al. 2018). Another study reported the presence of endophytes in tree species of the desert area (Gehlot et al. 2008). The pigmented fungi were also isolated from several cactus species (Suryanarayanan et al. 2005; Gehlot et al. 2008; Massimo et al. 2015). These studies showed that the isolates contained melanin and carotenoids such as lycopene. The increase in melanin content may help to overcome oxidative stress (Singaravelan et al. 2008). Such an adaptation in these endophytes (in the form of pigmentation) could provide protection against UV light, pathogenicity, solar radiation, high temperature, chemical and radioactive pollution, drought, and environmental stresses (Zhdanova and Vasil'evskaya 1988; Butler and Day 1998; Gupta et al. 2015). Ali et al. (2019)

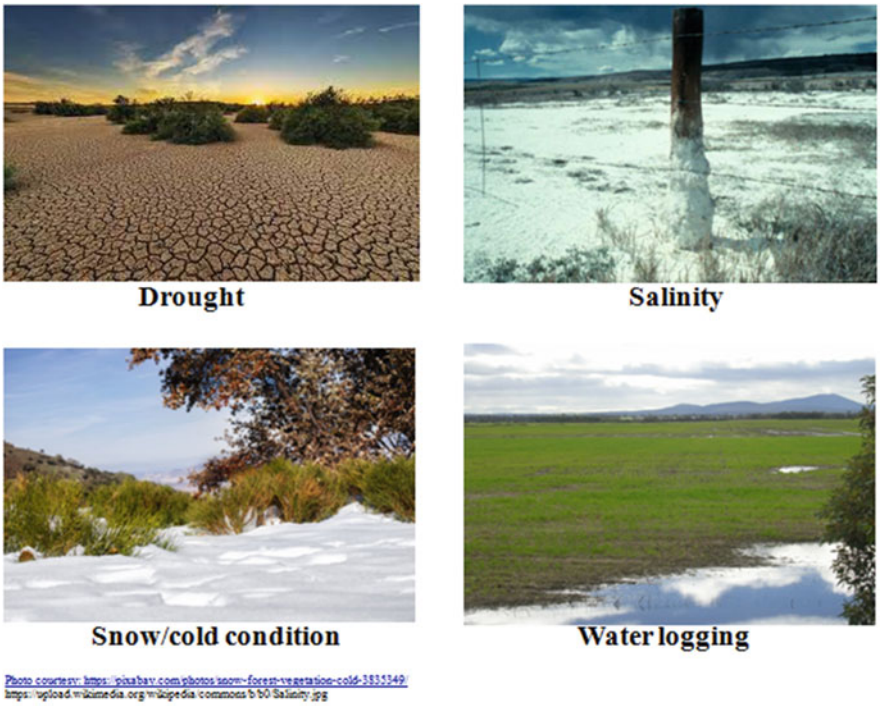

Fig. 10.5 Diverse environmental conditions on earth

showed that *Thermomyces lanuginosus* isolated from desert-adapted plant has the ability to sustain high temperatures. The thermal adaptation of *T. lanuginosus* originated from several DNA related pathways. Under the heat stress conditions the cell signaling and DNA repair is governed by ubiquitin degradation, histoneacetylation/deacetylation, and poly adenosine diphosphate ribosylation (Mchunu et al. 2013; Ali et al. 2019).

The drought conditions cause disruption of water potential gradients, limitations to gas diffusion, denaturation of proteins and enhanced production of Reactive Oxygen Species (ROS) (Rao and Chaitanya 2016). The decrease in intracellular CO_2 concentration results in production of ROS. It transfers electrons to photosystem I (Reddy et al. 2004). This results in oxidative damage due to lipid peroxidation (Rizhsky et al. 2004). It has been shown that *P. indica* an endophyte could counteract the ROS induced stress. It prevents the excess ROS formation under stress conditions and avoid lipid degradation (Sun et al. 2010).

A wide range of compounds are secreted by fungal endophytes. Various fungal endophytes are known for the production of plant hormones (Khan et al. 2011; Asaf et al. 2018). These hormones help to tolerate the salinity stress. During the salinity stress the presence of sodium chloride (NaCl) causes oxidative damage through production of ROS. Several studies reported that the endophytic fungi produce

antioxidant enzymes under salt stress and eliminate the free radicals to overcome the oxidative damage (Abogadallah 2011; Hashem et al. 2014; Ahmad et al. 2015; Yasmeen and Siddiqui 2017).

Cold and freezing is another important stress needs to be studied with respect to fungal endophytes. The fungal endophytes facing cold and freezing conditions may rely on possible physiological and morphological adaptations. The biosynthesis of unsaturated fatty acid and glycerol helps to maintain membrane fluidity and to accumulate glycerol as a cryoprotectant. Also, the plants inhabited by the fungal endophytes may help to survive the cold stress (Robinson 2001; Zhang et al. 2013).

Along with abiotic stresses the fungal endophytes have to face several biotic stresses as well.

One of biotic stresses faced by fungal endophytes is plant defense compounds (Bailey et al. 2005; Saunders and Kohn 2009). Over 10,000 secondary metabolites are produced by plants which have antifungal properties (Dixon 2001). Along with host plant defense compounds the fungal endophytes have to face the competitive interactions exerted by other fungal species. These competitive fungal species also produce biologically active compounds having antifungal activity (Schulz et al. 2002). The fungal endophytes adapt themselves to detoxify the toxic compound and overcome these barriers (Cooney et al. 2001).

There are three primary mechanisms using which fungal endophytes associate with plants and compete with fungal species. It includes tolerance: detoxification, structural alteration of the toxin target, and activation of membrane transporters (Carter et al. 1999; VanEtten et al. 2001; Saunders and Kohn 2009). The abiotic stresses such as salinity, low temperature, and heavy metal toxicity hamper the growth of plants. The adaptation of fungal endophytes to several environmental stresses not only benefits their own survival but also toward the fitness of host plant. Endophytic fungi facilitate plant growth through improved nutrient uptake, efficient water use and curtailing of environmental stresses (Fig. 10.6). Endophytes confer abiotic stress lenience in plants by triggering host stress response systems and by synthesizing antistress biochemicals (Sun et al. 2010; Husaini et al. 2012; Ansari et al. 2013; Gill et al. 2016; Lata et al. 2018). The fungal endophytes activate defense related genes, abiotic stress responsive genes of the host to mitigate the stress induced by high salinity and drought. They also enhance secretion of osmoprotectants (proline, glycine betaine) (Waller et al. 2005; Zarea et al. 2012; Ansari et al. 2013). This phenomenon is reported in *Triticum aestivum* (Zarea et al. 2012), Chinese cabbage (Sun et al. 2010) and strawberry (Husaini et al. 2012). The overproduction of ROS occurs in plants due to abiotic stress and fungal and viral infections. The excess ROS are removed from plant system with the help of fungal endophytes. The fungal endophytes modulate the expression of antioxidant defense enzymes other components of ROS-scavenging system of host plants (White and Torres 2010; Foyer and Shigeoka 2011; Hamilton et al. 2012; Waller et al. 2005; Sun et al. 2010). When the plants are exposed to pathogenic fungi, bacteria and virus the fungal endophyes upregulate various defense related genes of host plant (Waller et al. 2005; Serfling et al. 2007; Camehl and Oelmüller 2010; Molitor et al. 2011; Johnson et al. 2013; Gill et al. 2016). The fungal endophytes produce compounds

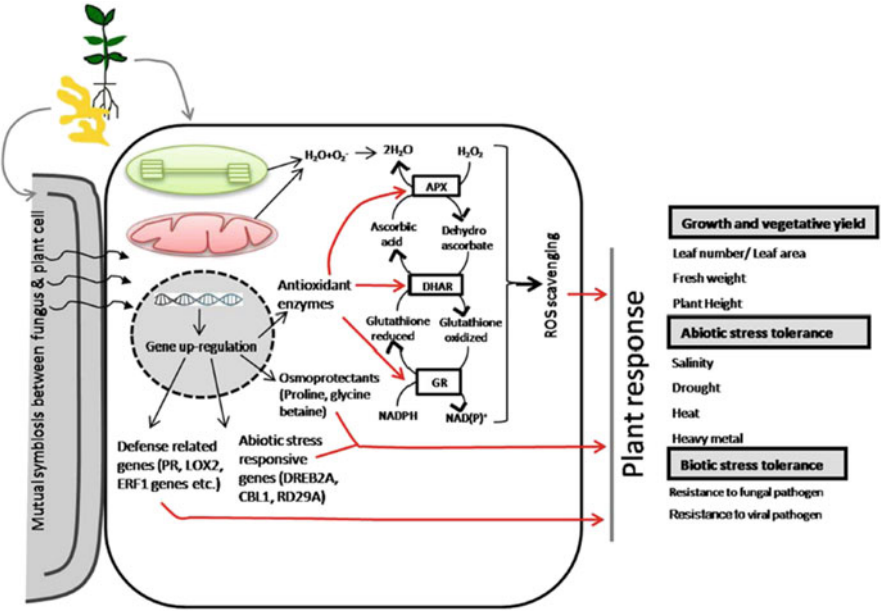

Fig. 10.6 Various mechanisms of stress tolerance in plants conferred by endophyte (Image courtesy Gill et al., 2016)

having antagonistic activities to increase host defense mechanism (Waller et al. 2005; Gill et al. 2016).

10.5 Conclusions

Almost all the plant species harbor a diverse range of microaerophilic fungi including endophytes. The research presented here showed various types of microaerophilic fungi, their classification and the habitats. Research documented here will help to understand endophyte ecology. Several factors influence the diversity of microaerophilic fungi including endophytes. These include type of host plant, competing species, and stress. The microaerophilic fungi including endophytes use various mechanisms to sustain various stresses and overcome the growth barriers. They use detoxification mechanisms. They produce several osmoprotectants. They are also capable of secreting the defense enzymes or antioxidant enzyme. These fungi not only protect themselves from several stresses but also confer these properties to the host plant and help it to sustain adverse conditions.

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Fungi in Hypoxic Soils and Aquatic Sediments

11

Irena Maček

Abstract

Under certain environmental conditions, O_2 availability in soils and aquatic sediments can become a limiting factor for the survival of aerobic organisms, including the majority of fungi. Here, hypoxia is presented in light of various aspects of fungal ecology. Fungi are diverse in many extreme environments, but many of these ecosystems have been poorly exploited to study general principles of fungal biology and ecology, and these include hypoxic environments such as submerged ecosystems and mofettes (natural CO_2 springs). Furthermore, with global change accelerating the frequency of extreme events, hypoxic environments are also becoming more common, with either permanent or temporary soil or sediment hypoxia caused by flooding or higher temperatures. Here, in addition to a range of aquatic hypoxic environments, we present some new insights and experiences on the response of fungi to hypoxia, derived from research on a specific extreme mofette ecosystem. Findings on the response of different groups of fungi in soil and sediments (e.g. yeasts, mycorrhizal fungi) and in particular on their patterns of community formation are presented. Finally, we report on the frontiers of fungal research in hypoxic environments, which include some little-studied topics such as the bioprospecting of new extremophile taxa, the study of fungal pathogens in humans, and extreme environments as natural models for long-term experiments in ecology and evolution.

I. Maček (✉)
Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia

Faculty of Mathematics, Natural Sciences and Information Technologies (FAMNIT), University of Primorska, Koper, Slovenia
e-mail: irena.macek@bf.uni-lj.si

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11.1 Introduction

Normally, soil air contains concentrations of O_2 similar to those in the atmosphere (just below 21%). However, under certain environmental conditions, O_2 availability in soil and also in aquatic sediments can become a limiting factor for the survival of aerobic organisms, including most plants, animals, and fungi. There can be several causes for hypoxia (low O_2 concentration compared to atmosphere) or even anoxia (no O_2). It can occur locally on a microscale or in a larger volume of soil or sediment. In the case of local (small-scale) hypoxia, larger animals, plant roots, and possibly some filamentous fungi may avoid it by moving or growing in another area where O_2 is more abundant. However, if hypoxia is present in a larger volume of soil or sediment, and long-term at that, avoidance is not possible and other mechanisms of O_2 supply must take place to support aerobic metabolism of aerophylls if they are still present in such a system (e.g. in permanently submerged environments, in terrestrial ecosystems during prolonged periods of flooding, or in some extreme hypoxic ecosystems such as natural CO_2 springs or mofettes, see the description in Sect. 3.1, Fig. 11.1).

In some cases (e.g. ecosystems with permanent soil hypoxia—natural CO_2 springs), specific terminology has been developed for plants based on their tolerance to hypoxia, but not yet in general for fungi or other microorganisms (Maček et al. 2016b, Fig. 11.2). Plants in mofettes form three categories: (1) mofettophobic, which strictly avoid geological CO_2 at concentrations above 2–3%; (2) mofettophilic, which grow above high CO_2 concentrations (which can be over 80% in soil); and (3) mofettovagous plants, which grow in both degassing and reference/control areas (Pfanz 2008, Pfanz et al. 2019, Fig. 11.1). A similar categorisation could be applied to other groups of organisms (animals and microbes) (Maček et al. 2016b) and has recently been used for some Collembolae found in mofettovage areas (Russell et al. 2011; Hohberg et al. 2015).

In this chapter, a number of hypoxic environments are presented with their specificities, in addition to insights into the response of different groups of fungi and, in particular, the patterns of their community structure. With the new molecular breakthroughs of the last decades, life in soils has become a new frontier of biological research in a number of different ecosystems and its response to hypoxia is an important topic to study. Hypoxia and its effects on various aspects of fungal ecology and physiology were chosen for presentation for several reasons:

Fig. 11.1 Terrestrial grassland (above) and aquatic forest mofette (below) sites near the village of Stavešinci, NE Slovenia. Reduced plant growth and altered plant community structure are seen at both mofette sites. Subsurface geogenic CO_2 release leading to continuous bubbling of water is seen in the forest-water aquatic mofette called Slepica (below). Dead animals that have suffocated in CO_2 traps near forest mofettes are often found at mofette sites (below). See also Maček et al. (2016b) for more details on mofettes, their flora, fauna and microbes. Photo: I. Maček

1. it is a common but little-studied stress in terms of its effects on the biology of various fungal groups (Maček et al. 2011, 2016b),
2. it is present in many natural ecosystems worldwide (Perata et al. 2011), both terrestrial and aquatic, with climate change models predicting that it will become even more common and severe in the future, due to larger floodplains and direct and indirect effects of higher temperatures (Hirabayashi et al. 2013), and.
3. the new knowledge and experience on this phenomenon coming from our research on a specific extreme hypoxic ecosystem—mofettes or natural CO_2 springs (e.g. Maček et al. 2011; Šibanc et al. 2014; Maček et al. 2016b; Šibanc et al. 2018, Fig. 11.1, Sect. 3.1).

In the first part, the definition of an extreme environment and extremophiles is presented. Then, different hypoxic ecosystems and their fungal inhabitants are

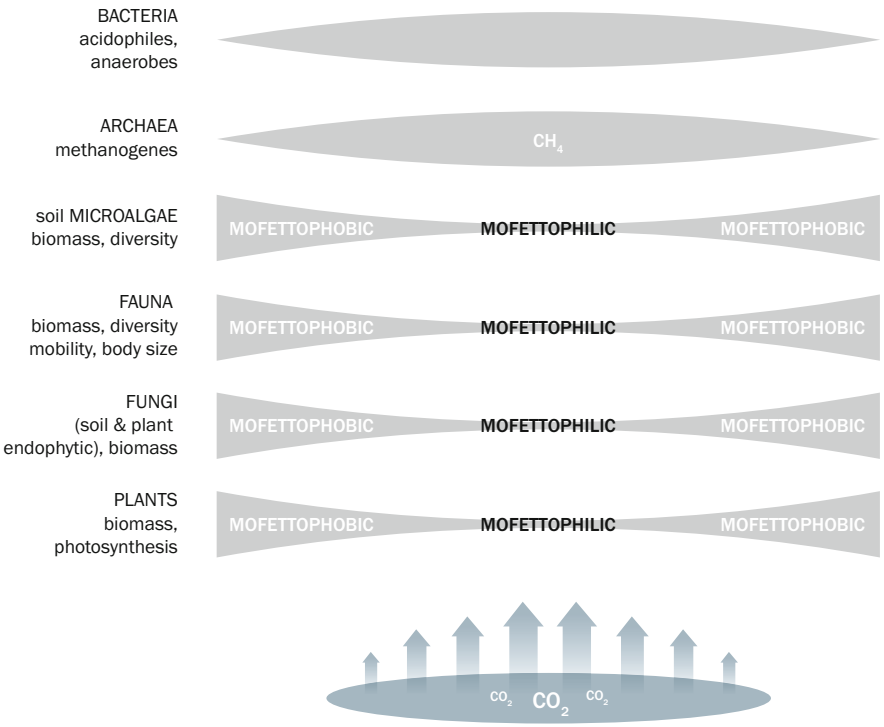

Fig. 11.2 Schematic representation of the effect of CO_2 venting at mofettes on biotic factors. The gradients of the different factors are indicated by the thickness of the lenticular shapes. A biconvex lens shape indicates a higher value of the specific parameter closer to the mofette centre with higher CO_2 concentration, while a biconcave shape indicates the opposite. Mofette specific terminology is presented (mofettophobic for organisms that strictly avoid geogenic CO_2 at concentrations above 2–3%, and mofettophilic based on their tolerance to hypoxia), see also Maček et al. (2016b) for details

presented, starting with a specific extreme ecosystem where soil hypoxia is induced by geogenic CO_2 exhalations in natural CO_2 springs or mofettes. These can be wet or dry (Fig. 11.1). Submerged ecosystems are then addressed (hypoxia in aquatic sediments), followed by less extreme, temporarily flooded and compacted soils with induced soil hypoxia. The chapter ends with a section addressing the new areas of studying fungi in hypoxic environments. This includes some little-studied topics such as bioprospecting for new taxa, exploring fungal pathogens in hypoxic environments, and a case study of mofettes that serve as natural long-term experiments in the fields of evolution and ecology.

11.2 Extreme Environments and Extremophiles

Extreme environments have been defined as environments that have one or more environmental parameters whose values are persistently close to the lower or upper limits known to support life (Torossian et al. 2016) and have long been studied as ecosystems of special interest. However, defining extreme environments remains difficult. Microorganisms are able to use a wider range of different metabolic pathways to survive in these environments. Therefore, it is clear that an extreme for humans or higher (multicellular) organisms may not be an extreme for microbes. In an analogous context, our current oxygen-rich atmosphere can be considered extreme for anaerobic organisms that dominated over a large period of life's existence.

Extreme environments are also hotspots of biological discovery that have served as rich sources of biotechnological compounds. Many initial studies have aimed to describe extreme systems from many different aspects, including diversity of abiotic factors and biological communities. By definition, an extremophile is an organism that thrives in extreme environments. This can include hypoxic environments. The ecology of extremophiles has long been a rich source of knowledge about the evolution and functions relevant to stress adaptation of microbes from different phylogenetic groups (e.g. Gostinčar et al. 2010). Ecological study, including biodiversity loss and the response of extreme ecosystems to global change, is becoming increasingly important. Fungi are diverse in many extreme environments, but many of these ecosystems have not been widely used to study general principles in fungal biology and ecology, and these include hypoxic environments such as submerged ecosystems and mofettes (Maček et al. 2016b; Maček 2017a, b). Extreme environments characterised by specific gas composition are increasingly used as natural experiments (e.g. Rothschild and Mancinelli 2001; Maček et al. 2005, 2011, 2016b). Environments that are locally extreme relative to average regional conditions (e.g. natural CO_2 springs or mofettes, see Sections 3.1 and 4) may be less promising in terms of biological discovery, yet they provide some of the most powerful natural experiments for studying ecology and evolution (Maček et al. 2016b, see Sect. 4.3).

11.3 Fungi in Hypoxic Soils and Aquatic Sediments

Fungi are a widespread, diverse and large group of eukaryotes with complex cell structures consisting of various forms ranging from filamentous fungi, such as mycorrhizal fungi, moulds and mushrooms to unicellular yeasts. Some fungi have the ability to form tissues and organs typical of higher organisms. Like other multicellular organisms, fungi are considered aerobes; however, their habitats can often be hypoxic or even anoxic due to, for example, infiltration of soil with water, metabolic activity in their environment, or during colonisation or infection of other organisms (Maček et al. 2011; Grahl et al. 2012; Maček et al. 2016b; Grossart et al. 2019; Maček 2019). Fungi play a crucial role in carbon mineralisation and cycling in

terrestrial environments, which are the habitat of the majority of known fungal taxa. Soil organisms, including fungi, are subject to occasional hypoxia, although the hypoxic response also begins in fungi (similar to multicellular eukaryotes) at oxygen levels of about 6% (Simon and Keith 2008), but they have evolved to tolerate low or rapidly changing oxygen levels (e.g. Maček et al. 2011). Yeasts are an exception in the fungal kingdom, as many are known to have the ability to ferment (e.g. Šibanc et al. 2018).

When hypoxia occurs in soils or aquatic sediments, respiration of roots and rhizosphere and root-associated endophytic fungi is under stress. Especially in severe hypoxia, root respiration is also impaired (Maček et al. 2005). Root respiration decreases significantly only when plant roots are exposed to CO_2 concentrations or water saturation in soil or sediment high enough to limit O_2 availability (above 50% CO_2 causes a decrease in O_2 saturation in liquids below 10%, Maček et al. 2005, 2016b). Active nutrient uptake is an energetically costly process that requires metabolic activity in all aerobic organisms (e.g. plants and fungi). Soil hypoxia can inhibit ion uptake by roots or fungal hyphae due to limited aerobic metabolism and higher dependence on less efficient anaerobic metabolism. In non-adapted plants, fermentation occurs in root cells due to the lack of O_2, leading to the production of lactic acid and ethanol.

Plants that have adaptive traits such as the ability to form aerenchyma or other systems for O_2 transfer to the roots and rhizosphere are more likely to survive in these environments and are also more likely to support and form ecological interactions with other aerobic organisms in the rhizosphere (Fig. 11.5). Aerenchyma are typical adaptive traits of plant species in wetlands, but can also form in response to flooding or mineral deficiency (Videmšek et al. 2006; Vodnik et al. 2009; Marschner 2012). In plants, the formation of aerenchyma can enable a relatively high O_2 content in the rhizosphere and maintain aerobic respiration of roots even in the context of very high soil CO_2 concentrations or flooded soils. In addition, plant-mediated changes in the rhizosphere (e.g. O_2 axial transport and escape from roots) can affect other organisms that live in hypoxic systems (e.g. soil and endophytic microbes and fauna) (Maček et al. 2016b, Fig. 11.5).

In some cases, hypoxic environments may be present locally, often aggregated within a small geographic area, with relatively little variance between sites in geophysical parameters. One such case of extreme environments with permanent soil hypoxia is mofettes or natural sources of CO_2, which may be terrestrial or aquatic, with geologic CO_2 outgassing locally and inducing soil, sediment, and water hypoxia. Apart from natural CO_2 springs (mofettes), hypoxia affects also a number of other ecosystems, e.g. flooded, submerged or compacted soils and sediments (Perata et al. 2011; Maček 2017a), which are specifically presented later in this book chapter, together with their fungal inhabitants.

11.3.1 Fungi in Mofettes

Terrestrial and aquatic mofettes (Fig. 11.1) are extreme ecosystems that occur in tectonically or volcanically active areas globally (Pfanz et al. 2004; Maček et al. 2016b). Mofettes are sites characterised by diffuse degassing of CO_2 of deep mantle origin (above 99%) at ambient temperature and traces of other gases, including methane (CH_4), nitrogen (N_2), hydrogen sulphide (H_2S), or noble gases. Geogenic CO_2 displaces O_2 from soil pores, so soil hypoxia or even anoxia is a common soil feature at mofettes. Being heavier than air, mofette CO_2 can accumulate in local landscape depressions, which, if large enough, can even form lakes of gases with concentrations ranging from 5% to almost 100% (Kies et al. 2015; Maček et al. 2016b, Fig. 11.1). The main characteristics of practically all natural CO_2 spring sites are high CO_2 concentrations and soil hypoxia (low O_2 concentration), while above-ground CO_2 concentrations are more variable and lower. Aquatic sites with mofettes usually exhibit subsurface CO_2 release, resulting in continuous effervescence of water (e.g. Šibanc et al. 2018; Fazi et al. 2019, Fig. 11.1). The environment of mofettes can therefore have both extremely high CO_2 levels and extremely low O_2 concentrations compared to the wider environment, in addition to low soil and water pH due to dissolved CO_2 and the formation of carbonic acid, which dramatically affects and limits the local ecology of these sites (e.g. Maček et al. 2005; Šibanc et al. 2014; Maček et al. 2011, 2016b; Beulig et al. 2016; Šibanc et al. 2018).

Mofettes can be found in several places, in Europe in Czechia (Cheb Basin), Germany (Eifel, Rhoen, Teutoburger Forest, NW Franconia), France (Massif Central), Hungary, Iceland (regions with volcanoes), Italy (Tuscany), Romania (Hargitha Mountains) and Slovenia (Radenci, Stavešinci area) (Maček et al. 2016b). Globally, they are found, for example, within the Yellowstone volcano caldera or in the Inyo crater range, in the Cascades range (United States), in geothermal fields of New Zealand (Bloomberg et al. 2012), Indonesia (Djeng Plateau), Japan and Kamchatka.

Mofettes have been used to study communities of soil microbes, with the majority of existing studies focusing on bacteria and archaea, but much less work published on soil fungi (Maček et al. 2011, 2016b; Šibanc et al. 2018). Soil gas composition in natural CO_2 springs, e.g. soil hypoxia, has a significant impact on communities of eukaryotic organisms that are predominantly obligate aerobes, such as soil fauna (e.g. Hohberg et al. 2015), plants (e.g. Maček et al. 2016b), and fungi (e.g. communities of arbuscular mycorrhizal fungi presented in Maček et al. 2011, 2016b; soil and aquatic yeasts as presented in Šibanc et al. 2018). Soil O_2 concentration was also the strongest abiotic predictor of archaeal and bacterial soil community composition in mofettes in Slovenia, while other soil factors were secondary, including concentrations of CO_2, pH, and availability of plant nutrients (Šibanc et al. 2014). Although most of the existing studies on natural CO_2 springs investigating community composition of different organisms represent single (snapshot) studies or at best a few time-points, this suggests a general and relatively stable pattern in the evolution of archaeal and bacterial communities in mofette soils. Moreover, this appears to extend to different groups of organisms, like fungi (Maček et al. 2011,

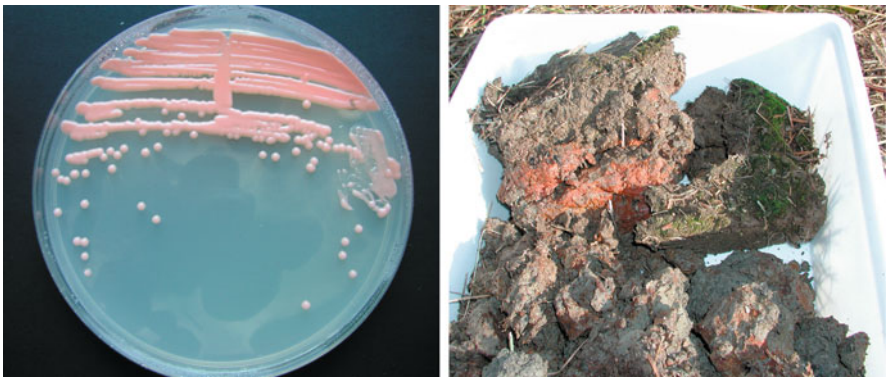

Fig. 11.3 *Occultifur mephitis* f.a., sp. nov. colonies in a Petri dish (9 cm diameter–left) and mofette soil with reductive processes (right). *Occultifur mephitis* is a newly described yeast species isolated from the Slovenian mofette soil in Stavešinci (NE Slovenia). The yeast was named after the Roman Mephitis (me.phiɕtis. L. fem. gen. n. mephitis), a goddess of gasses emitting from the soil. The photo of the yeast was taken from EXF-6436 (holotype) by N. Šibanc, the soil photo by I. Maček; see Šibanc et al. (2018) for details

2016b), with the recently described new species of yeast from soil, *Occultifur mephitis* sp. nov. (Šibanc et al. 2018) (see Sect. 3.1.1, Fig. 11.3), and invertebrates (Collembolae) (Schulz and Potapov 2010).

In contrast to more available published data on soil prokaryotes (e.g. Krüger et al. 2009; Oppermann et al. 2010; Krüger et al. 2011; Frerichs et al. 2013; Šibanc et al. 2014; Fazi et al. 2019), the diversity of fungi on mofettes is still largely unexplored (Maček et al. 2016b; Maček 2019), and some publications have reported that the majority of soil fungi are potentially excluded from mofette soil food webs due to their sensitivity to soil hypoxia (Beulig et al. 2016). Indeed, most fungi are considered aerobes, although their habitats can often be hypoxic (low in O_2) or even anoxic (no O_2), e.g. due to infiltration of soil with water or metabolic activity during infection of other organisms, in the biosphere (Simon and Keith 2008; Drake et al. 2017). However, existing studies show that diverse fungal communities also inhabit hypoxic mofette areas (Maček et al. 2011, 2016b; Šibanc et al. 2018), with fungal gene copy numbers decreasing only slightly at high CO_2 fluxes and sharply only at the most extreme sites, as reported for Spanish mofettes (Fernández-Montiel et al. 2016). There is limited published work on diversity of fungi in mofettes (e.g. Maček et al. 2011; Šibanc et al. 2018), and further research is urgently needed to understand the complex processes and ecological interactions of the soil biota in this extreme ecosystem, which include the fungal component.

In the study of soil fungi, there has been a particular focus on the ancient, ubiquitous symbiotic interaction between arbuscular mycorrhizal fungi and plants exposed to hypoxia in soil, while other filamentous groups of fungi from sites of natural CO_2 springs have not yet been investigated. There is also a single report on the diversity of yeasts in aquatic and terrestrial mofettes in Slovenia (Šibanc et al. 2018). Nevertheless, all existing studies confirm that natural CO_2 springs can be a

rich source of information on how organisms, populations and communities cope with long-term environmental stresses in their environment. There is extensive evidence that organisms in mofette fields are exposed to intense abiotic selection pressures, such as soil hypoxia (Maček et al. 2011, 2016b; Šibanc et al. 2014, 2018).

11.3.1.1 Yeasts

Recently, a report on yeast diversity from sites of natural CO_2 springs in northeastern Slovenia was published (Šibanc et al. 2018). Yeasts are known to be a group of fungi that are widely distributed and colonise both terrestrial as well as aquatic systems. In particular, yeasts are essential for ecosystem functioning in soils, as they are involved in the mineralisation of organic matter and assimilation of plant carbohydrates, as well as cycling of nutrients (Botha 2006, 2011).

The inventory of cultivable yeasts from soils (terrestrial) and water bodies (aquatic mofettes) (Šibanc et al. 2018, Fig. 11.1) includes a total of 142 isolated and identified strains from highly geologically CO_2-exposed soils and groundwater in a meadow, a forest pond and stream water. They were assigned to six yeast genera of the Basisiomicetes (6 species) and 11 genera of the Ascomycetes (18 species) (Šibanc et al. 2018). Using high dilution plating of a soil sample, 4 strains of an unknown basidiomycete species were isolated and described for the first time as *Occultifur mephitis* f.a., sp. nov. (Fig. 11.3) (based on phylogeny and phenotype criteria) (Šibanc et al. 2018). *Occultifur mephitis* did not show fermentative capabilities and was unable to grow under 100% CO_2 in anaerobic chambers (Šibanc et al. 2018). Nevertheless, it grew in an anaerobic vessel with a hypoxic atmosphere of 100% N_2 (Šibanc et al. 2018). This suggests that hypoxic conditions in soils of natural CO_2 springs likely include microenvironments with minimal O_2 supply, which are required for the survival of this species and likely others.

In Slovenia, the highest mofette yeast species richness (15 sp.) of ascomycetes was found in forest mofette water (pond with visibly bubbling CO_2) (Fig. 11.1), which contained yeast species found only in forest water: C*andida boleticola, Debaryomyces hansenii, Kazachstania exigua, Kluyveromyces dobzhanskii,* a representative of the *Metschnikowia pulcherrima* species complex (Lachance 2016), *Metschnikowia pulcherrima, Pichia kudriavzevii, Suhomyces* species and *Torulaspora delbrueckii* (Šibanc et al. 2018). Isolates identified as *Metschnikowia pulcherrima* species complex may represent another undescribed species as they differ in 11/462 nucleotide positions of D1/D2 (98% identity) from the sequence of the type strain of *Metschnikowia fructicola*. Of the taxa isolated, all ascomycetous yeasts, with the exception of *Debaryomyces hansenii*, were able to grow and ferment glucose under elevated CO_2. *Candida sophiae-reginae, Pichia fermentans* and *Candida vartiovaarae* were the dominant species in meadow and forest water with elevated CO_2 exposure. *Meyerozyma guilliermondii* and *Wickerhamomyces anomalus* dominated in highly CO_2-exposed soils (Šibanc et al. 2018, Fig. 11.3). The frequent occurrence of *Meyerozyma guilliermondii* and *Wickerhamomyces anomalus* and their in vitro ability to grow in high CO_2 and N_2 atmospheres, as well as their fermentative capacity, suggest that they might be well adapted to ecological niches characterised by elevated CO_2 and consequently decreased O_2.

The same could be true for the majority of other yeast species from the ascomycete group isolated from mofettes (Šibanc et al. 2018). Thereby, the most abundant yeasts found in the natural CO_2 spring soils of the meadow are also described in the literature as fermentative taxa (Kurtzman et al. 2011). Among the isolated yeast species, all ascomycetous taxa except *Debaryomyces hansenii*, which were able to ferment glucose, were also able to grow under elevated CO_2 (incubation under 100% CO_2 - initially). Strains that were tested and representing these species were also able to grow under 100% N_2 atmosphere (Šibanc et al. 2018). The low pH found in mofette environments with high CO_2 may also favour yeasts known to survive in environments with at least moderately reduced pH. This proves that mofette habitats provide new insights into microbial responses and adaptations to long-term changes in the abiotic soil environment and are a valuable source for the discovery of new taxa (Fig. 11.3, see also Sect. 4).

11.3.1.2 Arbuscular Mycorrhizal Fungi

A second group of fungi that has been studied intensively in mofette areas are arbuscular mycorrhizal fungi (Fig. 11.4). They were the first fungal group whose diversity and community were characterised from a CO_2 springs area, the Stavešinci mofette (Slovenia) (Maček et al. 2011). Arbuscular mycorrhizal fungi represent a ubiquitous soil group with high functional importance and form diverse communities even in hypoxic environments such as mofettes (Maček et al. 2011, 2016b). The widely distributed arbuscular mycorrhizal fungi are obligate biotrophic plant root endosymbionts. They are present in all terrestrial ecosystems and are estimated to colonise the roots of about two-thirds of plant species (Fitter and Moyersoen 1996; Brundrett and Tedersoo 2018). In addition to natural environments, they are also the most abundant fungal group in crops, which may be important for promoting sustainable agricultural practises (Helgason et al. 1998; Smith and Read 2008; Säle et al. 2015; Johnson et al. 2017). For their host plants, they are the main conduit for phosphorus uptake, and they can affect them in several other ways, including pathogen defence, improving water relations and soil structure, and micronutrient and nitrogen uptake (Smith and Read 2008; Johnson et al. 2017).

Roots of plants of several species growing in the most extreme sites in the Stavešinci mofette area (NE Slovenia) have consistently shown high colonisation by arbuscular mycorrhizal fungi, despite the persistent stress and carbon cost of colonisation (Maček et al. 2011, 2012; Maček 2013). It is not yet clear how these fungi cope, but soil mycelial growth is likely to be severely restricted in highly hypoxic soils, and it is unknown whether plants benefit from mycorrhiza in this environment (Maček et al. 2011; Maček 2017a). However, mofette fungal species are probably not subsidised by mycelium in the surrounding soil, which could explain how these fungal aerobes survive. Therefore, they must be adapted to hypoxic conditions or at least competitive, presumably either tolerating low O_2 or acquiring sufficient O_2 from roots; both explanations have profound implications for their biology (Maček et al. 2011).

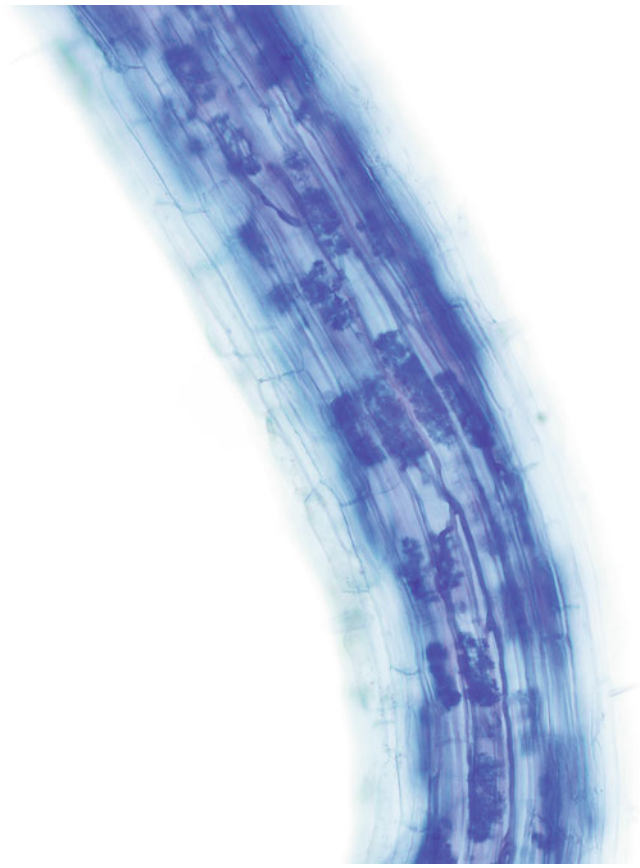

Fig. 11.4 Arbuscular mycorrhizal fungi colonising the roots of maize (*Zea mays* L.), with visible arbuscules (tree-like structures) important for nutrient exchange between the plant host and the fungi. Photo: I. Maček

Acquisition of sufficient O_2 from roots (Maček et al. 2011) is an unexplored concept of facilitation in the ecology of arbuscular mycorrhizal fungi (Maček et al. 2016b; Maček 2017a) that is relevant not only to mofette fungi (Maček et al. 2011) but also to microbes colonising plants in aquatic environments (see Sections 3.2 and 3.3, Fig. 11.5). Starting from physically extreme environments, plant-fungal interactions could therefore be extended beyond (trophic) nutrient interactions to include the additional benefit of a positive effect of one species on another, by reducing stresses in existing habitats and creating new habitats for arbuscular mycorrhizal fungi (Maček et al. 2016b; Maček 2017a).

With respect to the ecology of arbuscular mycorrhizal fungal communities, several studies suggest that under extreme environmental stress in soil, there are a small number of arbuscular mycorrhizal fungal lineages that are better able to tolerate these conditions, resulting in unique, adapted populations (Helgason and

Fig. 11.5 Water lily (*Nymphaea* sp.) and common reed (*Phragmites australis*) growing in a pond in Ljubljana, Slovenia. Some plants can grow in open water with only the floating leaves and flowers exposed to air, while rhizomes and roots with potential endophytic fungi are anchored deep in rocky sand or mud and remain constantly submerged. Plant tolerance to soil and sediment hypoxia may be directly related to aeration efficiency, which is achieved by the development of air spaces in the tissue (Justin and Armstrong 1987; Evans 2003). Water lilies, for example, grow rooted in water and mud and have a very well-developed aerenchyma of the same general type as in some *Rumex* species, termed "honeycomb" by Justin and Armstrong (1987) and classified as schizogenous (aerenchyma formed when intercellular gas spaces form during tissue development without cell death). Mature aerenchyma contains broad lacunae interrupted by diaphragms with small air spaces. Some of the diaphragms appear to be pierced by astrosclereids which presumably support longitudinal airflow (see Seago Jr et al. (2000) for more details on aerenchyma formation in wetland plants). On the other hand, in common reed (*Phragmites australis*), which can be seen in the background of the photo, the rate of gas flow from the atmosphere may be increased by wind-driven Venturi convection, which draws air into the subterranean system via dead culms cut above ground level (Videmšek et al. 2006). Photo: I. Maček

Fitter 2009; Dumbrell et al. 2010; 2011; Maček et al. 2011). Arbuscular mycorrhizal fungi form an extensive hyphal network in the soil and will therefore be subject to strong selection pressure from soil abiotic factors (e.g. Dumbrell et al. 2010; 2011; Maček et al. 2011). However, reports on community analyses and diversity studies of arbuscular mycorrhizal fungi in extreme ecosystems based on molecular studies remain scarce (e.g. Appoloni et al. 2008; Maček et al. 2011, 2016a, b; Maček 2017b). Maček et al. (2011) reported significant levels of arbuscular mycorrhizal

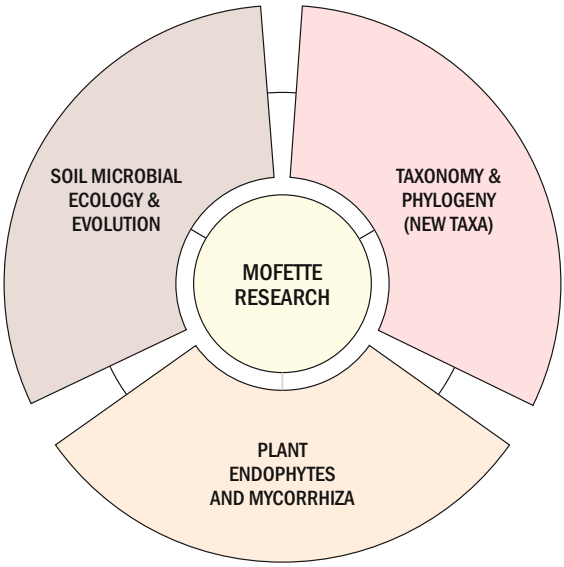

Fig. 11.6 Mofettes and research fields of applied microbial ecology for bioprospecting potential extremophilic taxa

fungal community turnover among soil types and numerical dominance of certain arbuscular mycorrhizal fungal species in hypoxic mofette soils. This work shows that direct environmental selection acting on arbuscular mycorrhizal fungi is a significant factor in regulating fungal communities and their phylogeographic patterns. Consequently, some arbuscular mycorrhizal fungi are more strongly associated with local variation in the soil environment than with the distribution of their host plants (Dumbrell et al. 2010; Maček et al. 2011). The higher temporal predictability (stability) also emerges from preliminary results on arbuscular mycorrhizal fungal communities, which suggest that under permanent (long-term) selection pressure, community composition is more constant compared to control sites (Maček et al. 2011, 2016b). The case of arbuscular mycorrhizal fungal community composition demonstrates the potential of mofettes to serve as model ecosystems to study some of the important unresolved questions in microbial community ecology (Maček et al. 2011, 2016b; Fig. 11.6). Importantly, the observed community stability at extreme sites may be more ubiquitous than currently recognised in many other environments with long-term disturbances or specific selection pressures (Maček et al. 2016b; Maček 2017b). The strong environmental gradients of mofette sites, which include extreme and lethal conditions, thus make them ideal models for further investigation of the rules of community establishment, temporal dynamics (e.g. interannual variability), facilitation (plant O_2 supply), and symbiosis in arbuscular mycorrhizal fungi, and provide insight into different pathways of plant mineral assimilation and the role of the fungal partner in this process in hypoxic environments (Maček et al. 2016b).

11.3.1.3 Other Filamentous Fungi

Apart from arbuscular mycorrhizal fungi and yeasts from the Slovenian mofette site in Stavešinci, there is only one published study reporting fungal abundance from mofette sites (Fernández-Montiel et al. 2016). Microbial communities were studied in a series of CO_2 fluxes from a natural volcanic vent in Campo de Calatrava (Spain). To assess changes in the diversity, abundance and functionality of the main groups of the soil microbiota (fungi, archaea and bacteria), the researchers used a number of different techniques, including quantitative polymerase chain reaction (qPCR), denaturing gradient gel electrophoresis (DGGE) and Biolog EcoPlatesTM. A general decrease for all variables studied was observed from control areas to high CO_2 areas. In contrast, at extreme CO_2 fluxes, bacterial and archaeal communities increased in abundance and activity but remained less diverse. The fungal community, on the other hand, showed a decrease in the number of fungal gene copies when CO_2 fluxes increased. In the case of fungi, extreme CO_2 fluxes decreased fungal gene copy number, with a significant decrease of three orders of magnitude in gene copy number in the sampling site with high CO_2 flux (Fernández-Montiel et al. 2016). No detailed data on general fungal community composition and taxa identity are available for this site.

11.3.2 Fungi in Aquatic Sediments—Submerged Environments

Sediment is an environmental substance consisting of any particulate material that can be transported by fluid flow and eventually deposited as a layer of solid particles on the bottom of a body of water or other fluid. Based on recently developed high-throughput molecular methods, datasets of fungal sequences come from both marine and freshwater samples (Panzer et al. 2015), as well as those associated with aquatic vegetation (e.g. rhizosphere of aquatic macrophytes, isoetid vegetation, mangroves) that may also represent hypoxic environments.

Molecular tools have helped to increase our understanding of fungal diversity in different marine and freshwater habitats (Pawlowski et al. 2021). They have shown that aquatic fungi are highly diverse and that they play fundamental ecological roles in aquatic systems (Panzer et al. 2015). More recently, aquatic fungal communities, including those in sediment (Orsi et al. 2013), have been shown to contribute to element cycling and mineralisation processes (Raghukumar 2012). Fungi can account for a large proportion of sediment DNA in aquatic ecosystems, but reports on their diversity in aquatic sediments, both marine and freshwater ecosystems, are still relatively scarce (e.g. Manohar and Raghukumar 2013; Panzer et al. 2015).

Environmental studies using molecular tools have revealed the presence of fungi from a wide range of marine habitats (Manohar and Raghukumar 2013; Panzer et al. 2015), such as deep-sea environments (Bass et al. 2007), hydrothermal vent ecosystems (Le Calvez et al. 2009), coastal regions (Gao et al. 2010), anoxic regions (Jebaraj et al. 2010), lakes (Monchy et al. 2011; Lefevre et al. 2012), rivers (Duarte et al. 2015), or in association with aquatic animals (Chukanhom and Hatai 2004; Amend et al. 2012), plants (Magnes and Hafellner 1991; Sakayaroj et al. 2010; Baar

et al. 2011; Sudová et al. 2015, Fig. 11.5) or algae (Zuccaro et al. 2003). However, it is not clear how many of the identified groups are true marine or aquatic fungi, as most of the clusters contain representatives from terrestrial regions (Manohar and Raghukumar 2013), which could be accidentally transferred to aquatic environments due to ecosystem connectivity.

With the intention of serving as a reference dataset for aquatic fungi, Panzer et al. (2015) used all publicly available fungal 18S ribosomal RNA (rRNA) gene sequences with the addition of new sequence data from a marine fungal culture collection and further enrichment of the dataset by adding validated contextual data (e.g. habitat type of samples assigning fungal taxa to ten different habitat categories). The combined data allowed the authors of the paper to infer fungal community patterns in aquatic systems (Panzer et al. 2015). Pairwise habitat comparisons revealed significant phylogenetic differences, suggesting that habitat strongly influences fungal community, with freshwater fungal community structure differing most from all other habitat types and dominated by basal fungal lineages (Panzer et al. 2015). For most communities, phylogenetic signals indicated clustering of sequences, suggesting that environmental factors are the main drivers of fungal community structure rather than species competition (Panzer et al. 2015). This includes potential hypoxia, which is an important abiotic driver of fungal communities in sediments. Several groups of Ascomycota, Basidiomycota, Chytridiomycota and other basal fungal lineages were reported in the sediments (Panzer et al. 2015). None of these formed a dominant group at higher taxonomic ranks. Fungi must adapt to conditions inherent to the sediment (Orsi et al. 2013), such as a strong oxygen gradient (potential hypoxia), a particular uptake capacity for nutrients and organic matter, or selective entrainment transport (Hedges et al. 1999; Avramidis et al. 2013). Therefore, fungal assemblages of sediments have been reported to differ significantly from assemblages of almost all other habitat categories (Panzer et al. 2015).

For the fungal groups associated with aquatic vegetation, the majority of studies on sediment fungi involve symbiotic arbuscular mycorrhizal fungi, but there are few reports on arbuscular mycorrhizal fungi in submerged environments associated with permanent inundation of plant root and aerial systems in continental areas (e.g. Baar et al. 2011; Kohout et al. 2012; Sudová et al. 2015) and marine environments—mangrove forests (Wang et al. 2011; Wang et al. 2015; Wang et al. 2016). Most studies on arbuscular mycorrhizal fungal diversity in submerged ecosystems (in the context of permanent inundation of plant root and aerial systems) come from aquatic macrophyte vegetation from the oligotrophic and ultraoligotrophic lakes of northern Europe (e.g. Baar et al. 2011; Sudová et al. 2015). Only now, and strengthened by newly developed molecular tools, have researchers investigated in more detail the composition of the arbuscular mycorrhizal fungal community in these specific ecosystems. Diverse arbuscular mycorrhizal fungal communities were found in the roots of the aquatic macrophyte *Littorella uniflora*, with several arbuscular mycorrhizal fungal taxa present, including taxa from the genera *Acaulospora, Archaeospora* and *Glomus* (Baar et al. 2011; Kohout et al. 2012). In addition, a new arbuscular mycorrhizal fungal species, *Rhizoglomus melanum*, was described

and isolated from the rhizosphere of aquatic macrophytes from the freshwater lake Avsjøen in Norway (Sudová et al. 2015). These plants have characteristic aeration systems (see also Fig. 11.5) that allow rapid O_2 diffusion from shoots to roots and are known to have high radial O_2 losses from their roots into the sediment (Smolders et al. 2002). Increasing plant nutrient uptake through arbuscular mycorrhizal symbioses seems to be particularly relevant for some submerged plants when nutrient availability in their environment is poor, e.g. for submerged vegetation of oligotrophic lakes in Norway (Møller et al. 2013). In the latter study, extensive extra-radical hyphal networks were found in the sediments of submerged isoetid plants (*Lobelia dortmanna* and *Littorella uniflora*) with a high mean hyphal density (6 and 15 m cm^{-3} for each plant species, respectively). This is comparable to the density typically found in terrestrial soils (Møller et al. 2013). The hyphal surface area exceeded the root surface area by 1.7–3.2 times with the highest density in the main root zone (Møller et al. 2013).

Arbuscular mycorrhizal fungi appear to be dependent on the high O_2 concentrations in the roots and surrounding root zones of aquatic plants (Wigand et al. 1998, Fig. 11.5), and this appears to be a consistent and important component of arbuscular mycorrhizal fungal habitats in hypoxic soils. Interestingly, in the context of an extreme environment, this means that some plant species modify conditions sufficiently to make life more hospitable to others that would otherwise be unable to survive in that environment. The concept is known in the plant literature as facilitation and is used to refer to beneficial (non-trophic) interactions that occur between physiologically independent plants and are mediated by changes in the abiotic environment (Brooker and Callaway 2009). The cross-trophic and cross-system interactions in this concept of facilitation are still challenging a current working definition of facilitation that is limited to plant-plant interactions, mostly in terrestrial environments (Brooker and Callaway 2009; Maček et al. 2016b).

Arbuscular mycorrhizal fungi are also common in mangrove forests (Radhika and Rodrigues 2007; D'Souza and Rodrigues 2013). Molecular analyses of arbuscular mycorrhizal fungal communities in the roots of mangrove trees were not conducted in a study of 16 mangrove species on riverine and fringing habitats in Goa, West India, (D'Souza and Rodrigues 2013). However, in a second study conducted in four semi-mangrove plant communities from the Qi'Ao Mangrove Forest Reserve in southern China, arbuscular mycorrhizal fungal taxa were determined by molecular approaches (e.g. Wang et al. 2011; Wang et al. 2015). The authors report six operational taxonomic units (OTUs) from the family Glomeraceae that could not be identified to the genus level and may represent new taxa, which is a common feature in hypoxic environments (see Sudová et al. 2015). A second group of fungi associated with marine aquatic vegetation belongs to the dark septate endophytes (DSE), culturable root mycobionts of the seagrass *Posidonia oceanica*. In *Posidonia*, roots have been reported to be colonised with new, undescribed taxa of DSE (Vohník et al. 2016). Therefore, the observations from seagrass, mangrove forests together with the new arbuscular mycorrhizal fungal species described from oligotrophic lakes suggest that submerged environments are still a rich potential source of new fungal taxa yet to be discovered.

11.3.3 Flooded Soils

As predicted by climate change models, the frequency and severity of flood events will increase dramatically in the future, with the greatest increase predicted by climate change models for the tropics and Western Europe (Hirabayashi et al. 2013). Therefore, to promote sustainable agriculture in the future, understanding the response of different organisms to soil hypoxia, including the interaction of crops with soil organisms, including symbiotic arbuscular mycorrhizal fungi, is becoming increasingly important (Maček 2017a). Despite the fact that temporarily flooded soils are not considered an extreme ecosystem but rather an environmental stress event, studies on arbuscular mycorrhizal fungal communities conducted in flooded soils show similar results as for submerged environments (see Sect. 3.2). For example, flooded rice fields and rice plants in general are one such system where considerable progress has been made in the interaction between plants and arbuscular mycorrhizal fungi and their joint response to flooding (e.g. Vallino et al. 2014). The latter includes adaptive traits such as the ability to form aerenchyma or other systems for O_2 transfer to roots. Aerenchyma are typical adaptive traits of wetland plant species (Fig. 11.5), but can also develop in response to flooding or mineral deficiency (Marschner 2012). The results of the Vallino et al. (2014) study on rice show that under flooding conditions arbuscular mycorrhizal fungal nutrient transporters are regularly expressed, but the functional markers of arbuscular mycorrhizal symbiosis show a significant decrease in the expression of plant and fungal nutrient transporters as flooding progresses (Vallino et al. 2014).

11.3.4 Compacted Soils

Apart from flooded and inundated substrates, hypoxic or even anoxic environments can also be found in other habitats, such as microenvironments in soil aggregates, subterranean borrows, and compacted soils. Here, hypoxia may be permanent or, more commonly, intermittent (Hourdez 2012). Due to the use of heavy field equipment and field traffic, soil compaction is common in agricultural fields, and soils are particularly susceptible to soil compaction in wet conditions. In addition to directly affecting soil structure, soil compaction can also affect roots (growth and activity) and soil microbial functions. Soil porosity is an important factor affecting soil aeration. Soil compaction reduces gas exchange in the soil through changes in pore space size and distribution and soil strength. When soil particles are compressed, the pore space between them is reduced, therefore compacted soils lack large pores (DeJong-Hughes et al. 2001), and because the pore space in the soil is reduced, bulk density increases. This can lead to local hypoxia or anoxia in the soil and affect soil life, including soil fungi.

11.4 Frontiers in Research of Fungi in Hypoxic Environments

In particular, long-term hypoxic soils (e.g. mofette soils) and submerged (aquatic) environments (e.g. hypoxic sediments) show much potential for further research on fungal biology in hypoxic environments in various fields, from soil ecology and biodiversity research to bioprospecting for new extremophile taxa (Fig. 11.6). The latter may have great potential for biotechnological applications, the study of hypoxia-tolerant human pathogens, and others. In the following, recent and future advances in the study of hypoxic soil and water environments and potential applications are described, with a view to further developments in this field in the future.

11.4.1 Bioprospecting for New Taxa

Extreme environments can serve as novel study systems to investigate how long-term abiotic selection pressures drive natural communities and their evolution, potentially leading to new specialised taxa (e.g. new yeast species *Occultifur mephitis* isolated from Slovenian mofettes, Šibanc et al. 2018). Different fungal groups have been studied at different depths in hypoxic environments. For example, reports on any aspect of the important and ubiquitous symbiotic arbuscular mycorrhizal fungal biology from extreme habitats or hypoxic environments are relatively scarce (Maček et al. 2011; Maček 2017a, b; Drake et al. 2017), whereas other fungal groups (e.g. yeasts) have been studied more extensively in extreme environments (see e.g. Cantrell et al. 2011; Rangel et al. 2018). Isolation of hypoxia- or stress-tolerant microbes and microbial communities can have great potential in biotechnology (e.g. new drug discovery). Since many biotechnological applications, such as industrial fermentation, require the ability to grow in high CO_2, low O_2 environments, mofettes (natural CO_2 sources), for example, along with other hypoxic soils and substrates, are likely ideal sites for bioprospecting for industrially important microbes (Figs. 11.1, 11.2, 11.3, and 11.6). To date, apart from initial studies on arbuscular mycorrhizal fungi and yeasts (Maček et al. 2011, 2016b; Šibanc et al. 2018), there are no reports on the diversity, ecology or function of fungi from mofette sites, while the biotechnological and medicinal potential of mofette sites and their biota remains largely unknown and unexploited, and similar is the case for submerged soils and aquatic sedimentary fungi.

In relatively recent reports, some endophytic fungi have also been isolated and identified from hydrophites. For example, more than 200 isolates of endophytic fungi of *Nymphaea* spp. (Fig. 11.5) have been reported growing at various sites in India and Thailand (e.g. Rajagopal et al. 2018; Supaphon et al. 2018), showing some antimicrobial activities, but there are no reports on the rhizosphere and root endophytes of water lilies.

11.4.2 Exploration of Human Fungal Pathogens in Hypoxic Environments

Since most eukaryotic human fungal pathogens are generally considered obligate aerobes, O_2 availability during fungal pathogenesis may play a critical role in the outcome of infection from the perspective of both the human host and the fungus (Grahl et al. 2012). Among the most resistant human fungal pathogens are hypoxia- and anoxia-tolerant microbes. In healthy tissues in the human body, O_2 levels of 2.5% to 9% are considered normal, whereas oxygen levels of 1%, as described in wounds and tumours, are considered hypoxic (Arnold et al. 1987; Simmen et al. 1994; Dewhirst 1998; Nizet and Johnson 2009). In the context of microbial pathogenesis, hypoxia is generally thought to occur at sites of infection and represents significant environmental stress for most host and microbial pathogen cells (e.g. Cramer et al. 2003; Peyssonaux and Johnson 2004; Nizet and Johnson 2009). The most common fungal pathogens that may be exposed to an oxygen-limited or even hypoxic microenvironment during fungal pathogenesis include *Candida albicans*, which is commonly found in the gastrointestinal tract, which contains significant regions of hypoxia (He et al. 1999; Karhausen et al. 2004), and *Cryptococcus neoformans*, which causes cryptococcal meningitis and is also exposed to reduced O_2 levels during infection in the human brain (Erińska and Silver 2001; Sharp and Bernaudin 2004). Human disease in immunodeficient individuals can be caused by various moulds typically found in soil and decaying organic material, such as *Aspergillus fumigatus* and *Fusarium oxysporum*, when O_2 levels in the tissues of their hosts are very low (Grahl et al. 2012). Although most moulds are traditionally considered obligate aerobes, *Aspergillus fumigatus* has been observed to tolerate O_2 levels as low as 0.1%, and some studies even suggest that *Aspergillus fumigatus* can survive and grow anaerobically (Tabak and Cooke 1968; Hall and Denning 1994). Moreover, *Fusarium* species appear to be particularly adept at tolerating hypoxic and even anoxic conditions, consistent with their resident ecological niche in the soil (Gunner and Alexander 1964; Hollis 1948). Therefore, soil environments where O_2 levels are low (e.g. submerged, flooded, compacted soils and mofettes) or can change rapidly with microbial metabolic activity (e.g. in compost piles) may be a source of human pathogenic fungi adapted to hypoxia (Grahl et al. 2012). Therefore, research on hypoxic environments can identify and further investigate potential risks for hypoxic habitats in nature that serve as reservoirs for pathogens.

11.4.3 Natural Long-Term Experiments in Ecology

Many natural phenomena and ecological processes occur very slowly, so that long-term observations and experiments are necessary to study them (Franklin 1989). However, the latter are still largely lacking for many microbial groups. Soil microbes are a key driver of many vital biogeochemical cycles, with soil being one of the most biodiverse habitats on the planet (Fitter 2005). Changes in the population density of

soil microbes in response to long-term environmental factors have the potential to influence plant community productivity and human health.

A natural study site with the potential to provide better predictions of ecosystem impacts due to induced long-term environmental change is mofettes, which can serve as natural long-term experiments in evolution and ecology (Maček et al. 2016b). Supported by advances in sequencing technologies in recent years (e.g. Dumbrell et al. 2016), more and more studies are also focusing on the subsurface. Not only the composition of soil communities, but also the various interactions (networks) that occur between taxa help monitor the response of taxa interactions to human changes in the environment, which is critical for ecosystem conservation (Vacher et al. 2016). Ecological networks are now becoming a standard method for representing and simultaneously analysing interactions among taxa (e.g. Coyte et al. 2015; Vacher et al. 2016; Tylianakis and Morris 2017). However, ecological networks in soils remain largely unknown. For example, the reduction of the soil community to the microbial component that tolerates permanent soil hypoxia induced by geogenic CO_2 makes mofettes a valuable model environment for studying diversity effects on specific soil functions. Indeed, mofettes are characterised by permanent exclusion of higher trophic levels and associated physical and ecological features from local food webs, as most eukaryotes require an aerobic environment (Maček et al. 2016b). Because a significant portion of the specific ecology of these systems is microbial, mofettes provide an ideal opportunity to explore network-based approaches to incorporate next generation sequencing-based data into the ecology of food webs in hypoxic environments (e.g. Maček et al. 2016b; Vacher et al. 2016).

Importantly, questions about long-term changes in soil microbial communities are relevant not only to the study of hypoxia as a stressor, but also to many other long-term anthropogenic drivers, including nutrient inputs, soil pollution, land-use change, and more (e.g. Maček et al. 2016a, b; Maček 2017b). The study of mofettes may be one of the ways to increase the knowledge of microbial and fungal ecology under long-term environmental changes, in this case long-term hypoxia.

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Chaotolerant Fungi: An Unexplored Group of Extremophile

12

Sanjay Sahay

Abstract

Chaophilic fungi constitute a small group of very special fungi that can complete their life cycle in the presence of macromolecule-destabilizing chemicals known as chaotropes, for example, $MgCl_2$, $CaCl_2$, etc. They, as concluded from the study of a limited number of isolated fungi of this group, exhibit halotolerance/halophilic and xerotolerance characters simultaneously. There is hardly any information about the unique physiological mechanism of chaotropicity that differentiates them from halotolerants. But the harsh conditions they are exposed to seem to be favorable for the evolution of exotic biomolecules in them. Thus, they may be potential sources of novel molecules of human uses.

Keywords

Chaotropicity · Water activity · Halotolerance · Kosmotropes · Osmotic adaptation

12.1 Introduction

Fungi inhabiting extreme environments such as extreme temperatures, pH, metal concentration, pressure, desiccation, and salinity are extremophilic fungi (Macelroy 1974). Of these, halophiles requiring >3% NaCl for growth (Wilson and Brimble 2009) and halotolerant are ecologically very important as they occupy and perform ecological functions in an osmotically very hostile environment such as sea and other saline waterbodies.

S. Sahay (✉)
Sarojini Naidu Government Postgraduate Girl's (Autonomous) College, Bhopal, Madhya Pradesh, India
e-mail: ss000@redffmail.com

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The saline water is broadly divided into two types, viz., NaCl-rich thalassohaline and $MgCl_2$- and $CaCl_2$-rich athalassohaline. Fungi have been reported in large numbers from thalassohaline water, but very limited efforts have been made to isolate them from athalassohaline environment (Sonjak et al. 2010; Oren and Gunde-Cimerman 2012; Zajc et al. 2014). The fungi isolated from bittern brines of solar saltern (Sonjak et al. 2010) contrasted earlier belief that chaotrope-rich saline water is devoid of life (Javor 1989). Chaotolerant fungi have also been reported from littoral Anchialine caves golubinka and medova buža (Croatia) (Chlebicki and Jakus 2019) and indoor air (Chlebicki et al. 2018).

The chaotolerant fungi from highly life-limiting environment are bioresources important from both ecological and biotechnological points of view.

12.2 Chaotropicity

The chaotropes (Greek chaos = disorder, tropes = behavior) consist of compounds that destabilize macromolecules. Kosmotropes, on the other hand, are compounds that provide stability to the macromolecules. As to its mechanism of action in living systems, there are two possible theories available. One theory believes it acts via breaking water (solvent) structure breaker vis-a-vis kosmotrope (water structure maker) (Collins 1997). Another theory relies on direct action of chaotropes on macromolecules via structural rearrangement of bulk liquid (Ball and Hallsworth 2015). They may extract water or take away hydrogen bonds and destabilize folding patterns, thus destroying the two-dimensional structure of biomolecules, or they may also enter hydrophobic parts, leading to their swelling or solubilization (Ball and Hallsworth 2015). Chaotropes such as ethanol may cause oxidative (Russo et al. 2001; Bhaganna et al. 2016) and osmotic stress (Cray et al. 2015). The results of direct action may be degradation of cell organelles such as membrane and ribosome, which is fatal to the organisms (Hallsworth et al. 2003; Duda et al. 2004; Lo Nostro et al. 2005; Bhaganna et al. 2016).

12.3 Chaotropicity Versus Water Activity

$MgCl_2$ is a highly water-soluble salt that exhibits chaotropicity as well as water activities. It has been used to test whether water activity or chaotropicity is more important a limiting factor to impact survival. Applying metagenomic technique, presence of mRNA (an indicator of life) has been tested along the $MgCl_2$ gradient of sea water (Hallsworth et al. 2007). It was found that life was available below 2.3 M $MgCl_2$ in the absence of kosmotroph. In pure culture, the highest $MgCl_2$ concentration that can allow growth was found 1.26 M at which corresponding water activity and chaotropicity values are 0.916 aw 26.1 kJ g^{-1}, respectively. Since the lowest value of water activity still allowing growth is 0.61 (equivalent to 3.7 M $MgCl_2$), explicitly indicates that it is the chaotropicity of $MgCl_2$ that limits life and not the water activity (Hallsworth et al. 2007). This finding was further verified using

environmental xerophytic fungi and chaotropic solutes glycerol and fructose (Williams and Hallsworth 2009). It was found that the xerophiles could not grow on fructose added medium (0.760 a_w), despite the water activity value (a_w) being above the minimum growth supporting one, viz., $\leq$ 0.710 a_w (Pitt and Christian 1968). Chaotolerant organisms for this reason are adapted to even more life-limiting conditions than halophiles and xerophiles.

12.4 Chaophilic Versus Chaotolerant

A very limited number of halophilic bacteria, archaea, and fungi tolerant to a higher concentration of kosmotropic (NaCl, KCl, and $MgSO_4$) and chaotropic (NaBr, $MgCl_2$, $CaCl_2$) solutes have been reported. There is also overlapping between halophiles and chaophiles; indeed, all chaophiles at the same time are halophiles (Zajc et al. 2014). There are halophilic species and strains that require NaCl as growth requirement (Gunde-Cimerman et al. 2009), but there has been no obligate chaophile reported so far. The most chaotolerant fungal taxa *Wallemia* sp. (e.g., *Wallemia mellicola*), *Talaromyces diversus,* and *Hortaea werneckii* can well grow on salt non-supplemented medium (Chlebicki et al. 2018), though *W. ichthyophaga* was reported to need 10% NaCl as a growth requirement (Zalar et al. 2005; Zajc et al. 2014). The reason may be a general tendency among microbiologists to use NaCl supplemented medium to isolate microbes from the saline environment. Hardly any dedicated search for chaotropic microbes has been made (Zajc et al. 2014), thus all the fungi so far reported as chaophiles are more appropriately the chaotolerant.

12.5 Chaotolerant Fungi

As chaotolerant microbes, fungi exhibit an edge over archaea since in the presence of the highest concentration of $MgCl_2$ (without compensating kosmotropes) the latter after 18 months of cultivation could continue to grow was 1.26 M (Hallsworth et al. 2007), which is lower than 1.5 M that the fungi could tolerate without compensating NaCl (Hallsworth et al. 2007). The majority of fungi show a preference for a relatively high concentration of kosmotropic salts, only a few of them having the ability to tolerate higher concentrations of chaotropic salts.

The first fungus *Xeromyces bisporus* capable of growing at the lowest water activity (Pitt and Hocking 2009) was described as exhibiting preference to chaotropic conditions/solutes and growth in the presence of high concentration (7.6 M) chaotropic glycerol (Williams and Hallsworth 2009). The fungus has also been tolerant to a very high concentration of kosmotropic salts (Pitt and Hocking 2009).

In one study, isolation of chaophilic fungi from bittern brines (Sonjak et al. 2010) of the Secovlje salterns (Slovenia) led to the identification of filamentous fungi, *Cladosporium* sp., black and other yeasts that were able to grow at higher

Table 12.1 Chaotolerant fungi and their tolerance to highest salt concentration (M) (Zajc et al. 2014)

	Kosmotropic salts			Chaotropic salts		
Fungal species/strains	NaCl	KCl	$MgSO_4$	$MgCl_2$	$CaCl_2$	NaBr
H. werneckii	5.0	4.5	3.0	2.1	1.7	4.0
Aureobasidium spp.		4.0	3.0	<1.5	≤ 1.2	
Cladosporium spp.	2.5–4.0	2.5–4.5	2.0–3.0			
C. tenuissimum EXF-1943						
C. Cladosporoides EXF-1824				2.0–2.1	1.7	
Wallemia ichthyophaga	>4.0	Sat	Sat	–	–	4.0
W. Ichthyophaga EXF-994				2.1	< 1	
Eurotium, *Emericella*						
Aspergillus and *Penicillium*	> 3.0	3.5	2.0	> 1.5	1.2	2.5

concentrations of $MgCl_2$ (1.5 M) (Sonjak et al. 2010), higher than that (1.26 M $MgCl_2$) reported earlier (Hallsworth et al. 2007).

In another survey, halotolerant rock-inhabiting fungi were isolated from Golubinka and Medova Buža littoral anchialine caves in Croatia (Chlebicki and Jakus 2019). There were six halotolerant isolates, viz., *Cladosporium psychrotolerans*, *C. delicatulum*, *Mucor circinelloides*, *Rhizopus stolonifer*, *Aureobasidium pullulans* var. *pullulans*, and *Talaromyces diversus* isolated of which *T. diversus* was found to be chaophilic as well (Chlebicki and Jakus 2019). *T. diversus* could thrive at 24% NaCl and 16% $MgCl_2$, the tolerance being more than that exhibited by *Wallemia mellicola*.

Another dedicated study on the tolerance of several halotolerant and/or xerotolerant fungal strains to chaotropic solutes resulted in identification of 37 strains of fungi 11 belonging to *Hortaea werneckii* and *Wallemia ichthyophaga* that can thrive in 1.8 M $MgCl_2$ concentrations (Zajc et al. 2014). Though both could tolerate the highest concentration of $MgCl_2$, *H. werneckii* could but *W. ichthyophaga* could not tolerate the highest concentration of $CaCl_2$. The study revealed chaotropic preferences of several known halotolerants such as *H. werneckii*, *Aureobasidium* sp., *Cladosporium* sp., *Wallemia* sp., *Eurotium, Emericella*, *Aspergillus*, and *Penicillium*. All of them were tolerant to higher concentration of kosmotropic salts and could grow in the presence 1.5 M $MgCl_2$ and 1.2 M $CaCl_2$ (Table 12.1). Some yeasts, provisionally named *Candida atmosphaerica*-like and *Pichia philogaea*-like, have also been isolated from the $MgCl_2$- rich athalassohaline habitat (bitterns) (Butinar et al. 2005).

12.6 Ecology

There is not much information about the various habitats that the chaophilic fungi occupy. However, they have been reported from solar saltern (Sonjak et al. 2010) and saline waterbodies containing a high concentration of chaotropes especially

$MgCl_2$ such as Dead Sea (Oren and Gunde-Cimerman 2012), etc. The ecological role the chaophilic fungi play in such areas with very limited life forms has been only scarcely explored. The chaotoleranr fungi, for example, *Talaromyces diversus,* have been found to produce mitorubrinic acid (Yilmaz et al. 2014) that can dissolve carbonate for, for example, limestone and thus seem to have an ecological role in lithosere (Chlebicki and Jakus 2019).

12.7 Physiology

There is hardly any dedicated study on the physiology of chaotolerant fungus. Since all chaotolerant fungi are at the same time halophilic, they should share many physiological adaptations (osmotolerance) of halophiles. From a comparative study on the salt tolerance mechanism in *H. werneckii*, a halotolerant fungus, and *W. ichthyophaga*, a halophilic fungus, for example, it has been reported that both accumulate small organic molecules (compatible solutes, e.g., glycerol) to balance the osmotic pressure of surroundings and avoid high concentration of intracellular toxic salts (NaCl, etc.) (Plemenitaš et al. 2014). Cells do have the option to use efflux and influx systems to discard surplus ions. The alkali-metal cation transporters in this wake become important for osmotic adaptations to highly saline conditions. For example in yeast, the Na^+-exporting ATPase (EnaA) is a major mechanism to tolerate salt (Ariño et al. 2010). Thus, the absence of EnaA in *X. bisporus* genome may be responsible for its poor growth in the presence of salt (Leong et al. 2014), and the presence of the same in multiple copies in *W. werneckii* (Lenassi et al. 2013), and differentially expressed in *W. ichtyophaga* (Zajc et al. 2013) impart them higher tolerance to salinity. In *W. ichthyophaga* cation-transporter genes are present in low number and passive barriers play a crucial role in providing it tolerance against high salinity conditions. Some of such barriers are unusually thick wall, clumping of cells, and the cell wall proteins, hydrophobins, which is expressed under saline condition (Zajc et al. 2013). Fungi may also apply such passive strategies as clumping of cells (Kralj Kunčič et al. 2010), painting extracellular polysaccharides over cell wall or thickening of cell wall (Kralj Kunčič et al. 2010), and pigmenting/melanizing the cell wall (Kogej et al. 2006).

Halotolerant or halophilic fungi employ high osmolarity glycerol (HOG) signal pathway to sense and respond to salt/osmolyte-led stress (Gostiňcar et al. 2011). The activation of this pathway leads to the production of glycerol, the latter then plays an active role in restoring the cell's osmotic balance (Hohmann 2009). The channels available in the cells are utilized to eject or take in glycerol as per requirement (Ferreira et al. 2005). This strategy thus enables fungi to enjoy considerable flexibility in adapting to salinity.

The comparative study on the HOG pathways present in *H. werneckii* and *W. ichthyophaga* by which both of these fungi synthesize glycerol and few other compatible solutes after exposure to high salinity environment has revealed that the key proteins of HOG pathway are conserved in both, but there is a difference in regulation (Plemenitaš et al. 2014). While the Hog1 kinase of *H. werneckii*

phosphorylates when the extracellular salinity is ≥3 M NaCl (Turk and Plemenitaš 2002), that of *W. ichthyophaga* remains phosphorylated constitutively under normal osmotic conditions and gets dephosphorylated under hyperosmotic or hypo-osmotic conditions (Konte and Plemenitaš 2013). Thus, Hog1 in *H. werneckii* may exercise differential activation or repression of osmoresponsive genes or regulation of chromatin and RNA polymerase II via physical interaction with them under salinity stress (Vaupotič and Plemenitaš 2007). The kind of the Hog1 regulation in *H. werneckii* seems to be compatible with its extreme halotolerance, the constitutive expression (phosphorylation) of the Hog1 in *W. ichthyophaga,* on the other hand, might be responsible for its obligate halophilicity. Furthermore, while all the HOG pathway components in *H. werneckii* are present in at least two copies, in *W. ichthyophaga* only Hog1 is present in two isoforms (Plemenitaš et al. 2014).

12.8 Biotechnological Potential

Over the past many decades, injudicious agricultural practices and global climate change have led to much deterioration of soil making it stressful for plants and microbes (Thomas et al. 2005; Seager et al. 2007). Chaotolerant fungi may be augmented to chaotrope (e.g., urea, NH_4NO_3, phenol, $MgCl_2$, $CaCl_2$)-polluted agriculture soil to restore microbial activity, leading to structure-fertility enhancement (Williams and Hallsworth 2009).

Pollution in the environment (land and water) especially with chemicals with chaotropic activities of xenobiotics is a challenge to microbes (Hallsworth et al. 2003). Fungi with chaotolerance and bioremediation potential will be boon to remediate such environments. The process can be more efficiently carried out at low temperature as low temperature prevents disordering impact of chaotropes on cellular structures (Hallsworth et al. 2003).

Chaotolerant fungi do show xerophytic feature, that is,. they have better survival ability under low water activity conditions. Food protection from food spoiler microbes is another area that may benefit from the work on chaotolerant fungi. A deep insight into the survival mechanisms under these conditions (packaged food products contain chaotropic stressors like sodium benzoate and are maintained under low water activity) will be useful in eliminating food spoiling microbes/fungi.

The human pathogen *Wallemia mellicola* (Chlebicki et al. 2018) is a chaotolerant fungus. Does chaotolerance provide this fungus with any pathogenic advantage? Such a conclusion needs a dedicated study. Although literatures are available in abundance on the biotechnological importance of whole cells and their products from halophilic fungi, there is hardly any data about the application of chaotolerant fungi per se.

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Xerophilic Fungi: Physiology, Genetics and Biotechnology

13

Sanhita Sarkar, Namita Ashish Singh, and Nitish Rai

Abstract

Xerophilic fungi are the distinctive organism which can grow under conditions of reduced water activity. The present work highlights the physiological adaptations of xerophilic fungi which include osmoregulation through membrane modifications, osmosensors-mediated sensing of low water activity (aw) and utilisation of alternate substrates, namely, salt and sugar. We have also covered the three unique strategies, namely combative, stress and ruderal, which is helpful for their survival in unfavourable conditions. In this chapter, we have tried to cover the molecular mechanism along with the genes expression responsible for the adaptation of xerophilic fungi under water stress conditions. Further, this chapter covers the various bioactive compounds produced by xerophilic fungi along with their potential bioactivity. In the last section, we have discussed the various aspects of xerophilic fungi such as enzyme and pigment production, air biofiltration, biodeterioration in museums and libraries, etc. We have also covered the health risks associated with the xerophilic fungi, namely fungal infections, food spoilage and mycotoxin production.

Keywords

Xerophilic · Physiology · Adaptation · Biodeterioration · Food spoilage

S. Sarkar · N. Rai (✉)
Department of Biotechnology, Mohanlal Sukhadia University, Udaipur, Rajasthan, India
e-mail: nitish.rai@mlsu.ac.in

N. A. Singh
Department of Microbiology, Mohanlal Sukhadia University, Udaipur, Rajasthan, India

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13.1 Introduction

Several microorganisms have the abilities to survive under low availability of water, but relatively few have become skilled at it through physiological adaptations that enable them to thrive under such an environment. One way in which the water availability could be expressed is water activity (aw), which is the ratio of the vapour pressure of water in a sample to that of pure water in the sample (aw = 1). Therefore, aw of pure water is 1.00 and as the free-water content of substrate drops so does its aw. Alternatively, water potential or Ψ, the sum of osmotic, matrix and turgor potentials, is also used as a measure whose units are in Pascal (Pa); and it is related to aw by formula $Water\ potential(\Psi) = RT/V\ log_n aw(+P)$; where R is the ideal gas constant, T the absolute temperature, P the atmospheric pressure and V the volume of 1 mole of water.

The fungi which can grow and divide in a condition of reduced water activity, that is, below 0.85 aw, is known as Xerophilic fungi. These fungi grow on a dried and concentrated substrate where there is low water available in the matrix due to the presence of high concentrations of soluble solids like salts and sugar (Su-lin et al. 2011; Rico-Munoz et al. 2019). Usually, these fungi produce solutes like glycerol to create an optimum osmotic pressure for reproduction and growth. The physiological adaptations in these fungi involve osmoregulation via membrane modifications, osmosensors-mediated sensing of low aw and utilising alternate salt and sugar substrates that unfortunately causes spoilage in a variety of processed food. The genera that come under xerophiles include *Eurotium, Penicillium* and *Aspergillus* species.

In this chapter, xerophilic fungi with their different aspect relating to physiology, genetics, adaptation, important bioactive secondary metabolites and biotechnological importance are discussed. The chapter attempts to discuss the molecular players which help the xerophilic fungi to adapt to the water stress by modulating the expression of various genes. Further, the chapter discusses the role of xerophilic fungi in various areas such as enzyme production, air biofiltration, food industry, museum, environment and health.

13.2 Overview of Xerophilic Fungi

In general, depending on the physiological characteristics, the fungi are classified into various categories such as thermophiles/psychrophiles (the ability to grow in high/low temperature), alkaliphiles/acidophiles (grow in high/low pH), halophiles (grow in high salt concentrations), oligotrophic (grow in low availability of nutrients), piezophiles (grow in high pressure) and radiophiles (grow in high radiation) and xerophiles (grow in low aw) (Abe and Horikoshi 2001; Rothschild and Mancinelli 2001; Van den Burg 2003). In nature, some environments are associated with one or more extreme condition and microorganisms inhabiting them are known as polyextremophiles (Rothschild and Mancinelli 2001).

The term "Xerophilic" is derived from Greek, meaning dry loving. Xerophilic fungi may be classified into two groups (Pitt and Hocking 2009), namely, moderate xerophile and extreme xerophile. Moderate xerophile, like *Paecilomyces variotii, Aspergillus pseudoglaucus, A. chevalieri, A. glaucus, A. montevidensis, A. ruber,* and *Penicillium* spp., are those that can grow at or below 0.85 aw values, and the extreme xerophiles are those filamentous fungi that either get completely inhibited under high aw or grow very slowly. Due to such physiology, moderate xerophiles are notably responsible for food spoilage (Pitt and Hocking 2009; Rico-Munoz et al. 2019). Some studies have also shown that xerophilic fungi may have a dominant existence in their habitat by producing toxic metabolite induced by a stress environment (Cray et al. 2013).

There are diverse groups of microorganisms present in the milieu of reduced aw including archaea, eubacteria, algae, cyanobacteria, yeasts and filamentous fungi. Most of these groups have species thriving in a highly saline environment, but only the yeasts and filamentous fungi grow effectively under a high sugar environment. Also, a far greater variety of fungi has been isolated from habitats with water activities below 0.85 as compared to other groups combined.

13.3 Physiology and Adaptation

A constant supply of water is inevitable to maintain normal cellular functioning for all life forms; however, often life is also found to thrive in minimal water conditions. Since all life processes take place in solution form, therefore the physiological adaptations used by the xerophilic fungus to adjust to osmotic alterations and aw below 0.85 are quite fascinating. In fact, in terrestrial ecosystems both abiotic and biotic factors spatiotemporally influence fungal activity, making the structure of fungal community and dominance possible.

Although certain disturbances are transient, some are permanent features of a habitat and communities inhabiting them employ three primal strategies and their combinations as secondary strategies to survive and even flourish in these unfavourable conditions. These strategies are known as *combative, stress* and *ruderal* strategies. While *combative* or C-selected strategies ensure maximum exploitation of resources in relatively ordinary conditions, *stress* or S-selected strategies involve the development of adaptability, resilience and endurance required for survival in continuous stress. *Ruderal* or R-selected strategies encourage a short life span but high reproductive potential enabling success in severely chaotic and poorly distributed nutrient conditions. Intercalation of these primary strategies in different combinations gives rise to secondary survival strategies that in continuum maximise survival potential of chaophilic organisms (Fig. 13.1).

Life exposed to low aw circumstances needs to check water loss and prevent desiccation by osmosis. A certain level of turgor pressure needs to sustain in order to support life. Two basic strategies followed are (1) achieving osmotic stability by using counter-balancing levels of inorganic ions (usually KCl) and (2) synthesis and accumulation of compatible solutes (a low molecular weight organic, polar,

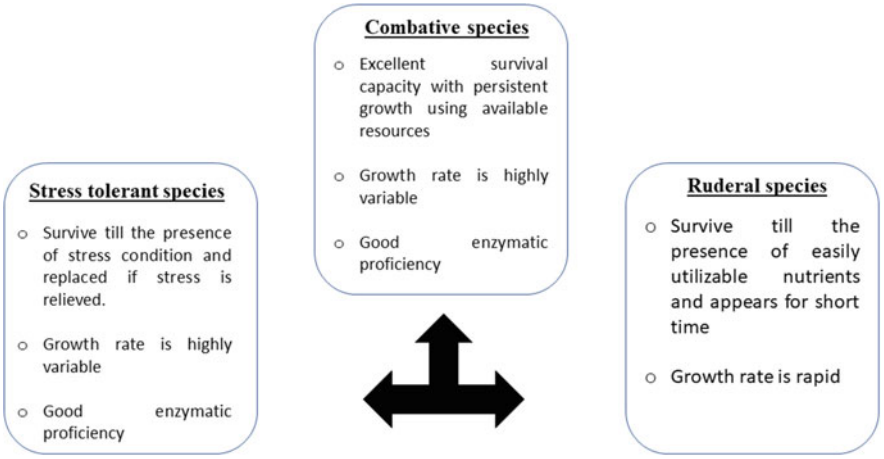

Fig. 13.1 Characteristic comparison of fungi in relation to the three main ecological strategies

uncharged osmolyte). These compatible solutes can easily pass through biomembranes and become zwitterionic in intra-cellular environments, which can make up for osmotic potential. The characteristic feature of compatible solutes includes some common structural motifs which may be poly hydroxyl groups, sugars, amino acid and their derivatives. Some suitable compatible solutes are polyols such as glycerol, arabitol, mannitol; sugars like trehalose and sucrose; and sugar derivatives like sulpho-trehalose and glucosyl-glycerol; betaines (trimethylammonium compounds) and thetines (dimethyl sulphonium compounds); amino acids, including proline, glutamate and glutamine; *N*-acetylated amino acids such as *N*-acetylornithine; glutamine amide derivatives such as *N*-carbamoylgutamine amide; ectoines, notably ectoine and β–hydroxyectoine. Fungi and yeasts have been found to make use of these compatible solutes for survival in low aw (Brewer 1999). Glycerol, being the smallest of sugar alcohols, plays a pivotal role in osmoregulation and combats the greatest reduction in aw on a molar basis compared with the other sugar alcohols accumulated by fungi during osmotic stress.

Growth at low aw requires continuous maintenance of cell turgor potential. However, what is noteworthy is that optimum growth is influenced not only by aw or turgor potential but also by interactions of other environmental factors like temperature, pH and gas composition (Magan et al. 2004). A shift towards high water potential affects all vital cellular processes such as nutrient uptake, protein biosynthesis and enzyme activities. To enable normal cell functionality, compatible solutes that can protect hydrated biopolymers and allow structural integrity under low-water potential conditions are used. Multiple physiological adaptations have been reported in different xerophilic fungi to combat physiological stresses like low aw, disturbed turgor pressure and high osmotic potential. One such physiological adaptation is modulation in the expression of enzymes that are crucial to glycerol metabolism. A study using media containing 10% salt or 7.0 MPa water potential

showed an almost 40-fold increase in the enzyme glycerol-3-phosphate dehydrogenase production (Leong et al. 2015).

Xeromyces bisporus is a model candidate for studying the molecular mechanisms utilised by typical xerophilic fungi, and its adaptation has been discussed in detail below. Various experiments have demonstrated that *X. bisporus* notably use stress-tolerant and ruderal strategies in response to competition whereas, combative strategies are shut down to minimise the production of secondary metabolites (Zak and Wildman 2004). It has been observed that *X. bisporus* at the genomic level is a true S-strategist. As it shows apparent loss of all gene clusters that produce secondary metabolites which are the key molecules for competition and interaction with other organisms, that is, abandonment of combative strategies. For example, analyses of non-transcriptionally regulated events that are under allosteric control show an increase in membrane fatty acid saturation and study of differentially expressed genes during osmotic stress reinforce events like phospholipid and cholesterol biosynthesis to achieve the best out of chaotropic situation.

They mainly apply two principal strategies in order to survive in extreme conditions. Firstly, *X. bisporus* constitutively expresses genes to produce surplus amounts of glycerol and increase their expression in hyper- or hypo-osmotic stress. Overproduction of these solutes leads to leaked or secreted solutes and their association with water comprise the bulk of the mycelial wet weight. This strategy employed by *X. bisporus* helps to modulate the micro-environment around its mycelium. Secondly, the low unsaturation index of membrane fatty acids, coupled with high glycerol content, and modifications of phospholipids, sterols and cell wall components, together lead to a unique 'Xerophilic' stress response. Very innovative self-conditioning physiological adaptations behind the success of *X. bisporus* include not only sensing low aw through osmosensors and accumulating, utilising and retaining compatible solutes for osmoregulation by membrane modifications, but also preferences for alternate salt and sugar substrates that makes a huge variety of processed food and stored commodities like jams and salterns susceptible to, what we will know from now on as fungal Xerophiles (Pitt and Hocking 2009). *X. bisporus* is an obligate ascomycete filamentous fungus, arguably the most 'dry-loving' organism known to mankind that grows mostly on sugary substrates where aw is as low as 0.61 (Pitt and Christian 1968; Leong et al. 2011). It has a genome size of 22 Mb and shows optimum growth in glycerol-supplemented growth media, at 30 °C and 0.653 aw, and this demarcates *X. bisporus* as a classified chaophile pointing to remodelling of cellular and subcellular structures. *X. bisporus* is known to manage the most successful living in such chaotropic conditions amongst all other xerophilic microorganisms.

The mechanisms deployed by xerophiles to achieve positive turgor potential for active hyphal growth even at aw as low as 0.61 against strong internal and external solute concentration gradients is pretty much alien and equally fascinating to the scientific community. Although few hypotheses have been proposed to address the above query, the underlying basic principles are apparently similar and we have tried to comprehensively explain the physiological adaptations in this section.

Decoding the transcriptomes of gene clusters of *X. bisporus* revealed a great deal of information about the strategies relinquished and employed by it to thrive in hypo and hyper-osmotic stress. Usually in fungus genes that code for secondary metabolites are present in clusters (Yu and Keller 2005). These clusters consist of a backbone gene (Khaldi et al. 2010) as well as some regulatory elements responsible for transcription of these backbone genes (Fox and Howlett 2008) and some enzyme coding genes required for modification of products of backbone genes (Andersen et al. 2013). In *Aspergillus nidulans* (a xerotolerant fungal species), 66 gene clusters have been annotated as genes for secondary metabolites; out of these, 63 clusters are associated to backbone enzymes (Inglis et al. 2013). However, in *X. bisporus,* only six out of *A. nidulans gene* clusters are conserved and five extra orthologues of backbone enzymes are found in isolated positions and not in orthologous loci. For example, *sidC,* a gene involved in peroxisome metabolism, is found to be conserved both in *A, nidulans* and *X. bisporus* (Gründlinger et al. 2013).

Xerophilic fungi that accumulate or synthesise compatible solutes have a rapid response system that reacts to a sudden erosion of the environment. Some compatible solutes are rapidly catabolised or polymerised to an osmotically inert state (Trüper and Galinski 1990). Normally, certain uncharged molecules like simple polyols may pass through bio-membranes pretty easily. The accumulation of polyhydric alcohols (produced from sugar fermentation) that enables crucial physiological processes alongside maintaining the osmotic balance between intracellular and extracellular environment is the primary feature of xerophilic fungi. This indicates either intrinsic differences in membrane structure of xerophiles or induced alteration of membrane permeability upon sensing of low aw. In fact, glycerol is quite efficient in decreasing mycelial water potential and a great choice for the compatible solute to combat stress generated by extremely low water availability. As a response to osmotic stress, some fungi increase glycerol production, while others manipulate membrane permeability and its transportation across biomembranes. Often, to prevent leakage of glycerol and counter-balance osmotic stress energy-dependent active transportation strategies are assigned. For example, to ensure optimum enzyme activities low intracellular Na+ is maintained and K+ and H+ fluxes initialise osmoregulatory signals to which the cells respond with glycerol production and accumulation. In fact, glycerol biosynthesis is triggered by rapid and transient lowering in osmotic potential, consequently followed by K+ depletion. Some species experience changes in membrane potential caused by Na+ efflux and K+ retention which leads to high internal K+: Na+ ratio as a response to disturbance in ionic gradient sensed by plasma membrane (Mager and Varela 1993). This loss in turgor pressure triggers a complex series of molecular events involving protein kinases, enzyme activation and gene expressions, for example, synthesis of trehalose and certain heat shock proteins (HSPs) necessary for cell recovery.

Genomic arrays have confirmed the impact of solute on physiological stress by making possible comparison of up- and downregulated genes under matric stress. This opened up possibilities for a better understanding of biosynthetic pathways responsible for active growth and survival in fluctuating ecosystems. In most fungi,

response to osmotic stress relies on a well-conserved mitogen-activated protein kinase or MAPK pathway. Differential transcriptome analysis of the filamentous fungi in steady state under optimal and minimal water conditions has revealed the following data. A few stress response elements were found to be upregulated, while baseline expression levels of signal pathways mediated by kinase activities and direct protein interactions remained rather unaltered. Seven transcriptional activators and eight downstream genes have been postulated to be involved in stress response event, including *MsnA (Msn2p, Msx4p)* as the key regulators (Causton et al. 2001). Several downstream genes are found to be upregulated like Transmembrane sensor *Msb2p; ROS* neutralizing enzyme, *SOD*, *CatA* and *CatB*; *GfdA*, a NAD + -dependent glycerol-2-phosphate dehydrogenase necessary for glycerol metabolism (Påhlman et al. 2001); *Pmp3*, a cation transporter (Navarre and Goffeau 2000); five genes putatively involved with DNA repair (orthologues of *RSC1*, *YAF9*, *RAD52/radC* and *bimD*); and the *Stl1* H+ symport/glycerol transporter (Ferreira et al. 2005).

Genes linked to glycerol metabolism were upregulated sevenfold at low aw, for example, glycerone kinase (orthologue of *dak1*) and NAD+ dependent glycerol-3-phosphate dehydrogenase, *GfdA*. Both these gene products use dihydroxyacetone phosphate (DHAP) as a substrate, which is the transition molecule from where the intermediates of glycolysis are directed to glycerol production either via dihydroxyacetone/glycerone or glycerol-3-phosphate pathways. An alternative FAD-dependent glycerol-3-phosphate dehydrogenase which is also orthologue of *gut2* was also found to be transcriptionally upregulated. In fact, an eightfold increase in expression of glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) and phosphoglycerate kinase (*pgkA*) had been accounted for. However, *X. bisporus* possesses only one copy of the genes responsible for glycerol synthesis. All of these evidence point towards the hypothesis that, although glycerol production is modulated post-transcriptionally by direct interactions of cytosolic signal transduction and enzymatic activations, reactions leading to DHAP and from DHAP play a major role in glycerol flux modulation (Bouwman et al. 2011). HogA along with other sensory and regulatory elements like transmembrane sensor ShoA and transmembrane mucin Msb2p that act in coordination with actin cytoskeleton also are some widely studied downstream response elements found in *X. bisporus* (Tatebayashi et al. 2007; Tanaka et al. 2014). NikA, a cytoplasmic putative sensor from the sln1 signalling pathway, is also upregulated in *X. bisporus* (Hagiwara et al. 2009).

Glycerol synthesis is regulated by a differentially expressing cluster of genes known as *hog* genes or high osmolarity glycerol genes via HOG-MAPK stress response pathway. Membrane osmosensor ShoA senses osmotic stress and activates a MAPK kinase, Pbs2 which, in turn, phosphorylates HogA, a stress-activated MAPK. Accumulation of phosphorylated HogA results in a cascade of reactions, leading to glycerol synthesis and accumulation in the presence of osmotic stress. However, upon return of favourable water conditions and with restricted energy source rapid depletion of glycerol by leakage has been accounted alongside conidia formation, indicating its utilisation as a metabolite (Hocking 1986).

Secretion of exopolysaccharide is another strategy deployed by extremophiles to adapt to their respective environments (Nicolaus et al. 2010). Secretion of rinsable

components by the filamentous fungus is one such strategic move. Experiments have demonstrated that the majority of the glycerol produced which is approximately twice the amount of glycerol per colony area leaches out into media when not required and is re-absorbed by ageing colonies (Hocking 1986), leading to an increase in mycelial density. In fact, no tight regulation of glycerol synthesis suggests its constitutive expression which is under allosteric control (Warringer et al. 2010). Examination of *X. bisporus* mycelium by scanning electron microscopy (SEM) has confirmed the presence of this viscous coating (Pettersson et al. 2011). Additionally, rinsable components (glycerol) secreted around these mycelia lead to 16% gain in a wet weight of the organism, justifying the 'mycelium density hypothesis' which is a sure shot strategy for extreme xerophilicity.

Just as important as production, accumulation and retention of compatible solutes, a certain transition of the membrane lipids and unsaturation index is also necessary to inhibit massive loss of water, ions and compatible solutes (Gostinčar et al. 2009). Glycerol retention is altogether a different story as it is dictated by cell membrane composition and hence permeability. The sterol to phospholipid ratio and saturation of fatty acids in the lipid bilayer vary greatly to modulate membrane fluidity in xerophilic fungi. In fact, lower sterol to phospholipid ratio and increased desaturation favours xerophilicity, as observed in the *X. bisporus*. This demonstrates a tendency to increase saturation as an adaptive response. A decrease in the amount of double-unsaturated linoleic acid and a corresponding increase in monounsaturated oleic acid helps it achieve a more rigid membrane. Only single copies of the genes that encode the key enzymes for fatty acid synthesis and modifications are found in *X. bisporus* genome, and they are actively transcribed. These are namely *fasA* and *fasB* that code for α-subunit and β-subunit of fatty acid synthase, *accA* acetyl-CoA carboxylase, two elongases similar to FEN1 and ELO1, *sdeA*Δ9-stearic acid desaturase and *odeA*Δ12-oleic acid desaturase. All the above-mentioned enzymes are under post-translational or allosteric control (Cossins et al. 2002; O'Quin et al. 2010).

However, altering the unsaturation index is just one way to regulate membrane fluidity in response to osmotic stress. Altering components like the lipid head group or modifying sterol-phospholipid ratio are some other strategies employed by fungi to achieve membrane rigidity. Differential expression of several genes have been accounted in *X. bisporus*, which have putative roles in biosynthesis and metabolism of phospholipid, sterols and sphingolipids. Ergosterol biosynthesis is modulated by downregulation of *IDI1,* an isopentenyl-diphosphate Δ-isomerase, and *ERG26,* a C3 sterol dehydrogenase/C4 decarboxylase and side-by-side upregulation of *ERG3* Δ-desaturase. A flotillin orthologue *floA* that putatively maintains sterol-rich plasma domains, and two ceramide metabolism genes are also found to be upregulated. In fact, differential expressions of genes engaged in biosynthesis and modulation of cell wall components like four mannosyl transferases contributing to further rigidity, *O*-glycosylation and anchorage of membrane proteins via glycosyl-phosphatidylinositol (Orlean 1990; Bourdineaud et al. 1998; Sutterlin et al. 1998) and further transcriptional regulation of β-glucanases*SCW11* and *DSE4*, respectively, again suggest fine control of cell wall remodelling genes (Fig. 13.2).

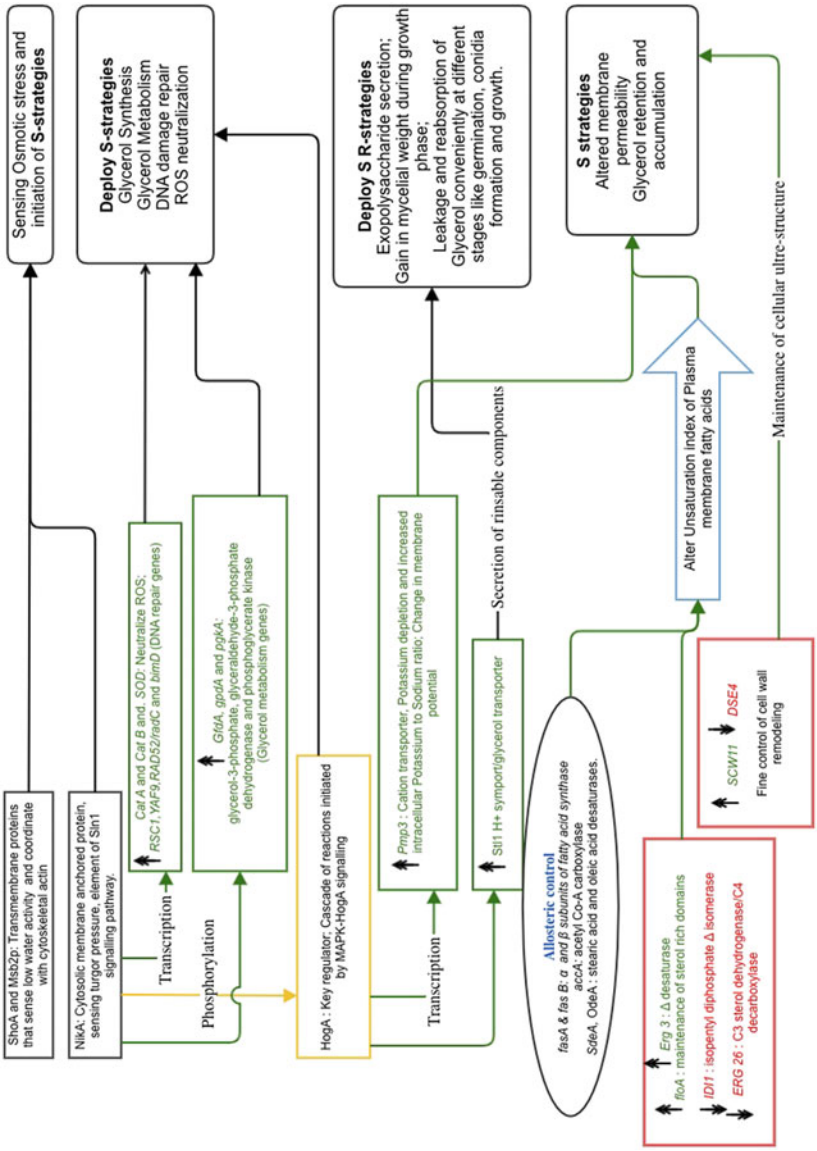

Fig. 13.2 Strategic physiological adaptations to water stress in *Xerophilus bisporus*. Transcriptionally regulated and allosterically controlled expression of genes and their respective physiological roles leading to osmo-adaptation. The symbol '↑' indicates upregulated genes and '↓' indicates downregulated genes

13.4 Bioactive Molecules from Xerophilic Fungi

The fungus *Aspergillus felis* isolated from rocks of the Atacama Desert were tested in a microdilution assay against paracoccidioidomycosis causing fungi *Paracoccidioides brasiliensis* Pb18. The fungi grown in Potato dextrose agar culture and extracted with dichloromethane showed a MIC of 1.9 μg/mL against *P. brasiliensis* and showed no cytotoxicity against normal mammalian cell line (Mendes et al. 2016). The xerophilic fungus *Chaetomium globosum,* thriving as an endophyte on *Ephedra fasciculata* (Mormon tea) in Sonoran Desert, is found to possess three novel esters of orsellinic acid, Globosumones A, B and C. The compounds Globosumones A and C were found to exhibit a moderate level of cytotoxic activities against four cancer cell lines, namely MIA Pa Ca-2, NCI-H460, SF-268 and MCF-7 with IC_{50} values ranging from 6.5 to 30.2 μM against doxorubicin as a positive control (IC_{50} 0.01–0.07 μM) (Bashyal et al. 2005).

Two new dioxopiperazine derivatives, Arestrictins A and B, are isolated from the xerophilic fungus *Aspergillus restrictus* A-17 isolated from house dust; however, their biological activity is not tested (Itabashi et al. 2006). The fungus *Penicillium citrinum* HGY1-5 isolated from the ash of extinct volcano Huguangyan in Guangdong, China, possessed 11 novel unique C25 steroid isomers with 20-*O*-methyl-24-*epi*cyclocitrinol, bicyclo[4.4.1]A/B rings named 24-*epi*-cyclocitrinol, 12*R*-hydroxycyclocitrinol, 20-*O*-methylcyclocitrinol, *erythro*-23-*O*-methylneocyclocitrinol, 24-oxocyclocitrinol, isocyclocitrinol B, *threo*-23-*O*-methylneocyclocitrinol, precyclocitrinol B and neocyclocitrinols B and D. Among them, the compounds bicyclo[4.4.1]A/B rings named 24-*epi*-cyclocitrinol, neocyclocitrinols B and *threo*-23-*O*-methylneocyclocitrinol induced the production of cyclic AMP in GPR12-transfected CHO cells at a concentration of 10 μM (Du et al. 2008).

13.5 Biotechnological Importance of Xerophilic Fungi

Xerophilic fungi have applications in diverse areas related to the industries, environment, biodeterioration, food spoilage, agriculture, etc.. in the biotechnology field. The xerophilic fungi have beneficial as well as adverse effects on the environment, agriculture, human beings, etc., as depicted in Fig. 13.3 and discussed in detail as follows.

13.5.1 Industrial Aspects

13.5.1.1 Enzyme and Pigment Production

A xerophilic fungus *Aspergillus niger* GH1 isolated from the Mexican semi-desert is used for the production of invertase enzyme using substrates molasses and sugarcane bagasse by solid state fermentation (Veana et al. 2014). Invertase is an important enzyme which is used as a catalytic agent in the food industry for the development

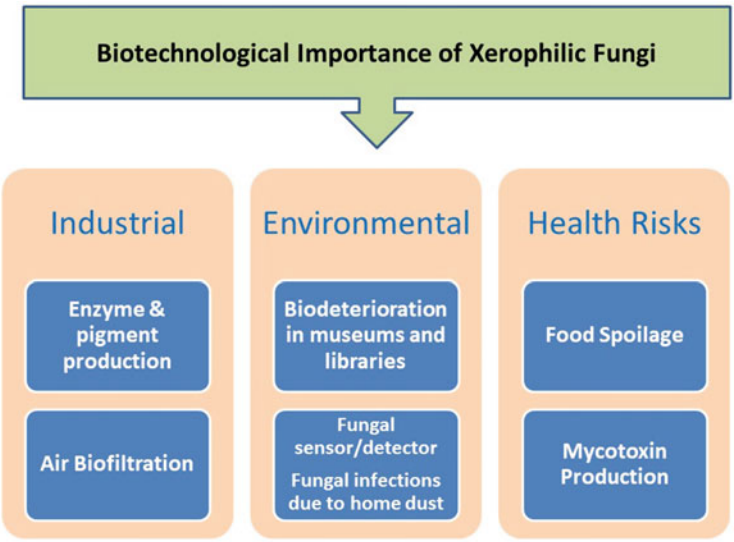

Fig. 13.3 Biotechnological importance of xerophilic fungi in diverse fields

artificial sweetener (Ashokkumar et al. 2001). Méndez et al. (2011) reported the red pigment production by xerophilic fungi *Penicillium purpurogenum* GH2 which can be used in the food and textile industry.

13.5.1.2 Air Biofiltration

Janda et al. (2009) have studied the extracellular enzyme activities of some rapid growing xerophilic fungi, that is, *Aspergillus flavus, A. fumigatus, A. melleus, A. nidulans, A. niger, A. parasiticus and Trichothecium roseum* isolated from the dried medicinal plants procured by herbal shops in Szczecin, Poland. They found that *A. melleus* had the utmost hydrolytic activity on milk, tributyrin, gelatin, starch, rapeseed oil and biodiesel oil agars and *A. nidulans* showed the highest hydrolase activity. Among the hydrolases, β-glucosidase activity was the highest, followed by others such as acid phosphatase, *N*-acetyl-β-glucosaminidase and naphthol-AS-BI-phosphohydrolase activities. These xerophilic fungi can utilise various substrates and therefore have high biodeterioration potential for biotechnological purposes, for example, in air biofiltration and waste or soil bioremediation. Prenafeta-Boldú et al. (2018) analysed the potential of xerophilic fungi *Cladosporium cladosporioides* for the biofiltration of indoor air. During their study, they found that *C. cladosporioides* can remove the total volatile organic compounds (VOCs) content by 96.1%.

13.5.2 Environmental Aspects

13.5.2.1 Biodeterioration in Museums and Libraries

Biodeterioration is the undesirable change in the properties of materials due to the biological agents. In museums as well as in libraries, xerophilic fungi play the most important role in biodeterioration. Fungal contamination occurs due to exposure of fungal spores in the air, by contact with contaminated materials or distribution by vector organisms (e.g. arthropods) (Trovao et al. 2013). The major components of paper used in books are fibre/fibrous material made up of hemp, cotton, linen, bagasse, rice straw and wood with functional additives, namely sizing, optical brighteners and consolidating agents such as gelatin, cellulose acetate, etc. Distinctive fungal infections found in libraries that colonizing documents are the species of slow-growing Ascomycetes as well as mitosporic xerophilic fungi of the genera *Aspergillus, Paecilomyces, Chrysosporium, Penicillium* and *Cladosporium* (Pinzari and Montanari 2011). Fungal spores may be viable for many years and will germinate when the environmental conditions favour (Gallo et al. 2003). A xerophilic fungus *Eurotium halophilicum* (anamorph *Aspergillus halophilicus*) has been identified from the Library of Humanities (BAUM), at Ca′ Foscari University, Venice (Italy). This fungus produces white mycelia growth forming scattered spots, mostly on books with leather or fabric bindings (Micheluz et al. 2015).

13.5.2.2 Xerophilic Fungi as Sensor/Detector

A fungal index which evaluates climates in the home environment was established using moderately xerophilic *Eurotium herbariorum* and extremely xerophilic *Aspergillus penicillioides.* The sensor fungus which exhibited the greatest response in a fungal detector (a device encapsulating spores of sensor fungi) provides a quantitative and qualitative indicator of the environment tested, representing the type of fungi that would contaminate the site. A fungal index would also be useful for detecting wetness which induces fungal contamination, which has side effects on human health (Abe 2012).

13.5.2.3 Fungal Infections Related to Home Dust

Fungal infections are mostly airborne with significant seasonal variations and high numbers of spores can gather in the layers of home dust, namely, *Penicillium/Aspergillus/Paecilomyces variotii* group, and *Aspergillus penicillioides, Aureobasidium pullulans, Cladosporium cladosporioides* and *Cladosporium herbarum* (Kaarakainen et al. 2009).

Xerophilic fungi have been isolated from Canadian and Hawaiian house dust which include 1039 strains out of which 296 strains belong to *Aspergillus*, representing 37 species. *Aspergillus sect. Aspergillus* was one of the most predominant groups which were isolated. Among all strains, two new species were found, namely, *A. mallochii* and *A. megaspores* (Visagie et al. 2017). The disease occurring due to fungal allergy is allergic bronchopulmonary aspergillosis usually caused by *A. fumigates* which is an omnipresent indoor and outdoor fungus. This is an inflammatory disease that mainly occurs in patients with asthma (up to 13% of

asthmatic patients) or cystic fibrosis due to the poor clearance of secretions from the airways (Agarwal and Chakrabarti 2013).

13.5.3 Health Aspects

13.5.3.1 Food and Food Products Spoilage

Eurotium species (previously known as '*Aspergillus glaucus* group') are the most universal foodborne genera with *Aspergillus* anamorphs (imperfect states). All the species of *Aspergillus glaucus* are xerophilic and cause spoilage in low aw foods as well as stored products (Hocking 2006). *Wallemia* spp. is now known as important spoilage organisms for foods with low aw (Pitt and Hocking 2009). Moderate xerophiles of the genera *Aspergillus, Eurotium* and *Penicillium* are the main spoilage organisms. Some xerophiles are isolated from both high-salt and high-sugar environments; others have a preference for either salt or sugar. Xerophiles can cause spoilage of high sugary products, high salted foods, as well as stored food and feed (Pettersson et al. 2011).

Ismail et al. (2012) analysed 55 samples from five baby food products chiefly made of cereal flour(s) and found that the incidence of xerophilic fungi was 88% of food samples. The highest contamination level by xerophiles was found in Mwebaza rice porridge (a component of rice flour) and the lowest in Mukuza (a product of maize, soyabean and sorghum flours). Eleven xerophilic species were found of which *Aspergillus* and *Eurotium* (four species each) were the predominant, resulting in 9.1% and 8.9% of the total CFU. Contamination of such foods is a matter of great concern as these foods are used for babies.

Wallemia sebi is a common xerophile in cereals and spices, and *Aspergillus penicillioides* as well as *Aspergillus restrictus* are early colonizers of stored products. Nuts are more vulnerable to attack by *Aspergillus* spp., especially *Aspergillus flavus, Aspergillus niger, Aspergillus candidus, Aspergillus ochraceus* and the xerophiles, namely, *Wallemia sebi* and *Aspergillus penicillioides* (Hocking 2014). The xerotolerant, xerophilic and halophilic *Wallemia* spp. are found in various osmotically challenging conditions, such as dry, salted or highly sugared foods, dry feed, salt crystals, indoor as well as outdoor air and agriculture aerosols globally. Recently, eight species were recognised for the genus *Wallemi*a, among which four are commonly associated with foods: *W. sebi, W. mellicola, W. muriae* and *W. ichthyophaga* (Zajc and Gunde-Cimerman 2018).

13.5.3.2 Mycotoxin Production

Mycotoxins are mainly present in the spores of fungal growth and in airborne dust which affect the human's respiratory system. These mycotoxins can cause the allergological problem known as 'farmer's lung disease' and can also promote infections in immunocompetent humans. Farmer's lung disease may be combined with pulmonary fibrosis which includes symptoms, that is, bronchial asthma, cough, tiredness, headaches and fever/night sweats which depend on the severity of the disease (Guarro et al. 2008; Gbaguidi-Haore et al. 2009). Xerophilic fungal species

are the prevailing species in crops under storage conditions when aw drops. After harvest xerophilic fungi, namely *Aspergillus* spp. and *Penicillium* spp., germinate and produce mycotoxins at a relative humidity of 80–90% and less (Manna and Kim 2017).

Al-Sohaibani et al. (2011) investigated black tea procured from the local markets of Tamilnadu, India, for fungal contamination and reported the presence of two xerophilic aflatoxigenic fungi, namely, *Aspergillus niger ML01* and *A. flavus* ML02. These xerophilic fungi are highly dangerous contaminants of tea because they are related to tea quality deterioration which ultimately leads to serious health risk. Ochratoxins are a group of mycotoxins produced by *Penicillium verrucosum* and various species of *Aspergillus* moulds, namely, *A. alliaceus, A. auricomus, A. carbonarius, A. glaucus, A. melleus* and *A. niger* which contaminate the crops in the field and during storage (Peraica 2016). Jančič et al. (2016) investigated the production of secondary metabolites produced by seven species of the genus *Wallemia,* namely, *W. sebi, W. muriae, W. mellicola, W. tropicali, W. canadensis, W. hederae* and *W. ichtyophaga* at hypersaline conditions. They found that the *Wallemia* spp. produce toxic metabolites, namely, walleminol, walleminone and wallimidione, and their production increases proportionally to increased concentrations of NaCl. Therefore, *Wallemia* spp. can be considered as a serious health risk associated with food having high salt concentration.

Two xerophilic fungus *Aspergillus chevalieri* and *Aspergillus amstelodami* have been isolated from peanuts which probably reduce their shelf life as well as the quality of the kernels. The incidence of these two fungi on peanuts provides favourable conditions for less xerophilic *Aspergillus* and other spoilage-related fungal genera, particularly mycotoxin-producing species which lead to contamination of mycotoxin (Kamarudin and Zakaria 2018).

13.6 Conclusion

Most of the environments on Earth are populated by fungi due to their broad adaptational amplitude. Xerophilic fungi are unique organism with the ability to grow under conditions of aw. Their life cycles are completed on substrates having high levels of soluble solids such as salts or sugars and as a result became dried or concentrated. Some xerophilic fungi like *Z. rouxii, H. werneckii* and *Wa. sebi* are so exceptionally adaptable that they can withstand water stress imposed by both salt and sugar, and also diverse ranges of water activities. Other species are more environment dependent and display a much narrower adaptation amplitude, with a strong preference for a particular substrate.

Despite their huge importance in various areas of biotechnology, various related aspects of xerophilic fungi are underexplored. It is also noteworthy that filamentous fungi are hugely ignored as compared to others in the same group like yeast and their adaptation to water stress. The area of xerophilic fungus and its importance demands more studies exploring the utility in industries like food and medicine. Also, it has a vital impact on the enterprises having recreational importance like museums and

music instruments. The chapter has discussed, in detail, the xerophilic fungi and their physiology, genetics, adaptation and industrially important bioactive metabolites. The biotechnological importance of xerophilic fungi is also discussed in the context of industry, environment and health.

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Conflict of Interest The authors declare no conflict of interest.

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Part III

Applications

Extremophilic Enzymes: Catalytic Features and Industrial Applications

14

Kanak Choudhary, Mangesh Kumar Mankar, and Sanjay Sahay

Abstract

Extremophilic microbes are those that are adapted to very harsh environmental conditions. Fungi constitute one of the important groups that by virtue of various adaptation strategies learn to live in extreme environment and serve important ecological functions. Their presence and activity are also essential for a large variety of flora and fauna of these harsh environments. Although microbes have developed a variety of strategies to successfully lead life in these extreme conditions, the enzymes with unique combination of catalytic features they have developed have especially enabled them to drive all metabolic and ecological under extreme conditions. The chapter highlights some important catalytic features found across a large number of important enzymes.

Keywords

Psychrophilic enzymes · Thermophilic enzymes · Alkaliphilic enzymes · Acidophilic enzymes · Halophilic enzymes · Radioresistant enzymes

14.1 Introduction

An organism needs physiological conditions that are essential for their survival. Physiological conditions such as moderate temperature (10–37 °C), near neutral pH (pH between 6 and 8), pressure (1 atm), salinity (0.15–0.5 M NaCl), and availability

K. Choudhary · M. K. Mankar
Department of Biotechnology, Barkatullah University, Bhopal, MP, India

S. Sahay (✉)
Sarojini Naidu Government Postgraduate Girl's (Autonomous) College, Bhopal, Madhya Pradesh, India
e-mail: ss000@redffmail.com

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of water are required. Organisms that grow and survive under these moderate environmental conditions are known as mesophiles or neutrophiles. However, many bacteria, archaea, and eukaryotic organisms are found to survive and thrive in toxic-heavy metal waste, refrigerated food, deep sea, solar salterns, hot and cold deserts, and soda lakes. These environmental conditions are known as extreme conditions, which are further categorized into physical and geochemical extremes. Physical extreme involves extreme temperatures (very cold or very hot), high pressure, and high level of radiations while geochemical extreme involves environment with high salinity and extreme pH (very low or very high). Extremophiles are the organisms that can thrive under these physically and geochemically extreme environments. The extremophiles can tolerate these nonphysiological conditions that are inhospitable or lethal for the survival of mesophiles. Extremophiles need these extreme conditions to grow while extremotolerants grow optimally at moderate environment but can tolerate extreme conditions. Extremophiles are distributed among all three domains of life that is bacteria, archaea, and eukarya. Most of the eukaryotic extremophiles belong to fungi and algae (Kumar et al. 2011; Rampelotto 2013; Orellana et al. 2018).

They have evolved or adapted to survive under these extreme conditions. They possess wide and versatile metabolic diversity. They are model organisms for the study of the existence of extraterrestrial life. Most extremophiles can thrive and survive under more than one type of extreme conditions, which are known as polyextremophiles such as thermoacidophiles that thrive under high temperature along with very low pH environment and haloalkaliphiles that thrive under high salt concentration along with high pH environment. Extremophiles are important for the study of mechanism of adaptation in extreme environment, metabolic pathways, and novel products or metabolites. Extremophiles are the source of extreme enzymes known as extremozymes and have ability to remain stable and active under these conditions. Extremozymes adapted themselves to function or catalyze a reaction optimally in extreme environment. When mesophilic enzymes are applied to non-natural or nonphysiological conditions, their stability and catalysis is reduced. In this case, extremozymes that have extreme stability become a key to overcome this problem. Extremophiles are able to maintain neutral pH in their internal cellular environment; thus, the intracellular enzymes do not possess any unique structural adaptations. Therefore, extracellular extremozymes with unique structural properties are used in many biotechnological industries. However, they do not possess any major structural variations as the residues that build the active site are conserved. Extremozymes make the biocatalysis specific, faster, and environmental friendly. One another advantage to study the properties of extremozymes is to design novel enzymes that possess similar properties with extremozyme (Gupta et al. 2014).

14.2 Types of Extremophiles

Extremophiles are classified according to the extreme conditions. Thermophiles are the organisms that can thrive under high-temperature environment with temperature range between 45 and 80 °C while hyperthermophiles can thrive under very high temperature, that is, >80 °C. To maintain the stability under high temperature, they possess high GC content and more hydrophobic core in protein. They protect the cell by synthesizing heat shock proteins. Psychrophiles are the organisms that can thrive under very low-temperature condition <10°C. They grow at temperature between -20 °C and 20 °C and possess optimal growth temperature < 15 °C. They maintain the membrane fluidity by increasing the amount of unsaturated fatty acids in their membrane. Accumulation and synthesis of cryoprotectants, anti-freeze proteins, cold-shock proteins, and chaperons protect their RNA and proteins. High catalytic activity, low thermostability at moderate temperature, and increased flexibility of psychrophilic protein make them feasible for industrial application at lower temperature. Psychrophilic enzymes are very economic as they decrease the energy consumption. Some extremophiles can thrive in extreme pH. Those that thrive under very high pH or alkaline environment are known as alkaliphiles while those that thrive under very low pH or acidic environment are known as acidophiles. Alkaliphiles acidify the cytosol as to neutralize the effect of high pH and protect the cell by preventing the entry of hydroxide ions. Acidophiles protect themselves from high proton by efficient proton pumping that rapidly remove the protons from the cytoplasm. Halophiles are the extremophiles that thrive under high salt environment or environment with high ionic strength (higher than of seawater). They prevent desiccation by increasing the osmotic concentration of the cell by accumulating osmoprotectants or compatible solutes and selective influx of ions in the cytoplasm. Some extremophiles thrive in environment rich in metals or heavy metals, and high level of radiations are known as metallophiles and radioresistants, respectively. Presence of heavy metals such as Zn, Cd, Hg, Pb, Ag, Co, and Cr make the environment very toxic. Metallophiles, natural habitants of metal-rich environment, utilize these toxic-heavy metals and remove them from various heavy metal contaminant areas. Generally high metal concentration inhibits the growth and functioning of microbes, but metallophiles adapt the strategies to function optimally at these conditions. Some metallophiles possess efficient efflux pumps for the rapid removal of toxic metals while others associate these metals by binding them with protein molecules. Metallophiles are used for ore-bioleaching, bioremediation, biomineralization, and biomining of expensive metals from industrial effluents. High level of radiations, mainly ultraviolet and gamma radiation, produces oxidative stress that causes protein denaturation and mutations through DNA damage. UV radiations are more lethal as they damage the nucleic acid of an organism. Radiophiles or radioresistant organisms protect themselves from this radiation by efficient DNA repair machinery, protecting substances such as chaperons or carotenoids and active defense system for radiation stress. Radioresistant organisms have the potential for bioremediation of radionuclide-contaminated sites and degradation of organopollutants in radioactive waste. Organisms that can thrive under

high hydrostatic pressure are known as piezophiles or barophiles. They can optimally grow under high pressure of 400 atm or more. Extreme barophiles grow optimally at hydrostatic pressure 700 atm or more. They survive by developing pressure gradient inside and outside of the cell and regulating membrane phospholipid fluidity. Piezophilic proteins are adapted to specific stabilization in the presence of higher pressure. This property of proteins is used by food industries for processing and sterilization of food materials at high pressure (Oarga 2009; Rampelotto 2013; Gupta et al. 2014; Parihar and Bagaria 2019).

14.3 Enzymes as Key to Metabolism

Metabolism is a series of biochemical reactions involved in the process of synthesizing and breaking down compounds enabling cells to survive and reproduce. Biochemical reactions occurring in cells are not catalyzed spontaneously. All cellular processes are carried out through biochemical reactions; the latter needs a specific protein or a catalyst to precede that is known as enzyme. These proteins speed up the rate of reaction by lowering the activation energy of the reaction without being changed during the whole process. They are very specific to a reaction they catalyzed. They do not shift the equilibrium of a reaction instead they help to attain the equilibrium quickly. Various enzymes work together to form a metabolic pathway that comprises a long chain of enzyme-catalyzed reactions. Metabolism involves two types of reactions: anabolic reactions and catabolic reactions. Anabolic reactions are usually endothermic that requires an input of energy for synthesizing compounds while catabolic reactions are exothermic releasing energy after the breakdown of complex organic molecules. Metabolism keeps the organism maintained. Enzymes are very essential in metabolism as they perform two important functions. They synthesize a large complex molecule from multiple substrates and synthesize multiple products from a large substrate. The rate of anabolic and catabolic reactions is dependent on enzyme and substrate concentration so that by altering their concentration the metabolic rate can be controlled. The metabolic rate can be regulated by feedback inhibition in which high product concentration signals to slow down the process (Molnar and Gair 2012).

14.4 Catalytic Features of Enzyme

14.4.1 Effect of Temperature

Enzymes are proteinous in nature, and proteins are very sensitive to thermal changes. They catalyze a reaction at optimum temperature, which is specific for each catalyst. The optimal temperature is a particular temperature at which an enzyme shows maximum activity. Initially, increase in temperature enhances the rate of reaction as the kinetic energy of enzyme molecule increases with slight increase in temperature. Elevated temperature increases the vibrational energy of enzyme molecules that

exert a pressure on hydrogen and ionic bonds present in enzyme. This pressure breaks the bonds that causes change in active site of an enzyme and increased kinetic energy denatures the protein's three-dimensional structure also. Substrate molecules are not able to bind with this altered active site. Thus at high temperature, the enzyme loses its catalytic properties and the structure gets denatured. While at lower temperature the enzyme becomes inactivated. However, rise in temperature allows enzyme to reactivate (Robinson 2015).

14.4.2 Effect of pH

Enzymes work at specific pH and show maximum activity at optimum pH. Enzymes are mostly active at or near neutral pH. Fluctuation in pH rapidly decreases the rate of reaction as the concentration of H^+ and OH^- ions interferes with the hydrogen and ionic bond leads to the ionization of the functional group present on active site that causes change in the shape of enzyme active site. The altered active site is no more complementary with substrate, hence rate of reaction decreases. Small changes do not cause permanent change as the broken bonds are reformed at original pH but major changes in pH cause enzyme denaturation and enzyme permanently lose its function (Robinson 2015).

14.4.3 Cofactors and Coenzymes

Most of the enzyme-catalyzed reactions cannot proceed without binding to a non-protein molecule. This nonprotein molecule is known as cofactor while enzymes in this kind of reaction are known as apozyme. Both apozyme and nonprotein part make a complete catalytic part known as holozyme. Apozyme alone cannot catalyze a reaction as their activity is dependent on nonprotein part. Cofactors can be organic or inorganic and help enzyme to perform a reaction. Cofactors are categorized into two parts: coenzymes that is organic molecule loosely binds to the active site of an enzyme and metalloorganic compounds in which metal ions bind with the enzymes and accelerate the enzyme-catalyzed reactions such as iron for catalase, zinc for DNA polymerase, and magnesium for hexokinase. They are recycled and participate in multiple enzymatic reactions. Some coenzymes bind reversibly with the active site such as thiamine pyrophosphate while some bind to the enzyme other than active site such as nicotinamide adenine dinucleotide. Coenzyme binds mostly by noncovalent linkages with the enzyme. However, those that tightly bind with the enzyme by covalent linkages are known as prosthetic groups such as heme group in hemoglobin (Broderick 2001). In brief, cofactors and coenzymes are small nonprotein molecules that enhance the enzyme's function.

14.4.4 Inhibitions

Some molecules bind to the catalyst and directly or indirectly interfere with the catalytic property of enzyme, thus reducing the activity of enzyme-catalyzed reaction. These molecules are known as inhibitors, and the process is known as inhibition. Although high concentration of substrate or products can also inhibit the enzyme activity. Competitive inhibition is a process of binding of enzyme substrate analog to the active site of enzyme. In noncompetitive inhibition, inhibitor binds with the enzyme at site other than active site. These inhibitors reduce the active enzyme concentration and Vmax value but Km value is not altered. Uncompetitive inhibition involves the binding of an inhibitor to the enzyme–substrate complex. This reduces the Vmax and Km value that interferes with the equilibrium. In irreversible inhibition, inhibitors permanently bind with the enzyme. This kind of inhibition acts as a potent toxin for enzymes (Robinson 2015).

14.4.5 Co-Cooperativity

For enzymes that contain more than one active site, the phenomenon of binding of a substrate to first active site influencing the binding of substrate of other site is known as cooperativity. This simply means that binding of one substrate changes the affinity for other substrate or fluctuations in affinity for binding of the other sites is affected by the original binding site. The influence may be positive or negative. In positive cooperativity, binding of first substrate increases the affinity for subsequent substrate. An example of positive cooperativity is found in globin chain of hemoglobin that facilitates the successive binding of oxygen molecule to alpha and beta globin chains. In negative cooperativity, binding of first substrate decreases the affinity for subsequent substrate (Bush et al. 2012).

14.5 Thermophilic Enzymes

14.5.1 Thermophiles

Thermophiles are the microorganisms that thrive under extreme temperatures. Moderate thermophiles optimally grow at 50–60 °C, extreme thermophiles optimally grow at 60–80 °C, and hyperthermophiles optimally grow at 80–110 °C. They are commonly found in thermal vents, hot springs, volcanic lakes, and boiling steam vents (Kumar et al. 2011).

14.5.1.1 Catalytic Features of Thermophilic Enzymes/Proteins

Thermophilic enzymes can tolerate many extreme conditions. They are active and stable even in high ionic strength medium, presence of denaturing agents, and organic solvents. Thermophilic proteins possess high content of helices. Arginine (propensity value 1.33) is a helix-favoring residue present in large amount in

thermophilic proteins while cysteine (propensity value 0.87) and histidine (propensity value 0.76) residues are present in negligible amount as they are unfavorable for helix formation. Proline frequency in thermophilic α-helices is 0.7% as compared to mesophilic protein for which the frequency of proline residue is 1.3%. This low frequency of proline residues protects the helix from kinks. Thermolabile amino acids asparagine, glutamine, methionine, and cysteine are present in very less amount as they possess the ability to undergo oxidation or deamidation at elevated temperature. Arginine and tyrosine are responsible for binding and folding of proteins at high temperature that provide the stability to thermophilic proteins (Kumar et al. 2000). Elevated temperature causes covalent modification in unstable amino acids leading to denaturation of proteins. Some amino acid replacements provide flexibility to these proteins due to their additional noncovalent interaction. The amino acids alanine, threonine, and arginine are more common in thermophilic proteins. Increased hydrophobicity is a feature of thermophilic protein. Hydrophobic core provides tight packing of protein that possesses smaller cavities, resulting in compact structure. Disulfide bonds form in between the cysteine residues. They are important in oligomerization of protein. They provide rigidity to overall structure and resist protein to unfold at high temperature. Increased number of disulfide bonds prevents the alteration in quaternary structure of protein. Salt bridging allows the interaction of charge–charge residues that increases the thermal stability of protein. An increased surface charge provides stabilization and prevents aggregation of protein (Reed et al. 2013). To prevent enzyme from nonspecific interactions, deep sea thermophilic enzymes have shorter loops. Comparative study of mesophilic and thermophilic proteins shows high alanine and leucine residues in thermophiles. Many metal-dependent structures are bound with the membrane that provides stability at high temperature (Li et al. 2019). Presence of tightly packed internal core, tandem repeats, thermo stabilizing domains, and disulfide bridges at the ends or α-helix region increases the activity of thermophilic enzymes (Collins et al. 2005). As the rigidity in the thermophilic enzymes is high, rate of exchange of amide protons becomes low. This will increase the resistance for proteolysis and denaturation. Thermophilic enzymes are only marginally temperature dependent. Active site amino acid residues are found to be conserved. Improved packing density and specific local interactions are responsible for the thermostability (Jaenicke 1991; Jin et al. 2019). Protein dynamics is inversely related with the protein stability. More dynamic structure of a protein leads to the lower stability of that protein (Cipolla et al. 2012). A study on thermophilic acylphosphatase suggests that the salt bridge present in the active site is responsible for increased enzymatic activity and shows stronger temperature dependency of enzymatic reaction (Lam et al. 2011). This is to be noted that a particular factor is not responsible for the stability of an enzyme because multiple factors contribute to the stability of a thermophilic enzymes.

14.5.1.2 Thermophilic Cellulases

Cellulases from thermophilic fungi have an approximately 30–250 kDa molecular weight protein that shows maximum activity at pH 4.0–7.0 and 50–80 °C temperature. Optimal thermal stability of this enzyme is at 60 °C temperature. In comparison

with mesophilic fungal cellulases, thermophilic cellulases have longer half-lives at 70, 80, and 90 °C. Catalytic efficiency of thermophilic cellulases is improved by the structure-based rational site-directed mutagenesis and random mutagenesis through directed evolution so that their stability at nonphysiological pH and elevated temperature can be increased. Three-dimensional structure analyzes the characterization of the different families of cellulases. The family 5 thermophilic cellulases were studied in thermophilic fungi *Trichoderma aurantiacus*. This family consists of endoglucanase type with β/α-barrel folding. Substrate binding cleft is present on the C-terminus of the barrel that possesses seven glucose residues binding site and has some extra barrel features. The family 6 cellulases studied in *Humicola insolens*. They consist of endoglucanases and cellobiohydrolases type of thermophilic cellulases that have distorted β/α-barrel structure where only seven parallel β-strands are present in central barrel. Strands I and VII form the substrate binding cleft that have six substrate binding sites. Around the active site, two extended surface loops are present in endoglucanase, which forms an open cleft enabling them to hydrolyze the bond internally in cellulose. The family 7 consists of endoglucanases and cellobiohydrolases type and was studied in *Trichoderma emersonii* fungi. The structure consists of two antiparallel β-sheets with six β-strains in each sheet. A substrate binding tunnel (approximately 50 Å long) is formed by the loop that connects both concave and convex faces of strands that have nine substrate binding sites. The family 12 of thermophilic cellulases was studied in *Humicola grisea*. The structure consists of 15 β-strands folded into two antiparallel β-sheets. Six antiparallel strands form the convex face while nine antiparallel strands form the concave face. This concave site forms a long substrate binding cleft that possess six substrate binding sites. The family 45 possesses endoglucanase type isolated from *Humicola grisea* and *Melanocarpus albomyces*. They are flattened sphere shape with dimensions about 32Å × 32Å × 22Å. The structure is composed of six stranded β-barrels with interconnected loops. These β-barrels and loop structures form the approximately 40 Å long, 10 Å deep, and 12 Å wide substrate binding cleft in between them (Li et al. 2019). Various thermophilic fungal cellulases from different thermophilic fungi were expressed in their heterologous hosts and their properties were evaluated (Table 14.1). Thermophilic fungi *Chaetomium thermophilum* secreted three forms of cellulases. These three forms of cellobiohydrolase are CBH1, CBH2, and CBH3. CBH1 and CBH2 consist of all four domains such as signal peptide domain, cellulose binding domain, hinge region, and catalytic domain while CBH3 consists of only signal peptide domain and catalytic domain. Deletion of cellulose binding domain causes reduction in enzymatic activity (Li et al. 2019). *Sporotrichum thermophile* produces hemoflavoprotein type of cellobiose dehydrogenase of 92–95 kDa molecular weights. This enzyme possesses N-terminal flavin domain, heme domain, and C-terminal cellulose binding domain. N-terminal flavin domain contains the active site. This enzyme possesses maximum activity at 60–65°C temperature and pH 4 (Maheshwari et al. 2000).

Thermophilic fungal species *Myceliophthora* sp. viz., fungal species *M. thermophila*, *M. heterothallica*, *M. hinnulea,* and *M. fergusii* optimally grow at 45°C secreted industrially valuable enzymes that have stability up to 70 °C. They

Table 14.1 Recombinant thermophilic fungal cellulases with their expression in heterologous hosts and their properties

Fungus	Gene	Family	Host	Optimal pH	pI	Optimal temperature (°C)	Thermal stability	Molecular mass (kDa)
Acremonium thermophilum	*cel7a*	7	*Trichoderma reesei*	5.5	4.67	60	NR	53.7
Chaetomium thermophilum	*cel7a*	7	*Trichoderma reesei*	4	5.05	65	NR	54.6
Chaetomium thermophilum	*cbh3*	7	*Pichia pastoris*	4	5.15	60	$T_{1/2}$: 45 min at 70 °C	50.0
Humicola grisea	*egl2*	5	*Aspergillus oryzae*	5	6.92	75	80% residual activity for 10 min at 75 °C	42.6
Humicola grisea	*egl3*	45	*Aspergillus oryzae*	5	5.78	60	75% residual activity for 10 min at 80 °C	32.2
Humicola grisea	*egl4*	45	*Aspergillus oryzae*	6	6.44	75	75% residual activity for 10 min at 80 °C	24.2
Humicola grisea var thermoidea	*eg1*	7	*Aspergillus oryzae*	5	6.43	55–60	Stable for 10 min at 60 °C	47.9
Humicola grisea var thermoidea	*cbh1*	7	*Aspergillus oryzae*	5	4.73	60	Stable for 10 min at 55 °C	55.7
Humicola insolens	*avi2*	6	*Humicola insolens*	NR	5.65	NR	NR	51.3
Humicola insolens	*cbhII*	6	*Saccharomyces cerevisiae*	9	NR	57	$T_{1/2}$: 95 min at 63 °C	NR
Melanocarpus albomyces	*cel7b*	7	*Trichoderma reesei*	6–8	4.23	NR	NR	50.0
Melanocarpus albomyces	*cel7a*	7	*Trichoderma reesei*	6–8	4.15	NR	NR	44.8
Melanocarpus albomyces	*cel45a*	45	*Trichoderma reesei*	6–8	5.22	NR	NR	25.0

(continued)

Table 14.1 (continued)

Fungus	Gene	Family	Host	Optimal pH	pI	Optimal temperature (°C)	Thermal stability	Molecular mass (kDa)
Talaromyces emersonii	*cel3a*	3	*Trichoderma reesei*	4.02	3.6	71.5	$T_{1/2}$: 62 min at 65 °C	90.6
Talaromyces emersonii	*cel7*	7	*E. coli*	5	4.0	68	$T_{1/2}$: 68 min at 80 °C	48.7
Talaromyces emersonii	*cel7A*	7	*Saccharomyces cerevisiae*	4–5		65	$T_{1/2}$: 30 min at 70 °C	46.8
Thermoascus aurantiacus	*cbh1*	7	*Saccharomyces cerevisiae*	6	4.37	65	80% residual activity for 60 min at 65 °C	48.7
Thermoascus aurantiacus	*eg1*	5	*Saccharomyces cerevisiae*	6	4.36	70	Stable for 60 min at 70 °C	37.0
Thermoascus aurantiacus	*bgl1*	3	*Pichia pastoris*	5	4.61	70	70% residual activity for 60 min at 60 °C	93.5
Thermoascus aurantiacus	*cel7a*	7	*Trichoderma reesei*	5	4.44	65	NR	46.9

Source: Li et al. (2019) Fungi in Extreme Environments: Ecological Role and Biotechnological Significance.2019:395–417

can grow and hydrolyze plant substrate efficiently, thus used in plant biomass degradation process or biological conversion of pretreated plant biomass to fermentable sugars. Higher thermostability of these enzymes allows the saccharification of plant biomass at increased temperatures resulting in shorter reaction time, higher mass transfer, and reduced substrate viscosity (van den Brink et al. 2013). *M. thermophila* produces thermophilic lignocellulolytic enzymes endoglucanase, β-glucosidase, xylanase, and avicelase that show maximum activity at 55–70 °C and maximum stability at 30–60 °C. Thermophilic avicelase efficiently hydrolyzes the crystalline cellulose and thermostable β-glucosidase is resistant to glucose inhibition making them specific to use in industries (de Cassia et al. 2015).

14.5.1.3 Themophilic Proteases

Thermophilic fungi *Chaetomium thermophilum* produce two thermostable proteases that have molecular weight 33 kDa and 63 kDa. Both proteases possess high thermal activity and optimal temperature 65 °C. The 33 kDa protease shows maximum activity at pH 10 along with Km value 6.6 mM and Vmax value 10.31 μmol/l/min. The 63 kDa protease shows maximum activity at pH 5 along with Km value 17.6 mM and Vmax value 9.08 μmol/l/min. The activity of proteases is stimulated by Ca^{2+} and inhibited by phenylmethanesulfonyl fluoride (Li et al. 2007). Proteases produced by thermophilic *Myceliophthora* species in solid state fermentation are alkaline and thermostable. Optimal temperature and pH for the activity of proteases are 50 °C and 9, respectively, in solid-state fermentation. These thermostable alkaline proteases have applications in leather processing, laundry detergents, brewing, food, and pharmaceutical industries. Thermal stability of proteases enhances when immobilized in alginate (Zanphorlin et al. 2010). Thermophilic fungus *Penicillium duponti* K1014 produces thermostable acid proteases that are stable at 50–60 °C. *Mucor pusillus* aspartic protease possesses aspartic acid at their active site. This enzyme shows maximum activity at 55 °C temperature and 3–6 pH. *Thermomyces lanuginosus* produces alkaline protease, which is basically a serine protease containing partially buried cysteine residue. This protease cleaves the substrate C-terminus end of hydrophobic amino acid residues (Maheshwari et al. 2000).

14.5.1.4 Thermophilic Lipases

Extracellular lipases from thermophilic fungi *Rhizopus oryzae* and *Rhizopus rhizopodiformis* isolated from palm oil mill effluent are active and stable at optimal pH 6.0 and optimal temperature 45 °C. Lipase from *R. oryzae* shows more activity at acidic pH and broader substrate specificity as compared to lipases from *R. rhizopodiformis* (Razak et al. 1997). Thermophilic fungus *Humicola lanuginosa* produce lipases that are heat stable. Its optimal temperature is 60 °C and optimal pH is 8.0. However, it is stable at pH range 7.0–10.0. Metal ions such as Mn^{2+}, Ca^{2+}, Ba^{2+}, Mg^{2+}, and Co^{2+} show stimulatory effect while Cu^{2+} and Hg^{2+} show inhibitory effect on the activity of lipases (Liu et al. 1973a, b). *Humicola* lipases possess high number of hydrophobic amino acids than hydrophilic amino acid that provides the compact structure to protect them from denaturation. Addition of sulfhydryl-

reducing agent and calcium ions does not affect the lipase activity. Addition of 8 M urea accelerated the heat inactivation of lipases. They possess six half cysteine residues per enzyme molecule (Liu et al. 1973a, b). *Rhizomucor miehei* produces heat-stable lipases and contains 269 amino acid residues. Serine, histidine, and aspartic acid present in the catalytic center are known as catalytic triad. Catalytic site is covered with a lid composed of short α-helical loop that regulates the entry of substrate and protects the site (Maheshwari et al. 2000).

14.5.1.5 Thermophilic Glucoamylases

Extracellular glucoamylases from thermophilic fungus *Ther. lanuginosus* are optimally stable in the pH range 6.5–8.0 and 50 °C temperature for 6 h. It shows optimal enzyme activity at pH 5.0, substrate concentration 0.2%, soluble starch or 0.4% maltose, and temperature 50 °C. This 57 kDa molecular weight protein is highly specific for α-D-1,4-glucosidic linkages and shows molecular activity of 4.76×10^3 min^{-1}. The rate of hydrolysis is significantly increased with increase in the chain length of substrates. The unique semi-random or random coiled well-hydrated structure provides thermostability to this enzyme (Basaveswara Rao et al. 1981).

Thermostable extracellular amylolytic enzyme glucoamylases produced by thermophilic fungus *C. thermophilum* is a 64 kDa molecular weight protein. The glucoamylase structure is composed of single polypeptide determined by Native PAGE. Optimal pH and temperature for this enzyme are 4.0 and 65 °C, respectively. The enzyme is stable in pH range of 3.5–8.0. Metal ions Hg^{2+}, Ag^{+}, and Fe^{2+} show inhibitory effect while Ca^{2+}, Na^{+}, and K^{+} show stimulatory effect on the enzyme activity. N-terminus amino acid sequence of this enzyme shows high similarity with glucoamylases from *Neurospora crassa*, *H. grisea,* and *Thielavia terrestris*. The half-life of glucoamylase is 20 min at 70 °C (Chen et al. 2005).

14.5.1.6 Thermophilic Xylanases

Thermophilic fungus *Talaromyces byssochlamydoides* strain YH-50 produces thermostable xylanase that shows stability at optimal temperature 70 °C and optimal pH 5.5. This native xylanase hydrolyze xylan to the extent of 90% and produces xylose in large amount along with arabinose, glucose, and galactose in small amount. This thermostable xylanase is used to degrade hemicelluloses to produce different monosaccharides (Yoshioka et al. 1981). Xylanase from *Thermoascus aurenticus* are highly homologous to mesophilic xylanase. This enzyme has 301 amino acid residues. Substrate binding site possesses glutamate and histidine residues that are linked with the negative charges. Proline residues present at N-terminus decrease the conformational freedom of enzyme that makes them stable under high temperature. Xylanase from *Ther. lanuginosus* possess a long cleft in which active site is present. The active site adjusted the heptaxylan molecules (Maheshwari et al. 2000). Comparative study of family 10 xylanase of *T. auranticus* with its mesophilic homolog shows increased hydrophobic packing and increased proline residues at the N-terminus in thermophilic xylanase. Although comparative study of family 11 xylanase of thermophile and mesophile shows high

threonine to serine ratio and an additional β-strand B1 at N-terminal in thermophilic xylanase (Collins et al. 2005). A thermophilic filamentous fungi *C. thermophilum* produce family 11 xylanase. Structure analysis shows that α-helix and highly twisted two β-sheets contribute the structure. Glycerol molecule is incorporated into the active site. Four sulfate ions and a calcium ion are present in this structure. Thermophilic xylanases contain more stable α-helix and an additional β-strand B1 at N-terminal. Ser/Thr surface incorporated with arginine residues increases the optimum temperature and thermotolerance of xylanase. Longer N-terminal as compared to mesophile is involved in the high activity (Hakulinen et al. 2003).

14.5.1.7 Thermophilic Laccases

Extracellular phenol oxidase produced by thermophilic ascomycete *T. aurantiacus* shows optimal oxidation temperature between 70 and 80 °C. Maximum enzyme activity is found at pH 6–8. Sodium azide and thioglycollic acid show highly inhibitory effect on enzyme activity (Machuca et al. 1998). Thermophilic actinomycetes *Thermobifida fusca* BCRC19214 produces 73.3 kDa laccase that possess trimeric structure and shows 4.96 U/ml laccase activity. pH stability ranges from 5.0 to 10.0. Optimal pH and temperature for laccase activity are 8.0 and 60 °C, respectively. Hg^{2+} (1 mM), SDS, and sodium azide partially reduce the enzyme activity while β-mercaptoethanol and EDTA strongly inhibit the laccase activity. Acetonitrile and dimethylformamide stimulate the activity of enzyme. Internal amino acid sequence analysis shows homology with laccase isolated from *Thermobifida fusca* YX (Chen et al. 2013). Laccase from *Myceliophthora thermophila* has heavy glycosylated structure. In total, 559 amino acid residues form a mature enzyme. It contains three tightly packed cupredoxin like domain. A and C domains form the trinuclear copper site while C domain contains T1 Cu site that coordinates two histidine His 431 and His 508 and one cysteine Cys 503 residue. T1 pocket shows high variability. A calcium ion involved in the crystal packing. Leu 363 carbonyl carbon and five water molecules coordinate with CA606 to form a stabilized loop structure (Ernst et al. 2018).

14.5.1.8 Thermophilic β-Glucosidase

A thermophilic fungi *C. thermophilum* produces thermostable cellulolytic enzyme β-glucosidase belonging to glycoside hydrolases family GH3. The crystal structure (Fig. 14.1) shows that it consists of three domains: catalytic triose phosphate isomerase barrel like domain, α/β sandwich domain, and fibronectin type III domain. Modifications are found in loops and linker regions. Linker region that connects domains 1 and 2 is found to be larger than other GH3 β-glucosidase except one from *N. crassa*. Sequence alignment study revealed that this β-glucosidase shows 72% similarity with *N. crassa* β-glucosidase. Glycosylated Asn 72 stabilizes the loop by making bond with N-glycans. Asn 504 was found as new glycosylation site. Active site is present near the first and second domain interface. Catalytic residue Asp 287 is situated at the N-terminal of first domain and functions as nucleophile while Glu 517 is situated in α/β sandwich domain and functions as an acid-base catalyst. Both catalytic residues are conserved. Second barrel β-strand are found shorter and

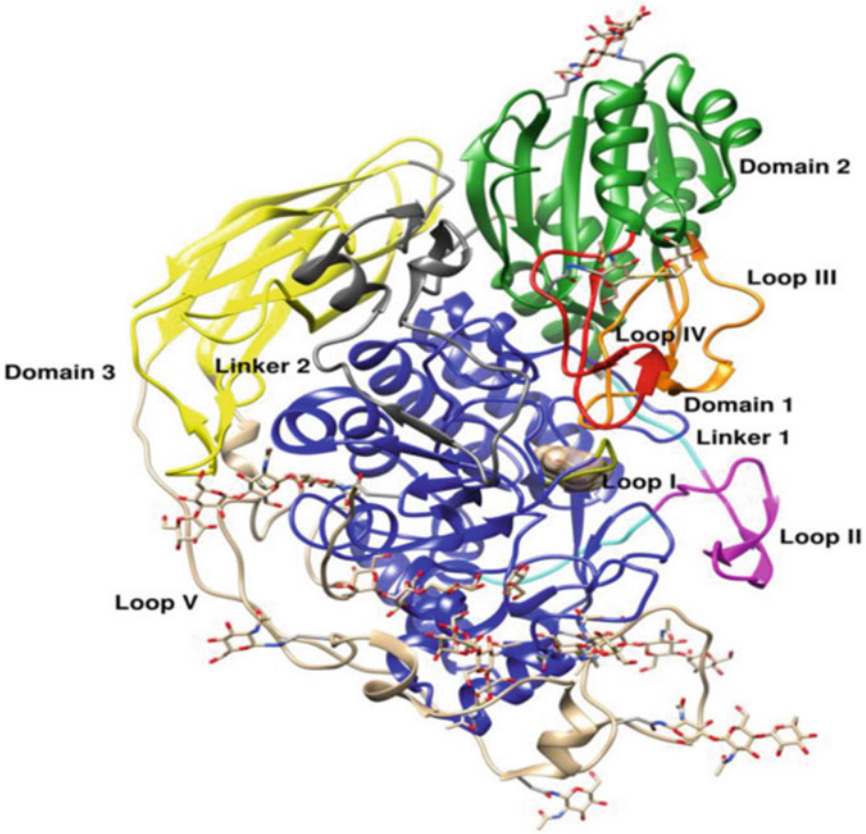

Fig. 14.1 The three domains of *Chaetomium thermophilum* β-glucosidase shown in its crystal structure. Different domains are indicated by blue, green, and yellow colors. Linker and loops are also shown by different colors. N-glycans and glucose in the active site are shown in the form of gray sticks. (Source: Mohsin et al. International journal of molecular sciences (2019) 20(23):5962)

antiparallel near the active site that makes active site wider and more accessible. Ramachandran plot shows 92.8% residues found in the most favorable region while 1% residues are found in the disallowed region. This enzyme has an optimum activity at 50 °C temperature. This enzyme retains half of the enzymatic activity after incubation for 55 minutes at 65 °C. Two thermostability parameters are responsible for its stability. One of them is glycosylation and the other is the charged residues that form electrostatic interactions. Thermophilic cellulases are preferred in cellulose degradation process as high temperatures make the process easy due to swelling of cellulose molecule (Mohsin et al. 2019).

14.5.1.9 Thermophilic α-Amylases

The α-amylase isolated from thermophilic fungus of genus *Aspergillum* STG3 and STG6 is thermostable that shows maximum enzyme activity at 55 °C when incubated for 10 min. Enzyme activity of STG3 and STG6 is 1.202 U/mg and 1.052 U/mg, respectively. pH optima for this enzyme is 6.0 for both strains. Ca^{2+}, Mg^{2+}, and Zn^{2+} metal ions strongly inhibit the amylase activity (Teklebrhan Welday et al. 2014). With increasing temperature, stability also increases. Increased enzyme stability leads to decrease in entropy of enzymatic reaction and low flexibility. Catalytic center of thermostable enzyme is compact and rigid due to which they are not flexible. Decreased conformational flexibility increases the stability of thermophilic enzyme. Thermophilic enzyme's catalytic domains contain numerous interactions that make them high temperature dependent, thus low temperature decreases the enzymatic activity of thermophilic enzymes (D'Amico et al. 2003). α-amylase from thermophilic actinomycete *Thermobifida fusca* is chloride dependent. Removal of chloride ligand causes low affinity of enzyme. Kcat value is found higher than its mesophilic and psychrophilic homolog. It possesses compact conformation due to its higher affinity (Cipolla et al. 2012).

14.5.1.10 Thermophilic Phytase

Phytase is the enzyme that catalyzes the breakdown of phytic acid. One such phytase is isolated from thermophilic fungus *Ther. lanuginosus* that is cloned and heterologously expressed to form a 60 kDa recombinant phytase. This phytase shows superior catalytic efficiency. Maximum enzyme activity shows at optimum temperature 65 °C. pH stability of this enzyme is 3.0–7.5 and active at near neutral pH. Specific activity of this recombinant phytase is 110 μmol/min/ mg of protein. This enzyme is used in feed industries to reduce the high phosphate waste and also reduces the phosphate content in manure (Berka et al. 1998).

14.6 Psychrophilic Enzymes

14.6.1 Psychrophiles

Psychrophiles are the organisms that can thrive under very low-temperature condition <10 °C. They grow at temperature between -20 °C and 20 °C and possess optimal growth temperature < 15 °C. Common habitats of psychrophiles are Arctic and Antarctic regions, icebergs, glaciers, snow fields, refrigerated food, etc. (Rampelotto 2013).

14.6.2 General Adaptations

Psychrophiles protect themselves from cold by increasing the amount of unsaturated and short chain fatty acids to regulate the membrane fluidity. They synthesize cold shock proteins and chaperons to protect the nucleic acid and proteins. Some

antifreeze proteins are found that has essential role in protecting the cell from cold. Accumulation of cryoprotectants or compatible solutes enables them to survive and tolerate extreme cold (Rampelotto 2013). But the highly studied adaptive strategy to survive in extreme cold is their cold-adapted enzymes.

14.6.3 Catalytic Features of Psychrophilic Enzymes

Biochemical reaction in psychrophilic environment or very low temperature is possible in the presence of psychrophilic enzymes, which is cold active and heat labile. Psychrophilic enzymes enhance the mobility and flexibility of the active site by decreasing the temperature dependence of the reaction. Catalytic center of psychrophilic enzymes is identical to mesophilic enzyme, but local interactions are different that provides high activity and improved catalysis at low temperature. Destabilization of active site structure and reduced number of various weak interactions enhances the dynamics of active site residues (Feller 2013). These enzymes have very flexible structure. As temperature decreases rate of reactions, it slowly decreases due to reduction in activation enthalpy, low kinetic energy, and more negative activation entropy. Lower arginine to lysine ratio, more glycine residues, reduced proline residues in loops, and increased proline residues in α-helices are some amino acid replacements present in psychrophilic enzymes. Reduced core hydrophobicity, decreased disulfide bridges, reduced oligomerization, and less electrostatic interactions make them very flexible. When compared to its mesophilic and thermophilic homologue; psychrophilic enzymes are more productive due to high catalytic efficiency. The active sites of these enzymes are the most heat-labile structure that enables them to function at low temperatures (Cavicchioli et al. 2011). Low proline content, less disulfide bond, and higher glycine clusters increase the activity of cold-active enzymes at low temperature (D'Amico et al. 2003). A figure represents some modifications in structure (insertions and extensions) and interactions of psychrophilic alkaline phosphate and psychrophilic α-amylase respectively to understand the adaptations of psychrophilic enzymes to cold environment (Fig. 14.2) (Santiago et al. 2016). From mutational studies, it is revealed that mutation in the site of enzyme away from the active site increases the enzyme activity. This also suggests that the local unfolding in psychrophilic enzymes increases the entropy that causes high enzymatic activity (Deniz 2018). For any biochemical reaction, activity constant is directly dependent on temperature.

Thus, lower temperature causes gradual decrease in enzyme activity of mesophiles. Cold active enzymes have tenfold higher specific activity to cope with the low temperature. Weak stability of psychrophilic enzymes and unfolding or inactivation at moderate temperature make them responsible for its maximal activity. Active site of cold-active enzymes is more heat labile as the enzyme inactivation starts before protein unfolding. This means that active site is more flexible and active structure. Noncatalytic part is stable as it is nonflexible. Large active site of cold-active enzymes mitigates the effect of rate-limiting step (Feller 2013). Increased conformational flexibility and solvent interactions are the most observed features of

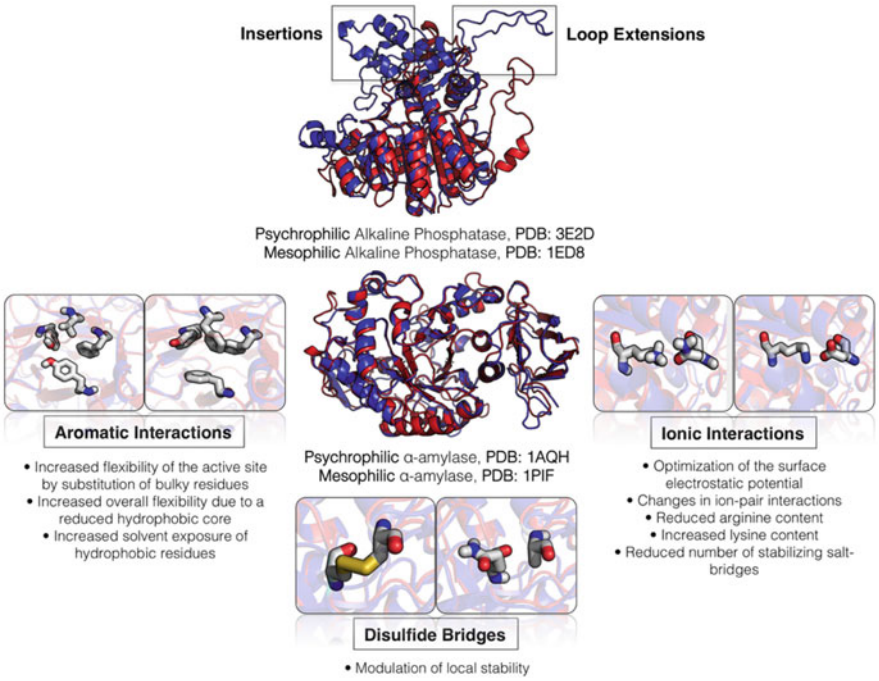

Fig. 14.2 Some important structural adaptations in psychrophilic enzymes vis-a-vis their mesophilic counterparts (Source: Santiago et al. 2016)

psychrophilic enzymes to cold environment (Karan et al. 2012). Deep sea cold-active enzymes possess more α-helix as compared to mesophilic enzymes, which is important for maintaining the flexibility of these enzymes. Their capacity of binding to solvent molecule is very high and strong that is responsible for its high catalytic rate at low temperature (Jin et al. 2019). Psychrophilic enzymes have high turnover rate due to high value of catalytic constant Kcat. However, they also have high value of K_M as psychrophilic enzymes have weak substrate binding affinity due to their flexible active sites. Although the catalytic efficiency of psychrophilic enzymes is higher because Kcat value is relatively higher than K_M value. Flexibility in psychrophilic enzymes is more important than stability (Ichiye 2018). At low temperature, kinetic energy of reacting molecules is low due to less number of weak interactions, thus the rate of reaction is slow. Viscosity of the solvent increases at low temperature; higher viscosity weakens the affinity of the enzymes for their substrate. Hence, psychrophilic enzymes have high specific activity, low thermostability, and increased interactions with the solvent molecules. Lower activation enthalpy in psychrophilic enzymes reduces the stability but enhances their flexibility that reduces the additional energy cost and enhances the activity at low temperature (Collins et al. 2007). The flexibility of protein is understood by its B-value, which is a value that measures dynamic motions of atoms in a protein crystal. Psychrophilic

enzymes possess high B-value in β-sheet along with turns. They possess significantly high amount of buried water, which suggests that psychrophilic enzyme core are more solvated than mesophilic counterpart. Their cavity is surrounded by high number of acidic amino acid that contains more water molecules than mesophilic homolog. It can be considered that psychrophilic enzymes either possess fewer cavities with large cavity volumes or more cavities with equivalent cavity volume as in mesophilic enzymes (Paredes et al. 2011).

14.6.4 Industrial Application

High catalytic efficiency of these enzymes enables them to use in biotechnological industries. Solvent tolerance of these enzymes is useful for cleaning purposes and removal of ester components in biofilms. These cold-active enzymes are used in food and feed industry to prevent the changes in flavor, nutritional value, and food spoilage. Transformation of heat-labile product by psychrophilic enzyme is beneficial. Cold-adapted lipases and esterases are used in chiral drug production due to high stereospecificity (Cavicchioli et al. 2011). They are used to stimulate ripening process of slow ripened cheese. They are used in the removal of macromolecular stains from fabrics and protect color of cloths during washing process at low temperature. Peeling process of leather consumes much energy. Use of cold-active proteases saves the energy as they work at the temperature of tap water. Cold-active enzyme reduces the energy consumption of a process that needs low temperature. Pulp and paper industries also need cold-active enzymes for the degradation of polymers (Joshi and Satyanarayana 2013).

14.6.4.1 Psychrophilic Lipases

Psychotropic fungus *Penicillium canesense* BPF4 and *Pseudogynmoascus roseus* BPF6 produce alkaline metallolipases BPF4 and BPF6, respectively, that is stable at 4 °C, 20 °C, and 40 °C as they retained its 80% and 90% residual activity after 2 h and 1 h, respectively. pH optima for BPF4 and BPF6 are 9 and 11, respectively. Cu^{2+} and Fe^{3+} ions stimulate the activity of BPF4 and BPF6 lipases while the activity of both lipases is inhibited by tween-20, $HClO_3$, and H_2O_2. EDTA and Sn^{2+} do not affect BPF4 activity but enhance BPF6 activity. SDS reduces the activity of BPF6 while slightly enhances the activity of BPF4 lipase. These properties make them potential to use in detergent formulation (Sahay and Chouhan 2018). *Moesziomyces antarcticus* produces two types of cold-active lipases. Antarctic fungus *Geomyces* species P7 lipases active and stable at optimum temperature 8–35 °C, and at 0 °C this lipase shows 15% of its maximum activity. These cold-active lipases are used in biodiesel production, wastewater treatment at low temperature, and cleaning products (Duarte et al. 2018).

14.6.4.2 Psychrophilic Protease

Penicillium chrysogenum FS010 produces cold-active lipase that has 35 °C optimum production temperature. It shows high activity between 15 and 35 °C and retained

41% residual proteolytic activity at 0 °C temperature. The activity of cold-active proteases is increased by addition of Ca^{2+}, Na^{+}, Mg^{2+}, K^{+}, and NH_4^{+} while it reduces by the addition of Cu^{2+} and Co^{2+}. Fe^{3+} and EDTA strongly inhibit the activity (Joshi and Satyanarayana 2013). *Rhodotrula mucilaginosa* L7 produces extracellular acid proteases that are active at temperature between 15 and 60 °C. Proteases from *Vanrija humicola* show activity at temperature 0–45 °C. Proteases from Antarctic fungus *Glaciozyma antarctica* 107 are very cold active as they exhibit activity from -10 °C to 25 °C. They are used in food industries to preserve the quality of heat-sensitive nutrients, softening of refrigerated meat products, cleaning of contact lenses, and cheese ripening process. They are used in the textile industry for cold washing process (Duarte et al. 2018).

14.6.4.3 Psychrophilic Amylases and Glucoamylases

Structural analysis of cold-active α-amylases from *G. antarctica* Pl12 shows that they possess binding sites for conserved and nonconserved calcium ion and sodium ion. *Moe. antarcticus* CBS6678 α-amylase and glucoamylase utilize high molecular mass substrates. They are applicable in wine production in brewing industry and juice clarification process at low temperature (Duarte et al. 2018). Psychrophilic α-amylase possess strictly conserved catalytic cleft made up of 24 residues. The catalytic cleft has large opening as compared to mesophilic homolog (Feller 2013). Cold-active α-amylase from *G, antarctica* PI12 possesses binding sites for conserved and nonconserved calcium ion and sodium ion (Duarte et al. 2018). Low activation enthalpy of cold-active enzymes reduces the temperature dependency of enzymatic reactions. Lesser extent of enthalpy-driven interaction makes them heat labile. Low stability of active site of cold-active enzymes leads to the high activity at low temperature. The active site of cols active enzymes is found to be more heat labile than overall structure. Free and active state of active site is more flexible due to which they possess more fluctuating structure. Activity of cold-active enzyme increases by reducing the temperature dependency by destabilizing the active site domain (D'Amico et al. 2003).

14.6.4.4 Psychrophilic Chitinases

Chitinolytic enzyme from fungus *Lecanicillium muscarium* CCFEE-5003 is a complex structure made up of five proteins of molecular weight 20–75 kDa with isoelectric point between 4.5 and 5.0 pH. Flexibility in this enzyme is due to replacements of some amino acids in surface and loop regions identified in *G. antarctica* PI12 chitinase. They are useful in chitin-rich waste treatment at low temperature, preventing contamination in refrigerated foods and biocontrolling of microbial spoilage (Duarte et al. 2018).

14.6.4.5 Psychrophilic β-Galactosidases

Marine Antarctic yeast *Tausonia pollulans* produces high amount of extracellular β-galactosidase that shows high activity in cold environment. They are used to reduce sugars in food and drugs, and reduce industrial effluents during

bioconversion of milk to whey and production of animal and human diet additives (Duarte et al. 2018).

14.6.4.6 Psychrophilic β-Glucanase

β-Glucanase from marine psychrophilic yeast *G. antarctica* PI12 contains a conserved motif G-E-x-D-x-x-E. Glu 214 and Glu 219 are highly conserved catalytic residues of glucoside hydrolases family 16 performs acid–base catalysis and nucleophile reactions. It shows optimum temperature 20 °C and maximum activity at pH 7. It comprises 28.6% α-helices and 42.8% β-strands. It possesses longer external loops. Long broken β-sheets are connected by a loop. Salt bridges and hydrogen bonds are lower in this enzyme as compared to mesophilic enzymes. The enzyme activity is higher for lichenan. Recombinant β-glucanase shows optimum activity at 15 °C and pH 7; however, it retains its activity at lower temperature and alkaline pH (Mohammadi et al. 2021).

14.6.4.7 Psychrophilic Xylanases

Cold-active xylanases are used in biofuel production and chemical production from lignocelluloses. Psychrophilic xylanase from Antarctic fungus *Naganishia adeliensis* shows lower activation energy and higher catalytic efficiency at 0–20 °C temperature. Structure analysis shows the presence of less compact and more flexible molecular structure (Duarte et al. 2018). Psychrophilic xylanase possess threefold higher activities than mesophilic xylanase at 5 °C temperature. They have short half-life and low denaturation temperature. Decreased stability of cold-active xylanase increases its flexibility that is responsible for the catalysis at low temperature (Collins et al. 2005).

14.6.4.8 Psychrophilic Laccases

An Antarctic soil fungi *Penicillium commune* AL2, *Aspergillus fumigates* AL3, and *Penicillium rugulosum* AL7 produce laccases by utilizing phenol that is active at 5 °C temperature. These fungi degraded polycyclic aromatic hydrocarbons. Laccase from Antarctic yeast is active at 15 °C temperature. These enzymes are used in textile industry and lignin degradation (Duarte et al. 2018).

14.6.4.9 Psychrophilic Cellulases

Many Antarctic filamentous fungi and yeast are found to produce cold-adapted cellulases. *Penicillium roqueforti, Cadophora malorum, Geomyces* species, and *Mrakia blallopsis* produce cellulase at 4-22 °C temperature. These cold-adapted enzymes are tolerant to organic solvents. Psychrophilic cellulases have the potential to be used in the production of volatile and heat-sensitive compounds (Duarte et al. 2018).

14.6.4.10 Psychrophilic Catalases and Superoxide Dismutase

Antarctic fungus of genus *Aspergillus, Cladosporium,* and *Penicillium* produces cold-active catalases. An Antarctic fungus *Aspergillus glaucus* 363 produces high

amount of superoxide dismutase at 10 °C. This cold-adapted enzyme is useful in cosmetic formulation to prevent skin injury (Duarte et al. 2018).

14.6.4.11 Psychrophilic Tannases

These cold-active tannases are useful in the production of instant tea, garlic acid, and fruit juice. *Verticillium* species P9 produces two types of extracellular tannase, TAH1 and TAH2, which has temperature optima 25 and 20 °C (Duarte et al. 2018).

14.7 Halophilic Enzymes

14.7.1 Halophiles

Organisms that live in high salt environment are known as halophiles. They grow and survive at high salt concentration. Optimum growth of these organisms lies between the salt concentration ranges 0.2–0.3 M NaCl. High concentration of ions, toxic metals, and low water activity is the characteristic feature of hypersaline environment (Edbeib et al. 2016).

14.7.2 Protein Adaptation

At high salt concentration, water activity is very low, which means water is less available to the proteins. The low water activity causes hydrophobic amino acids in a protein to lose hydration and aggregate. Non-halophilic proteins aggregate, precipitate, or denature at high salt environment, but halophilic proteins remain functionally active in that environment because of their haloadaptation. Halophiles adapted to high salt remodel the protein at different levels. At cell physiology level, they have large number of salt bridges allowing to stable at high salt. Another mechanism includes large number of acidic or polar chain amino groups on their protein surfaces and less bulky hydrophobic residues. At proteomics level, some enzymes like cysteinyl-tRNA synthatases and P45 proteins provide additional stability to haloproteins. At genomic level, high DNA—protein interaction protects the protein from denaturation at high salt concentration (Gomes and Steiner 2004; Kumar et al. 2018).

Stability of these proteins is also ensured due to the negative charges of acidic amino acids on the surface of protein, hydrophobic groups on protein core, and their hydration due to carboxylic group. High negative charges maintain the confirmation of protein and prevent aggregation at high salt concentration through binding with the hydrated cation and reduce the surface hydrophobicity of protein. Lysine amino acid percentage is fewer in halophilic proteins (Kumar et al. 2018).

14.7.3 Catalytic Features

Haloenzymes are the enzymes produced by the halophiles. They have similar characteristics to nonhalophilic homologues but have different structural properties. Halophilic enzymes are the protein of halophilic origin (secreted by halophilic microorganism). These enzymes are structurally adapted to function under high saline environment. Nonhalophilic enzymes under this extreme condition lose their structure and ability to perform catalysis efficiently. Halophilic enzymes function in high salt because they have some unique features in their structure (Charlesworth and Burns 2016). Low water activity and limited salvation in saline environment disrupt the structure of water molecule near proteins, resulting in high hydrophobicity. The scarcity of water molecule affects the solubility and stability of protein. Thus, high salinity causes aggregation and precipitation of nonhalophilic proteins (Reed et al. 2013).

Halophilic enzymes are more soluble in the presence of salt. They cannot perform catalysis in low saline or nonsaline environment as they lose their enzymatic activity due to the absence of salts. The role of salt is to maintain the native structure of enzyme. Salt maintains the rigidity of secondary and tertiary structure of halophilic enzymes against denaturants (Siglioccolo et al. 2011). Halophilic enzymes prevent their unfolding with the help of Ca^{+2} and regulate their catalytic activity with the help of Na^{+}. Thus, both the cations are essential for halophilic enzyme activity (Sinha and Khare 2014). Halophilic enzymes are not limited to function under high salt concentration but also they can be able to function under high temperature and organic solvents. They can overcome the problems encountered during the industrial processes such as high salt, high temperature, and high pH (Reed et al. 2013). At high salt concentration, water is present in hydrated ionic form, which makes them less available to the proteins. It is necessary to make protein molecule hydrated so that halophilic enzymes contain large and multilayered hydration shell. There is prevalence of glutamate residues in halophilic enzymes as they possess high water binding capacity. Lysine residues are present in very negligible amount in protein surface. Reduced surface hydrophobicity along with increased surface hydration enables halophilic enzymes to function in low water activity environment (Karan et al. 2012). The main problem in front of halophilic enzymes is to deal with the high concentration of internal and external electrolytes. So they prefer highly hydrated amino acid residues, especially glutamic acid and arginine, in their structure. They possess low ratio of nonpolar to polar residues. Halophilic enzymes maintain its flexibility at high salt concentration by reducing the hydrophobicity; thus, they prefer amino acid of low hydrophobic nature in their structure (Jaenicke 1991). Carboxylic groups of aspartic and glutamic acid make the protein surface hydrated. The activity of halophilic enzymes is dependent on salt concentration so that low salt concentration causes reduction in enzymatic activity and makes them less useful in industrial process. This can be overcome by using these enzymes in nonaqueous media as high salt concentration that leads to low water activity. Thus, they are useful for pharmaceutical industry (Kumar et al. 2011; Jin et al. 2019).

14.7.3.1 High Acidic Residues on their Surface

The halophilic enzymes contain high proportion of acidic amino acids such as glutamic and aspartic acid residues on its surface. These acidic amino acid residues help in the solvation (Gomes and Steiner 2004). An increased acidic residue provides negative charges to the surface. These negative charges repel each other and increase the flexibility of enzymes. Acidic surface is also essential for the solvation of the biocatalyst (Kumar et al. 2018).

14.7.3.2 Low Hydrophobicity at the Core

These enzymes contain small proportion of aromatic hydrophobic amino acid in the core. A lower bulky hydrophobic residue makes the hydrophobic interaction weaker in these proteins. Thus, decreased hydrophobicity in halophilic proteins provides them flexibility at high salt concentration (Siglioccolo et al. 2011; Reed et al. 2013; Kumar et al. 2018).

14.7.3.3 Increased Salt Bridge Formation

Salt bridges are the electrostatic attraction between oppositely charged residues. The presence of greater number of salt bridges in halophilic enzymes provides stability to them (Sinha and Khare 2014; Kumar et al. 2018).

14.7.3.4 Salt-Dependent Folding

Salt-dependent folding is another catalytic feature of halophilic enzymes. Halophiles utilize the salt to fold the protein and enable them to function under saline environment. Thus, they have salt-dependent catalytic activity (Reed et al. 2013; Sinha and Khare 2014). In salt-free medium, proteins remain unfolded. This will reduce the enzyme activity. However, addition of salt into medium induces refolding (Sinha and Khare 2014) (Fig. 14.3).

In a study on the effect of salt on the catalytic activity of bacterial (*Haloferax volcanii*) Ligase N, an NAD^+-dependent DNA ligation enzyme, it was found that high salt (KCl) concentration maintains its folded (active) structure and activates it. The enzyme, however, remained inactive (unfolding of enzyme) in the presence of NaCl. The enzyme has three domains, viz., DNA binding domain, an adenylation domain with two sub-domains (Fig. 14.3b), and an OB fold domain. During catalysis, adenylation of enzyme (transfer of an AMP molecule from NAD^+ to the protein or adenylation of enzyme) occurs that close the subdomains.

14.7.3.5 Peptide Insertions

A peptide insertion consists of high number of acidic amino acid. This negatively charged peptide insertion in halophilic enzyme is responsible for its flexibility (Reed et al. 2013).

14.7.3.6 Low Lysine Content

Halophilic enzymes possess low frequency of lysine amino acid for stabilization (DasSarma and DasSarma 2015).

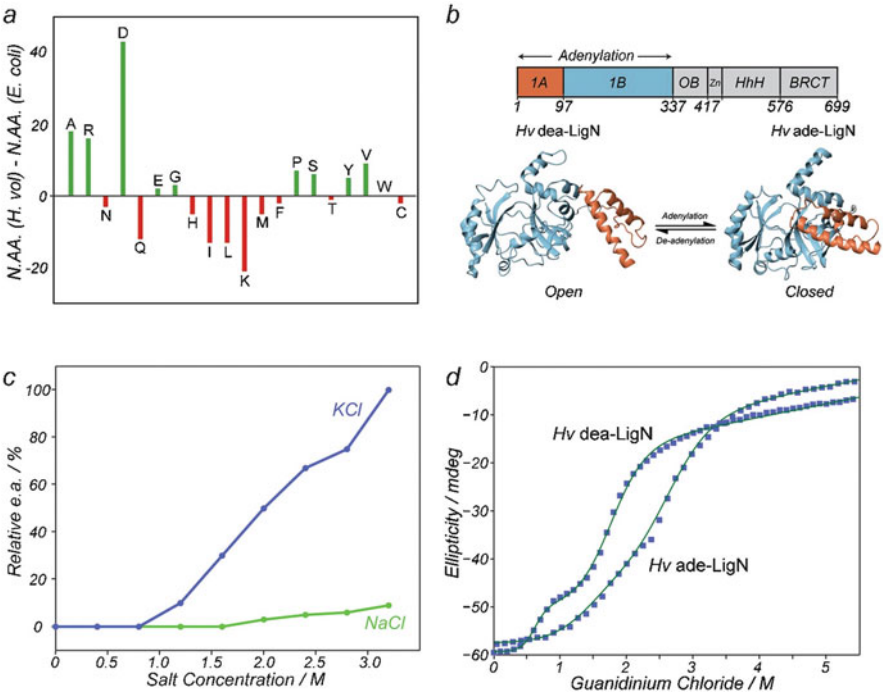

Fig. 14.3 (**a**) Impact of salt (KCl) on ligase N structure and activity. (**b**) Note the adenylation domain and its two subdomains in orange light blue: enzyme structure; active (folded) and inactive (open) are related with adenylation and de-adenylated, respectively. (**c**) Relative enzyme activity depends on KCl (not NaCl) concentration. (**d**) Representative examples of the chemical denaturation profiles monitored by CD, for *Hv* dea-LigN and *Hv* ade-LigN. (Source: Ortega et al. 2011)

14.7.3.7 Thermal Stability

Halophilic microorganisms have optimal growth at temperature range between 45 °C to 60 °C. Hence, halophilic enzymes are soluble and thermostable at high salt environment. Presence of ion–pair network in its structure is responsible for its thermal stability. The decreased hydrophobicity and hydrogen bonding are important parameters to maintain the stability of these enzymes at high temperature (DasSarma and DasSarma 2015).

14.7.3.8 Stability and Activity in Organic Solvents

As the halophilic enzymes are adapted to function under low water activity environment, they are able to maintain their property in organic solvent too. Presence of acidic residues on the surface and disulphide bonds in these enzymes enables them to stable and active in organic solvents (DasSarma and DasSarma 2015). The enzymatic activity of halophilic enzymes increases in the presence of some salt such as NaCl or KCl. Halophilic enzymes bind the water tightly due to which its solvation and solubility is regulated under extreme salinity (Kumar et al. 2018).

14.7.4 Industrial Applications

The unique catalytic features and multi-resistant properties of these halophilic enzymes make them stable and active under a wide range of salt concentration. Halophilic hydrolases such as amylase, protease, lipase, cellulase, xylanase, and laccase are the largest group of industrially important enzymes (Kumar et al. 2011).

14.7.4.1 Halophilic Protease

Proteases are the enzymes that catalyze hydrolysis of peptide bonds present in protein. They have wide application in industries. They are used in leather industries for skin dehairing. They have an important role in bioremediation process in the treatment of highly saline wastewater. They enhance the protein stain removal action of detergent, thus used in detergent industries. Salt stable proteases produced by *Nocardiopsis prasina* are active at pH range 7–10 and temperature range 20–42 °C (Solanki and Kothari 2011).

14.7.4.2 Halophilic Xylanases

Xylanases are the xylan degrading enzymes that cleave the β-1,4 linkage of this polysaccharide. In pulp and paper industries, cellulase-free xylanases are used for pulp biobleaching. Xylanases produce by the *Phoma* species of mangrove fungi was cloned and expressed in *Pichia pastoris*. This recombinant xylanase was found to be active at pH 5 and 45 °C temperature. This enzyme shows high degree of salt resistance up to 4 M NaCl (Wu et al. 2018).

14.7.4.3 Halophilic Cellulases

Cellulases from halophilic microorganisms have higher catalytic activities as they are stable at high salt and high temperature. These enzymes are used in the production of bioethanol. Cellulases from a moderate halophile *Aspergillus caesiellus* are active in salinity up to 2 M NaCl concentration. This cellulases has two isoforms of approximately 50 and 35 kDa molecular mass. Optimal temperature and pH range for enzyme activity are 60 °C and 5–6 pH, respectively. These thermostable and halostable cellulases are used in biorefinery process for the degradation of lignocellulosic materials (Batista-García et al. 2014). Cellulases from *Emericella nidulans* and *Cladosporum cladosporioides* are highly active at pH 4 and 10% NaCl and pH 10 and 10% NaCl, respectively (Moubasher et al. 2016).

14.7.4.4 Halophilic Lipases

These enzymes catalyze the hydrolysis of triglycerides and produce glycerol and fatty acids. Halophilic lipases are very useful in biodiesel production, bioremediation, and detergent formulation. Halophilic extracellular lipase produced by halophilic *Fusarium solani* using palm oil mill effluent is a very efficient approach for low-cost production of halophilic lipase (Geoffry and Achur 2018).

14.7.4.5 Halophilic Amylase

Halophilic fungi *Engyodontium album* produce polyextremophilic α-amylases, which have enzymatic activity at pH range between 5 and 9 and temperature range between 40 and 60 °C. They have optimal enzyme activity at pH 9 and temperature 60 °C. However, they require 6–30% NaCl for their activity, optimal enzyme activity being at 30% NaCl concentration. The catalytic activity is stimulated by $BaCl_2$, $CaCl_2$, and $HgCl_2$ and inhibited by β-mercaptoethanol, EDTA, $FeCl_2$, and $ZnCl_2$ (Ali et al. 2014a, b). An obligate halophile *Aspergillus gracilis* produces extracellular α-amylases, which are approximately 35 kDa molecular mass protein and are of polyextremophilic nature. This enzyme has specific enzymatic activity 131.02 U/mg and optimal enzymatic activity is found at pH 5, temperature 60 °C, and salt concentration 30% NaCl. Thus, these salt-adapted enzymes are used in bioremediation process for the treatment of saline and low water activity wastewater (Ali et al. 2014a, b). An obligate halophile *Aspergillus penicillioides* TISTR3639 strain produces extracellular α-amylase that has approximately 42 kDa molecular mass and utilizes soluble starch as a substrate. Specific activity, Vmax value, and Km value of catalyst are 118.42 U/mg, 1.05 μmol/min, and 5.41 mg/mL, respectively. This amylase is optically active at pH 9, 80 °C temperature, and 300 g/L NaCl. Enzyme activity increases with the addition of $CaCl_2$ at 2 mM concentration. Enzyme activity of amylase is found strongly inhibited by $ZnCl_2$ and $FeCl_2$ while EDTA at 2 mM concentration moderately inhibits the activity of amylase (Ali et al. 2015).

14.7.4.6 Halophilic Laccases

Laccases constitute a group of enzymes consisting of lignin peroxidase, phenol peroxidase, phenol oxidase, manganese peroxidase, and tryrosine. They degrade the lignocellulosic materials efficiently in the presence of salt. Halotolerant fungus *Pestalotiopsis* species degrade lignin and toxic waste in elevated temperature and salt. They are used in the treatment of 29 henolics wastewater and pulp-making processes (Arfi et al. 2013). Halotolerant laccase from *Chaetomium* species, *Xylogone sphaerospora,* and *Coprinopsis* species are chloride tolerant and used in the transformation of aromatic pollutants like polycyclic aromatic hydrocarbon (Qasemian et al. 2012).

14.7.4.7 Halophilic Chitinase

A thermohalophilic fungus *Aspergillus flavus* produces 30 kDa molecular mass chitinase. This enzyme shows maximum catalytic activity at substrate concentration 0.9 g/l, pH 7.5, temperature 60 °C, and NaCl concentration 0.8 M. The enzymatic activity is stimulated by $MnCl_2$ and $FeSO_4$. The kinetic parameter of this enzyme such as Km value and Vmax value was found to be 0.18 g chitin/ml and 274.31 U/l, respectively (Beltagy et al. 2018).

14.8 Acidophilic Enzymes

14.8.1 Acidophiles

Organisms that thrive at extremely low pH and exhibit optimal growth below pH 3 are known as acidophiles. They maintain their cellular pH near neutral by regulating the pH gradient.

14.8.2 Habitat of Acidophiles

Acidophiles are found in acid mine drainage, acidothermal hot springs, coal spoils, solphataric fields, and bioreactor (Sharma et al. 2016).

14.8.3 Characteristics of Acidic Environment

Acidic environment is characterized by low pH value, high concentration of protons, and low salinity. Some toxic-heavy metals and aggressive oxidative agents are also present in acidic environment (Christel 2018).

14.8.4 Catalytic Features of Acidophilic Enzymes

The intracellular enzymes of acidophiles do not need to develop any strategy to counter low pH as the organism maintains its internal pH near neutral. However, the extracellular enzymes show adaptations against low pH. The acidophilic enzymes have increased number of negatively charged amino acid residues on the surfaces (negatively charged surface). They have glutamic acid and aspartic acid in excess amount so that they can form a highly negative surface, which provides them stability in acidic environment (Hassan et al. 2019). Reduced solvent access and binding of metal co-factors enable them to catalyze at high acid environment (Hassan et al. 2019). Enriched acidic amino acid residues along with low solvent exposure makes them acid stable. They reduce the electrostatic repulsion by altering charged amino acid with neutral polar amino acid. Isoleucine residues are also present in high amount in acidophilic enzyme as compared to mesophilic enzymes (Jaenicke 1991).

14.8.5 Industrial Applications

Acid-stable enzymes are used in many industries such as paper, leather, and food and feed industry. Biobleaching, food industry, starch and baking industry, fruit juice industry, wine industry, and animal feed are some industries that need acidophilic

enzymes (Charlesworth and Burn 2016; Sharma et al. 2016; Dumorné et al. 2017; Christel 2018; Narayanasamy et al. 2018).

14.8.5.1 Acidophilic Laccase

Laccase from marine fungi *Trematosphaeria mangrovei* have maximum activity at 65 °C temperature and pH 4.0. Presence of $FeSO_4$ and NaN_3 inhibits the activity of enzyme (Atalla et al. 2013). A white rot fungus *Ganoderma lucidium* produces laccase that have optimal activity at pH 3.5 and temperature 20 °C (Ko et al. 2001). Laccase from *Aspergillus* species HB-RZ4 has molecular weight approximately 62 kDa and optimal enzyme activity is 8.125 U/mL. This enzyme is stable and active under 4.6–6.0 pH range and 20–60 °C temperature; however, optimal pH is 4.5 and optimal temperature is 34 °C. Glucose in the media highly increases the productivity and glycerol in the media has very low productivity. Sodium azide, halides, and fluorides inhibit the laccase activity. Cu^{2+}, Mo^{2+}, Mn^{2+}, and Zn^{2+} stimulated the activity of laccase. Kinetic parameters, Km and Vmax values, are 26.8 mM and 7132.6 mM/min, respectively. The attractive property is that during the bleaching process it decolorizes the dye in the absence of a laccase mediator system, which ultimately reduces the production cost (Sayyed et al. 2020). *Fomitella fraxinea* acidophilic laccase has optimal activity at pH 3 and temperature 70 °C (Hassan et al. 2019).

14.8.5.2 Acidophilic Manganese Peroxidase

Manganese peroxidase from *Phanerochaete chrysosporium* is made up of two domains. It possesses an extra disulfide bond between cys341 and cys 348 that is essential for the formation of Mn^{2+} binding site. Ten major and one minor helix contribute the structure. Proximal His 173 of active site and Asp 242 make a hydrogen bond responsible for iron low negative potential. His 46 and Arg 42 form H_2O_2 binding pocket. Cd^{2+} is found as a reversible competitive inhibitor of Mn^{2+}. Their activity in low pH makes them industrially important (Chandra et al. 2017).

14.8.5.3 Acidophilic Chitinase

A fungal species of genus *Microbispora* produces chitinases, which has acidophilic and thermophilic property. The isolated enzymes have around 35 kDa molecular mass. They have optimum enzymatic activity at pH 3.0 and temperature 60 °C. Although they retain their activity at pH range 3–11. The maximum activity is found in the presence of p-nitro-β-D-N,N′-diacetylchitobiose (Nawani et al. 2002).

14.8.5.4 Acidophilic Xylanase

Acidophilic xylanase from acidophilic fungus *Bispora* are very stable under acidic condition. The enzyme activity graphs of this acidophilic xylanase show two activity peaks at pH 3 and 4.5. They are acid stable and resistant to Co^{2+}, Mn^{2+}, Cr^{2+}, and Ag^{2+} (Wang et al. 2010). Acid stable xylanases are used in pulp and paper industries for biobleaching process. As they have optimal activity at low pH, the metal ions in the pulp are removed out easily. So that there is no requirement of the additional

processes for metal ion removals such as application of chelating agents like EDTA which lowers the production cost. In food and feed industry, these enzymes are used for the release of nutrients from the diet, resulting in better digestibility. In biofuel production process, the pretreatment requires the addition of dilute sulphuric acid for making the catalysis easy. Thus, use of these acidophilic xylanases reduces the cost of biofuel production as there is no need of pH adjustment (Rao and Li 2017; Sharma et al. 2017; Dey and Roy 2018). An extracellular xylanase is isolated from acidophilic *Aspergillus flavus* MTCC9390 is active and stable at pH 6 along with 60 °C temperature. Addition of metal salts or additives inhibits the activity of xylanase while polyethylene glycol inhibits and interferes with the enzyme activity in a concentration-dependent manner (Bharat et al. 2014). *Trichoderma reesei* produces xylanase made up of 178 amino acid residues. Glu 75 and Glu 164 are catalytic residues. Glu 75 is highly conserved and functions as nucleophile while Glu 164 is less conserved and functions as acid base catalyst. It contains two β-sheets and one α-helix. Three subsites are present at active site: one subsite at reducing end while remaining two subsites at the nonreducing end near ligand binding. Xylanase C of *Aspergillus kawachii* possess nucleophilic residue Glu79 and acid–base catalyst residue Glu170. More acidic amino acid residues at the edge of catalytic cleft and Asp 37 are responsible for low pH optima. Xylanase 1 from *Scytalidium acidophilum* possess Glu 104 and Glu 192 catalytic residue and Asp 60 residue near the acid base catalyst forms hydrogen bond with an angle of 2.45 Å. Xylanase I of *Aspergillus niger* possess same catalytic and aspartic acid residues as in the xylanase of *A. kawachii*. This enzyme functions at pH 3. Asp 37 makes hydrogen bond with Glu 170 with a bond angle 2.8 Å that is responsible for low pH activity (Dey and Roy 2018). Family 11 acidophilic xylanase contain aspartic acid residue makes hydrogen bond with acid–base catalyst. They possess high number of acidic residues and low number of salt bridges as compared to alkaliphilic xylanase (Collins et al. 2005).

14.8.5.5 Acidophilic Polygalactouronse

Acidic polygalactouronase are used in food industries for clarification of vegetables and fruits juices by the degradation of pectin and lowering the viscosity. They are used in combination with the other hydrolases to produce the animal feed-in-feed industries. The acid-stable hydrolases of *A. kawachii* is used in the fermentation process (where acidic medium is required) as they are highly active under low pH range 2.0–3.0 (Hassan et al. 2019).

14.8.5.6 Acidophilic β-Mannanase

β-Mannanases are enzymes that break β-mannosidic bonds in mannan. It has optimal pH range of 2.4–6.0 that makes it very suitable for feed industries. A novel acidic β-mannanase produced by *Penicillium pinophilum* C1 isolated from tin mine has maximum activity at pH 3 and temperature 28 °C (Hassan et al. 2019). Mannnanase from acidophilic *Bispora* MEY-1 has high activity and stability over the pH range 1.0–6.0 and optimal temperature 65 °C. They have strong resistance for proteases.

14.8.5.7 Acidophilic α-Amylases

Acidophilic α-amylases from acidophiles are used for the saccharification of starch and hydrolysis of polysaccharides for the production of bioethanol. Acid-stable α-amylases reduce the cost and time of the process that makes them economic and time saving. These enzymes possess high number of basic residues that makes the enzyme surface positive, which is responsible for the acid stability (Parashar and Satyanarayana 2018).

14.9 Alkaliphilic Enzymes

14.9.1 Alkaliphiles

Extremophiles that grow optimally at pH value above 10 classified as alkaliphiles (Dumorné et al. 2017).

14.9.2 Classification of Alkaliphiles

Organisms that grow at pH above 9 are alkaliphiles while those grow at pH above 9 along with the high salt concentration are referred to as haloalkaliphiles. Alkaliphiles that grow only at alkaline pH above 9 but cannot grow at neutral pH are obligate alkaliphiles while those grow at alkaline pH but can also grow at neutral pH are known as facultative alkaliphiles. Microorganisms that can actively grow at pH range 7–9 but can also survive in alkaline environment with pH value above 9 are known as alkalitolerant (Gupta et al. 2014).

14.9.3 Habitat

Hypersaline water, high CaOH ground water, soda lake, soda desert, alkaline drainage water, and alkaline carbonate lakes are native locations of alkaliphilic microorganisms (Ulukanli and DIĞRAK 2002).

14.9.4 Challenges in Alkaline Environment

At high pH, the cytoplasm of a mesophile becomes alkaline as a result of influx of hydroxyl ions in the cell or efflux of hydrogen ions from the cell. Proton motive force is disrupted at high pH, which interferes with the substrate uptake, resulting in low ATP synthesis. At alkaline condition, cell membrane structure becomes disturbed. This decreases the specific permeability of cell membrane. Thus, maintenance of internal pH is very essential for survival in alkaline environment (Gomes and Steiner 2004).

14.9.5 Catalytic Features of Alkaliphilic Enzymes

The intracellular enzymes of alkaliphiles do not use any strategy to counter high pH as the organism maintains its internal pH near neutral. However, the extracellular enzyme shows adaptations against high pH.

14.9.5.1 Negatively Charged Amino Acid Residues

The alkaliphilic enzymes have increased number of negatively charged amino acid residues on its surface (Kumar et al. 2018).

14.9.5.2 Increased Hydrogen Bonds

Higher number of hydrogen bonds and hydrophobic interactions in alkaliphilic enzymes makes them alkaline stable (Kumar et al. 2018).

14.9.5.3 Fewer Hydrophobic Residues

The number of hydrophobic residues exposed to the solvent is very low, essential for its activity in alkaline environment (Krulwich et al. 2010).

14.9.6 Industrial Applications

Alkaliphilic enzymes are active and stable at high pH. This property of enzyme is very useful in biotechnological industries. Alkaliphilic proteases are used in hide deharing processes and removing clogs in drain pipes. Alkaline cellulases are used in saline wastewater treatment. Alkaline xylanases and pectinases are used in paper manufacturing and wastewater treatment. Alkaline mannanase are used in food processing. Use of alkaliphilic enzymes in detergent industries makes the product stable and reduces the cost of production (Ulukanli and DIĞRAK 2002).

14.9.6.1 Alkaliphilic Laccase

Alkaliphilic laccases from Alkalitolerant fungi *Myrothecium verrucaria* have optimal activity at pH 9 and temperature 70 °C. Substrate specificity of this enzyme is toward phenolics and aniline compounds (Sulistyaningdyah et al. 2004). Laccase from basidiomyceteous fungus *Coprinopsis cinerea* is engineered and expressed in *Pichia pastoris*. This mutated laccase is an alkali stabile and shows optimal pH 7–7.5 for dye decolorization process. They are active and stable at alkaline pH, thus highly desirable in textile industries (Yin et al. 2019).

14.9.6.2 Alkaliphilic Xylanase

The alkaliphilic xylanases readily break down xylan that is soluble in alkaline solution. Alkaliphilic extracellular xylanases isolated from *Aspergillus nidulans* show optimal activity at pH 8.0 and temperature 55 °C. However, they can be active and stable over a wide range of pH (4.0–9.5) and temperature (40–55 °C). The enzyme activity is stimulated by the presence of Na^{2+} and Fe^{3+} while its activity is inhibited by the presence of acetic anhydride, SDS, Hg^{2+}, and Pb^{2+}. Substrate

binding affinities of xylanases are higher for the xylans from hardwood. Studies on inhibition show that the cysteine residues are present on the catalytic site. Positively charged amino groups are essential for the catalytic activity of this enzyme (Taneja et al. 2002). *Penicillium citrinum* extracellular xylanases have optimal enzymatic activity at pH 8.5, temperature 50 °C, and PI 3.6. Molecular mass were found to be 25 kDa. Their activity is stimulated by β-mercaptoethanol, dithiotheritol, cysteine, NaCl, urea, and SDS. Hg^{2+} and Mn^{2+} inhibit the activity of enzymes. There is no metal ion present in the active site (Dutta et al. 2007). Xylanase from an alkalitolerant fungus *Aspergillus niveus* RS2 has approximately 22.5 kDa molecular mass and shows maximum activity of 18.2 U/ml at pH 8. Enzymatic activity is optimal at pH 7 and 50 °C temperature. Km and Vmax values of enzyme are 2.5 mg/ml and 26 μmol/mg/min, respectively. Hg^{2+} strongly inhibits the enzyme activity while it is moderately stimulated with the presence of Mn^{2+} (Sudan and Bajaj 2007). Alkaliphilic xylanase of family 11 type contains high number of salt bridges and less number of acidic residues as compared to acidophilic xylanase. In alkaliphilic xylanase, asparagine makes hydrogen bond with acid–base catalyst (Collins et al. 2005).

14.9.6.3 Alkaliphilic Proteases

Alkaliphilic protease increases the smoothness and dye affinity of wool (Kumar et al. 2011). Alkaliphilic protease from *A. niger* shows maximum activity at 45 °C and pH 8.5. It is thermostable and has a molecular mass of 38 kDa. The combination of 50 °C temperature and pH 10 is mandatory for optimal enzymatic activity. Presence of detergents stimulates the enzyme activity while Cu^{2+}, Hg^{2+}, Zn^{2+}, EDTA, and sodium azide inhibit the activity of protease (Devi et al. 2008). An alkaliphilic strain of *Streptomyces albidoflavus* produces extracellular proteases, which are capable of hydrolyzing keratin. This protease has optimal enzymatic activity at pH 9 and temperature 60–70 °C. Alkaline proteases have optimal pH range 9.0–11.0 and optimal temperature range 50–70 °C. They have molecular mass between 15 and 30 kDa. To maintain their active confirmation, they require metal ions or divalent cations. Combination of metal ions or cations maximizes the enzyme activity. Hg^{2+}, phenylmethylsulphonylfluoride, and diisopropyl fluorophosphates are some inhibitors of protease activity. They are more specific against aromatic or hydrophobic amino acid residues (El-Shafei et al. 2010).

Alkalitolerant *P. citrinum* produces 32.27 kDa halotolerant alkaline serine proteases that are stable in acidic condition along with high salinity. They have optimal activity at pH 8.0, temperature 40 °C, and on substrate casein. They are stable at broad range of pH 6.0–8.0 and temperature 4–30 °C. The Km and Vmax values of protease for casein were found as 1.93 mg/mL and 56.81 μg/min/mL, respectively. They require salinity for their activity but higher NaCl concentration interferes with their activity. However, many ions such as K^{+}, Ca^{2+}, Zn^{2+}, Mg^{2+}, Fe^{2+}, and Fe^{3+} inhibit the protease activity. Phenylmethyl sulfonyl fluoride strongly inhibited the activity of protease while presence of Mn^{2+} stimulated the activity of this enzyme (Dutta et al. 2007). *Aspergillus* strain KH17 produces alkaline protease

that has maximum enzyme activity (215 U/ml) on casein. Enzymatic activity is optimal at pH 9 and 25–30 °C temperature (Palanivel et al. 2013).

14.9.6.4 Alkaline Cellulases

They catalyze a reaction under alkaline condition. In detergent industry, alkaline cellulases are used as a laundry detergent additive, which enhances the cleaning effect of detergent. These cellulases are known for their thermostability as they have excellent heat resistance. Some *Aspergillus* and *Penicillium* strains isolated from rainforest of Peru produce alkaline cellulases that are active at pH 9.4 (Vega et al. 2012; Rao and Li 2017).

14.9.6.5 Alkaline Pectinases

A class of enzyme that catalysis the breakdown of pectin substrates. Pectinases from *Penicillium italicum* is stable at pH 8 and temperature 50 °C. *Amycolata* species secreted highly alkaline pectinase having optimum activity at pH 10.25 and temperature 70 °C. Pectinases from *Streptomyces* species QG-11-3 is active at pH 3–9 and stable at optimal temperature 60 °C. Alkaline pectinases are used in textile processing, bioscouring of cotton fibers, degumming of plant bast fibers, retting of plant fibers, and pretreatment of pectic wastewaters (Hoondal et al. 2002).

14.10 Piezophilic Enzymes

14.10.1 Piezophiles

Piezophiles also known as barophiles are the organisms that thrive under high hydrostatic pressure often exceeding up to 400 atm. Extreme barophiles optimally grow at 700 atm or more. Piezophiles are found in deep sea sediments, marine trenches, hydrothermal vents, ocean floors, and deep rocks. Piezophilic microorganisms requires high pressure to grow optimally while piezotolerant microorganisms grow optimally at atmospheric pressure or high pressure but do not obligately require high pressure (Ichiye 2018; Jin et al. 2019).

14.10.2 Structural/Catalytic Features of Piezophilic Proteins

High pressure causes reduction in lipid fluidity and membrane permeability, denaturation of DNA and protein, compactness in protein structure, protein unfolding, and reduction in enzyme activity. Proteins from piezophiles do not exhibit any adaptation to counter pressure effect. Compression and unfolding are the two changes observed in proteins due to high pressure. Studies on protein domains and internal cavities of different proteins show compaction at high pressure. Elevated pressure reduces the volume in proteins resulting in the modification of interactions between the polypeptide and water molecule leads to unfolding of protein. Protein denaturation occurs at or more than 400 MPa hydrostatic pressure. At genomic level,

these organisms primarily adapt strategies to protect the DNA and protein from damage and secondary adaptation involves the high repair rate. Some organisms possess pressure-regulated operons. Transfer of mobile genetic element is very significant as they possess pressure-adaptive genes. Cellular adaptations include reduction in cell division, production of compatible osmolyte (piezolytes) to restrict the changes caused by high pressure, and presence of increased number of polyunsaturated fatty acids in membranes to increase the membrane fluidity. Increase in 1 kbar pressure causes reduction in reaction rate by 15% that indicates the sensitivity of proteins to pressure unfolding. At proteomic level, these piezophilic proteins include the high amount of arginine amino acid and increased number of small amino acids such as glycine, lysine, and valine. Proline residues are present in very less amount. Low stability of piezophilic enzymes provides high flexibility. High-pressure structural stability is due to small volume and high amount of hydrophobic interaction in protein. Arginine is known to be a barophilic amino acid as it is more commonly found in piezophilic enzyme. Smallest volume and high percentage of hydrophobic interactions in piezophilic enzymes makes them stable in high-pressure environment. Small cavities present in piezophilic proteins allow the penetration of water molecule at increased pressure. They possess high compressibility, which protects them from pressure-induced deformation. High absolute activity and high relative activity at high pressure increase its catalytic efficiency and make them stable. Kcat values are relatively higher in most of the deep sea piezophilic enzymes. Piezophilic enzymes and their mesophilic homolog show no structural variations. However, variations are found in the flexibility and hydration of protein. Yeast *S. cerevisiae* cope with the deep sea pressure by altering its membrane composition. This is to be noted that higher and positive the value of change in free energies of unfolded and folded state, greater will be the protein ability to prevent itself from denaturation (Kumar et al. 2011; Ichiye 2018; Castell et al. 2019; Jin et al. 2019).

14.10.3 Industrial Application

Piezophilic proteins are used in processing and sterilization of food products that need high pressure. High pressure provides better flavor to food products. At high pressure, food products decolorize but use of piezophilic protein preserves the color of food products. The high-pressure stable enzymes are very useful in high-pressure bioreactors for food processing and production of antibiotics and other drugs. They are also used in microbial-assisted oil recovery process (Ichiye 2018; Jin et al. 2019). Yeast found in deep sea of Japan trench produces two types of polygalacturonase that is active at 24 °C temperature and 100 MPa hydrostatic pressures (Arifeen and Liu 2008). These pressure-tolerant enzymes are also thermostable and hence catalyze a biological reaction more efficiently under high pressure and high temperature. During the fermentation process, microorganism produces certain undesirable by-products that are reduced with increased pressure as the microorganism modifies the metabolic pathway and produces the desirable products. Piezophiles are used in the biopurification process of antigens where high pressure applied that increases the

volume and causes dissociation of antigen–antibody complex to purify the desired antigen. High pressure induces the dissociation of complexes. Therefore, piezophiles and their enzymes are used in protein recovery process to purify protein from the aggregate of incomplete folded protein. High pressure reduces the allergenicity of protein. Hence, piezophiles are used to produce food products with very low or no allergenicity. High pressure enhances the hydrolysis of inaccessible site of protein that increases the digestibility of food products. Therefore, food products obtained by piezophiles or with the help of their enzymes possess greater shelf life and increased digestibility. Due to its thermostability and high-pressure resistance, piezophiles and their enzymes are very useful in various biotechnological and pharmaceutical processes. They are useful in the formation of gels and starch granules (Castell et al. 2019). Piezophilic α-amylases from deep sea piezophiles are important because they produce trisaccharides from maltooligosaccharides at high pressure instead of producing maltobiose and tetrasaccharide. This low-energy consumption process is very useful for food processing industries (Jin et al. 2019).

14.11 Metallophiles and Metallophilic Enzymes

14.11.1 Metallophiles

Metallophiles are the organisms that thrive under metal-rich condition or environment with high metallic concentration. They are able to tolerate and detoxify high concentration of heavy metals. Most of the metallophiles are acidophiles, thus enhancing their survival 1000-fold than mesophiles and efficiently tolerate the high level of heavy metals (Anahid et al. 2011; Gupta et al. 2014).

Fungal metalloenzymes have hardly been studied from catalytic mechanism point of views. There are reports of their finding from the study on models (synthetic) of metalloenzymes (Ohta et al. 2012; Sunghee et al. 2012).

14.12 Radioresistants and Their Enzymes

14.12.1 Radioresistants

Radioresistants or radiophiles are the extremophiles that are highly resistant to high level of ionizing and ultraviolet radiation. Radioresistant organisms tolerate extreme radiations for longer period of time while radiotolerant organisms tolerate extreme radiations for only a short period of time. Ionizing radiation such as gamma radiation and nonionizing radiation such as ultraviolet radiation are the two major radiations that cause lethal effect on an organism. Radiophiles are polyextremophiles as they can tolerate extreme cold, dehydration, vacuum, and high acidic concentration (Coker 2019).

14.12.2 Radioresistant Enzymes

Catalytic features of radioresistant fungi have not been deeply studied. They are mainly studied for their adaptation to radiation mutations. Biofilm forming radioresistant fungi are especially adapted to high mutation rate and are more resistant to ionizing radiation than other radioresistants (Ragon et al. 2011). Genome-wide radiation resistance analysis of *Cryptococcus neoformans*, a radioresistant yeast, explains the upregulation of DNA repair machinery for reducing the radiation stress. Rad53 protein kinase regulates the transcription factor Bdr1 and controls the transcription (Jung et al. 2016).

14.12.3 Industrial Application

Radioresistants possess high potential for the treatment of radioactive environmental waste. Radiophiles are useful in ore-bioleaching and biomineralization process. Extracellular polymeric substances produced by this extremophile are used as adhesive, used in cosmetic, pharmaceutical products as antitumor, antiviral, or anti-inflammatory agent and oil recovery processes (Kazak et al. 2010; Parihar and Bagaria 2019). Melanized fungi show increased growth in the presence of ionizing radiation due to the changes in the electronic properties of melanin. Thus, these melanized fungi can be used in high radiation-affiliated processes (Dadachova et al. 2007). Fungal hyphae possess the ability to absorb or adsorb radionuclides, which are very useful in the treatment of industrial effluent contaminated with heavy metals and radionuclides. Some melanized fungi such as *Cladosporium sphaerospermum* optimally grow in radiation-rich environment such as radioactive nuclear plant and show the ability to prevent ionizing radiations. They perform radiosynthesis using melanin as an analog to energy harvesting pigment such as chlorophyll to convert gamma radiations into chemical energy. This unique property is useful for industries for making a passive radiation shields and used in deep space exploration mission to protect against radiations (Shunk et al. 2020).

Radiation-tolerant fungi can be used as the indicators of radioactive contamination. Occurrence of fungal species such as *Chaetomium aureum* and *Penicillium liliacinus* shows the high radiation environment ($3.7x10^6$–$3.7x10^8$ Bq/Kg) (Matusiak 2016). An acid-tolerant and gamma radiation-resistant yeast *Rhodotorula taiwanensis MD1149* isolated from an acid mine drainage possess the ability to cope with the environment of high radiation, low pH, and high level of heavy metals. This strain grows under 66 Gy/h of radiation exposure at pH 2.3 and high concentration of mercury and chromium, which makes them very suitable for bioremediation of acidic radioactive waste (Tkavc et al. 2018).

A study on filamentous fungi irradiated with gamma radiation was investigated for their lignolytic enzyme production. The fungi *Aspergillus awamori*, *Aspergillus terreus,* and *Penicillium* species produce lignin peroxidase, Manganese peroxidase and laccase in high amount at 500 Gy dose. Lignolytic enzyme production can be increased in these fungi through gamma radiation exposure (de Queiroz Baptista

et al. 2015). Studies suggest that many fungal hyphae grow toward the ionizing radiation source when they are exposed to high radiation environment. The fungi exposed to radiations show positive radiotropism and some of them involved in the decomposition of ribonuclide containing organic debris (Zhdanova et al. 2004). Radioresistant microorganisms are used for making photoprotective devices. Microbes that have the ability to withstand high radiation environment are very useful for pharmaceutical industries. Microbial metabolic reserves or extremolytes of these organisms can be used in the production of anticancer drugs as extremolytes are resistant to UV radiations and possess antioxidant property. Thus, it can prevent the skin from damage caused by harmful radiations (Singh and Gabani 2011).

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Biotechnological Application of Extremophilic Fungi

15

Aneesa Fasim, H. K. Manjushree, A. Prakruti, S. Rashmi, V. Sindhuja, Veena S. More, K. S. Anantharaju, and Sunil S. More

Abstract

White biotechnology (BT), a sustainable and eco-friendly technology, has taken precedence over chemical industries in the last few decades. It has revolutionized the industrial BT sector by exploiting abundant natural resources for the production of important commodities benefiting mankind. Industries employ microorganisms or biomolecules extracted from them for production and processing in various industrial areas such as food and feed, beverages, agriculture, pharmaceutical, textile, leather, paper, detergent, polymers, cosmetics, waste management, etc. Despite the advantages, the use of biomolecules is not substantial because they cannot tolerate harsh industrial conditions, which in turn affects the production process. In the last decade, the industrial research focus has shifted toward extremophiles, organisms that can survive extreme conditions. These organisms have evolved defense mechanisms to survive severe conditions such as high or low temperature, salinity, pressure, pH, radiation, and desiccation. Biomolecules extracted from these organisms have robust characteristics to retain optimum activity even under unnatural conditions. A class of eukaryotes called extremophilic fungi are at the crux of this research focus as they are a reservoir of sturdy biomolecules with many industrial applications. Fungal extremozymes can be easily cultured on agro-industrial waste and also easily purified. All these factors make fungal extremozymes an attractive resource for large-scale, cost-effective, and eco-friendly industrial processes. In addition to extremozymes, extremophilic fungi are an abundant resource of potent cytotoxic, antimicrobial

A. Fasim · H. K. Manjushree · A. Prakruti · S. Rashmi · V. Sindhuja · S. S. More (✉)
School of Basic and Applied Sciences, Dayananda Sagar University, Bengaluru, Karnataka, India

V. S. More
Department of Biotechnology, Sapthagiri College of Engineering, Bangalore, Karnataka, India

K. S. Anantharaju
Department of Chemistry, Dayananda Sagar College of Engineering, Bangalore, Karnataka, India

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drugs. This chapter focuses on various extremophilic fungi used in the BT industry. It also covers the different extremozymes, biomolecules, and secondary metabolites secreted by them and their potential biotechnological applications.

Keywords

Extremophilic fungi · Extremozymes · Natural products · Bioactive compounds · Biotechnological applications

15.1 Introduction

A sustainable bio-based economy is a ray of hope in response to the present environmental crisis such as population expansion, climatic changes, exhaustion of nonrenewable resources, global warming, pollution, etc. The advent of bioprocess technology, also known as white biotechnology, has revolutionized the industrial sector by exploiting natural resources for the production and processing of value-added products that positively impact the global economy and environment. This contemporary technology employs enzymes or microorganisms such as yeast, bacteria, fungi, and plant extracts in numerous industrial applications. Fungal sources have been the major contributors in this field as many enzymes, organic acids, antibiotics, etc., are produced on a commercial scale (Meyer et al. 2016). The discovery of penicillin, fungal antibiotics along with the commercial production of citric acid by *Aspergillus niger,* marked a milestone in the era of fungal biotechnology, and since then many more discoveries have steadily transformed it into a powerful and proficient technology. Fungi play a vital and irreplaceable role in energy recycling of the ecosystem by helping in the decomposition and recycling of organic matter. This versatile class of eukaryotes are omnipresent and can be found in soil, desserts, glaciers, sea, freshwater bodies, and various other environments including the stratosphere (van der Giezen 2011). Fungi have proven to be a valuable resource to humanity from being consumed as food to combating infectious diseases and many biomolecules with important industrial applications. Besides, helping in the fermentation processes of baking, brewing, etc., they aid in the production of enzymes, antibiotics, organic acids, pigments, vitamins, lipids, and numerous other products that are economically important (Adrio and Demain 2003). Their fast growth rate, short life cycles, ease of culture, and purification are highly favorable attributes that benefit the industrial production processes (Hooker et al. 2019).

Fungi are highly resilient organisms that can adapt to diverse habitats and due to their ecological plasticity, they can survive harsh environments precluded to most life forms. They dwell in virtually all types of extreme habitats ranging from extremely dry and cold deserts in the Antarctic and other very cold areas worldwide to highest mountain peaks (Selbmann et al. 2008) to deep permafrost soils (Ozerskaya et al. 2009; Selbmann et al. 2015), geothermal and fumarole soils in volcanic areas, acid mine drainages with sulfuric acid (Selbmann et al. 2008), or in highly alkaline sites (Gunde-Cimerman et al. 2009; Selbmann et al. 2013). Under

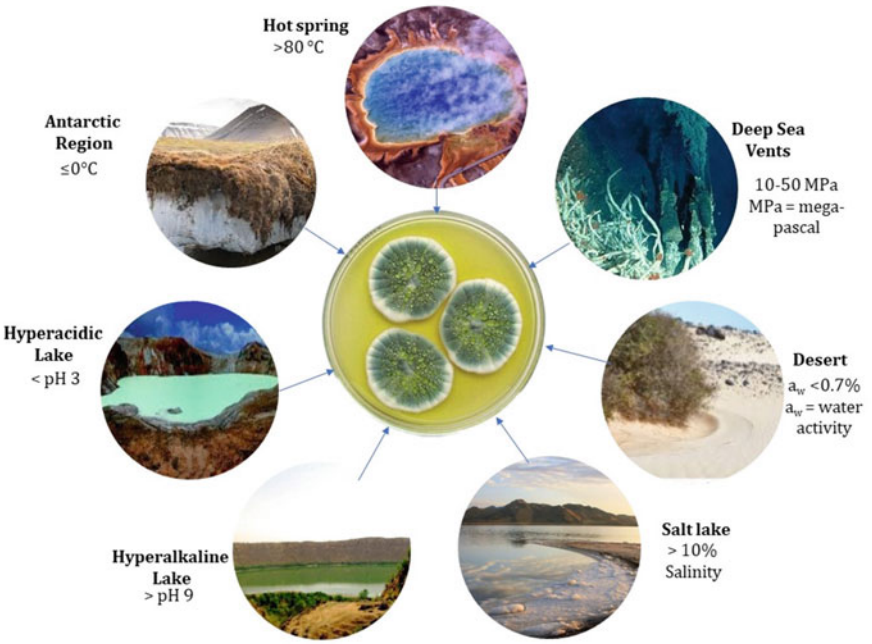

Fig. 15.1 Extreme environments of the earth

severe conditions and high competition, fungi acquire peculiar skills to exploit natural or xenobiotic resources and such fungi are termed as extremophilic fungi (Zhang et al. 2018).

These fungi have evolved defense mechanisms in the form of regulation and expression of specific genes or production of robust enzymes that help them to survive conditions such as high or low temperature, salinity, pressure, pH, radiation, and desiccation. Biomolecules extracted from these organisms have robust characteristics and retain optimum activity even under harsh industrial conditions. All these factors make fungal extremozymes an attractive resource for large-scale, cost-effective, and eco-friendly industrial processes, and the scope to use extremophilic fungi for biotechnological applications is increasing with time (Sarmiento et al. 2015).

The term "extremophile" was first proposed by MacElroy in 1974 to describe a broad group of organisms that can live optimally under extreme conditions. They belong to all three domains of life —Eucarya, Bacteria, and Archaea. Extremophiles are classified into seven categories based on the extreme habitats they inhabit (Fig. 15.1). Piezophiles can survive high hydrostatic pressure and have been isolated from deep sea sediments (>3000 m deep). Thermophiles or hyperthermophiles are organisms that inhabit hot springs, deep sea hydrothermal vents, and can tolerate very high temperatures varying from 50 to 80 °C or over 80 °C (Raddadi 2015). Some halotolerant fungi can tolerate high salt concentration and abiotic stress

(Gunde-Cimerman et al. 2003). This is why many fungi inhabit marine environments. Alkalophiles can tolerate a pH range between 9 and 12, whereas acidophiles can survive extremely low pH of 1–2 (Jin and Kirk 2018). Psychrophiles are the next class that can tolerate extreme cold conditions of the Antarctic zone (Selbmann et al. 2008) and some yeasts can survive ultraviolet rays (UV-B) exposure even at lethal doses (Selbmann et al. 2011). Due to their uncommon adaptability, fungi may also easily colonize stressful and extreme environments created by anthropogenic activities, such as those polluted with heavy metals, toxic chemicals, sewage, etc. (Ceci et al. 2019). Therefore, polluted sites are a rich source to screen for extremophilic fungi. Fungal strains isolated from these environments are strongly adapted to high toxicity and extreme physical parameters (i.e., high salt concentration and high pH). These strains are potentially useful in biotechnological applications such as the biodegradation of the pollutants (Gomes and Steiner 2004; Selbmann et al. 2013) or they can be considered as sources of important bioactive compounds, specific enzymes, biosurfactants, and antioxidants, useful for applications in medicine or food, cosmetics, and chemical industry (Adrio and Demain 2003). They are also employed in biofuel and bioenergy industries since solar cells of specialized pigments work only under extreme conditions like polar caps.

15.2 Biotechnological Applications

Biotechnological industries are exploiting a variety of enzymes as solutions to numerous industrial processes. Fungi from the extreme environment are considered a vital source of commercial hydrolytic enzymes due to their exceptional properties of high catalytic activity, stability, high enzyme yield, ease of culture, and retention of activity even under high-stress conditions. Lipases, amylases, proteases, cellulases, xylanases, etc., are highly used in industries that require efficient breakdown of lignocellulosic biomass in the processing and production of good quality biobased products. Hence, fungal extremozymes help in large-scale, cost-effective, and eco-friendly industrial processes that could significantly affect the growth of the biotechnology sector (Shukla and Singh 2020). Some of the important fungal extremozymes are listed in Table 15.1. The important fields that use these enzymes include decolorization of dyes in the textile industry, detoxify pesticides, degrade agricultural waste to valuable by-products, delignify biomass for biofuel production, bleach the kraft pulp in the paper industry, processing and stabilization of juice, wine, bakery products in the food industry, bioremediation, and many other processes (Baldrian 2006; Brijwani et al. 2010). Along with extremozymes, secondary metabolites and bioactive peptides are also products of extremophilic fungi. Their potential role in preventive medicine as antimicrobials, antivirals, cytotoxic agents, antitumorigenic, antidiabetic, anti-inflammatory, lipid-lowering activities is also illustrated in this chapter (Fig. 15.2).

Table 15.1 Extremophilic enzymes sources and uses in industries

Enzymes	Organisms	Applications in industries	References
Proteases	*Penicillium buponti, Malbranchea pulchella var. sulfurea, Humicola lanuginose Rhodotorula mucilaginosa L7 Leucosporidium antarcticum Acremonium sp. L1–4B Pseudogymnoascus pannorum Candida humicola*	Food, detergents, leather, pharmaceutical, agricultural industries	(Maheshwari et al. 2000) (Lario et al. 2015) (Turkiewicz et al. 2003) (Evaristo da Silva Nascimento et al. 2015) (Krishnan et al. 2011) (Ray et al. 1992)
Laccases	*Chaetomium thermophilium Corynascus thermophiles aspergillus oryzae Aigialus grandis, Cirrenalia pygmea, Gliocladium sp., Hypoxylon oceanicum, Halosarpheia ratnagiriensis, Gongronella sp., Sordaria flmicola, Verruculina enalia and Zalerion varium. Cladosporium halotolerans, Cladosporium sphaerospermum, Penicillium canescens. Cerrena unicolor (MTCC 5159)* and *Penicillium pinophilum (MCC 1049)*	Paper and pulp, Textile industry, agriculture, Food and beverages	(Chefetz et al. 1998) (Babot et al. 2011; Berka et al. 1997; Bulter et al. 2003; Xu et al. 1996) (Raghukumar et al. 1994) (Jaouani et al. 2014) (D'Souza-Ticlo et al. 2009)
Cellulases	*Trichoderma resei Chaetomiumthermophile, Sporotrichum thermophile, Humicola grisea var thermoidea, Humicola insolens, Myceliopthera thermophila, Thermoascus aurantiacus and Talaromyces emrsonii Cadophora, Pseudeurotium, Geomyces, Wardomyces, Pseudogymnoascus,*	Biofuel production, paper and pulp, Textile	(Mandels and Weber 1969) (Maheshwari et al. 2000) (Krishnan et al. 2011; Tsuji et al. 2014; Vaz et al. 2011; Wang et al. 2013)

(continued)

Table 15.1 (continued)

Enzymes	Organisms	Applications in industries	References
	Verticillium, Cryptococcus and Mrakia		
Xylanases	*Aureobasidium pullulans varmelangium,* *Pencillium occitanis PO16,* *Aureobasidium pullulans* *Pencillium oxalicum* *Pencillium citrinum,* *Aspergillus fumigatus* *Humicola insolensY1,* *Sporotrichum thermophile* *Rhizomucor pusillus,* *Aspergillus gracilis,* *Aspergillus penicillioides* *Naganishia adeliensis.*	Paper and pulp, Animal feed, Textile, Food and brewery	(Ohta et al. 2001) (Driss et al. 2011) (Yegin 2017) (Muthezhilan et al. 2007) (Dutta et al. 2007) (Deshmukh et al. 2016) (Du et al. 2013) (Sadaf and Khare 2014) (Robledo et al. 2016) (Ali et al. 2012) (Gomes et al. 2003)
Lipases	*Rhizomucor miehei* *Kurtzmanomyces sp. I-11* *Moesziomyces antarcticus* *Leucosporidium scottii L117* *Mrakia blollopis SK-4* *Geomyces sp. P7*	Biofuel, detergent, food, and beverages	(Maheshwari et al. 2000) (Kakugawa et al. 2002; Goto et al. 1969) (Goto et al. 1969) (Duarte et al. 2015) (Tsuji et al. 2013) (Tsuji et al. 2013)
Amylases	*Rhizomucor pusillus,* *Humicola lanuginose,* *Myriococcum thermophilum,* *Thermomyces ibadanensis,* *Thermomyces lanuginosus* *Candida antarctica* *Geomyces pannorum*	Starch processing, food and beverage, paper and pulp, Textile, and pharmaceutical	(Adams 1994; Arnesen et al. 1998; Barnett and Fergus 1971; Bunni et al. 1989; Fergus 1969; Jayachandran and Ramabadran 1970; Sadhukhan et al. 1992) (Mot and Verachtert 1987) (Gao et al. 2016)
Pectinases	*Aspergillus Niger* *Cryptococcus albidus var. albidus,* *Aspergillus Niger MTCC478,* *Saccharomyces cerevisiae,* *Penicillium sp. CGMCC 1669* *Rhizomucor pusilis* *Thermomucor indicae-seudaticae* *Arthrobotrys,* *Aureobasidium,* *Cladosporium,* *Leucosporidium* *Tetracladium*	Biofuel production, oil extraction, paper and pulp, food, and beverage	(Lara-Márquez et al. 2011) (Federici 1985) (Anand et al. 2017) (Gainvors et al. 2000) (Yuan et al. 2011) (Siddiqui et al. 2012) (Martin et al. 2010) (Fenice et al. 1997) (Carrasco et al. 2016)

(continued)

Table 15.1 (continued)

Enzymes	Organisms	Applications in industries	References
Chitinases	*Trichoderma, Oenicillium, Penicillium, Lecanicillium, Neurospora, Mucor, Beauveria, Lycoperdon, aspergillus, Myrothecium, Conidiobolus, Metharhizium, Stachybotrys, Agaricus Talaromyces emersonii, Thermomyces lanuginosus Dioszegia, Glaciozyma, Lecanicillium, Leuconeurospora, Mrakia, Metschnikowia Phoma, Sporidiobolus, Verticillium lecanii* *Glaciozyma antarctica* PI12	Pharmaceutical and agricultural industry	(Hamid et al. 2013; Karthik et al. 2014) (McCormack et al. 1991) (Zhang et al. 2012) (Barghini et al. 2013; Carrasco et al. 2012; Fenice et al. 2012, 1998, 1997; Onofri et al. 2000) (Ramli et al. 2011)
Phytases	*Aspergillus Niger Myceliophthora thermophila, Talaromyces Papiliotrema laurentii AL27* R*hodotorula mucilaginosa strain JMUY1*	Bread making and animal feed	(Haros et al. 2001) (Maheshwari et al. 2000) (Pavlova et al. 2008) (Yu et al. 2015)

15.2.1 Food and Beverage Industry

Use of enzymes instead of chemicals improves the quality of the processed food and creates superior products with improved yields. In addition, enzymes also play key role in enhancing the nutrition and appeal of the products. Enzymes are used in baking, making sugar syrups, cheese and dairy making, extraction and clarification of juices, oil, as sweeteners, for flavor development, meat tenderizing, etc., and in many other processes. From making food products to storage of food and beverage all require extreme conditions making extremozymes an essential ingredient to achieve food quality at low costs in this industry.

Cold-active enzymes produced by psychrophiles are flexible, resulting in higher catalytic activity at low temperatures (Arora and Panosyan 2019). These enzymes can be used to soften frozen meat products, preserve the heat-sensitive nutrients, accelerate cheese ripening, and they are also effective against wine and juice clarification. *Rhodotorula mucilaginosa L7* is a yeast strain from the Antarctic region that produces acid protease with an activity range between 15 °C and 60 °C and pH 5 (Lario et al. 2015). A similar discovery of a psychrophilic and halotolerant serine protease from Antarctic region resulted in isolation of *Leucosporidium antarcticum* fungal strain where the enzyme was found most active at 10–25 °C

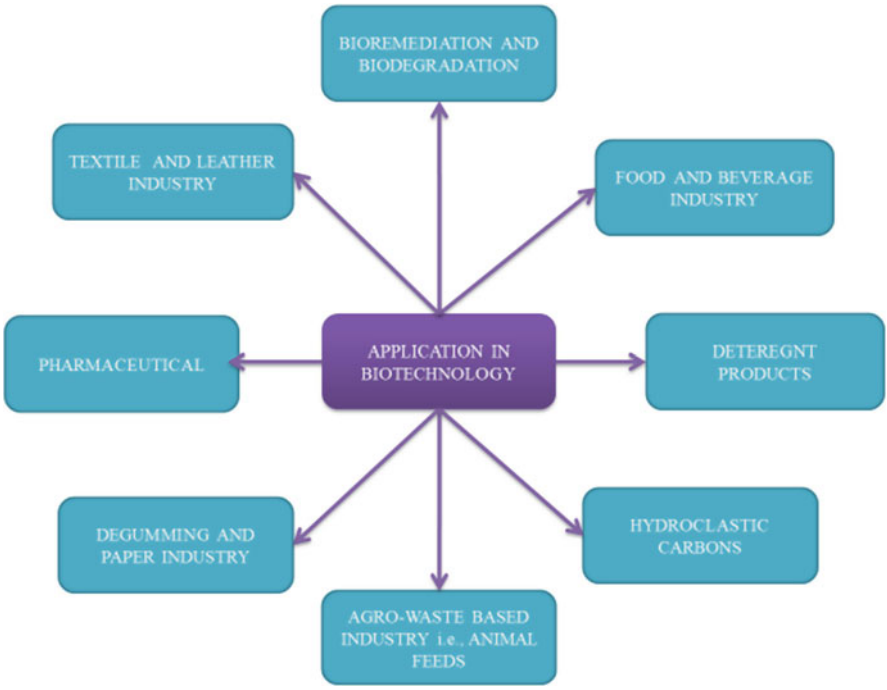

Fig. 15.2 Representation of extremophilic fungi biotechnological applications

and 3.5% marine salt (Turkiewicz et al. 2003). Additionally, Laccases have many applications like processing and stabilization of juice, wine, bakery products in the food industry, and many other processes (Baldrian 2006; Brijwani et al. 2010).

Amylases are another class of enzymes highly used in food industry; they are also used in various other industries such as starch processing, textile, food and beverage, paper, pharmaceutical, and many other industries (Pandey et al. 2000). Extremophilic fungal α-amylases have achieved an important place in industrial enzymes. Many thermophilic fungal species studied so far are capable of secreting amylases. *Rhizomucor pusillus,* and *Humicola lanuginose*, *Myriococcum thermophilum, Thermomyces ibadanensis,* and *Thermomyces lanuginosus* are a few of the thermophilic fungi found to produce amylase enzyme (Sadhukhan et al.1992; Jayachandran and Ramabadran 1970; Fergus 1969; Bunni et al. 1989; Barnett and Fergus 1971; Arnesen et al. 1998; Adams 1981; Adams 1994). Psychotolerant fungi are also a good source of amylases. *Candida antarctica* from Antarctic region was observed to produce both α and γ amylases. Both enzymes were active on high molecular weight polysaccharides with α-amylase showing activity even on cyclodextrins (Mot and Verachtert 1987). Extremophilic fungal xylanases and pectinases also have many benefits such as pulping, juice and wine clarification, oil extraction etc. (Soni et al. 2017). *Trichoderma sp, Aspergillus sp, Penicillium sp,* and *Acido bacterium spp.* are the major extremophilic fungal genera

that contribute to the production of xylanases. Similarly, many acidic fungal pectinases like *Aspergillus niger* between pH 3 and 5.5 (Lara-Márquez et al. 2011). *Cryptococcus albidus* var. *albidus,* pH 3.75 (Federici 1985), *Aspergillus niger* MTCC478, pH 4 (Anand et al. 2016), *Penicillium* sp. CGMCC 1669, pH 3.5 (Yuan et al. 2011), and *Saccharomyces cerevisiae* pH 3–5.5 (Gainvors et al. 2000) have been screened. Novoshape (novozymes), pectinase 62 L (biocatalysts), and lallzyme (lallemand) are few commercially available food-based companies that use pectinase enzyme (Dumorné et al. 2017; Sarmiento et al. 2015). Acidic pectinases are one such enzyme used in the clarification of fruit juices, beer, and wine as well (Kashyap et al. 2001). Recent research has indicatedS screening of bacterial strains known to produce alkaline and thermophilic pectinases. Anand et al. 2016 purified and characterized an alkaline pectinase from *Aspergillus fumigatus MTCC 2584* having a pH optima of 10. In another study, thermophilic pectinase was purified from *Rhizomucor pusilis* having temperature optima of 55 °C was isolated (Siddiqui et al. 2012). Martin et al. 2010 also isolated a thermophilic pectinase producing fungal strain *Thermomucor indicae-seudaticae* that could grow at 45 °C. Recently, psychrophilic and pectinolytic fungi were isolated from Antarctic region. The representative genera are *Arthrobotrys, Aureobasidium, Cladosporium,* and *Leucosporidium* showed the pectinase activities even at 5 °C (Fenice et al. 1997). A cold-adapted pectinase-producing fungi was also isolated from *Tetracladium* sp. with highest activity at 15 °C (Carrasco et al. 2019).

15.2.2 Detergents

Extermophilic fungal lipases are sought-after enzymes in detergent industries as they possess robust properties. Particularly esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3) are important as they catalyze the cleavage of esterbonds and also help in reverse reactions in organic solvents (Fuciños et al. 2012). Lipases help in acidolysis, alcoholysis, aminolysis, esterification hydrolysis, interesterification, etc. (Daiha et al. 2015), making them versatile and having many applications in organic and fine chemical synthesis, and cleaning products. A thermostable lipase from *Humicola lanuginosa* strain Y-38 was isolated from compost in Japan. The enzyme was thermophilic having temperature optima of 60 °C and alkalophilic with pH optima of 8.0. *Rhizomucor miehei*, formerly called *Mucor miehei*, also produced active lipase (Maheshwari et al. 2000). Kakugawa et al. (2002) reported a thermostable and acidophilic lipase-producing yeast strain *Kurtzmanomyces sp. I-11* with optimum activity at 75 °C and pH 2–4. Another noteworthy example of thermostable and alkalophilic lipase is produced by *Thermomyces lanuginosus*, known as TLL showing maximum lipase activity between 60 and 85 °C and pH 10 (Avila-Cisneros et al. 2014). Lipolase, Lipoclean, and Lipex are few of the genetically improved lipases from the fungus *Thermomyces lanuginosus* included in detergent formulations by Novozymes (Jurado-Alameda et al. 2012). Cellulases are the next class of enzymes that have found applications in the detergent industry to increase brightness and dirt removal from cotton mixed garments (Kuhad et al. 2011). Many

commercially available detergents have been reported where enzyme such as lipase, protease, amylases, cellulases, and mannanases are included in the formulations (Sarmiento et al. 2015).

15.2.3 Paper and Pulp Industry

In the paper and pulp industry, the significant application of enzymes is in the prebleaching of kraft pulp. Xylanases, hemicellulases, and cellulases are the commonly used enzymes for this purpose due to its displayed efficiency. Enzymes have also been used to raise water retention, pulp fibrillation, and decrease the beating time in virgin pulps. Enzymes are also involved in increasing the freeness and in the deinking process (Dumorné et al. 2017; Bajpai 1999). Fungal laccases are involved in lignin degradation due to displayed efficiency (Alcalde 2007; Thurston 1994). Due to high enzyme yield and higher redox potential, fungal laccases are preferred over the plant or bacterial enzymes in the biotechnology sector (Thurston 1994). *Corynascus thermophilus* is a fungal strain secreting highly active thermostable laccase that was used to delignify eucalypt pulp. This laccase was heterologously expressed in *Aspergillus oryzae*, characterized, and commercialized (Xu et al. 1996; Berka et al. 1997; Bulter et al. 2003; Babot et al. 2011). Cellulases are also heavily used in this industry. *Penicillium roqueforti, Cadophora malorum, Geomyces sp.,* and *Mrakia blollopis* are few of the cold-adapted cellulase-producing fungal strains (Carrasco et al. 2016; Duncan et al. 2006; Duncan et al. 2008). *Trichoderma sp, Aspergillus sp, Penicillium sp,* and *Acidobacterium spp* are the major extremophilic fungal genera that contribute to the production of xylanases.

15.2.4 Agricultural Applications

Many cellulolytic and xylanolytic fungi are acknowledged to have applications in the field of agriculture by boosting the seed germination, improved root system and flowering, increased crop yields, and rapid plant growth (Ahmed and Bibi 2018). Fungal xylanases such as *Pencillium oxalicum* (Muthezhilan et al. 2007)*, Pencillium citrinum* (Dutta et al. 2007)*, Aspergillus fumigatus* (Deshmukh et al. 2016)*,* and *Humicola insolensY1*(Du et al. 2013) are isolated showing optimum activity between pH 8–9 and 45–55 °C with *H.insolensY1* also being highly thermophilic with a temperature optima of 70–80 °C. Other thermophilic xylanase-producing fungi include *Chaetomium sp. CQ31, Sporotrichum thermophile* isolated from composting soil having activity at neutral pH and 60–70 °C temperature (Jiang et al. 2010; Sadaf and Khare 2014). *Rhizomucor pusillus* and *Aspergillus fumigatus* screened from the maize silage showed optimum xylanase activity at 75 °C and pH 6 (Robledo et al. 2016). Many thermophilic xylanase-producing fungi such as *Chaetomium thermophilum, Humicola insolens, Melanocarpus sp., Malbranchea sp.,* and *Thermoascus aurantiacus* were reported by Ghatora et al. 2006. Halotolerant fungal xylanase *Phoma sp* isolated from mangrove sediments having

enzyme activity at pH 5, 45 °C, and a high salt concentration of 4 M NaCl (Wu et al. 2018). *Aspergillus gracilis* and *Aspergillus penicillioides* were screened from man-made solar saltern (Ali et al. 2012) and psychrophilic fungal xylanases were isolated from Antarctic soils, marine sponges, etc. *Cladosporium sp.* from marine sponge showed high xylanase activity at low temperatures (Del Cid et al. 2014). *Naganishia adeliensis* are isolated from Antarctica (Gomes et al. 2003). Phytases are another class of enzymes involved in seed germination, but they are also considered antinutrients because they act as strong chelators of divalent mineral ions such as calcium, magnesium, iron, and zinc. Chitinases have many applications, especially as antiphytopathogenic and antifungal agents. They are used to protect crops to control pathogens. Cola-active extremozymes are used in agriculture to enhance the water management by plants, which are under deficiency stress (Dumorné et al. 2017).

15.2.5 Animal Feed Industry

Cellulases and xylanases have advantage in the animal feed industry in the treatment of agricultural silage, grains, and seeds to enhance nutritional value. Cold-adapted phytases have advantages as they can be directly included in the feed of monogastric animals and also in aquaculture.

15.2.6 Bioremediation and Biodegradation: Major Application of Extremozymes

Bioremediation and biodegradation employ microbes in the elimination of pollutants, contaminants, and toxins from water, soil, and other environments. Waste from any kind of industry is hazardous. It is highly acidic or alkaline, and contains all kinds of biomass and proteinaceous waste. It also has a high content of metal ions and many other toxins, dyes, chemicals, radioactive material, etc., making it very harmful to the flora and fauna around it.

Certain microbes can be used to recycle and degrade pollutants as they produce hydrolytic enzymes that can degrade and help clean up the contaminated sites. Fungal extremozymes are extremely useful in these processes as they can sustain harsh conditions and still work on organic toxins. Thermophiles convert recalcitrant materials in bioprocessing and favor the in situ bioremediation process (Castro et al. 2019). As the solubility of the pollutants increases, the metabolic activity of thermophiles also increases (Zeldes et al. 2015). Thermophilic fungi such as *Pyrodictium, Clostridium,* and *Methanopyrus* can metabolize naphthalene, anthracene, and phenanthrene (Ghosal et al. 2016). White rot fungi are the chief representatives of the biodegradation of lignin substances (Deshmukh et al. 2016). 21 PAH degrading fungi were isolated from PAH-contaminated soils that could efficiently degrade PAH. A*spergillus niger*, *Diaporthe sp., Coriolopsis byrsina, Pestalotiopsis sp.,* and *Cerrena* are known to treat and bioremediate textile mill

effluents (Rani et al. 2014). *Stenotrophomonas maltophilia* strain AJH1 has been isolated from Arabia, which was able to degrade low and high molecular weight PAHs such as anthracene, naphthalene, phenanthrene, pyrene, and benzo(k)-f[2]uoranthene (Rajkumari et al. 2019). *D. radiouridans* is another important fungus used in bioremediation of radioactively contaminated sites (Brim et al. 2006). *Sulfolobus sulfataricus* secrete lactonase enzyme that acts against organophosphates (Hawwa et al. 2009). *Thermoascus aurantiacus,* another fungal strain, can secrete phenol oxidase and target phenolic hydrocarbons (Machuca et al. 1998).

Rajkumari et al. (2019) studied different approaches of degradation of hydrocarbon waste. *Candida, Aspergillus, Chlorella*, and *Penicillium* were found to be most suitable in the elimination of these wastes. A marine fungal laccase-mediated detoxification and bioremediation of anthraquinone dye called reactive blue was reported (Verma et al. 2012). These laccases could work under very high salinity. Similarly, laccase from *Fusarium incarnatum* was able to degrade bisphenol A, which is a endocrine-disrupting chemical (Chhaya and Gupte 2013). Other studies indicated heavy metal and chloropyriphos bioremediation can be achieved by using *Aspergillus sp, Curvularia,* and *Acrimonium sp*. (Akhtar et al. 2013; Silambarasan and Abraham 2013); likewise, polychlorinated biphenyl degradation can be degraded by *Phoma eupyrena, Doratomyces nanus, Myceliophthora thermophila,* and *D. verrucisporus* (Barghini et al. 2013). Lugowski et al. 1998 has reported that *Pseudomonas sp* is used for degradation of aromatic hydrocarbons. *Halomonas* sp. and *Pseudomonas aeruginosa* strain is used for cleaving of aliphatic hydrocarbons.

15.2.7 Bioactive Peptides from Marine Fungi

Oceans are the biggest resource for novel therapeutic compounds. Thousands of secondary metabolites such as polyketides, lactones, alkaloids, steroids, and peptides having pharmacological significance are discovered from marine fungal strains (Jin et al. 2016). Sessile marine microorganisms usually harbor the fungal strains in a symbiotic relationship where the marine fungi protect the host against predators and disease by releasing bioactive compounds (Schueffler and Anke 2014). The unique structural and functional diversity of the marine bioactive compounds is attributed to the extreme conditions of salinity, pressure, and temperature that also give immense stability from all kinds of degradation to these peptides, making them promising candidates for drug discovery. Thus, isolating and characterizing novel bioactive peptides and metabolites from marine fungi with therapeutic properties is a promising avenue to explore in the prevention of human diseases. To date, thousands of compounds have been isolated from many marine fungi, but curating them all is not feasible. So, the data from two latest reviews covering last 15 years of research (Ibrar et al. 2020; Youssef et al. 2019) on the fungal bioactive peptides and compounds is adapted and a comprehensive summary is presented in Table 15.1 with additions and modifications made according to the relevance and scope of this chapter.

15.2.7.1 Peptides

In the last five decades, a significant number of marine bioactive peptides are discovered that either fall in the class of synthetic, non-ribosomally produced peptides such as bacitracins, polymixins, glycopeptides, or gramicidins, etc., or natural, ribosomal peptide class. The synthetic peptides are mostly produced by bacteria, but natural peptides are produced by many species including marine fungi with potent activities (Saleem et al. 2007). Many fungi belonging to various genus produce potent peptides showing antimicrobial, antiviral, cytotoxic, antitumorigenic, antidiabetic, anti-inflammatory, lipid-lowering activities. These peptides are structurally diverse from being cyclic to N-methylated. Some are dipeptides, nonapeptides, depsipeptides, or pentadecapeptides having complex backbones and many side chains. Genus *Aspergillus* is found to be a rich source of bioactive peptides with Aspergellicins A–E, Cyclodipeptide, Sclerotide A–B, Terrelumamide A–B, Psychriphillin E–G, Aspersymmatide A, Cotteslosin A, Diketopiperazine dimer, cyclic tetrapeptide, Aspergellipeptide D–E, and 14-hydroxycyclopeptine being produced by them showing cytotoxic, anticancer, and anti-inflammatory properties (Table 15.1). Cordyhehptapeptides and efrapeptins are certain other bioactive peptides isolated from *Acremonium sp* with cytotoxic and antibacterial activities. Lajollamide A from *Asteromyces*, Dictyonamide A from *Certodictyon*, Clonostachysins from *Clonostachys,* and Ungusin A, Emercellamide from *Emericella sp,* and Rostratins from *Exserohilium* are cytotoxic, antidinoflagellate, and antimicrobial in nature. Similarly, peptides from *Microsporum, Penicillium, Scytalidium, Simplicillium, Stachylidium, Talaromyces,* and *Zygosporium* fungi also show various toxic effects on cancers and microbes.

The general procedure for isolating fungal peptides involves culturing of fungi under appropriate conditions and extraction of peptides using solvents such as ethyl acetate. The extracted sample is lyophilized and further purified using chromatographic techniques until pure forms of peptides are obtained. 1D and 2D NMR techniques in combination with mass spectrometry are used to determine the structure of the peptides and Marfey's and Mosher's reactions are used to elucidate the absolute configuration, amino acid composition, and structural modifications (Wang et al. 2017) Biological activity of the purified peptide is measured using IC_{50} or MIC (minimum inhibitory concentrations) values against cancer cell lines, pathogenic bacteria, and many other microbes.

15.2.7.2 Bioactive Compounds

Marine secondary metabolites have gained a lot of attention in the recent past due to their potent pharmacological properties. The accidental discovery of cephalosporin C antibiotic from the marine *Cephalosporium sp.* fungus in 1949 started a trend to explore marine habitats for bioactive compounds. Many other marine fungi-derived products are currently available in the market such as antibacterial terpenoid fusidic acid, polyketide griseofulvin antibiotic, penicillins, cephalosporins, macrolides, statins, many alkaloids, glycosides, isoprenoids, lipids, etc. (Chandra and Arora 2009; Hamilton-Miller 2008), that exhibit potent toxicity towards tumors, cell proliferation, microtubule formation, pathogenic bacteria, viruses, nematodes, foul

smells, and also exhibit photo-protective activities (Rateb and Ebel 2011). Bioactive compounds are produced by all kinds of extremophilic fungi from psychotolerant, to thermophiles, piezophiles, acidophiles, halotolerant, and xerophiles. Table 15.2 recapitulates different secondary metabolites and their biological activities. Many bioactive compound-secreting fungal strains are discovered by exploring extremely toxic environments such as Berkeley acid lake, hot springs, salt salterns, fumaroles, deep sea sediments and vents, mangroves, Antarctic permafrost, etc. These places have become rich biodiversity for the exploration of such value-added compounds (Ibrar et al. 2020).

Bioactive compounds are also extracted and purified in the same way as peptides, although the characterization techniques will differ. A bioassay-guided fractionation procedure is employed to obtain pure compound fractions, where the potential activity of the fractions is assessed. Most marine compounds have different chemical composition so different polar compounds have to be used for the fractionation method so that the active compound can be separated from the inactive fractions depending on the partition coefficients of the analytes. Polyketides alkaloids, sugars, steroids, and saponins are generally found in aqueous fractions, whereas peptides need mildly polar solvents, and terpenes, hydrocarbons, and fatty acids are found in low-polar fractions. The bioactive fractions are next subjected to gel permeation chromatography to further purify the molecules. The purified compounds are then structurally and chemically characterized by sophisticated techniques such as mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. High-resolution 1D and 2D NMR spectroscopy are routinely used for the structural characterization of the bioactive compounds.

15.3 Conclusion

Biotechnological industries are using a variety of extremophilic fungi as solutions to diverse industrial processes. The survival strategies of extremophilic fungi are unique and associated with the production of extremozymes and various secondary metabolites with robust qualities, making them a rich and abundant resource. Despite their potential, a very small percent of extremophilic fungi are discovered. Exploration of extremophilic organisms will make a huge impact and open new avenues in biotechnology research. With the advancement in various technologies like metagenomics, genetic engineering, in silico analysis, and technology that can access uninhabitable and inaccessible places on earth, it is now possible to identify, isolate, and extract potent compounds that can cater to the needs of almost every sector of the biotech industry to help form a sustainable and efficient biobased economy.

Table 15.2 Bioactive peptides and secondary metabolites isolated from marine fungi, their structure, sources, and biological activities

Bioactive peptides	Marine fungi	Features	Source	Biological activity	References
Peptides					
Cordyheptapeptide C Cordyheptapeptide C	*Acremonium persicinum SCSIO 115*	Cyclic heptapeptides	Marine fungus	Cytotoxic and antitumor activity	(Chen et al. 2012)
Efrapeptin Eα	*Acremonium*	Pentadecapeptides	Marine fungus	Cytotoxic activity	(Gupta et al. 1992)
Efrapeptin F Efrapeptin G RHMI	*Tolypocladium niueum* *Acremonium sp* *Acremonium sp.*	Polypeptides Polypeptide N-methylated linear octapeptides	Fractionated extract of *T. niueum* Cultured from a marine sponge	Cytotoxic activity Cytotoxic and antibacterial activity Antibacterial activity	(Boot et al. 2006) (Boot et al. 2007)
Aspergillicins A-E	*Aspergillus carneus*	Depsipeptides	Estuarine sediment in Tasmania	Cytotoxic activity	(Capon et al. 2003)
Cyclo-(L-Trp-L-Tyr)	*Aspergillus Niger EN-13*	Cyclic dipeptide	Isolated from the marine brown alga *Colpomenia sinuosa*	Cytotoxic activity	(Zhang et al. 2010)
Sclerotide A Sclerotide B	*Aspergillus sclerotiorum PT06–1*	Cyclic hexapeptide	Putian Sea salt field, China	Antifungal activity Antifungal, antibacterial, and cytotoxic activity	(Zheng et al. 2009)
Similanamide	*Aspergillus similanensis KUFA 0013*	Cyclohexapeptide	Ethyl acetate extracts of marine unknown sponge	Cytotoxic and antitumor activity	(Prompanya et al. 2015)
Terrelumamide A Terrelumamide B	*Aspergillus terreus*	Linear lumazine peptides	Marine sediments	Improved insulin sensitivity	(You et al. 2015)
Psychrophilin E	*Aspergillus sp*	Cyclic tropeptide	Isolated from marine brown algae *Sargassum*	Cytotoxic activity	(Ebada et al. 2014)
Psychrophilin G	*Aspergillus* Versicolor ZLN-60	Cyclic peptides with anthranilic acid	Marine-derived fungi	Lipid-lowering activity	(Peng et al. 2014)
Aspersymmetide A	*Aspergillus* Versicolor	Cyclic hexapeptide	Isolated from a gorgonian coral *Carijoa* sp.	Cytotoxic activity	(Hou et al. 2017)

(continued)

Table 15.2 (continued)

Bioactive peptides	Marine fungi	Features	Source	Biological activity	References
Cotteslosin A	*Aspergillus* versicolor (MST-MF495)	Cyclic pentapeptides	Isolated from Australian beach sand	Cytotoxic and antitumor activity	(Fremlin et al. 2009)
Diketopiperazine dimer Cyclic tetrapeptide	*Aspergillus Violaceofuscus*	Diketopiperazine dimer Cyclic tetrapeptide	Isolated from marine sponge *Reniochalina* sp.	Anti-inflammatory activity	(Liu et al. 2018)
Aspergillipeptid D Aspergillipeptid E	*Aspergillus*sp. SCSIO 41501	Cyclic pentapeptide Tripeptide	Isolated from marine gorgonians	Antiviral activity	(Ma et al. 2017)
14-Hydroxy-cyclopeptine	*Aspergillus sp. SCSIOW2*	Cyclic dipeptide	Isolated from deep sea (1000 m depth) fugus	NO inhibition activity	(Zhou et al. 2016)
Clavatustides A Clavatustides B	*Aspergillus clavatus* C2WU	Cyclodepsipeptides	*Xenograpsus testudinatus* Sulphur-rich hydrothermal vents in Taiwan	Cytotoxic and Antitumor activity	(Jiang et al. 2010)
Lajollamide A	*Asteromyces Cruciatus*	Pentapeptide	Isolated from the Coast of La Jolla, USA	Antibacterial	(Gulder et al. 2012)
Dictyonamide A	*Fungus KO63*	Linear dodecapeptides	Isolated from marine red alga *Ceratodictyon spongiosum*	CDK-4 inhibition	(Komatsu et al. 2001)
Clonostachysin A Clonostachysin B	*Clonostachys rogersoniana* strain HJK9	N-methylated cyclic nona peptides	Isolated from a sponge, *Halicondria japonica*	Antidinoflagellate activity	(Adachi et al. 2005)
Unguisin A Emericellamide B	*Emericella* CNL-878	Cyclic depsipeptides	Isolated from co-culture with marine *Salinispora arenicola*	Antibacterial activity	(Oh et al. 2007)
Microsporin A Microsporin B	*Microsporum* cf. *gypseum*	Cyclic tetrapeptides	Isolated bryozoan *Bugula* sp., Virgin Islands USA	Inhibition of histone deacetylases, cytotoxic and antitumor activity	(Gu et al. 2007)

Penicimutide	*Penicillium purpurogenum G59*	Cyclic dipeptide	Isolated from a neomycin-resistant mutant marine *Penicillium purpurogenum G59*	Cytotoxic activity	(Wang et al. 2016)
Psychrophilin D	*Penicillium algidum*	Cyclic nitropeptide	Soil under a Ribes sp. east of Oksestien, Greenland	Antimicrobial, antiviral, anticancer, and antiplasmodial	(Dalsgaard et al. 2005)
Cis-Cyclo (Leucyl-Tyrosyl)	*Penicillium F37*	Dipeptide	Isolated from marine sponge *Axinella corrugate*	Antibiofilim activity	(Scopel et al. 2013)
Halovir A Halovirs B–E	*Scytalidium CNL240* *Scytalidium*	Linear, lipophilic Peptides,	Deep sea-derived fungi	Antiviral activity	(Rowley et al. 2003, 2004)
Simplicilliumtide A Simplicilliumtide D Simplicilliumtide E, G and H Simplicilliumtide J	*Simplicillin obclavatum EIODSF 020*	Linear peptides	Deep sea-derived fungi	Cytotoxic activity Antifouling activity Cytotoxic and antitumor activity Antifungal and antiviral activity	(Liang et al. 2017, 2016)
Endolide A Endolide B	*Stachylidium sps.*	N-methylated peptides	Isolated from a Marine sponge	Binding to vasopressin receptor Binding to seratonin receptor	(Almeida et al. 2016)
Talaropeptide A Talaropeptide B	*Talaromyces sps,*	N-methylated linear peptides	Isolated from a marine Tunicate.	Antibacterial activity	(Dewapriya et al. 2018)
Zygosporamide	*Zygosporium masonii*	Cyclic depsipeptide	Marine-derived fungus	Cytotoxic and antitumor activity	(Oh et al. 2007; Torres-García et al. 2014)

(continued)

Table 15.2 (continued)

Bioactive peptides	Marine fungi	Features	Source	Biological activity	References
Secondary metabolites					
Fuscin, dihydrofuscin, dihydrosecofuscin, and secofuscin	*Oidiodendron griseum* UBOCC-A-114129	Polyketide	765 m below the seafloor	Antibacterial, inhibited CLK1 kinase	(Navarri et al. 2017)
Cytochalasin D	Endophytic fungi *Xylaria* sp	Polyketide amino acid hybrid	From marine seaweed *Bostrychia tenella*	Antitumor and antibiotic	(de Felício et al. 2015)
Pentacyclic cytochalasin	*Diaporthaceae sp PSU-SP2/4*	Polyketide amino acid hybrid	Isolated from the marine sponge	Antibacterial	(Khamthong et al. 2014)
Sterigmantocystin	*Aspergillus sp*	Polyketide derivative	Marine algae derived, Germany	Cytotoxic	(Ebada et al. 2014)
Rugulosin and skyrin	*Pencillium Chrysogenum*	Polyketides	Marine benthic-derived Antarctic lake	Antimicrobial	(Brunati et al. 2009)
Malbranpyrroles A–F	*Malbranchea sulfurea*	Polyketides	Fumerole soil	Cytotoxic	(Yang et al. 2009)
Myceliothermophins A–E	*Myceliophthora thermophila*	Polyketides containing tetramic acid	Fumarole soil	Cytotoxic	(Yang et al. 2007)
Xanthone and chromone	*Penicillium sp. SCSIO Ind16F01*	Xanthones and quinolones	Deep sea sediments	Cytotoxic and antimicrobial	(Liu and Kokare 2017)
Anthraquinone	*Penicillium sp. OUCMDZ 4736*	Polyketide	Sediment roots of mangrove	Antiviral	(Jin and Kirk 2018)
Anthraquinone	*Aspergillus Versricolor*	Polyketide	Deep sea	Antimicrobial	(Wang et al. 2017)
Azophilones	*Pleurostomophora sp*	Polyketides	Acidic Berkeley lake	Antimicrobial	(Stierle et al. 2015)

Berkeley lactones	*Penicillium fuscum and P. camembertii / clavigerum*	Cyclic macrolides	Acidic Berkeley lake	Antimicrobial	(Stierle et al. 2017)
Purpurquinones A-C	*Penicillium purpurogenum JS03–21*	Polyketides	Red soil from Yunnan, China	Antiviral	(Wang et al. 2017)
Pennicitrinone C	*Penicillium citrinumB-57*	Citrinin dimers	Jilantai salt field	Antioxidant	(Lu et al. 2008)
Terraquinone	*Aspergillus sp*	Curvularian derivative	Sonoran Desert	Cytotoxic	(He et al. 2004)
Paecilin E	*Neosartorya fennelliae KUFA 0811*	Dihydrochromone dimer	Marine sponge-associated fungus	Antimicrobial	(Kumla et al. 2017)
Curvularin derivatives	*Penicillium sp. Sf-5859*	Lactone polyketide	Marine sponge-associated fungus	Anti-inflammatory	(Ha et al. 2017)
Graphostrin A	*Graphostroma sp. MCCC 3A00421*	Chlorinated polyketide	Deep sea hydrothermal sulfide deposits	Anti-allergic	(Niu et al. 2018)
Berkeleydioneand berkeleytrione	*Penicillium sp*	Polyketide-terpenoid hybrid	Acidic Berkeley lake	Cytotoxic	(Stierle et al. 2004)
Berkazaphilones A-C and many other polyketides	*Penicillium rubrum*	Polyketide metabolites	Acidic Berkeley lake	Cytotoxic	(Stierle et al. 2012)
Phomopsolides	*Penicillium clavigerum*	Polyketides	Alga associated from acidic Berkeley lake	Cytotoxic	(Stierle et al. 2014)
Eremophilanetype sesquiterpenes	*Penicillium sp. PR19N-1*	Sesquiterpenes	Prydz Bay, Antarctica	Cytotoxic	(Lin et al. 2014) (Wu et al. 2013)
Purpurides B and C	*Penicillium purpurogenum JS03–21*	Sesquiterpene esters	Red soil from Yunnan, China	Antiviral	(Wang et al. 2017)

(continued)

Table 15.2 (continued)

Bioactive peptides	Marine fungi	Features	Source	Biological activity	References
Indole-diterpinoids	*Penicillium camemberti OUCMDZ-1492*	Terpenes	Rhizospora apiculata roots from acid niche, China	Antiviral	(Fan et al. 2013)
Penicilliumin B	*Penicillium sp. F00120*	Methylcyclopent-enedione sesquiterpene	Deep sea sediment	Antioxidant and antiallergic	(Lin et al. 2014)
Spirograterpene A	*Penicillium granulatum MCCC3A00475*	Spiro tetracyclic diterpene	Deep sea sediment	Antiallergic	(Niu et al. 2017)
Bisabalone sesquiterpenes, and coumarin	*Penicillium sp.*	Terpenes	Acidic Berkeley lake	Cytotoxic	(Stierle et al. 2004)
Berkeley acetals A–C	*Penicillium sp.*	Meroterpenes	Acidic Berkeley lake	Cytotoxic	(Stierle et al. 2007)
Berkidrimanes A and B	*Penicillium solitum*	Drimane sesquiterpenes	Acidic Berkeley lake	Cytotoxic	(Stierle et al. 2012)
Gliotoxin	*Aspergillus* SCSIO Ind09F01	Diketopiperazine alkaloids	Deep sea	Cytotoxic and antibacterial	(Luo et al. 2017)
Aspochalasins I, J, and K	*Aspergillus flavipes*	Cytochalasans alkaloids	Rihizospere of plant Sonoran Desert	Cytotoxic	(Zhou et al. 2004)
Globosumones A − C	*Chaetomium.Globosum*	Orsellinic acid esters alkaloids	Endophytic fungi from Sonoran Desert	Cytotoxic	(Bashyal et al. 2005)
Terremides A–B	*A terreus PTO6–2*	Terremides	Putian salt field	Antimicrobial/antiviral	(Wang et al. 2011)
Indole 3 ethanamide	*Aspergillus.Sclerotiorum sp. PT06–1*	Alkaloid	Putian salt field	Cytotoxic	(Wang et al. 2011)
Variecolorquinones A–B	*Aspergillus.variecolorB-17*	Quinone alkaloid	Jilantai salt field	Cytotoxic	(Wang et al. 2007)
Asperentin B	*Aspergillus sydowii*	Alkaloid	Deep sea	Tuyosine phosphatase inhibitor	(Wiese et al. 2017)

Tyrosine derivatives	*Pithomyces sp*	Aromatic alkaloids	Acidic Berkeley lake	Antihypertensive and antimigraine	(Stierle et al. 2007)
Berkeley amides A–D	*Penicillium sp.*	Amide	Acidic Berkeley lake	Cytotoxic	(Stierle et al. 2008)
Glionitrin A	*Aspergillus fumigatus KMC-901and A. sphinogomonas KMK 001*	Diketopiperazine disulfide	Acid mine drainage	Cytotoxic and antimicrobial	(Park et al. 2009)
Talathermophilins A and B	*Talaromyces. Thermophilus YM1–3*	Prenylated Indol alkaloids	Hot spring	Nematocidal	(Chu et al. 2010)
Thermolides	*Talaromyces. Thermophilus YM3–4*	Macrocyclic PKS-NRPS hybrids	Hot spring	Nematocidal	(Guo et al. 2012)
Dichotomocejs A–D	*Dichotomomyces.Cejpii F31–1*	NRPS hybrid dichotomocej A	Marine-lobophytum crissum derived	Cytotoxic	(Chen et al. 2017)
Brevianamides/ Mycochromenic acid	*B.brevicompactum DFFSCS025*	Alkaloids	Deep sea sediment	Cytotoxic/antifouling	(Xu et al. 2017)

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Extremophilic Fungal Cellulases: Screening, Purification, Catalysis, and Applications

16

Sangita Chouhan, Rajkumar Ahirwar, Tejpal Singh Parmar, Ashiq Magrey, and Sanjay Sahay

Abstract

Cellulases constitute a consortium of enzymes that by their coordinated activity hydrolyze cellulose to glucose. Commercially they are very important with their varied industrial applications. Currently, *Trichoderma* and *Aspergillus* are the main sources of commercially available cellulases, but for certain applications more suitable enzymes are required that can be provided by extremophilic microbes including fungi. During the last few decades cellulases from extremophilic fungi exhibiting desirable features for specific applications such as psychrophilic enzymes for textile and detergent applications and highly sturdy polyextremophilic cellulases for biofuel application have been reported that they have paved the way for the exploration and finally applications of cellulases from extremophilic fungi.

Keywords

Extremophilic microfungi · Extremocellulases · Cellulases production · Cellulases activity · Extremocellulases employability

16.1 Introduction

Cellulases are a group of enzymes which by their concerted action hydrolyze cellulose. At least three members of cellulases, viz., endo-1,4-glucanase (also named carboxymethyl cellulase; EC 3.2.1.4), an exo-β-1-4, glucanase

S. Chouhan · R. Ahirwar · T. S. Parmar · A. Magrey
Department of Biotechnology, Barkatullah University, Bhopal, MP, India

S. Sahay (✉)
Sarojini Naidu Government Postgraduate Girl's (Autonomous) College, Bhopal, Madhya Pradesh, India

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(EC 3.2.1.91), and a β-glucosidase (also called cellobiase; EC 3.2.1.21), are required in cellulose hydrolysis. Cellulose itself is the most abundant glucan composed of D-glucose-linked β-1,4 glycosidic bonds (Kotchoni et al. 2006). It is the most abundant renewable bioresource and raw material for various products such as biofuel, textile, animal feed, adhesive, bioplastic, biopolymer, bionanomaterials, and phenolics (Sahay 2020) produced by the plants through photosynthesis. Cellulose is found intricately complexed with lignin and hemicelluloses (called lignocelluloses) in the plant biomass (Sahay 2020). Current technology to utilize lignocellulose envisages its pretreatment followed by its enzymatic hydrolysis. Apart from this, cellulases have multiple applications such as in textile, food processing, and detergent formulation.

Cellulases are present in plant, animal, and microbes, but microbes have been found to be a better source for commercial exploitation because they can be easily cultured in large quantities in a relatively short time period in a cost-effective way (Acharya and Chaudhary 2012). Microbes do produce at a higher rate and their enzymes are more stable than those from plants or animals (Headon and Walsh 1994). Among microbes, fungi are more amenable to solid-state fermentation, a relatively cheaper method of enzyme production. Moreover, fungal cellulases are found mostly as extracellular and free molecules rather than as complex cellulosome that the bacteria produce.

Enzymes in general work optimally under a specific set of physicochemical conditions which are indeed not always available in the industrial setup. Enzymes from extremophilic microbes however in many instances are able to work under harsh conditions because of various adaptations they developed during living in hostile conditions (Gomes and Steiner 2004; Sujatha et al. 2005; Acharya and Chaudhary 2012). Cellulases being the third largest enzyme from an industrial point of view have multiple applications with each application requiring specific set of catalytic features. Cellulases from extremophilic fungi are thus attractive.

16.2 Cellulases

Cellulases comprise a group of enzymes that coordinately catalyze hydrolysis of cellulose into glucose. Except a few fungi, e.g., *Neocallimastix*, *Piromyces*, and *Orpinomyces* (Tatsumi et al., 2006; Watanabe and Tokuda, 2010), that produce cellulases in a complex form called cellulosomes, all other fungi produce cellulases in free forms. Fungal cellulases comprise three hydrolytic enzymes viz., endo-(1,4)-β-D-glucanase (EC 3.2.1.4), exo-(1,4)-β-D-glucanase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) (Goyal et al. 1991).

Commercially, cellulases are obtained from fungi like *Aspergillus* and *Trichoderma* spp. *Aspergillus* strains are also known for their ability to produce β–glucosidase or cellobiohydrolase with much higher yields than *Trichoderma* spp. (Damisa et al. 2011). The *Trichoderma reesei* produces cellulases at a high rate, but its cellulases contain β-glucosidase in inadequate quantity affecting the efficiency of cellulose hydrolysis into glucose. This in turn results in the accumulation of

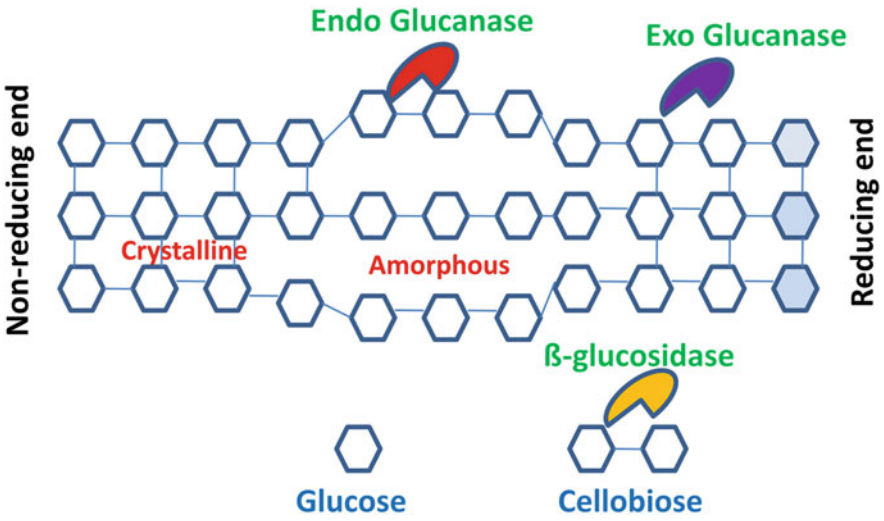

Fig. 16.1 Three members of cellulases and their sites of activity on substrate

cellobiose (dimer of glucose) which is a strong inhibitor of endo- and exoglucanases and ultimately overall process of hydrolysis (Gilkes et al. 1991). The addition of exogenous β-glucosidase is thus indispensable to ensure efficient cellulose hydrolysis with *T. reesei* cellulases (Ting et al. 2009). The activities of the three enzymes are as below (Fig. 16.1):

(a) Endo-β-1-4, glucanase: Catalyzes random breaking of internal β-1-4, linkages.
(b) Exo-β-1-4, glucanase: Catalyzes the removal of glucose units from the non-reducing end of the cellulose chain and successively. The enzyme is inhibited by glucose.
(c) β-glucosidases or cellobiases: It acts on cellobiose and catalyzes its hydrolysis into glucose. The enzyme is also inhibited by glucose.

16.3 Screening

16.3.1 Rapid Test

The rapid cellulase screening method (Smith 1977) uses autoclaved soft agar (0.75% w/v) medium without any carbon source dispensed in a screw-capped bottle. This is to be overlaid by cellulose-azure containing medium. As recommended originally, the cellulose-azure containing medium is prepared at first in two parts, first containing only recipe of medium but with two-thirds volume of water required and second with only cellulose-azure and one-third water required. After sterilization, the two are mixed while they are still warm, and then a small volume of it is pipetted onto the medium kept in a screw-capped bottle. Originally, the medium

used was the Patterson medium (Coutts and Smith 1976), but it can be replaced with another suitable medium as per the requirement of a specific microbe. The finally prepared medium is inoculated with spores or cork-borer cut disc of fresh mycelium. After suitable incubation, cellulolytic microbe is identified by its ability to release blue color in the medium.

16.3.2 Congo Red Test

The fungal isolates are screened for their ability to produce cellulases according to the method given earlier (Teather and Wood 1982) with minor modification by various workers. One of the methods applies Czapek–Dox medium containing (g/l): sucrose – 30, $NaNO_3$–2, K_2HPO_4 – 1, $MgSO_4$–0.05, KCl – 0.5, $FeSO_4$–0.01, carboxymethyl cellulose – 1%, agar agar – 20; pH of the medium has to be adjusted according to fungal requirement viz., 3–4 (for acidophilic), 5–6 (thermo/psychrophilic), and 9–11 (alkaliphilic). Medium may be supplemented with 5–10% NaCl as per the requirement of halophilic fungus. After autoclaving, the medium is to be poured into Petri plates and allowed to solidify. Cavities of definite size (e.g., 6 mm) are made in the solidified medium and inoculated with 0.1 mm size of the fungal colony (fungi forming mat) or spore suspension (10^6/ml) from log phase culture. Generally, the plate is incubated at around 30–37°C (mesophilic), 20 °C (psychrotrophic), and >40°C (thermophilic) for 3–4 days, slow grower extremophiles may need a longer incubation period. After incubation, 10 ml of 1% Congo Red staining solution is added to the plates and shaken at 50 rpm for 15 min. The Congo Red staining solution is then discarded, and 10 ml of 1 N NaOH is added and shaken again at 50 rpm for 15 min. Finally NaOH solution is also discarded and the staining of the plates is analyzed by noticing the formation of clear or yellowish zones around the fungal inoculated wells.

16.4 Production of Enzymes

There are two approaches to be followed viz., solid-state fermentation (SSF) and submerged fermentation (SF). In SSF the growth medium contains agro-residue such as wheat or rice straw as a carbon source. This is supplemented with a mixture of inorganic compounds to supply various growth requirements. In SF the carbon source used in the growth medium is microcrystalline cellulose (Avicel), which is used generally. This is supplemented with other inorganic compounds. The pH and salinity condition of the medium is adjusted as per the requirements of the fungus used. Also, the incubation temperature depends upon the requirement of the fungus for optimal growth. The submerged fermentation is carried out on an incubator shaker. After the completion of fermentation a test is made for the presence of enzyme activity in the medium. If expected enzyme activity is detected, the SF medium is directly filtered through a four-layered cheese cloth and the filtrate obtained is centrifuged at 5000 rpm for 10 min at 4 °C and supernatant is used as

a source of enzyme. In SSF after the completion of fermentation the medium is washed with sterile distilled water. The wash is used as the source of enzyme.

16.5 Purification of Cellulases

Cellulases can be obtained in reasonably pure form applying the following steps:

Ammonium sulfate fractionation.

Supernatant is progressively subjected to increasing saturation with ammonium sulfate, i.e., 0–10%, 10–20%, 20–30%, 30–40%, 40–50%, 50–60%, 60–70%, 70–80%, 80–90% following overnight incubation at 4°C the precipitates after each saturation are spun down. Precipitates thus obtained for each saturation are assayed for each of the cellulase subunits, i.e., CMCase, FPase, and glucosidase. Finally, enzymatically active fractions are pooled, dissolved in a buffer usually 1 M phosphate buffer (pH 6.5), and kept at 4 °C until further purification steps.

16.5.1 Dialysis

Dissolved precipitates of CMCase, FPase, and glucosidase are to be desalted by dialyzing against the same buffer overnight at 4 °C using a dialysis membrane of cut-off 14,000 Da. Alternatively, G-50 column may be used to desalt the enzyme preparation.

16.5.2 Chromatographic Purification

Further purification may be carried out applying ion exchange (DEAE) chromatography (Garsoux et al. 2004) or gel exclusion column chromatography (Bakare et al. 2005) or affinity chromatography applying swollen Avicel as affinity matrix (Ncube 2013).

Before packing in the suitable column, the chromatographic material (matrix) is pretreated with a suitable buffer (e.g., phosphate buffer). The sephadex G-100 is incubated in phosphate buffer overnight and warmed in boiling water bath for 4–5 h to swell it before packing. The packing is carefully done to avoid entrapment of any air bubbles in the matrix. A suitable sample size of dialyzed samples, i.e., CMCase, FPase, and glucosidase, is loaded onto the column, washed with the same buffer followed by elution of bound protein (s) with suitable elution buffer usually the same buffer containing a linear gradient of ammonium sulfate or NaCl (usually 0–0.5 M). Fractions of a specified volume, e.g., 3 ml, are collected manually or applying automatic collector with constant rate, e.g., ½ ml per minute. The fractions tested for the presence of protein directly by measuring absorbance at 280 nm. Positive fractions are then assayed for the presence of enzyme activity. Finally positive fractions are pooled and again dialyzed and lyophilized. Finally, the enzymes are

characterized by applying sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE).

Enzyme purification is currently done using fast and automatic system such as FPLC (e.g., FPLC Mono Q Sepharose column) (Dutta et al. 2008).

16.6 Enzyme Assays

16.6.1 Total Cellulase or Filter Paperase (FPase)

It is determined as described earlier (Ghose 1987). The assay mixture (total volume of 2 ml) contains 50 mg of Whatman No.1 strip (1 × 6 cm) in 1 ml of 50 mM citrate buffer (pH 4.8) and 0.5 ml of diluted crude enzyme and is incubated at a suitable temperature (varies for thermophilic, mesophilic, and psychrophilic enzymes) for 30 min. The buffer may be replaced with suitable ones in case of alkaliphilic/acidophilic enzymes. The reaction mixture may be supplemented with suitable concentration of salt in case of halophilic enzymes. Standard curve was prepared with glucose. Overall cellulase activity is expressed as filter paper unit (FPU) per ml of supernatant.

16.6.2 CMCase

Carboxy methyl cellulose (CMCase) activity is determined as described by Mandels et al. (1975). The assay mixture, in a total volume of 2 ml, contains 0.5 ml of 1 mM of carboxyl methyl cellulose (CMC) in 50 mM citrate buffer (pH 4.8) and 0.5 ml of diluted crude enzyme. The reaction mix may be prepared with suitable buffer and supplementation of salt and is incubated at a suitable temperature for 30 min as in the case of FPase.

16.6.3 Cellobiase (β-Glucosidase)

Cellobiase activity is assayed by incubating 0.5 ml of supernatant with 0.5 ml of 2% cellobiose in 0.05 M sodium citrate buffer (pH 4.8) at a suitable temperature for 30 min. The reaction mix may be prepared with suitable buffer and supplementation of salt and is incubated at a suitable temperature for 30 min as in the case of FPase.

After incubation in each case, DNS mixture is added, boiled for 5 min, and transferred immediately to a cold water bath. Then 20 ml of distilled water is added to the tubes, mixed, and the developed color is measured at 540 nm to estimate the amount of reducing sugars released. The enzymatic activity of CMCase, total FPase, and endoglucanase is defined in international units (IU). One unit of enzymatic activity is defined as the amount of enzyme that released 1 μmol reducing sugars (measured as glucose) per ml per min.

The activity is calculated by the following formula:

$$\text{CMCase/FPase/Cellobiase activity (IU/ml)} = \times(\text{mg glucose produced}) \times 5560 \times 22 \times 2/30$$

$$\text{Enzyme concentration} = \frac{1 \text{ volume of enzyme sample in dilution}}{\text{Total volume of dilution}}$$

$$1/\text{dilution} = 1/20 = 0.05$$

$$\text{CMCase and FPase (IU/ml)} = 0.37/\text{Enzyme concentration that releases 2.0 mg glucose}$$

$$\text{Cellobiase} = 0.0926/\text{enzyme concentration that releases 1.0 mg glucose}$$

16.6.3.1 Chromogenic Method for β-Glucosidase

β-glucosidase activity can also be determined using chromogenic substrates such as p-nitrophenyl-β-D-glucoside, p-nitrophenyl β-D-1,4-glucopyranoside,-β-naphthyl-β-D-glucopyranoside, 6-bromo-2-naphthyl-β-D-glucopyranoside, and 4-methylumbelliferyl-β-D-glucopyranoside. The enzyme acts on these substrates and releases colored or fluorescent products. The most common substrate among these is p-nitrophenyl β-D-1,4-glucopyranoside (pNPG) which releases p-nitrophenyl that can be detected at 430 nm. pNPG (5 mM) is prepared by adding 0.1576 g of pNPG in 100 ml acetate buffer. 1.0 ml of 5 mM pNPG is mixed with 1.8 ml of suitable buffer and 0.2 ml of appropriately diluted enzyme and is incubated at a suitable temperature. After adding 4.00 ml of glycine buffer to stop the reaction, absorbance is read against blank at 430 nm. Standard curve is made with 4-nitrophenol (Zhang et al. 2009).

High-throughput multicolored chromogenic test has been developed for cellulases assay by Kračun et al. (2015).

16.6.3.2 Effect of Glucose and Ethanol on β-Glucosidase Activities

β-glucosidase activity was quantified with the addition of glucose (100–1000 mmol) or ethanol (50–400%) to the reaction mixture, according to Leite et al. (2008). The assays were carried out in 100 mmol sodium acetate buffer, pH 50, at 50 °C.

16.6.4 Protein Assay

Protein is assayed applying Lowry et al. (1951) method.

16.7 Enzyme Production

The commercial dominance of cellulases from the fungus *Trichoderma* spp. is due to its higher production rate which in turn has resulted from tremendous research efforts to improve strain and optimize production rate (Seiboth et al. 2011), even

then it suffers from very low β-glucosidase activity (Knapp 1985). It has been reported that the thermophilic fungi *Sporotrichum thermophile* (Coutts and Smith 1976) and *Talaromyces emersonii* (Folan and Coughlan 1978) match cellulase production level with *Trichoderma reesei*. Moreover, the enzymatic activity of cellulases from thermophilic fungi *Chaetomium thermophile*, *Sporotrichum thermophile,* and *Thermoascus aurantiacus* has been found to be two- to three-fold higher as compared to those of *Trichoderma viridae* (Tansey 1971). Halophilic fungus *A. terreus* UniMAP AA-6 gas been found to produce highly sturdy ionic liquid tolerant CMCase. Production of this enzyme has been optimized (2.2-fold) applying Plackett–Burman experimental design (Gunny et al. 2015) implying the potential of exploration of suitable extremophilic cellulases for various applications and their production optimization.

16.8 Applications

Commercially cellulases hold a prominent place among industrially important enzymes. They have applications in various industries such as textile, detergent, pulp and paper, food, animal feed, biofuel, and molecular biology. In a market research report, cellulases hold 32.84% of the Global market share among enzymes in 2016. The same report forecasted a 5.5% compound annual growth in demand for cellulases during 2018–2025 reaching 2300 million USD.

Currently, mesophilic cellulases produced from *Trichoderma* spp. and *Aspergillus* spp. are commercially sold. *Trichoderma* spp. produces cellulases very efficiently, but the proportion of β-glucosidase in them is low so they cannot hydrolyze cellulose completely and efficiently (Knapp 1985). The addition of *Aspergillus* β-glucosidase is so required in the enzyme cocktail. Moreover, some applications require some special characteristics of enzyme, for example, food industries require processing at a lower temperature to preserve nutritional values and aroma which is possible if cold-active enzyme is available. Extremophilic microbes, e.g., fungi produce cellulases with special features valuable for various industries (Table 16.1).

16.8.1 Food Industries

The food industry is one of the biggest industries worldwide that depends on huge supply of enzymes. The following activities are especially dependent on enzymes.

16.8.2 Juice Clarification

Fruit and vegetable juice in the beginning looks hazy because of floating fibrous materials of mostly carbohydrate nature. This is unattractive for consumers and thus has to go. This is dealt with a cocktail of enzymes containing pectinases, cellulases, and hemicellulases. The treatment not only clarifies the juice but also reduces the

Table 16.1 Representative extremophilic cellulases with essential features from fungi

Fungal species	Isolation site	Enzymes	Optimum			Reference
			pH	Temp(°C)	Salinity	
(A) Halophilic enzyme						
Aspergillus flavus	Solar saltern	CMCase	10	60	200 g l^{-1}	Bano et al. (2019)
Aspergillus ZJUBE-1	Marine	All three	4–5	65	–	Liu et al. (2012)
A. niger	Marine				8%	Liang and Xue (2017)
Penicillium chrysogenum FU42	Marine	CMCase	7.5	60	0.1 M	Lee et al. (2015)
Stachybotrys microspora	Sodic soil	CMCase	8.0	70	5.0 M	Hmad et al. (2017)
(B) Acidophilic						
Pleurotus ostreatus	–	CMCase	4.0	55	–	Okereke et al. (2017)
Rasamsonia emersonii	Plant litter	CMCase	3.0	–	–	Thanh et al. (2019)
A. Fumigates	CMCase	2.0	65	–	–	Grigorevski-Lima et al. (2009)
A. niger	CMCase	4.0	70	–	–	Li et al. (2012)
A. nidulans	CMCase	4.0	50		–	Tavares et al. (2013)
(C) Alkaliphilic/tolerant						
Sodiomyces alkalinus	Soda soil		6.0–10	–	–	Grum-Grzhimaylo et al. (2017)
P. citrinum	Soil	CMCase	5.5/8.0	–	–	Dutta et al. (2008)
(D) Thermophilic						
Myceliophthora thermophila JCP 1–4		CMCase	5.5	65		
		Cellobiase	5.5	70		
Thermoascus aurantiacus		CMCase	4.5–5.0	60–75	–	Tong et al. (1980)
		Cellobiase	Do	70		
Penicillium *funiculosum*		All three	4.9	52–58		de Castro et al. (2010)
Aspergillus wentii		CMCase	4.2	60		Giorgi (2017)
A. versicolor			4.0	60		Do
Chaetomium thermophile			4.5	55		Do
Sporotrichum pulverulentum			4.5	65		Do

(continued)

Table 16.1 (continued)

Fungal species	Isolation site	Enzymes	Optimum			Reference
			pH	Temp(°C)	Salinity	
Acremonium thermophilum		CMCase	5.5	60		Voutilainen et al. (2008)
Humicola grisea var thermoidea		Cellulase	5.0	55–60		Takashima et al. (1996)
Humicola insolens		CMCase	5.0	50		Hayashida and Yoshioka (1980)
Talaromyces emersonii		Cellobiohydrolase	5.0	68		Grassick et al. (2004)
Thermoascus aurantiacus		Cellobiohydrolase	6.0	65		Hong et al. (2003)
Humicola grisea		Cellobiase	5.0	75		Takashima et al. (1999)
Humicola grisea		Do	5.0	60		Do
E. Psychrophilic						
Glaciozyma Antarctica		β-1,3-glucanase	7.0	20		Mohammadi et al. (2020)
Cladosporium spp.			5.0	60		Abrha and Gashe (1992)
Tausonia pullulans		β-Galactosidase	4.0	50		Song et al. 2010
Verticillium sp. AnsX1		FPase	5.3	38		Wang et al. (2013)
Mrakia blollopsi		CMCase	5.4	4–22		Carrasco et al. (2016)
Cadophora Malorum						Berg (1978)
Penicillium spp.		All three	4.9	52–58		Jorgensen et al. (2002)
A. terreus AKM-F3		CMCase	4.0	35		Maharana and Ray (2015)
F. Endophytes						
Fusarium oxysporum			5–6	28		Dar et al. (2013)
A. niger DR02		CMCase				Robii et al. 2013
Trichoderma atroviride						
DR17,DR19		Cellobiase				
Alternaria sp. DR45						
Annulohypoxylon stigyum DR47						
Talaromyces wortmannii DR49.						
Penicillium glabrum		All three				Cabezas et al. (2012)
Induratia spp.		All three				Pereira et al. (2020)

viscosity. Juice especially of citrus fruits is valued for vitamins (Vit C) which is degenerated at higher temperatures. Thus, cold-active enzymes that preferably work at storage temperature (<4°C) are highly desired as they enable storing juice immediately after extraction with enzymes. This practice preserves not only the nutritional quality but also the aromatic profile of the juice.

In wine industry, cellulases in combination of pectinases are used to enhance the release of juice, tannins, and color from grapes (Sahay 2019). Enzymes working optimally at lower pH and temperatures (cold-active) are highly desirable.

16.8.3 Pigment Extraction

Natural pigments such as carotenoid are highly in demand nowadays as they are used as safe food colorants. The peels of colored fruits are the sources of these pigments whose extraction is highly accelerated in the presence of the cocktail of the cell wall acting enzymes including cellulases.

16.8.4 Olive Oil Extraction

During extraction olive oil is subjected to the mixing step to facilitate the aggregation of small oil droplets into bigger ones. The addition of cellulases with or without pectinases accelerates the process and also enhances the release of phenolic and antioxidant compounds in the oil (Jayasekara and Ratnayake 2019).

16.8.5 Bakery

The quality of bakery products such as the softness of crumb, loaf volume, flavor, and shelf life is enhanced substantially if the enzyme cocktail including cellulases is added to the flour (Illingworth and Cook 1998).

The cold-active enzymes are especially useful in the food industries as they can enable processing at lower temperatures which in turn ensures heat labile compounds of nutritional values (e.g., vitamins and antioxidant compounds) and aroma to be preserved.

16.8.6 Textile Industry

Textile comprises the most important part of the world's one of the hugest fashion industries. Cloths of unique feel, texture, shine, and colors are in huge demand. Many of these qualities in the cloth depend on enzymatic treatments. Cellulases form the third largest group of enzymes used in textile industries to catalyze processes involved in improving these qualities (Xia and Cen 1999).

16.8.6.1 Biostone Washing

Cellulases are used to replace conventional three-stepped biostone washing. The conventional process involves the removal of starch coating from fabric with amylases (desizing), subjecting the cloth to rubbing with pumice stone in washing machine, and treating the cloth with sodium hypochlorite or potassium permanganate. After the process is over pumice stones are removed manually which is tiresome and pumice stone itself is abrasive to machine. The application of cellulases deals with these problems. Cellulase (CMCase or endoglucanase) removes small exterior fibers containing most of the indigo stains thus giving a faded look to the cloth. One more problem, i.e., back staining whereby removed stain again binds to treated cloth that remains even after treatment with cellulase. One of the solutions is to use the cocktail of amylase, cellulase, and laccase; the last enzyme decolorizes the removed stain preventing back staining (Cortez et al. 2001). *T. reesei* endoglucanase II is found useful for biostone washing (Maryan and Montazer 2013).

16.8.6.2 Biopolishing or Biofinishing

Cotton and linen cloths look fuzzy due to protruding microfibers. If they are left untreated, they may turn into an even worse ball-like appearance. All these are unattractive themselves and also reduce the shine and brightness of color. Cellulases are used to remove these microfibers thereby giving a glossy look, improved hand feel, and brightness to color. Additionally enzyme also removes dirt spots and stains trapped within fibers (Sreenath et al. 1996; Anish et al. 2007).

16.8.6.3 Bioscouring

The softness of the cloth can also be enhanced by removing non-cellulosic materials (cuticle) from the surface. This is done with cellulase alone or in combination with pectinases (Ibrahim et al. 2011).

16.8.6.4 Biocarbonization and Wool Scouring

Removal of extra cellulosic and other vegetative materials from raw wool, cotton, or cotton blend fabric (carbonization) is also required to improve the quality of the fabric. Earlier sulfuric acid was used for this that made the process costly, corrosive, and hazardous. Now the enzyme cellulases have replaced the acid making the process safe.

16.8.6.5 Defibrillation of Lyocell

Lyocell is a generic name given to pulp fabric, obtained by treating wood pulp with amine oxide followed by pushing the dissolved materials through spinnerets. The fabric material is regarded as sustainable but requiring the removal of superficial tangled fibers (fibrillation) before use. The fibers are digested with cellulases giving the fabric a glossy look and a smooth hand feel.

Textile enzymatic treatment requires exo- and endoglucolytic activities. Therefore, *T. reesei* cellulase is mostly used in the treatment. There are now reports for the use of cold-active alkaliphilic cellulases for textile because of certain advantages. Cold-active enzymes enable process to be carried at low temperature under mild

condition that would reduce wear and tear during processing. Cold-active enzymes are generally heat labile, thus it is possible to denature the enzyme after heat treatment that would reduce water use during the washing step.

16.8.7 Paper and Pulp

16.8.7.1 Pulping

Paper and pulp industry is also one of the largest industrial sectors globally. The industry applies the most basic steps of cutting wood into pieces and their mechanical pulping which are energy intensive processes and generate stiff pulps laden with fines and bulk (Kuhad et al. 2011). Biopulping applying cellulases has been found to reduce energy investment by 20–40% (Statista 2018), reduce fiber coarseness and viscosity, and enhance bleachability (Pere et al. 1995; Mansfield et al. 1996). Moreover, pulp generated through biopulping gives smaller particles during the subsequent refining process which exhibits a lesser drainage rate during paper making (Kuhad et al. 2011). Thus the quality of pulp/paper is improved and energy cost and environmental impact are also reduced (Sharma et al. 2016).

16.8.7.2 Deinking

Recycling and reuse of papers require deinking of the used papers. Traditional deinking process involves expensive and hazardous chemicals (alkali) that generate toxic by-products (Zhang et al. 2013). Replacing them with celluloses called bio-deinking is environment-friendly process that does yield whiter recycled paper as it is done at acidic pH avoiding alkaline yellowing (Kuhad et al. 2011).

Pulping is done under more or less severe conditions due to the presence of wood-generated phenolics, salts, and metals. So, sturdy and polyextremophilic or polyextremotolerant (metallophilic, halophilic, thermophilic, and acidophilic) cellulases may be more suitable to be used.

16.8.7.3 Detergent Industry

Use of enzymes in detergent formulations is now a common practice. Because of this, the detergent industry was found to be the largest user of enzymes by 2014 according to the market report (Zhang et al. 2013). The major enzymes used in the detergent are protease, lipase, cellulase, and amylase and the purpose behind their use is to remove stains of various chemical natures under mild condition to preserve cloth quality and longevity.

Cold-active, alkaliphilic, and heat labile cellulase is the most desirable one as it would enable cold washing saving energy especially in cold regions. Because the heat labile enzyme loses activity at a higher temperature, cloth after drying would hardly be expected to retain residual activity thus giving health benefits.

16.8.7.4 Animal Feed

Cellulase-treated cereal-based animal feed has been found to benefit an animal in proper digestion of food and subsequent growth and productivity (e.g., milk yield) of

animals (Baker and Wicker 1996; Boutte et al. 2009; Vasco-Correa et al. 2016). Cellulases have also been implicated in promoting cecal fermentation processes via enhancing propionic acid production, which inhibits colonization by pathogenic bacteria (Pascual 2001; Fortun-Lamothe et al. 2001; Pazarlioglu et al. 2005).

Cellulosic materials' hydrolysis encounters phenolics, metals, and salt; thus, sturdy and polyextremophilic or polyextremotolerant cellulases may be more suitable to be used. Apart from them, acid-tolerant cellulases may remain active in the intestine and give positive impact there.

16.9 Agriculture

Generation of protoplast and somatic hybridization are experiments which are extensively used globally in improving plant. This is enzyme-dependent process applying cocktail of cellulases, hemicellulases, and pectinases.

16.9.1 Medical Applications

People with metabolic disorder or weak digestion are prescribed enzyme blends including cellulase. Cellulases enable digestion of cellulose-rich fibers available in most of the food articles of plant origin. Apart from this direct use, cellulases have also been reported to be useful in combination with chitinase to partially digest chitin to get chitosan. Chitosan and its derivatives are used in various medical purposes such as production of artificial skin, bone rebuilding, hemostatic dressing, anticoagulant, and production of biopharmaceutics (Garcia-Ubasart et al. 2013).

16.9.2 Bioremediation

Waste water from paper, detergent, and textile industries containing cellulosic residues and high concentration of salt requires halophilic and alkaliphilic cellulases for bioremediation.

16.9.3 Biofuel Application

To mitigate current pace of climate change and ensure energy security, there is a global effort to use bioethanol as gasoline blend. Also to avoid food vs fuel conflict, second generation (2G) bioethanol technology is expected to use agro-forest-municipal residues instead of crop as feedstock. Currently, the most potential 2G bioethanol technology envisages three-step process viz., pretreatment (by autohydrolysis, dilute acid, alkali, or ionic liquid) of biomass to yield relatively pure cellulose, enzymatic (cellulases) hydrolysis of residual cellulase to generate glucose, and fermentation of thus obtained glucose.

Cellulose obtained from pretreatment step thus may be acidic, alkaline, or with residual ionic liquid. It does have degradation by-products of sugars (e.g., furfural and hydroxymethyl furfural), lignin (array of phenolic compounds), and organic acids (acetic acid, etc.). To hydrolyze this cellulose, very sturdy cellulases tolerant to many of these inhibitory conditions are required. There are several reports where the nonspecific and irreversible adsorption of cellulase to lignin has been observed (Bernardez et al. 1993; Yang and Wyman 2004) that reduces the enzyme activity and recovery. Moreover, currently available commercial cellulases work optimally near 60°C which is not compatible with yeast fermentation temperature (25–30°C) and thus with these cellulases simultaneous saccharification and fermentation (SSF) which is considered to be cost-effective technology is not possible.

Taking into consideration of all of the facts there is a great scope to explore suitable extremophilic cellulases that could commercialize 2G bioethanol technology and enable various Governments to achieve stipulated bioethanol blending with fossil fuels. Highly sturdy in nature that works optimally at 25–30°C are the most desirable features of the cellulase to be used in 2G bioethanol technology. Additionally, if the cellulosic material is to be obtained by ionic liquid pretreatment of biomass, then cellulases must also be tolerant to ionic liquid (Xu et al. 2014). Ionic liquid disrupts hydrogen bonds and hydrophobic interaction and affects water hydration cell of the protein thereby inhibiting enzyme activity. Cellulolysis from lime pretreated biomass requires halophilic cellulases (Woolard and Irvine 1995). Cellulases from obligate halophilic fungus *A. flavus* have been reported to be sturdier than available commercial cellulases under alkaline and saline conditions (Bano et al. 2019). The global demand for such cellulases would be unexpectedly much higher surpassing all other industrial uses.

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Extremophilic Fungal Xylanases: Screening, Purification, Assay, and Applications

17

Aneesa Fasim, A. Prakruti, H. K. Manjushree, S. Akshay, K. Poornima, Veena S. More, and Sunil S. More

Abstract

Lignocellulosic biomass is an abundant natural resource that can be utilized for the production of commercial products beneficial to mankind. Biomass degradation and processing is the first step to harness its potential and this is achieved by hydrolase enzymes. Xylanases are one such enzyme that plays a vital role in the degradation of xylan, a major component of lignocellulose. During the last decade, demand for xylanases has markedly increased due to its wide applications not only in the biofuel industry but also in various other industries such as baking, beverage, degumming, paper and pulp, and animal feed. Xylanases are produced by many microorganisms but fungal xylanases are preferred due to their high enzyme production, ease of culturing on cheap agro-industrial substrates, and easy purification. The latest advancement in xylanase research is the discovery of extremophilic fungi (EF) xylanases that have robust characteristics and can retain activity even under harsh industrial conditions such as high or low pH, salt concentrations, pressure, and temperatures. These qualities can drastically benefit the global economics of biofuel production, paper and food industries, and many other industrial production processes.

Thus, a detailed review of several techniques of isolation, screening, and characterization of EF xylanases is discussed here. A comprehensive summary of purification and different assay techniques has also been listed to better understand and optimize enzyme extraction. Improved understanding of the biochemical properties of fungal xylanases allows the exploration of xylanases for various inventive industrial and biotechnological uses.

A. Fasim · A. Prakruti · H. K. Manjushree · S. Akshay · K. Poornima · S. S. More (✉)
School of Basic and Applied Sciences, Dayananda Sagar University, Bengaluru, Karnataka, India
e-mail: drsunil@dsu.edu.in

V. S. More
Department of Biotechnology, Sapthagiri College of Engineering, Bangalore, Karnataka, India

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Keywords

Lignocellulosic biomass · Xylan · Biomass degradation · Agroindustrial substrates · Industrial applications

17.1 Introduction

Biomass, an abundant natural resource, has the power to end the world energy crisis and create a sustainable economy, provided we have the right tools to harness its untapped potential and produce value-added, chemical-free, and eco-friendly products. Trees, woody plants, grasses, crops, agro-wastes, wood wastes, etc. are all forms of terrestrial lignocellulosic biomass that can be degraded and processed to harness the potential. An evolving trend is to employ hydrolytic enzymes secreted by microorganisms to process lignocellulose which is a major component of the plant cell walls. The cell wall is a multifaceted, molecular system that provides structural integrity, rigidity, and protection to the cell. It allows the diffusion of nutrients and intercellular signals within the cell via the plasma membrane. Growing plant cells are surrounded by a thin layer of primary cell wall, but once the cell reaches its maximum growth lignin starts depositing and a thick secondary cell wall starts developing (Buchanan et al. 2000). The secondary plant cell wall is a highly resolute and active network composed of hemicellulose (30–35%), lignin (15–20%), and cellulose (40–50%) (Singla et al. 2012) held with covalent and non-covalent interactions (Sánchez 2009). Hemicellulose is further made up of xylans (xyloglucans and arabinoxylans) and mannans (glucomannans) with xylan forming the major component of the lignocellulosic plant cell wall. Xylan makes up approximately 30% dry weight of the terrestrial plants. It is impermeable to water and hydrolytic enzymes, thus protecting the plant cell from enzymatic degradation. Xylan is a heteropolysaccharide made up of β-xylopyranose units forming the backbone. Additionally, D-galactose, L-arabinose, D-mannose, α-glucuronic acids, and α-arabinofuranose are also present as side branches to the backbone. Organic acids such as acetic acid, ferulic acid, and glucuronic acid are found interwoven together with the help of glycosidic and ester bonds to the xylan main chain (Collins et al. 2005; Ahmed et al. 2007; Motta et al. 2013; Sharma 2017).

The percent and composition of xylan differ in different plants as well as different parts of the plant. It is observed that the content of xylan is 10–35% in hardwood and 10–15% in softwood. Also, the content of acidic xylan is higher in hardwood compared to softwood due to the presence of acetyl groups. In hardwood xylan, one acidic group is present per 5–6 D-xylose units, while in softwood it is one acidic group per 9–12 D-xylose units (Bastawde 1992). Reports also claim that 95% of the side chains of xylan consist of 4-*O*-methyl-α-D-glucuronic acid residues. Owing to the complexity of the xylan, complete hydrolysis can only be achieved by the synergistic action of many xylanases that depolymerize xylan to a simple monosaccharide xylose that can be further utilized as a carbon source (Walia et al. 2017).

17.2 Xylanases

Xylanases are a group of hydrolase enzymes that specifically target xylans. As xylans are structurally diverse, a set of xylanases act on different parts of the xylan substrate and break it down. The enzymes that belong to this group consist of endo-1,4-β-D-xylanase (EC number 3.2.1.8), β-D-xylosidases (EC number 3.2.1.37), α-glucuronidase (EC number 3.2.1.139), acetyl xylan esterase (EC number 3.1.1.72), and ferulic acid esterase (EC number 3.1.1.73). Endo-1,4-β-D-xylanase and β-D-xylosidases cleave the xylan backbone to release xylose monomers, and the removal of side chain acid groups is catalyzed by α-L-arabinofuranosidases. Likewise, α-D glucuronidases and acetylxylan esterases act on the phenolic and acetyl side branches; thus, these enzymes assist in complete xylan degradation (Walia et al. 2017). Figure 17.1 gives an overview of the xylan degradation by the xylanase enzymes.

Previously, xylanases were classified based on the crystal structure and kinetics (Jeffries 1996), biochemical properties (molecular weight, isoelectric point) (Wong et al. 1988), or the substrate specificity and product profile (Motta et al. 2013). But with the discovery of novel enzymes, a more preferred comprehensive classification of glycoside hydrolases (GH) of carbohydrate hydrolyzing enzymes (Cazy database) was developed, and under this classification, xylanases were grouped under GH 5, 7, 8, 9, 10, 11, 12, 16, 26, 30, 43, 44, 51, and 62 families. The xylanases belonging to 16, 51, and 62 are bifunctional with two catalytic domains, whereas 5, 7, 8, 10, 11, and 43 have differences in their catalytic domain (Collins et al. 2005). GH10 and

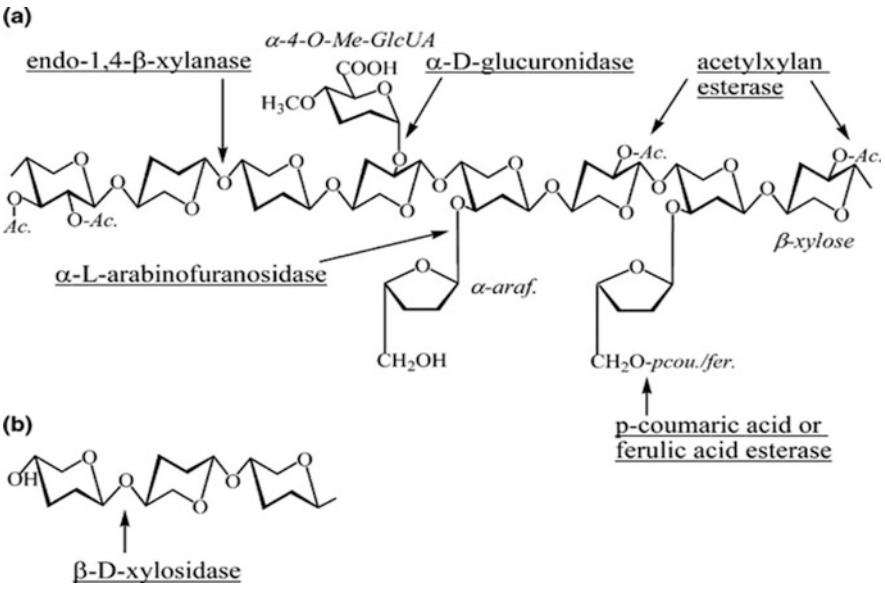
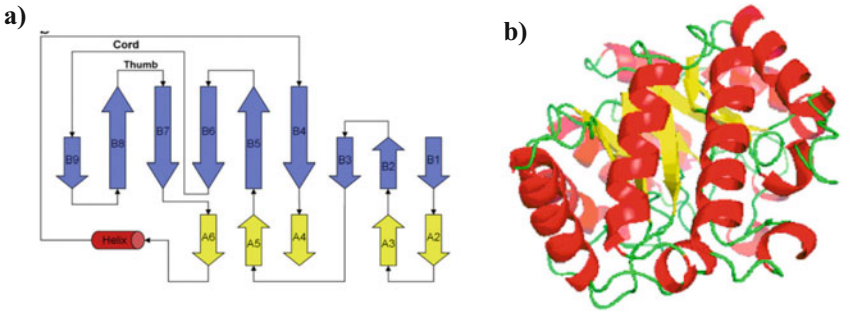


Fig. 17.1 Cleaving site of different xylanases. (Adapted from Selvarajan and Veena 2017)



Cartoon representation of a typical GH-11 member

β- 1,4 Endo Xylanase (1TUX) Xylanase from *Thermoascus aurantiacus*

Fig. 17.2 (**a**) Cartoon representation of a typical GH-11 member. (Adapted from Paes et al. 2012) and (**b**) β-1,4 Endo Xylanase (1TUX) Xylanase from *Thermoascus aurantiacus*. (Adapted from Liu and Kokare et al. 2017)

GH11 are the most extensively studied GH groups with plant, fungal, and bacterial xylanases categorized under GH10 family and fungal, and few bacterial enzymes included in the GH11 family (Chakdar et al. 2016). GH11 members are considered as "true xylanases" as the endo-β-1, 4-xylanases belonging to this group catalyze preferential cleavage of xylan backbone to release D-xylose (Henrissat and Bairoch 1993). GH11 group enzymes exhibit interesting characteristics such as optimum activity at a wide range of temperature and pH, low molecular weight (<30 kDa), high substrate selectivity, and catalytic effectiveness making them suitable for many industrial applications (Paes et al. 2012). Overall, the structure of the GH11 family comprises two large β-pleated sheets and a single α-helix that forms a partially closed right hand (Torronen and Rouvinen 1997). Paes group conducted an exhaustive study on 25 GH11 family xylanases where they combined structure and sequence data along with biochemical properties to gain insights into the relationship between the structure and enzyme properties. The superimposition studies revealed a conserved β-jelly-roll domain with most residues in catalytic cleft also being conserved. Based on the findings, a cartoon representation of GH11 family xylanase structure was deduced (Fig. 17.2a). In another study, X-crystallography studies of a thermostable xylanase from *Thermoascus aurantiacus* provided an understanding of its structure and the presence of one disulfide and more than 10 salt bridges contributed toward its stability at elevated temperatures (Fig. 17.2b) (Natesh et al. 1999), but Paes et al. (2012) concluded that every enzyme has unique properties to acquire thermostability and the presence of disulfide bonds did not essentially confer thermostability.

17.3 Types of Xylanases

17.3.1 Endo-β-1,4-Xylanase

Endo-β-1,4-xylanase hydrolyzes O- as well as S-glycosyl compounds. Endo-β-1,4-xylanases cleave the glycosidic linkage of xylan residues releasing xylooligosaccharides (XOS) and xylose. XOSs are two to five xylose molecules (xylobiose, xylotriose, and xylotetrose) linked by α1–4 glycosidic bonds. They are used as prebiotics with many health benefits (Linares-Pastén et al. 2018; Ma et al. 2017). **XOS** has a huge market in the food industry where they are used to fortify many food products (Aachary and Prapulla 2011).

17.3.2 β-D-Xylosidases

β-D-Xylosidases are exo-glycosidases. This class of enzymes hydrolyzes short xylooligomers into single xylose residues. During xylan hydrolysis, short β-D-xylopyranosyl oligomers get accumulated leading to inhibition of the endoxylanase activity. β-xylosidases degrade these oligomers thereby enhancing the degradation of xylan by endoxylanases (Manju and Singh Chadha 2011).

17.3.3 α-Glucuronidases

α-Glucuronidases are a class of hydrolases capable of hydrolyzing 4-*O*-methyl-D-glucuronic acid not only in non-reducing ends of xylopyranosyl units but also cleaves the internal xylosyl residues of xylan molecules (Dimarogona and Topakas 2016). α-Glucuronidases possess two groups within GH family 67 well-defined either by bacterial or fungal origin. The α-glucuronidases of bacterial or fungal origins differ in their molecular weight and quaternary structures. However, the enzyme has a highly conserved active site which makes them different from other class of xylanases (Yeoman et al. 2010).

17.3.4 Acetylxylan Esterase

Acetylxylan esterase is another group of xylanase that cleaves the *O*-acetyl group on second and third positions of the beta-D-xylopyranosyl residues of xylan molecule (Bajpai 2014). When acetyl side groups are removed from the xylan chain, it facilitates the endoxylanase for the efficient hydrolysis of xylan. Therefore, acetyl xylan esterase removes the acetyl groups. Biely (1985) first reported the existence of acetylxylan esterase in hemicellulolytic and cellulolytic fungi such as *Aspergillus niger*, *Trichoderma reesei*, and *Schizophyllum commune*.

17.3.5 Ferulic Acid Esterases

Ferulic acid esterases are classified under EC number 3.1.1.73. It cleaves the ester linkage between the fifth carbon(O) position of arabinose in hydroxycinnamate and hemicellulose. This linkage is denoted as FAX. It primarily forms a covalent linkage between lignin and hemicellulose. FAE is used for the investigation of plant biomass for biofuels, transesterification reactions utilizing hydroxycinnamic esters, and as animal feed (Hunt et al. 2017).

Fungal xylanases are secreted by many microorganisms such as bacteria, fungi, yeast, protozoa, crustaceans, snails, and insects to degrade the cell wall xylan, either to get entry into the plant cell or to utilize the plant biomass as a carbon source (Murashima et al. 2003). *Bacillus*, *Micrococcus*, *Paenibacillus*, *Microbacterium*, *Rhodothermus*, *Arthrobacter*, *Staphylococcus*, and *Pseudoxanthomonas* are a few of the bacterial genera that are reported to yield xylanases (Chakdar et al. 2016). Similarly, *Aspergillus* spp., *Trichoderma* spp. are some of the fungal xylanase producers (Sakthiselvan et al. 2015). Comparative studies prove that fungi secrete much higher levels of this enzyme than bacteria and yeasts (Topakas et al. 2013). Higher enzyme production along with ease of culturing fungi on agro-industrial waste has helped industries bring down the production and processing costs drastically. Also, the utilization of agro-waste indirectly helps in building an eco-friendly and sustainable bioprocess technology.

Although the fungal xylanase production is high, they are found to be optimally active at temperatures around 42–65 °C and neutral or slightly acidic pH implying that they cannot tolerate harsh conditions, which can directly impact the industrial production process. These problems lead scientists to focus on a novel group of organisms called extremophilic fungi (EF). These fungi can survive extreme conditions such as high or low pH, salt concentrations, pressure, and temperature as they have evolved survival strategies. Xylanases from such fungi have robust characteristics that can sustain harsh conditions, and the most extensively studied EF xylanases are thermophiles, halophiles, alkalophiles, and acidophiles. *Trichoderma* sp., *Aspergillus* sp., *Penicillium* sp., and *Acidobacterium* spp. are the major EF species that contribute to the production of EF xylanases. Table 17.1 summarizes the biochemical properties, sources, and purification techniques of different EF fungal xylanases isolated to date.

17.4 Isolation and Screening of Extremophilic Xylanase Producing Fungi

Bioprospecting is the first step toward finding efficient biomolecules for the production and processing of industrial bioproducts. In recent years, screening and isolation of EF fungal xylanases have received much attention. Many different sources are explored to screen and isolate these EF fungi.

Table 17.1 Extremophilic fungi: screening, purification methods, and application

Fungi	Optimum temperature/ pH	Sources	Purification techniques	References
Acidophilic fungi				
Aureobasidium pullulans var. *melangium*	50 °C, pH 5.0	Soil sample from the crude sugar industry	Ultrafiltration, DEAE-Cellulofine A-500 Sephacryl S-200 HR	Ohta et al. (2001)
Penicillium occitanis PO16	45 °C, pH 3	Purchased from Cayla corporation, France	Ammonium sulfate Precipitation Biogel P-100 gel filtration chromatography Mono Q anion exchange chromatography	Driss et al. (2011)
Aureobasidium pullulans	50 °C, pH 4, 0–20% NaCl	Agriculture research service, USA	Ammonium sulfate precipitation Cation exchange chromatography—SP Sepharose fast column	Yegin (2017b)
Alkaliphilic fungi				
Penicillium oxalicum	45 °C, pH 8	Pichavaram mangroves region soil, Tamil Nadu	–	Muthezhilan et al. (2007)
Penicillium citrinum	50 °C, pH 8.5	Soil samples from Dhapa situated in East Kolkata	Ammonium sulfate Precipitation Phenyl Sepharose	Dutta et al. (2007)
Aspergillus fumigatus	55°C, pH 8	Soil samples from decayed paper and wood material	Ammonium sulfate precipitation Biogel P-100 gel filtration chromatography	Deshmukh et al. (2016)
Humicola insolens Y1	70–80 °C, pH 9	Commercially obtained	Ultrafiltration Dialysis FPLC Gel permeation chromatography	Du et al. (2013)
Thermophilic fungi				
Rhizomucor pusillus Aspergillus fumigatus	75 °C, pH 6	Maize silage, Mexico	–	Robledo et al. (2016)
Fusarium sp. *Aureobasidium* sp.	50 °C, pH 7	Leaf debris, across the Luzon Island, Philippines	–	Torres and Dela Cruz (2013)

(continued)

Table 17.1 (continued)

Fungi	Optimum temperature/ pH	Sources	Purification techniques	References
Chaetomium sp. *CQ31*	60–70 °C, pH 7	Composting soil from Weihai, China	SDS electrophoresis	Jiang et al. (2010)
Sporotrichum thermophile	60–75 °C, pH 7	Soil composts	–	Sadaf and Khare (2014)
Chaetomium thermophilum, *Humicola insolens*, *Melanocarpus* sp., *Malbranchea* sp. and *Thermoascus aurantiacus*	55–65°C, pH 7	Composting soil samples	–	Ghatora et al. (2006)
Halophilic fungi				
Phoma sp.	45 °C, pH 5, 0.5 M–4 M NaCl	Mangrove sediments from National Nature reserve, China	Ultrafiltration Affinity 1 chromatography—Nickel NTA column	Wu et al. (2018)
Aspergillus gracilis Aspergillus penicillioides	–	Soil samples around a man-made solar saltern, Thailand	–	Ali et al. (2014)
Aspergillus nidulan sp.	55 °C, pH 7.8	Industrial effluents of paper industry, Punjab	Ammonium sulfate precipitation	Taneja et al. (2002)

17.4.1 Sources

Xylanases are one of the important enzymes secreted by saprophytic fungi to utilize biomass as the source of their food. Extreme environments with high availability of biomass form a rich source to explore for the xylanase producing EF fungi. Screening samples from such environments enable isolation of robust xylanase producers. Table 17.1 also summarizes xylanases secreting EF fungi isolated from different environments

Mangrove forests are a preferred and most widely explored source for isolation of the extremophilic fungi. Mangroves are a complex and interesting ecosystem, usually present around the intertidal areas where the terrestrial and marine aquatic ecosystems merge. These ecosystems have fluctuations in their salinity, temperature, and pH creating an ideal extreme environment (Kathiresan and Bingham 2001). Carbon recycling in the mangrove region is due to the decomposition of leaves and roots, and this feat is accomplished by the hydrolytic enzymes secreted from the microfauna, especially fungi that are efficient biomass degraders (Duarte and

Cebrian 1996, Kathiresan et al. 2011). Torres group collected leaf debris from 44 different locations across the Luzon Island in the Philippines and screened for xylan degrading fungi. They were able to isolate and identify many fungal species such as *Aspergillus* sp., *Fusarium* sp., *Aureobasidium* sp., *Paecilomyces* sp., *Penicillium* sp., *Colletotrichum* sp., and *Phomopsis* sp. (Torres et al. 2016). In another study, Shankou mangrove, National Nature reserve in China, was explored and novel halotolerant xylanase secreting fungi was isolated from the sediments of mangrove and identified as *Phoma herbarum* (Wu et al. 2018). Similarly, 69 fungal strains were isolated from Pichavaram mangroves, Tamil Nadu, India, and identified as *Aspergillus* followed by *Rhizopus*, *Alternaria*, *Mucor*, and *Penicillium*. However, *Penicillium oxalicum* was the highest xylanase producer among all the strains isolated (Muthezhilan et al. 2007)

Another excellent source of xylanase producing fungi is the soil and effluent samples around the industrial region. *Aspergillus nidulans*, an alkalophilic xylanase producing fungus, was isolated from the Shreyas Paper Industry, Ahmedgarh, Punjab effluents (Taneja et al. 2002). *Penicillium citrinum* was another extremophilic strain isolated from soil samples from Dhapa, situated in east Kolkata (Dutta et al. 2007)

Aureobasidium pullulans var. *melanigenum* (ATCC 20524), an endo-beta-1,4-xylanase producing fungus, was screened from soil samples collected from the crude sugar industry (Ohta et al. 2001), whereas *Aspergillus fumigatus* was isolated from the wood material and decayed paper soil samples collected near Puducherry, India (Deshmukh et al. 2016). Screening soil samples around a man-made solar saltern situated in Phetchaburi province of Thailand resulted in the isolation of two obligate halophilic fungal strains *Aspergillus gracilis* and *Aspergillus penicillioides* (Ali et al. 2014). Likewise, *Chaetonium* sp. was one more fungal strain isolated from composting soil from Weihai city of Shandong province, China (Jiang et al. 2010).

Silage is another important source of xylanase producers. Maize silage is a type of fermented fodder made from corn cobs and maize plants with excess sugar content. It was collected from a local farm in the province of Chihuahua, Mexico and 21 fungal strains were isolated. The best xylanase producers *Rhizomucor pusillus* and *Aspergillus fumigatus* were chosen, identified, and further characterized (Robledo et al. 2016).

17.4.2 Screening Techniques

Screening plays a vital role in the isolation of a producer strain. Although samples are collected from rich biodiversities, without screening it is impossible to isolate specific biomolecule producing microorganisms. Generally, screening involves the incorporation of specific nutritional requirements such as carbon or nitrogen source essential for growth and propagation of the specific producer strain. For example, if the growth media is supplemented with xylan, only those fungal strains that can secrete the xylanase enzyme will be capable to utilize xylan as a carbon source and survive.

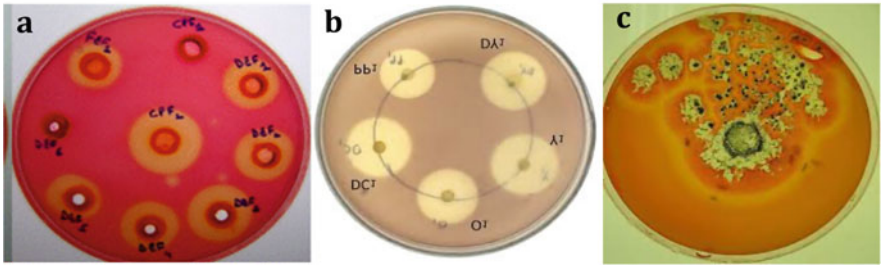


Fig. 17.3 The representative agar plates show different screening techniques: (**a**) Congo red staining, (**b**) iodine staining, and (**c**) Congo red stain infused agar plates showing zone of clearance with the growth of colonies

Oat spelt xylan is one of the extensively used substrates in the primary screening of xylanase producers. While the strains grow and degrade xylan on solid agar plates, it is very difficult to identify the colonies. So dyes like iodine or Congo red are added to the agar plates to visualize the xylanase producers. The principle behind the Congo red or iodine assays is that these stains specifically stain only polysaccharides. Xylanase produced by the microorganism hydrolyzes xylan in its vicinity to produce xylose and xylooligosaccharides that do not take up the stain, thus giving a clear zone of hydrolysis around the enzyme-producing colonies (Sakthiselvan et al. 2015). Hence, most reports have followed staining as the primary screening protocol. Imran A group performed screening for xylanase enzyme by inoculating *A. gracilis* and *A. penicillioides* on potato dextrose agar (PDA) containing agar plates with 1% xylan substrate. Small wells were drilled in the agar plate using cock borer, and 50 μL of cell-free supernatant extracted from culturing the fungi was added to the wells and incubated for 24 h at 37 °C. After incubation, the plates were flooded with iodine to observe the zone of clearance that indicated xylanase activity (Ali et al. 2013). Screening of *Penicillium oxalicum* was performed by flooding Congo red stain on the oat spelt agar plates aiding in the formation of orange colored halos around the enzyme-producing colonies (Muthezhilan et al. 2007). Another fungal strain, *Aspergillus fumigatus*, was also screened for xylanase production by using 0.1% Congo red stain on xylan agar plates for 10–15 min followed by 10-min treatment with 1 M sodium chloride and 5% acetic acid. The best enzyme producer was isolated based on the biggest diameter of the hydrolysis zone (Deshmukh et al. 2016) (Fig. 17.3).

An alternative technique is to infuse the dye in the agar media plate itself so that clear zones appear as the colonies grow and the enzyme is produced. *Rhizomucor* and *Aspergillus* fungal isolates were identified using this technique. The fungal species were inoculated on agar plates containing 0.5% birchwood xylan, 0.1% yeast extract, 0.5% Congo red, and 1.5% agar and incubated for 5 days at 55 °C. The formation of clear zones indicated the xylanase production (Yoon et al. 2007) (Fig. 17.3).

17.4.3 Growth Media

Depending on the fungal growth requirements, various types of growth media are utilized for culturing. Potato dextrose agar (PDA) is a well-known media often used to culture fungi. Robledo group used serially diluted silage samples on PDA plates and isolated thermophilic fungi after incubation for 5 days at 55 °C (Robledo et al. 2016). In another study, halophilic fungi were primarily screened using wheat bran agar media made with 50% seawater, 1 g wheat bran, 1 g $NaNO_3$, 1.5 g agar, 0.5 g $MgSO_4.7H_2O$, and streptomycin antibiotic to maintain the salinity of the sample. The culture was grown at 28–30 °C for 3–4 days and once the isolates were identified, they were preserved on PDA slants containing seawater (potato infusion – 200 g, agar 15 g, dextrose 20 g, and 50% seawater) (Muthezhilan et al. 2007). *Penicillium occitanis PO16* mutant, a xylanase producing fungi purchased from Cayla Co., France, was cultured in a liquefied medium with oat spelt xylan as carbon source. For the production of the enzyme, Mandel's medium (g/L) (KH_2PO_4 – 2; $NaNO_3$ – 5; $MgSO_4.7H_2O$ – 0.3; $CaCl_2$ – 0.3; yeast extract – 1; trace elements solutions – 1 mL/L, and tween 80 – 1 mL/L, pH 5.5) supplemented with 2% glucose was used (Driss et al. 2011). One more frequently used media component is birchwood xylan. *Aspergillus fumigatus* was grown on the minimal media supplemented with birchwood xylan (KH_2PO_4 – 0.5; $MgSO_4$ – 0.25; NH_4Cl – 1; yeast extract – 0.1, birchwood xylan – 0.1). The media was inoculated with soil samples and incubated for 5–7 days at 45 °C in a shaker. Production of the enzyme was carried out using rice bran, an agro-industrial waste used as a solid substrate fermentation media (Deshmukh et al. 2016). *Phoma herbarum*, a halo tolerant fungus, was also isolated and cultured using birchwood xylan medium for 5–7 days at 25 °C (Wu et al. 2018). Yeast mold (YM) is another medium of choice. It was used to grow an extremely acidophilic and halophilic yeast-like fungus *Aureobasidium pullulans*. The YM media is made of (g/L) yeast extract – 3.0 g, peptone – 5.0 g, malt extract – 3.0 g, glucose – 10 g, and agar – 20 g, and cultures were grown at 24°C for 3 days. Growth of fungi and production of xylanase enzyme were carried out at 28 °C at 150 rpm in the media composed of (g/L) xylose – 10 g, asparagine – 2 g, KH_2PO_4 – 5, yeast nitrogen base – 6.7 g, KH_2PO_4–5 g, and pH 5 (Yegin 2017b).

17.5 Purification Techniques

The next step in enzyme extraction is purification. The enzymes are either completely/partially purified or used directly as crude cell-free supernatant for various applications. Most purification techniques to extract extracellularly secreted enzymes follow a similar routine. After the growth of the fungi on submerged or solid substrates, cultures are centrifuged to separate supernatant from the cell mass and macromolecular debris followed by filtration using filter membranes or ultrafiltration techniques. Once the cell-free supernatant is relatively pure, it is subjected to lyophilization. The lyophilized samples are then dissolved in the right buffer and

subjected to complete purification using ion exchange followed by gel permeation chromatography techniques. The fractions are collected based on the absorbance at 280 nm or 220 nm, and appropriate assays are conducted to identify the xylanases.

Some examples of purification and characterization of extremophilic xylanases are described below.

17.5.1 Acidophilic Xylanases

Xylanase from *Aureobasidium pullulans* var. *melanigenum* (ATCC 20524) showed optimum activity at pH 2.0 and 50°C making it a highly acidophilic enzyme. It has a molecular weight of 24 kDa and a pI of 6.7. Anion exchange chromatography using DEAE-cellulofine column combined with gel permeation chromatography using Sephacryl S-100 was used to purify the enzyme (Ohta et al. 2001). Five days of incubated culture was centrifuged at 2000 *g* for 30 min and then subjected to ultrafiltration using 3 kDa cutoff membrane in a stirred cell to concentrate the supernatant. The concentrate was loaded onto DEAE-cellulofine A-500 column pre-equilibrated with 20 mM Tris–HCl buffer, pH 8.5 buffer. To elute the protein, 0–0.5 M linear gradient of NaCl was used. Fractions displaying enzyme activity were pooled and subjected to Sephacryl S-200 gel permeation chromatography to separate proteins based on mass; 512 U/mg of specific activity was observed in a 4.2-fold purified enzyme with 62.2% yield recovery after the final purification step.

In a similar study, another acidophilic xylanase from *Penicillium occitanis* POl6 having optimal xylanase activity at pH 3 and 45 °C was purified using anion exchange and size exclusion chromatography. They used a five-step purification process where the sample was subjected to Biogel P-100 size exclusion column twice. First, the cultures were filtered using a 0.45 μm nitrocellulose membrane followed by dialysis and lyophilized. The sample was dissolved in 20 mM citrate-phosphate buffer pH 3 and loaded on to Biogel P-100 column pre-equilibrated with 20 mM Tris–NaCl, pH 8 buffer. Fractions that exhibited xylanase activity were pooled and loaded on to Mono-Q Sepharose anion exchanger. Again the fractions exhibiting xylanase activity were pooled and loaded onto Biogel P100 for final separation. All the purification steps lead to a recovery of 0.135% enzyme with 5.2-fold-purity and 358 U/mg specific activity (Driss et al. 2011).

Deshmukh RA group followed a slightly different protocol where partial purification by ammonium sulfate precipitation was employed followed by gel permeation chromatography using Biogel-P60 on acidic xylanase from *Aspergillus fumigatus*. They achieved a very high specific activity of 38196.22 U/mg from a 3.4-fold purified enzyme with 54.81% recovery after the final purification step. The cell-free supernatant obtained after culturing the fungus was centrifuged and subjected to 30–55% fractional ammonium sulfate precipitation followed by centrifugation at 10,000 *g* for 10 min at 4 °C. After extensive dialysis to remove salt, the sample was lyophilized followed by separation on Biogel-P60 column equilibrated with 50 mM phosphate buffer, pH 7. Absorbance at 280 nm was used as a reference to collect and measure the activity of the fractions. Although the optimum pH and temperature of

the purified xylanase were 5 and 50 °C, respectively, the enzyme was also found to be stable at an alkaline pH of 8 and 9 (Deshmukh et al. 2016).

17.5.2 Alkaline Xylanases

Extracellular alkaline xylanase was purified from the fungi *Penicillium citrinum* with a pH and temperature optima of 8.5 and 50 °C, respectively. The fungus was cultured on wheat bran solid substrate for 5 days before extracting the enzyme. Wheat bran beds were subjected to agitation in 50 mM acetate buffer for 1 h at 150 revs/min, and the supernatant was collected by centrifugation at 3000 *g* for 30 min. Partial purification of the enzyme was achieved using 80% ammonium sulfate $(NH_4)_2SO_4$ precipitation. The precipitate was suspended in 50 mM sodium acetate buffer, pH 5.5, and dialyzed to remove the salt. The sample was loaded onto a Phenyl Sepharose hydrophobic column pre-equilibrated with 50 mM acetate buffer, pH 5.5. Active fractions were pooled and concentrated using Centricon-10 ultrafiltration cups. A 14-fold purification with a yield of 28% and a specific activity of 361 U/mg was achieved using this purification protocol (Dutta et al. 2007).

Next, alkalophilic xylanase was partially purified from *Aspergillus nidulans* (Taneja et al. 2002). The cell-free supernatant was obtained by centrifugation of the culture broth grown on wheat bran substrate. To partially purify the enzyme, 40% saturated ammonium sulfate precipitation was used. The precipitate was dialyzed and used for further studies. The optimum pH and temperature of the partially purified enzyme were found to be 8 and 55 °C, respectively.

17.5.3 Halotolerant Xylanase

One of the simplest purification techniques to achieve complete purification is affinity chromatography using histidine-tagged recombinant proteins. Halotolerant recombinant xylanase from *Phoma* sp. MF13 was purified using this technique. The gene xynMF13A was cloned into pPICZα-C vector and heterogeneously expressed in *Pichia pastoris.* Xylanase activity was detected in the supernatant after induction with methanol for 120 h. The culture supernatants were concentrated using a PES5000 ultrafiltration membrane and then loaded onto Ni^{2+}-NTA agarose gel column. A gradient of 20–200 mM imidazole in a Tris–NaCl buffer pH 7.6 was used to elute the protein. The optimum pH and temperature of the recombinant xylanase were found to be 5 and 45 °C, respectively. The enzyme was highly active in 0.5 M NaCl, and it retained approx. 54% of the activity even in the presence of 4 M NaCl (Wu et al. 2018).

An extremely halophilic and acidophilic xylanase was purified from *Aureobasidium pullulans* using a single step chromatographic assay. The cell-free supernatant obtained after centrifugation of the 126 h culture was concentrated by lyophilization and desalted using PD-10 columns as the conductivity of the crude sample was very high. The sample was then resuspended in 20 mM citrate buffer,

pH 5, and loaded onto the SP Sepharose cation exchange column. Elution was performed using a gradient of 1 M NaCl in the same buffer, and the enzyme eluted at 28% of the NaCl gradient with an 80% activity yield. The fractions with xylanase activity were pooled and further characterized. The molecular weight of the enzyme was found to be 21.6 kDa with pH and temperature optima at 4 and 50 °C, respectively. Even 20% NaCl did not hinder the activity of the enzyme making it extremely halophilic xylanase (Yegin 2017b).

17.5.4 Thermophilic Xylanase

Thermophilic xylanases are stable at very high temperatures. To purify these enzymes, similar chromatographic and gel permeation procedures are utilized. In a study conducted by Y Du group, direct purification of one xylanase and heterologous expression of three more xylanases were reported from *Humicola insolens* Y1 strain. For direct purification, 6-day-old *H. insolens Y1* wheat bran cultures were harvested by centrifugation at 12,000 *g* 4 °C for 10 min. The supernatant was concentrated using a 5 kDa vivaflow 200 ultrafiltration membrane, dialyzed and loaded on to HiTrap Q-Sepharose XL FPLC column pre-equilibrated with 20 mM Tris–HCl buffer at pH 7. A gradient of 0–1 M NaCl was used to elute the proteins at a 4 ml/min flow rate. Fractions showing xylanase activity were pooled, concentrated, and subjected to size exclusion by loading onto Superdex 75 column. Elution was carried out using 50 mM McIlvaine buffer with pH 6.0 at 0.5 mL/min flow rate. For heterologous expression, XynA, XynB, and XynC xylanase encoding genes were amplified from *H. insolens Y1* cDNA, cloned into the pPIC9 vector, and transformed into *Pichia pastoris* GS115 cells for extracellular xylanase expression. The expression was induced by methanol for 72 h with 90% xylanase found in the supernatant. Purification was carried out using the same protocol and HiTrap Q Sepharose XL FPLC column of anion exchange column. The xylanases showed optimal activity between 70 and 80 °C (Du et al. 2013). The specific activities of directly purified xylanase were 236.2 U/mg, and recombinant xylanases of XynA, XynB, and XynC were 527.3 U/mg, 278.1 U/mg, and 244.9 U/mg, respectively.

17.6 Assay Methods

Many different assay methods are employed to determine the xylanase enzyme activity. These assays either quantify the released reducing sugars due to the action of the enzyme on the substrate or estimate the viscosity/turbidity of the enzyme-substrate reaction mixture before and after the assay.

The dinitrosalicylic acid (DNS) and Nelson–Somogyi (SN) methods are routine and simple assay methods used to determine the xylanase activity, whereas other methods listed here are not so common but are rapid, efficient, and accurate when compared to conventional methods.

17.6.1 DinitroSalicylic Acid (DNS) Method (DNS)

The DNS method is a simple colorimetric estimation of reducing sugars released due to the action of the enzyme on the substrate. The free carbonyl group present on the released sugars reduces the dinitrosalicylic acid reagent to 3-amino 5-nitrosalicylic acid, a reddish-brown colored complex formed under alkaline conditions that can be measured colorimetrically or spectrophotometrically at 540 nm absorbance. An appropriate concentration of enzyme is incubated with the substrate under optimum conditions of pH and temperature for a given amount of time. The reaction is halted by the addition of DNS followed by boiling the samples for 10–15 min. Once the mixture is cooled, absorbance is measured using an enzyme or substrate blank as controls (Gusakov et al. 2011). The absorbance is then converted to enzyme activity using a standard product curve where the color produced is directly proportional to the concentration of released sugars. This method is frequently used to estimate the xylanase activity using xylan, oat spelts, larchwood, or birchwood as substrates. However, this assay has certain disadvantages such as lack of sensitivity and linearity, and it cannot be used to estimate samples containing high sugar content, e.g., bread enhancing mixtures and chicken feeds (Bailey 1988).

17.6.2 Nelson–Somogyi (NS) Method

The NS method also relies on the principle of redox reaction by the released reducing sugar to give a blue-colored molybdenum compound that can be quantified by measuring the absorbance at 610 nm. After the enzyme-substrate reaction, Somogyi's alkaline copper tartrate reagent is added to stop the reaction followed by boiling. The carbonyl group on the sugar reduces the reagent to cuprous oxide which in turn reacts with Nelson's arsenomolybdate reagent giving a blue-colored molybdenum compound. The enzyme activity is calculated with the help of controls where the blue color is directly proportional to the sugar released due to the enzyme action (Gusakov et al. 2011). NS method is considered to be sensitive and more accurate than the DNS method. However, the drawback of this method is the use of arsenomolybdate, a highly toxic reagent (Nelson 1944). Phosphomolybdate reagents are other alternative chromophores, but they lack color stability and are also toxic to the environment (Hatanaka and Kobara 1980). According to McCleary and McGeough (2015), NS is the best method suitable to determine the release of reducing sugars by the endoxylanases as color intensity directly correlates with the glycosidic bond cleavage liberating the reducing sugar. Another improvement to the NS method is Somogyi's iodometric method (Somogyi 1952). Somogyi's alkaline copper tartrate is added to the reaction mixture followed by the addition of 5 N sulfuric acid. This solution is then titrated using 0.0025 N $Na_2S_2O_3$ and measured at 510 nm (Ghose and Bisaria 1987). The accuracy is very high in the iodometric titration such that the error of analysis chiefly depends on the composition of the copper reagent and condition affecting its sensitiveness and reproducibility during the oxidation of sugar (Shaffer and Somogyi 1933).

17.6.3 Bicinchoninic Acid (BCA) Method

This assay is based on the methods of Fox and Robyt (1991) and Meeuwsen et al. (2000) with a few modifications such as the assay volume, incubation time, and wavelength of the sample mixture. BCA reagent is added to the enzyme-substrate reaction mixture followed by incubation at 80 °C for 1 h. The sample is then cooled to room temperature before measuring the absorbance at 562 nm. The concentration is determined by plotting the absorbance against the known substrates standard, e.g., birchwood xylan (sigma-Aldrich), beechwood xylan (Sigma-Aldrich), wheat arabinoxylan (Megazyme), and xyloglucan (Megazyme) (Sydenham et al. 2014). BCA method is considered to be much more accurate and sensitive compared to the DNS method. The sensitivity of BCA assay is about 1–20 nmol per assay compared to DNS which is 500 nmol per assay (Anthon and Barrett 2002; Moretti and Thorson 2008). Another major advantage of this assay is that it can quantify reducing sugars with different chain lengths, unlike DNS or SN method where only mono or disaccharides can be quantified. Many different debranching xylanase enzymes are present and these enzymes can cleave the substrate and release a mixture of long-chain oligosaccharides that can be quantified easily (Utsumi et al. 2009). Besides these advantages, BCA assay method produces stable colored products upon reaction with chromogenic reagents making it a reliable and accurate assay method.

17.6.4 Viscosity Assay (VA)

As the enzyme degrades the polymer, viscosity also decreases. The enzyme-substrate reaction is carried out in a C-type viscometer that is pre-equilibrated with buffer. The substrate solution is pipetted in it followed by the addition of the enzyme that is mixed well and incubated at appropriate conditions. After the incubation period, the rate of decrease in viscosity is measured by taking five falling time readings (in s) for approximately 30 min. Each reading is taken as the elapsed time from combining the enzyme/substrate solutions to the mean of the falling time. The viscosity of the reaction is directly proportional to the falling number. Murphy group has successfully used this method to estimate the xylan activity purified from *Pichia pastoris* and *Schizosaccharomyces pombe* using arabinoxylan as the substrate (Murphy et al. 2009). Viscosity assay is accurate and reliable, but very tedious. However, from the industrial use perspective, it can be used as a reference method for other assay methods (Buckee and Baker 1988).

17.6.5 Xylazyme AX Test

This is another simple test used to quantify the enzyme activity using commercial substrate xylazyme AX tablets. The tablet contains azurine, an active constituent that is cross-linked to wheat arabinoxylan. Upon hydrolysis by xylanase, a water-soluble dyed fragment is generated which can be measured at 590 nm absorbance. The rate

of release of these fragments (increased absorbance) is directly related to the enzyme activity that degrades the xylazyme AX tablets. The xylanase enzymes extracted by *A. niger* and *T. longibrachiatum* were accurately quantified using this method (Megazyme). It was observed that this test was sensitive and precise compared to viscometric assay methods (European Symposium on Enzymes in Grain Processing 2000).

17.7 HPAEC-PAD Technique

This is one of the recent methods employed to estimate enzyme activity using HPLC coupled to anion exchange chromatography (Cürten et al. 2017). The enzyme activity was carried out in a temperature controlled Dionex™AS-AP autosampler using HPLC vials. After the reaction, a gradient elution facilitated the separation of soluble short-chain xylooligomers such as xylose, xylotriose, and long-chain xylooligomers that were liberated throughout enzymatic hydrolysis. The enzyme activity of the xylanase extracted from *Bacillus subtilis*, *Bacillus stearothermophilus*, and *Aspergillus niger* was estimated using this technique (Rothenhöfer et al. 2015). This method was accurate and the separation of even very short chain oligomers could be achieved. It also helped in the detection and distribution of separated molecules based on their chain length (Ballance et al. 2005).

17.8 Applications

During the last decade, demand for xylanases has markedly increased due to its wide applications not only in the biofuel industry but also in various other industries such as baking, beverage, degumming, paper and pulp, animal feed, and waste management (Beg et al. 2001; Kuhad and Singh 1993; Virupakshi et al. 2005). Xylooligosaccharides extracted from xylanase degradation are further used as food additives (Pellerin et al. 1991). EF fungal xylanases are the latest tools in the repertoire of hydrolytic enzymes used to unleash this untapped potential (Fig. 17.4).

17.8.1 Biofuel Industry

Degradation and saccharification of lignocellulosic biomass is the first step in the generation of biofuels. Simple sugars released after the degradation process are allowed to ferment in the presence of suitable microorganisms to produce biofuel, bioethanol, and other useful products. Hence, biomass is subjected to pre-treatment with hydrolytic enzymes that either modify lignocellulose structure or help in the removal of hemicellulose to get access to the polysaccharide substrate (Jorgensen et al. 2007). At this stage, enzymes like xylanases help in the release of carbohydrates from the cell wall polysaccharides, and these sugars are then subjected

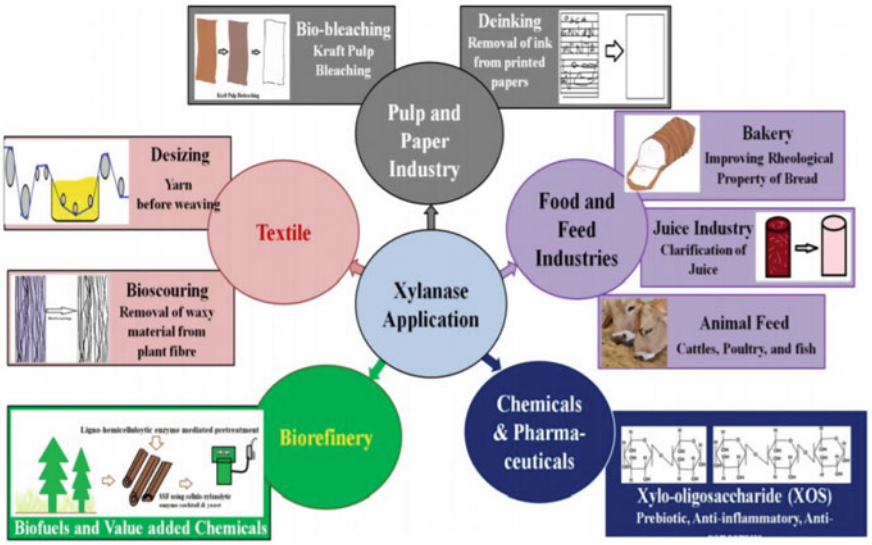


Fig. 17.4 Applications of xylanase. (Adapted from Bhardwaj et al. 2019)

to fermentation for the production of bioethanol. Halophilic fungi *A. gracilis* and *A. penicillioides* are both successfully employed in the biofuel production process (Ali et al. 2014). *Aureobasidium pullulans* 477 NRRL Y-2311-1 is another ethanol tolerant fungus suitable for the bioethanol production process. This fungus was able to grow in the presence of ethanol which made it possible to co-culture this fungus in the fermentation step itself (Yegin 2017a).

17.8.2 Paper and Pulp Industry

Paper is made from the wood pulp, and the quality of the paper is determined by low lignin content and whiteness. First, the wood is subjected to pulping where it is treated with organic solvents at very high temperatures to break down the lignocellulosic biomass. This process is followed by bleaching, which is the removal of residual lignin by the use of corrosive chemicals like chlorine dioxide/hypochlorite (Singh et al. 2019). However, these procedures apart from being hazardous have other disadvantages like the generation of toxic by-products and less yield. Effluents released from these industries enter water bodies and adversely affect the environment and marine life (Singh et al. 2019). To combat these problems, biobleaching and bio-pulping are introduced. Bio-pulping involves pre-treatment of wood with hydrolytic enzymes produced from fungi. Debarked and chipped wood is first decontaminated by steaming and then subjected to lignin degradation by fungi (Scott et al. 1998). After this initial treatment, the chips are pulverized by either mechanical or chemical means (López et al. 2017). In the biobleaching process,

xylanase enzyme is used to remove residual lignin that imparts brightness to the paper (Viikari et al. 1994). Xylanases used in biobleaching process should be able to tolerate high temperatures and pH. Therefore, enzymes from extremophilic fungi give us a huge advantage to manufacture paper in an efficient, eco-friendly, and economical manner.

Sridevi et al. reported that biobleaching of paper pulp at 50 °C with stable alkaline xylanase from *Trichoderma asperellum* improved the brightness by 4 points due to the release of chromophores present on lignin and also reduced the kappa number (signifies the residual lignin) by 4.2 points. One of the reasons for the increase in brightness was the breakdown of the bond by alkalophilic xylanase that retained its activity even under alkaline conditions (Sridevi et al. 2017). Similarly, xylanase from *T. longibrachiatum* and *P. corylophilum* effectively reduced the kappa number and release of chromophores when kraft pulp from Eucalyptus was subjected to biobleaching (Medeiros et al. 2007). In another study, it was observed that xylanase extracted from *Aspergillus sydowii* SBS 45 retained 90% of its activity even after 5 h of incubation at 40 °C and pH 8.2 (Nair et al. 2010). A comparative study, using thermostable and alkalophilic xylanases from *C. thermophilum*, *Melanocarpus* sp., *Humicola insolens*, *Thermoascus aurantiacus*, and *Malbranchea* sp. in the biobleaching process of decker pulp, showed maximum brightness was achieved by *Malbranchea* sp. Brightness increased by 2.04 ISO units with maximum release of chromophores (Ghatora et al. 2006). Xylanase from alkalophilic fungus *Aspergillus nidulans KK-99* bleached and released chromophore at 1.01 U/g of pulp, 55 °C, and 3 h of incubation time. The kappa number was reduced by 5% with 1.7-fold increase in the concentration of reducing sugars (Taneja et al. 2002).

17.8.3 Textile Industry

Desizing, scouring, and bleaching are the important processes involved in fabrication development. Non-cellulosic impurity of pectic substances, wax, ashes, and lignin containing protein are eliminated by a chemical intensive process called as scouring. Conventional scouring solutions affect non-precisely on cellulosic material of fiber which results in loss of strength in the fabric. Xylanase positively performs over the hemi-cellulosic materials by efficiently removing them. Enzymatic treatment does not lead to any loss of strength in the fiber, rather the fiber will be developed to be more soft and smooth after desizing. During the later stages of bleaching and finishing, fungi like *Trichoderma reesei*, *Trichoderma longibrachiatum* produce xylanase which offers partial hydrolysis of seed coat fragments by increasing the accessibility of the chemicals (Dhiman et al. 2008).

Garg et al. (2010) reported the enzyme pre-treatment resulted in the liberation of additional reducing of sugar and weight loss when compared with that of control at 54 °C incubation conditions. 120 min of incubation period was adequate to intensify the whiteness and brightness of fabric up to 3.93 and 10.19%, respectively, and also diminished the yellowness by 5.57%.

17.8.4 Baking Industry

Although bread making is an ancient process carried out in the conventional method, many modifications are made to improve the overall quality and shelf life of bread. One such modification is the use of enzymes (Butt et al. 2008). Wheat flour, the basic ingredient in bread, contains 2–3% of arabinoxylan that negatively impacts the quality of the bread. When arabinoxylan is treated with xylanase enzyme, it helps in the release of sugars that influence the growth of yeast and production of CO_2 and, at the same time, it makes arabinoxylan more water extractable to form gluten network which drastically influences the quality of bread (Courtin and Delcour 2001; Wang et al. 2003; Yegin et al. 2018). The xylooligosaccharides formed by the breakdown of arabinoxylan act as a prebiotic and help in the growth of beneficial bacteria in the gut (Chapla et al. 2012; Yegin et al. 2018). Yegin et al. used xylanase from *Aureobasidium pullulans* NRRL Y-231 strain to study its effect on bread-making process. An increase of 35–40% in water absorption by the dough was observed along with an increase in growth time and stability, whereas the firmness of the crumb reduced. An overall improvement in dough and bread quality was observed when xylanase was used in the bread preparation (Yegin et al. 2018).

17.8.5 Animal Feed

More than 900 agricultural ingredients are added to an animal feed that includes straw, husk, grains, cereals, legumes, and many other agro-waste constituents. Excess gluten in wheat, oats, etc. as well as β-glucans and arabinoxylans in the cell walls of cereal grains linked with glucans, celluloses, galactans, and mannans make digestion difficult in domestic animals. Enzymatic treatment of the feed greatly refines the quality, and it also results in better digestion and growth rates in animals. The addition of xylanase to the feed results in improved availability of polysaccharides and increased nutrient absorption by thinning out the gut contents. Xylanases also facilitate the conversion of hemicellulose to sugars that assists in gaining sufficient energy from lesser feed to the animals, particularly chickens (Garg and Tripathi 2017).

17.8.6 Beverage and Brewing Industry

Juice yield from fruits or vegetables can be increased when treated with xylanases. Xylanases can also reduce the viscosity of the fruit juices along with improved filterability. Xylanases also help in the extraction of sugars from barley when added to the beer malt during the fermentation process (Garg and Tripathi 2017). Yanlong Du et al. used three heterologously expressed xylanases from *Humicola insolens Y1* in the mashing process. He observed a positive effect on the mashing process as the xylanases reduced the viscosity and specific filtration rate of the mash. However, the

cocktail mixture of xylanases was far more effective than individual preparation (Du et al. 2013).

17.8.7 Fruit Ripening and Seed Germination

Germinating seeds naturally yield xylanases that aid in providing the required nutrition for the growth of the sapling. Softening of fruits like papaya is one of the major applications of xylanases. As the fruit begins to soften by ripening, xylanases exhibit a significant part by altering the XOS in cell wall matrix. Consequently, xylanases display a commercial role in the ripening of fruits (Kalim et al. 2015).

17.8.8 Degumming

Traditional water retting for fiber separation in the textile industry is a laborious and harmful process. It also leads to low-quality fiber impacting the quality and cost of the product. Bio-degumming is a process of degradation of non-cellulosic components using hydrolytic enzymes (Duan et al. 2016). Xylanases are extensively used in this process, and it is found that the degumming performed by a combination of mild chemical pre-treatment along with enzymatic treatment resulted in an enhanced yield of fiber. It is also reported that xylanases play a significant role in the elimination of non-cellulosic as well as gummy substances from the bamboo parts (Fu et al. 2008).

17.8.9 Pharmaceutical Industry

Xylooligosaccharides are the non-digestible sugar units composed of xylose monomers. XOS has numerous purposes in the pharmaceutical and biotechnology industry. XOS intensifies the growth of vital good bifid bacteria in the colon and thereby limits the growth and proliferation of other harmful bacteria. Thus, XOS acts as a prebiotic since it is not hydrolyzed or absorbed. XOSs are also found to have anticancerous, immunomodulatory antimicrobial, anti-inflammatory, antioxidant, and antihyperlipidemic activities (Bhardwaj et al. 2019). Therefore, XOSs are used in beverages like soy milk, tea, and coffee, dairy products, desserts like pastries, cakes, biscuits, puddings, jellies, and jams, and also as an active component of symbiotic provisions. The action of the xylanase enzyme on animal feedstock makes it easier to digest releasing the entrapped nutrients and thereby improving the digestion in domestic animals. XOS can also be subsequently converted into simple sugars, i.e., glucose and xylose that are useful in bioethanol production (Pellerin et al. 1991).

The discussed applications thus prove that the employment of highly stable extremophilic fungal xylanases in various industrial productions leads to the

Table 17.2 Applications of extremophilic xylanases

Microorganism	Extremophilicity	Applications	References
Aureobasidium pullulans 477 NRRL Y-2311-1	Acidophilic Ethanol tolerant Halophilic	Fruit juice production Wine-making and brewing Bioethanol production and processing of seafoods	Yegin (2017a)
Aureobasidium pullulans NRRL Y-2311-1	Extremophilic	Bread making	Yegin et al. (2018)
Phomopsis sp. *MACA-J* *Fusarium* sp. *KAWIT-A* *Aureobasidium* sp. *2LIPA-M*	Alkaliphilic Thermophilic Thermophilic	Enzymatic pre-treatment of recycled paper and pulp	Torres and Dela Cruz (2013)
Sporotrichum thermophile	Thermophilic	Xylooligosaccharide synthesis (food industries)	Sadaf and Khare (2014)
Chaetomium sp. *CQ31*	Thermophilic	Chinese steamed bread	Jiang et al. (2010)
Humicola insolens Y1	Thermophilic	Brewing industry	Du et al. (2013)
A. gracilis and *A. penicillioides*	Halophilic	Biofuel production	Ali et al. (2014)
Chaetomium thermophilum, *Humicola insolens*, *Melanocarpus* sp., *Malbranchea* sp. and *Thermoascus aurantiacus*	Thermophilic	Pulp bleaching	Ghatora et al. (2006)
Aspergillus nidulans KK-99	Alkalophilic	Pulp bleaching	Taneja et al. (2002)

development of value-added, chemical-free, and environmentally friendly products (Table 17.2).

17.9 Conclusion

Biodegradation of biomass involves the action of numerous enzymes, among which xylanases play a significant role. It has numerous applications in various industries, and it is used in the production of value-added, chemical-free, and eco-friendly products. Out of innumerable microorganisms isolated from different sources, fungal strains are found to exhibit high potential for the production of xylanase enzyme. The majority of xylanases from the fungal origin show optimal activity at mesophilic temperatures (around 40–60 °C) and neutral to slightly acidic pH. So exploring extremophilic environments for robust fungal xylanases that can sustain harsh industrial conditions is the focus of the current research. This chapter gives a detailed review of the extremophilic fungal xylanase isolation, screening, and purification techniques along with the industrial applications. Its role in biofuel production, paper and pulp industry, animal feed, baking, and beverage industry is discussed in detail

here. However, further studies need to be conducted to gain knowledge about this particular class of enzymes. New approaches, standardized and optimized protocols to increase production will assist in deciphering more of its applications in the future. The use of new technology involving genetic engineering, sequencing programs, in silico approach to study the extremophilic xylanase, and xylanase family of enzymes will further improve our understanding of these enzymes so that they can be employed for an efficient, economical, and eco-friendly bioprocess technology.

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Extremophilic Fungal Lipases: Screening, Purification, Assay, and Applications

18

J. Angelin and M. Kavitha

Abstract

Extremophiles are microorganisms which require or tolerate extreme environmental conditions like high and low temperature, extreme acidic or basic pH, high exposure to radiations, high salinity, low and high pressure, growth in the presence of toxic wastes, organic solvents, heavy metals, and other habitats for their survival. Among different fungal enzymes produced by these extremophiles, lipases are considered to be more valuable natural resource to replace chemical agents for industrial applications. Recombination and immobilization have greatly improved the biocatalytic properties than the native lipases of extremophilic fungal origin. It is more important to screen the molds or yeasts using appropriate methods to determine their lipase producing capability. The most crucial process is the purification of fungal lipases which aid to characterize and distinguish their potential for utilization in various fields of interest. Evaluation of lipolytic activity using different methods provides information about their hydrolyzing property. Extremophilic fungal lipases play indispensable roles in biotechnological applications such as detergent, food and feed, pharmaceuticals, bioremediation, oleochemistry, fine chemicals, textiles, leather, pulp, and paper industries. In this chapter, diverse methods for screening of lipases from fungi isolated from different extreme regions, purification scheme, different lipase assays, and the industrial applications of extremophilic fungal lipases are discussed in detail.

Keywords

Extremophiles · Fungal lipases · Screening · Purification · Lipase assay · Industrial applications

J. Angelin · M. Kavitha (✉)
School of Biosciences and Technology, Vellore Institute of Technology, Vellore, Tamil Nadu, India

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18.1 Introduction

Extremophiles are microorganisms which have the ability to survive and flourish in harsh environmental conditions while normal organisms are incapable to confront such situations. An organism which prefers harsh environments for their growth and survival are known to be extremophiles whereas the organisms that withstands these extreme conditions are known as extremotolerants. Interestingly, an organism which endures more than one extreme environmental condition is known as poly-extremophiles (Jin et al. 2019; Salwan and Sharma 2020). Based on their affinity toward diverse regions, they are categorized on the basis of pH (acidophile and alkaliphile), pressure (piezophile), radiation (radioresistant), redox potential (xerophile), salinity (halophile), and temperature (psychrotolerant, psychrophile, mesophile, thermophile, and hyper-thermophile) (Sahay et al. 2017). The extremophiles comprise bacteria, archaea, fungi, yeast, and few protozoans. These extremophiles are discovered in places such as hot springs, deep-sea hydrothermal vents, decaying plant matter, Antarctic, Arctic, glaciers, ashes, and deserts. Some of the marine fungal species are found in Mediterranean and Red Sea DHABs (Deep-Hypersaline Anoxic Basins) (Zhang et al. 2018). Figure 18.1 represents the prevalence of extreme environments around the world. In order to withstand in these extreme conditions, extremophiles undergo certain modifications at structural, biochemical, and molecular levels. The enzymes secreted by these extremophiles are called as extremozymes that include amylases, cellulases, esterases, keratinases, lipases, pectinases, peroxidases, proteases, and xylanases (Varela et al. 2012). Especially, halo-tolerant fungi harbors stable and valuable enzymes compared to enzymes isolated from terrestrial ecosystem. These extremozymes are known for their tremendous attributes like high catalytic efficiency and high stability under varied temperature and pH conditions, salinity, low water activity, low oxygen, and more shelf life than enzymes of other origin. Among these extremozymes, lipases are appraised as the third largest enzyme group, after proteases and carbohydrases based on their market value. They occupy up to 10% enzyme market among the other hydrolases (Basheer et al. 2011; Sharma et al. 2016a).

Lipases (EC 3.1.1.3) are triacylglycerol acyl hydrolases with an important physiological role toward the hydrolysis of triglycerides into mono- and diglycerides, fatty acids, and glycerol at oil–water interface; they break down carboxylic ester bonds through catalytic triad composed of serine, histidine, and aspartate/glutamate to perform various important reactions such as hydrolysis, esterification, and transesterification reactions (alcoholysis, acidolysis, aminolysis, and inter-esterification) (Gopinath et al. 2013). Lipases of microbial origin have attracted the scientific communities than plant- and animal-derived lipases with profitable benefits and functional ability at extreme conditions, stability in organic solvent, chemoselectivity, enantioselectivity, and they do not require any co-factor (Hasan et al. 2010; Singh and Mukhopadhyay 2012). Lipases acquire significant features to perform at the interface between an aqueous and a non-aqueous phase (Kour et al. 2020). Lipases have a characteristic folding pattern of α/β-hydrolase with mostly parallel β-sheets, flanked on both sides by α-helixes in the structure. The two distinct

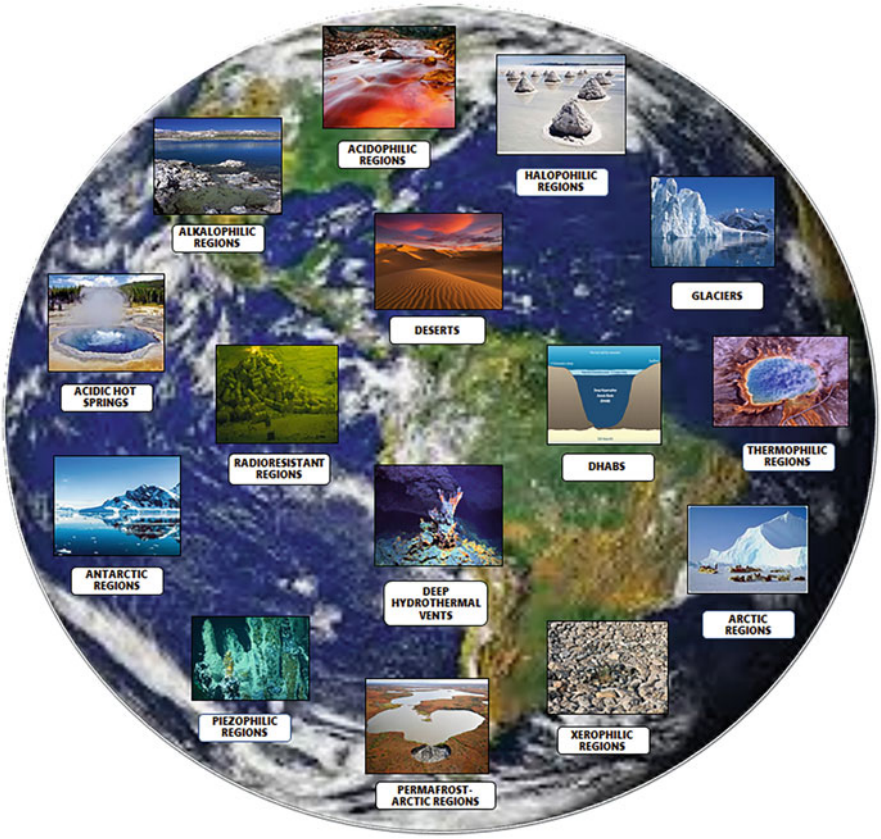

Fig. 18.1 Prevalence of extreme environments around the world

characteristics of lipases are (a) a lid covering the active site and (b) interfacial activation. The lid influences the activity, specificity, enantioselectivity, and stability of the enzyme. Lipases share a consensus sequence of G (glycine)-X_1 (histidine)-S (serine)-X_2 (glutamic or aspartic acid) –G (glycine), whereby X may be any amino acid residue. Lipase exhibits interfacial activation whereby it acts only on emulsified substrates. The active site of lipase is covered by a lid-like α-helical structure. The lid moves away upon binding to a lipid interface, causing the active site of lipase fully accessible, enhancing hydrophobic interaction between the enzyme and lipid surface. This lid experiences conformational rearrangement at the lipid water interface when the substrate interacts with the active site of the enzyme which is termed as "interfacial activation" (Balan et al. 2012; Sarmah et al. 2018). Lipase producing microorganisms are ever-present to be isolated from soil, marine environment, wastewater from fish industry, agro-industrial waste, waste volatile substances, air, palm-oil mill effluent, intestine of silkworm, and human skin (Ilesanmi et al. 2020). Figure 18.2 depicts the frequent sites for the isolation of extremophilic fungal lipases.

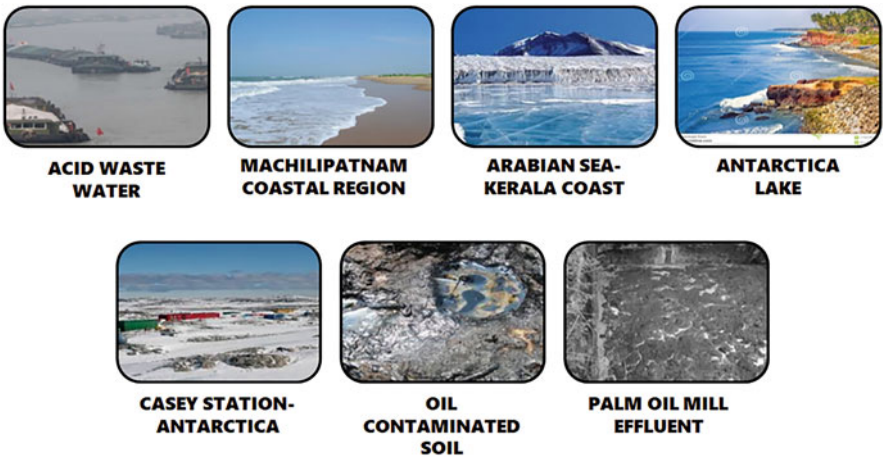

Fig. 18.2 Frequent sampling sites for extremophilic fungal lipases

Lipases are produced by plants, animals, bacteria, fungi, and archaea. Fungal extremozymes are secreted by fungi belonging to the groups ascomycota and basidiomycota that include *Mucor*, *Penicillium*, *Candida*, *Aspergillus*, etc. Antarctic fungi encompasses the genera *Beauveria*, *Candida*, *Cryptococcus*, *Geomyces*, *Leucopsoridium*, *Moesziomyces*, *Mrakia*, *Pseudozyma*, *Penicillium*, *Phoma*, *Pseudogymnoascus*, *Verticillium*, and *Trichosporon* and have been recognized as lipase producers (Martorell et al. 2019; Duarte et al. 2018). On the other hand, lipase producing yeasts include *Candida rugosa*, *Candida antarctica*, *Yarrowia lipolytica*, *Pichia* sp., *Rhodotorula* sp., *Trichosporon* sp., etc. In particular, among microbial sources, filamentous fungi are good lipase producers and the extraction, purification, and processing steps are relatively simple. Filamentous fungi are employed for industrial purposes because it produces extracellular lipases which is much easier to be recovered from the production medium than bacterial lipases and reduces production expenses (Contesini et al. 2017). Lipases play significant role in multi-faceted industrial applications such as pharmaceuticals, food, biodiesel, agrochemicals, dairy and textile, detergent, and surfactant productions (Dumorné et al. 2017; Raveendran et al. 2018). Cold-adapted lipases (CLPs) have developed specific structural features which provide thermal flexibility around the active site and high specific activity at low temperatures (Kavitha 2016; Joseph et al. 2008). On the other hand, lipase producing microorganisms isolated from thermal environments produce thermo-stable lipases with other unique properties including solvent tolerance and resistance to chemical inactivation (Salihu and Alam 2015; Casas-Godoy et al. 2012). Therefore, lipases from psychrophilic microorganisms are some of the most widely used classes of enzymes in biotechnology applications, organic chemistry, detergent industry, for bioremediation purposes, and in the food industry (Dalmaso et al. 2015). In 2012, world lipase demand was US$255 million, and this demand is expected to increase 6.2% annually to US$460 million in 2022, driven by the growth in industrial and specialty markets (Group 2009).

Some researchers have increased and improved the effectiveness of the fungal extremozymes employing recombination through heterologous expression using other potential microorganisms such as bacteria (*Escherichia coli*) and yeast (*Pichia pastoris*) (Xing et al. 2020; Ichikawa et al. 2020). In addition to that effort, lipases are immobilized to ameliorate the technical and economic advantages which lower the cost and enhance stability especially of psychrophilic lipases. Immobilization facilitates the separation of products, lipase properties such as thermal stability and activity, and provides more flexibility with enzyme/substrate contact by using various reactor configurations (Yadav et al. 2017). For example, enzymatic synthesis of glyceryl monoundecylenate (GMU) was achieved through immobilized *Candida antarctica* lipase B preparation by esterification and could be referred for industrial synthesis of fatty acid esters of glycerol (Yadav et al. 2017). Metagenomic technologies have been developed to bypass the requirement for the isolation or cultivation of microorganisms, and they could prove to be a powerful tool for discovering novel genes and enzymes directly from uncultured microorganisms (Madhavan et al. 2017). In the sequence-based approach, the colony hybridization technique is used for screening metagenomic clones using an oligonucleotide primer or probes for the target gene, and the desired gene may also be amplified by PCR using specific or degenerate primers and subsequently cloned into suitable expression vectors (Kim et al. 2010). This chapter encompasses traditional as well as novel methods for screening lipases from fungi of varied extreme regions, purification schemes, different lipase assays, and industrial applications.

18.2 Screening from Extreme Environments

The lipid solubilizing microorganisms are retrieved from different environmental sources to isolate, identify, and screen for hydrolyzing property. It is difficult for the researchers to recreate the same environment for fungi obtained from harsh environment. But various attempts are made to retain the characteristics of the potent organisms to be cultivated at laboratory level (Navvabi et al. 2018). Lipid materials could be dissolved by the lipases produced by bacteria, yeasts, filamentous fungi, and few protozoans (Gopinath et al. 2013). The samples obtained from various extreme sources are processed by serial dilution followed by spread plating on solid agar medium with a lipid source. Varied strategies for screening have been proposed for identifying fungi producing lipases which involve culturing of fungi on solid agar medium, a direct screening plate assay containing lipase substrates, or in liquid media containing substrates which act as inducers and later test the filtrates for the lipase activity (Kotogán et al. 2014). Table 18.1 and Fig. 18.3 show the various screening techniques of lipase producers from different extremophilic fungi.

Table 18.1 Screening strategies for fungal lipases from extreme sources

S. no	Type	Fungus	Source	Screening medium/substrate	Reference
1.	Alkalophile	*Aspergillus fumigatus*	Oil contaminated soil—Himachal Pradesh	Tributyrin agar	Mehta et al. (2018a)
2.	Mesophilic	*Rhizopus* JK-1	Oil contaminated soil samples	Rhodamine–olive oil agar plates	Kantak et al. (2011)
3.	Mild acidophile	*Penicillium aurantiogriseum*	Soils and oil refineries	Tributyrin agar	Pandey et al. (2018)
4.	Osmo-tolerant	*Debaryomyces hansenii*	Dry-salted olives	Rhodamine–olive oil agar	Papagora et al. (2013)
5.	Psychrophile	*Aspergillus awamori*	Arabian Sea water of Kerala coast	Rhodamine B–olive oil agar	Basheer et al. (2011)
6.	Organic solvent tolerant	*Trichosporon* sp.	Soil sample (oil contaminated sites)	PDA plates with olive oil and rhodamine B	Cao et al. (2020)
7.	Halotolerant	*Engyodontium* sp.	Marine water (Machilipatnam coastal region)	Phenol red olive oil agar	Lanka and B (2018)
8.	Thermo-stable–organic solvent stable	*Rhizopus* sp.	Palm kernel cake (west oil mill)—Malaysia	Phenol red olive oil agar and tween 80 agar	Riyadi et al. (2017b)
9.	Thermophilic fungal strain	*Neosartorya fischeri* P1	Acid wastewater—China	Olive oil–rhodamine B agar	Sun et al. (2016)
10.	Organic solvent tolerant	*Candida tropicalis* SD7	Oil contaminated soil—China	Tributyrin supplemented medium	Peng et al. (2016)
11.	Cold active yeast	*Crytococcus* sp. Y32	Nella lake—Antartica	Tributyrin agar base	Kumar Maharana and Mohan Singh (2018)

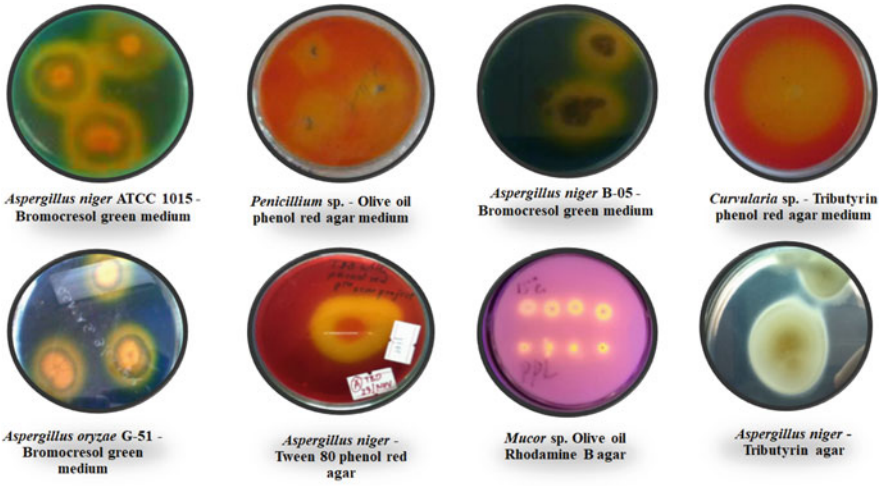

Fig. 18.3 Screening of lipase producing extremophilic fungi on solid agar media

18.2.1 Direct Screening Methods

The direct screening methods are qualitative or semi-quantitative in nature. Researchers have identified many screening methods to determine lipolytic activity. Most frequently used method is solid medium-based screening with clear zone or turbid appearance around the fungal colonies after certain period of incubation. The substrates used to identify lipase production by extremophilic fungal stains as well as other fungal sources include vegetable and fish oils, animal fats, synthetic triacylglycerides, and Tween 20, 40, 60, and 80. Chromogenic substrates that include rhodamine B, phenol red, Victoria blue, etc. are also used in addition with the major substrates to recognize lipase activity. The most commonly employed direct screening methods are listed below.

18.2.1.1 Olive Oil–Rhodamine B Agar Method

Olive oil contains high amount of oleic acid, which is a suitable substrate for lipase production for both bacteria and filamentous fungi. It plays a vital role in the detection and induction of lipase production and also serves as a carbon source for growth. Olive oil-agar plate method is the best method to screen true lipase producing fungi. Lipases can be screened using carbon sources, such as olive oil added with rhodamine B (Eugenia et al. 2016; Wagner et al. 2018; Singh et al. 2012). When exposed to UV light of wavelength 350–370 nm, the hydrolyzed compounds such as fatty acids, monoglycerides, and diglycerides combine with rhodamine B and form orange fluorescence around the individual colonies (Akbari et al. 2016; Adan Gökbulut and Arslanoğlu 2013; Selvam et al. 2011). *Trichoderma harzianum* was identified to show best lipolytic activity on olive oil–rhodamine B plates which is the preliminary method of screening fungal species (Canseco-Pérez et al. 2018; Li et al.

2018). *Aspergillus carbonarius* obtained from rotten cassava tuber was observed for lipase production on rhodamine B agar to form orange halo around the fungal colonies under UV light at 350 nm (Ire and Ike 2014). The true lipase secretion from *Penicillium* sp. DS-39 was confirmed through fluorescence zone formation on olive oil–rhodamine B agar under UV irradiation (Dsm et al. 2011). Cold active *Wickerhamomyces psychrolipolyticus*, a novel yeast species producing two kinds of lipases with activity at different temperatures of 25 °C and 4 °C, utilizes olive oil and rapeseed oil as sole carbon source and shows lipase activity on the rhodamine B medium (Shimizu et al. 2020). The three strains of *T. lanuginosus* (GSLMBKU-10, GSLMBKU-13, and GSLMBKU-14) exhibited high lipase activity on rhodamine B agar medium as evidenced by maximum orange fluorescent halo (Sreelatha et al. 2017).

18.2.1.2 Tributyrin Agar Method

Tributyrin is a fatty substance that can be digested by lipases, thus rendering it the preferred assay for testing lipase-producing microorganisms. Tributyrin is a surface active substance which is most appropriate and labor-saving substrate because it is well dispersed in water by shaking or stirring without the addition of any emulsifiers (Griebeler et al. 2011). Tributyrin is also supplemented to the agar medium through filter sterilization to screen the lipases production (Ülker and Karaoğlu 2012). Lipase activity using tributyrin added agar plates was identified by hydrolysis indicating that extracellular enzyme production and diameter of the clear zone around the colonies are measured (Cho et al. 2007; Gopinath et al. 2013; Maharana and Ray 2015). In some cases, the samples were transferred to the enrichment culture medium supplemented with olive oil. After certain period of incubation, the cultures were diluted and then added to the medium containing tributyrin indicating lipase hydrolysis around the colonies (Peng et al. 2016; Mehta et al. 2018a). *Alterneria* sp. and *Aspergillus flavus* isolated from oil contaminated sites were reported as best extracellular lipase producers on tributyrin agar plates than other fungi (Wadia and Jain 2020). Since tributyrin added medium shows positive results for both lipase and esterases, it is reported as a non-specific method (Kumar et al. 2012a). Cold active zygomycetal fungal strains such as *Dissophora*, *Gamsiella*, *Gilbertella*, *Mortierella*, *Mucor*, *Rhizomucor*, *Rhizopus*, and *Umbelopsis* were also screened with respect to their ability to hydrolyze tributyrin (Kotogán et al. 2014). The *Cladosporium langeronii* isolated from southern Caspian Sea showed higher lipolytic activity on tributyrin agar plates (Sadati et al. 2015). *Candida boidinii* KF156789 alkaline cold-adapted lipase obtained from spent olive derived from olive fruits showed lipolytic activity (20 mm) on the tributyrin agar plate (Insaf et al. 2014). 17 lipase positive fungi were determined using qualitative tributyrin phenol red agar method with various degrees depending on the intensity of the produced yellow color. Among them, *Curvularia* sp. DHE 5 producing alkaline lipase was considered as the most prominent strain (El-Ghonemy et al. 2017).

18.2.1.3 Tween 80 Agar Method

Screening using Tween 80 agar plates were also used as an indication of extracellular lipase activity, where it exhibited opaque zone of precipitation around the fungi. This is due to the deposition of insoluble calcium crystals salt formed by the liberation of the fatty acid as the fungi grow on the Tween 80 agar plates (Akmoussi-Toumi et al. 2018; Riyadi et al. 2017a). Halotolerant and halophilic fungi isolated from Great Sebkha of Oran (Algeria) such as *P. vinaceum*, *G. halophilus*, *Wallemia* sp. and *Ustilago cynodontis* showed better appearance of an opaque precipitation around the thallus on Tween 80 supplemented medium (Chamekh et al. 2019). Bromocresol green agar medium containing Tween 80 was used in the isolation of lipolytic *A. niger* ATCC 1015 on the basis of its ability to hydrolyze lipid in the medium which resulted in the color change of the surrounding medium from green to yellow, thus becoming acidic due to the release of fatty acids and glycerol (Bamitale Osho and Quadri Adio 2015). Phenol red medium containing olive oil and tween 80 as carbon source was suitable to identify lipolytic activity of *Aspergillus aculeatus* (Triyaswati and Ilmi 2020).

18.2.1.4 Olive Oil Phenol Red Method

Fungi are screened qualitatively on chromogenic medium such as phenol red olive oil agar plates for lipase production. Phenol red agar plate is a pH-based detection method to detect lipase in plates and gels. Phenol red appeared reddish at pH above neutrality, with a reddish end-point at pH 7.3–7.4. A slight decrease in pH (7.0–7.1) due to the hydrolysis of the lipid substrate, which liberated fatty acids, turned the agar plate color to yellow, denoting lipolysis. This change in color of phenol red was used as an indicator of the enzyme activity. Psychrotrophic fungi were screened using olive oil agar and palm oil agar with phenol red showed halo zones indicating lipase production (Sahay and Chouhan 2018). *Aspergillus* sp. isolates that were able to hydrolyze olive oil using the phenol red agar medium were further analyzed depending on their ability to hydrolyze tributyrin (Alabdalall et al. 2020).

18.2.2 Other Direct Screening Methods

Aspergillus tamarii JGIF06 isolated from rhizosphere soil was monitored for its lipolytic activity by observing deep blue color around the fungal colony on Spirit blue agar (Das et al. 2016). For screening cold-adapted lipases, Victoria blue agar plates were also used to evaluate the lipolytic activity of added substrates and color change in indicator dye after incubation (Salwoom et al. 2019).

18.2.3 Quantitative Screening Methods

Aspergillus aculeatus was quantitatively screened through submerged fermentation using the medium containing 1% glucose and 1% olive oil at pH 7.0 and temperature of 30 °C. After 96 and 120 h of incubation the enzyme activity was estimated to be

5.13 ± 0.30 U/mL and 5.22 ± 0.59 U/mL, respectively (Triyaswati and Ilmi 2020). In cold-tolerant zygomycetal fungal strains, addition of mineral salts and olive oil to the solid fermentation medium resulted in at least 1.5-fold increment in the enzyme activities of the crude extracts. Tween 80 proved to be a good inductor for lipase production since most of the investigated fungi displayed high enzyme activity when this substrate was applied (Kotogán et al. 2014). *Aspergillus japonicas* (MTCC no. 1975) strain was able to grow in SSF of castor bean waste and demonstrate substantial lipase production. The maximum lipase activity reached was 24.8 U/g and 18.9 U/g, about 48% and 47% greater when compared to activities reached before the optimization process (Jain and Naik 2018). *Fusarium solani* isolated from Arabian Sea has been reported to produce halophilic lipase using palm oil mill effluent as substrate reveals a significant 3.2-fold increase with enzyme activity 7.8 U/mL (Geoffry and Achur 2018). The production medium containing olive oil has promoted *Fusarium solani* strain SKWF7 to express its extracellular lipolytic production with maximum activity of 36.9 U/mL (Kanmani et al. 2013). The novel *Penicillium* sp. LBM 088 was the best producer of lipase isolated from Paranaense rainforest with highest activity (1224 U/mL) (Ortellado et al. 2020). Two fungal strains *Arthrographis curvata* and *Rhodosporidium babjevae* were isolated and found to produce higher levels of lipases showing optimal activity at 40 °C and pH 9.0 for *A. curvata* and at 40 °C and pH 8.0 for *R. babjevae* (Aamri et al. 2020).

18.2.4 Purification of Extremophilic Fungal Lipases

In general, enzyme purification plays a principle role in the determination of primary amino acid sequence and three-dimensional structure that leads to X-ray studies of pure lipases to enable the examination of structure–functional relationships. Purification also contributes to transparency in kinetic mechanisms of lipase action on hydrolysis, synthesis, and group exchange of esters (Mehta et al. 2017). Purification of the lipase depends on the microbial origin and intracellular or extracellular nature of enzyme from the filamentous fungi. Intracellular enzymes require cell disruption to release the enzyme in fluid phase. The common techniques of cell wall disintegration are ultrasound disintegration, homogenization in bead mill, application of chemicals of various types, and osmotic shock. The enzyme purification scheme includes extraction or concentration followed by purification by a combination of different chromatographic methods and extraction protocols. Table 18.2 and Fig. 18.4 present the purification strategies of lipases extracted from extremophilic fungi.

18.2.5 Extraction

Lipases are initially subjected to pre-purification steps before proceeding with main purification procedures. The cell-free supernatant obtained from the production medium after a certain period of incubation undergoes filtration or centrifugation

Table 18.2 Purification scheme for extremophilic fungal lipases

S. no	Fungus	Extraction method	Purification method	Specific activity	Purification fold	Molecular weight	Yield	References
1.	*P. chrysogenum* SNP5	Ammonium sulfate precipitation	DEAE-cellulose ion-exchange resin	40.7 U/mL	10.6 and 26.28	40 kDa	39.6%	Kumar et al. (2012b)
2.	*Aspergillus awamori*	Ammonium precipitation	Sephadex G100 ion exchange chromatography	1164.63 U/mg	–	90 kDa	33.7%	Basheer et al. (2011)
3.	Recombinant *Trichosporon coremiiforme* V3 lipase	Ultrafiltration	DEAE sepharose fast flow column	2549 U/mg	2.75	70 kDa	–	Wang et al. (2015)
4.	*Trichosporon coremiiforme* V3	Ammonium sulfate precipitation	DEAE sepharose anion exchange chromatography	8.0 U/mL	3.96	32.6 kDa	36.64%	Wang et al. (2015)
5.	*Mortierella alliacea* YN-15	Acetone precipitation and filtration	DEAE sepharose column Phenyl-sepharose fast flow column Superdex 200 column	179 U/mg	–	11 kDa	4.0%	Jermsuntiea et al. (2011)
6.	*Mucor hiemalis f. corticola*	Ammonium sulfate precipitation and dialysis	Gel filtration column chromatography and ion exchange chromatography	–	12.63	46 kDa	27.7%	Ülker and Karaoğlu (2012)
7.	*Neosartorya fischeri* P1	Ultrafiltration	HiPrep 26/10 desalting column	870 U/mg	–	~70 kDa	–	Sun et al. (2016)
8.	*Aspergillus tamari*	Ammonium sulfate precipitation and dialysis	DEAE Sepharose ion exchange and Sephadex G200 column chromatography	260,210.46 U/mg	7.9	50 kDa	43.1%	Das et al. (2016)

(continued)

Table 18.2 (continued)

S. no	Fungus	Extraction method	Purification method	Specific activity	Purification fold	Molecular weight	Yield	References
9.	*Penicillium* sp. DS-39	Ammonium sulfate precipitation	Sephacryl® 100-HR–I Q Sepharose® HP Sephacryl® 100-HR–II	308.73 U/mg	129.72	43 kDa	8.82%	Dsm et al. (2011)
10.	*Aspergillus japonicus* LAB01	Ammonium sulfate precipitation	Superose 12HR gel filtration chromatography Fast protein liquid chromatography (FPLC)	3.5×10^4 U/mg	3.9	25 kDa	44.2%	Souza et al. (2014)
11.	*Aureobasidium pullulans*	Ultrafiltration	DEAE sepharose fast flow column	17.7 U/mg	18.2	39.5 kDa	7.2%	Li et al. (2019)
12.	*Aspergillus fumigatus*	Ammonium sulfate precipitation	Octyl sepharose column	14.34 U/mg	6.96	35 kDa	11.03%	Mehta et al. (2018b)
13.	Recombinant lipase LK1 *(Pichia pastoris)*	–	Ni^{2+}–NTA column chromatography	1.82 U/mg	11.6	35.5 kDa	31.75%	Nurul Furqan and Akhmaloka (2020)

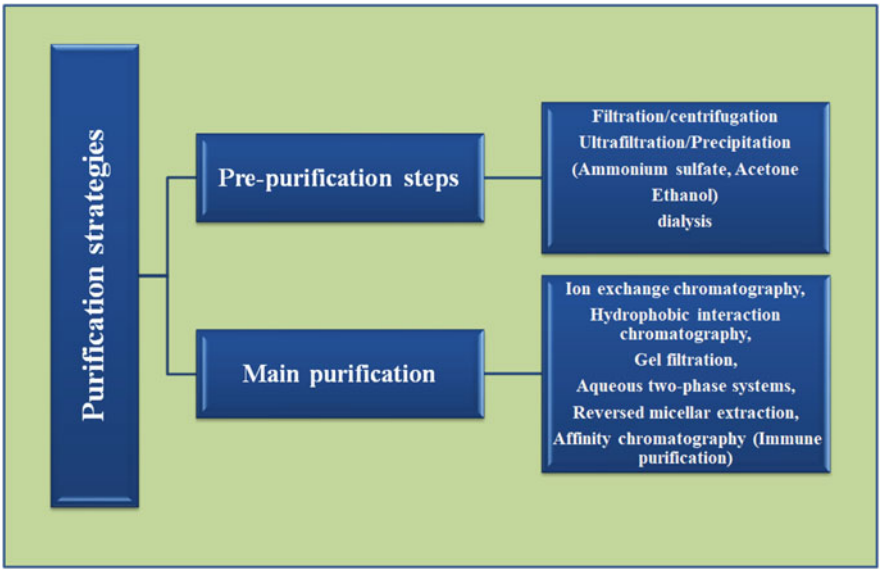

Fig. 18.4 Purification strategies for extremophilic fungal lipases

followed by concentration by means of ultrafiltration or precipitation using ammonium sulfate, acetone, or ethanol or extraction with organic solvents (Gaur et al. 2017). Precipitation enables modification in the solubility of proteins and favors the formation of protein aggregates. Ammonium sulfate is a neutral salt which is economically feasible with high solubility and lack of denaturing properties toward most proteins. It exhibits stabilizing effect on many proteins and protects the biological properties of proteins (Brígida et al. 2014). The addition of different polar organic solvents to a protein solution can also promote precipitation; as such solvents lower the di-electric constant of the aqueous solution. The hydro-organic solution enables an increase in the electrostatic attraction between bodies with opposite charges, such as proteins, leading to their precipitation (Melani et al. 2019). The protein precipitation is precipitation by ammonium sulfate which is used about 60% of the time, while 35% use ethanol, acetone, or hydrochloric acid. In general, precipitation is followed by chromatography which yields less protein when compared to precipitation (Yagmurov et al. 2017). The increase in lipase activity depends on the concentration of the ammonium sulfate used during partial purification (Palekar et al. 2000). Partial purification of extracellular alkaline lipases from novel fungus *Curvularia* sp. DHE 5 upon ammonium sulfate precipitation and dialysis resulted in 3.1-fold purification with 50.6% recovery and 8.1–24.7 U/mg specific activity (El-Ghonemy et al. 2017). Ultrafiltration is also a concentration method recruited as an alternative or combined with other extraction methods where separation is determined by particle size (Melani et al. 2019). After precipitation, the enzymes are subjected to dialysis to remove salts and metal ions. Sometimes, phenylmethylsulfonyl fluoride was added to the crude enzyme extract in order to

prevent enzyme degradation during the purification process. The concentrated lipase was then filtered in order to remove larger molecules that could potentially clog the gel filtration column (Tišma et al. 2019).

18.2.6 Purification

Chromatographic methods are separation processes where chromatographic matrices (columns) are usually packed with hydrophilic materials that demonstrate no interactions with the biomolecules (Melani et al. 2019). The selection of chromatographic techniques are determined in terms of source of microorganisms, size of the proteins, and protein interaction with chromatographic resins, such as liquid ionic charge, molecular weight, hydrophobicity, and specificity to purify lipases (Bharathi and Rajalakshmi 2019; De Carvalho et al. 2019). The common chromatographic techniques include hydrophobic interaction chromatography, gel filtration, ion exchange chromatography, aqueous two-phase systems, reversed micellar extraction, immune purification, and affinity chromatography (Jares et al. 2010; Mehta et al. 2017). In some cases, purification may reduce the final yield of lipase but enhances the lipolytic activity. Industrial applications such as in medical and pharmaceutical industries demand high degree purity of enzymes. Ion-exchange chromatography is the most frequently employed method, and the main anion and cation exchangers are the diethyl-aminoethyl cationic group and carboxymethyl anionic group, respectively. As lipases are well known for its hydrophobic nature that possesses hydrophobic surfaces around the active site, affinity chromatographic techniques are preferred. The most predominantly used chromatographic technique involves columns packed with QAE sephadex, CM cellulose, DEAE cellulose, phenyl-sepharose, etc. (Joseph et al. 2008). Psychrotrophic fungal lipases were purified by ammonium sulfate precipitation, dialysis, and DEAE cellulose column chromatography and the purity tested on SDS-PAGE gel electrophoresis to determine molecular mass of the lipase (Sahay and Chouhan 2018). The partial purification of *Geotrichum candidum* lipase has been reported to achieve higher recovery factors upon ammonium sulfate precipitation and reduces concentration period of lipase with ethanol precipitation (Resende et al. 2015). 39 kDa monomeric lipase from the newly isolated fungus *Talaromyces thermophilus* was purified from the culture supernatant using ammonium sulfate precipitation, gel filtration, and anion exchange chromatography (Romdhane et al. 2010). The routine chromatographic techniques used in fungal lipase purification are dealt below.

18.2.6.1 Ion Exchange Chromatography

Ion exchange chromatography exhibits the principle of attraction between oppositely charged stationary phase, known as an ion exchanger, and analyte. It is frequently chosen for the separation and purification of proteins, peptides, nucleic acids, polynucleotides, and other charged molecules, mainly because of its high resolving power and high capacity. There are two types of ion exchanger, namely cation and anion exchangers. Cation exchangers possess negatively charged groups and these

will attract positively charged cations. These exchangers are also called acidic ion exchangers because their negative charges result from the ionization of acidic groups. Anion exchangers have positively charged groups that will attract negatively charged anions. The term basic ion exchanger is also used to describe these exchangers, as positive charges generally result from the association of protons with basic groups (Lim et al. 2020; Maldonado et al. 2016; Tan et al. 2015). Cold active lipase obtained from mesophilic yeast *Pichia lynferdii* NRRL Y-7723 was purified by DEAE anion exchange chromatography column and further purified with Sephacryl S-200 size exclusion chromatography column representing 33.1 purification fold and 0.31% recovery (Bae et al. 2014). Lipase from Antarctic krill, with a molecular weight of 71.27 kDa, was purified with ammonium sulfate precipitation and a series of chromatographic separations over ion exchange (DEAE) and gel filtration columns (Sephacryl S-100), resulting in 5.2% recovery with a 22.4-fold purification ratio (Chen et al. 2020).

18.2.6.2 Gel Permeation Chromatography

This chromatographic technique is used for the separation of molecules on the basis of their molecular size and shape and exploits the molecular sieve properties of a variety of porous materials. The terms exclusion or permeation chromatography or gel filtration describe all molecular separation processes using molecular sieves. The general principle of exclusion chromatography is quite simple. A column of microparticulate cross-linked copolymers generally of either styrene or divinylbenzene and with a narrow range of pore sizes is in equilibrium with a suitable mobile phase for the analytes to be separated. Large analytes that are completely excluded from the pores will pass through the interstitial spaces between the particles and will appear first in the eluate. Smaller analytes will be distributed between the mobile phase inside and outside the particles and will therefore pass through the column at a slower rate, hence appearing last in the eluate (Lim et al. 2020; Tan et al. 2015). The fungus *Cunninghamella verticillata* lipases were reported as a monomeric proteins with molecular masses of 49 and 42 kDa as determined by SDS–PAGE and gel filtration chromatography (Gopinath et al. 2002). *Yarrowia lipolytica* LgX64.81 lipase was purified by gel filtration on Sephacryl S-100 to receive overall yield of 72% and a 3.5-fold increase in the specific lipase activity (Turki et al. 2010). The lipase from *Mucor hiemalis f. corticola* was purified to 12.63-fold with a final yield of 27.7% through following purification steps; ammonium sulfate precipitation, dialysis, ion exchange chromatography, and gel filtration column chromatography respectively (Ülker and Karaoğlu 2012). The lipase produced by *Aspergillus niger* was purified through ammonium sulfate precipitation followed by Sephadex G-100 gel filtration. Molecular mass of the purified lipase was 57 kDa as evident on SDS-PAGE analysis (El-Ghonemy et al. 2021).

18.2.6.3 Affinity Chromatography

Affinity chromatography has been exclusively developed for the purification and separation of enzymes based on extremely specific biological interactions and

reduces the number of steps necessary for lipase purification with greater yields of purified enzymes (Mehta et al. 2018b). Affinity column chromatography purifies proteins according to their specific affinity toward a ligand. Such chromatography is also known as immobilization, which is normally called immobilized metal affinity chromatography. When the analyte molecules in the crude enzymes interact with the solid resin, which has a covalent linkage with a polydentate metal-chelating group binding to a metal ion, e.g., nickel (Ni^{2+}), surface-exposed amino acid residues of the enzyme of interest will exchange with the water molecule in the metal coordination site, thus the enzyme is immobilized (Lim et al. 2020). Immunoaffinity chromatography or immunopurification is a type of affinity chromatography when a protein of interest is purified by applying an antibody–antigen principle. Immunopurification as of today is considered one of the strongest and most selective methods of protein purification, with the purification factor ranging from 1000- to 10,000-fold in one single step procedure (Yagmurov et al. 2017). The choice depends on the availability of monoclonal antibody against the target protein and the composition of the crude preparation, namely, the type and the concentration of the contaminants (Venkatanagaraju and Divakar 2017). Recombinant lipase produced by *Trichosporon asahii* MSR54 was purified by affinity chromatography and the molecular mass was recorded as monomeric 27 kDa with 1.7 purification fold (Kumari and Gupta 2015). A recombinant lipase obtained from *Aspergillus niger* GZUF36 purified on Ni^{2+} (Nickel resin)-NTA affinity chromatography column and estimated relative molecular mass of 35 kDa was recorded (Xing et al. 2020). A recombinant acidic lipase purified by Ni^{2+}-NTA affinity chromatography column observed to have molecular weight of 60 kDa as determined by SDS-PAGE analysis (Zhang et al. 2019).

18.2.6.4 Hydrophobic Interaction Chromatography

Since lipases are known to be hydrophobic in nature, with large hydrophobic surfaces around the active site, the purification of lipases could be significantly achieved by opting for hydrophobic interaction chromatography. The hydrophobic amino acid residues of lipases would be exposed by addition of salt ions, and then allowed to interact with hydrophobic groups such as butyl, octyl, and phenyl attached to a matrix facilitating protein–matrix interaction. Lipases produced by *Aspergillus fumigatus* was purified by ammonium sulfate precipitation and octyl sepharose column chromatography, which resulted in sevenfold purification, and the molecular weight found to be 35 kDa indicating the enzyme was homo-dimer (Mehta et al. 2018b). The specific activity of *Aspergillus fumigatus* lipase was observed to be 14.34 U/mg with a fold purification of 6.96 through purification using octyl sepharose column (Mehta et al. 2020). The dialyzed extracellular filtrate from *Penicillium* sp. section Gracilenta CBMAI 1583 was subjected to octyl sepharose chromatography using ammonium acetate buffer and 52.9 kDa protein presented esterification activity on octyl oleate (Turati et al. 2019). The lipases from *C. rugosa* and *G. candidum* were purified using a single step purification via interfacial adsorption on strongly hydrophobic support with the yield of 9.7% and 10.9% after complete purification (De Morais et al. 2016). Recombinant lipases

produced by *Rhizopus chinensis* were sequentially purified via Q Sepharose™ Fast Flow anion exchange column chromatography and Phenyl-Sepharose 4 fast flow hydrophobic chromatography column chromatography (Jiang et al. 2020). Lipase recovered from *Aspergillus fumigatus* was purified by octyl sepharose column chromatography to 6.96 purification fold and purified to homogeneity confirmed by SDS and Native PAGE (Kaur et al. 2019).

18.2.6.5 Aqueous Two-Phase Systems

Aqueous two-phase system has been viewed as a powerful purification technique used in the separation and purification of biomolecules. Upon mixing of aqueous solutions containing two different incompatible polymers, they will separate into two distinct phases because of steric exclusion. If it is a mixture of a polymer and a high ionic strength salt, the phase separation phenomenon also occurs since the salt retains huge amount of water. This phenomenon is used to separate enzymes, without compromising its activity. The most used pairs are polyethylene glycol and dextran and PEG–potassium phosphate (Carvalho et al. 2017). Aqueous two-phase system composed of a hydrophilic organic solvent and an inorganic salt solution has many advantages, which include rapid phase-separation, high extraction efficiency, low viscosity, high polarity differences between the phases, a gentle aqueous environment, and may be formed by inexpensive chemicals easy to recycle. These systems, formed by adding a salt solution to an aqueous solution of an organic compound, have been recently proposed and used for the partitioning of different biomolecules, such as proteins, amino acids, and other natural products (Souza et al. 2015). The mutant *Trichosporon laibacchii* lipase was partially purified using aqueous two-phase systems with activity recovery of 80.4%, and purification factor of 5.84 (Zhang and Liu 2010). The research conducted in 2017 has proved that aqueous two-phase system is evaluated as promising method for purification of extracellular lipase from *Yarrowia lipolytica* than traditional methods such as precipitation and ultrafiltration (Carvalho et al. 2017). Purification factor of *Aspergillus carbonarius* lipase has been increased using PEG/potassium phosphate aqueous two-phase system (Panajotova et al. 2017).

18.2.6.6 Reverse Micellar Extraction

A reverse micelle is a system that combines an aqueous dispersed phase and a non-aqueous continuous phase with the assistance of amphiphilic molecules (Chen et al. 2020). Reverse micellar extraction of the protein is an attractive liquid–liquid extraction method for the downstream processing of the enzymes. Reverse micelles are used as a reaction system for enzymatic catalysis, liquid–liquid extraction of proteins, and protein refolding in the field of biotechnology (Basheer and Thenmozhi 2010). Selective extraction of the target biomolecule from mixture of enzymes/proteins in these reverse micelles can be achieved by varying parameters both in organic phase and in aqueous phase (Gaikaiwari et al. 2012). More importantly, the formation of reverse micelles could activate lipase, allow lipase to molecularly disperse so as to interact with substances efficiently, and provide a large interface for the reaction. The water/sodium 1,4-bis-2-ethylhexylsulfosuccinate /isooctane

reverse micelle system was set up as a reaction medium for *Candida rugosa* lipase AY30 to synthesize β-sitosterol laurate (Chen et al. 2020).

18.3 Assay Methods

Lipase activity is examined by the release of either free fatty acids or glycerol from triacylglycerol. Since lipases act at the oil–water interface, change in properties of interface is an important criterion for measuring lipolysis (Nagarajan 2012). The assay methods are involved for measuring the hydrolytic activity as well as the detection of lipase. The methods are classified as: (1) titrimetry, (2) spectroscopy (photometry, fluorimetry, and infrared), (3) chromatography, (4) interfacial tensiometry, (5) radio activity, (6) conductimetry, (7) turbidimetry, (8) immunochemistry, and (9) microscopy (Singh and Mukhopadhyay 2012). The most commonly used lipase assay protocol is the titrimetric assay using olive oil as a substrate because of its accuracy, simplicity, and reproducibility. The titrimetric assays are performed in emulsion containing synthetic triacylglycerols (triacetin, tributyrate, tricaprylate, tripalmitate, or tristearin). Other assays reported for lipase activity include fluorescence, gas chromatography, HPLC-based assay, monomolecular film technique, oil drop method, atomic force microscopy, and IR spectroscopy (Nagarajan 2012). Both bacterial and fungal lipases are able to hydrolyze para-nitrophenyl (p-NP) esters having C2–C16 (p-NP acetate to p-NP palmitate) in their fatty acid chain. Kinetics of lipases for substrate hydrolysis depends on different esters. It is determined by Michaelis constant (Km), that is substrate concentration at which the rate of reaction is half of the maximum rate (Vmax). Vmax is the maximum rate when an enzyme is fully saturated with substrate concentration (Shamim et al. 2018). Table 18.3 and Fig. 18.5 provide the detailed information on different types of lipase assays. The common lipase assays employed are discussed below in detail.

18.3.1 Titrimetry

In this method, the free fatty acids released as a result of lipase hydrolysis with suitable substrate were titrated against alkali. Lipase activity of filamentous fungi can be evaluated through titrimetric method using olive oil as substrate and phenolphthalein as a pH indicator (Griebeler et al. 2011; Resende et al. 2015; Gutarra et al. 2009). Triton X-l00 and gum arabic were used in the assay mixture, and both act as emulsifiers (Ayinla et al. 2017; Kantak et al. 2011). *Penicillium canesense* and *Pseudogymnoascus roseus* produced BPF4 and BPF6 lipases which were found to be alkaline metallolipase, showing maximum activity at pH 11 and 9 respectively and at the temperature 40 °C were assessed by titrimetric method (Sahay and Chouhan 2018). *Talaromyces thermophilus* lipase showed specific activity of about 7300 ± 122 and 9868 ± 139 U/mg using Tributyrin and olive oil emulsion as substrates, respectively, at pH 9.5 and 50 °C (Romdhane et al. 2010). *Engyodontium* sp. was found to produce maximum lipase of 7.2 U/mL using olive

Table 18.3 Assays for quantification of extremophilic fungal lipases

S. no	Fungus	Substrate	Method	pH	Temp	Km	Vmax	Enzyme activity	Reference
1	*Aspergillus tamarii*	Olive oil	Titrimetric method	4	37 °C	330.4 mg	53,690 U/mL/min	23,666.66 U/mL/min	Das et al. (2016)
2	*Aspergillus fumigatus*	p-NP benzoate	Spectrometric method	9.0	40 °C	6.59 mM	7.29 mmol/min/mL	–	Mehta et al. (2018a)
3	*Penicillium chrysogenum*	p-NP palmitate	Spectrometric method	–	–	0.4 mM	47.61 U/mL	–	Kumar et al. (2012b)
4	*Debaromyces hansenii*	Olive oil	Titrimetric method	–	–	–	–	7.44 U/mL	Papagora et al. (2013)
5	*A. awamori*	p-NP caprylate	Spectrometric method	7	40 °C	–	–	495.0 U/mL	Basheer et al. (2011)
6	*Candida rugosa*	p-NP butyrate	Spectrometric method	5.0 & 6	30 °C and 40 °C	129.21 μM	0.034 μmol/min	12.3 ± 0.2 U/mL	De Morais et al. (2016)
	Geotrichum candidum	p-NP butyrate	Spectrometric method			465.44 μM	0.384 μmol/min	11.9 ± 0.2 U/mL	
7	*Sporisorium reilianum* SRZ2	p-NP hexanoate	Spectrometric method	8.0	65 °C	0.14 mmol/L	–	–	Shen et al. (2020)
8	*Aspergillus japonicus* LAB01	p-NP palmitate	Spectrometric method	–	–	0.13 mM	12.58 μmol/min)	–	Souza et al. (2014)
9	*Candida antarctica* ZJB09193	p-NP acetate	Spectrometric method	8.0	52 °C	0.34 mM	7.36 μmol / min/mg	–	Liu et al. (2012)

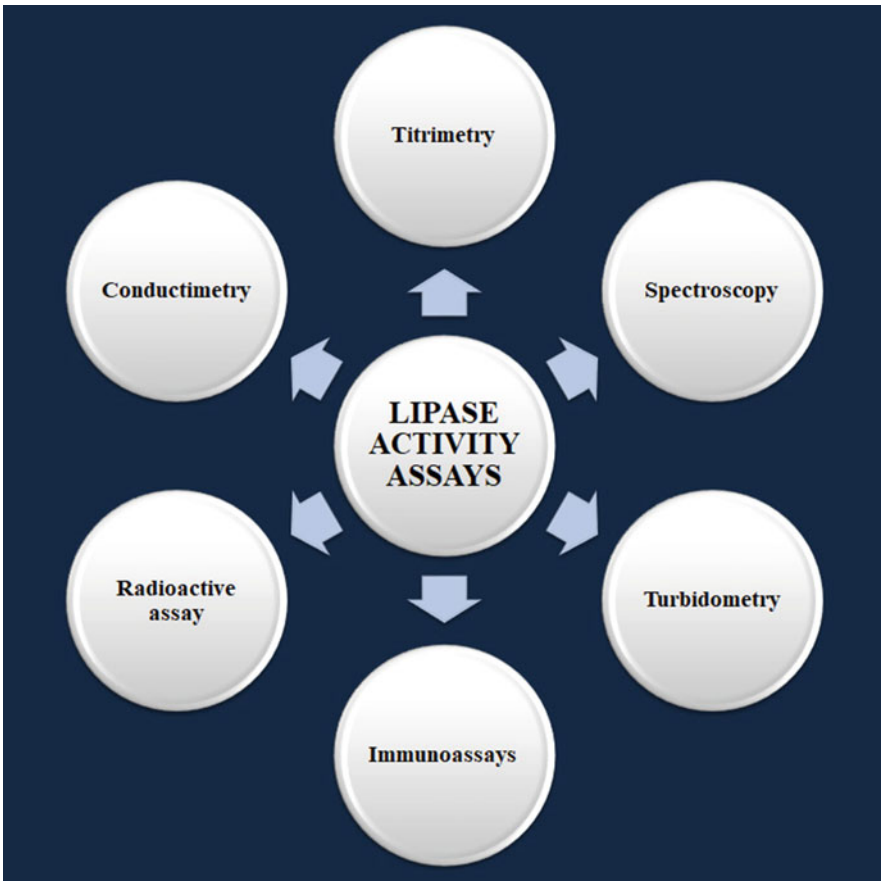

Fig. 18.5 Distinct assays for lipase activity

oil as substrate compared to other fungal members by titration method (Lanka and B 2018). An Antarctic basidiomycete yeast *Guehomyces pullalans* showed maximum lipase activity using olive oil as inducer at pH 8.0 and 40 °C (Demera et al. 2019). Purified lipase from *Aspergillus fumigatus* showed utmost enzyme activity at optimum temperature of 40 and pH 9.0 with kinetic parameters of V_{max} and K_m as 10.42 μmol/min/mg and 9.89 mM, respectively (Mehta et al. 2018b). A recombinant lipase from *Aspergillus niger* GZUF36 utilized olive oil as substrate to exhibit highest activity of 7.02 ± 0.05 U/mL at 35 °C and pH 4.0 using alkali titration method (Xing et al. 2020). The extracellular lipase produced by *Trichoderma harzianum* displayed maximal lipase activity of 1.58 IU/mL/min ± 0.11 with olive oil as substrate (Rihani and Soumati 2019). The major advantage of the titrimetric assay is the resistance to turbidity. The assay also has a disadvantage as the strong buffers make it hard to hold a stable pH, thus it is tough for the assay to be performed (Pohanka 2019). *Aspergillus niger* acidic lipase when fused with small

ubiquitin-related modifier possessed a higher catalytic efficiency toward p-NP caprylate and long-chain triglycerides (olive oil, triolein, and tripalmitin) than *Aspergillus niger* acidic lipase (Zhang et al. 2019). Despite its disadvantages like long analysis time (two determinations per hour), low sensitivity (1 μmol/mL), tedious measurements, and errors due to incomplete titration, the titrimetric method for lipases activity determination remains in use as a reference method (Stoytcheva et al. 2012).

18.3.2 Spectrophotometry

The spectrophotometric methods for lipase activity determination make use of synthetic lipase substrates transformed upon enzyme catalyzed hydrolysis into products able to be detected spectrophotometrically. The predominant substrates are p-nitrophenyl and naphthyl esters of the long chain fatty acids, and thioesters. The lipolysis of the p-NP esters (laurates, palmitates, and oleates) gives rise to the yellow colored p-nitrophenol, measured at 405–410 nm. In the assay, p-NP acetate, p-NP butyrate, p-NP valerate, p-NP caproate, p-NP decanoate, p-NP dodecanoate, p-NP myristate, and p-NP palmitate are used as substrates for lipase to determine the substrate specificities (Pohanka 2019). The deficiency of this method is related to the pH dependence of the p-NP absorption coefficient and the total absence of absorption at acidic pH values. In addition, p-NP esters could undergo a non-enzymatic hydrolysis. The cleavage of the naphthyl esters (naphthylcaprylate, naphthylacetate, and naphthylpropionate) yields naphthol, which is complexed with diazonium salts to produce a red colored complex measured at 560 nm. p-NP palmitate hydrolysis was maximal at 40 °C and pH 7.0 for the *Rhizomucor miehei* lipase, and at 30 °C and pH 5.2 for the *Rhizopus oryzae* enzyme. The enzymes showed almost equal affinity to p-NP palmitate, but the Vmax of the *R. oryzae* lipase was about 1.13 times higher than that determined for *R. miehei* using the same substrate (Km 2017). Lipase from *R. miehei*, *R. stolonifer*, and *M. echinosphaera* produced by solid-state fermentation were able to catalyze trans-esterification reactions in organic media (Kotogán et al. 2014; Maharana and Ray 2014, 2015). The purified *Aspergillus niger*-lipase displayed the maximal activity at 20 °C and pH 6.5 with the specific activity of 1293 U/mg determined by spectrophotometric method (Cong et al. 2019). Purified cold active lipase recovered from *Pichia lynferdii* Y-7723 exhibited Km and Vmax values of 1.68 mM and 8.32 μmol/min/mg respectively by spectrophotometric method using p-NP butyrate as substrate (Bae et al. 2014).

18.3.3 Fluorimetry

Fluorimetry is a sensitive analytical technique allowing continuous monitoring of enzyme activity. The fluorimetric methods for lipase activity are classified as methods using chromogenic substrates and methods based on the quantification of the fatty acids released after their conversion into chromogenic products. A variety

of fatty acid esters derived from parinaric acid, coumarin (umbelliferone), pyrenic compounds, resorufin, fluorescein, etc. are used as chromogenic substrates (Stoytcheva et al. 2012). Fluorescence spectroscopy analysis is used to monitor the biomolecular interactions between surfactants and proteins. Lipase secreted by *Y. lipolytica* (YlLip2) contains three tryptophan residues Trp233, Trp271, and Trp285 at active site. The fluorescence spectrum of YlLip2 in HEPES buffer shows one peak at 337 nm. The addition of lipo-peptides significantly decreased the fluorescence intensity of YlLip2, but without significant blue shift (Janek et al. 2020).

18.3.4 Turbidimetry

Turbidimetry is a method for determining the concentration of a substance in a solution by measuring the change of intensity of light in the direction of propagation of the incident beam, with reference to a standard solution. Lipase activity quantification is performed by monitoring the decrease with time in the absorbance of a triacylglycerol emulsion, due to its de-emulsification with the release of free fatty acids (Stoytcheva et al. 2012). The two main strategies employed are (1) the use of labeled lipase substrates and quantification of the liberated products, and (2) the use of unlabeled substrates and determination of free fatty acids labeled once released. The radioactive substrates commonly used are oleoyl glycerols labeled with ^{14}C or ^{3}H, as well as ^{131}I triglyceride analogues. Radioactive assays are specific and sensitive analytical methods. However, they do not allow continuous monitoring and are time-consuming, because of the implicated extraction steps to remove the fatty acids in addition to the use of hazardous radioactive substances (Stoytcheva et al. 2012).

18.3.5 Immunoassays

Immunoassays are well known for their high specificity and sensitivity for lipase activity. These methods are of primary importance for clinical diagnostics where they are applied for lipases quantification in serum and plasma, tissues and cell culture lysates, and duodenum. A number of ELISA-based clinical test kits have been developed and are useful in the range up to 500 ng/mL lipase. These techniques are not suitable for the evaluation of the activity of lipases originated from other sources, because they require the selection of a wide range of specific antibodies. In addition, the enzyme could form aggregates, which limits the accuracy of the immunological assays (Stoytcheva et al. 2012).

18.3.6 Conductimetry

Conductimetric evaluation of lipase activity is based on the measurement of the variation in the solution conductance due to electrical charge concentration change as a result of the release of free fatty acids. Triacetin is a suitable substrate due to its water solubility. The limiting equivalent conductivity of the liberated acetate anions is higher in comparison with that of the long-chain fatty acids, increasing the sensitivity of the determinations. The drawback of the technique is that triacetin is not a specific lipase substrate and conductimetric measurements suffer from high temperature dependence (Stoytcheva et al. 2012).

18.4 Industrial Applications

Lipases are produced by plants, animals, and microorganisms of extreme environmental conditions. Specifically, hydrolytic lipases of filamentous fungi have grabbed more attention due to high yield in production, many varieties of catalytic activities that satisfy industrial procedures, and ease of genetic manipulation (Hasan et al. 2006). Lipases are exploited in waste water treatment (degreasing of lipid clogged drains), pharmaceutical (resolution of racemic mixtures), dairy (hydrolysis of milk, fat), leather (removal of lipids from hides and skin), detergent (removal of oil/fat stains), and medical (diagnostic tool in blood triglyceride assay) industries (Verma and Sharma 2014). Lipases are able to catalyze acidolysis (where an ester and carboxylic acid are involved), alcoholysis (where an ester and an alcohol are involved), aminolysis (where an ester is allowed to react with an amine), and inter-esterification (where two acyl groups are exchanged between two esters) (Sharma et al. 2016a). The industrial process demands biocatalysts that can resist a range of harsh conditions, including temperature, pH, salinity, and pressure, while exhibiting high conversion rates and reproducibility (Jin et al. 2019). The original physicochemical properties of fungal lipases are altered through various attempts so that the efficiency of the enzymes would satisfy the industrial strategies. It is more important to be aware about the characteristics of a particular enzyme before indulging in modification techniques. These issues include the type and size of the protein, the structure and size of the modifying reagent, the chemical reactions involved in the modification procedures, and the conditions of such modifications (Dalmaso et al. 2015). Lipases which are able to combat during industrial processes are recruited for the development of several products. A major requirement for commercial lipases is thermal stability which would allow enzymatic reaction to be performed at higher temperatures and would be helpful to increase conversion rates and substrate solubility. Industrial and biotechnological processes also require thermo-stable lipases for use in processes such as grease esterification, hydrolysis, trans-esterification, inter-esterification, and organic biosynthesis (Jin et al. 2019). *Aspergillus fumigatus* is a potential lipase producing candidate for industrial applications such as bioremediation, detergent, leather, and pharmaceutical industries (Mehta et al. 2018a). *Rhizopus chinensis* produced novel mutant lipases

Table 18.4 Industrial applications of extremophilic fungal lipases

S. no	Fungus/Lipase	Mechanism	Properties	Applications	References
1.	CALB-type (*Sporisorium reilianum* SRZ2) SRL	Short-chain flavor ester synthesis	High thermo-stability, pH stability, and good organic solvent tolerance	Used as flavor and fragrance compounds in food, beverage, cosmetic, personal care, chemical, and pharmaceutical industries	Shen et al. (2020)
2.	*Mucor hiemalis f. corticola*	–	Stable in organic solvents	Useful in organic synthesis and biodiesel production	Ülker and Karaoĝlu (2012)
3.	*Candida* sp. 99–125	Wax esters from oleic acid and cetyl alcohol	Dynamic viscosity, flash point, solidifying point, pour point, and acid value of wax esters are similar to those of jojoba oil	Widely used in lubricant, pharmaceutical, cosmetic, and plasticizer industries	Deng et al. (2011)
4.	*Trichosporon laibachii*	Synthesis of ((R)-5-acetoxy-1,3-oxathiolan-2-yl)ethyl benzoate using reversible hemi-thioacetal transformation	–	Useful in pharmaceutical industries	Zhang et al. (2020)
5.	CALB	Butyl oleate synthesis by immobilization	Thermal stability and operational stability	Biodiesel production	Silva et al. (2012)
6.	*Aureobasidium pullulans*	Hydrolysis of oils	Resistance to some organic solvents, surfactants, and ions	Detergent production, biodiesel synthetization, and food manufacturing	Li et al. (2019)
7.	*Thermomyces lanuginosus*	Fish oil ethanolysis through adsorption	Improved stability	Useful in food industry, biodiesel, and fine chemical production	Jorge and Ninow (2016)
8.	*Thermomyces lanuginosus*	Synthesis of isoamyl oleate (biolubricant) by esterification reaction	High catalytic activity and reusability	Hydraulic and metal working fluids, hydraulic of harvesters, drilling oils, slab and gear oils, and lubricants for power saw chains	Lage et al. (2015)

9.	*Rhodotorula glutinis* HL25	Utilization of waste frying oils	–	Biodiesel production and organic synthesis reactions	Taskin et al. (2016)
10.	*Aspergillus fumigatus*	Esterification process for the synthesis of ethyl acetate and ethyl lactate	–	Useful in food industry as artificial flavor enhancers	Mehta et al. (2020)
11.	*Aspergillus niger*	Synthesis of flavor ester (ethyl lactate, butyl butyrate, and ethyl caprylate) in soybean-solvent system	Cold, acid, and alkaline tolerance	Useful in food processing, such as cheese ripening, alcoholic beverage production, and other fermented foods production	Cong et al. (2019)
12.	*Penicillium* sp. Section Gracilenta CBMAI 1583	Esterification reaction	Thermo-tolerant and acid pH tolerance	Treatment of dairy and industry effluents, resolution of esters in the pharmaceutical industry or in the food industry	Turati et al. (2019)
13.	*Rhizomucor endophyticus*	Trans-esterification	Regioselectivity	Biofuel industry	Yan et al. (2016)

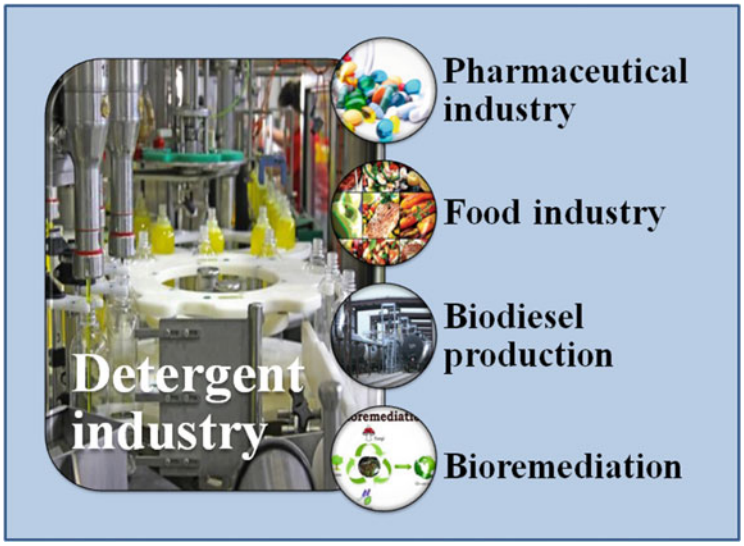

Fig. 18.6 Principal industrial applications of extremophilic fungal lipases

(Lipr27RCL-K64N and Lipr27RCL-K68T) with improved thermo-stability through a combination of B factor analysis and site-directed mutagenesis, and these resultant enzymes represent attractive candidates for use in industrial applications (Jiang et al. 2020). Table 18.4 and Fig. 18.6 display the industrial application of fungal lipases. The applications of fungal lipases in various industries are explored here in detail.

18.4.1 Detergent Industry

Laundry detergents are widely used because they are required for washing purposes but chemical agents deposited in the environment leads to contamination of ground water. These pollutants are responsible for health-related issues which should be replaced by other harmless stain removal agents. The enzyme-based detergents have disclosed superior cleaning properties over synthetic agents that are active at low washing temperatures and environmentally friendly. Additionally, enzyme carrying detergents upgrade the fabric quality and keep the color bright (Hasan et al. 2010). Lipases extend profit-oriented services in laundry detergents where thermo-stability and tolerance in the alkaline environment are highly recommended. An estimated 1000 tons of lipases are added to approximately 13 billion tons of detergents produced each year (Hasan et al. 2010; Verma and Sharma 2014). During laundering, lipases adsorb onto the fabric surface to form a stable fabric–lipase complex and then hydrolyze oil stains present on the fabric. The complex is resistant to the harsh wash conditions and is retained on the fabric during laundering (Rigoldi et al. 2018). To be detergent compatible, lipases should convince the following attributes: stability at alkaline pH, solubility in water, tolerance to detergent proteases and

surfactants, and low substrate specificity (Sharma et al. 2018; Maharana and Ray 2015). *Talaromyces thermophilus* produced thermo-alkaline lipase which could be used as commercial wash and bleach agent in detergent industry (Romdhane et al. 2010). Cold active lipase produced by *Cryptococcus* sp. Y-32 established low temperature and high pH stability, which has the ability to degrade lipid wastes in cold regions and useful in detergent formulations for cold temperature washing of delicate clothes (Kumar Maharana and Mohan Singh 2018). Lipases from psychrotolerant fungi *Penicillium canesense* and *Pseudogymnoascus roseus* were characterized as detergent compatible alkaline lipases that facilitate cold-washing if included in detergent formulations (Sahay and Chouhan 2018). *Aspergillus tamarii* JGIF06 lipase exhibited oil-de-staining efficiency in hot water when tested on cotton fabric pieces stained with peanut oil (Das et al. 2016).

18.4.2 Medical and Pharmaceutical Industries

The main application of fungal lipases is in the treatment of diseases such as dyspepsia, gastrointestinal disturbances, cutaneous manifestations of digestive allergies, and cancer. Lipases also serve as a diagnostic tool in medicine, thereby justifying the growing demands (Yagmurov et al. 2017). Immobilized lipase B from *Candida antarctica* (Novozyme 435) catalyzes direct esterification of n-butanol and lactic acid and the lactate esters thus produced are key intermediates in chemical preparations for medical and pharmaceutical purposes (Knez et al. 2012). The three recombinant lipases produced by *Trichosporon asahii* found to be enantioselective which was determined by esterification of racemic 1-phenylethanol and capric acid. These enantioselective lipases showed nearly 40% enantiomeric excess that is indispensable in the field of pharmaceuticals for the chiral resolution of drugs (Singh and Gupta 2016). The synthesis of both enantiomers of four new phenylthiazole-based amines by enantiomer-selective acylation of racemic amines and by hydrolysis of the corresponding racemic amides using lipase B from *Candida antarctica* (Novozyme 435) as chiral catalyst was performed with good yields and excellent enantioselectivities (Radu et al. 2014). A novel alkali tolerant recombinant lipase TALipA produced by *Trichosporon asahii* MSR54 was found to be enantioselective, regioselective, and long chain fatty acid selective which is the major requirement of pharmaceutical industries (Kumari and Gupta 2015). *Candida antarctica* lipase Awas investigated for its potential in trans-esterification of the phenolic OH group of capsaicin and several capsaicin analogues using *Capsicum* oleoresin for pharmaceutical purposes (Diaz-vidal et al. 2020). The efficient green enzymatic process for L-ascorbyl palmitate synthesis by stable indigenously immobilized *Candida antarctica* lipase B (CALB) lipase using underivatized substrates with high space time yield of 15 g/L/h, and no by-product formation with recycling of substrates makes the overall process clean and environment-friendly (Yadav et al. 2018). Medium-chain, oleic (18:1n-9), and medium-chain fatty acid and structured lipids were produced by acidolysis reaction in solvent-free medium with capric (10:0) and lauric (12:0) free fatty acids and triolein or olive oil,

using *Yarrowia lipolytica* lipase as biocatalyst. This lipase showed promising properties as a potential biocatalyst that may be effectively used in the production of bioactive structured lipids, which might be applied for the prevention of metabolic and inflammatory disorders related to obesity (Akil et al. 2020).

18.4.3 Food Industry

The majority of the food industries search for enzymes that exhibit catalytic specificity, thermo-stability, high catalytic activity in a wide range of pH and temperature, and structural properties which can be immobilized with high catalytic efficiency (Sharma et al. 2016b; Contesini et al. 2017). The literature presents several studies that address lipase application in food industry in dairy processing—baking, oil, meat, fish, and beverages (Coelho and Orlandelli 2020). The chemically synthesized products cannot be regarded as "natural," which is a fatal blow to the increasing demand for "natural" flavor esters. In contrast, the enzymatic synthesis of flavor esters catalyzed by lipases is a more efficient, economically benign, and promising alternative approach to traditional methods (Shen et al. 2020). *Thermomyces lanuginosus* lipase immobilized on styrene–di-vinyl benzene beads was observed to produce butyl butyrate which could be employed as an artificial fruit flavor ester in food industry (Martins et al. 2013). *Thermomyces lanuginosus* lipase immobilized on PEGylated polyurethane particles was further coated with polyethyleneimine to increase the stability of lipase enzyme. Therefore, it was efficiently used in the production of ethyl esters from fish oil compared to the free enzyme (Jorge and Ninow 2016). Since the enzymatic technique is milder and greener, and can minimize oxidation, polymerization, and cis–trans isomerization of oil containing polyunsaturated fatty acids, lipase catalysis has been a promising technique among the enrichment methods. *Trichosporon* sp. lipase F1–2 showed many good characteristics for industrial applications, including high catalytic activity and stability under alkaline conditions, high activity without metal ions as cofactors, tolerance to various organic solvents and sn-1,3 regioselectivity. Since the lipase preferred short- and medium-chain fatty acid esters and discriminated against long-chain fatty acid esters, its potential application in enrichment of eicosapentaenoic acid and docosahexaenoic acid in fish oil was investigated (Cao et al. 2020). *Aspergillus fumigatus* lipases are involved in the synthesis of two esters (ethyl acetate and ethyl lactate) which could be used in high value-added products due to their low toxicity. They can also be used in artificial fruit essence and give artificial flavors such as pineapple, bananas, and strawberry in confectionary, ice-cream, cakes, etc. (Mehta et al. 2020). The sn-1,3 selectivity of extracellular lipase from *A. niger* GZUF36 has great potential in the synthesis of functional oils (Xing et al. 2020). Immobilization of CALB onto hydrophobic anion exchange resin Purolite MN102 provided esterification catalytic mechanism to yield two valuable esters such as isoamyl acetate and L-ascorbyl oleate to be used as food ingredients (Milivojevic 2016). Soybean oil deodorizer distillate, a by-product of vegetable oil refining industries, can be hydrolyzed and esterified using Amano-30 (a crude lipase from

Candida rugosa) and NS-40013 (an immobilized lipase from *Candida antarctica*) to prepare functional foods like sterols and tocopherols. (Bengal and Bengal 2019). The appropriate combination of *S. lactis* and *Lactobacillus* and the addition of lipase (*Aspergillus oryzae*) efficiently improved the quality of yogurt-flavored bases. In addition, the addition of fungal lipase to fermentation conducted by mixed lactic acid bacteria significantly enhanced the physicochemical properties, especially total volatile organic acids (Huang et al. 2020).

18.4.4 Biodiesel Production

Biodiesel is composed of methyl-esterified fatty acids derived from trans-esterification of triglycerides by enzymatic action, providing a number of advantages such as the reduction in the operational process in the manufacture and separation of glycerol by-products (Dalmaso et al. 2015). The enzymatic trans-esterification is more promising as it offers advantages with an environment-friendly option compared to the chemical processes, such as mild reaction condition, less energy intensity, higher yield in esters, as well as better recovery of glycerol and the trans-esterification glycerides with high free fatty acid contents (Yan et al. 2016). Lipases are versatile biocatalyst for biodiesel synthesis that requires relatively simple downstream processing steps for the purification of biodiesel and its by-product glycerol. Immobilized lipases obtained from filamentous fungi and yeasts are more advantageous than free lipases because they are reusable, cost effective and also possess improved catalytic property (Aguieiras et al. 2015). Extremophilic fungal lipases produced by *Yarrowia lipolytica*, *Candida antarctica*, *Candida rugosa*, *Penicillium camembertii*, *Rhizomucor miehei*, and *Thermomyces lanuginosus* are immobilized on solid support for biodiesel production by the following methods such as cross-linking, physical adsorption, covalent bonding, and entrapment (Zhao et al. 2015). Among fatty esters of industrial interest, 1-butyl oleate is used as biodiesel additive to decrease the viscosity of diesel in winter use, polyvinyl chloride plasticizer, water-resisting agent, and in hydraulic fluid (Séverac et al. 2011). Usually, the synthesis of butyl oleate is conducted in the presence of concentrated sulfuric acid through a chemical route, which has many disadvantages, such as formation of by-products, disposal of acid wastewater, and equipment erosion by the concentrated acid (Wang et al. 2010). Therefore, the enzymatic synthesis becomes more and more interesting, since enzymes have high selectivity, specificity, and activity in mild reaction conditions. Furthermore, lipases have broad availability, low cost, mild reaction conditions, no need for co-factors, and substrate specificity. Since halophilic lipases are highly desirable for biodiesel production, halophilic lipase obtained from *Fusarium solani* has been suggested to be utilized for industrial purposes (Geoffry and Achur 2018). Lipases from *Candida antarctica*, *Thermomyces lanuginosus*, and *Rhizomucor miehei* were covalently immobilized on epoxy-functionalized silica and used to produce biodiesel by trans-esterification of canola oil with methanol (Babaki et al. 2015). A recombinant CALB immobilized on core-shell polymeric supports enables production of esters using residual fatty acids

as substrates which could be used for biodiesel and cosmetic production (Cipolatti et al. 2018). A recombinant cold-adapted lipase belonging to *Rhizomucor endophyticus* that possess high yield and excellent properties was conferred for its great potential for biodiesel production in bioenergy industry (Yan et al. 2016). *Aspergillus niger* lipase immobilized on magnetic nanoparticle was reported to produce high quality biodiesel at 85.3% (Jambulingam et al. 2019).

18.4.5 Bioremediation

Due to escalated human population ultimately our environment has been contaminated with improper disposal of waste discharges from various industrial sectors such as dairy, food industries, oil refinery, poultry house, pesticides, and wool processing factories. Therefore, biocatalysis process came into existence which is cost-effective and energy-efficient targeting lipid-containing waste material and conversion into value-added products (Kumar et al. 2019). The recombinant lipase constructed by cloning lipase gene from *Trichosporon coremiiforme* V3 and functionally expressed in *Pichia pastoris* X33 exhibited several significant industrially important properties such as high temperature, pH stability, wide organic solvent tolerance, and broad hydrolysis range on vegetable oils, which could be successfully used in bioremediation of oil polluted sites (Wang et al. 2015). Lipase producing *Alternaria* sp. and *Aspergillus flavus* were suggested for bioremediation of oil contaminated sites (Wadia and Jain 2020). The lipase produced by *Penicillium chrysogenum* showed promising results in the remediation of used cooking oil (Kumar et al. 2012b). *Aspergillus awamori* BTMFW032 isolated from seawater has potential for use in industries for the production of extracellular lipase under submerged fermentation, which could be used in bioremediation of oil laden effluent (Basheer et al. 2011). *Penicillium* sp. DS-39 lipase exhibited a broad substrate range with distinct specificity for oils and triacylglycerols of long unsaturated fatty acids and was found to be significantly stable in the presence of non-polar hydrophobic solvents. These features render *Penicillium* sp. DS-39 lipase suitable for potential applications in non-aqueous biocatalysis such as biodiesel production and enzymatic restructuring, by inter-esterification of different oils and fats and biodegradation of oil spills in the environment (Dsm et al. 2011). An alkaline lipase produced by *Cladosporium langeronii* has been suggested for potential application in degradation of oil and cleaning up the environment (Sadati et al. 2015). The fungal strains of *Thermomyces lanuginosus* were stimulated to produce lipase in the presence of either triacetin or olive oil suggesting the adaptive nature and contribute to the deterioration of oil seeds during storage (Sreelatha et al. 2017). *Mrakia blollopis* CBS8921 (Antarctic yeast) lipase assumed to be promising bio-remediation agent for cleaning up unwanted milk fat curdles from dairy milk wastewater under low temperature conditions (Tsuji et al. 2013). Lipase produced by *Fusarium incarnatum* KU377454 was immobilized on Fe_3O_4 nanoparticles and was found to be efficient for degradation of waste cooking oil which pollutes environment to a greater extent (Joshi et al. 2019).

18.4.6 Cosmetic Industry

Active lipases can mainly be found in cosmetics for surficial cleansing, anti-cellulite treatment, or overall body slimming, where they are responsible for the mild loosening and removal of dirt and/or small flakes of dead corneous skin (i.e., peeling) and/or assist in breaking down fat deposits, often in combination with further enzymes such as proteases (Ansorge-Schumacher and Thum 2013). Flavor and fragrance compounds are important chemical ingredients of cosmetic products, with further applications in the food, feed, chemical, and pharmaceutical industries. The lipase retrieved from *Aspergillus fumigatus* synthesized methyl butyrate which is an essential flavor enhancer in perfumes. Methyl butyrate is the methyl ester of butyric acid having a characteristic sweet and fruity odor similar to that of apples and pineapples. It can also be used as emulsifier in the food and cosmetic industries (Kaur et al. 2019). The fatty amides (9Z,12Z)-N-dodecyloctadeca-9,12-dienamide (3) and *N*-dodecyl-2-hydroxybenzamide (5), respectively derived from linoleic acid and salicylic acid were synthesized through aminolysis reactions catalyzed by CALB. These amphiphilic compounds receive greater attention from cosmetic industry due to a range of beneficial properties for skin (Mouad et al. 2016).

18.4.7 Leather Industry

In leather processing, lipases are used in the removal of natural fat present in animal skin. Separate degreasing step is required for animal skins with high fat content. Insufficient removal of natural fat during processing prevents the chemicals from penetrating into the leather which leads to negative impacts on the quality of finished leather such as hardness without sufficient internal softness, fatty spew formation, stained appearance due to chrome soap formation, weak bonding of the finishing layer, and bad odor. Traditionally excess fat was removed using solvents and emulsifiers but they add to pollution. Alternatively, lipases of microbial origin can be used in degreasing to reduce pollution. Research on the use of lipase for degreasing dated a few decades ago. Use of acid lipase of fungal origin in degreasing on pickled pelts was reported in 1978 (Yeshodha et al. 1978). Acid lipase from *Rhizopus nodosus* along with commercial degreaser was used in degreasing in 1982 (Muthukumaran and Dhar 1982). Recently, with the availability of commercial lipases, the effectiveness of acid and alkaline lipases of commercial origin in degreasing at various stages of leather processing was also reported (Afsar and Cetinkaya 2008). In leather industries, tannery fleshing is a waste generated after de-hairing of animal hides which are rich in proteins and fats. These wastes create pollution which should be recovered through lipases and utilized as poultry feed and bio-fertilizer. Application of extracellular lipase using *A. tamarii* MTCC 5152 on tannery fleshing shows 92% of fat solubility. The enzymatically recovered fat can be used for biodiesel manufacture and the residual protein as a fish or poultry feed (Dayanandan et al. 2013).

18.4.8 Biosensors

A biosensor can be defined as a device resulting from the association between a sensitive biological element and a transducer, which converts the biological signal into a measurable physical signal. Biosensors are strong candidates for pesticide residue determination and are becoming more relevant in environmental and food analysis (De Moura et al. 2019). In the last few decades, the development of enzymatic biosensors has attracted increasing attention and offered a new approach to organophosphorus quantification. Many enzymes have been used in various studies to develop biosensors for the detection of organophosphorus compounds in aquatic environments. Biosensor system was developed with lipase as an alternative to detect organophosphorus compounds which are toxic substances in polluted water leading to serious health issues such as digestive diseases and damage of liver and kidney (Kartal et al. 2007; Zehani et al. 2014b). Two novel impedimetric biosensors for highly sensitive and rapid quantitative detection of diazinon (pesticide) in aqueous medium were developed using two types of lipase, from *Candida rugosa* (microbial source) and from porcine pancreas (animal source) immobilized on functionalized gold electrode. Lipase is characterized to specifically catalyze the hydrolysis of ester functions leading to the transformation of diazinon into diethyl phosphorothioic acid and 2-isopropyl-4-methyl-6-hydroxypyrimidine. These biosensors are very promising analytical tool for the detection of organophosphate pesticides. It also used in the monitoring of detoxification processes for the treatment of wastewaters generated by the industrial production of organophosphate pesticides (Zehani et al. 2014a). A novel and simple optical biosensor to detect triglycerides has been successfully constructed by using pectin hydrogel membrane as the indicator pH and chromoionophore ETH 5294, with lipase (*Candida antarctica*) as the catalyst (Hasanah et al. 2019). Chemical modification of elastin-like recombinamers is carried out via enzymatic catalysis with CALB in a mild and effective one-pot reaction. The coupling reaction with p-phenylazoaniline allowed to obtain photo-responsive and electromagnetic radiation-sensitive biomaterials that could find a use as sensors in devices for controlled drug delivery, whereas coupling with 4-[(2-amino) carbamoyl]phenylboronic acid led a glucose-responsive elastin-like recombinamers with potential for the design of glucose-sensitive biosensors and actuators for use in drug-delivery systems, for instance as insulin-delivery systems for patients with diabetes mellitus (Testera et al. 2019).

18.5 Immobilization of Lipases

In general, enzymes have some drawbacks in their free form such as difficulty in recovery, insoluble in some media, and unstable in organic solvents in pH and temperature ranges. Thus enzymes are immobilized to improve the stability of enzymes to heat and extreme pH conditions and allow their recovery, thereby reducing the cost of enzymatic processes. Immobilization is a powerful tool not only for the reuse of expensive enzymes in industrial processes, but also to improve

catalyst stability, reaction selectivity, catalytic activity, resistance to inhibitors, product purity, among others. Lipases are immobilized by the methods including adsorption and covalent attachment, cross-linking, adsorption followed by cross-linking, and physical entrapment using commercial carriers. Immobilization of lipases has been performed by adsorption on hydrophobic adsorbents, including glass beads coated with hydrophobic materials, methylated silica, phenyl-sepharose, poly-(ethylene glycol)-sepharose, polypropylene particles, polypropylene hollow-fibers and nonwoven fabric, and nitrocellulose membranes. Immobilized enzymes are used in many commercialized products for higher yields (Sharma and Kanwar 2014). 2-Ethylhexyl oleate was synthesized using *Candida antarctica* lipase immobilized on magnetic poly (styrene-co-divinylbenzene) particles in a continuous packed-bed bioreactor. 2-Ethylhexyl oleate is an example of a commercially important ester with applications in the cosmetics, pharmaceutical, food, and chemical industries. These emollient esters are widely used as skin softening and moisturizing agents in creams, lotions, sunscreen products, makeup, and antiperspirants (da Silva et al. 2020). Esterification of lauric acid with n-butanol was catalyzed by immobilized *Candida antarctica* lipase in aqueous-organic biphasic solvent system (Shankar et al. 2013). Optimized glyceryl monoundecylenate synthesis can be used as a useful reference for industrial synthesis of fatty acid esters of glycerol by the immobilized CALB (Yadav et al. 2017).

Immobilization of *Yarrowia lipolytica* lipase on macroporous resin improved reusability of lipase and provided a chance to expand the application of marine microbial lipase in organic system to catalyze hydrolysis and esterification in harsh condition (Sun et al. 2015). Green synthesis of pentaerythritol monoricinoleate was carried out using CALB immobilized on hydrophobic adsorbent via interfacial activation (Yadav et al. 2019). *Rhizomucor miehei* lipase (RML) immobilized on octyl agarose–polyethylenimine–dextran sulfate has a reduced release of enzyme to the medium under drastic conditions and enables reusability (Virgen-ort and Fernandez-lafuente 2016). Biocompatible hybrid blend of cellulosic copolymers made of hydroxypropyl methylcellulose and chitosan is designed for immobilization of RML, in order to construct the robust biocatalytic system to synthesize industrially important dodecanoate compounds. Butyl dodecanoate is a fatty acid ester, which is a colorless liquid oil having pleasant fruity smell and is widely used in pharmaceuticals, cosmetics, perfumery, food, and beverages as a flavoring ingredient (Badgujar et al. 2017). RML was immobilized on montmorillonite K-10 by adsorption and in polyvinyl alcohol by entrapment to obtain a more stable and active lipase preparation. The thermal stability of the enzyme was significantly enhanced after immobilization and it showed 23.5-fold higher catalytic efficiency than that of the free enzyme. It also exhibited better reusability performance than that of polyvinyl alcohol-based lipase (Ece et al. 2019).

18.6 Conclusion

Extremophilic fungal lipases retrieved from diverse extreme resources have exhibited more remarkable performance than other microbial lipases in industrial sectors. This book chapter explored the diverse screening approaches to discover novel extremophilic fungi with true lipase producing ability. In order to exploit extremophilic fungal lipases to their exact potential and sketch out their inherent characteristics, purification to homogeneity is inevitable. Detailed purification schema was designated. Various assays used in the quantification of extremophilic fungal lipases were described comprehensively. The operational behavior of extremophilic lipases was strengthened through immobilization which is absolutely necessary for successful industrial applications. Recombination had ameliorated lipase yield with high specific activity and renders favorable roles in different biotechnological applications. Extremophilic fungal lipases are extensively used in industries such as dairy, beverage, food, detergent, pharmaceuticals, textile, cosmetics, fuel, fat and oil, and agrochemicals, and in pollution control and production of personal care products. The expansive research conducted on fungal lipases of extreme origins and their immense potential for diverse industrial applications evidences that these lipases are dominant over other counterparts and holds a highly prospective future in commercial, health, and environment sectors.

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Extremophilic Fungal Proteases: Screening, Purification, Assay, and Applications

19

Sourav Bhattacharya and Arijit Das

Abstract

The use of extremophilic fungal proteases in industries has been an integral event for decades together and some of these fungal cultures are efficient producers of many hydrolytic enzymes. The fact that such proteases exhibit specific range of action and vast diversity in terms of being active over a very wide range of experimental conditions has made them attractive models for industrial usage. Besides being widely distributed in nature, extremophilic fungi are preferred over their mesophilic counterparts owing to their ability to tolerate harsher growth conditions and to overproduce hydrolytic enzyme with extraordinary properties. The search for novel extremophilc fungi as producers of proteases is a thrust area of research since such extremophilc proteases may find major applications in modern day food processing, beverage production, animal nutrition, leather and textiles processing, detergent manufacture, etc. This chapter addresses the basic characteristics and advantages of using extremophilc fungal proteases in industries. The major focus has been on discussing the potential fungal sources, screening strategies, assay techniques, purification regime of these valued macromolecules, and already documented roles of these extremophilic proteases in food, pharmaceutical, and beverage manufacturing sector.

Keywords

Extremophilic · Fungal protease · Thermophilic · Psychrophilic · Cold adaptive

S. Bhattacharya (✉) · A. Das
Department of Microbiology, School of Sciences, JAIN (Deemed-to-be University), Bangalore, Karnataka, India

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19.1 Introduction

Proteases (peptidases or proteolytic enzymes) constitute a variety of enzymes that hydrolyse peptide bonds in other proteins leading to degradation of proteinaceous substrates into their constituent amino acids, or it can be specific, resulting in selective protein cleavage for post-translational modification and processing (Yu et al. 2020).

Proteases hold central importance in industrial applications by involving selective degradation of proteins. The present cost for the sale of industrial enzymes is about $8 billion of which 65% is contributed by proteases alone (Omrane Benmrad et al. 2016; Barzkar et al. 2018). The specific hydrolytic behaviour of proteases imparts applications in leather, food, pharmaceutical, detergent, silk-degumming, silver recovery, waste management, and peptide synthesis (Razzaq et al. 2019).

19.2 Protease Types

Depending on their site of action, proteases are subdivided into exopeptidases and endopeptidases. The exopeptidases act only near the ends of polypeptide chains. Based on their site of action at the N or C terminus, they are classified as amino and carboxypeptidases (de Souza et al. 2015) (Fig. 19.1).

Aminopeptidases (EC 3.4.14) act at a free N terminus of the polypeptide chain and liberate a single amino acid residue, a dipeptide, or a tripeptide. The carboxypeptidases act at C terminals of the polypeptide chain and liberate a single amino acid or a dipeptide. On the basis of the nature of the amino acid residues at the active site of the enzymes, carboxypeptidases involve three major groups: serine peptidases (EC 2.4.16), metallopeptidases (EC 2.4.17), and cysteine peptidases

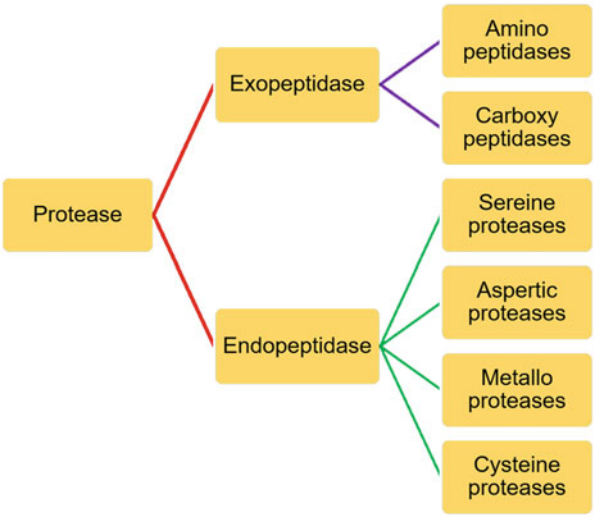

Fig. 19.1 Types of proteases

(EC 2.4.18). Endopeptidases are characterized by their preferential action at the peptide bonds in the inner regions of the polypeptide chain. Considering their catalytic mechanism, the endopeptidases are of four subgroups, i.e. serine proteases (EC 2.4.21), cysteine proteases (EC 2.4.22), aspartic proteases (EC 2.4.23), metalloproteases (EC 2.4.24) (Mótyán et al. 2013).

Serine proteases are characterized by the presence of a serine group in their active site. They are generally active at neutral and alkaline pH, with optima at pH 7–11, low molecular mass (18–35 kDa) and have applications in a number of industries (Sundus et al. 2016). Aspartic acid proteases, commonly known as acidic proteases, are the endopeptidases that depend on aspartic acid residues for their catalytic activity. The activity of all cysteine proteases depends on a catalytic dyad consisting of cysteine and histidine. Generally, cysteine proteases are active only in the presence of reducing agents such as HCN or cysteine. Papain is the best-known cysteine protease. Metalloproteases are the most diverse of the catalytic types of proteases. They are characterized by the requirement for a divalent metal ion for their activity (Nageswara et al. 2019).

19.3 Natural Sources of Proteases

Proteases occur in animals, plants, and microorganism and have critical role in many physiological and pathological processes such as protein catabolism, blood coagulation, cell growth and migration, tissue arrangement, morphogenesis in development, inflammation, tumour growth and metastasis, activation of zymogens, release of hormones and pharmacologically active peptides from precursor proteins, and transport of secretory proteins across membranes (Gaur and Bartariya 2020). Extracellular proteases catalyse the hydrolysis of proteins into smaller peptides and amino acids for subsequent absorption into cells, constituting a very important step in nitrogen metabolism (de Souza et al. 2015).

19.4 Microbial Proteases

Proteases play an important role in physiological processes like growth and differentiation, metabolic processes, gene expression, and cell signalling (Banerjee and Ray 2017). Although plants and animals produce proteases, microbes are considered as promising candidates because of their diverse biochemical properties, limited space requirement, and ease of genetic modifications (Saggu and Mishra 2017).

Among the proteases produced by plants, animals, and microbes, about 40% of the total sale is contributed by microbes alone. The microbial proteases serve as preferred driver of increasing economy in the area of white biotechnology because of their desired characteristics needed in the industrial processes. Bacteria produce majority of the proteases as neutral and alkaline in nature, but proteases from fungi are neutral, acidic, or alkaline in nature, and ~60% of these have been commercialized till 2009 (Inacio et al. 2015). Various neutral proteases such as

Umamizyme find their use in food industry due to their specificity in breaking hydrophobic bonds at neutral pH (Bagnasco et al. 2013).

19.5 Fungal Proteases

Filamentous fungi are used in many industrial processes for the production of enzymes and metabolites. Among the many advantages offered by the production of enzymes by fungi are low material costs coupled with high productivity, faster production, and the ease with which the enzymes can be modified. Further, enzymes, being normally extracellular, are easily recoverable from the media (Meyer et al. 2016). Proteases production of fungal origin has an advantage over bacterial protease as mycelium can be easily removed by filtration. Besides, the use of fungi as enzyme producer is safer than the use of bacteria, since the former are normally recognized as generally regarded as safe (GRAS) (de Souza et al. 2015). Many filamentous fungal species including *Aspergillus*, *Chrysosporium*, *Fusarium*, *Penicillium*, *Pleurotus*, *Rhizopus*, *Scedosporium*, and *Trichoderma* are known to produce proteases (Omrane Benmrad et al. 2019). Besides these, proteases from basidiomycetes such as *Agaricus bisporus*, *Armillaria mellea*, *Flammulina velutipes*, *Grifola frondosa*, *Pleurotus ostreatus*, *Pleurotus eryngii*, *Phanerochaete chrysosporium*, and *Schizophyllum commune* have also been reported for protease production (Gurung et al. 2013).

Fungal proteases have high industrial demand due to high stability and catalytic activity, broad diversity, and substrate specificity required for various bioengineering and biotechnological applications. Moreover, the extracellular enzyme production from fungal species leads to cost-effective production because of easy downstream processing and recovery of enzymes which is a major obstacle in industrial processes (Singh and Bajaj 2017).

19.6 Extremophilic Fungal Proteases

19.6.1 Thermophilic Proteases

19.6.1.1 Sources

Fewer studies exist that report extremophilic fungi expressing proteases. *Aspergillus niger*, *Chaetomium thermophilum*, *Fusarium oxysporum*, *Penicillium oxalicum*, *Thermomonospora fusca* YX, and *Trichoderma harzianum* are the significant cultures with promising protease production ability to satisfy industrial demand (Maťaťa et al. 2019).

The fungi with minimum and maximum temperatures for growth as 20 and 50 °C, respectively, are known as thermophilic fungi. Whereas the ones that can tolerate temperature below 20–55 °C are referred to as thermotolerant. Thermotolerant fungi are easily found to be associated with stacks of plant biomass and agricultural products with trapped humidity, oxygen, and plentiful supply of organic residues.

Table 19.1 Protease characteristics from prominent thermophilic fungi (Li et al. 2007)

Organism	Nature	Thermostability (°C)	pH
Malbranchea pulchella var. *sulfurea*	Alkaline protease	50	8.5
Torula thermophila	Alkaline protease	60	8.0
Penicillium duponti K-1014	Acid protease	60	2.5
Humicola lanuginosa	Thiol protease	55	8.0
Chaetomium thermophilum PRO33	Serine protease	60	10.0
C. thermophilum PRO63	Serine protease	60	5.0

Fungal tolerance for high temperature is a less frequent event when compared to bacteria (some tolerating up to 100 °C) as among the 50,000 known fungi, a mere 30 species can tolerate 40–45 °C. *Talaromyces thermophilus*, *Thermoascus aurantiacus*, *Thermomyces ibadanensis*, and *T. lanuginosus* grow optimally at 42–52 °C but are reported to tolerate till 61 °C (Maheshwari et al. 2000).

The fungal thermophiles' ability to produce extracellular proteases with less microbial contamination during protease production demands attention towards understanding temperature tolerance of these fungi which comparatively has been less explored (Chen et al. 2004). Proteases of thermophilic *Humicola lanuginose*, *Malbranchea pulchella* var. *sulfurea*, *Mucor pusillus*, and *Penicillium duponti* are active within 45–55 °C and pH 3–6. Thus for their high specific activity and thermal stability, proteases from thermophilic fungi hold promise for industrial applications (Li and Li 2009) (Table 19.1).

Thermophilic fungi produce protein hydrolases with higher thermostability, hydrolysis rates, and appreciable activity at elevated temperatures. A few fungal species like *Thermoascus aurantiacus* and *Thermomyces lanuginosus* are considered as a source of thermostable acid protease. The *T. aurantiacus* enzyme production involved SSF system (with wheat bran, at 60 °C) (Merheb et al. 2007). Optimum protease activity of the thermophilic fungi *Thermomyces lanuginosus* is frequently reported to be 70, 50, and 45 °C, while for *Thermomucor indicae-seudaticae* it is 70 °C (Macchione et al. 2008; Merheb-Dini et al. 2010). Proteases from *Aspergillus oryzae* and *Penicillium* sp. are reported to show optimum activities at 45 °C and 55 °C, respectively (Germano et al. 2003; Vishwanatha et al. 2010).

19.6.1.2 Screening

Strains of *T. lanuginosus* were isolated from composting site, soil, and dung on Emerson's yeast extract soluble starch agar. All strains isolated were evaluated for their protease profile by inoculation onto yeast extract agar plates (with 1% skim milk). Following their growth on the screening medium (for 72 h), a proteolytic zone was observed around the colonies of *T. lanuginosus*. Among the 500 isolates, P_{134} strain produced the widest proteolytic zone and was hence considered for further evaluation (Li et al. 1997).

While studying the protease production by thermophilic fungi (*Chaetomium thermophile* var. *dissitum*, *Humicola lanuginosa*, *Malbranchea pulchella* var. *sulfurea*, and *Sporotrichum thermophile*), the primary screening (preliminary

indication of proteolysis) involved growth on casein agar (containing 1% w/v casein). As a part of the secondary screening procedure, spore suspensions (prepared using a detergent solution) from the cultures (grown on yeast–glucose agar) were used as inoculum for modified Czapek-Dox medium (composed of 4% w/v casein), maintained at pH 7.4. The culture was grown at 45 °C under shaking condition. By periodic sampling of the medium the maximum production of extracellular protease was determined by assaying for caseinolytic activity at three different pH values (pH 3, 7, and 9). During the maximum extracellular protease activity, the cultures were harvested and the cell-free extracts prepared and the evaluated for protease activity. The best producers were found to be *H. lanuginosa* and *M. pulchella* var. *sulfurea* (Ong and Gaucher 1973).

Evaluation of protease production under solid-state and submerged fermentation, respectively, by thermophiles (*Aspergillus flavus* 1.2, *Aspergillus* sp. 13.33, *Aspergillus* sp. 13.34, *Aspergillus* sp. 13.35, *Rhizomucor pusillus* 13.36, *Rhizomucor* sp. 13.37, *T. aurantiacus* Miehe, *T. lanuginosus,* and *T. lanuginosus* TO.03) reports using milk powder, soybean flour, soybean milk, rice, and wheat bran. The most satisfactory screening results were observed in solid-state fermentation involving wheat bran. Under solid-state fermentation, *T. lanuginosus*, *T. lanuginosus* TO.03, *Aspergillus* sp. 13.34, *Aspergillus* sp. 13.35, and *Rhizomucor* sp. 13.37 produced protease at appreciable levels. While under submerged fermentation, the significant proteolytic cultures were *T. aurantiacus*, *T. lanuginosus* TO.03 and 13.37, respectively (Macchione et al. 2008).

19.6.1.3 Purification

A thermotolerant protease produced by a *Fusarium oxysporum* was purified to homogeneity by Sephadex G-200 gel filtration column and α-casein agarose gel affinity chromatography. The purified *F. oxysporum* protease was reported to have a specific activity of 93.88 U/mg protein. The purification magnitude was 7.7 and the total yield was 20%. Purified protease had an optimum pH of 5.0, while the optimum temperature was 40 °C (Ja'afaru et al. 2020).

The crude protease (later found to be thermostable) from *A. flavus* was precipitated by ammonium sulphate saturation (70%) and dialyzed (24 h, 40 °C) against phosphate buffer (50 mM, pH 7.0). The filtrate was passed into a DEAE-cellulose column (equilibrated with phosphate buffer, 50 mM, pH 7.0) and eluted with a linear NaCl concentration gradient (0–0.4 M) in the same buffer and 3.0 ml fractions were collected (flow rate 20 ml/h). Following the purification steps, a 284 U of protease activity, 0.37 mg of total protein, 170 U/mg of specific activity, 5.8-fold purification, and 3.2% recovery, respectively, were recorded (Muthulakshmi et al. 2011).

While purifying a novel thermotolerant fibrinolytic protease from *Fusarium* sp. CPCC 480097, the culture supernatant is reported to be treated using a two-step ammonium sulphate salting-out procedure (first at 40%, and then at 60%). The crude precipitate of the 60% saturation centrifuged (8000 × *g* for 30 min at 4 °C) and dissolved in Tris–HCl buffer (20 mmol/l, pH 7.4) is then loaded onto a G-25 column (16 × 25 mm). The protease-containing fraction was applied to

a MonoQ column (10 × 100 mm). The active fraction was concentrated by lyophilized after desalting. The concentrated sample was loaded onto a Superdex 75 column (16 × 600 mm). After the three purification steps, the enzyme was purified 158.5-fold, with a protein content of 1.8 mg, total activity of 13,700 U, specific activity of 76,111 U/mg and a final yield of 6.8%, respectively (Wu et al. 2009).

A novel thermostable protease (SPPS) from *Pleurotus sajor-caju* CTM10057 is known to be purified to homogeneity by heat-treatment (80 °C for 20 min), followed by ammonium sulphate precipitation (35–55%)-dialysis, FPLC based ion-exchange chromatography (UNO Q-6 column), and finally gel filtration chromatography (HPLC-ZORBAX PSM 300 HPSEC). Following purification, enzyme activity, 79×10^4; total protein, 10 mg; specific activity, 79,000 U/mg of protein; protein recovery, 15%; nine-fold purification, respectively are reported (Omrane Benmrad et al. 2019).

A serine protease with a pH optimum from 7 to 9 and activity over the range of pH 3–10 is isolated and purified from culture filtrates of *Penicillium charlesii* after 16 days of inoculation. The enzyme can be purified by the following sequence of procedures: (1) gel permeation chromatography through Sephacryl S-200, (2) DEAE-Sepharose anion-exchange chromatography, and (3) fast protein liquid chromatography over Superose 12 (Abbas et al. 1989).

19.6.1.4 Assay

The protease activity of the crude proteases from the thermophile *T. lanuginosus* was estimated using the modified protocol of Rick (1974). The enzyme (0.5 ml) was added to casein (2.5 ml, 0.4%) dissolved in Tris-HCl (0.2 M, pH 9.0) or 0.2 M CH_3COOH/CH_3COONa (pH 5.0) buffer and incubated (50 °C, 60 min). Trichloroacetic acid (2 ml, 10%) was added and incubated (room temperature, 30 min). The solution was filtered and to the filtrate (1 ml), water (5 ml) was added, and absorbance was determined spectrophotometrically at 280 nm (Rick 1974).

Crude cell extracts of thermophilic fungi (*Chaetomium thermophile* var. *dissitum*, *C. thermophile* var. *coprophile*, *Humicola lanuginosa*, *H. insolens*, *H. grisea* var. *thermoidea*, *Penicillium duponti*, *Malbranchea pulchella* var. *sulfurea*, *Sporotrichum thermophile,* and *Talaromyces thermophilus*) were prepared by subjecting the slurry of lyophilized cultures to ultrasonication for protease release. Centrifugation (48,000 × *g*, 30 min) of the sonicate yielded supernatants to be considered as the crude cell-free extracts. Before protease assay, the supernatants were diluted with the buffers (0.2 M glycine-HCl, pH 3.0; 0.2 M phosphate, pH 7.0; or 0.2 M glycine-NaOH, pH 9.0). The enzyme (0.25 ml) was added to casein (1.75 ml, 0.5% w/v) in an appropriate buffer and incubated (37 °C, 1 h). Trichloroacetic acid (3 ml, 5%) was then added, and after standing at room temperature for 30 min, the solution was filtered. To the filtrate (1 ml), NaOH (5 ml, 0.4 N) and diluted phenol reagent (1 ml, 1:2 v/v) were added consecutively and incubated (room temperature, 30 min) for determining the absorbance at 660 nm (Ong and Gaucher 1973).

The extracellular thermostable acidic protease activity of *Aspergillus terreus* NCFT4269.10 was determined according to the method of van den Hombergh et al. (1995). Each protease sample (450 µl) was incubated with BSA (50 µl, 1% w/v) in sodium acetate buffer (0.1 M, pH 4.0) at 37 °C. After 30 min of incubation, the reaction was terminated with trichloroacetic acid (500 µl, 10% w/v). After incubation at 0 °C for 30 min, the precipitated proteins were removed through centrifugation (6000 rpm, 5 min) and the absorbance of the TCA-soluble fraction was estimated as per Lowry et al. (1951).

19.6.2 Applications

Talaromyces emersonii thermozyme cocktails are in use as baking enzymes as they impart a positive effect on the final product. Enzyme screening studies reveal the presence of hemicellulases, amylolytic enzymes, and proteases in all cocktails (in various relative amounts) is essential for the manipulation of dough. The results show that small quantities of crude enzyme mixtures positively affect the antistaling properties, volume and softness of bread, thereby improving baking. This makes *T. emersonii* a novel source of efficient thermozymes for the baking industry (Waters et al. 2010).

Thermostable proteases, acting in temperature range of 65–85 °C successfully convert proteins into amino acids and peptides, thus finding applications in baking, brewing, detergents, and the leather industry (Haki and Rakshit 2003; Merheb et al. 2007) (Fig. 19.2).

19.6.3 Psychrophilic Proteases

19.6.3.1 Sources

Fungi can flourish under low temperature locations and inhabit permafrost, banks of polar waters, glaciers, icebergs, and freshwater ice (Tojo and Newsham 2012). The fungi that survive and are able to tolerate extreme low temperature conditions (optimum growth temperature being 10 °C but may grow at lower temperature) are referred to as psychrophilic fungi. Further, fungal forms that grow maximally at 20 °C are psychrotrophic fungi (Hassan et al. 2016).

Extracellular proteases are documented from psychrophilic fungi (*Candida humicola*, *Glaciozyma antarctica,* and *Rhodotorula mucilaginosa*) and mesophilic fungi (*Aspergillus niger* and *Trichoderma harzianum*) (Duarte et al. 2018). Species pertaining to the genus *Aspergillus ustus*, *Cryptococcus gilvescens*, *Humicola marvinii*, *H. fuscoatra*, *Mrakia gelida*, and *Rhodotorula laryngis* are significant protease producers (Damare et al. 2006; Turchetti et al. 2008). Several deep-sea fungi are capable of producing psychrophilic protease including *Aspergillus terreus*, *Beauveria brongniartii,* and *Acremonium butyric* (Srilakshmi et al. 2014).

Protease producing fungi from Antarctic locations are from the genera *Acremonium*, *Candida*, *Cryptococcus*, *Chrysosporium*, *Embellisia*, *Exophiala*,

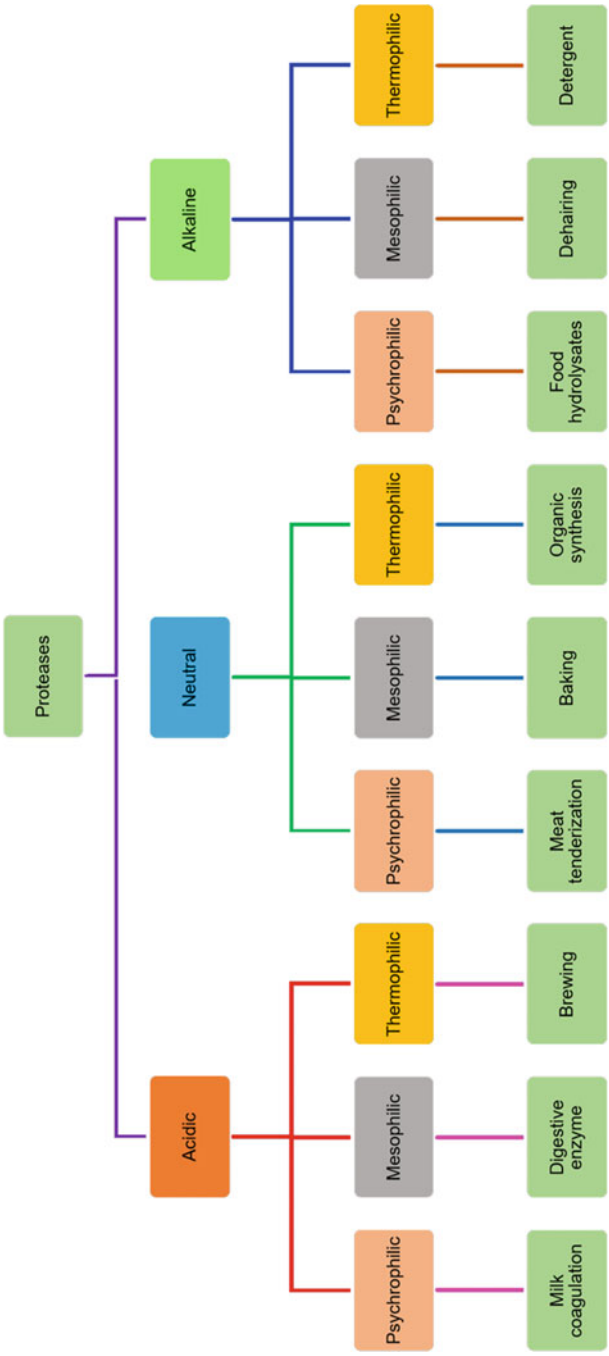

Fig. 19.2 Applications of various extremophilic proteases

Geomyces, *Glaciozyma*, *Glomerella*, *Leuconeurospora*, *Leucosporidium*, *Mrakia*, *Phoma*, *Pseudogymnoascus*, *Rhodotorula*, *Trichoderma*, *Vanrija*, and *Wickerhamomyces* (Fenice et al. 1997).

The protease of *Candida humicola* is reported to be active at 0–45 °C and is resistant to freeze-thaw cycles (Ray et al. 1992). The serine protease of *Leucosporidium antarcticum* 107 is highly active at 25 °C and even at 10 °C (Turkiewicz et al. 2003). Proteolytic enzyme of *Geomyces pannorum* exhibits the greatest activity at 4 °C (Krishnan et al. 2011).

19.6.3.2 Screening

For the determination of proteolytic activity from cold-tolerant *Rhodosporidiobolus*, *Cystofilobasidium,* and *Yamadazyma*, the isolates were inoculated onto a basal medium containing skimmed milk as a protein source: (g/l) skimmed milk 1.0; yeast extract 1.0; agar-agar 18.0; KH_2PO_4 1.0; $MgSO_4{\cdot}7H_2O$ 0.1; $CaCl_2.H_2O$ 0.05; NaCl 5.0; Na_2CO_3 1.0. Proteolytic activity was determined by the observation of clear halo around the colony (Yadav et al. 2016).

While studying the enzymatic activity of halotolerant and halophilic fungi from the Great Sebkha of Oran in Northwestern Algeria, protease activity was detected on milk agar (containing 30% skim milk and 2% agar). After incubation, the degradation of casein was reflected by a clear zone around the thallus (Sarath et al. 1989). *Aspergillus subramanianii*, *A. terreus*, *A. calidoustus*, *Penicillium egyptiacum,* and *Cladosporium ramotenellum* were the significant cold-tolerant fungal forms to show proteolysis (Chamekh et al. 2019).

19.6.3.3 Purification

The cold adaptive protease of *Candida humicola* isolated from the soil of Schirmacher Oasis can be concentrated by ultrafiltration followed by anion-exchange chromatography purification (Ray et al. 1992). *Glaciozyma antarctica* isolated from the sub-glacial waters (200 m deep) in Admiralty Bay when subjected to concentration by acetone precipitation followed by purification in Sephadex G75 anion-exchange chromatography Sephacryl S-100 column is reported to result in 1560-fold purity and 22.7% yield (Turkiewicz et al. 2003). *Rhodotorula mucilaginosa* L7 from the Antarctic marine macroalgae when concentrated by ultrafiltration followed by purification in cation-exchange chromatography Sephacryl S-100 column results in 15.6-fold purity and 29.7% yield (Lario et al. 2015).

19.6.3.4 Assay

While studying the molecular cloning of cold-adapted serine protease expression in Antarctic yeast *Glaciozyma antarctica* PI12, the proteolytic activity involves estimation by the modified protocol of Alias et al. (2014) using azocasein. Azocasein (0.5%) was solubilized in Tris-HCl (0.1 M, pH 7) and enzyme action initiated by adding enzyme sample (100 µl). Incubation conditions involved 20 °C for 30 min. Same volume of trichloroacetic acid (10% w/v) was added to stop the reaction and absorbance read (450 nm).

The alkaline, cold-tolerant protease activity of deep-sea isolate of *Aspergillus ustus* NIOCC#20 was assayed using crude culture filtrate (150 µl) and azocasein (250 µl, 2% azocasein prepared in 0.1 M boric acid-borax buffer, pH 9). The reaction mixture was allowed to incubate (5 and 30 °C, 30 min). The reaction was stopped by addition of trichloroacetic acid (1.2 ml, 10% w/v). Any developed turbidity was removed by centrifugation (8000 rpm, 10 min). To the supernatant, NaOH (1.4 ml, 1 N) was added and the absorbance read (440 nm). One azocasein digestion unit is defined as the increase in absorbance (0.001/min) under the assay conditions (Damare et al. 2006).

19.6.3.5 Applications

Aspartic protease is one of the most versatile enzymes in food processing. Owing to the increasing demand in cheese products, microbial aspartic proteases serve as beneficial complements for animal-derived milk coagulant. A novel aspartic protease gene (P10) from an Antarctic psychrophilic fungus *Geomyces pannorum* can be successfully expressed in *Aspergillus oryzae* when cultured at 20 °C. Its potential application in cheese-making provides novel insights into application of psychrophilic fungal protease in the food industry (Gao et al. 2018).

Cold-adapted fungal proteases find their applications in the food industry to fasten the ripening of cheese, making frozen meat products tender, protecting the status of thermosensitive nutrients, and as an effective treatment for haze-associated proteins in wine and malted beverages. In laundries, cold-adapted fungal proteases can be incorporated in detergents powder for 'cold washing' to wash fabrics at room temperature (Peterson et al. 2013) (Fig. 19.2).

19.6.4 Acid/Aspartic Proteases

19.6.4.1 Sources

While studying the acid protease production in fungal root endophytes, multiple isolates of *Phialocephala fortinii*, *Meliniomyces variabilis*, *Umbelopsis isabellina*, *Hebeloma incarnatulum*, *Laccaria bicolor*, and *Irpex lacteus* can be considered. *H. incarnatulum* and *L. bicolor* secrete metalloproteases having pH optima above 4, while other fungi produce aspartic proteases with lower pH optima. *M. variabilis* and *P. fortinii* exhibit intermediate levels of protein utilization and *M. variabilis* exhibits a very low pH optimum (Mayerhofer et al. 2015).

Aspartic proteases are sourced from moulds and yeasts and rarely from bacteria. Aspartic protease enzymes from fungal sources mainly fall into two groups: pepsin-like enzymes produced by *Aspergillus*, *Penicillium*, *Rhizopus*, and *Neurospora* and rennin-like enzymes produced by *Mucor miehei*, *M. pusillus*, and *Endothia parasitica* (Mamo and Assefa 2018).

19.6.4.2 Screening

Primary screening for protease produced by *Aspergillus* Z1BL1 can be tested using skim-milk agar medium for the production of the clear zone (Saran et al. 2007). The

detection medium is prepared using skim milk, agar–agar dissolved in 200 ml distilled water, and 600 ml of phosphate buffer (0.2 M, pH 5.0). The plates can be subsequently inoculated with previously purified fungal isolates and incubated (30 °C, 2 days). The plates are examined for the formation of the clearing zone by flooding them with a solution of trichloroacetic acid or tannic acid (10% v/v). The relative enzyme activity is calculated using the inhibition zone diameter and colony diameter.

In order to detect the acid protease activity of sixty-one fungal endophytes (*Alternaria* sp., *Aureobasidium pullulans*, *Beltrania rhombica*, *Chaetomium fibripilium*, *Colletotrichum acutatum*, *Fusarium* sp., *Curvularia* sp., etc.) a qualitative dot blot method is used. A gel is prepared by mixing a solution containing acrylamide/bisacrylamide (1 ml, 30%); substrate (0.4 ml, 2% autoclaved gelatin); Tris-HCl buffer (2.3 ml, 50 mM, pH 9.0); TEMED (0.003 ml, 100%); and ammonium persulphate (0.003 ml, 40%) and poured in a gel cassette. After polymerization, it is topped with Bis-Tris buffer (pH 7.0) or sodium acetate buffer (pH 5.0). Thus, a composite gel made of gel strips of pH 5.0, pH 7.0, and pH 9.0 is obtained. Lyophilized crude enzyme (10 mg) mixed with 1 ml of appropriate buffer (as mentioned above) is centrifuged (14,000 rpm, 5 min, 20 °C) and the enzyme solution (5 μl) was spotted on the appropriate gel fields of the composite gel and incubated (10 h), stained with Coomassie Brilliant Blue (R 250, 0.025%) for 3 h, washed with distilled water, and observed for the presence of clear zones on the deep blue gels (indicating enzyme activity). Protease action is clearly visible as a colourless spot on the dark blue background of the gel. Twenty-five isolates out of the 61 endophytes elaborated acidic protease (Thirunavukkarasu et al. 2017).

19.6.4.3 Purification

In order to collect the cell-free extracts from *Aspergillus terreus* and *Aspergillus niger* (3-days old) fungal mats, the biomass were washed twice with 1.0 mM dithiothreitol (DTT), KCl (50 mM), and phosphate buffer (150 mM, pH 7.5). The resulting homogenates were cheesecloth filtered, centrifuged (1500 rpm, 5 min), and the supernatant considered as crude protease. The crude protein was precipitated with ammonium sulphate (70% w/v) and suspended in phosphate buffer (20 ml, pH 7.5) followed by treatment with calcium phosphate (4 °C, for 24 h). The supplement was centrifuged (5000 rpm, 15 min, 4 °C) and supernatant added to Sephadex G-200 affinity chromatography column. Elution was done with phosphate (50 mM, pH 7.5). Results indicated that for *A. terreus*, following the purification steps, the total protein, specific activity, and yield were 1.9 mg of protein, 294.7 U/mg protein, and 16%, respectively. While for *A. niger*, after purification the total protein, specific activity, and yield were 7.8 mg of protein, 179.4 U/mg protein, and 16%, respectively. A 81.9 and 28.9-fold increase in purification was recorded for *A. terreus* and *A. niger*, respectively (El-Shora and Metwally 2008).

Penicillium duponti K1014, a thermophile under submerged fermentation produced an acid protease that was purified by the combination of alcohol precipitation, DEAE-cellulose column chromatography, batchwise treatment with *O*-carboxymethyl-cellulose, and Sephadex G-200 gel filtration chromatography.

These procedures were carried out at 0–4 °C. After purification the total activity, specific activity, and yield were 279,842 U, 193 U/mg protein, and 25.5%, respectively (Hashimoto et al. 1973).

The *Aspergillus hennebergii* HX08 mat growth on wheat bran was extracted with acetic acid–sodium acetate buffer (0.1 mol/l, ratio of solvent to bran, 10:1 w/v, pH 5.4) for 12 h. The mixture was centrifuged (12,000 rpm, 15 min), and the supernatant collected as crude enzyme. After membrane filtration (0.22 μm), the crude enzyme solution was further purified by precipitation, ion-exchange chromatography, and Sephacryl S-300 gel chromatography. All of the purification steps were carried out at 4 °C. After purification the total activity, specific activity, and yield were 2257.07 U, 103.67 U/ml, and 16.09%, respectively. A 22.94-fold increase in purification was recorded (Huang et al. 2017).

Purification of the acid protease from *Aspergillus brasiliensis* strain BCW2 was based on a three-step procedure involving initial precipitation with ammonium sulphate (80% concentration), the precipitated fraction was centrifuged (10,000 rpm, 4 °C, 10 min), and the pellet re-suspended in phosphate buffer (0.1 M, pH 7). Finally, the solution was subjected to dialysis overnight (membrane cut-off 12,000 Da) and the dialyzed sample subjected to a Sephadex G-200 gel filtration column (equilibrated with 0.1 M phosphate buffer, pH 7.0) to give a 7.7-fold protein purification and 29% recovery, respectively (Chimbekujwo et al. 2020).

While purifying the acid protease of *A. niger*, the purification steps involved ammonium sulphate precipitation (60–90% saturation), suspension of precipitate in sodium sulphate buffer (0.02 M, pH 6.8), and solution dialyzed overnight against the same buffer and the solution was loaded on DEAE-cellulose column (2 × 30 cm) previously equilibrated with sodium phosphate buffer (0.02 M, pH 7). The column elusion involved the same buffer with increase in morality of NaCl (0.02–0.5 M) (Ahmed 2018).

The milk clotting acid protease of *Rhizopus oryzae* was precipitated between saturation of ammonium sulphate (20–60%). The precipitate obtained after centrifugation (10,000 × *g*, 30 min) was suspended in phosphate buffer (50 mM, pH 6.0) and dialyzed overnight against the same buffer. The obtained enzyme preparation was further purified on a pre-equilibrated (with 50 mM phosphate buffer, pH 6.0) DEAE-cellulose column (25 × 2.6 cm) and size-exclusion chromatography involving Sephadex G-100 column (65 × 1.5 cm). After purification the total activity, specific activity, and recovery were 8279 U, 759.5 U/mg protein, and 26%, respectively. A 91-fold increase in purification was recorded (Kumar et al. 2005).

An aspartic protease (Peptidase R) sourced from *Rhizopus oryzae* was purified to homogeneity by anion-exchange chromatography (HiLoad 26/10 Q Sepharose High-Performance column, equilibrated with 50 mM sodium phosphate buffer, pH 7.0) followed by the hydrophobic interaction chromatography column (HiTrap Phenyl Sepharose column, equilibrated with 50 mM sodium phosphate buffer, pH 7.0) that resulted in total protein content of 87 mg, total activity of 5×10^6, specific activity of 57.5×10^6, and 3.4-fold increase in purification fold, respectively (Hsiao et al. 2014).

19.6.4.4 Assay

Aspergillus Z1BL1 acid protease activity was assayed according to the method of Kembhavi et al. (1993) using haemoglobin as substrate. Enzyme solution (0.5 ml), suitably diluted, was mixed with haemoglobin (1 ml, 2% w/v, in 100 mM glycine-HCl, pH 3.0) and the mixture was incubated (50 °C, 10 min). The reaction was terminated by adding trichloroacetic acid (2 ml, 5% w/v). The mixture was allowed to incubate (room temperature, 15 min) and then centrifuged (10,000 × *g*, 15 min) to remove the precipitate. The absorbance of the soluble fraction was measured at 280 nm. A standard curve was generated using tyrosine solutions (0–50 mg/l). One unit of protease activity is defined as the amount of enzyme required to liberate 1 μg of tyrosine per min under the experimental conditions.

Rhizopus oligosporus acid protease activity was performed by method as described by Arima et al. (1970) with little modifications. Casein (2.5 ml, 1% w/v) in acetate buffer (0.02 M, pH 4.0) and crude enzyme extract (0.5 ml) were mixed and incubated (37 °C, 10 min) and the reaction was terminated by adding trichloroacetic acid solution (2.5 ml, 0.44 M). The precipitate formed was removed by filtration through Whatman No 1 filter paper. Folin Ciocalteau reagent (1 ml) and sodium carbonate solution (2.5 ml, 0.55 M) were added to the clear filtrate (1 ml) and incubated (20 min, 37 °C) for colour development. The absorbance at 660 nm expressed the activity of enzyme in terms of proteolytic units.

Acid protease activity of 14 days cultures of fungal endophytes (grown on BSA media) was quantified by the fluorescently labelled casein assay. The experimentation involved mixing of culture or control media (50 ml) with fluorescently labelled casein (50 ml) in citrate–citric acid buffer in black 96-well microplates. The casein buffer was adjusted to a pH range (pH 2.0–6.0). The microplates were incubated (in dark, 18 h) and fluorescence read (excitation at 590 nm, emission at 645 nm) (Mayerhofer et al. 2015).

19.6.4.5 Applications

(a) Food industry

The appreciable actionf of acid proteases in coagulating milk proteins advocates their high demand in food processing where they are majorly applied to curdle milk proteins, from which cheese is manufactured following the removal of whey (Neelakantan and Mohanty 1999). The prominent forms of milk-curdling enzymes include animal rennets, microbial proteases, recombinant chymosin, and plant rennet (Ward et al. 2009). Worldwide, the dairy industry suffered a scarcity of animal rennet, due to the increase in cheese demand and the resistance from animal rights protection bodies (Furia 1980). This ushered the use of alternate milk coagulation enzymes, primarily of microbial origin that would cleave the specific peptide bond (Phe105-Met106 in bovine casein) in casein to liberate macromolecules and para-casein (Rani et al. 2012). In similar lines, the recombinant rennin (calf chymosin) is expressed in *A. niger* var. *awamori* for dairy industry needs. Industrially, the most important protease for cheese making is sourced from *Rhizomucor miehei* (Ward et al. 2009).

Fresh cheese produced using an ochratoxin-free extracellular acid protease from *Aspergillus niger* FFB1 and reconstituted cow milk as a substrate show similar basic characteristics (pH 4.5, acid taste, and colour) as cheeses produced with calf rennet (Fazouane-Naimi et al. 2010). The concentration of free amino acid (FAA) and physiochemical characteristics is similar in the Turkish, white, brined cheese produced using calf rennet and microbial rennet from *Rhizomucor miehei* (Çepoğlu and Güler-Akın 2013).

Besides their widespread involvement in the dairy industry, fungal-sourced acid proteases have also been extensively harnessed in food seasonings and the improvising protein-rich cereal based foods. Gluten (an insoluble protein found in wheat flour determines the dough properties) can be enzymatically treated to facilitate the handling of dough and mixing time reduction. Furthermore, increased loaf volume and the accumulation of a wider range of products result from wheat gluten following the action of acid proteases from *A. oryzae* (Rao et al. 1998).

(b) Medical and pharmaceutical industry

Acid (aspartic) proteases are commercially available for tackling certain lytic enzyme deficiency syndromes and in the formulation of digestive syrups (Chanalia et al. 2011). The major aspartic proteases secreted *in vitro* by *C. albicans*, *C. parapsilosis*, and *C. tropicalis* are Sap2, Sapp1, and Sapt1, respectively (Rao et al. 1998). Several candidal aspartic proteases contribute to human infections and the search for suitable aspartic protease inhibitors is of interest for treating these infections (Aoki et al. 2012) (Fig. 19.2).

(c) Beverage industry

Industrially processed fruit-based beverages receive clarification steps to prevent turbidity from haze. Acid proteases from *A. saitoi* (aspergillopepsin I) are used in the manufacture of certain fruit juices and alcoholic beverages, to degrade the proteins contributing to turbidity (Sumantha et al. 2006). In the fermentation of sake (Japanese alcoholic beverage), *A. oryzae* acid proteases decide the final taste of the product where they bring protein hydrolysis from the steamed rice to release amino acids and peptides (Shindo et al. 1998).

Haze formation in fresh and fermented fruit and cereal based beverages is due to the aggregation and precipitation of proteins that usually is associated with microbial spoilage (Falconer et al. 2010). Addition of *A. niger* aspartic proteases to kiwi fruit juice reduces the visible turbidity and prevents cold storage associated with haze formation (Dawes et al. 1994). Addition of a commercial fungal acid protease from *A. niger* to cherry juice resulted in a significant reduction in the immediate turbidity. Results also indicated that during the cold storage of the same there was a minimal impact on clarification (Pinelo et al. 2010). Similar observations were also associated with the black currant juice production where commercially available amino acid protease A (a form of acid protease) from *A. niger* and *Mucor miehei* acid protease was used (Landbo et al. 2006).

In beer manufacture, haze development is an outcome of the proteins from autolyzed yeasts, dead bacteria from malt, presence of glucan from modified

malt, oxalate from calcium deficient worts, residual starch and carbohydrates. In the breweries, fungal acid proteases are investigated to degrade proteins (related to haze formation) during storage. *A. niger* acid proteases effectively prevent chill-haze formation in beer, due to the hydrolysis of proline-rich proteins, thereby liberating a peptide fraction that fails to form a complex with the polyphenols (Steiner et al. 2010).

In a recent investigation, an acid protease (aspergillopepsin I and II) from *A. niger* var. *macrosporus* was used together with flash pasteurization to degrade haze proteins in white wine. Results indicate that addition of the aspergillopepsin directly to the fermentation wort results in a 20% reduction protein (Marangon et al. 2012). At wine-making temperatures, an acid protease (BcAP8) from *Botrytis cinerea* effectively reduces haze and retains its activity after completion of fermentation (Van Sluyter et al. 2013) (Fig. 19.2).

19.6.5 Alkaline Proteases

19.6.5.1 Sources

Olivieri et al. (2002) identified and characterized a serine protease from *Fusarium solani* f. sp. *eumartii* that is found to be associated with potato tuber infections. The synthesis of alkaline proteases by *Fusarium culmorum, F. poae*, and *F. graminearum* was reported in grains of barley (Kudryavtseva et al. 2013). When soil sample was cultured from Charm-Shahr, Iran, researchers successfully isolated *Aspergillus oryzae* CH93 that is documented to produce an alkaline protease active at pH 8 and 50 °C (Salihi et al. 2017). The production of alkaline protease by an *Aspergillus flavus* strain using solid-substrate fermentation is considered as a depilatory agent (Malathi and Chakraborty 1991).

Aspergillus clavatus was isolated from the Atlantic forest soil, Peruíbe city, SP, Brazil. This strain shows the highest extracellular proteolytic activity that is primarily an alkaline protease (Tremacoldi and Carmona 2005). An alkaline metalloprotease produced by *Aspergillus niger* C-15 was purified and the optimum pH and temperature for the protease activity are pH 8.0 and 60 °C, respectively (Kim 2004). When cultivated in the presence of collagen (200 μg/ml) as sole nitrogen and carbon source, *Aspergillus fumigatus* secretes an inducible alkaline protease (AlPase) with maximum activity at pH 9.0 against azocollagen (Monod et al. 1991) (Table 19.2).

19.6.5.2 Screening

Aspergillus nidulans, *A. glaucus*, *A. terreus*, and *A. fumigatus* isolated from mushroom compost and cow manure were screened for their alkaline protease activity on Czapek-Dox agar media containing gelatin. Following incubation (45 °C, 48 h) resulting in the growth of organism, the plate was flooded with mercuric chloride solution. Presence of a clear zone around the colony was an indication of an alkaline protease activity (Singhania et al. 2018).

Table 19.2 Fungal sources of alkaline protease and (Sharma et al. 2019)

Organism	Source	pH	Temperature (°C)
Myceliopthora sp.	Poultry soil	9.0	40–45
Penicillium sp.	Soil	9.0	45
Aspergillus niger C-15	Poultry soil	8.0	60
Aspergillus clavatus	Forest soil	9.5	40
Aspergillus parasiticus	Sputum sample	8.0	40
Aspergillus terreus gr.	Agricultural soil	11.0	50
Aspergillus nidulans HA-10	Poultry soil	8.0	35
Pleurotus citrinopileatus	Fruiting bodies	10.0	50

In order to screen *Trichoderma longibrachiatum* and *A. niger* for their ability to produce alkaline protease, the cultures were spot inoculated at the centre of casein agar, skimmed milk agar, and gelatin agar (supplemented with 1 mg/100 ml streptomycin), respectively. pH of these media was maintained at pH 8.5. Following incubation at room temperature for 3 days, results indicated that both the isolates resulted in >20 mm zone of clearance in the three media (Suryawanshi and Pandya 2017).

Production of alkaline protease by *A. flavus* and *A. niger* was detected by using the plate assay method of Sudarkodi et al. (2015), using gelatin (1% w/v) as a protein supplement in the growth medium. The fungal isolates were spot inoculated on the growth medium. Following inoculation, the petridishes were incubated (28 ± 1 °C, 3 days) and gelatinolysis was observed (as a clearing zone upon flooding the plate with aqueous saturated solution of mercuric chloride reagent) around fungal colonies. The gelatinolysis zone was measured and enzyme activity was calculated using the concept of zone index.

19.6.5.3 Assay

Protease activity in the crude enzyme extract was determined according to the method of Sudarkodi et al. (2015) by using casein as substrate. Casein solution (5 ml, 0.65%) was added as a substrate and the enzyme (1 ml)–substrate mixture was incubated (37 °C, 30 min). The reaction was terminated by addition of trichloroacetic acid solution (5 ml) and the solution was filtered using Whatman No 1 filter paper. The filtrate was mixed with sodium carbonate (5 ml) and followed by addition of Folin Ciocalteus phenol reagent (1 ml of two fold diluted). The resulting solution was incubated in dark (30 min, room temperature) and the absorbance measured at 660 nm against a reagent blank using tyrosine standard.

The *Aspergillus flavus* alkaline protease activity was assayed by the method of Lovrien et al. (1985). Reaction mixture (3 ml) containing casein (0.5% in 2.95 ml of 0.1 M Tris-HCl buffer, pH 8.0) and enzyme (0.1 ml) was incubated (40 °C, 30 min). The reaction was stopped by adding cold trichloroacetic acid (3 ml, 10%). After 1 h, each of the culture filtrate was centrifuged (8000 rpm, 5 min) to remove the precipitate and absorbance of the supernatants was read (540 nm). The amount of

amino acids released was calculated from a standard curve plotted against a range of known concentrations of tyrosine.

19.6.5.4 Purification

While purifying an alkaline proteinase of *Fusarium culmorum*, the concentrated culture medium was centrifuged (1700 × *g*, 5 min) and the supernatant was applied to a P-30 size-exclusion column (2.5 × 70 cm) equilibrated with ammonium acetate (20 mM, pH 5.0). The column was eluted with the same buffer and the fractions that voided the size-exclusion column were combined and subjected to carboxymethyl cellulose cation-exchange chromatography column (1 × 6 cm). Immediately prior to using, the enzyme was subjected to a final HPLC-cation exchange purification step with a Shodex IEC CM-825, 8 × 75 mm column. Following the purification steps, the purified alkaline protease had a protein content of 0.06 mg, enzyme activity of 150 U, specific activity of 2500 U/mg, 11% yield, and 8.3-fold purification, respectively (Pekkarinen et al. 2002).

With the objective to purify an alkaline protease (SAPTEX) of *P. chrysogenium* X5, the broth culture was centrifuged (20 min, 10,000 × *g*). The clear supernatant was heat-treated (10 min, 80 °C), and by centrifugation (9000 × *g* for 30 min) all insoluble materials removed. Ammonium sulphate saturation (between 30 and 50%) was used for supernatant precipitation. Recovery of the precipitate was done by centrifugation (20 min, 9000 × *g*) followed by its resuspension in Tris-HCl (50 mM, pH 8.5) and dialyzed overnight. The obtained supernatant was loaded to a previously equilibrated UNO Q-12 FPLC column (12× 53 mm). The proteins were eluted with the same buffer containing an increasing concentration of NaCl (0–500 mM). The pooled active fractions were concentrated in centrifugal micro-concentrators (30-kDa cut-off membranes). The purification profile of SAPTEX is as follows: enzyme activity, 157 × 10^4; total protein, 20 mg; specific activity, 78,500 U/mg of protein; protein recovery, 35%; 9.3-fold purification, respectively (Omrane Benmrad et al. 2018).

Broth culture of *Trametes cingulata* CTM10101was centrifuged (30 min, 10,000 × *g*); the supernatant was used as the crude serine alkaline protease preparation and submitted to the following purification steps. It was initially saturated up to 40% ammonium sulphate, centrifuged (10,000 × *g*, 20 min), and the supernatant re-saturated with 60% with ammonium sulphate, re-centrifuged, re-suspended in NaCl buffer (10 mM), and dialyzed overnight. The sample thus obtained was loaded on a fast protein liquid chromatography system (7× 35 mm UNO S-1 column previously equilibrated with 20 mM Histidine buffer supplemented with 2 mM $CaCl_2$, pH 5.5). The proteins were eluted with the same buffer containing an increasing concentration of NaCl (0–500 mM). Pooled fractions containing protease activity were concentrated in centrifugal micro-concentrators with a cut-off membrane (10 kDa). The purification profile is as follows: total activity, 141 × 10^4 U; total protein, 15 mg; specific activity, 94,000 U/mg; purification fold, 6.27 and protein recovery, 20%, respectively (Omrane Benmrad et al. 2016).

A halotolerant alkaline serine protease from *Penicillium citrinum* YL-1 (isolated from traditional Chinese fish sauce) when purified by ammonium sulphate

precipitation, dialysis, and DEAE 52-Cellulose column results in a 4.66-fold increase in specific activity (110.68 U/mg) (Xie et al. 2016).

An alkaline protease is reported to be purified from culture broth of *Penicillium expansum* by fractioning with acetone and column chromatography on Sephadex G-100 and DEAE-Sephadex A-50. The purification profile is as follows: total activity, 744 PU; total protein, 12.08 mg; specific activity, 61.59 PU/mg; purification fold, 96.23 and protein recovery, 48%, respectively (Umar 1994).

19.6.5.5 Applications

Fungal alkaline proteases are involved in a variety of applications, primarily in the detergent and food industries. In view of the recent trend of developing eco-friendly technologies, fungal alkaline proteases are envisaged to have extensive applications in leather treatment and in several bioremediation processes. These enzymes are also used to develop high value added products (Kumar and Takagi 1999) (Fig. 19.2).

(a) Detergent industry.
Proteases are one of the standard ingredients of all kinds of detergents ranging from those used for household laundering (Nehra et al. 2002) to reagents used for cleaning contact lenses (Anwar and Saleemuddin 2000) or dentures. The ideal detergent protease should possess substrate specificity to facilitate the removal of a large variety of stains due to food, blood, and other body secretions. Activity and stability at high pH and temperature and compatibility with other chelating and oxidizing agents added to the detergents are among the major prerequisites for the use of proteases in detergents. A number of published reports are available on the compatibility of the fungal alkaline proteases with detergents (Pundir et al. 2012). The fungal alkaline protease *Conidiobolus coronatus* was consistent with commercial detergents used in India while maintaining 43% of its activity in the presence of calcium (25 mM) and glycine (M). These data imply that protease obtained from *C. coronatus* has potential for use in laundry detergents (Lourdes et al. 2014) (Fig. 19.2).

(b) Leather industry.
Alkaline proteases with elastolytic and keratinolytic activity have been used in leather processing, especially for the dehairing and debating of skins and hides. The enzymatic process is easy to control, less time consuming, and also helps in waste management and is therefore eco-friendly. In addition enzymatic treatment destroys undesirable pigments and increases the skin area, thereby producing clear hide (Arora 2003). Few fungal alkaline proteases have been reported to be suitable and find application in leather industry (Malathi and Chakraborty 1991; Pal et al. 1996) (Fig. 19.2).

(c) Dairy industry.
The major application of proteases in the dairy industry is in the manufacture of cheese, proteolysis is responsible for characteristic of most varieties and is indispensable for good flavour and textural development (Fox 1982). Perea et al. (1993) have used alkaline protease for the production of whey hydrolysate from the cheese whey although rennet is generally the enzyme of choice for

cheese making. In the dairy industry, bovine rennet is still widely used in making cheese; fungi such as *Rhizomucor miechie*, *R. pusillus*, and *A. oryzae* are extensively used for the production of proteases for use as milk coagulants (Neelakantan and Mohanty 1999). Another extract with the powder of coagulating milk, already produced industrially, is derived from the fungus *A. niger var. awamori* (Neves Souza and Silva 2005) (Fig. 19.2).

(d) Pharmaceutical industry.
The wide diversity and specificity of fungal protease are used to great advantage in developing effective therapeutic agents. Oral administration of protease from *Aspergillus oryzae* has been used as a digestive aid to correct certain lytic enzyme deficiency syndromes (Rani et al. 2012). Alkaline proteases have also been used for developing production of medical use, such as for the treatment of burns and purulent wound. *A. niger* LCF 9 alkaline protease has a high collagenolytic activity and is being used for therapeutic application (Kumar and Takagi 1999). Proteases from the tested *Aspergillus* strains exhibited promising hydrolytic activities towards fibrinogen, fibrin, and blood clot (El-Shora and Metwally 2008). *Aspergillus* protease can be used as digestive aids in gastrointestinal disorders such as dyspepsia (Fig. 19.2).

(e) Brewing industry.
In brewing, proteases have two major applications. They can be used during the cereal mashing process to increase the yield of extract. Though papain, bromelain, and papsin are the traditionally used proteases in chill proofing process, microbial proteases also have been reported to be useful. The rennet produced by *Mucor pusillus* has been reported to be effective for beer clarification (Nelson and Witt 1973) (Fig. 19.2).

(f) Waste management.
Waste from poultry and leather industry is keratin rich whose polypeptide is densely packed and stabilized by several weak interactions in addition to disulphide bonds. Fungal keratinases from *Aspergillus oryzae*, *Chrysoporium inducum*, *Trichophyton* sp., *A. terreus*, *Microsporum gypseum*, *Fusarium oxysporum* have also been studied towards the degradation of keratin (Kim 2003; Ali et al. 2011; Sharma and De 2011).

19.7 Conclusions

The use of extremophilic fungal proteases in industries has been an integral event for decades together and some of these fungal cultures are efficient producers of many hydrolytic enzymes. The fact that such proteases exhibit specific range of action and vast diversity in terms of being active over a very wide range of experimental conditions has made them attractive models for industrial usage. Besides being widely distributed in nature, extremophilic fungi are preferred over their mesophilic counterparts owing to their ability to tolerate harsher growth conditions and to overproduce hydrolytic enzyme with extraordinary properties. The search for novel extremophilc fungi as producers of proteases is a thrust area of research

since such extremophilc proteases find major applications in modern day food processing, beverage production, animal nutrition, leather and textiles processing, detergent manufacture, etc. However, only 5% of the protease producing extremophilc fungi have been studied in great detail and applied in large scale processing facilities. Thus in future, there lies a great opportunity to engineer proteases using recombinant DNA technology for heterologous expression, site-directed mutagenesis, and directed evolution of proteases with improved properties and catalytic efficiency to meet newer industrial demands.

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Extremophilic Fungal Amylases: Screening, Purification, Assay, and Applications

20

Ragini Bodade and Krutika Lonkar

Abstract

Extremophiles are the microorganisms that grow under extreme environments of high and low pH, high and low temperatures, deficient nutrient conditions, high atmospheric pressure, high radiations, and low water availability. Development of adaptation mechanism including strategies for survival, protection, perpetuations, and metabolic diversity makes them to survive under these extreme conditions. Moreover, studies through advanced techniques viz. proteomics, genomics, and metabolomics revealed the production of many extremozymes and extremolytes and their role in survival strategies. Microorganisms including bacteria and archea are explored for extremolytes/extremozymes compounds for their commercial production; however, fungi needs more attention being equally metabolic diverse and easy to cultivate. Extremozymes are stable and more catalytically active at these extreme conditions, and thus have immense biotechnological applications. Amylase, an important enzyme responsible for catalysis of starch to monomeric sugars, thereby plays an important role in many industrial applications. This chapter describes the status of isolation, screening, purification, and industrial applications of fungal amylases in the last few decades.

Keywords

Extremophilic fungi · Extremozymes · Amylases · Isolation · Purification · Assay · Applications

R. Bodade (✉) · K. Lonkar
Department of Microbiology, Savitribai Phule Pune University, Pune, Maharashtra, India
e-mail: ragini.bodade@unipune.ac.in

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20.1 Introduction

"Everything is everywhere: but, the environment selects," a well-known formulated quote was described for the distribution of extremophilic microorganisms around the world by the microbiologist Lourens Baas Becking in his book *Geobiologie of Inleiding Tot De Milieukunde* in 1934. This revealed the ubiquitous nature of microorganisms. From an anthropocentric point of view, physicochemical parameters that deviate from the supporting mammalian or terrestrial life forms have considered as extreme viz. pH (above 9 and below 4), temperature (range of −2 to 20 °C and 60 to 300 °C), salt concentration (3–30% NaCl), UV-gamma radiations (above 5 gy), and other factors. In 1974, MacElroy proposed the term "extremophile" to define a broad group of organisms that thrive optimally under these above-mentioned extreme physiochemical and geochemical conditions, however, still has Cavitate and lies in the eye of beholders. Some organisms can tolerate these hostile conditions to some extent and are labeled as extremotolerant. Moreover, an organism growing in more than one extreme condition is termed as polyextremophiles. Various adaptive features togetherly at the cellular level make the extremophiles sustain in one or more of these extreme conditions (Selbmann et al. 2013; Ma et al. 2010; Zhang et al. 2018).

Amylases are widely distributed in microorganisms, plants, and animals. Being a hydrolase enzyme, amylase is responsible for the conversion of starch into simple sugars or oligosaccharides. Structurally starch is a branched polysaccharide of glucose monomer and consists of two polymers amylopectin and amylose. Amylopectin constitutes 70–80% of starch containing branched glucose chain at every 15–45 glucose units linked by α-1,6-glycosidic bonds, while amylose creates 20–25% of starch made by linear chain of glucose with α-1,4-glycosidic bonds. There are two types of hydrolases: endo-hydrolase and exo-hydrolase. Endo-hydrolases cleave the chain from the interior of the substrate molecule, whereas exo-hydrolases cleave from the terminal non-reducing ends. Nearly about 300 amylases are known to date. Depending upon the site of action, structure, and mode of starch hydrolysis, amylases are of three types: α-amylase, β-amylase, and γ-amylase (Table 20.1). TakkamylaseA (TAA) from *Aspergillus oryzae* is studied first time for its catalytic and gene regulatory function (Sundarram and Murthy 2014).

α-Amylases (α-1,4-glucan-4-glucanohydrolase; EC 3.2.1.1), an endo-enzyme belonging to glycoside hydrolase family-13 (GH), catalyze the hydrolysis of internal α-1,4-glucosidic linkage in glycogen, starch, and various malto-oligosaccharides to produce glucose, maltose, and maltotriose units. Other amylases belong to GH family no. 57 and 119. α-Amylase is a metalloenzyme and contains Ca^{2+} that maintains most of the amylase structure and activity while other amylase forms require divalent ions such as Mn^{2+}, Zn^{2+}, and Fe^{2+}. Fungal α-amylases are of great significance for bioconversion processes such as liquefaction, saccharification, and isomerization in food, fermentation, detergent, textile, and paper industries and thus share 30% of the total market. It is also used for desizing processes, such as bio-finishing, stone washing, and surface fibril stain removal along with cellulases

Table 20.1 Types of amylases

Type of amylase	Reaction catalyzed	Product	Source	Reference
α-Amylase (EC 3.2.1.1)	α-1,4-Glycosidic linkages	Glucose and small chain oligosaccharides	Animals, plants, microbes	El-Enshasy et al. (2013)
β-Amylase (EC 3.2.1.2)	α-1,4-Glycosidic linkages	Maltose and β-limit dextrins	Bacterial, fungal, plants	
Glucoamylases or γ-amylase (EC 3.2.1.3)	α-1,4-Glycosidic linkages α-1,6-Glycosidic linkages	Glucose, maltose, isomaltose	Animals, plants, microbes	

(Han et al. 2013; Kato et al. 2007). β-Amylases (EC 3.2.1.2) are exo-hydrolase of plant origin responsible for catalyzing the non-reducing end of the substrate to form maltose and β-limit dextrins and are incapable to hydrolyze the branched linkages in glycogen or amylopectin. γ-Amylases (EC 3.2.1.2) cleave α-1,6-glucosidic linkage as well as the last α-(1–4) glycosidic linkages at the non-reducing end of amylose and amylopectin to form glucose. A wide variety of microorganisms are isolated, screened, and studied from different niche with novel characteristics (Sundarram and Murthy 2014; El-Enshasy et al. 2013; Kour et al. 2019).

20.2 Isolation and Screening

Extremophiles are found almost in all three domains such as Archea, Eubacteria, and Eukarya, while fungi revealed most versatile entity among all the eukaryotes (Berde et al. 2019). A plethora of research on distribution and adaptation of extremophilic fungi confirmed their existence in hypersaline, extreme cold, and hot environments of the earth. They have been isolated from deep oceans, dry rock surfaces, snow and ice, permafrost, salterns, and lakes and compost soil, heated soil, manure, nuclear reactor effluents, mines, agricultural residues, and vegetation associated with these extreme environments. Many investigators have isolated extremophilic fungi for commercial production of extremozymes and extremolytes (Gostincar et al. 2009; Patel and Rawat 2021).

Temperature is one of the extremely important environmental factors that plays a pivotal role in the survival, growth, distribution, and diversity of microorganisms on the surface of the earth. Those fungi that show growth up to 60 °C and grow optimum between 45 and 50 °C temperatures are called thermophilic fungi. Also, few fungi grow up to 50–60 °C but optimally at or below 20 °C, termed thermotolerant. Historically scientist Hugo Miehe isolated thermophilic fungi from self-heating damp haystacks during his study on stored agricultural products followed by scientist Kurt Noak from other natural substrates. Diverse types of the thermophilic fungi have been reported from different soil types, pond sediments,

composting systems, power plant cooling system, heaped masses of plant materials, and piles of agricultural and forestry products belonging to phylum Phycomycetes, Zygomycota, Ascomycota, fungi imperfecta, and Basidiomycota. These habitats are mainly characterized with required temperature, water activity, humidity, and aerobic environments that support the fungal development. Thermophilic fungi from taxonomic genus *Canariomyces*, *Chaetomium*, *Coonemeria*, *Corynascus*, *Dactylomyces*, *Malbranchea*, *Melanocarpus*, *Myceliophthora*, *Myriococcum*, *Paecilomyces*, *Rhizomucor*, *Remersonia*, *Scytalidium*, *Stilbella*, *Talaromyces*, *Thermoascus*, *Thermomyces*, and *Thielavia* are described from advanced sequencing methods viz. 18S rDNA, metagenomics, and ITS sequencing. In laboratory conditions the thermophilic fungi can be cultivated in media supplemented with yeast extract, ammonium sulfate, glucose, and an inducer (L-asparagine and succinic acid) at pH 5.5–7 under aerobic conditions. Adaptations in thermophilic fungi exhibited at cytoplasmic, nuclear, and cell membrane levels. Thermophilic fungi incorporate high content of saturated and long fatty acids in their phospholipid monolayer with ether linkages. At cytoplasmic level formation of heat shock proteins, thermostable enzymes, and low metabolite transportation across the membrane revealed as prominent adaptation as compared to mesophilic fungi. Moreover, adaptation for reduced genome size, high G:C content, small sized introns, loss of transposable elements, upgradation of hyphal melanization gene, and protein coding genes carried at nucleus level. A wide industrial application of extremophiles described for the production of extremozymes. Extremozymes are biocatalysts that work at high salt concentrations and low and high temperature conditions, thus having commercial interest. Thermophilic fungi produces thermostable enzyme viz. cellulases, xylanases, amylases, lipases, and proteases with great catalytic activity and stability at high temperatures due to their ionic interaction, self-aggregation capability, hydrophobicity, and compact conformations. The enzymes are therefore applied for reducing the viscosity of media, for hydrolysis of complex plant biomass at extreme culture conditions (salt and temperature), and to avoid the contaminations (Maheshwari et al. 2000; de Oliveira et al. 2015; Patel and Rawat 2021).

In contrast, psychrophiles are a group of microorganisms that exhibit growth at or below 0 °C and optimum growth between 15 °C and ≤20 °C cold temperature. However, psychrotolerant fungi grow close to 0 °C and require an optimum temperature at ≤15 °C and a maximum temperature above 20 °C for development. Psychrophiles may be obligate or facultative in nature. Earth covers diverse cold habitats comprising deep sea to high mountains and from Antarctica to Arctic region with 5 °C temperature seasonally or permanently. Out of 85% of these biosphere most other habitats are covered by oceans (~71%), terrestrial environments covering snow (~35%), frozen ground (~24%), sea ice (~13%), and glacier (~10%) with −1 to 4 °C temperature. Moreover, other low temperature environments comprise permafrost, cold soils, lakes, caves, cryoconite glacier holes, and cold deserts. A wide variety of yeasts, water molds (Chromista group), and filamentous fungi (Helotiales/Pleosporales/Articulospora and Varicosporium) were isolated from cryoconite sediments and Antarctica. The isolated fungal/yeast genus are from *Thielavia*, *Geomyces*, *Apiosordaria*, *Cadophora*, *Penicillium*, *Aspergillus*,

Fusarium, *Curvularia*, *Cladosporium Cylindrocarpon*, *Golovinomyces*, *Phoma*, *Antarctomyces*, *Thelebolus*, *Mortierella*, *Pythium*, *Sordariomycetes*, *Mycopappus*, *Mrakia*, *Tetracladium*, *Phaeosphaeria*, *Venturia*, *Eurotiomycetes*, *Leotiomycetes*, *Tremellomycetes*, *Alternaria*, *Lecanoromycetes*, *Arthoniomycetes*, *Geomyces*, *Phialophore*, *Acremonium*, *Rhizosphaera*, *Candida*, *Cyptococcus*, *Rhosospoidium*, *Pichia*, *Rhodotorula*, *Sporobolomyces*, and *Trichosporon*. In most of these marine ecosystems and at cold environment, psychrophilic fungi/yeast contributes in recycling of nutrients and organic matter mineralization (geochemical cycles). Adaptation in psychrophiles under a cold environment involved the production of antifreeze proteins, cold shock proteins, nucleation proteins, compatible solutes (glycerol and mannitol), cryoprotectant (trehalose and polyols), photo-protective pigments (melanin), exopolysaccharide, and unsaturated branched fatty acid chains in the cell membrane. The main adaptation of cold adapted enzymes from psychrophiles is increase in flexibility and high site complementarity for substrate with great catalytic activity. The structural flexibility can be achieved by more glycine residues in surface loops, increased α-helices secondary structure, increased surface hydrophobicity, reduced number of core hydrophobic amino acid (lysine, arginine, and aromatic amino acids), and reduced ionic and electrostatic interactions. Various extracellular enzymes like DNase, protease, phosphatase, amylase glucose oxidase, lipases, and polygalacturonase have been reported with promising catalytic activity than mesophilic enzyme (Hassan et al. 2016; Duarte et al. 2018).

Halophiles are the extremophiles capable of surviving in salt rich environments. The word halophile is derived from the Greek, meaning "salt loving." Fungi showing optimum growth in hypersaline habitats, i.e., at NaCl concentrations above 1.7–3 M salt concentration are categorized as halophiles. They may be slight halophiles (0.3–0.8 M NaCl), moderate halophiles (0.8–3.4 M NaCl), and extreme halophiles (3.4–5.1 M NaCl) depending upon the salt requirement for optimum growth. These microorganisms can sustain in high salt concentrations as well as other ions, UV radiation, and extreme pH. Most of the halophile microorganisms are isolated and characterized from saline water, saline soil, salt lakes, soda lakes, salted foods, and salterns. Moreover, different foods are supplemented with salts to enhance flavor and as a preservative, thereby supporting the growth of halophiles and halotolerant microorganisms. Halophilic fungi can grow in a wide salinity range from freshwater to saturated NaCl solutions (1.7–3 M). Fungal halophiles have been mostly isolated and characterized from the above salty habitats from genus *Cladosporium*, *Aspergillus*, *Penicillium*, *Emeicella*, *Alternaria*, *Scopulariopsis*, *Eurotium*, *Aureobasidium*, *Debaryomyces*, *Phaetotheca*, *Trimmatostroma*, and yeast like *Wallemia* and *Hortaea*. Adaptation in fungi including accumulation of cytoplasmic compatible solutes (erythritol, ribitol, arabinitol, xylitol, sorbitol, mannitol and galacticol, glycerol, trahlose, glutamic acid, alanine, and mycosporin), enhanced secondary transporters/ionic pumps (Na^+/H^+ and Na^+/K^+) and low sterol-to-phospholipid, short fatty acid chains, ration in membrane are few important strategies. Halophilic fungi mostly maintain the intracellular osmotic balance using glycerol, trahlose, and other organic compatible solutes in their cytoplasm. Proteins with high hydrophobicity, increased acidic residues, and reduced helices make

proteins and enzymes more stable in high salt concentrations. Many of the halophilic fungi are reported for the production of bioactive compounds, enzymes, in bioremediation (Gunde-Cimerman et al. 2009; Ali et al. 2019).

pH is another important parameter for the growth of microorganisms. Most of the microorganisms easily thrive at neutral pH conditions. However, pH condition is not the same throughout the surface of earth. There are places where the pH ranges from 0 to 4 and 11 to 14, and still some microorganisms are seen thriving with ease. Acidophiles, a class among extremophiles, are capable of growing under acidic conditions, i.e., below pH 4. Acidic natural environments having pH range from 3 to 4 are relatively common and include acid sulfate soils, acidic lakes, hydrothermal systems, mining effluents, coal tips, drainage water and industrial waste water, metal and coal mines, swamps, and peat bogs. Acid-tolerant yeasts like *Rhodotorula* spp., *Candida*, *Cyptococcus*, and *Leucosporidium*, from acid mine drainage (AMD) and from sandy soils are reported. Filamentous fungi *Bispora* sp., *Purpureocillium lilacinum*, *Schizoblastosporion*, *Trichosporoncerebriae*, *Fusarium*, *Aspergillus*, *Penicillium*, *Verticillium*, *Bipolaris*, *Acontiumcylatium*, and *Cephalosporium* sp., *Teratosphaeria acidotherma*. Under these acidic conditions the fungi maintain the pH toward neutrality by pumping the protons out of the cell and having low proton membrane permeability (Gross and Robbins 2000; Johnson 1998).

The microorganisms exhibiting growth in alkaline conditions where the pH is between 8 and 11 and optimally at 9 or above but not at neutral pH are grouped under alkaliphiles. Alkaliphiles are found mainly in hypersaline soda lakes; hallow hydrothermal systems, alkali thermal hot springs, soda soils, and sewage. Different isolated fungi viz. *Acremonium* sp., *Stilbella*, *Veticeillium* sp., *Paecilomyces*, *Metarhizium* sp., *Fusarium* sp., *Phialophora* sp., *Scopulariopsis* sp., *Mucor* sp., *Gliomastric* sp., *sodiomyces* sp., *Acrostalagmus* sp., *Emericellopsis* sp., *Thielavia* sp., *Alternaria* sp., *Chordomyces* sp., *Acrostalagmus* sp., *Scopulariopsis* sp., and *Cladosporium* sp. are reported from soda lakes and soda soils using cultured media buffered at pH 5–11. Neutralizing the cytoplasm pH by Na^+/H^+ and K^+/H^+ anti-ports with continuous H^+ influx through ATPase revealed prominent adaptation in alkalophiles. Accumulation of compatible solutes mannitol and arabitol are also an adaptation for survival of microbes (Kladwang et al. 2003; Grum-Grzhimaylo et al. 2016).

The adaptations in the extreme conditions of such organism are some extent due to well-adapted cellular biomolecules and product of the metabolic pathways. Interestingly, some of these compounds show polyextremophilicity, i.e., stable and catalytically active in more than one extreme condition. The products are referred to as extremolytes/extremozymes viz. form of polysaccharides, proteins, primary/secondary metabolites, and enzymes. Though more than 3000 different enzymes for biotechnological and industrial applications have been identified till date, still the present enzyme stock fail to meet all demands like increased catalytic activity and stability at extreme conditions (pH, temperature, salinity, pressure, and organic solvents), enhancing the solubility of substrates, high mass transfer rate, and having low risk of pollution during the process. The efficiency of these enzymes is improved by genetic engineering and chemical modifications as well as by immobilization

methods. Still search of novel enzymes from different niches with desired characteristics for various indusial and biotechnological process is required. Therefore, characterization of extremophiles that are capable to withstand in extreme environment has drawn great attention. Such extremophiles can be a valuable source of novel extremozymes (Niehaus et al. 1999; Van Den Burg 2003; Singh et al. 2016; Dumorne et al. 2017).

Many investigators are carrying out a plethora of research to find novel fungal cultures for amylase producer from different niches. Fungal amylase producers are preferred for use in food and pharmaceutical industries due to their acceptable characteristics like saccharifying efficiency, optimal conditions, and wide substrate specificity and product. Fungi can grow on enrichment media like potato dextrose agar, starch-yeast extract agar, malt extract agar, Saboraud dextrose agar, agricultural waste materials, starchy foodstuffs, and wastewater. Several methods and technologies are reported for studying the dynamic of extremophiles, such as quantitative polymerase chain reaction (PCR), RNA stable isotope probing, proteomics, genomics and metagenomics approach, microarray techniques, genetic engineering, and recombinant technology (Singh et al. 2019).

The isolates of filamentous fungi were collected from different locations and were enriched in their respective conditions. The selected fungi were examined for amylase activity in starch agar medium, potato dextrose agar, yeast nitrogen base agar, malt extract agar, Saboraud dextrose agar, and Czapek dox agar containing 0.5–2% starch. Halophilic fungal culture isolation required 5–10% of NaCl salt. After the incubation, the amylolytic activity was assessed by flooding the plates with potassium iodide solution. The activity of α-amylase can be investigated based on the principle that starch and iodine react to form a blue colored complex. The ratio of clear zone around the growth to colony indicates amylase-producing isolates (Niknejad et al. 2013; Mukherjee et al. 2019).

20.3 Assay of Amylase

Alpha-amylase cleaves internal −1,4-glycosidic linkages of starch to produce glucose, maltose, or dextrins, while glucoamylase cuts −1,4- and −1,6-glycosidic linkages to release glucose from the non-reducing ends of starch. Both the enzymes are widely used in the industries for the conversion of starch into sugars. Amylases and glucoamylases are characterized by paper chromatography, high-performance liquid chromatography, and thin-layer chromatography. Amylase activity is detected using broth supernatant or crude extract by qualitative and quantitative assays. Qualitative detection of starch hydrolysis mediated by α-amylase or glucoamylase was done by paper chromatography. Using Whatman no.1 filter paper at room temperature. The chromatogram is spot inoculated with hydrolysate, developed by solvent butanol:acetic acid:water (4:1:5 v/v), and visualized by silver nitrate dip (Spencer-Martins and Van Uden 1979).

Quantitative enzyme assay measures the reducing sugar formation as an action of amylase on starch (Dinitrosalicylic acid assay or the Nelson–Somogyi assay).

Reducing sugars are sugars that contain aldehyde or ketone group (glucose, galactose, lactose, and maltose). Different substrates viz. glycogen, pullulan, amylopectin, and amylose can also be used to check the hydrolysis by amylase and glucoamylases (Xiao et al. 2006).

20.3.1 DNS Method

In the 3,5-dinitrosalicylic acid method (DNSA), the aliquots of the substrate (1–2% starch) solution is incubated with amylase enzyme for 10–15 min at 30–50 °C. DNSA reagent (chromogen) is added after incubation in the reaction mixture and placed in boiling water bath for 5–10 min. Under alkaline condition (pH 7.5–8.5) the 3,5-dinitrosalicylic acid is reduced to 3-amino-5-nitro salicylic acid .Conversion of yellow to brick red color indicates enzyme catalytic activity. After cooling to room temperature, 10 mL of water was added and the absorbance of the reaction mixture is read at 540 nm. One unit (U) of enzyme activity was defined as the amount of enzyme that produced 1 μmol of glucose per minute under standard assay condition (Ali et al. 2015; Gusakov et al. 2011).

20.3.2 Nelson–Somogyi Method

The Nelson–Somogyi method is also used for the quantitative determination of reducing sugars. The enzyme is mixed with aliquots of 0.5% starch solutions in 50 mM Tris–HCl buffer (pH 9) and whole reaction mixture is incubated at 30–50°C temperature for 10 min. After incubation, Somogyi reagent (alkaline copper tartrate) is added and heated in boiling water bath for 20 min. The reaction is terminated by addition Arsenomolybolic acid reagent. Formation of yellow green to blue green color indicates positive enzyme activity or starch hydrolysis, which can be estimated spectrophotometrically at 620 nm. When the reducing sugar is heated with alkaline copper tartrate solution, it reduces copper to cupric to cuprous (cuprous oxide). Cuprous oxide reacts with arsenomolybdic acid and forms blue–green colored molybdenum (Kobayashi et al. 1992; Nelson 1944; Somogyi 1945).

20.3.3 Glucose Oxidase-Peroxidase Assay (GOPOD)

A reliable, simple, micro-volumetric, rapid, and high-throughput analytical method was developed for the detection and quantification of amylase/glucoamylase activity using the glucose oxidase-peroxidase assay (GOPOD). The reaction mixture contains amylase enzyme, aliquots of buffered starch, or maltose (0.2%) and incubated at 30 °C for 20 min. The formed maltose/glucose from starch hydrolysis can be detected by glucose oxidase-peroxidase method. The maltose/glucose was catalyzed by glucose oxidase (GOD) to form gluconic acid and hydrogen peroxide. The product hydrogen peroxide was estimated by 4-aminoantipyrine and phenol in

the presence of peroxidase enzyme after converting it to a quinone. The formed chromogenic compound quinone is estimated by absorbance at 520 nm. The test is recommended for screening amylase inhibitors and for glucoamylase activity detection (Visvanathan et al. 2016; Spencer-Martins and Van Uden 1979).

20.3.4 Hugget and Nixon Method

In this method glucoamylase/amylase activity was determined using p-nitrophenyl-α-D-glucopyranoside, the chromogenic substrates. The substrate is reacted with crude enzyme in buffered reaction mixture (0.05 M citrate phosphate buffer, pH 5.2) for 30 min at 50°C. The released glucose is measured by GOPOD method. α-Glucosidase/amylase unit was defined as the amount of enzyme which liberates 1 μM of glucose per minute (Gautam and Gupta 1992). Other substrate ethylidene-pNP-G7 is cleaved by α-amylase results in the release of the chromophore (p-nitro phenol), and that can then be measured at 405 nm (Carrasco et al. 2017). All these different methods are based on estimation of formed glucose by amylase/glucoamylase catalytic activity. Details of other methods used to estimate glucose concentration directly from the different biogenic samples are Otoluidine reagent, Folin Wu reagent, glucose oxidase (GOD), and hexokinase enzyme-based methods. Following addition of *O*-toluidine reagent (6% v/v in glacial acetic acid) in reaction mixture and heating at 100 °C for 10 min formation of stable green color is estimated by reading absorbance at 630 or 635 nm that gives information about the glucose concentration (Passey et al. 1977).

20.3.5 Sandstedt, Kneen, and Blish (SKB) Method

It is used for detection of amylases in bakery industry. The reaction mixture contains buffered starch solution (0.5%) and enzyme solution, and the mixture is incubated at 30 °C for 5 min. After incubation, 1 mL of iodine reagent was added. Transmittance (T) at 660 nm was measured. One SKB unit is the amount of enzyme that will dextrinize starch under assay conditions. The method expresses diastatic strength of the malt (Ambade et al. 1998; Attia and Ali 1977). A similar method for determining amylase activity in cereals and cereal products is described by Indian pharmacopeia (ISI). Different aliquots of buffered starch and fixed volume of enzyme are incubated at 40 °C for 1 h. Addition of iodine solution confirms the enzyme activity. The starch aliquot without blue colorization indicates the enzyme activity in terms of grams of starch digested (Gupta et al. 2003).

20.3.6 Fuwa Method

Another assay method is based on the decreased staining value of blue starch–iodine complexes, i.e., Fuwa method. In the Fuwa method, the reaction mixture contains

2.5 mL of buffered-amylase and 2.5 mL amylose (2 g/L), which is incubated at 37 °C for 30 min. After incubation, 5 mL of 1 N acetic acid is added to terminate the reaction. After cooling, the mixture is then transferred into a 250 mL flask and diluted to nearly 200 mL with water, followed by the addition of 5 mL of iodine reagent (0.2% iodine and 2% potassium iodide). The amount of color development is later determined by measuring the absorbance at 700 nm. Dextrinizing activity is then calculated using the formula $D = (D_0 - \mathrm{D}) \div D_0 \times 100 \div 10$, where D is the absorbance of the enzyme sample and D_0 is the absorbance of the amylo-sugars degraded by amylase action. Control is without the addition of enzyme (Fuwa 1954).

20.3.7 Spencer-Martins Method

According to Spencer-Martins and Van Uden's reported method, 1 mL of the culture filtrate is incubated with 3 mL of 1% buffered soluble starch solution (0.05 M citrate-phosphate buffer, pH 5.5). The reaction mixture is heated to 40 °C for 15 min, and then 0.5 mL is pipetted into 5 mL of iodine solution (0.15 iodine in 0.5% potassium iodide solution) and diluted with distilled water to 20 mL. The formed blue color read at 550 nm. An α-amylase unit is defined as the quantity of enzyme, mediating 0.1 ΔE 1 cm/550 nm under standard assay conditions (Spencer-Martins and Van Uden 1979; Smith and Roe 1949; Gautam and Gupta 1992).

20.3.8 Viscometric Assay Method

In viscometric assay method decrease in viscosity of starch solutions during enzymatic reaction is measured. At low concentrations, starch solutions have high viscosity. In the assay, the viscosity is reduced as the molecules are degraded by the action of amylase. This method requires rapid visco analyzer. Viscosity of cassava starch resulting after enzyme addition was rapidly measured with respect to time. The enzyme activity in terms of decreasing viscosity of starch suspension determined by falling number (FN) method and amylograph/farinograph test. In the falling number method, the enzyme substrate preparations are assayed at 100 °C. The amylograph test employs the principle of the relationship between peak viscosity of starch slurry and the enzyme activity. The lesser the viscosity of the starch slurry, the more the activity of the enzyme (McCleary et al. 1997; Gonzalez et al. 2002).

20.4 Purification of Amylases

The industrial production of α-amylase was carried out by submerged fermentation (SmF) and solid-state fermentation (SSF). SmF has been the choice of production because of the ease of control of different parameters such as pH, temperature, aeration and oxygen transfer, and moisture. However, SSF systems provide a

resemblance to the natural habitat for culture to grow. Purification of enzymes is a crucial step and is application specific. Enzymes used for industrial purposes generally require less downstream processing, whereas enzymes used in the application of pharmaceutical or clinical applications are highly purified. Purity is also requiring for enzymatic study, i.e., for structure–function relationships and biochemical properties. Purification methods frequently employed are salt or solvent precipitation (acetone, ethanol, 2-propanol, and ammonium sulfate), ultrafiltration, chromatography (ion exchange, gel filtration, hydrophobicity interactions, and reverse phase chromatography), and liquid–liquid extraction. A combination of all the above methods is followed in a series of steps to yield high grade of enzyme purity. In a study, the 5–14 days grown fungal culture broth was subjected to centrifugation and the supernatant collected. This was followed by ammonium sulfate precipitation with 20–90% salt saturation. This resulted in the precipitation of α-amylase and then followed by series of dialysis, i.e., salting out. The dialysate was then subjected to ion exchange and gel filtration. Different fractions were collected and tested for *α*-amylase activity and total protein content (Fig. 20.1).

Although certain steps require expensive equipment at each step, making them laborious, time consuming, barely reproducible, and may result in increasing loss of the desired product, however, this process is widely employed in the chemical industry due to its simplicity, low costs, and ease of scale up (Souza 2010; Sundarram and Murthy 2014; Ali et al. 2015; Far et al. 2020).

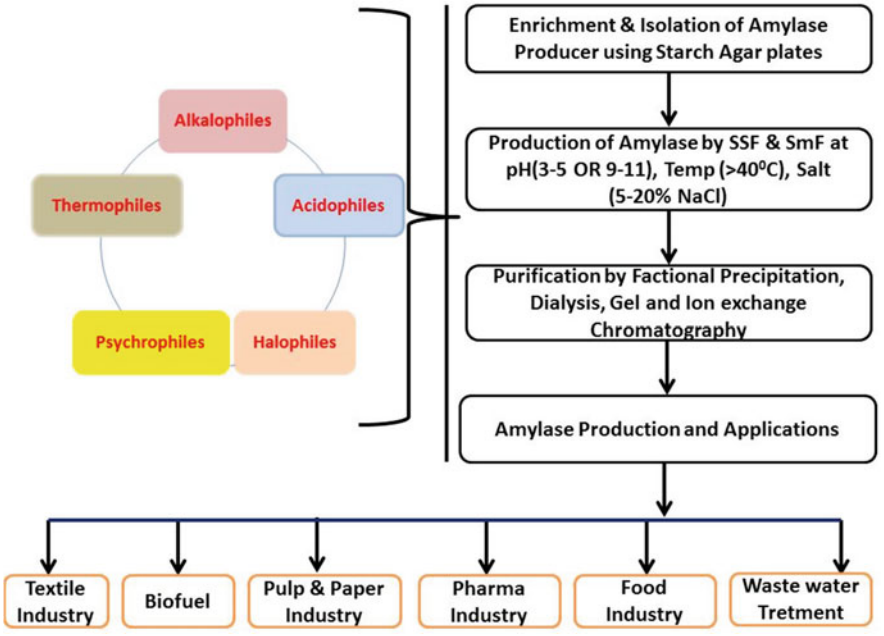

Fig. 20.1 Isolation, purification, and application of α-amylases

Aspergillus species produce a large variety of extracellular enzymes including amylases. *Aspergillus gracilis* TISTR 3638a halophilic fungus was isolated from hypersaline man-made saltern from Thailand for α-amylase production using 10% starch and 10% NaCl. The enzyme revealed polyextremophilic nature with maximum specific activity (131.02 U/mg) at pH 5, temperature 60 °C, and 30% NaCl concentration. Purification of enzyme using ammonium sulfate fractional precipitation (up to 90%) followed by dialysis and Sephadex G-100 columns elution with Tris buffer (100 mM, pH 6) revealed 35 kDa MW protein using SDS-PAGE. Being active at high salt and temperature conditions, it could have application in bioremediation (Ali et al. 2014a). Ali et al. (2015) reported another halophilic *Aspergillus penicillioides* TISTR3639 strain revealed for amylase production (42 kDa protein; specific activity 118.42 U/mg) with maximum activity at pH 9 and temperature 80 °C. The metal $CaCl_2$ was found as activator while $ZnCl_2$ and $FeCl_2$ as inhibitors. As the enzyme is stable at high salt concentration (300 g/L NaCl) and extreme pH and temperature, it has a great potential in detergent industry. The enzyme was purified by Sephadex G100 gel column after eluting with 25 mM Tris–HCl buffer (pH 8) (Ali et al. 2015). Amylase production by *Aspergillus niger* strain UO-1 was carried out in media supplemented with brewery (BW) and meat (MPW) wastewaters along with different starch concentrations. The highest amylase activity was obtained (70.29 and 60.12 EU/mL) in the BW and MPW media supplemented with 40 g of starch/L after 88 h of fermentation respectively. Media supplemented with casamino acids, peptone, or yeast extract further enhances the amylase production. The enzyme was stable 50 °C and pH 4.95 (Hernandez et al. 2006). Unal (2015) isolated thermophilic *Aspergillus niger*, *Aspergillus oryzae*, and *Aspergillus terreus* from soil and water samples collected form Afyon and Eskisehir in Turke. *A. niger* showing the highest activity at optimum temperature 65 °C and pH was 8 (Unal 2015). Acidophilic *Aspergillus niger* RBP7 was isolated from municipal dumping area of Midnapore town, West Bengal, India for the production of α-amylase. Enzyme was produced by SSF using potato peel. The cell-free supernatant significantly hydrolyzes raw starchy foodstuff (taro, yam, malanga, and sweet potato) to form glucose and maltose. Response surface methodology (RSM) enhanced enzyme titer up to 1112.25 U/gds with an initial medium pH 3.0. These characteristics of the enzyme may be suitable for use to treat digestive dysfunction (Mukherjee et al. 2019). Thermophilic two fungal isolates of genus *Aspergillum* produced amylase were isolated from hot spring water and soil samples near to afar Ethiopia. Both the isolates produce amylase at 55 °C after 7 days of incubation with enzyme activity 2.73 ± 0.0 U and 2.51 ± 0.0 U. The enzyme activity was revealed at optimum temperature between 45 and 65 °C and pH 4–10. Enzyme activity was strongly inhibited by Ca^{2+}, Mg^{2+}, and Zn^{2+} (Teklebrhan Welday et al. 2014).

Thermotolerant *Aspergillus fumigatus* isolated from soil samples of Eastern Nigeria produced amylase after 96 h at 30 °C in a mineral medium containing 1% starch and 1.5% organic nitrogen. Sorghum starch as carbon source and inorganic (NH_4Cl)/organic (soybean meal) as nitrogen sources led to higher concentration of amylase in culture fluid. Amylase was optimally active at pH 6.0 and 60 °C temperature. The amylase enzyme was repressed by Fe^{2+} and Mn^{2+} but not inhibited

by Cu^{2+}, CO^{2+}, and Hg^{2+} at a concentration of 2 mM. The highest hydrolytic activity of the *A. fumigatus* amylase was recorded for yam, followed by potato and cassava starches. Another isolate *Fusarium* sp. produced amylase using 1% soybean meal, 0.2% NH_4Cl, and 2.5% corn starch and elicited the highest amylase yield. Optimum pH for the enzyme was 6.5 and at 50 °C. Mg^{2+}, Ca^{2+}, and Zn^{2+} stimulated catalytic function of the crude amylase at 2 mM concentrations. The enzyme also hydrolyzed cassava, potato, and yam starches effectively (Nwagu and Okolo 2011a, b). Ali et al. (2014b) reported *Engyodontium album* TISTR 3645 polyextremophiles fungi from man-made salterns for amylase production. The considerable enzyme specific activity 132.17 U/mg was observed for this 50 kDa amylase. The enzyme was well purified by ammonium sulfate precipitation (90%) and gel elution by Sephadex G 100 with 25 mM Tris–HCl buffer. The enzyme gives maximum activity at temperature 60 °C and pH 9. The enzyme strongly inhibited by $FeCl_2$, $ZnCl_2$, and activated by $BaCl_2$, $CaCl_2$, $HgCl_2$, and $MgCl_2$ (Ali et al. 2014b). *Rhizomucor pusillus* isolated from compost heap, Thailand, produces glucoamylase on solid wheat bran medium at 45 °C. The crude enzyme was purified by ammonium sulfate fractional precipitation (20–60%), followed by Sephadex G-75 gel filtration and DEAE-Sephadex A-50 column eluted with 0.15–0.5 M NaCl solution and finally by Bio-Gel P-100gel column (specific activity 57.7 U/mg). The obtained purified enzyme was 68 kDa with highest activity at temperature 65 °C and pH 4.6 (Kanlayakrit et al. 1987). Thermotolerant *Rhizopus microsporus* isolated from the soil of the Brazilian cerrado region produced high levels of amylase (specific activity 88.25 U/mg) by submerged fermentation using CarvalhoPeixoto (CP) medium at 45 °C and pH 6 after 72 h incubation. Purified enzyme revealed maximum activity at optimum temperature 65 °C and pH 5.0 (Peixoto et al. 2003). Chakraborty et al. (2009) reported amylase from marine *Streptomyces* sp. D1. The enzyme revealed maximum activity (specific activity 113.64 U/mg) at optimum temperature at 85 °C and pH 9. The enzyme has molecular weight of 66 kDa. The enzyme retained 70–35% of its activity up to 7–90 days in the presence of commercial detergent, and 80–100% in the presence of oxidizing agents (sodium hypochlorite and H_2O_2), activated by Ca^{2+} and inhibited by Hg^{2+} and Fe^{3+} ions, and thus has application in detergent industry (Chakraborty et al. 2009). Gaur et al. (1993) reported a comparative amylase production from *Humicola* and *Paecilomyces* sp. *Humicola* sp. produces thermostable amylase enzyme (activity 3.9 U/mL) at 50 °C, while *Paecilomyces* sp. with enzyme activity 4.2 U/mL and optimum stability at 35 °C (Gaur et al. 1993). Thermophilic fungus *Malbranchea cinnamomea* S 168 was studied for novel α-amylase production using 3.5% glutinous rice flour by shake flask culture method after 3 days of incubation at 35 °C temperature with a specific activity 514.6 U/mg. The enzyme was purified with 60–70% ammonium sulfate followed by DEAE gel column elution with Tris–HCl buffer (20 mM, pH 8). The enzyme has a molecular mass of 60.3 kDa, exhibiting maximal activity at pH 6.5 and temperature 65 °C. The enzyme hydrolyses starch, amylopectin, amylose, pullulan, cyclodextrin, and maltooligosaccharides. The enzyme was crystallized and revealed resolution of 2.25 A° with 10 α-helices and 14 β-strands (PDB code 3VM7). The enzyme was activated by Mn^{2+} and Co^{2+} and inhibited by Cr^{3+} and Hg^{2+} (Han et al. 2013).

Another species *Malbranchea sulfurea* was isolated from manuring cow dung and demonstrated for extracellular amylase activity. The enzyme was produced in starch yeast extract medium after 7 days of incubation at 45 °C temperature with crud enzyme activity 21.279 U (Gautam and Gupta 1992). A thermophilic fungi *Scytalidium thermophilum* produced α-amylase of molecular weight 36 kDa and maximum activity at pH 6 and temperature 60 °C by submerged fermentation method. Enzyme purification was carried out by dialyzed crude extract using DEAE-cellulose column, eluted by gradation of 0–500 mM sodium chloride containing 10 mM sodium acetate buffer, pH 5.5, followed by CM-cellulose column and a Sepharose CL-6B gel filtration column. The enzyme was inhibited by $HgCl_2$/$CuCl_2$ and did not show any increased activity by other metals including Ca^{2+}. The α-amylase preferentially hydrolyzed starch, followed by amylopectin, maltose, amylose, and glycogen (Aquino et al. 2003). Another thermophilic fungus *Paecilomyces varioti* produced α-amylase showed optimum enzyme activity at temperature 60 °C and pH 4.0. The enzyme was activated by Ca^{2+} and CO^{2+} with a molecular mass of 75 kDa and pI value of 4.5. The enzyme acts on starch, amylose, and amylopectin as substrate with specific activity 81.1 U/mg. Enzyme purification was done using DEAE cellulose ion exchange column followed by Sephadex 100 G gel filtration (Michelin et al. 2010). In another study, thermophilic fungi were isolated from Apete and Bodija refuse dump sites at Ibadan, Nigeria by Olagoke for amylase production and identified as *Humicola* sp., *Absidia corymbifera*, *Chaetomium elatum*, *Gilmaniella humicola*, *Talaromyces helicus*, *Chaetomium* sp., and *Rhizomucor pusillus*. The amylase enzyme activity was observed at 45 °C temperature and pH 6.9 by all the isolated cultures (Olagoke 2014). In a parallel study from Ethiopia, 61 amylase producers at 45 °C temperature have been isolated from hyperthermal springs named Arbaminch, Awassa, Nazret, Shalla and Abijata, Wendo Genet, and Yirgalem (Haki and Gezmu 2012). Petrova et al. (2000) studied the comparative production of amylase production using a wild and mutant type of thermophilic fungus *Thermomyces lanuginosus* ATCC 34626. The molecular weight of the purified α-amylases was 58 kDa by SDS-PAGE and pI 3. The optimal enzyme activity at pH revealed 5.0 and 4.5 for the wild and mutant respectively. Only Cu^{2+} and Fe^{3+} act as inhibitor and Mn^{2+}, Ca^{2+}, Mg^{2+}, Ba^{2+}, and Cd^{2+}as activator for enzyme activity. Mutant culture revealed 349 mg/U specific enzyme activity. During enzyme purification culture supernatant was precipitated with ice-cold 2-propanol (1:3 v/v), followed by ion exchange chromatography using Mono Q HR 5/5 for FPLC system and lastly by Superose 12 column (Petrova et al. 2000). Mishra and Maheshwai, 1996 isolated similar culture from manure heap. The culture *Thermomyces lanuginosus* IISC91 produces 40 U amylase at 50 °C temperature and is a homodimer protein of MW 42 kDa. The enzyme is active maximally at pH 5.6 and at 65 °C. Enzyme purification was done by ultrafiltration followed by separation using DEAE-Sephadex A-50, UltrogelAcA 54 column eluted with gradation of 0–400 mM NaCI in 50 mM, pH 5 acetate buffer, and 50 mM sodium phosphate pH 8 buffer system. The purity of the protein was enhanced by separation by Bio-Gel P-30 column (Mishra and Maheshwari 1996). Similarly Kunamneni et al. (2005) reported extracellular amylase production by same culture

by solid-state fermentation using wheat brane, rice brane, maize meal, millet cereal, barley bran, and crushed maize. Maximum enzyme activity was obtained using wheat brane (534 U/mg) after 120 h at 50 °C temperature and pH 6 (Kunamneni et al. 2005). A thermotolerant endophytic fungal isolate, *Fusicoccum* sp. BCC4124, showed α-amylase production (25.9 U/mg specific enzyme activities) using agro-industry substrates (wheat-bran soybean, Cassava pulp-soybean, cassava pulp, and mixed media). The crude enzyme filtrates after membrane filtration were purified by gel chromatography using DEAE-Sepharose column with MOPS buffer (50 mM, pH 6.8). The enzyme worked optimally at 70 °C in a neutral pH in the presence of Ca^{2+}. The hydrolyzed product of substrate starch was identified as maltose, matotriose, and maltotetraose as well as glucose, maltose, and maltotriose after prolonged incubations (Champreda et al. 2007). Thermophilic yeast *Cryptococcus flavus* exhibited the highest amylase production (specific activity 842.85 U/mg) in yeast nitrogen base media supplemented with 2% starch. The enzyme purified by Sephacryl S-100 column was equilibrated and eluted with 50 mM acetate buffer (pH 5.5), containing 1 mM $CaCl_2$ and 100 mM NaCl. The enzyme revealed 75 kDa MW having optimum enzyme activity at pH 5.5 and 50 °C temperature. The catalytic activity on starch substrate gives major products amylose and amylopectin; pullulan and glycogen were maltose and maltotriose. Enzyme activity was inhibited by Cu^{++}, Fe^{++}, and Hg^{++} (Wanderley et al. 2004). Fossi et al. (2005) isolated amylolytic yeast strains from starchy soils (flour mills environment, flour market, and cassava farms) by enrichment media using 1% starch (pH 3). Enzyme was produced on different starchy media viz. wheat, corn, potato, rice, cassava, and starch. Eight percent wheat starch gives maximum activity, i.e., 298.5 ± 0.1 U/mL at 30°C and pH 4.5. The enzyme activity revealed maximum at temperature 70 °C and pH 5.5 (Fossi et al. 2005). Cold-adapted yeast *Tetracladium* sp. was isolated and biochemically characterized. The enzyme was produced in yeast extract–malt extract medium (pH 7) supplemented with soluble starch (1%). The culture filtrate was used for ammonium fractional precipitation (20–80%) followed by desalting using a HiTrap desalting column. The enzyme optimal activity at pH 6.0 and 30 °C confirms its cold-adapted nature and has no dependency on Ca^{2+} for its hydrolytic activity. The enzyme can be used for biofuel production (Carrasco et al. 2017). Enzyme α-amylase and a glucoamylase were also reported from *Candida antarcticus* (*Moesziomyces antarcticus*) by De Mot and Verachtert (1987). The amylase enzyme was purified by protamine sulfate treatment followed by ammonium sulfate precipitation, gel chromatography using Sephadex G-75, and UltrogelAcA 54, DEAE Sephacel chromatography, and hydroxyapatite chromatography. While, for glucoamylase the final step was carried out with affinity chromatography on acarbose–AH-Sepharose 4B. Both the enzymes (amylase and glucoamylase) were found to be glycoproteins with molecular weight of 50 kDa and 48.5 kDa, optimum temperatures of 62 ¯C and 57 ¯C, and pH of 4.2 and 4.7, respectively (De Mot and Verachtert 1987).

An Antarctic psychrotolerant fungi *Geomyces pannorum* produces amylase enzyme. The α-amylase gene was cloned and overexpressed in *Pichia pastoris* and studied for its production and purification. Enzyme purification was carried out by

ammonium sulfate precipitation (20–70%) followed by DEAE-Sephacel and Sephadex G-100 column chromatography, equilibrated with 0.05 M phosphate buffer at pH 6.0 and eluted with a linear gradient of 0–0.5 M NaCl and Sephadex G-100 column). The SDS-PAGE confirms 54 kDa MW of the separated protein. The enzyme was catalytically optimum at pH 6.0 and 70 °C, and the specific activity was 9.78×10^3 U/mg (Gao et al. 2016). Psychrophilic fungi *Glaciozyma antarctica* PI12 (*Leucosporidium antarcticum*) has also been confirmed for amylase production (Ramli et al. 2013). Another cold adapted yeast *Rhodotorula glacialis* has reported for higher amylase activities at 10–22 °C (Carrasco et al. 2016). Halotolerant fungal species *Penicillium chrysogenum*, *Fusarium incarnatum*, and *Penicillium polonicum* were isolated from saltwater Lake Urmia, Iran for amylase activity using starch agar plates. Among the three selected isolates, only *P. chrysogenum* was the most tolerant isolate that grew up to 25% salinity, whereas *P. polonicum* isolate U4 was the most potent producer of amylase with a yield of 260.9 U/L in yeast nitrogen base media supplemented with 5% NaCl and 1% starch after 7 days of incubation at 27 ± 1 °C temperature (Niknejad et al. 2013).

Mohapatra et al. (1998) reported amylase activity from marine sponge *Spirastrella* sp., associated *Mucor* sp. grown at 30 °C. The optimum catalytic activity was observed at pH 5.0 and temperature 60 °C. The metals NaCl, Ca^{2+}, and Mg^{2+} have no effect on enzyme activity but strongly inhibited by EDTA (Mohapatra et al. 1998). A halotolerant fungus *Penicillium* sp. was studied for amylase production by submerged fermentation using 10% NaCl and 1% starch. The purified enzyme revealed maximum activity at pH 9 and 11. Highest enzyme production using maize meal obtained at 30 °C and pH 11 was obtained followed by other materials (barley grains, wheat grains, wheat bran, crushed wheat grains, birds feed grains, and wheat meal). The enzyme activity was stable up to 10% NaCl, pH 9 and 30 °C temperature (Gouda and Elbahloul 2008).

20.5 Applications of Amylase

Presently the usage of extremophilic amylases has increased because of its suitable characteristics over mesophilic enzymes in food industry such as sweeteners, syrups, beverages production, cheese ripening, starch liquefaction, bakery production, and animal feed supplement process. Moreover, it has wide scope in biofuel production, detergents production, laundry and textile industry, and paper industry. Apart from these conventional applications, it is reported for wastewater purification, hydrogel formation, in controlled drug delivery system, digestive syrup preparation, and as a biofilm inhibitor in pharmaceutical industry (Paul et al. 2021). The commercial production of enzyme was begun by chemist Christian Hansen in 1874 who for the first time extracted the rennet from dried calves' stomachs for industrial use. Soon the microbial enzyme demand was significantly increased in the twenty-first century and still rising as it has enormous potential in the feed, food, pharmaceutical, beverages, detergent, paper/pulp, and leather industries. The current global market

for the enzyme has reached $6.2 billion in 2020 and still rising (Niehaus et al. 1999; Van Den Burg 2003; Singh et al. 2016; Dumorne et al. 2017).

20.5.1 Pharmaceutical Industry

α-Amylase has been used as an ingredient in the preparation of digestive syrups to treat digestive disorders. Acidophilic amylases are suitable for digestive syrup preparation that can withstand the stomach pH. Along with polymer dextran or dextrin, it forms copolymers useful for enzymatically controlled drug delivery system (ECDR) (Rahmouni et al. 2001).

20.5.2 Starch Conversion

Amylase has been widely used in starch industry for hydrolysis to form glucose and fructose syrup. The enzymatic conversion of starch comprises gelatinization (i.e., dissolution of starch granules to form a viscous solution), liquefaction (i.e., partial hydrolysis and reduction in viscosity), and saccharification (involves formation of glucose, maltose, and fructose). Thermophilic fungal cultures have remarkable thermostability and thus have interest in large scale biotechnological industry (Souza 2010).

20.5.3 Bioethanol Production

Demand for the renewable sources is increasing rapidly in current situation due to fuel escalation, and bioethanol production from the starch can be a good choice. Starch solubilization and liquefaction using microbial amylase can be carried out in industry at large extent, followed by fermentation of sugars to ethanol. Microorganisms are screened for conversion of sugars to bioethanol formation. Natural polymers like starch, cellulose, pullulan, and lignocellulose are good source of glucose; however, they require pretreatment either chemically or enzymatically at high temperature. Genetically modified microbial culture further assists to enhance the fermentation process (Rana et al. 2013).

20.5.4 Detergent Industry

Enzyme application in detergent industry is useful to remove the tough stains, thus making the detergent environmentally safe. It is used in laundry and dishwasher detergents to convert residual starch to dextrins and smaller oligosaccharides. Amylase is fortified with detergent to remove the starchy food stains (gravies, potatoes, and chocolate) from the surface and to increase the brightness of the cloths. Almost all the detergent contains amylase enzymes as important ingredients. A

characteristic amylase working at low temperature, alkaline condition, and oxidatively resistant is suitable for detergent industry (Rana et al. 2013).

20.5.5 Textile Industry

Starch is widely used in textile industry as sizing agent because it is cheap and easily available. It is applied to the yarn for increasing its strength during weaving process before fabric production. Amylase is used for removing this starch after fabric production called as desizing to give a smooth texture (Souza 2010).

20.5.6 Paper Industry

In the pulp and paper industry, starch is used as sizing agent for improving the writing quality and erasability of the paper. The natural starch has high viscosity and need pretreatment using the amylases, to convert it into low viscous by partial hydrolysis. Sometimes starch coats on the paper to make it sufficiently smooth and strong at first followed by hydrolysis using the amylase (Far et al. 2020).

20.5.7 Baking Industry

α-Amylases have been widely used in the baking industry to produce flavored, sweet, soft bakery products by enhancing the fermentation rate and reducing the viscosity of dough. During dough making, the addition of amylase degrades the starch into dextrins, which can be fermented easily by yeast to form sugars, thereby enhancing the product texture quality, volume, and shelf-life. In food industry, it is used for digestive syrup preparation that improves the metabolism of the patient and in the preparation of fruit juices it degrades starch into sugars giving a natural sweet taste and clear appearance. Pretreatment of animal feed with amylase improves the digestibility of fiber. Thermostable and cold adapted amylase thus has great potential in breaker and food industry respectively (Sundarram and Murthy 2014).

20.5.8 Wastewater Treatment

The effluents from various industries like textile, paper, and food and sewage water plant contain many organic chemicals and starch that can cause water pollution. Amylases from extremophilic fungi can be able to degrade these pollutants and starch, thus alleviating the pollution. (Priya and Renu 2018).

20.6 Conclusion

Extremozymes, the metabolites of extremophilic microbes, have a great potential in many industrial processes, including agricultural, chemical, and pharmaceutical industries as catalyst, therapeutic drug, analytic reagents, and diagnostic tools. Researchers are keen to understand the physiology and metabolic pathway running in extremophiles to apply them for industrial applications. Various studies proved their stability at extreme conditions of temperature, acidic and alkaline pH, and high salt concentration that are requisite for industrial processes. Isolation and screening of potential extremophiles for novel extremozymes are continuous processes in research. Moreover, genetically modified mesophilic natural strain by cloning extremophiles gene is of great interest in industrial research and development sector. Amylases from extremophilic fungi will have increased demand in coming years to avoid chemical methods and non-extreme enzymes in food, textile, paper, food, biofuel, pharma industries, and wastewater treatment. Being genetically and metabolically more stable, a systematic investigation in isolation and enzyme production by extremophilic fungi is pivotal to open the new applications in health and environmental sector.

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Extremophilic Fungi as a Source of Bioactive Molecules

21

Annada Das, Kaushik Satyaprakash, and Arun Kumar Das

Abstract

Extremophiles are the organisms that survive the harshest and extreme environments on earth, notably deep-sea sediments, permafrost, deserts, hypersaline water, etc., of the extremophiles, various fungal species like *Aspergillus* spp., *Emericella* spp., *Eutypella* spp., *Microsporum* spp., *Penicillium* spp., *Trichoderma* spp., *Wallemia* spp., etc. produce a number of bioactive molecules categorized into polypeptides, polyketides, terpenoids, alkaloids, sterols, etc. These metabolites are proven to possess antibacterial, antiviral, antifungal, anti-inflammatory, anticancer, and anti-diabetic activities. These compounds have significant scope in biomedical research and can be explored as potential candidates for new drug discovery. Apart from it, they can be substantially applied in the fields of environmental, industrial, and food technology. There is a need to isolate, propagate, and conserve these novel microorganisms and their active metabolites. This chapter provides an overview of various bioactive molecules obtained from different genera of extremophilic fungi isolated from extreme habitats on this planet.

Keywords

Extremophiles · Extreme environments · Fungi · Bioactive molecules · Drug discovery

A. Das (✉)
Department of Livestock Products Technology, Faculty of Veterinary and Animal Sciences, West Bengal University of Animal and Fishery Sciences, Kolkata, West Bengal, India

K. Satyaprakash
Department of Veterinary Public Health and Epidemiology, Faculty of Veterinary and Animal Sciences, Banaras Hindu University, Mirzapur, Uttar Pradesh, India

A. K. Das
Eastern Regional Station, ICAR-Indian Veterinary Research Institute, Kolkata, West Bengal, India

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21.1 Introduction

Emergence of antimicrobial resistance to conventional drugs has prompted scientists and researchers to search for various alternatives in the unexplored sources, viz. the extreme environments. A number of literatures published over time have revealed about the microorganisms surviving and growing in extreme habitats by producing specialized metabolites that have some inherent bioactive properties.

If we look at extremophiles, these are the organisms which can survive and/or multiply in physically and chemically extreme environmental conditions which are detrimental to most of the life forms on this planet. Most of the extremophiles are microorganisms—fungi constitute one of the big classes among them. Extremophiles can be classified according to the environmental condition in which they survive and reproduce and these are thermophiles (60–80 °C), hyperthermophiles (>80 °C), psychrophiles (<20 °C), acidophiles (pH < 3), alkalophiles (pH > 9), piezophiles (>500 atm. pressure), halophiles (>0.2 M NaCl), oligotrophic (low nutrient availability), xerophiles (very dry climate), metallophiles (high concentration of metals), and endolithic (within rock or within pores of mineral grains). Some extremophiles are tolerant to a combination of extreme physicochemical factors, hence called polyextremophiles, viz. thermoacidophiles and haloalkalophiles (Gupta et al. 2014).

Extremophilic organisms have developed robust defense mechanisms to survive under extremely harsh environments, leading to the synthesis of biomolecules with diverse biological activities. The ability of extremophiles to adapt to adverse environmental factors is probably due to the regulation and expression of specific genes in their genome. The strategy for survival in extremes is due to the production of enzymes and other secondary metabolites. Such compounds have many biotechnological applications, viz. environmental (bioremediation, biodegradation, and biocontrol), industrial (biomining, biofuel, and food), and medical (antibiotics, antiviral, antifungal, anti-tumor compounds) (Dhakar and Pandey 2016; Zhang et al. 2018). In order to survive the extreme pressure, temperature, salinity, water availability, and pH conditions, the biosynthesis of various novel natural products with diversified biological activities takes place, which could lead to their industrial application (Rogozhin et al. 2018). Biotechnological process frequently happens in inhospitable conditions to microbes in the industrials setup like extreme temperature, salinity, and pH which could be comparable to that of the natural habitats of extremophiles (Elleuche et al. 2015). Many bioactive compounds are produced by extremophilic fungi, yet their potential has not been fully evaluated. Keeping this in view, this chapter focuses on the bioactive molecules produced by different extremophilic fungi which can provide a plethora of novel biomolecules for the industry to work with.

21.2 Bioactive Compounds from Extremophilic Fungi

The abundant presence of diverse genera of fungi in extreme habitats makes them ideal sources for search of bioactive compounds which could be useful in designing new drugs. Fungi living in extreme environments might have made adjustments at genome level to sustain life by augmenting the chemical defense and communication (Deshmukh et al. 2018). Cephalosporin C was the first compound obtained from deep-sea fungus *Cephalosporium* spp. in 1949. Thereafter a number of important biochemicals have been isolated from marine fungi.

Of the specialized compounds obtained from extremophilic microorganisms, antibacterial activities are exhibited by 33.6% of compounds followed by anticancer (25.6%), antifungal and anti-inflammatory (9.6% each), and antiviral (8.0%) activities which possess the potential to be future candidates of lifesaving drugs (Sayed et al. 2019).

21.2.1 Desert Fungi

Fungi are the most stress-resistant eukaryotes (Sterflinger et al. 2012). In the challenging desert environments, they have exhibited a wide range of adaptive mechanisms (Onofri et al. 2007; Stevenson et al. 2017; Santiago et al. 2018). Early isolation studies from soil of Negev Desert in Southern Israel and Sonoran Desert in North America revealed extensive fungal diversity (Taylor-George et al. 1983). The Atacama Desert in Chile has been the hunting ground for scientist in search of microflora in desert habitats (Bull et al. 2016, 2018; Bull and Goodfellow 2019) among the studies in all non-polar deserts (Kurapova et al. 2012; Tiwari et al. 2015; Ouchari et al. 2018) of the world. Although novel fungal species have been discovered from desert ecosystem, yet their utility in bioprospecting campaigns is limited. For instance, 13 distinct fungal genera, notably that of *Alternaria* spp. and *Ulocladium* spp. were isolated from Atacama Desert soil (Conley et al. 2006); 135 novel species dominated by *Ascomycetes* from Makhtesh Ramon Desert soil in Israel (Grishkan and Nevo 2010); 77 lichenoid fungal species including four novel species (*Amandinea efflorescens*, *Diploicia canescens*, *Myriospora smaragdula,* and *Rhizocarpon simillimum*) from two altitudinal transects of Alto Patache in Atacama Desert (Castillo and Beck 2012); *Cladosporium*, *Neucatenulostroma,* and *Penicillium* spp. from high altitude rocks of Atacama Desert (Gonçalves et al. 2016); three novel fungal species (*Diversispora omaniana*, *Septoglomus nakheelum,* and *Rhizophagus arabicus*) from an Omani Desert (Symanczik et al. 2014) and two novel halophilic fungi (*Aspergillus atacamensis* and *Aspergillus salisburgensis*) from a cave at the Atacama Desert (Martinelli et al. 2017) have been isolated. The deserts of the Middle East are also home to some extensive diverse fungal species (Murgia et al. 2018). The chemical structures of some bioactive compounds obtained from desert fungi are given in Fig. 21.1.

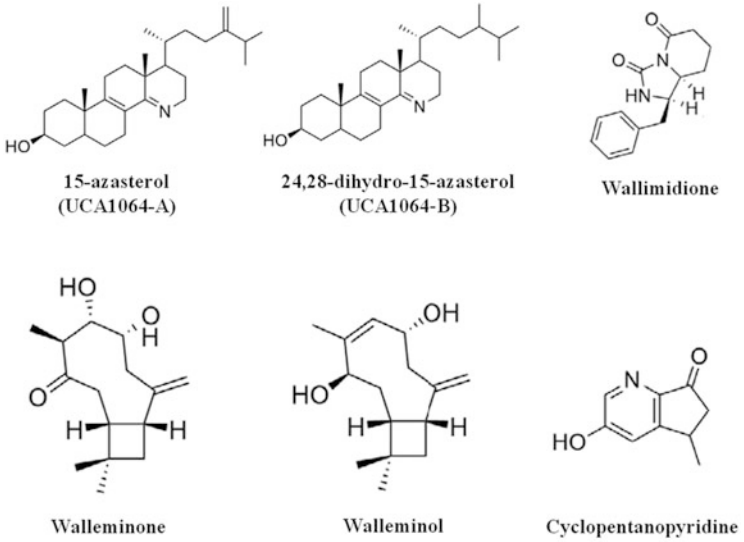


Fig. 21.1 Chemical structures of compounds obtained from desert fungi (Sayed et al. 2019)

21.2.1.1 N-Containing Compounds

Recent studies on fungi from desert ecosystem underline their potential as a prolific source of novel bioactive chemicals (Santiago et al. 2018). *Wallemia sebi,* isolated from the Atacama Desert was found to produce wallimidione (Desroches et al. 2014), 15-azasterol, and 24, 28-dihydro-15-azasterol (Jančič et al. 2016). Molecules UCA 1064-A and UCA 1064-B exhibited in vivo anti-tumor activity against mammary tumor in mouse and in vitro anti-proliferative activity against HeLa cells, respectively. Further, antifungal activity against *Saccharomyces cerevisiae* and inhibition of G+ve bacteria were also exhibited by the above compounds (Jančič et al. 2016). Cyclopentenopyridine (an alkaloid from a halophilic strain of *W. sebi*) was found to inhibit *Enterobacter aerogenes* (Peng et al. 2011).

21.2.1.2 Terpenes

The unique terpenes, Walleminone and walleminol have been produced by *W. sebi* (Jančič et al. 2016).

21.2.2 Permafrost Soil Fungi

The layer beneath the earth's crust which remains at a sub-zero temperature for at least 2 consecutive years is known as permafrost layer (Jansson and Tas 2014). Water availability, temperature, high viscosity, and low thermal energy are the most important abiotic factors for the microorganisms in the permafrost (Jansson and Tas 2014). Normal cell cycle is inhibited under such conditions and the transmembrane and intracellular proteins can denature leading to loss of cell structure and cell

membrane fluidity (Chattopadhyay 2006; D'Amico et al. 2006). These cryophilic microorganisms have developed survival strategies, viz. initiating a state of dormancy, generation of specialized metabolites and proteins, storing energy in the form of polyphosphates, triglycerides, wax, esters, and glycogen, thereby reducing the cellular metabolism (Unell et al. 2007; Bowman 2008; Bakermans et al. 2009).

In Arctic permafrost, the fungi of genera *Aspergillus*, *Cladosporium, Geomyces,* and *Penicillium* (Ozerskaya et al. 2009) are predominant. Of nearly 400 taxonomically distinct fungal genera isolated from Antarctic soil (Bridge and Spooner 2012), *Cladospora*, *Geomyces,* and *Thielava* from Cape Royds, Antarctica (Blanchette et al. 2010) are the important ones. Fungal diversity in the Antarctic lichens of King George Island includes *Arthoniomycetes*, *Eurotiomycetes*, *Lecanoromycetes*, *Leotiomycetes*, *Sordariomycetes* of Ascomycota, and *Cystobasidiomycetes* and *Tremellomycetes* of Basidiomycota (Park et al. 2015). The chemical structures of some bioactive compounds obtained from permafrost fungi are given in Fig. 21.2.

21.2.2.1 N-Containing Compounds

Fungi of polar region have the ability to metabolize rapidly a variety of substrates. *Oidiodendron truncatum*, a psychrophilic fungus found in Antarctica, was found to produce two novel epipolythiodioxopiperazines (chetracins B and chetracin C) and five novel diketopiperazines (oidioperazines A–D and chetracin D). The sulfide bridge in chetracin B and chetracin C is responsible for their anticancer activity against human cancer cell lines (Li et al. 2012). Cytochalasins Z_{24} (moderate cytotoxicity towards human breast cancer cells (MCF-7)), cytochalasins Z_{25}, and cytochalasins Z_{26} were recovered from *Eutypella* sp. D1, isolated from Arctic soil on London Island of Fongsfjorden (Liu et al. 2014). Antibacterial activity against G+ve and G−ve bacteria was observed for Libertellenone G (a N-containing diterpene), obtained from *Eutypella* spp. Another N-containing diterpene, eutypenoid B obtained from the same organism was found to be a potent immunosuppressant (Lu et al. 2014; Zhang et al. 2016a, b). Similarly, Lindgomycin and ascosetin obtained from Arctic sponge-derived fungal strain of family Lindgomycetaceae have exhibited potent antimicrobial activity against methicillin-resistant *Staphylococcus aureus* and *Candida albicans* (Ondeyka et al. 2014; Wu et al. 2015).

21.2.2.2 Polyketides

Penilactone A and penilactone B (highly oxygenated polyketides), isolated from an Antarctic deep-sea-derived fungus *Penicillium crustosum* were found to exhibit inhibitory action against nuclear factor-κB (Wu et al. 2012). *Pseudogymnoascus* spp., isolated from an Antarctic marine sponge was found to produce pseudogymnoascin A–C and 3-nirtoasterric acid, but they failed to exhibit antifungal or antibacterial activity may be due to the presence of nitro-group (Figueroa et al. 2015). Again, ochraceopones A–E, isoasteltoxin, and asteltoxin B (highly oxygenated polyketides) were obtained from *Aspergillus ochraceopetaliformis*, of which anti-influenza activity against H1N1 and H3N2 viruses was observed for ochraceopones A and isoasteltoxin (Wang et al. 2016).

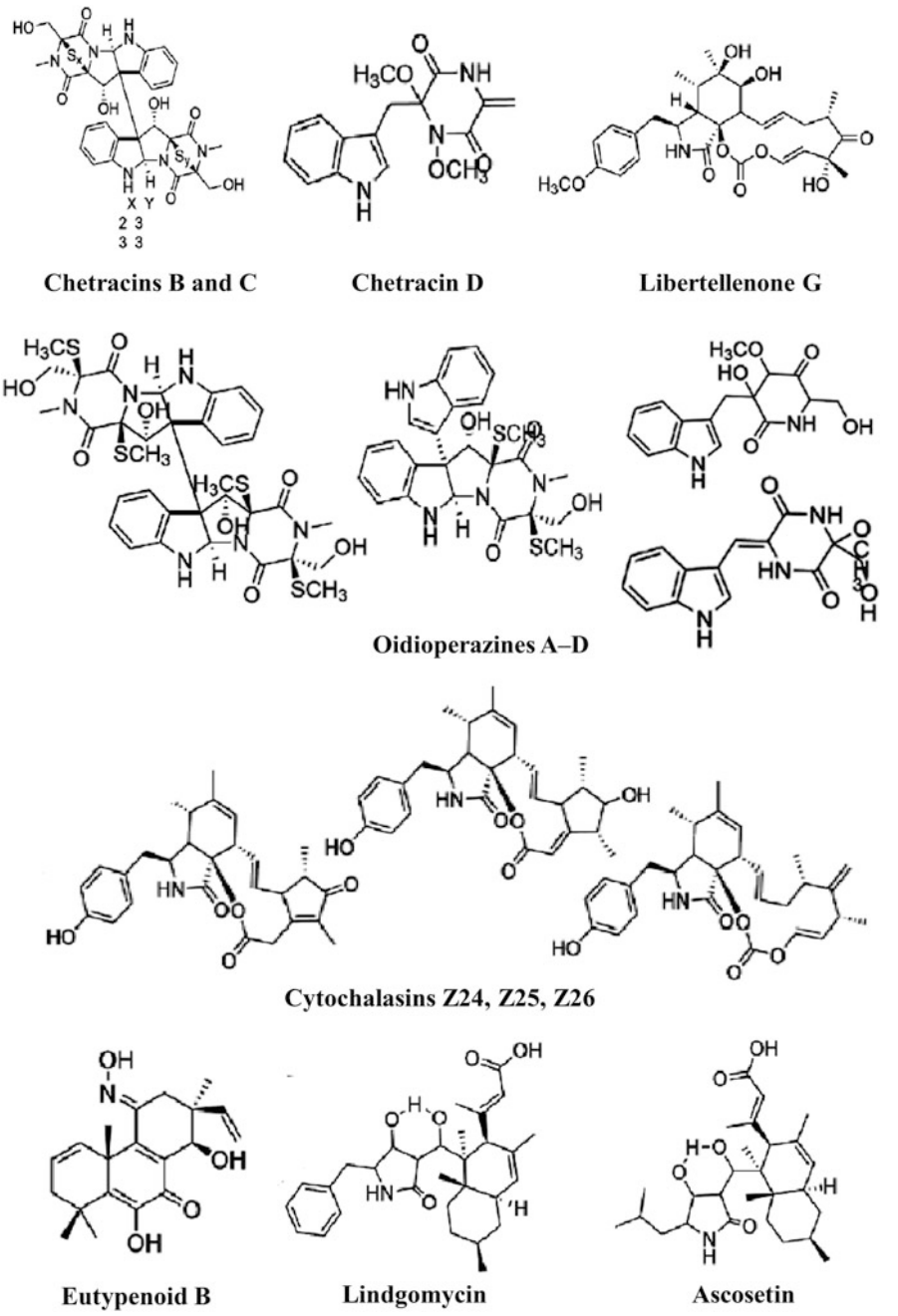


Fig. 21.2 Chemical structures of compounds obtained from permafrost soil fungi (Sayed et al. 2019)

21.2.2.3 Terpenes

Four new diterpenes, scoparasin B, libertellenone H, and eutypenoids A and C, were isolated from *Eutypella* strain from Arctic soil on London Island (Liu et al. 2014). Antarctic moss-derived fungus *Penicillium funiculosum* has been found to contain the meroterpenoids, chrodrimanin I and chrodrimanin J along with five known structurally related chrodrimanins, but the novel chrodrimanins revealed a weak inhibitory activity against H1N1 virus (Zhou et al. 2015).

21.2.3 Deep-Sea Sediments Fungi

The ocean is home to taxonomically distinct microorganisms. Less attention has been given to the deep-sea sediments especially in the Polar Regions and deep trenches, may be due to the difficulty in accessing the samples. The deep-sea environment (water depths below 1000 m) is a potentially difficult habitat for sustenance of life with respect to variation in temperature, lack of dissolved oxygen, limited or no penetration of sunlight, high hydrostatic pressure (up to 400 atm.), and limited nutrient availability (Danovaro et al. 2017; Barone et al. 2019). Organisms surviving and growing in these environments must have developed some sort of mechanisms to tolerate the extremes of temperature, pH, salinity, osmolarity as well

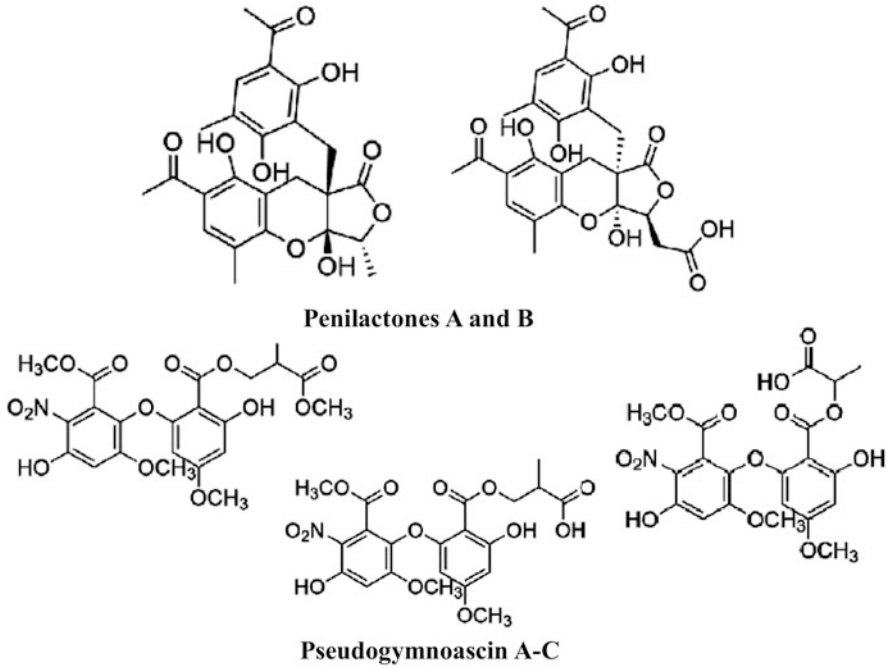


Fig. 21.2 (continued)

as production of certain bioactive molecules to protect themselves in these harsh conditions.

Some of the important fungi isolated from deep-sea sediments include *Graphium* spp. from Northern Antarctic Peninsula (Gonçalves et al. 2017), filamentous fungi belonging to the phylum Ascomycota from the Central Indian Basin (Singh et al. 2010, 2012) and *Candida*, *Cryptococcus*, *Pichia,* and *Rhodotorula* spp. from deep Polar sea (Nagano et al. 2013). The chemical structures of some bioactive compounds obtained from deep-sea sediment fungi are given in Fig. 21.3.

21.2.3.1 N-Containing Compounds

Eremophilane (a lactam-type metabolite) from Antarctic deep-sea fungus *Penicillium* sp. PR19N-1 (Lin et al. 2014) and circumdatin K and circumdatin L (benzodiazepine alkaloids), 10-epi-sclerotiamide and 5-epi-sclerotiamide (indole alkaloids) along with aspergilliamide B (a novel amide) from *Aspergillus westerdijkiae* DFFSCS013 (Peng et al. 2013) have been isolated. Meleagrins B–E, roquefortines F–I, and breviones F–H (all alkaloids) isolated from deep-sea sediment-derived *Penicillium* spp. have displayed cytotoxic activity against MOLT4, HL60, A549, BEL7402, and HeLa cell lines (Li et al. 2012).

21.2.3.2 Terpenes

A moderate cytotoxic effect against cancer cell lines HL-60 and A549 has been exhibited by chlorinated eremophilane sesquiterpenes obtained from the *Penicillium* spp. PR19N-1 isolated from Antarctic region (Wu et al. 2013). Further, five new cytotoxic eremophilane-type sesquiterpenes have been identified from the above strain (Lin et al. 2014).

21.2.3.3 Polyketides

Two novel polyketides, penilactone A and penilactone B, with unusual highly oxygenated structures were identified from an Antarctic deep-sea fungus *Penicillium crustosum* PRB-2 (Wu et al. 2012).

21.2.4 Marine Fungi

(a) Halophiles

Halophiles are organisms that can thrive in saline environments with concentrations higher than 0.2 M NaCl (Gupta et al. 2014). Halophiles can be found in hypersaline environments like tide pools, deep-sea, salt lakes, etc. In order to cope with the osmotic stress, the halophilic eukaryotes have developed a mechanism for intracellular accumulation of compatible solutes or osmoprotectants or osmolytes like polyols, soluble sugars, amino acids, and quaternary ammonium compounds in contrast to salts (Lentzen and Schwarz 2006). *Debaryomyces hansenii*, *Hortaea werneckii,* and *Wallemia ichthyophaga* have been isolated from natural hypersaline environments (Prista et al. 1997; Gunde-Cimerman et al. 2009).

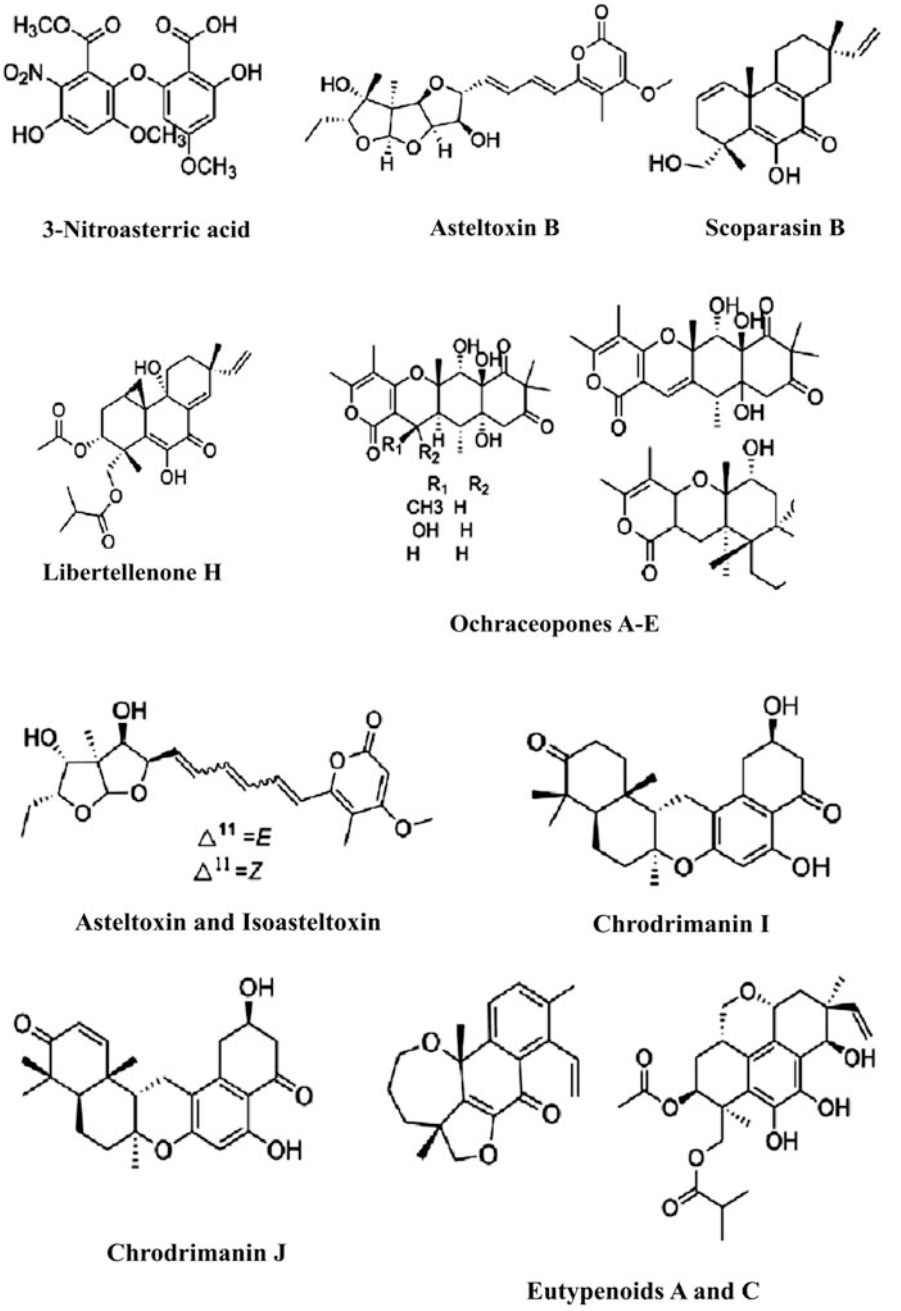


Fig. 21.3 Chemical structure of compounds obtained from deep-sea sediment-derived fungi (Sayed et al. 2019)

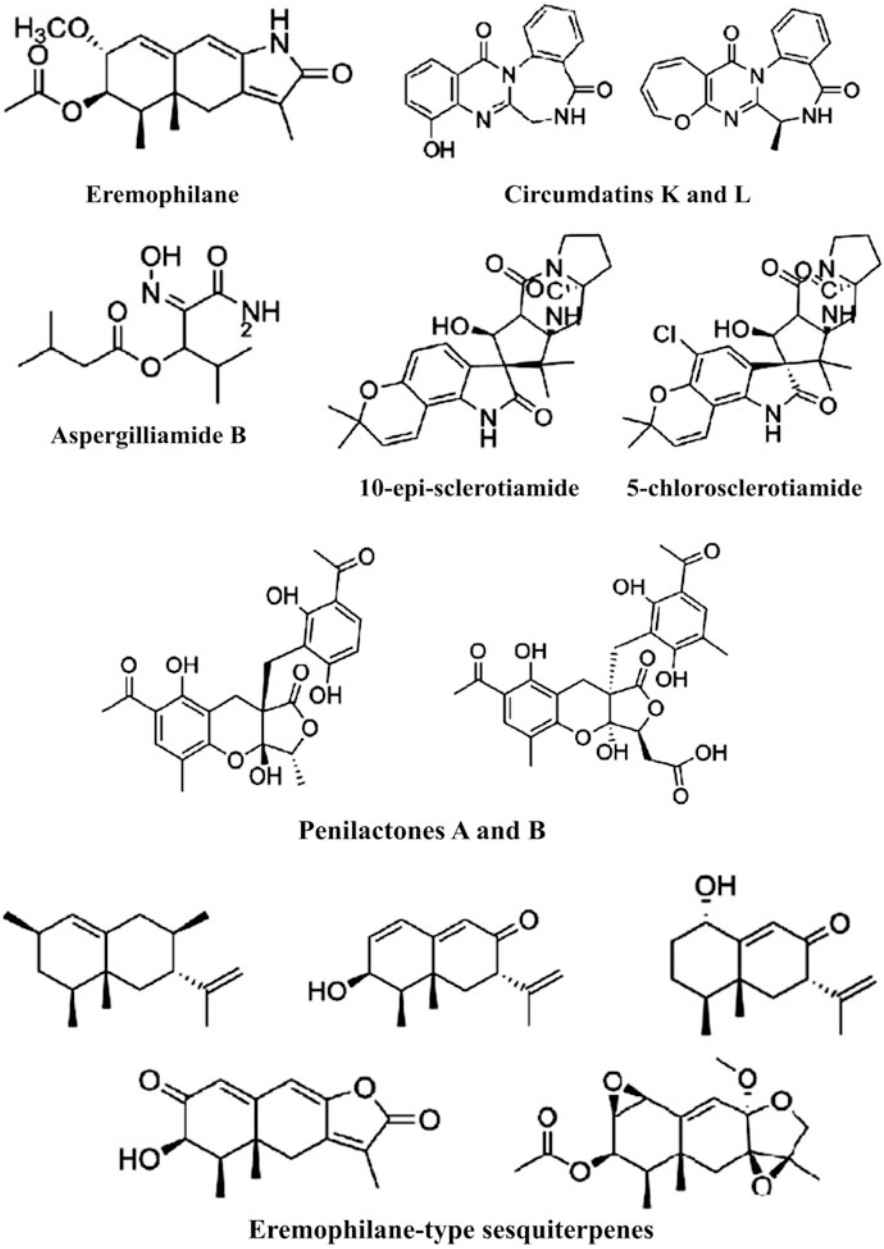


Fig. 21.3 (continued)

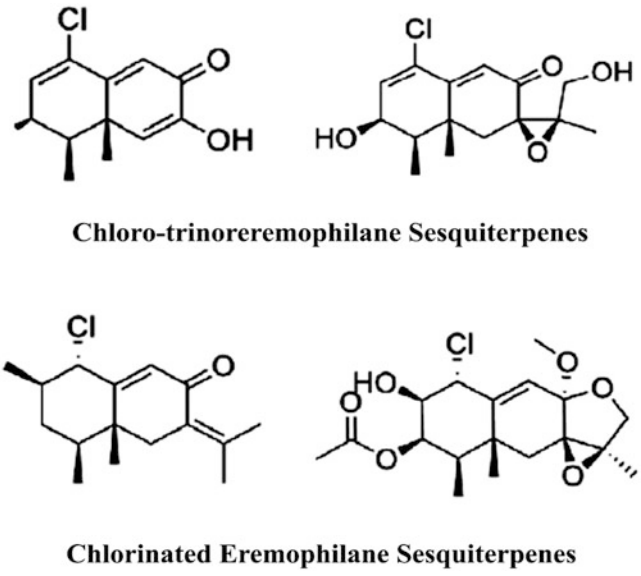


Fig. 21.3 (continued)

H. werneckii and *Trimmatostroma salinum* have been found to produce extracellular hydrolytic enzymes having xylanolytic, lignolytic, and cellulolytic activities (Plemenitǎs et al. 2008; Zalar et al. 2005). Salt (NaCl) concentration affects the production of some secondary metabolites (wallimidione, walleminol, walleminone, UCA 1064-A, and UCA 1064-B) from *W. sebi* with substantial biological activities (Botíc et al. 2012; Jančič et al. 2016). The marine-derived fungus, *Emericellopsis* spp. produces an array of peptide antibiotics, viz. emericellipsin A, zervamicins, bergofungins A, B, C, and D, heptaibin, and emerimicines with antibacterial and antifungal activities with emericellipsin A also exhibiting a significant cytotoxic effect against HepG2 and HeLa tumor cell lines (Rogozhin et al. 2018).

The compounds (22R,23S)-epoxy-3β,11α,14β,16β-tetrahydroxyergosta-5,7-dien-12-one and 6-(1H-pyrrol-2-yl)-hexa-1,3,5-trienyl-4-methoxy-2H-pyran-2-one (existed as a pair of epimers), along with nine other compounds were isolated and identified from the fermentation broth of *Aspergillus flocculosus* PT05-1, a halotolerant fungus obtained from sediments of Putian saltern of Fujian Province of China which exhibited cytotoxic activity against HL-60 and BEL-7402 cells, as well as antimicrobial activity against *Enterobacter aerogenes*, *Pseudomonas aeruginosa,* and *Candida albicans* (Zhang et al. 2013). Alternaroside A–C (cerebrosides) and alternarosin A (diketopiperazine alkaloid) obtained from the halotolerant fungus, *Alternaria raphani* isolated from Hongdao sea salt field, Qingdao, China were found to possess weak antibacterial activity (Wang et al. 2009).

(b) Piezophiles

The piezophilic fungus (previously known as barophilic fungus) is a class of extremophiles that can thrive and grow in high-pressure habitats, especially the oceans (Pettit 2011). Microorganisms in the deep-sea environment are affected by elevated hydrostatic pressure, low temperature, and low nutrients. The high pressure alters the fluidity and permeability of cell membranes, the stability of H-bonds in DNA, thereby affecting the cellular integrity and cell division (Burgaud et al. 2015).

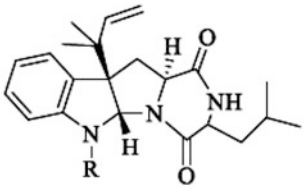
The piezophilic fungus *Phialocephala* spp. FL30r was reported as a natural producer of trisorbicillinone A (a sorbicillin trimer) and oxosorbiquinol and dihydrooxosorbiquinol (bisorbicillinoids). All sorbicillin compounds showed cytotoxicity against P388, A549, HL60, BEL-7402, and K562 cell lines (Li et al. 2007a, b). A total of 16 steroids with bicycle[4.4.1] A/B rings were obtained from *Penicillium citrinum* HGY1-5 from Huguangyan, China of which 24-epi-cyclocitrinol, cyclocitrinol, neocyclocitrinol C, and threo-23-*O*-methylneocyclocitrinol were found to enhance cAMP production in GPR12-transfected CHO cells (Du et al. 2008). *Penicillium* spp. isolated from Berkeley Pit Lake of Bute (Montana) produced the bioactive compounds like berkeleydione and berkeleytrione (having anticancer activity, inhibiting MMP-3 and caspase-1), preaustinoid A and preaustinoid A1, berkeleyones A–C (Stierle et al. 2011), while *Pleurostomophora* spp. isolated from the same lake was found to produce the secondary metabolites berkchaetoazaphilones A–C, the red pigment berkchaetorubramine, and 4-(hydroxymethyl) quinolone (Stierle and Stierle 2014). Berkchaetoazaphilone B inhibited the production of IL-1β, TNF-α, and IL-6 and showed cytotoxicity on human retinoblastoma cell line Y79, leukemia cell lines CCRF-CEM and SR, and the melanoma cell line LOX IMVI (Stierle and Stierle 2014). Dicitrinone B, obtained from *P. citrinum* HGY1-5 from a dead volcano in Huguangyan, China was observed to induce apoptosis in tumor cells. The fungal strain *Leptosphaeria* spp. isolated from Tanabe Bay, Japan produced leptosins A-N1, of which leptosins I, J, M, M1, N, and N1 exhibited cytotoxicity against a panel of 39 human cell lines (Takahashi et al. 1994; Yamada et al. 2002).

(c) Psychrophiles

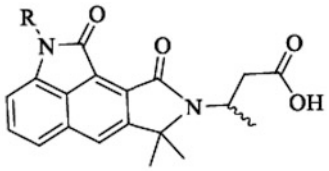
Psychrophiles are cold-adapted microorganisms with an optimum growth temperature of 15 °C or lower. The survivability of microorganisms in cold environment has been very challenging. At least, the temperature of 90% of the ocean volume is below 5 °C (Anwar et al. 2020). The decrease in temperature may damage the cell membrane and generate reactive oxygen species (ROS) which can degrade the genetic materials, proteins, and lipids (Gocheva et al. 2009). Microorganisms residing in cold climates especially at the Antarctic region must have made some structural and functional adaptations at the cellular constituents, cell membrane, metabolic pathways, production of radical scavenging molecules, and mechanisms to inhibit the formation of intracellular ice crystals. The production of different metabolites in response to these extreme situations has a tremendous scope for the biotechnological and pharmaceutical industries to search for potent bioactive molecules.

A variety of polyketides, steroid, and indole-derivatives, sesquiterpenoids, alkaloids, aromatic compounds, fatty acids, pyrone analogs, sorbicillin, breviane-derivatives, and compounds containing amino acid structures have been found to exhibit anticancer activities, while some other compounds, viz. prenylxanthones, depsidone-based analogs, triple benzene compounds, and citromycetin analog were shown to possess antibacterial activity (Wang et al. 2015). A hybrid polyketide, cladosin C, produced by *Cladosporium sphaerospermum* 2005-01-E3 showed antiviral activity against H1N1 influenza virus (Wu et al. 2014). The deep-sea fungus *Aspergillus versicolor* CXCTD-06-6 was described as a source of antifungal and antifouling agents, viz. diketopiperazine, brevianamide W, diketopiperazine V, brevianamide Q, R, K, and E (Kong et al. 2014). *P*-hydroxyphenopyrrozin and terphenyl isolated from deep-sea fungi *Chromocleista* spp. (Park et al. 2006) and *Aspergillus candidus* (Liu et al. 2013), respectively, have shown antifungal activity against *Candida albicans*. The compound terphenyl also showed antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis,* and *Vibrio* spp. (Liu et al. 2013). The β-diversonolic ester obtained from deep-sea fungus *Penicillium* spp. SCSIO 06720 from the Indian Ocean showed moderate antibacterial activity against *S. aureus* ATCC 29213 and methicillin-resistant *S. aureus*-shh-1 (Guo et al. 2020). An Antarctic strain of *Geomyces pannorum* has been associated with non-enzymatic antioxidant response and production of phenolic compounds (Maggi et al. 2013). Rugulosin and skyrin (*bis*-anthraquinones) from *Penicillium chrysogenum* isolated from a saline lake in Vestfold Hills were to electively inhibit *S. aureus*, *Enterococcus faecium,* and *E. coli* (Brunati et al. 2009). *Aspergillus sydowii*, *Penicillium allii-sativi*, *Penicillium brevicompactum*, *P. chrysogenum,* and *Penicillium rubens* were shown to produce antibacterial, antifungal, anticancer, herbicidal, and antiprotozoal compounds. Extracts from *P. allii-sativi*, *P. brevicompactum,* and *P. chrysogenum* exhibited a high antiviral activity against dengue virus 2 and antiprotozoal activity against *Trypanosoma cruzi*; strong antifungal activity against the phytopathogen *Colletotrichum gloeosporioides* as well as herbicidal activity (Godinho et al. 2015). *P. chrysogenum* obtained from the endemic Antarctic macroalga *Palmaria decipiens* revealed to possess selective antifungal and trypanocidal activities (Godinho et al. 2015). *Penicillium tardochrysogenum* isolated from McMurdo Dry Valley in Antarctica produces penicillins, secalonic acids D and F, asperentins, and the uncharacterized extrolite met Ø (Houbraken et al. 2012). The extracts from the endophytic fungi belonging to the genera *Alternaria*, *Antactomyces*, *Cadophora*, *Davidiella*, *Hegardia*, *Herpotrichia*, *Microdochium*, *Oculimacula*, *Phaeosphaerica* isolated from plants *Deschampsia antarctica* and *Colobanthus quitensis* collected in Antarctica showed leishmanicidal activity and a specific cytotoxic activity was found for *Microdochium phragmitis* extract on UACC-62 cell line (Santiago et al. 2012).

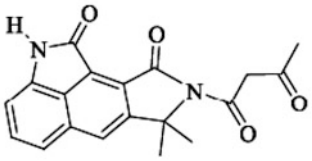
The chemical structures of different bioactive compounds obtained from marine fungi are given in Figs. 21.4, 21.5, 21.6, 21.7, 21.8, and 21.9.



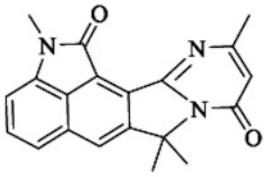
Brevicompanines D, R=CH_2OCH_3
Brevicompanines E, R=$COCH_2CH_3$
Brevicompanines F, R=$CO(CH_2)_2CH_3$
Brevicompanines G, R=$COCH_3$
Brevicompanines H, R=CH_2COCH_3



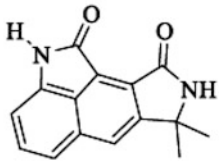
Cyclopiamide B, R = CH_3
Cyclopiamide C, R = H



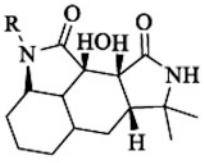
Cyclopiamide D



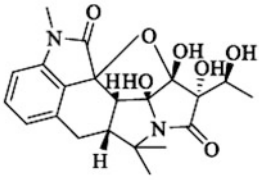
Cyclopiamide E



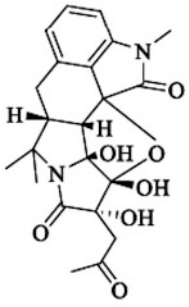
Cyclopiamide F



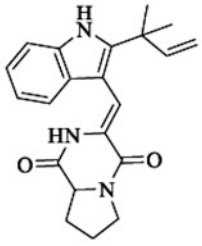
Cyclopiamide G, R = H
Cyclopiamide H, R = CH_3



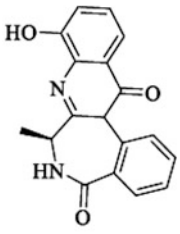
Cyclopiamide I



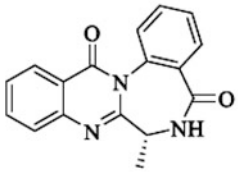
Cyclopiamide J



(±)Brevianamide R



Circumdatin G



Circumdatin F

Fig. 21.4 Chemical structures of N-containing compounds obtained from marine fungi (Arifeen et al. 2020)

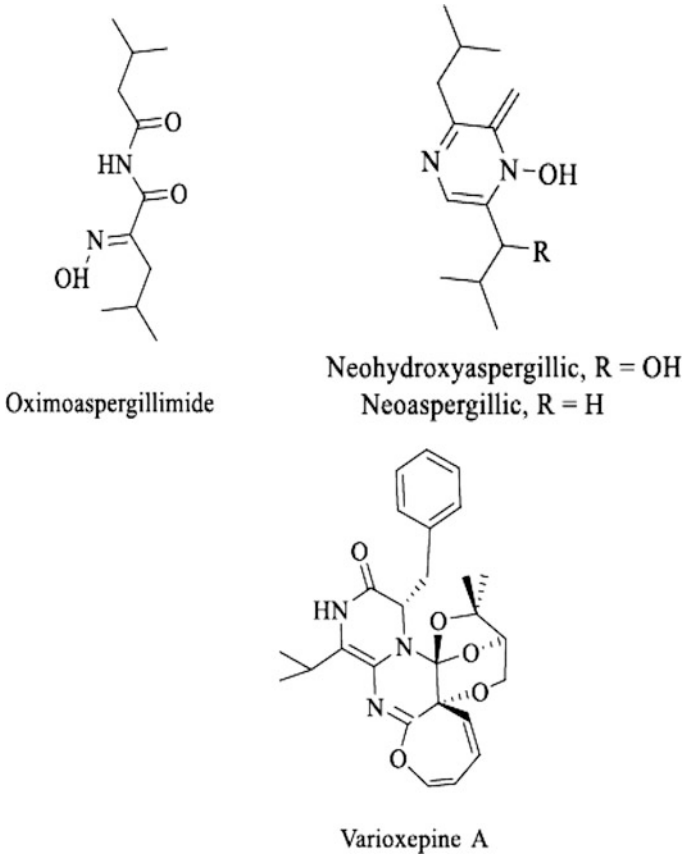


Fig. 21.4 (continued)

21.2.4.1 N-Containing Compounds

The majority of bioactive compounds from marine fungi have been isolated from two fungal genera, i.e., *Penicillium* (41.2% of compounds) and *Aspergillus* (28.1% of compounds). Some noteworthy novel alkaloids from deep-sea *Penicillium* spp. include brevicompanines D–H and cyclopiamide B–J which exhibited cytotoxicity against BV2 cells, brine shrimp, SMMC-7721, and BEL-7402 cell lines (Cardoso-Martínez et al. 2015; Fredimoses et al. 2015; Hu et al. 2019). The novel alkaloids from deep-sea *Aspergillus* spp. include brevianamide R, circumdatin G, circumdatin F, oximoaspergillimide, neohydroxyaspergillic acid, and neoaspergillic acid, of which the first one and the last two exhibited antibiotic activities against BCG, *B. subtilis*, *S. aureus*, *P. aeruginosa*, *B. cereus*, *Klebsiella pneumonia,* and *E. coli*. Varioxepine A and Neoechinulin A extracted from fungi other than Penicillium or Aspergillus showed antimicrobial activity against *E. coli*, *Aeromonas hydrophila*, *Micrococcus luteus*, *S. aureus*, *Vibrio anguillarum*, *Vibrio harveyi,*

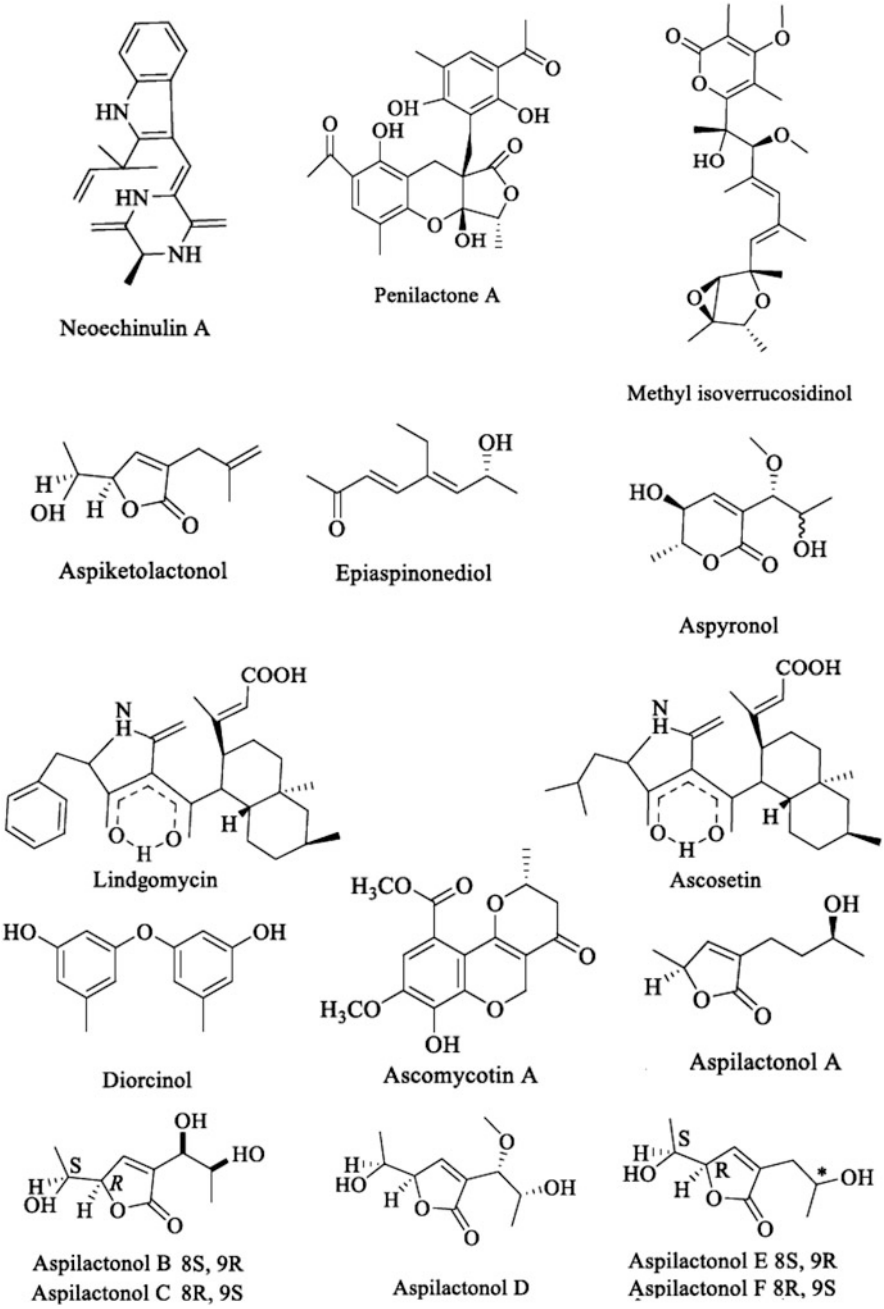


Fig. 21.5 Chemical structure of polyketide compounds obtained from marine fungi (Arifeen et al. 2020)

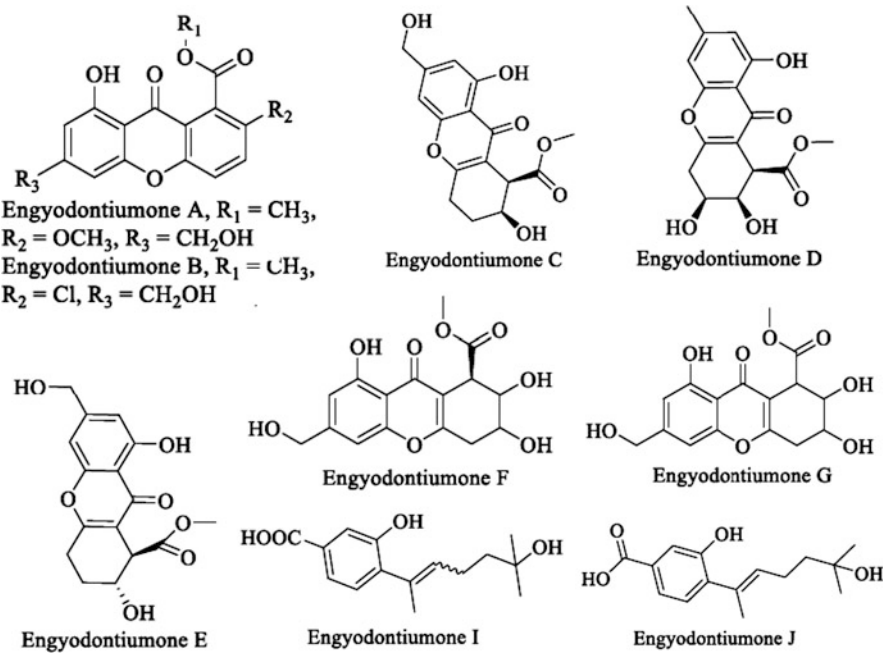
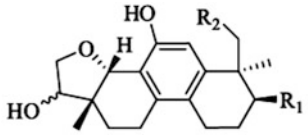


Fig. 21.5 (continued)

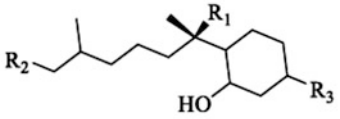
and *Vibrio parahaemolyticus* and cytotoxic activity against HeLa cells, respectively (Li et al. 2011; Wijesekara et al. 2013; Zhang et al. 2014, 2018; Xu et al. 2015a, b).

21.2.4.2 Polyketide Compounds

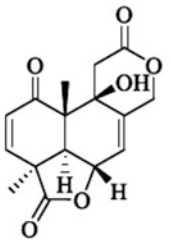
Some noteworthy polyketide compounds from deep-sea *Penicillium* spp. include methylisoverrucosidinol, showing antimicrobial activity against *B. subtilis* and penilactone A showing NF-κβ inhibition activity (Wu et al. 2012; Pan et al. 2016). Aspiketolactonol, aspilactonol A–F, aspyronol, and epiaspinonediol obtained from *Aspergillus* sp. 16-02-1 exhibited cytotoxicity against various cancer cell lines, viz. K562, HL-60, HeLa, and BGC-823 (Chen et al. 2014a, b). Ascomycotin A and diorcinol from *Ascomycota* sp. Ind19F07 and lindgomycin and ascocetin from Lindgomycetaceae strains KF970 and LF327 were found to exhibit strong antibiotic activity against *B. subtilis*, *Acenetobacter baumanii*, *E. coli*, *S. aureus*, *Enterococcus faecalis*, *Staphylococcus epidermidis,* and *Propionibacterium acnes*, whereas engyodontiumone A–J obtained from *Engyodontium album* DFFSC021 exhibited strong cytotoxic activity against U937 cells (Yao et al. 2014; Tian et al. 2015; Wu et al. 2015).



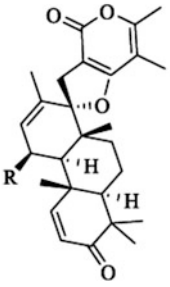
Asperethers A, R_1=OH, R_2=H
Asperethers B, R_1=H, R_2=H
Asperethers C, R_1=H, R_2=OH
Asperethers D, R_1=OH, R_2=OAc
Asperethers E, R_1=OH, R_2=H



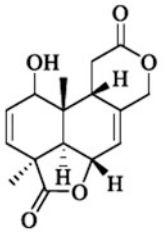
(7S)-(+)-7-O-methylsydonol,
R_1 = OMe, R_2 = H, R_3 = CH_2OH
(7S,11S)-(+)-12-hydroxysydonic acid,
R_1 = OH, R_2 = OH, R_3 = COOH
7-deoxy-7,14-didehydrosydonol,
R_1 = OH, R_2 = H, R_3 = CH_2OH



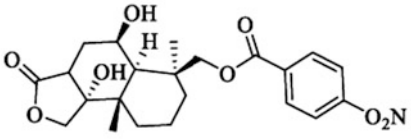
Asperolides D



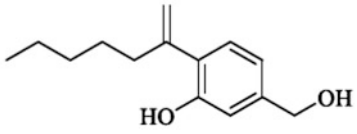
Brevione F, R=H
Brevione I, R=OH



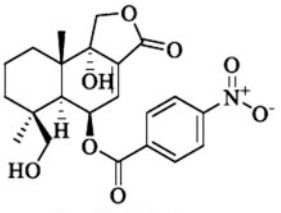
Asperolides E



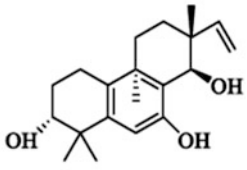
6b, 9a-Dihydroxy-14-p-nitrobenzoylcinnamolide



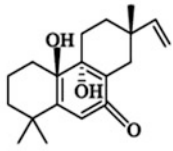
(S)-(+)-Sydonol



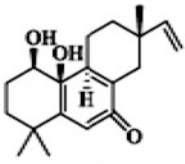
Insulicolide A



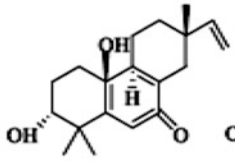
Aspewentin A



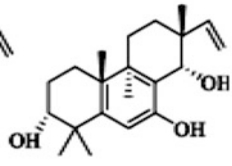
Aspewentins D



Aspewentins F



Aspewentin G



Aspewentin H

Fig. 21.6 Chemical structures of terpenoid compounds obtained from marine fungi (Arifeen et al. 2020)

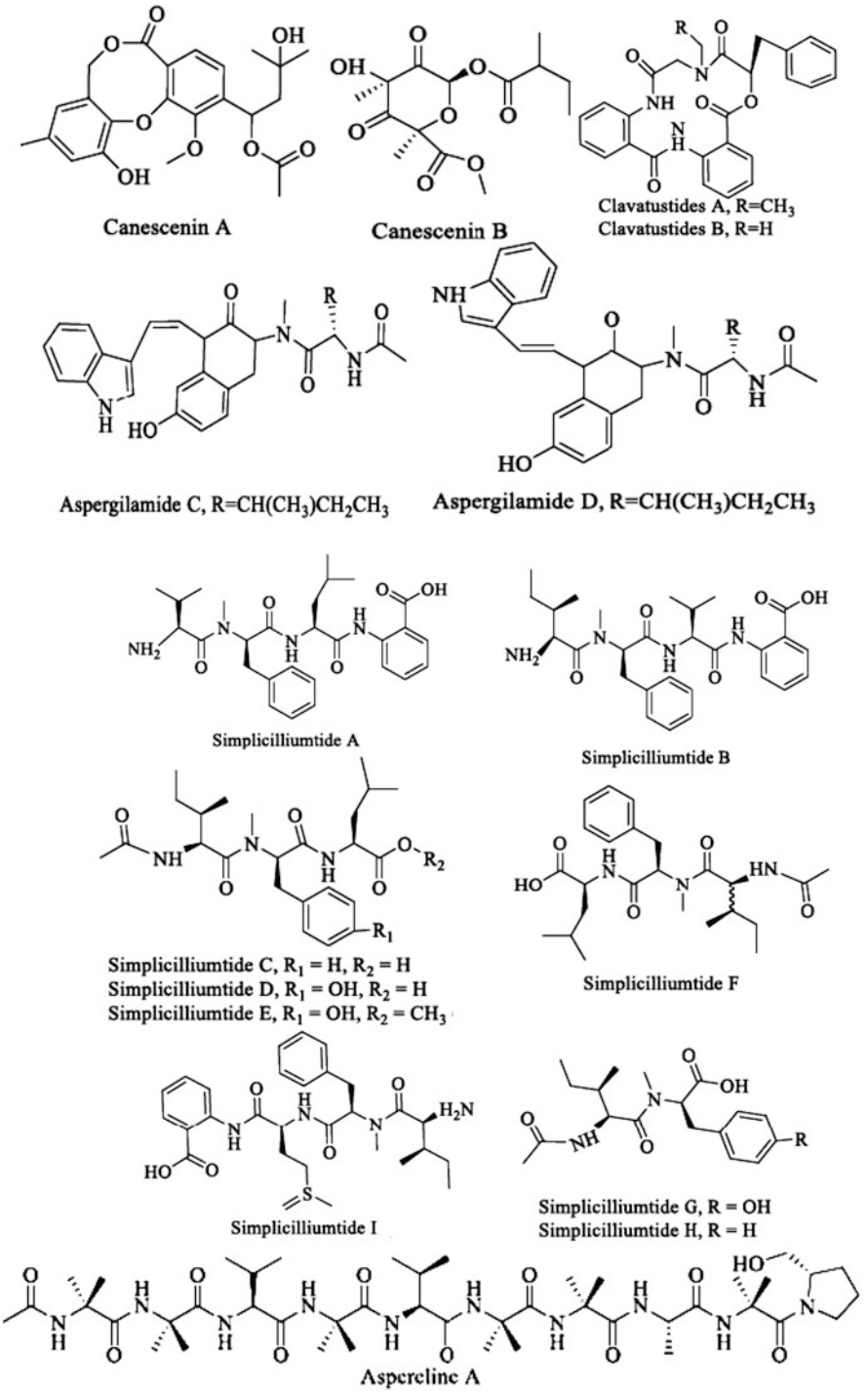


Fig. 21.7 Chemical structure of polypeptide compounds obtained from marine fungi (Arifeen et al. 2020)

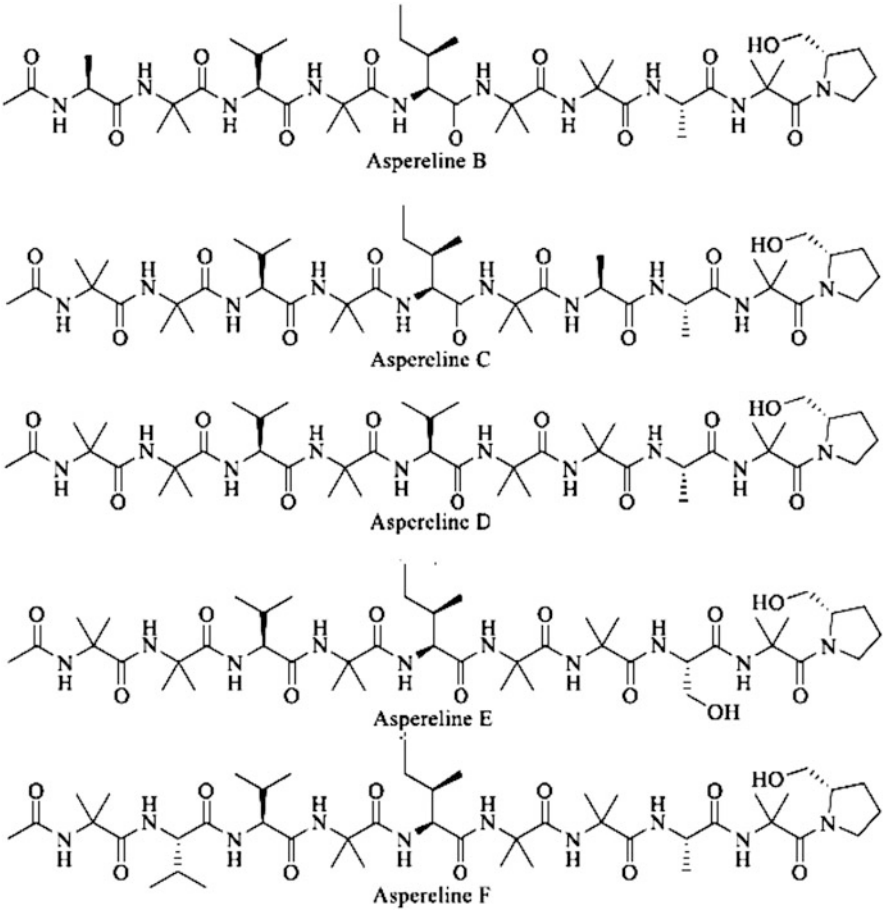
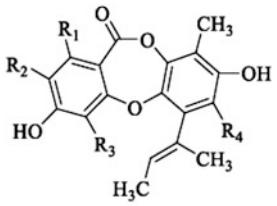


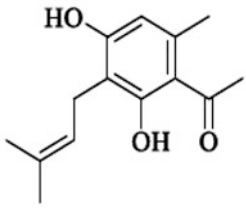
Fig. 21.7 (continued)

21.2.4.3 Terpenoids

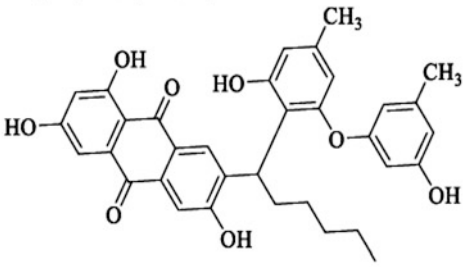
Brevione F–I, obtained from sediment-derived fungus *Penicillium* spp. has shown cytotoxic activity against HeLa, MCF-7, and A549 cells and growth inhibition of HIV-1 against C8116 cells (Li et al. 2012; Zhang et al. 2018). Aspewentin A, aspewentin D–H, asperether A–E, asperoloid D–E from *Aspergillus wentii*, and 6b,9a-dihydroxy-14-*p*-nitrobenzoylcinnamolide and insulicolide A from *Aspergillus ochraceus* Jcma1F17 showed antimicrobial, anti-inflammatory, antiviral, and cytotoxic activities (Fang et al. 2014; Li et al. 2016a, b, c). Sesquiterpenoids (sydonol and its derivatives) obtained from *Aspergillus sydowii* have shown anti-inflammatory and anti-diabetic activities (Chung et al. 2013).



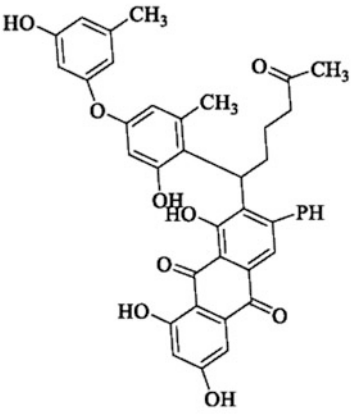
7-Chlorofolipastatin, R_1=CH(CH_3)CH(CH_3),R_2=H, R_3=CH_3, R_4=Cl
Folipostatin, R_1=CH(CH_3)CH(CH_3),R_2=H, R_3=CH_3, R_4=H
Unguinol, R_1=CH_3,R_2=H, R_3=CH_3, R_4=H
2-Chlorounginol, R_1=CH_3,R_2=Cl, R_3=CH_3, R_4=H
2,7-Dichloroung, R_1=CH_3,R_2=Cl, R_3=CH_3, R_4=Cl
Nornidulin, CH_3, R1=CH_3, R_2=Cl, R_3=Cl, R_4=Cl



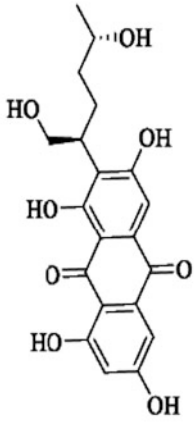
Pestalotionol



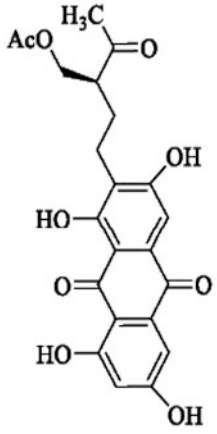
Asperigilol G



Aspergilol H



Aspergilol I



Coccoquinone A

Fig. 21.8 Chemical structures of esters and phenolic compounds obtained from marine fungi (Arifeen et al. 2020)

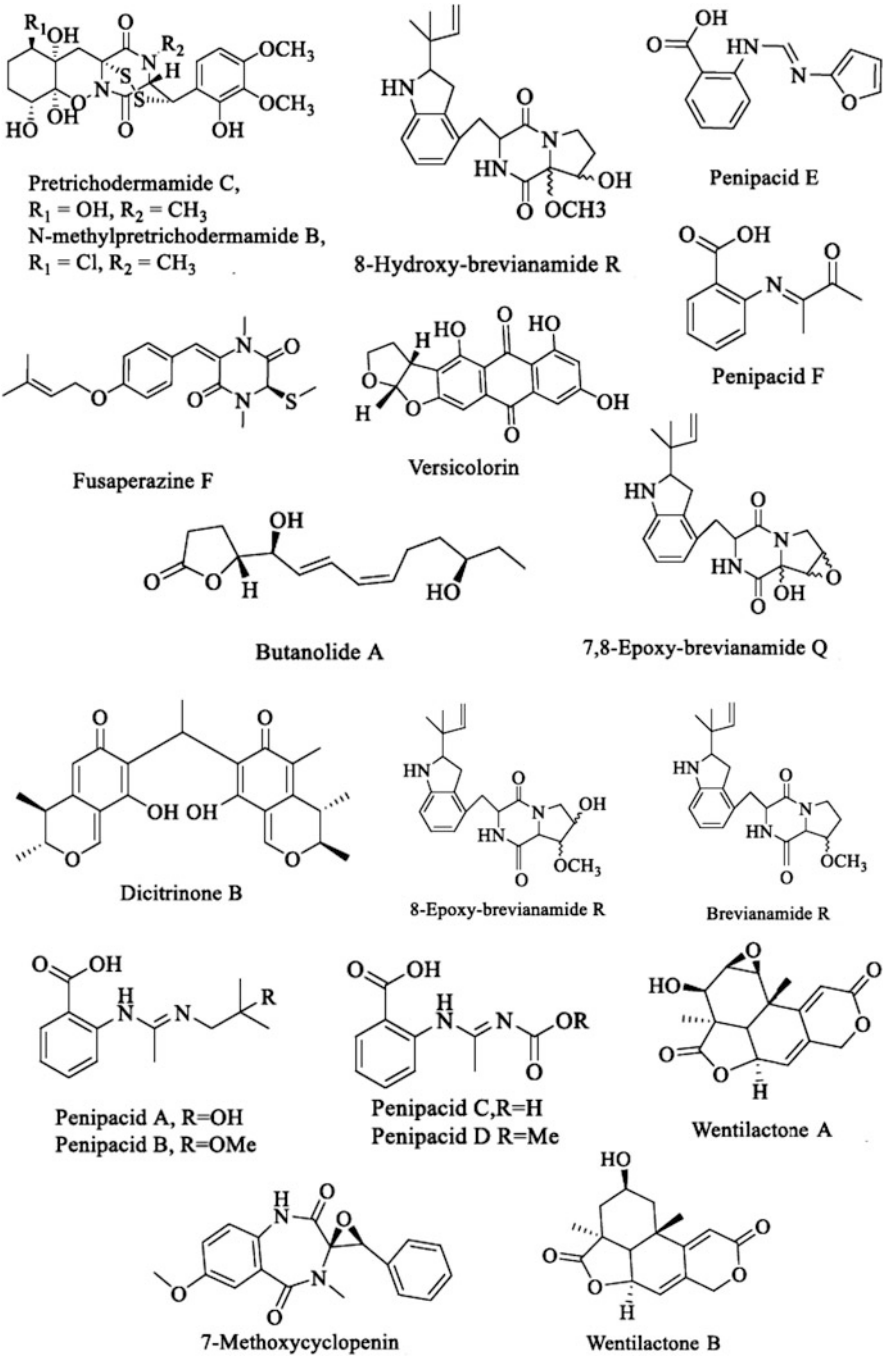
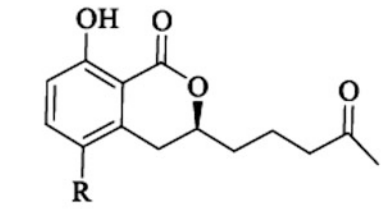
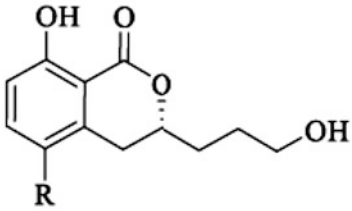


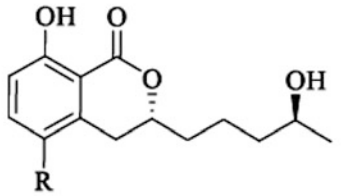
Fig. 21.9 Chemical structure of piperazine and other compounds obtained from marine fungi (Arifeen et al. 2020)



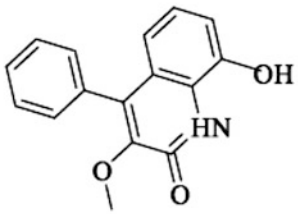
Penicillisocoumarin A, R = OH
Penicillisocoumarin B, R = H



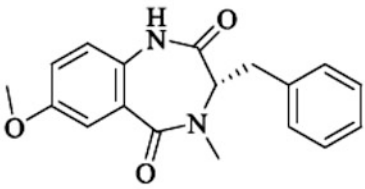
Penicillisocoumarin C



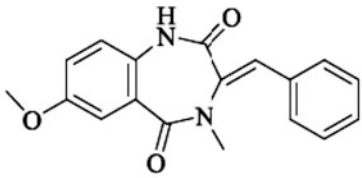
Penicillisocoumarin D, R=OH
Aspergillumarins B,R=H



9-Hydroxy-3-methoxyviridicatin



7-Methoxydehydrocyclopeptin



7-Methoxydehydrocyclopeptin

Fig. 21.9 (continued)

21.2.4.4 Polypeptides

Canescenin A and canescenin B obtained from Penicillium canescens SCSIO z053 revealed antibacterial activities against *Bacillus amyloliquefaciens* and *P. aeruginosa* (Dasanayaka et al. 2020); clavatustide A and clavatustide B from *Aspergillus clavatus* C2WU and aspergillamide C & aspergillamide D from *Aspergillus terreus* SCSIO 41008 showed cytotoxic activity against HepG2, SMMC-7721, Bel-7402 and human glioma U87 cell lines (Jiang et al. 2013a, b; Luo et al. 2019); simplicilliumtides A–I from *Simplicillium obclavatum* EIODSF 020 displayed cytotoxicity against human leukemia HL-60 and K562 cell lines (Liang et al. 2016) and asperelines A–F from *Trichoderma asperellum* were found to exhibit antibacterial activity against *B. amyloliquefaciens*, *S. aureus*, *P. aeruginosa,* and *E. coli* (Ren et al. 2009).

21.2.4.5 Ester and Phenolic Compounds

The esters (7-chlorofolipastatin, folipostatin B, Unguinol, 2-chlorounginol, 2,7-dichlorounguinol, and nornidulin) extracted from *Aspergillus ungui* NKH-007 were found to be potent candidates for anti-atherosclerotic agents along with showing some antibiotic and cytotoxic activities (Uchida et al. 2016). The phenolic compounds (pestalotionol, aspergilol G–I and coccoquinone A) extracted from *Penicillium* sp. Y-5-2 and *Aspergillus versicolor* SCSIO 41502 showed potent antimicrobial activity against *S. aureus* and *B. subtilis* along with some anti-HSV-1, antioxidant, and antifouling properties (Huang et al. 2017; Pan et al. 2017a).

21.2.4.6 Piperazine Derivatives

Piperazine derivatives, viz. fusaperazine F, brevianamides, and versicolorin B obtained from deep-sea sediment-derived marine fungi of genera *Penicillium*, *Aspergillus,* and *Dichotomomyces* showed strong cytotoxicity against K562 and mouse lymphoma cell lines and also exhibited antibacterial activity against *S. aureus* (Hu et al. 2019; Liu et al. 2019).

21.2.4.7 Other Compounds

Some other secondary metabolites, viz. steroloic acid, dicitrinone B, penipacids A–F, butanolide A obtained from deep-sea sediment-derived *Penicillium* spp. showed cytotoxic activities against RKO, MCF-7, PTP1B, and A375 cancer cell lines (Li et al. 2012, 2013; Chen et al. 2014a, b; Zhou et al. 2018). Isocoumarins (penicillisocoumarin A–D) from *Penicillium* and another isocoumarin, aspergillumarin B have exhibited antibacterial activities (Pan et al. 2017b). Wentilactones having anti-tumor activities were obtained from *Aspergillus* spp. (Xu et al. 2015a, b; Pan et al. 2017a).

21.2.5 Highly Acidic Habitat Fungi

Acidophiles are the microorganisms that grow below the optimum pH of 3.0 (Johnson and Quatrini 2016).

21.2.5.1 Polyketides

Penicillium rubrum obtained from a lake of acid mine in Montana, USA has been associated with production of various polyketides, viz. azaphilone-type polyketides, berkazaphilones A, B, and C, berkedienoic acid, berkedienolactone, vermistatin, dihydrovermistatin, penisimplicissin, and methylparaconic acid. Berkazaphilones B, berkazaphilones C, and penisimplicissin were found to inhibit leukemia cancer cell lines by inhibiting caspase-1 enzyme (Stierle et al. 2012a, b).

21.2.5.2 Terpenes

Two new drimane sequiterpene lactones, berkedrimane A and berkedrimane B and a new carboxylic acid were obtained from *Penicillium solitum*, isolated from an acid mine waste lake of Montana, USA. Berkedrimanes A and B exhibited in vitro

anti-inflammatory activity by inhibiting caspase-1 and caspase-3 enzymes (Stierle et al. 2012a, b).

21.2.6 Saline and Hypersaline Habitat Fungi

A habitat having salt concentration similar to that of sea water (3.5% w/v of total dissolved salts) can be considered as a saline habitat (Díaz-Cárdenas et al. 2017). Hypersaline habitats are the environments having salt concentration >100 g/l (Enache et al. 2017), viz. some lakes and biomes of Antarctic region where the microorganisms have to sustain the osmotic pressure and low water activities (Oren 1999; Gunde-Cimerman and Zalar 2014; Waditee-Sirisattha et al. 2016).

Extremely halotolerant and halophilic fungi, viz. *Aspergillus* spp., *Cladosporium* spp., *Penicillium* spp., *Emericella* spp., *Eurotium* spp., and *Wallemia* spp. have been isolated from solar salterns across the world (Gunde-Cimerman and Zalar 2014).

21.2.7 High-Temperature Environments

Thermophilic (optimum growth temperature of 55 °C) and hyperthermophilic (optimum growth temperature of 80 °C) microorganisms are often found in hot-springs and deep-sea hydrothermal vents (Rastogi et al. 2010; Urbieta et al. 2015). The polyamines present in hyperthermophiles are responsible for their survival at high temperatures (Hidese et al. 2018).

21.2.7.1 Polyketides and N-Containing Compounds

Malbranpyrroles A–F, the photosensitive polyketides were discovered from the thermophilic fungus *Malbranchea sulfurea* from Sihchong River Hot Spring Zone of Taiwan. Two cyclodepsipeptides (clavatustides A and B) were obtained from the fungus *Aspergillus clavatus* C2WU isolated from a crab, *Xenograpsus testudinatus*, that was collected at the sulfur-rich hydrothermal vents in Taiwan. They exhibited anticancer activity against hepatocellular carcinoma cell lines HepG2 (Jiang et al. 2013a, b). Nematicidal activity of two prenylated alkaloids (talathermophilins A and B) obtained from the fungus *Talaromyces thermophilus* was observed against the parasite *Panagrellus redivevus* (Chu et al. 2010).

The extracts from fungal strains isolated from the Atacama Desert, Chile have been shown to be effective against *Paracoccidioides brasiliensis* Pb18, the causative agent for South American blastomycosis. The antifungal agent cytochalasin was obtained from organic extract of *Aspergillus felis* from the same area (Mendes et al. 2016).

21.2.7.2 Thermozymes

Thermozymes, the extracellular enzymes from thermophilic fungi have been explored commercially in bioprocesses due to their intrinsic thermostability and catalytic properties (Maheshwari et al. 2000; Fotouh et al. 2016). Different

extracellular enzymes, viz. proteases from *Achaetomium, Chaetomium, Penicillium, Rhizopus, Torula* spp. (Dhakar and Pandey 2016); a proteolytic enzyme with milk-curdling activity at 50 °C from *Mucor pusillus* (Nouani et al. 2009); lipase from *Humicola lanuginose* strain Y-38 (Arima et al. 1972); α-amylase from *Thermomyces lanuginosus* (Arnesen et al. 1998) and *Myceliophthora thermophila* D14 (ATCC 48104) (Sadhukhan et al. 1992); glucoamylase from *T. lanuginosus* (Taylor et al. 1978) and *Humicola grisea* var. thermoidea (Tosi et al. 1993); exocellulase, endocellulase, and cellobiase from *Thermomyces aurantiacus* (Khandke et al. 1989); cellobiose dehydrogenases from *Sporotrichum thermophile* (Coudray et al. 1982; Schou et al. 1998) and *Humicola insolens* (Schou et al. 1998); xylanases from *Melanocarpus albomyces* (Prabhu and Maheshwari 1999), *Humicola grisea* var. thermoidea (Monti et al. 1991) and *Talaromyces emersonii* (Tuohy et al. 1993); polygalacturonases (exo-TePG28a and endo-TePG28b) from *Talaromyces leycettanus* JCM12802 (Li et al. 2017); laccases from *Chaetomium thermophilum* (Chefetz et al. 1998); phytases (myo-inositol hexakisphosphate phosphohydrolases) from *Myceliophthora thermophila* and *Talaromyces thermophilus* (Wyss et al. 1999) have been isolated, purified, and characterized.

21.3 Conclusion

The growing concern over antimicrobial resistance in human and animal pathogens has led to the search for alternative strategies to combat them. This has resulted in the discovery of some novel bioactive substances produced by microbial species isolated from extreme habitats. These compounds can be exploited to control multidrug-resistant pathogens and treat chronic ailments like cancer, diabetes, epilepsy, blood pressure, etc. As per the available literatures, taxonomically diverse extremophilic and extremotolerant fungi (e.g., *Penicillium* spp. and *Aspergillus* spp.) were the source of several novel specialized metabolites, of which the N-containing compounds and polyketides were the most frequently isolated classes of metabolites. It is observed that the wide range of bioactive compounds obtained from extremophilic fungi showed several activities, but majority of them showed antibacterial and anticancer activities. Fungal strains have been isolated from all the extreme biomes like highly acidic habitats, saline and hypersaline habitats, high-temperature environments, etc. Terpenoid derivatives from extremophilic fungi showed stronger antibacterial and cytotoxic potentials and have the possibility of being future candidates for anticancer drug development.

The major challenges in growth, production, and extraction of extremophilic fungi in laboratory scale are the difficulty in achieving the optimum conditions for growth and high cost involvement. There remains a huge difficulty in producing enough biomass for large scale industrial production. The knowledge on extremophiles is increasing but is still insufficient considering the hugeness in diversity of compounds and variety of organisms present. More research in this area can lead to identification of novel bioactive compounds of clinical importance.

In general, extremophilic fungi can be considered as "chemical factory." Hence, it can be presumed that the extremophilic fungi as source of bioactive compounds could be the new arsenal for modern science.

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Piezophilic Fungi: Sources of Novel Natural Products with Preclinical and Clinical Significance

22

Tuyelee Das, Puja Ray, Samapika Nandy, Abdel Rahman Al-Tawaha, Devendra Kumar Pandey, Vijay Kumar, and Abhijit Dey

Abstract

Extremophiles span through all taxonomic ranges starting from prokaryotes to Eucarya as well as Archaea. Extremophiles are categorized into seven groups on the basis of their various extreme habitats. Piezophiles withstand high hydrostatic pressure and reside at deep-sea sediments or are isolated from the bottom-dwelling animals' guts. Piezophilic fungi are known to produce plethora of natural compounds with tremendous preclinical and clinical significance. Deep-sea-derived fungi such as different strains of *Phialocephala* sp., *Penicillium* sp., *Ascomycota* sp., *Aspergillus* sp., and *Emericella* sp. have been known to produce an array of pharmacologically active compounds such as diketopiperazine and oxindole alkaloids, sorbicillin-type compounds, diterpenes like brevianespiroditerpenoids, prenylxanthones, hydroxyphenyl acetic acid, etc. exhibiting antimicrobial, antiviral, anticancer, α-glucosidase inhibitory, and anti-oxidant properties in a number of preclinical investigations. The present review comprehensively retrieves the reports on novel piezophilic fungi-derived natural products mainly focusing on the recent literature emphasizing on their structures, biological activities, and structure–activity analyses with a note on future clinical implications of such piezophiles-derived drugs.

T. Das · P. Ray · S. Nandy · A. Dey (✉)
Department of Life Sciences, Presidency University, Kolkata, West Bengal, India
e-mail: abhijit.dbs@presiuniv.ac.in

A. R. Al-Tawaha
Department of Biological Sciences, Al-Hussein Bin Talal University, Maan, Jordon

D. K. Pandey · V. Kumar
Department of Biotechnology, Lovely Faculty of Technology and Sciences, Lovely Professional University, Phagwara, Punjab, India

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Keywords

Extremophiles · Piezophilic · Natural Products · Structure-activity · Bioactivity

22.1 Introduction

Extremophiles are a wide group of organisms present in extreme conditions. Extremophiles include bacteria, archaea, and eukarya (Woese et al. 1990). Based on different extreme environmental conditions, extremophiles are categorized into piezophiles (high hydrostatic pressure) (Skropeta 2008; Yayanos 1995; Horikoshi 1998), thermophiles (50–80 °C or more than 80 °C) (Wilson and Brimble 2009), psychrophiles (present in extreme habitats like in the Antarctic, the Arctic, and glacial regions) (Deming 2002), halophiles (requires >3% NaCl for growth) (Wilson and Brimble 2009), xerophiles (ashes and deserts) (Evans and Johansen 1999), acidophiles (present in an environment with pH values less than 4), and alkaliphiles (pH values more than 9) (Wilson and Brimble 2009). Due to this extreme ecosystem's extremophiles possess some unique strategies for their reproduction and survival, which needs the involvement of metabolic pathways and genetic regulation. Metabolic pathways that involve in their survival produced some noteworthy and precious compound, which can be used as natural products alternative to chemically synthesised drugs for the treatment of some most important human diseases (Stetter 1999; Madern et al. 2000; Rothschild and Mancinelli 2001; Soldatou and Baker 2017). Natural products include bioactive secondary metabolites, which are produced by all ranges of organism prokaryotes to eukaryotes. These compounds exhibit several biological activities including antioxidant, antibacterial, antifungal, antiviral, cytotoxic, and anti-inflammatory. Most of the species from extreme ecosystems are still unexplored. Fungi from such environment hold undiscovered metabolites.

Deep-sea is defined by the depths of 200–300 m beyond the euphotic zone (van Dover et al. 2002). Some of the world's remote ecosystems are present in the ocean more than 1000 m deep, with every 10 m of depth 1 atm hydrostatic pressure increases (Skropeta and Wei 2014; Takami et al. 1997). World's 71% of marine environment and polar region is cold. Piezophiles are present in the sea below the thermocline layer where cold environment is constant throughout the year. Water present below the thermocline layer is having extreme hydrostatic pressure (Moyer et al. 2017). Piezophilic organisms can grow at 70–80 MPa, though unable to sustain below 50 MPa (Kato et al. 1998). Oger and Jebbar 2010 described that piezophiles grow and complete reproduction under high pressure $\leq$100 MPa (Oger and Jebbar 2010). These organisms habituated from deep-sea obtain their nutrients from the deep-sea sediment or isolated waters. However, wide varieties of deep-sea fungi are present but proper sampling methods of those fungi are the major limitations for the discovery of new bioactive chemicals. ZoBell describes "barophile" (also known as piezophile) term for the first time and Roth et al. (1964) studied and published the first paper on deep-sea piezophilic fungi in 1964 (ZoBell and Johnson 1949; Roth

et al. 1964). The piezophilic fungi are a novel and sustainable source for the specific and wide varieties of bioactive molecules that can treat cancer, bacterial, viral, and fungal diseases.

Deep-sea fungi (piezophiles) can be isolated from the different sources such as from the sediment, free suspension, any attached flocculated material, any surfaces, or from association with alga, plant, or other living partners without changing the pressure and temperature. After collection, this sample can be preserved in liquid nitrogen for as long as decades. The extraction of fungi from the mycelium of broth culture settled into different solvents such as acetone, hexane, n-butanol, chloroform, ethyl acetate (EtOAc), methanol (MeOH), carbon tetrachloride, and DCM. Bioactive compounds are generally categorized in six classes: alkaloids, polyketides, peptides, shikimates, sugars, and terpenes. Because of the mixed and wide diversity of bioactive compounds, separation is difficult. TLC, HPLC, and other types of column chromatography are good choices for the separation of the extracted compounds (Zhang et al. 2011; Parkes et al. 2009). Structures of all the bioactive compounds are generally screened by spectroscopy analysis (NMR, MS, and FTIR) and X-ray crystallographic studies. The present chapter focuses on piezophillic fungal bioactive compounds, their source, structures, biological activities, and structure–activity relationship analysis.

22.2 Diversity of Deep-Sea Fungi

The ecosystem of deep-sea rich in different microbial organisms not only contains bacteria and Achaea but also contains a wide variety of fungi. The investigated deep-sea environments include South Atlantic Ocean, South China Sea, Suruga-Bay, Japan Prydz Bay, Antarctica Gulf of Mexico Hsinchu, Taiwan Bohai Sea, West and South Pacific Ocean, Paracel Islands, and Indian Ocean. Deep-sea fungi included in this chapter are *Ascomycota*, *Acremonium*, *Aspergillus*, Cladosporium, *Chromocleista*, *Dichotomomyces*, *Diaporthe*, *Emericella*, *Engyodontium*, *Penicillium*, *Phialocephal*, *Phoma*, *Spiromastix*, and *Simplicillium*. In this review, we summarized new and important bioactive compounds obtained from deep-sea fungi during the last 15 years (Table 22.1). Chemical structures of antibacterial and anticancer fungi derived natural products and some other bioactivity of fungi derived natural products are described in Figs. 22.1 and 22.2. Figure 22.3 presents some other bioactivity of fungi derived natural products.

22.3 Biological Activities

22.3.1 α-Glucosidase Inhibitory Activity

α-Glucosidase inhibitors are broadly used for the treatment of type 2 diabetes. Natural products could be also an important source for α-glucosidase inhibitors. Deep-sea fungus *Dichotomomyces cejpii* FS110 (South China Sea, depth 3941 m)

Table 22.1 Piezophilic fungi bioactive compounds and their activities

Species	Habitat	Location of collection	Bioactive compounds	Structures were elucidated by	Activity	Active against bacteria/fungus/virus/larva or inhibited cell line	Activity value (MIC/IC_{50}/EC_{50}/LC_{50})	References
Acaromyces ingoldii FS121	Deep-sea sediment	South China Sea	Acaromycin A, cryptosporin	Spectroscopic analysis, ECD	Cytotoxicity	MCF-7, NCI-H460, SF-268, HepG-2	IC_{50}: < 10 μM	Gao et al. (2016)
Arthrinium sp. UJNMF0008	Deep-sea sediment	South China Sea	4-Hydroxy-2-pyridone alkaloids arthpyrones D–K, apiosporamide, arthpyrone B	Spectroscopic analysis	Antibacterial	*M. smegmatis*, *S. aureus*	IC_{50}: 1.66–42.8 μM	Bao et al. (2018)
					Cytotoxicity	U2OS, MG63	IC_{50}: 19.3 μM (U2OS) 11.7 μM (MG63)	
Ascomycota sp. CYSK-4	Mangrove endophytic *Pluchea indica*	Guangxi Province, China	Desmethyldichlorodiaportintone	Spectroscopic analysis	Anti-inflammatory	LPS-induced RAW 264.7 cells	IC_{50}: 15.8 μM	Chen et al. (2018)
			Dichlorodiaportintone, desmethyldichlorodiaportin, dichlorodiaportin	Spectroscopic analysis	Anti-inflammatory	LPS-induced RAW 264.7 cells	IC_{50}: 41.5 μM (dichlorodiaportintone), 33.6 μM (desmethyldichlorodiaportin), 67.2 μM (dichlorodiaportin)	
					Antibacterial	*S. aureus*, *B. subtilis*, *E. coli*, *K. pneumoniae*, *A. calcoaceticus*	MIC: 25–50 μg/mL	
Aspergillus sp. SCSIO Ind09F01	Deep-sea sediment	Indian Ocean	Sydoxanthone C, acremolin B, diorcinol, cordyol C, 3,7-dihydroxy-1,9-dimethyldibenzofuran	1D, 2D-NMR, HRESIMS	Cytotoxicity	HeLa, DU145, U937	IC_{50}: 2.4 μM (diorcinol), 7.1 μM (cordyol C), 10.6 μM (3,7-dihydroxy-1,9-dimethyldibenzofuran)	Tian et al. (2015)
Aspergillus fischeri FS452	Deep-sea sludge	Indian Ocean	Fiscpropionates A–D	J-HMBC, ECD	Inhibitory	*M. tuberculosis* protein tyrosine phosphatase B	IC_{50}: 5.1, 12, 4.0, 11 μM	Liu et al. (2019)
Aspergillus ochraceus Jcma1F17	Marine alga *Coelarthrum* sp.	Paracel Islands,	Nitrobenzoyl sesquiterpenoids	NMR, MS, CD, optical rotation analysis	Cytotoxicity	H1975, U937, K562, BGC-823, Molt-4, MCF-7,	IC_{50}: 1.95–6.35 μM	Fang et al. (2014)

		South China Sea				A549, Hela, HL60, Huh-7		
					Antiviral	H3N2, EV71	IC_{50}: 17.0 μM, 9.4 μM	
Aspergillus sp. SCSIO Ind09F01	Deep-sea sediment	–	Gliotoxin, 12,13-dihydroxyfumitremorgin C, helvolic acid	NMR, ESIMS	Anti-tuberculosis	*M. tuberculosis*	MIC_{50}: <0.03, 2.41 and 0.894 μM	Luo et al. (2017)
			Gliotoxin	NMR, ESIMS	Cytotoxicity	K562, A549, Huh-7	IC_{50}: 0.191 μM, 0.015 μM, 95.4 μM	
Aspergillus sydowii YH11–2	Deep-sea sediment	Guam	(2R)-2, 3-Dihydro-7-hydroxy-6, 8-dimethyl-2-[(E)-propl-enyl] chromen-4-one; 2, 4-dihydroxy-3, 5, 6-trimethylbenzaldehyde	Spectroscopic analysis	Cytotoxicity	P388	IC_{50}: 0.14, 0.59 μM	Tian et al. (2007)
Aspergillus sydowii C1-S01-A7	Deep-sea sediment	West Pacific Ocean	2-Hydroxy-6-formyl-vertixanthone, 12-O-acetyl-sydowinin A, aspergillus one A, AGI-B4, 8- demethoxy-10-methoxy-wentiquinone C, emodin	Spectroscopic analysis	Antibacterial	MRSA	MIC: 15–32 μg/mL	Wang et al. (2019)
			AGI-B4	Spectroscopic analysis	Cytotoxicity	A549	IC_{50}: < 10 μM	
Aspergillus terreus YPGA10	Deep-sea water	Yap Trenc, West Pacific Ocean	Aspernolide A, Aspernolide E	NMR, MS	Inhibitory	α-Glucosidase	IC50: 6.98 (aspernolide A), 8.06 μM (aspernolide E)	Cheng et al. (2019)
Aspergillus versicolor MF030	Deep-sea sediment	Bohai Sea, China	Brevianamide S	Spectroscopic analysis	Antibacterial	Bacille Calmette-Gu?rin	MIC: 6.25 μg/mL	Song et al. (2012)
Aspergillus versicolor A-21-2-7	Deep-sea sediment	Southern China Sea	Aspergilols A–F, cordyol C, methylgerfelin, violaceol II, lecanoric acid	Spectroscopic analysis	Antioxidant	Activates Nrf2-regulated gene	–	Wu et al. (2016)
Aspergillus versicolor SD-330	Deep-sea sediment	South China Sea	Sesquiterpenoid, aspergoterpenin C, engyodontiumone I	NMR, HRESIMS, X-ray crystallographic analysis, ECD	Antimicrobial	*A. hydrophilia*, *E. coli*, *E. tarda*, *V. harveyi*	MIC: 1.0–8.0 μg/mL	Li et al. (2019)

(continued)

Table 22.1 (continued)

Species	Habitat	Location of collection	Bioactive compounds	Structures were elucidated by	Activity	Active against bacteria/fungus/ virus/larva or inhibited cell line	Activity value (MIC/IC_{50}/ EC_{50}/LC_{50})	References
Aspergillus versicolor SCSIO 41502	Deep-sea sediment	South China Sea	4-Carbglyceryl-3,3′-dihydroxy-5,5′-dimethyldiphenyl ether, sydowiol B, sydowiol E, sydowiol D, 2,4-dihydroxy-6-(4S-hydroxy-2-oxopentyl)-3-methylbenzaldehyde	Spectroscopic analysis	Antifouling activity	*B. neritina*	EC_{50}: 1.28 μg/mL, 2.61 μg/mL, 5.48 μg/mL, 1.59 μg/mL, 3.40 μg/mL	Huang et al. (2017)
Aspergillus versicolor SCSIO 05879	Deep-sea-derived	Indian Ocean	Versicoloid A, versicoloid B, versicone A, cottoquinazoline A	NMR, X-ray single crystal diffraction	Antifungal	*C. acutatum*	MIC: 1.6 μg/mL	Wang et al. (2016)
Aspergillus westerdijkiae SCSIO 05233	Deep-sea sediment	South China Sea	Circumdatin G	NMR, optical rotation analysis	Cytotoxicity	K56, HL-60	IC_{50}: 25.8 μM (K56), 44.9 μM (HL-60)	Fredimoses et al. (2015)
			Circumdatin F	NMR, optical rotation analysis	Antifouling	*Balanus amphitrite*	$EC_{50:}$ 8.81 μg/mL	
Aspergillus sydowii	Deep-sea sediment	Hsinchu, Taiwan	Diorcinol, bisabolane-type sesquiterpenoids, AGI-B4, sydowinin B	NMR, HPLC, MS-ESI	Anti-inflammatory	Inhibits superoxide anion and elastase release	–	Chung et al. (2013)
Chaetomium globosum	Deep-sea sediment	Indian Ocean	Cytoglobosins H and I, cytochalasan alkaloids	1D, 2D NMR, MS	Cytotoxicity	MDA-MB-231, B16F10	IC_{50}: 0.62 (MDA-MB-231), 2.78 μM (B16F10)	Zhang et al. (2016c)
Chromocleista sp.	Sediment	Gulf of Mexico	p-Hydroxyphenopyrrozin	NMR, y X-ray crystallography	Antifungal	*C. albicans*	MIC: 25 μg/mL	Park et al. (2006)
*Cladosporium cladosporioides*HDN14–342	Deep-sea	Indian Ocean	Clindanones A and B, cladosporols F and G	MS, NMR, TD-DFT, ECD	Cytotoxicity	HeLa	IC_{50}: 3.9 μM	Zhang et al. (2016b)
*Cladosporium sphaerospermum*2005–01-E3	Deep-sea sludge	Pacific Ocean	Cladosin C	NMR	Antiviral	Influenza A H1N1 virus	IC_{50}: 276 μM	Wu et al. (2014)

Dichotomomyces cejpii FS110	Deep-sea sediment	South China Sea	Dichotocejpins A	HRESIMS, NMR, X-ray crystallography, ECD	Inhibitory activity	α-Glucosidase	IC_{50}: 138 μM	Fan et al. (2016)
			6-Deoxy-5a,6-didehydrogliotoxin, gliotoxin, acetylgliotoxin	HRESIMS, NMR, X-ray crystallography, ECD	Cytotoxicity	MCF-7, NCI-H460, HepG-2, SF-268	IC_{50}: 0.08–1.52 μM	
Diaporthe phaseolorum FS431	Deep-sea sediment	Indian Ocean	Phaseolorin A–E	Spectroscopic analysis, single-crystal X-ray diffraction	Cytotoxicity	HepG-2, MCF-7, SF-268	–	Guo et al. (2019)
Emericella sp. SCSIO 05240	Deep-sea sediment	South China Sea	Emerixanthones A, B	NMR, HSQC, HMBC, 1H-1H COSY, MS, CD	Antibacterial	*E. coli*, *K. pneumonia*, *S. aureus*, *E. faecalis*, *A. baumannii*, *A. hydrophila*	–	Fredimoses et al. (2014)
			Emerixanthone D	NMR, HSQC, HMBC, 1H-1H COSY, MS, CD	Antifungal	*Fusarium* sp., *Penicillium* sp., *Aspergillus niger*, *Rhizoctonia solani*, *Fusarium oxysporium*	–	
Engyodontium album DFFSCS021	Deep-sea sediment	South China Sea	Engyodontiumone H	Spectroscopic analysis	Cytotoxicity	U937	IC_{50}: 4.9 μM,	Yao et al. (2014)
					Antibacterial	*E. coli*, *B. subtilis*	MIC ≤64 μg/mL	
Phialocephala sp. FL30r	Deep-sea sediment	East Pacific site W2003–03	Dihydrotrichodermolide, dihydrodemethylsorbicillin, phialofurone	Spectroscopic analysis	Cytotoxicity	P388	IC_{50}:11.5 ± 1.4 μM (dihydrotrichodermolide), 0.1 ± 0.1 μM (dihydrodemethylsorbicillin), 0.2 ± 0.01 μM	Li et al. (2011a, b)
						K562	IC_{50}: 22.9 ± 0.8 μM (dihydrodemethylsorbicillin,	

(continued)

Table 22.1 (continued)

Species	Habitat	Location of collection	Bioactive compounds	Structures were elucidated by	Activity	Active against bacteria/fungus/ virus/larva or inhibited cell line	Activity value (MIC/IC_{50}/ EC_{50}/LC_{50})	References
							phialofurone), 4.8 ± 0.3 μM (dihydrodemethylsorbicillin), 22.4 ± 0.9 μM (phialofurone)	
Phialocephala sp. FL30r	Deep-sea sediment	–	Trisorbicillinone A	Spectroscopic analysis	Cytotoxicity	P388	IC_{50}: 9.10 μM	Li et al. (2007b)
						HL60	IC_{50}: 3.14 μM	
Penicillium sp. SCSIO 06720	Deep-sea sediment	Indian Ocean	b-Diversonolic ester	NMR, MS HPLC, ECD	Antibacterial activity	MRSA	MIC: 10.4 ± 3.7 μg/mL	Guo et al. (2020)
						S. aureus-shh-1	MIC: 46.9 ± 29.7 μg/mL	
Penicillium sp. PR19N-1	Deep-sea sediment	Prydz Bay, Antarctica	Chloro-trinor eremophilane sesquiterpene	IR, HRMS, 1D, 2D NMR	Cytotoxic activity	HL-60,	IC_{50}: 11.8 ± 0.2 μM,	Wu et al. (2013)
						A549	$IC_{50:}$ 12.2 ± 0.1 μM	
Penicillium sp. JMF034	Deep-sea sediment	Fujikawa, Suruga-bay, Japan	Gliotoxin, gliotoxin G	ESIMS, ^{1}H NMR	Cytotoxic activity	P388	0.024 μM (gliotoxin), 0.020 μM (gliotoxin G)	Sun et al. (2012)
Penicillium brevicompactum DFFSCS025	Deep-sea sediment	South China Sea, Sansha City, Hainan Province	Brevianamides, mycochromenic acid	Spectroscopic analysis	Antifouling	*Bugula neritina*	EC_{50}: 13.7 μM	Xu et al. (2017)
					Cytotoxicity	HCT116	IC_{50}: 15.6 μM	
Penicillium chrysogenum MCCC 3A00292	Deep-sea sediment	South Atlantic Ocean	Peniciversiols A	HRESIMS, NMR, ECD	Cytotoxicity	BIU-87	IC_{50}:10.21 μM	Niu et al. (2019)
			Decumbenone A, decumbenone B, 3,3′-dihydroxy-5,5′-dimethyldiphenyl ether, violaceol-II,3,8-dihydroxy-4-(2,3-dihydroxy-1-hydroxymethylpropyl)-1-	HRESIMS, NMR, ECD	Cytotoxicity	ECA109, BIU-87, BEL-7402	IC_{50}: 7.70 to >20 μM	

			methoxyxanthone, asperdemin, cyclopenol					
Penicillium commune SD-118	Deep-sea sediment	South China Sea	Xanthocillin X	NMR	Antimicrobial	*S. aureus*	MIC: 2 μg/mL	Shang et al. (2012)
						E. coli	MIC: 1 μg/mL	
					Antifungal	*A. brassicae*	MIC: 32 μg/mL	
					Cytotoxicity	MCF-7	IC_{50}: 12.0 μg/mL	
						HepG2	IC_{50}: 7.0 μg/mL,	
						NCI-H460	IC_{50}: 10.0 μg/mL, 10.0 μg/mL	
						HeLa, MDA-MB231, DU145,	IC_{50}: 8.0 μg/mL	
*Penicillium fellutanum*HDN14–323	Deep-sea sediment	Indian Ocean	Peniphenylanes	Spectroscopic analysis	Cytotoxicity	HeLa	IC_{50}: 9.3 μM	Zhang et al. (2016a)
*Penicillium paneum*SD-44	Deep-sea sediment	South China Sea	Anthranilic acid, penipacids A-E	NMR, MS	Cytotoxicity	RKO	IC_{50}: 8.4 μM, 9.7 μM	Li et al. (2013)
						Hela	IC_{50}: 6.6 μM	
Phomopsistersa FS441	Deep-sea sediment	Indian Ocean	Tersone E, citridone A	Spectroscopic analysis, single-crystal X-ray diffraction experiments, ECD	Antibacterial	*S. aureus* ATCC 29	MIC: 31.5 μg/mL	Chen et al. (2019)
			Tersone E		Cytotoxicity	SF-268	IC_{50}: 32.0 μM	
						MCF-7	29.5 μM	
						HepG-2	39.5 μM	
						A549	33.2 μM	
Simplicillium obclavatum EIODSF 020	Deep-sea sediment	East Indian Ocean	Simplicilliumtide J, verlamelins A and B	Spectroscopic analysis	Antiviral	HSV-1	IC_{50}: 14.0 μM (simplicilliumtide J), 16.7 μM (verlamelin A), 15.6 μM (verlamelin B)	Liang et al. (2017)
					Antifungal	*A. versicolor*, *C. australiensis*	MIC: 14.0 μg/disc (simplicilliumtide J), 16.7 μg/disc (verlamelin A), 0.156 (verlamelin B) μg/disc	

(continued)

Table 22.1 (continued)

Species	Habitat	Location of collection	Bioactive compounds	Structures were elucidated by	Activity	Active against bacteria/fungus/ virus/larva or inhibited cell line	Activity value (MIC/IC_{50}/ EC_{50}/LC_{50})	References
Spiromastix sp., *Simplicillium*	Deep-sea sediment	South Atlantic Ocean	Spiromastixones A–O	NMR	Antibacterial	MARS, MRSE	MIC: 0.1–8.0 μg/mL	Niu et al. (2014)
Spiromastix sp. MCCC 3A00308	Deep-sea sediment	South Atlantic Ocean	Spiromastols A–K	NMR, MS	Antibacterial	*X. vesicatoria*, *P. lachrymans*, *A. tumefaciens*, *R. solanacearum*, *B. thuringensis*, *S. aureus*, *B. subtilis*	MIC: 0.25–4 μg/mL	Niu et al. (2018)

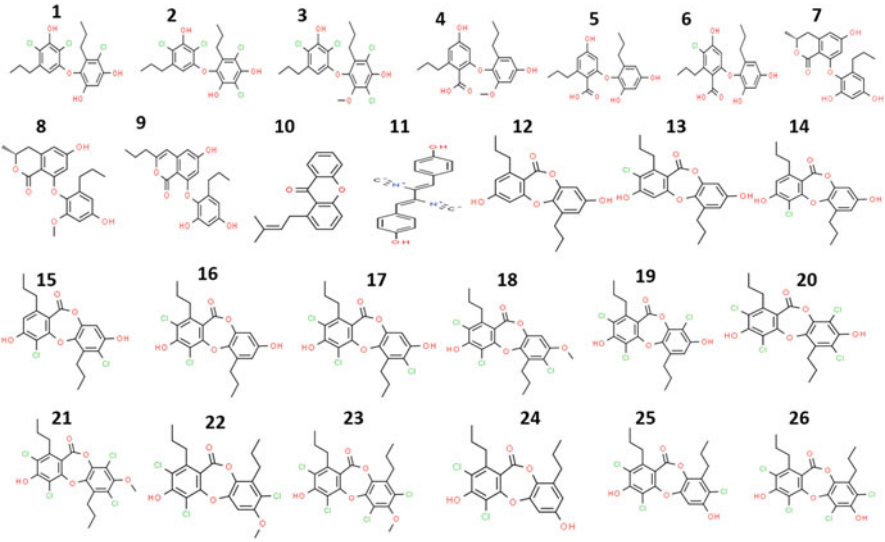

Fig. 22.1 Chemical structure of antibacterial natural products from piezophilic fungi. 1. Spiromastol A, 2. Spiromastol B, 3. Spiromastol C, 4. Spiromastol D, 5. Spiromastol E, 6. Spiromastol F, 7. Spiromastol G, 8. Spiromasto H, 9. Spiromasto l, 10. Prenylxanthone, 11. Xanthocillin X, 12. Spiromastixones A, 13. Spiromastixone B, 14. Spiromastixone C, 15. Spiromastixone D, 16. Spiromastixone E, 17. Spiromastixone F, 18. Spiromastixone G, 19. Spiromastixone H, 20. Spiromastixone I, 21. Spiromastixone J, 22. Spiromastixone k, 23. Spiromastixone L, 24. Spiromastixone M, 25. Spiromastixone N, 26. Spiromastixone O

derived biologically significant dichotocejpin A compound showed inhibitory activity against α-glucosidase with IC_{50}: 138 μM (Fan et al. 2016). One recent study by Chen et al. (2019) reported that ethyl acetate extract of deep-sea water *Aspergillus terreus* YPGA10 fungus produced butyrolactone I, butyrolactone VII, aspernolide A, and aspernolide E which also inhibited α-glucosidase (Cheng et al. 2019). This study could be helpful for the development of new potential inhibitors of α-glucosidase.

22.3.2 Antibacterial Activity

Antibacterial drugs are one of the most important drugs against human pathogenic bacterial diseases. Deep-sea fungal strains and its bioactive compounds are new and noteworthy potential alternative source, which by screening can be designed as drugs against bacterial diseases.

New polyphenols spiromastols A–K were isolated from the fermentation broth of the deep-sea-derived fungus *Spiromastix* sp. MCCC 3A00308 (South Atlantic Ocean). All these polyphenols inhibited the growth of *Staphylococcus aureus* ATCC 25923, *Xanthomonas vesicatoria* ATCC 11633, *Ralstonia solanacearum* ATCC11696, *Pseudomonas lachrymans* ATCC11921, *Bacillus thuringensis*

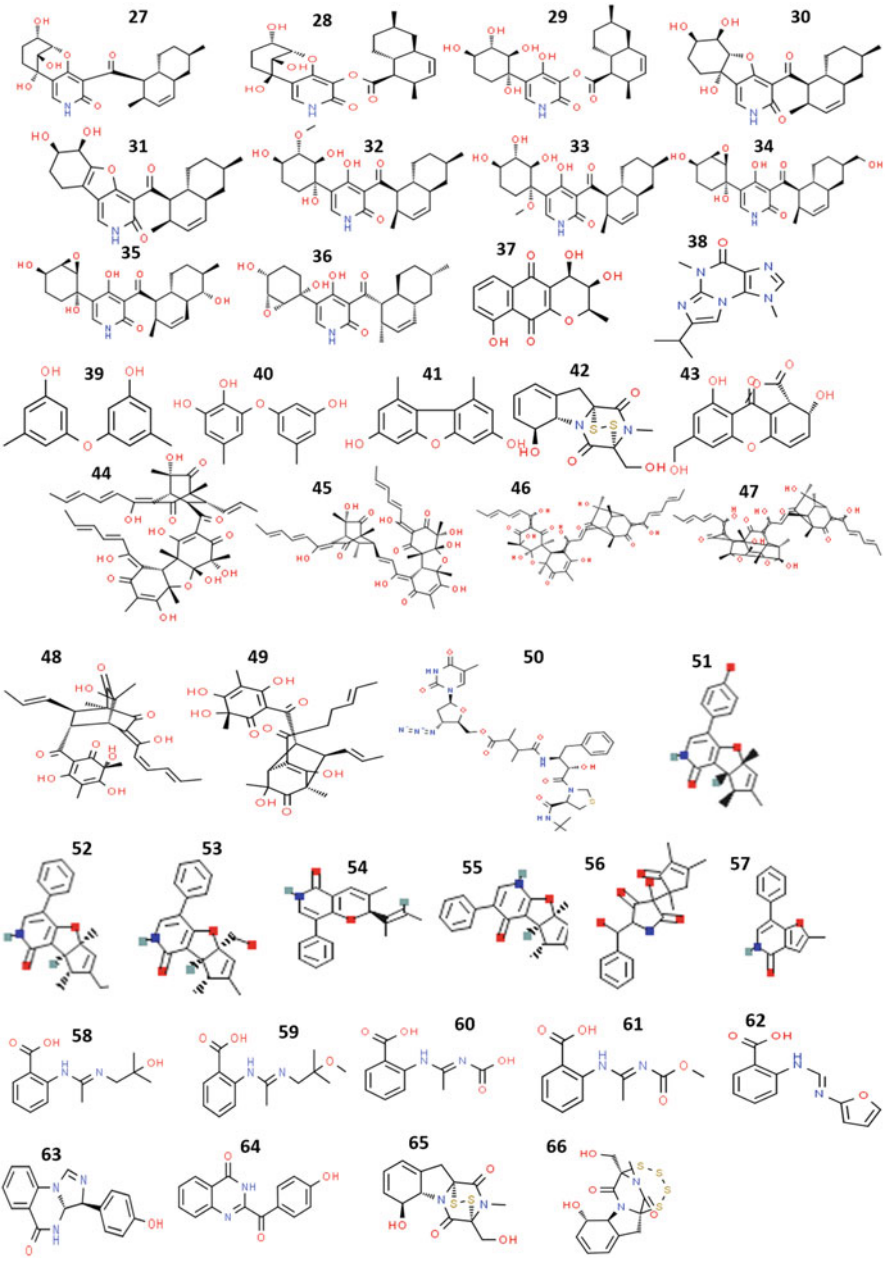

Fig. 22.2 Chemical structure of natural products from piezophilic fungi with cytotoxic activity. 27. Arthpyrone B, 28. Arthpyrone D, 29. Arthpyrone E, 30. Arthpyrone F, 31. Arthpyrone G, 32. Arthpyrone H, 33. Arthpyrone I, 34. Arthpyrone J, 35. Arthpyrone K, 36. Apiosporamide, 37. Cryptosporin, 38. Acremolin B, 39. Diorcinol, 40. Cordyol C, 41. 3,7-Dihydroxy-1,9-dimethyldibenzofuran, 42. Gliotoxin, 43. Engyodontiumone H, 44. Trisorbicillinone A, 45. Trisorbicillinone B, 46. Trisorbicillinone C, 47. Trisorbicillinone D, 48. Oxosorbiquinol, 49. Dihydrooxosorbiquinol, 50. Racemates, 51. Tersone A, 52. Tersone B, 53. Tersone C, 54.

ATCC 10792, and *Bacillus subtilis* CMCC 63501 with MIC values between 0.25 and 4 μg/mL (Niu et al. 2015). Prenylxanthone obtained from EtOAc extract of *Emericella* sp. SCSIO 05240 collected from depth 3258 m in the South China Sea displayed week antibacterial activity against *Escherichia coli*, *Klebsiella pneumoniae*, *S. aureus*, *Enterococcus faecalis*, *Acinetobacter baumannii*, and *Aeromonas hydrophila* (Fredimoses et al. 2014). One isocyanide compound xanthocillin X have been described as metabolite from *Penicillium commune* SD-118 (South China Sea) fungus that showed antibacterial activity against *S. aureus* and *E. coli*. The yield of xanthocillin X compound from *P. commune* was 361.55 mg/L in the liquid fermentation method (Shang et al. 2012). Deep-sea-derived fungi are also useful for the treatment of several strains of multidrug-resistant bacteria. *Penicillium* sp. SCSIO 06720 reported for the production of b-diversonolic ester, which displayed antibacterial activity against methicillin resistant (MR) *S. aureus*-shh-1 and *S. aureus* with MIC (microbial inhibitory concentration) values 46.9 ± 29.7 μg/mL and 10.4 ± 3.7 μg/mL, respectively (Guo et al. 2020). Additionally, depsidone-based analogues spiromastixones A–O from *Spiromastix* sp. displayed inhibitory effects against methicillin resistant strain of S. *aureus* ATCC 29213 and *Staphylococcus epidermidis* with MIC: 0.1–8.0 μg/mL. Spiromastixones J also inhibits VRE (vancomycin-resistant enterococci) strains of *E. faecalis* and *E. faecium* (Niu et al. 2014). Pyridone alkaloids and isocoumarins obtained from marine sediment and mangrove endophyte *Arthrinium* sp. UJNMF0008 and *Ascomycota* sp. CYSK-4 are active against *B. subtilis*, *E. coli*, *K. pneumoniae*, *Acinetobacter calcoaceticus*, *Mycobacterium smegmatis*, and *Staphylococcus aureus* bacteria. MIC values ranges between 25 and 50 μg/mL for isocoumarins (Chen et al. 2018). (+)-tersone E and ent-citridone A have been isolated from the *Phomopsis tersa*, which was collected from the deep-sea sediment in the depth of 3000 m. Results showed strong antibacterial activity against *S. aureus* ATCC 29 with the MIC value 31.5 μg/mL (Chen et al. 2019).

22.3.3 Anticancer Activity

Worldwide, cancer becomes a significant health-related problem, with a high mortality rate, with approximately 9.6 million deaths in 2018 (https://www.who.int/cancer/PRGlobocanFinal.pdf). In 2020, approximately 1.8 million new cancer cases and 600,000 cancer deaths were recorded in the USA. Cancer treatments available are chemotherapy, immunotherapy, radiation therapy, and targeted therapy; however, chemo-resistance is still a problem, which we need to overcome. However, several bioactive plant or microbes metabolites could be a good choice for treatment

Fig. 22.2 (continued) Tersone D, 55. Tersone E, 56. Tersone F, 57. Tersone G, 58. Penipacid A, 59. Penipacid B, 60. Penipacid C, 61. Penipacid D, 62. Penipacid E, 63. Penipanoid B, 64. Penipanoid C, 65. Gliotoxin, 66. Gliotoxin G

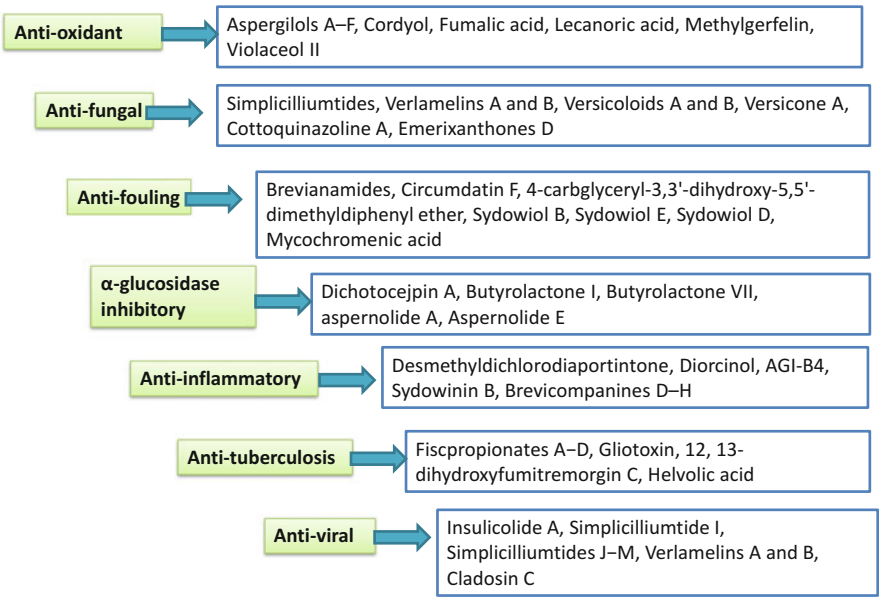

Fig. 22.3 Some other bioactivity of fungi derived natural products

of cancer. Below we discussed the possibilities of piezophillic fungi in cancer treatment.

One new compound acaromycin A and one known compound (+)-cryptosporin were obtained from the fermentation broth of *Acaromyces ingoldii* FS121, and collected from the South China Sea from the depth of 3415 m that displayed cytotoxic activities against MCF-7, NCI-H460, SF-268, and HepG-2 with IC_{50} values less than 10 μM (Gao et al. 2016). Marine *Arthrinium* fungi are distributed throughout the world and known for its cytotoxicity, antimicrobial, and AChE inhibitory activities. Bao et al. (2018) studied UJNMF0008 strain of *Arthrinium* sp. (South China Sea, depth 3858 m) fungi and reported the presence of new eight 4-hydroxy-2-pyridone alkaloids arthpyrones D–K compound with apiosporamide and arthpyrone B analogs. These compounds displayed cytotoxicity against U2OS and MG63 cell lines (Bao et al. 2018). Bioactive compounds of deep-sea-derived strains of *Aspergillus* sp. revealed significant cytotoxicity activity. One xanthone sydoxanthone C, one alkaloid acremolin B, diphenyl ether diorcinol, cordyol C, 3,7-dihydroxy-1,9-dimethyldibenzofuran, nitrobenzoyl sesquiterpenoids, diketopiperazine, fumiquinazoline, 2-hydroxy-6-formyl-vertixanthone, and 12-O-acetyl-sydowinin A are some bioactive compounds isolated from deep-sea-derived *Aspergillus* sp. Tian et al. (2015) reported that diorcinol, cordyol C, and 3,7-dihydroxy-1,9-dimethyldibenzofuran displayed COX-2 inhibitory activity against HeLa, DU145, and U937 cell lines. COX-2 produces enzymes, which activates tumor-inducing promoters, cytokines, and mitogens. Gliotoxin induces

apoptosis (McDougall 1969). Luo et al. (2017) reported the presence of cytotoxic gliotoxin in deep-sea-derived *Aspergillus* sp. SCSIO Ind09F01 fungus showed inhibitory activity against K562, A549, and Huh-7 cell lines with IC_{50} values of 0.191, 0.015, and 95.4 μM, respectively. Engyodontiumone H was obtained from *Engyodontium album* DFFSCS021 collected from deep-sea sediment of South China Sea in 3739 m depth. Engyodontiumone H revealed cytotoxic activity against U937 cells with IC_{50} value of 4.9 μM (Yao et al. 2014).

Phialocephala sp. FL30r obtained from the underwater sample (depth 5059 m, the east Pacific) was a powerful producer of diverse fungal polyketides such as sorbicillin-type compounds (cyclohexanone ring with a sorbyl side chain), sorbicillinoids, benzofuranone derivatives (Li et al. 2011a, b), oxosor-biquinol, dihydrooxosorbiquinol (Li et al. 2007a), and trisorbicillinone A, B, C, and D (Li et al. 2010, 2007b). Sorbicillinoids and phialofurone compounds revealed cytotoxic effects against P388 and K562 cell lines (Li et al. 2011a, b). Bisorbicillinoids (oxosor-biquinol and dihydrooxosorbiquinol) displayed cytotoxic effects on P388, A-549, HL60, BEL7402, and K562 cell lines evaluated by the MTT method. Oxosorbiquinol showed moderate cytotoxicity against the A549 cell line (Li et al. 2007a). Trisorbicillinone A exhibited cytotoxicity against P388 and HL60 cells. IC_{50} value for Trisorbicillinone A was 9.10 μM and 3.14 μM, against P388 and HL60 cells, respectively (Li et al. 2007b). Trisorbicillinone B, C, and D also showed cytotoxicity against P388 and K562 cell lines which were detected by the MTT method. The IC_{50} values for P388 cells were 77.1 μM, 78.3 μM, and 65.7 μM and for K562 cells were 88.2 μM, 54.3 μM, and 51.2 μM respectively (Li et al. 2010).

Deep-sea-derived FS441 strains of *Phomopsis tersa* (depth 3000 m, Indian Ocean) are known for the production of novel metabolites such as phenylfuropyridone racemates, (±)-tersones A, B, C, D, E, F, and G phenylpyridone racemate, pyridine alkaloid, and phenylfuropyridone. All these compounds exhibited in vitro cytotoxic activity against SF-268, MCF-7, HepG-2, and A549 cell lines (Chen et al. 2019). After the discovery of penicillin from *Penicillium*, it became an attraction to scientists for the production of several metabolites. Deep-sea-derived *Penicillium* (*P. paneum*, *P. fellutanum*, *P. brevicompactum*, and *P. chrysogenum*) become attracted from the last decades for the presence of novel metabolites which showed cytotoxic activity against RKO, BIU-87, ECA109, BEL-7402, PANC-1, HCT116, Hela-S3, HeLa, HL-60, A549, and HCT-116 cell lines. Triazole carboxylic acid (penipacids A–E), penipanoids B and C, quinazolinone alkaloids, and quinazolinone derivative reported from deep-sea sediment fungi *P. paneum* SD-44 (South China Sea). Structures were screened and elucidated by NMR and MS analysis. Penipacid A, penipacid E, and quinazolinone derivative displayed cytotoxicity against the human colon cancer RKO cell line, while compound 6 displayed cytotoxic activity against SMMC-7721 and RKO cell line (Li et al. 2011a, b, 2013). Penipacid A inhibits SMMC-7721 and RKO cell line with an IC_{50} value of 54.2 μM (Li et al. 2013) and 8.4 (Li et al. 2011a, b), respectively, while penipacid E inhibits the RKO cell line with IC_{50} value 9.7 μM (Li et al. 2011a, b). Trimeric peniphenylanes A–B and dimeric peniphenylanes C–G were produced by deep-sea sediment derived fungus *P. fellutanum* HDN14–323

(depth 5725 m, Indian Ocean). These new 6-methylsaligenin derivatives exhibited cytotoxic activity against HeLa, HL-60, and HCT-116 cell lines (Zhang et al. 2016a). Chloro-trinoreremophilane sesquiterpene 1, chlorinated eremophilane sesquiterpenes 2–4, and eremofortine C were produced by *Penicillium* sp. PR19N-1 (Prydz Bay, Antarctica). Among them chloro-trinoreremophilane sesquiterpene displayed moderate cytotoxicity against HL-60 and A549 cell lines with IC_{50} values of 11.8 μM and 12.2 μM, respectively (Wu et al. 2013). Additionally, five new eremophilane-type sesquiterpenes were also isolated from *Penicillium* sp. PR19N-1 fungus showed cytotoxicity against similar cell lines but with a low IC_{50} value5.2 μM against the A549 cell line (Lin et al. 2014). *Penicillium chrysogenum* MCCC 3A00292 fungus from the South Atlantic Ocean, depth of 2076 m, contain peniciversiols A, B, C, and penicilactones A and B, where speniciversiols A inhibited BIU-87 cells with IC_{50} value of 10.21 μM (Niu et al. 2019). Gliotoxin and gliotoxin related compounds (bis(dethio)bis-(methylthio)-5a,6-didehydrogliotoxin, 6-didehydrogliotoxin, and gliotoxin G) were isolated from deep-sea sediments of the *Penicillium* sp**.** JMF034 fungus (Suruga-Bay, Japan). Among them, gliotoxin and gliotoxin G reported the most potent one which inhibited P388 cell with IC_{50} 0.024 and 0.020, respectively (Sun et al. 2012).

22.3.4 Antifungal Activity

Simplicilliumtides, verlamelins A and B isolated from the deep-sea sediment fungus *Simplicillium obclavatum* EIODSF 020 (East Indian Ocean) and screened based on spectroscopic analysis. The MICs of these compounds range between 0.156 and 16.7 μg/disc against *Aspergillus versicolor* and *Curvularia australiensis* (Liang et al. 2017). Versicoloids A and B, versicone A, and cottoquinazoline A have been isolated from the deep-sea-derived *A. versicolor* SCSIO 05879 (Indian Ocean, depth 3927 m) and characterized based on NMR, and structure elucidates by X-ray single-crystal diffraction. These compounds showed antifungal activity against *Colletotrichum acutatum* fungi. A phytopathogenic *C. acutatum* fungus is known for global economic loss in agricultural crops. Wang et al. (2016) reported that versicoloids A and B showed strong antifungal activity and could be a promising candidate for the production of natural products (Wang et al. 2016). Emerixanthones D are also active against agricultural pathogens *Fusarium* sp., *Fusarium sporium* f. sp. *cucumeris*, *Aspergillus niger*, *Fusarium oxysporium* f. sp. *niveum*, *Penicillium* sp., and *Rhizoctonia solani* with the diameter of zone of inhibition ranges between 3 and 4 mm. Emerixanthones D derived from deep-sea fungus *Emericella* sp. SCSIO 05240 (depth 3258 m, South China Sea) (Fredimoses et al. 2014). *Candida albicans* is a harmful human pathogenic bacteria which can be inhibited by p-hydroxyphenopyrrozin metabolite from deep-sea-derived *Chromocleista* sp. with MIC 25 μg/mL (Park et al. 2006).

22.3.5 Antifouling Activity

Fredimoses et al. (2015) reported the presence of circumdatin F isolated from EtOAc extract of deep-sea-derived *Aspergillus westerdijkiae* SCSIO 05233 (depth 4593 m, South China Sea). Circumdatin F showed antifouling activity against *Balanus amphitrite* with EC_{50} value 8.81 μg/mL (Fredimoses et al. 2015). Strong antifouling activity against *Bugula neritina* larva took place by 4-carbglyceryl-3,3′-dihydroxy-5,5′-dimethyldiphenyl ether, sydowiol B, sydowiol E, sydowiol D, 2,4-dihydroxy-6-(4S-hydroxy-2-oxopentyl)-3-methylbenzaldehyde metabolites isolated from *Aspergillus versicolor* SCSIO 41502 (South China Sea) fungus with EC_{50} values of 1.28, 2.61, 5.48, 1.59, and 3.40 μg/mL, respectively (Huang et al. 2017). Two new mycochromenic acid derivatives brevianamides and mycochromenic acid metabolites from *Penicillium brevicompactum* DFFSCS025 (South China Sea, Sansha City, 3928 m depth) also displayed antifouling activity against *B. neritina* but with high EC_{50} value than that Huang et al. (2017) reported (Xu et al. 2017).

22.3.6 Anti-Inflammatory Activity

Desmethyldichlorodiaportintone was obtained from *Ascomycota* sp. CYSK, which was collected from mangrove endophytic *Pluchea indica*. Desmethyldichlorodiaportintone showed LPS-induced anti-inflammatory activity in RAW 264.7 cells with IC_{50} value of 15.8 μM (Chen et al. 2018). Diorcinol, bisabolane-type sesquiterpenoids, AGI-B4, sydowinin B isolated from the deep-sea fungus *Aspergillus sydowii* and characterized on the basis of NMR, HPLC, MS-ESI studies. All these compounds act as an anti-inflammatory compound by inhibiting superoxide anion generation and elastase releases. This study also reported that the addition of a 5-azacytidine, enhanced the production of diorcinol, (7S)-(+)-7-O-methylsydonol, (S)-(+)-sydonol (4), (S)-(+)-sydonic acids. 5-azacytidine act as epigenetic modifiers (Chung et al. 2013). New diketopiperazine alkaloids brevicompanines D–H obtained from deep-sea sediment fungus *Penicillium* sp. F23–2 showed anti-inflammatory activity on LPS-challenged BV2 cells with IC_{50} values of 27 and 45 μg/mL, respectively (Du et al. 2010).

22.3.7 Anti-Oxidant Activity

Oxidative stress leads to a wide range of human diseases including cancers, aging, Alzheimer's disease, Parkinson's disease, and inflammations. Antioxidants are neutralizing free radicals. New phenolic bioactive compounds Aspergilols A–F obtained from ethyl acetate extract of deep-sea fungus *Aspergillus versicolor* (A-21-2-7) which, collected from the South China Sea from 3002 m depth, displayed antioxidant activity that detected by the DPPH assay. Cordyol, violaceol II, 1-methylpyrogallol, and fumalic acid showed strong free-radical scavenging effects. Aspergilol E, cordyol C, methylgerfelin, violaceol II, and lecanoric acid compounds

displayed significantly activated Nrf2, which is a potent therapeutic factor for neurodegenerative diseases (Wu et al. 2016).

22.3.8 Anti-Tuberculosis Activity

Polypropionate derivatives (Fiscpropionates A–D) were obtained from the deep-sea sludge of *Aspergillus fischeri* FS452 fungus (Indian Ocean, depth 3000 m) and displayed anti-tuberculosis activity against MptpB protein of *M. tuberculosis* bacteria with IC_{50} ranges between 5.1 and 11 μM (Liu et al. 2019). Another strain of *Aspergillus* sp. SCSIO Ind09F01 produced gliotoxin, 12,13-dihydroxyfumitremorgin C, helvolic acid which also showed anti-tuberculosis activity with $MIC_{50} < 0.03$, 2.41, and 0.894 μM, respectively (Luo et al. 2017).

22.3.9 Antiviral Activity

Nitrobenzoyl sesquiterpenoid (6β,9α-dihydroxy-14-p-nitrobenzoylcinnamolide) and its analogue insulicolide A obtained from marine alga *Coelarthrum* sp. derived *Aspergillus ochraceus* Jcma1F17 (Paracel Islands, South China sea) showed antiviral activity against H3N2 and EV71 virus with 50% inhibitory effects 17.0 μM and 9.4 μM, respectively (Fang et al. 2014). Another deep-sea-derived fungus *Simplicillium obclavatum* EIODSF 020 (East Indian Ocean) produces natural bioactive compound peptides like simplicilliumtide I, simplicilliumtides J–M, and verlamelins A and B which displayed antiviral activity against HSV-1 virus (Liang et al. 2017). Cladosin C is a polyketide which was reported by Wu and colleagues from the *Cladosporium sphaerospermum* 2005–01-E3, a deep-sea fungus displayed a decent inhibitory activity of H1N1 influenza A virus (Wu et al. 2014).

22.4 Structure–Activity Relationships

Structure–activity relationship is an influential technology that predicts the biological activity from the molecular structure of compounds, which leads to the development of new desirable drugs. Very few studies are present about SAR for bioactive compounds of deep-sea piezophilic fungi. Comparing the structures and bioactivities of simplicilliumtides J–M, verlamelins A and B showed that the lactonized 5-hydroxytetradecanoic acid residue played an important role in the antifungal and antiviral activities. When lactone linkage is open, bioactivities will lose. Simplicilliumtides J and verlamelins A and B showed more antiviral and antifungal activity because of the absence of C-13/C-14 of the 5-hydroxytetradecanoic acid residue, which is present in simplicilliumtides K, L, and M (Liang et al. 2017). Niu et al. (2015) reported that antibacterial activities of spiromastols were based on ring A and B substitution by the analysis of the SAR. This study also reported that isocoumarin is more potent than dihydroisocoumarin

scaffold for bacterial inhibition. Ester bonds that connected A and B rings displayed stronger effects than ether bond. *Agrobacterium tumefaciens*, *B. thuringensis*, *B. subtilis*, *Pseudomonas lachrymans*, *R. solanacearum*, *S. aureus*, and *X. vesicatoria* inhibited because of dichlorinated ring A, while carboxylic acid analogues significantly decreased antibacterial activity at C-1 (Niu et al. 2015). Meleagrin B is already known for its significant cytotoxic activity for cancer cell lines. Acetate–mevalonate-derived C5 or C9 side chains addition on N-17 suppressed the meleagrin alkaloids activities. However, diterpene moiety substitutions on imidazole ring enhance the cytotoxic activities of meleagrin alkaloids (Du et al. 2010). Brevicompanine E and brevicompanine H showed average activities that inhibited lipopolysaccharide (LPS)-induced nitric oxide (NO) production in BV2 microglial cells with IC_{50} values of 27 mg/mL and 45 mg/mL. Substitution at the N-6 position contributes to anti-inflammatory activities by inhibition of NO production (Du et al. 2010).

22.5 Conclusion and Future Prospectives

Deep-sea fungi known as piezophilic fungi are an important and novel source of bioactive secondary metabolites including mostly in alkaloids, lactones, polyketides, terpenoids, peptides, and steroids categories. This chapter reviewed the chemical structures and biological activities of deep-sea fungi genera including *Ascomycota*, *Acremonium*, *Aspergillus*, *Cladosporium*, *Chromocleista*, *Dichotomomyces*, *Diaporthe*, *Emericella*, *Engyodontium*, *Penicillium*, *Phialocephala*, *Phoma*, *Spiromastix*, and *Simplicillium*, where *Aspergillus*, *Penicillium*, and *Phialocephala* are extensively reviewed. Our chapter highlighted that fungi from the deep-sea extreme ecosystems are the most diverse source for the production of natural products. Research on deep-sea piezophillic fungi are limited due to challenges in sample collection and fungal culture techniques. Meleagrin increases cytotoxicity by the addition of acetate–mevalonate-derived C5 or C9 side chains, whereas LPS induced NO inhibition capacity of brevicompanine E and brevicompanine H increases by substitution at the N-6 position. Additionally, simplicilliumtides J and verlamelins A and B antimicrobial activity depend on lactone linkage. All the new isolated compounds screened and detected by 1D and 2D NMR, HRMS, HRESIMS, TD-DFT, IR ESIMS, and CD. Most of the isolated compounds showed cytotoxic and antimicrobial activity with few shows antioxidant, antifouling, anti-inflammatory, and anti-tuberculosis activity. Synopsis of bioactive compounds from deep-sea fungi, their sources, biological activities, and references will help readers to better understand the bioactive compounds' prime potential as drug candidates that further could provide solutions to new drug discovery.

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Biotechnological Applications of Microaerophilic Species Including Endophytic Fungi

23

Beenish Sarfaraz, Mehwish Iqtedar, Roheena Abdullah, and Afshan Kaleem

Abstract

Fungi are very diverse in nature and can be found in any ecosystem. Until now around 10,000 species have been characterized and many remain to be identified. Fungi play an integral role in the stability of an ecosystem as they perform a multitude of environmental tasks including but not limited to the decomposition of organic matter, forming mutualistic mycorrhizal association with plants, and cause of some diseases. They are capable of growing in different environmental conditions, i.e., low and high oxygen environments, temperatures, and pH values. Fungi can grow in a wide range of oxygen gradients. Their unique characteristics combined with extensive metabolic activity under different oxygen conditions make them valuable in many biotechnological applications. In this chapter, different fungal species have been explored for their biotechnological potential under microaerophilic conditions. Microaerophilic conditions are deemed most suitable for ethanol and hydrocarbon production. Under microaerophilic conditions fungi can effectively degrade industrial dyes and produce cheese, carotenoid, and even nanoparticles. Different fungal species have been studied in this regard and further research is required to explore their potential under these conditions.

Keywords

Fungi · Extremophiles · Microaerophilic fungi · Fungal biodegradation · Fungal enzymes

B. Sarfaraz
Department of Biology, Lahore Garrison University, Lahore, Pakistan

M. Iqtedar (✉) · R. Abdullah · A. Kaleem
Department of Biotechnology, Lahore College for Women University, Lahore, Pakistan

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23.1 Introduction

The biosphere comprises a multitude of ecosystems and biomes. Each ecosystem has a characteristic unique environment that caters to its biotic community with appropriate living conditions, i.e., habitat, food, energy, biomass, soil, oxygen, etc. (Haines and Potschin 2005). Oxygen is one of the key requirements of all living organisms. Each habitat has different oxygen gradients which contribute to diverse microbial communities. Bacterial and fungal communities thrive in diverse oxygen concentrations. Some prefer high O_2 levels, while some are habituated to oxygen-free environments. Fungi are considered chief decomposers in the biosphere along with bacteria. They are present in different ecosystems thriving in deep marine sediments of the Indian Ocean (Raghukumar and Raghukumar 1998), in air over the Atlantic ocean (Pady and Kapica 1955), in volcanic landscape (Tadiosa and Briones 2013), in rivers (Pascoal and Cássio 2004), in soil (Garrett 1963, 1981), and in plant roots as mycorrhizal fungi (Miller and Jastrow 1992, 2000; Miransari 2011; Zhang et al. 2020).

They are powerful contributors to global carbon cycling, biogeochemical cycles, decomposition cycles, and in mobilizing macroelements (Gadd 2006). They form mutualistic relationships with plant roots on one hand (Zhu and Miller 2003) and act as notorious pathogens on other hand (Anaissie et al. 1989). Microaerophiles are generally defined as organisms that require less than 21% of O_2 for their maximum growth (Krieg and Hoffman 1986), but that term is open to interpretation for microbiologists and considered too restrictive. Based on oxygen demand microbes are divided into aerotolerant anaerobes, facultative anaerobes, obligate aerobes, obligate anaerobes, and nanoaerobes (Baughn and Malamy 2004). The term "microaerobe" has been recently used by Morris and Schmidt (2013) which is defined as any microbe that can respire oxygen using high-affinity terminal oxidase within microoxic environments.

Oxygen affects microaerophiles in two ways. It could be deleterious to them or beneficial as it can be used for generating energy in the respiration process. Some microaerophiles can grow anaerobically or using fermentative pathways, but their preference of using O_2 as a terminal electron acceptor during low O_2 levels sets them apart from other categories. Also, they can survive in low O_2 levels (Krieg and Hoffman 1986).

Fungi thrive in various oxygen conditions, most of the fungal species flourish in aerobic conditions and they have been long considered obligate aerobes requiring oxygen for their growth and metabolism (Tabak and Cooke 1968). However, the relationship of fungi with oxygen varies significantly from species to species and some can grow in a wide range of oxygen conditions based on their desired function. Some studies have demonstrated that *Blastocladiomycota* clades in aquatic environments can survive in low oxygen environments (Naranjo-Ortiz and Gabaldón 2019). Some studies have shown that filamentous fungi and yeast species can grow in microaerophilic conditions as well (Brown 1922; Durbin 1955; Fellows 1929; Tabak and Cooke 1968). Various studies have proved that fungi were able to grow at low oxygen conditions like genus *Trichocladium* which is a saprophytic

fungus found in aquatic environments and soil. It can display excellent cellulolytic activity under microaerophilic conditions and certain *Trichocladium* species can convert up to 96% of cellulose to ethanol (Eichorst and Kuske 2012). Fungal pathogens are considered obligate aerobes requiring oxygen for pathogenesis. *Candida albicans*, one of the common human fungal pathogens, is located in the gastrointestinal tract which has low oxygen tensions. Similarly, *Cryptococcus neoformans* also survive in low oxygen conditions in the brain. Apart from fungal pathogens, typical soil fungi are also present in poor oxygen environments and thrive there and perform all essential metabolic activities, which proved that fungi could survive in low O_2 conditions due to their adaptations to survive in rapidly changing oxygen conditions. *Aspergillus fumigatus* can tolerate up to 0.1% oxygen and it can even grow anaerobically (Grahl et al. 2012). Although most of the studies are focused on the growth of fungi in aerobic or anaerobic conditions, different studies have been done on fungal growth and metabolism in microaerophilic conditions. As fungi can grow across a range of O_2 levels, studies are included on the basis of the fact that maximum activity and growth were observed under microaerophilic conditions.

23.2 Biotechnological Applications

The potential interactions between microorganisms and the O_2 in their environment provide a useful basis for the physiological and ecological characterization of microorganisms. Microaerophilic fungal species are ecologically diverse with unique features. Below are some of the major biotechnological applications of fungi that are grown under microaerophilic conditions. Tabak et al. (1968) isolated the fungal species of *Fusarium solani*, *Mucor hiemalis*, *Geotrichum candidum*, *Fusarium oxysporum*, *Phoma herbarum*, *Rhodotorula mucilaginosa*, *Candida parapsilosis*, *Aspergillus niger*, *A. fumigatus*, *Aureobasidium pullulans*, *Phialophora jeanselmei*, *Penicillium oxalicum*, and *Penicillium brevi* from the enriched soils, polluted water, and sewage sludge samples. It was observed that many fungal species needed vitamins for their growth in microaerophilic and anaerobic conditions apart from *Geotrichum candidum*, *Fusarium oxysporum*, and *F. solani*. Different fungi metabolized organic substrates and produced cell biomass under microaerophilic and anaerobic conditions which could help to understand their role in bioremediation, especially in sewage treatment systems.

23.2.1 Bioethanol Production

Lignocellulosic material degradation is a widely studied topic. Various organisms have been explored for their ability to carry out enzymatic degradation of these complex compounds. Fungi are mostly nominated for performing this task owing to the presence of an extensive enzyme system capable of carrying out this degradation process. *Fusarium* species, *Basidiomycete* sp., *Geotrichum* sp., and *Trichocladium*

canadense produce enzymes to hydrolyze sawdust and sugarcane bagasse under both aerobic and microaerophilic conditions efficiently. However, these microbes preferred low oxygen environment (5–15% O_2) for enzyme production and growth whereas variable lignocellulosic activity. Fungal CMCase, avicelase, and β-glucosidase activity have been reported highest in both combined and microaerophilic conditions (Pavarina et al. 1999; Pavarina and Durrant 2002).

Lignocellulosic biomass is widely degraded in aerobic conditions but sometimes low O_2 conditions also support the degradation process in the soil or mud. O_2 mostly determines the fate of lignin degradation where higher concentration accelerates the decay process, but tree logs and trunks inhabiting fungi also take part in the decay process despite the presence of low O_2 conditions which means low oxygen concentration can also support the process (Blanchette et al. 1989). White rot fungi degrade lignin inside wooden substrates under different gas levels (Tuor et al. 1995). Some fungi have the capability to grow on a wide range of substrates. *Trichocladium canadense* and *Basidiomycete* species exhibited high levels of xylanase, avicelase, lactase, β-glucosidase, and CMase activity that could carry out cellulose fermentation by successfully consuming lignin, xylan, cellobiose, pentose, and hexose under microaerophilic and aerobic growth conditions. However, the efficiency of both strains achieved faster cellulose utilization under microaerophilic conditions (Durrant 1996a, b).

Some other fungi that are equipped with biosynthetic machinery to actively degrade recalcitrant lignin compounds in both anaerobic and microaerophilic conditions by producing lignin-degrading enzymes are *Verticillum* sp. that are microaerophilic and can tolerate 10–15% O_2 with 10% CO_2. *Basidiomycete* sp. has demonstrated higher lignin-peroxidase, manganese-peroxidase, and laccase activities under microaerophilic conditions. Lignosulfonic acid was degraded to 27.5% by *Verticillum* sp. in microaerophilic conditions, whereas *Fusarium oxysporum* and *Aspergillus* sp. showed higher LiP, Lac, and MnP activities under microaerophilic conditions (Silva et al. 2009).

Different fungal species such as *Pichia stipitis* Y7124, *Pachysolen tannophilus* Y2460, *Kluyveromyces marxianus* Y2415, and *Candida shehatae* Y12878 exhibited a different rate of ethanol production under different O_2 levels, whereas under microaerophilic conditions, *Pichia stipitis* produced the highest amount of ethanol: 3.5 g of ethanol per gram dry cell per day (Delgenes et al. 1986). *Pichia stipitis* NRRL Y-7124 was also able to produce high sugar and ethanol from hemicellulose acid hydrolysate of *Eichhornia crassipes* under microaerophilic conditions (Nigam 2002). *Pichia stipites* has unique physiology; it not only produces a high amount of ethanol (41 g ethanol per liter) but also helps in bioremediation process. It has a thick cell wall, simple nutritional needs, and high resistance to contaminants. This species grows ideally in microaerophilic conditions and has a low sugar consumption rate (Agbogbo and Coward-Kelly 2008).

23.2.2 Hydrocarbon Production

Endophytes are fungal or bacterial species capable of surviving inside plants. They could form a highly beneficial mutualistic relationship or a pathogenic relationship with plants. Endophytic fungi, which are estimated to be 1.5 million fungal species, reside in plants forming a symbiotic relationship with their hosts. Fungal strains could not only degrade complex carbon but also can produce hydrocarbons. One study analyzed the cellulose and hydrocarbons degrading ability of endophytic fungi belonging to the genus *Gliocladium* under the microaerophilic conditions which could help to synthesize pretreatment-free microbial biofuels. Endophytic fungi produce various hydrocarbons including benzene, heptane, tridecane, 3,4-dimethyl, m-xylene, hexane, hexadecane, heptane, and others by using cellulosic biomass. Cocultures of *Escherichia coli* and *Gliocladium* sp. demonstrated 100 times higher hydrocarbon production ability than the single inoculum culture of *Gliocladium* sp. owing to the stability of dry mycelial mass in stationary phase in a coculture (Ahamed and Ahring 2011).

In a host plant, *Eucryphia cordifolia* volatile hydrocarbons were produced from the growth of *Gliocladium roseum* (NRRL 50072). This fungus synthesized extensive varieties of acetic acid esters of straight-chained alkanes including those of pentyl, hexyl, heptyl, octyl, sec-octyl, and decyl alcohols. It also produced undecane, 2,6-dimethyl; decane, 3,3,5-trimethyl; cyclohexene, 4-methyl; decane, 3,3,6-trimethyl; and undecane, 4,4-dimethyl, and VHCs including benzene, heptane, and octane were also produced. Fungal endophyte was also producing lipids and fatty acids. This elaborate hydrocarbon profile of *G. roseum* helped to obtain a new name "myco-diesel" for its volatiles (Strobel et al. 2010). Increased production of VHCs under microaerophilic conditions was due to similar conditions in host tissue as endophytes frequently develop intracellularly in plant tissues. VHCs could possibly be produced under microaerophilic conditions in the plant apoplast (Stadler and Schulz 2009).

An endophyte *Nodulisporium* sp. (designated TI-13) produced volatile organic compound (VOC) under microaerophilic conditions in a solid state reactor utilizing agricultural waste beet pulp as a substrate. Ester compound production rate escalated under microaerophilic conditions. These conditions are similar to the natural growing condition of fungi inside plants, and these VOCs are a kind of protective gear of fungi against invading organisms (Schoen et al. 2017).

23.2.3 Dye Degradation

The industrial revolution has changed human life. Industrial products are produced using toxic chemicals which end up being discharged into water bodies, affecting the freshwater and marine flora and fauna. Microbes have been commonly studied for their bioremediation potentials. One possible application of microbes would be the natural degradation of these chemicals for reducing pollution hazards. In a similar context, a study was conducted using a consortium of fungal and bacterial species

(*Aspergillus ochraceus* NCIM-1146 and *Pseudomonas* sp. SUK1) to assess their ability to biodegrade and decolorize azo dye Rubine GFL and textile effluent. This developed consortium-AP helped to obtain the decolorization rate of 95% and 98% in dye and effluent, respectively, in aerobically grown *A. ochraceus* NCIM-1146 and *Pseudomonas* sp. grown in microaerophilic conditions possibly due to involvement of oxygen-sensitive reductase. Individually both microbes showed reduced levels of decolorization as compared to consortium-AP. Microbes grown in aerobic conditions showed 46% and 34% decolorization of dye, whereas microbes grown in microaerophilic conditions demonstrated 43% and 63% dye decolorization. Biological oxygen demand (BOD; 82%), chemical oxygen demand (COD; 96%), and total organic carbon (TOC; 48%) were also reduced in consortium-AP-treated textile effluent in microaerophilic conditions (Lade et al. 2012).

Azo dyes are stable compounds with various applications in textile and other industries. They are not easily degraded due to their recalcitrant xenobiotic nature, but different microbes can perform this important task under different oxygen conditions. A new consortium GG-BL comprising of fungus *Galactomyces geotrichum* MTCC 1360 and bacterium *Brevibacillus laterosporus* NCIM 2298 cultures could produce reusable water by converting toxic form of Golden Yellow HER (GYHER), a mono azo dye to fewer toxic forms demonstrating higher decolorization activity (98%) under microaerophilic conditions as compared to individual microbial decolorization. Even 100% dye was degraded in consortium in the mixed aeration condition (12 h aerobic followed by 12 h microaerophilic). COD and TOC levels' reduction was also reported in addition to reduced phytotoxicity to dye metabolites after decolorization in *Phaseolus mungo and Sargassum vulgare* plants (Waghmode et al. 2011).

The ability of bacterial and fungal-based consortium to degrade textile effluents under microaerophilic conditions using *Aspergillus ochraceus* NCIM 1146 and *Providencia rettgeri* strain HSL1 was also observed by Lade et al. (2016). Consortium successfully degraded 92% of ADMI textile effluents in 30 h under microaerophilic conditions, whereas consortium only degraded 4% textile effluents in aerobic conditions at 30 °C. This study again proved the need for carrying out the degradation of azo dyes under microaerophilic conditions. These low oxygen conditions are present during effluent treatment plants and coculture accelerated the degradation process due to efficient enzyme activity which also reduced phytotoxicity of dyes (Lade et al. 2016).

Azo dye degradation under microaerophilic conditions was also exhibited from a yeast species *Issatchenkia occidentalis* which is an ascomycete. More than 80% of the dye degraded under microaerophilic conditions in 15 h. Yeast species degraded dye during late log phase and in acidic pH. *I. occidentalis* managed to decolorize dyes but in lower quantities. Reduction mostly depends on the stage of microbial growth and enzyme activity where 1-amino-2-naphthol and *N*,*N*-dimethyl-*p*-phenylenediamine, which are dye degradation products, were used as carbon and nitrogen sources (Ramalho et al. 2004).

23.2.4 Cheese Production

Cheese production is a billion dollar industry with more than 1000 varieties of cheese around the world. Bacterial and fungal species form a rind on cheese surface imparting unique properties to each cheese. More than 30 yeast species have been found to be prevalent on the ripened cheese surface including *Geotrichum candidum* and *Debaryomyces hansenii*. Yeast helps in the development of flavor and texture due to lipolytic, proteolytic, and deacidifying activities. *Geotrichum candidum* is a microaerophilic fungi with vast applications in cheese industry (Fröhlich-Wyder et al. 2019). In the study of Livarot cheese, researchers found out that different yeast species are involved in the fermentation process but *Geotrichum candidum* being the most prevalent although it is a salt-sensitive species. It helped in curd deacidification and aroma compound production (Larpin et al. 2006). *Geotrichum candidum* yeast is able to carry out primary and secondary proteolysis while degrading casein at the cheese surface. This action increased the concentration of free amino acids in cheese from early weeks till late ripening (Boutrou et al. 2006).

23.2.5 Other Applications

Selenium is a trace element required in human diet for maintaining growth and health. Elemental selenium is a nontoxic form which has great health benefits. Selenium nanoparticles can be synthesized using different green chemistry methods for use in the health sector. *Saccharomyces cerevisiae* was used in a study that produced extracellular SeNPs ideally under microaerophilic conditions. Selenium/protein NPs were fabricated in vivo and moved outside of the cell in vesicles in low oxygen conditions. These NPs were approximately 100 nm in diameter and spherical in shape. Yeast proteins act as a capping agent for NPs formation. FTIR analysis confirmed the presence of proteins and other biomolecules in SeNPs. Microaerobic environment supported the reduction of selenite to elemental selenium (Zhang et al. 2012).

Yeast species *Phaffia rhodozyma* can produce carotenoid pigments. The ability to produce pigments depends on the culture conditions and astaxanthin was synthesized under exponential growth phase of yeast. Xanthophyll pigment significantly improved from 90 to 406 concentration μg (g yeast)$^{-1}$ during fermentation. Oxygen concentration dictated the optimum yields of yeasts and pigments. Under microaerophilic conditions yeast accumulated β-carotene and the monoketone echinenone (Johnson and Lewis 1979).

Microaerophiles are an integral part of an ecosystem and should be further explored in order to find their applications. Due to their different nutritional requirement and metabolic processes, these fungi can be successfully applied to processes where microaerophilic to anaerobic conditions prevail.

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Whole Cell Application Potential of Extremophilic Fungi in Bioremediation

24

Sunil Bhapkar, Rushikesh Pol, Deeksha Patil, Anupama Pable, and Umesh U. Jadhav

Abstract

Various microorganisms are known for their potential to metabolize complex pollutants and this ability of microorganisms has been utilized in remediation processes. The bioremediation process in which the fungi are used is referred to as myco-remediation. When it comes to the whole cell application of microorganisms at extreme contaminated sites, fungi are a good choice because of their wide habitats and ability to digest complex pollutants. They either catalyze the contaminants through extracellular secretion of active components or may uptake them inside the cells. The source sites for such kind of extremophiles are either natural sources like hot springs, cold deserts, soda lakes, or man-made contaminated sites. These fungi are isolated from such sites and enriched for their potential to metabolize the contaminants. Many of such fungal organisms have been identified and applied for their potential to remove the contaminants like heavy metals, radioactive elements, hydrocarbons, phenols and phenolic derivatives, etc. from the polluted sites. This chapter reviews various reports on extremophilic fungi and their whole cell application potential in bioremediation.

Keywords

Fungi · Bioremediation · Extremophiles · Heavy metals · Radioactive elements · Hydrocarbons · Phenols

S. Bhapkar · R. Pol · D. Patil · A. Pable · U. U. Jadhav (✉)
Department of Microbiology, Savitribai Phule Pune University, Pune, Maharashtra, India
e-mail: ujadhav@unipune.ac.in

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24.1 Introduction

Increasing globalization and population explosion gave rise to increasing industrialization which ultimately led to tremendous increase in pollution in developed as well as developing countries. Various pollutants introduced into the environment may include polyaromatic hydrocarbons (PAHs), phenol and phenol derivatives, heavy metals, etc. Many of them are toxic as well as recalcitrant providing harm to biosphere in different ways. There are various methods like physical, chemical, and biological methods to remove or clean up these toxic compounds from the contaminated sites including soil and water.

Each method has its own advantages and limitations. When biological agents are used to serve the purpose, it is called as bioremediation (Fig. 24.1). Bioremediation is defined as the use of living organisms to reduce or eliminate environmental hazards resulting from accumulation of toxic chemicals and other hazardous compounds (Gibson and Saylor 1992). In other words, it is a process in which the waste and hazardous materials are transformed into non-hazardous or less-hazardous

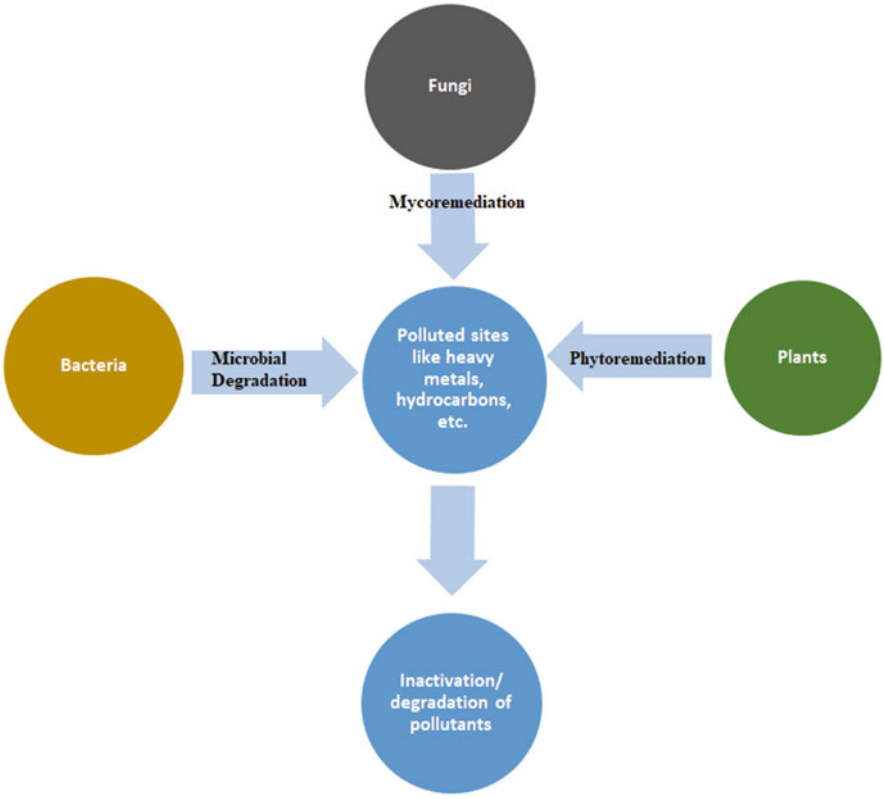

Fig. 24.1 The concept of bioremediation

substances by microorganisms such as bacteria, fungi, and algae. Nowadays, they are deliberately released into the polluted environment to clean up the pollutants.

24.2 Bioremediation

Different life forms like plants, bacteria, fungi, and algae have been shown potential for bioremediation. Depending on the contaminated site (like soil, water, etc.) their selection is favored. When the plants are used to serve the purpose, it is referred to as phytoremediation while the use of fungi is called mycoremediation. Most of the agents are isolated from contaminated sites or natural reserved sites. A study reported that a number of microorganisms are widely distributed in nature which can be used for pollutant degradation. These microorganisms can be enriched to remove various contaminants from the contaminated sites (Zobell 1946). Figure 24.2 shows the various strategies used by microorganisms to remove the contaminants and includes the following processes. (a) Biosorption is metabolically independent process and is based on ionic interactions between the extracellular surface of biomass and metal ions. (b) In bioaccumulation, microorganisms use proteins to absorb metal ions inside their intracellular space, (c) while bioprecipitation is a process where soluble metal ions are immobilized through redox reactions, enzymes, and metabolites present on the extracellular surface of microorganisms. (d) Bioreduction is a transformation process where toxic metals/metalloids are altered to non-toxic elements through biological reduction and oxidation process. (e) Lastly bioemulsification process makes use of proteins or metabolites to form emulsions in two immiscible liquid phases (Jeong and Choi 2020). A number of

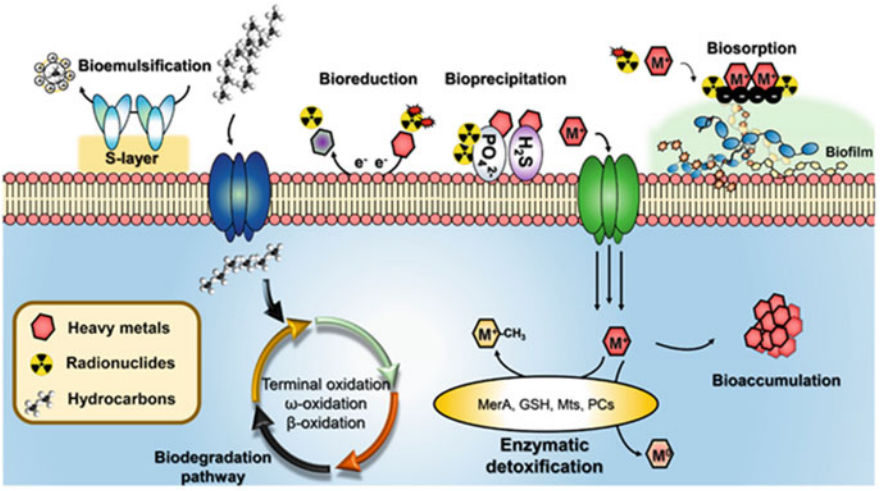

Fig. 24.2 Microbial bioremediation strategies for the removal of diverse toxic pollutants (Jeong and Choi 2020)

microorganisms are capable of using toxic organic compounds as the sole source of carbon and energy to drive their metabolism. This ability of transforming or hydrolyzing toxic compounds into nontoxic or less toxic compounds is by the virtue of their biochemical pathways. Since, the emergence of genetic engineering, various tools and techniques are being used to enhance the natural remediation potential of these agents. These organisms may perform their metabolism under aerobic, anaerobic, or facultative aerobic/anaerobic conditions. The applications of fungal microorganisms in bioremediation have been well known as fungal bioremediation or mycoremediation. Fungi are competent for metabolizing various environmental chemicals and utilize its product for survival without any additional need for nutrition. The fungi decompose the biomass residues and chemical pollutants by producing several enzymes. These microorganisms are used for the biotransformation of organic waste materials and the removal of pollutants from the environment (Balabanova et al. 2018). They are also adapted to survive in diverse conditions continuously. Though a wide range of fungal species is used for the treatment of various pollutants like heavy metals, various hydrocarbons and pesticides, radioactive compounds, etc., this chapter focuses on the applications of only extremophilic fungi used in bioremediation.

24.3 Extremophilic Fungi for Bioremediation

Many species belonging to fungi can able to survive and sustain themselves in extreme habitats such as extreme temperature and pressure, high salinity, acidic and alkaline substrate or soil, and limited nutrient conditions (Abdel-Hafez 1982). Along with natural extreme habitat, fungal species have also been isolated from man-made extreme habitats such as the acidic and alkaline industrial effluent plant of wastewater (Kumar and Dwivedi 2019), heavy metal contaminated soil, and hazardous chemicals contaminated industrial (Bordjiba et al. 2001) and agricultural (Zafar et al. 2007) areas. Considering the harsh environment of contaminated soil and water, it is very essential for organisms to sustain within a harmful environment of pollutants, toxins chemicals, and heavy metals, and to remove the hazardous effect of contaminants. The ability to grow in the above-mentioned extreme conditions thus makes extremophilic fungi a potential eco-friendly candidate for bioremediation. Various strategies are used by fungi for their survival in these extreme conditions and those properties are essential for the selection of these fungi in the bioremediation process. The enzymes produced by extremophiles are considered superior than mesophilic enzymes as they are able to perform reaction under extreme and adverse conditions in which conventional proteins are completely denatured. The common extremophilic enzymes are the oxidative enzymes such as peroxidases, oxygenases, laccases, etc., and these enzymes producing fungi have shown a role in the bioremediation of contaminated water and soil. Peroxidases catalyze the degradation of lignin and phenolic compounds. The role of fungal cytochrome P450 monooxygenase has been studied for detoxification of toxic compounds from soil and water (Hernández-López et al. 2016). Laccases are oxidases, the enzymes

capable of oxidization of a large number of phenolic and non-phenolic compounds (Arregui et al. 2019). Fungi are one of the organisms having ability to produce higher amount of laccase and have been applied for bioremediation of phenolic compounds, aromatic hydrocarbons (Tavares et al. 2009), and dyes (Iark et al. 2019; Yin et al. 2019). Filamentous fungi are suitable and low cost candidates for biosorption and adsorption of heavy metals by using either living or inactivated biomass (Ting and Choong 2009; Cai et al. 2016). Further, use of halophilic fungi increases the efficiency of biosorption under the conditions found at heavy metal contaminated sites (Bano et al. 2018; Ali et al. 2019). The application of filamentous fungi may be advantageous for those situations where translocation of essential factors (nutrients, water, the pollutant itself, and so on) is required for the transformation or detoxification of the pollutant (Harms et al. 2011).

24.4 Need of Extremophilic Fungi in Bioremediation

Different methods applied for bioremediation include natural attenuation, biostimulation, and bioaugmentation, or a combination thereof.

These methods are environmentally friendly as well as cost-effective and can be managed easily. When the catalysis process can be controlled in a single step, only single cells can be applied but when it comes to the catalysis of complex environmental pollutants through multiple steps, the application of consortia of different species is must. Together, they metabolize pollutants. The degradation of the contaminants may occur intracellularly or extracellularly depending on the nature of the contaminant and the type of biological agent (Fig. 24.3). Considering the example of recalcitrant compound, Fig. 24.4 depicts the various mechanisms used by fungi to remove/detoxify the recalcitrant compound from the affected site (Deshmukh et al. 2016).

However when it comes to the recreation of contaminated sites having extreme conditions (like pH, salinity, radiations, or temperature) of normal species cannot perform their function efficiently. This raises the need for extremophiles which can grow easily at the extreme sites and carry out bioremediation more efficiently. Microorganisms from extreme natural or man-made environments provide robust enzymatic and whole cell biocatalytic systems that are attractive under conditions that limit the effectiveness of typical bioconversions. A variety of extremophilic systems adapted to specific niche environments have been shown in Fig. 5.

These bioconversion operations attempt to augment naturally occurring remediation capacity and accelerate reactive processes by removing limitations on microbial action. Many environmental sites are contaminated with multiple pollutants and support multiple modes of extremophily that operate in concert to facilitate chemical transformation. In order to apply the extreme microorganisms, scientists have deepened their knowledge and understanding of molecular systems that regulate extremozyme action, stability, and expression. Similarly, they are focusing on insights into metabolic strategies for whole-cell catalysis under extreme conditions. Technological advances in the recruitment of extremophilic systems for remediation

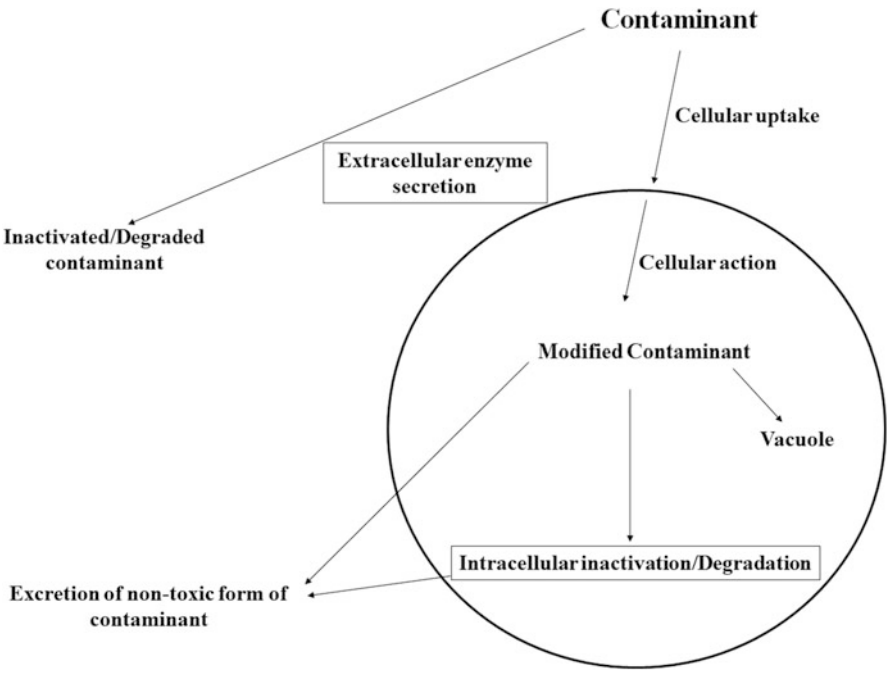

Fig. 24.3 Schematics of intracellular and extracellular inactivation/degradation of contaminants

operations are made through evaluating selective conditions that dominate contaminated environments, identifying extremophilic organisms that can grow and adapt in contaminated environments, and enhancing the remediation capacity with the help of genetic engineering into organisms that can survive under extreme selective pressures (Peeples 2014). Various extremophilic organisms along with their sources are mentioned in Fig. 24.5. These sources include deep-sea hydrothermal vents, hot springs, sulfataric fields, soda lakes, inland saline systems, solar salterns, hot and cold deserts, environments highly contaminated with nuclear waste or heavy metals as well as lithic or rock environments.

24.5 Heavy Metal Tolerant Fungi

24.5.1 Metal Tolerants from Coastline, Mangroves, and Salterns

Nazareth and Marbaniang (2008) from India isolated four different heavy metal tolerant species based on morphological differences. Their isolates were collected from a well-situated close to copper smelting plant near the coastline (WP), from the mangroves (MP), and from salterns (SP) of Goa, India. Based on their penicillial heads morphology, WP1 was biverticillate symmetric, MP2 observed as

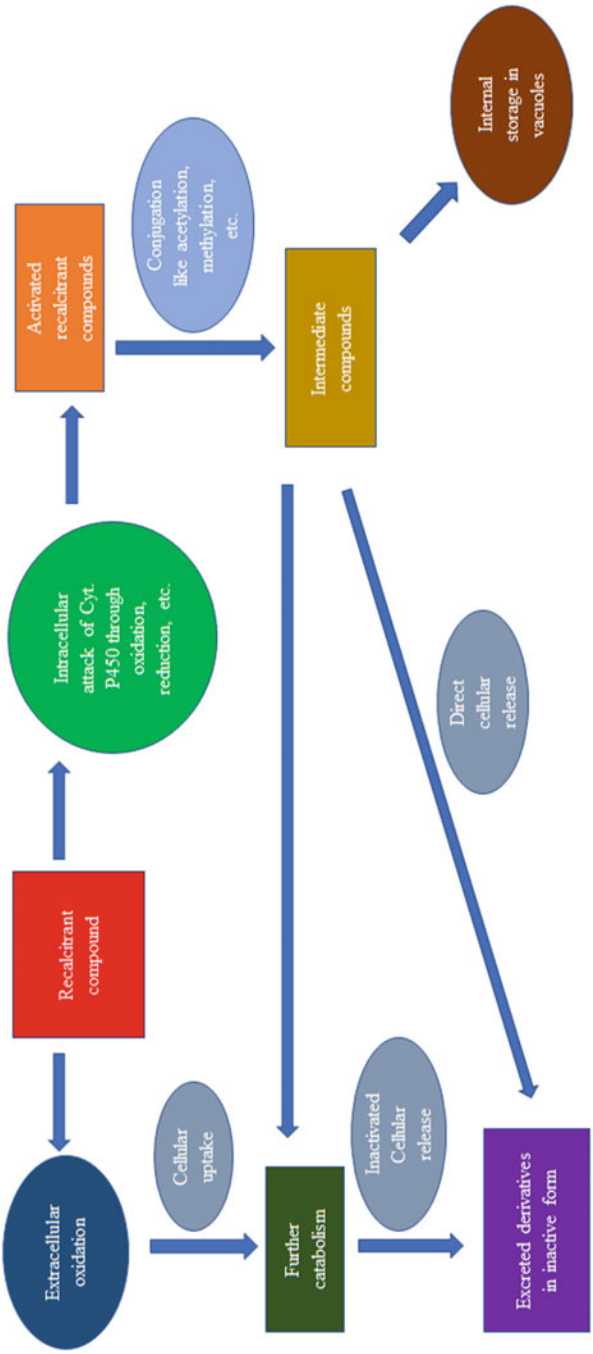

Fig. 24.4 Mechanisms adopted by fungi for bioremediation of toxic, recalcitrant compounds (Deshmukh et al. 2016)

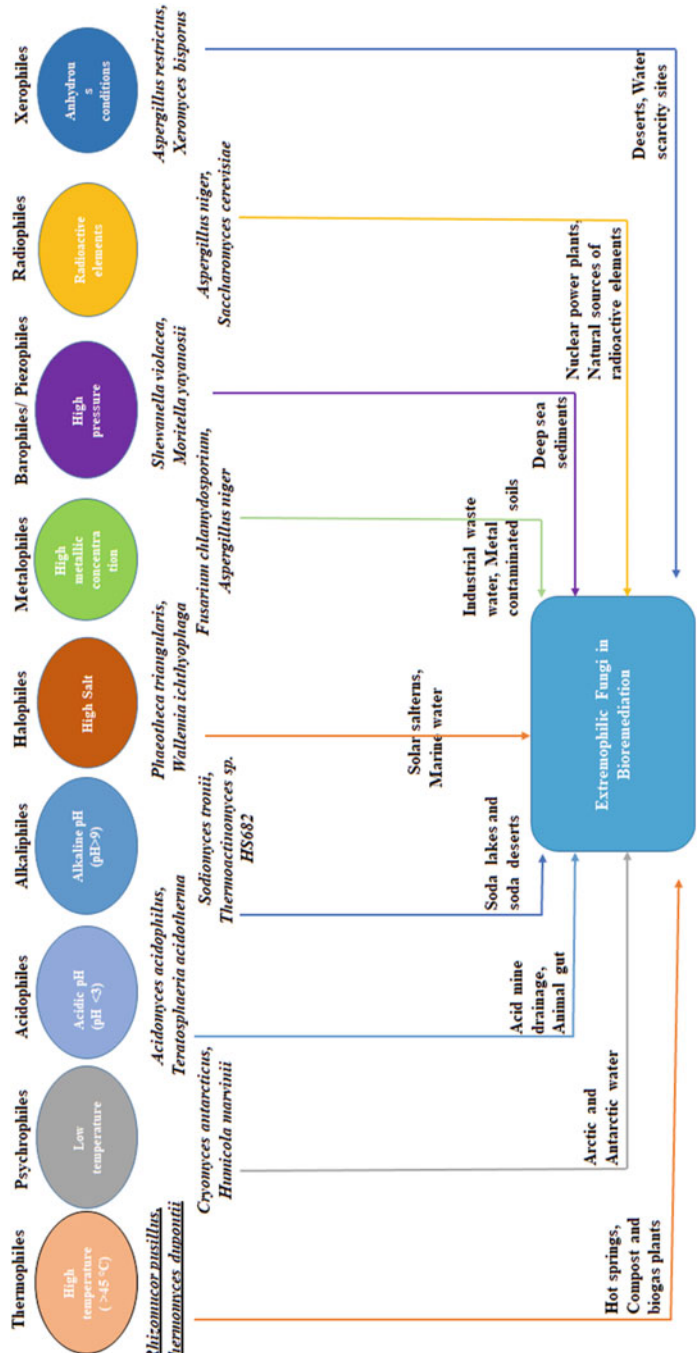

Fig. 24.5 Various types of extremophiles applied in the process of bioremediation

biverticillate asymmetric, MP4 terverticillate, and SP10 monoverticillate. Further, they were compared for heavy metal resistance against the presence of lead, copper, and cadmium salts. The cultures were grown on Czapek-Dox agar supplemented with concentrations of $Pb(NO_3)_2$, $CuSO_4.5H_2O$, and $Cd(NO_3)_2.4H_2O$. The growth was examined in terms of colony diameter. All four cultures were resistant to Pb^{2+} at a concentration of 7.5 mM, with no decrease in growth up to 5 mM; WP1 and MP2 were resistant to 2 mM Cu^{2+} and very slightly to 3 mM Cu^{2+}, and MP4 was resistant to 5 mM Cu^{2+}, while SP10 showed no resistance to Cu^{2+}; WP1 could resist Cd^{2+} as $Cd(NO_3)_2$ up to 1 mM but not as $CdSO_4$, while MP2 and SP10 could resist only $CdSO_4$ at 1 mM concentration; MP4 tolerated both nitrate and sulfate salts of cadmium up to 5 mM and 4 mM, respectively. Thus, lead was the most easily tolerated of the heavy metals tested and caused the least variations in cultural and morphological characteristics, while cadmium was the most toxic, causing significant cultural and morphological variations. More striking was the observation that resistance to the heavy metals was highest by the terverticillate *Penicillium*, decreasing in the biverticillate isolates, the monoverticillate isolate showing the least resistance (Nazareth and Marbaniang 2008). Massaccesi et al. (2002) worked on Cd removal using filamentous fungi isolated from heavily polluted streams near to some Argentinian industrial area. The identified species included *Aspergillus terreus*, *Clodosporium clodosporioides*, *Fusarium oxysporum*, *Gliocladium roseum*, *Penicillium* spp., *Talaromyces helices*, and *Trichoderma koningii*. The concentration of Cd in the sediments from where these fungal species were isolated ranges between 0.25 and 0.50 mg/L. They were isolated in Cd basal medium. *Penicillium* spp. was able to grow and remove a 100-fold higher Cd level after 13 days of incubation by an absorption process. Most of the above-mentioned species were found highly efficient in detoxifying Cd and helping in remediation of chronically contaminated sites (Massaccesi et al. 2002).

24.5.2 Metal Biosorption by Obligate Halophilic Fungi

Bano et al. (2018) studied a halophilic group of fungal species having the ability to perform biosorption of heavy metals from contaminated sites. The obligate halophilic fungal species comprised of *Aspergillus flavus*, *Aspergillus gracilis*, *Aspergillus penicillioides* (sp. 1), *Aspergillus penicillioides* (sp. 2), *Aspergillus restrictus*, and *Sterigmatomyces halophilus.* The heavy metals included cadmium, copper, ferrous, manganese, lead, and zinc. During studies, the metals were supplemented as salts in media for the growth of obligate halophilic fungi and incubated for 14 days. Amongst all the fungi, *A. flavus* (86%) and *S. halophilus* (83%) exhibited best average adsorption of all the heavy metals. The rest of all the tested fungi showed moderate to high adsorption of heavy metals. On an average, Fe and Zn were best removed from the liquid media with an average of 85 and 84%, respectively. The study involved the use of inexpensive media in stagnant conditions which provides a cost-effective environmental solution for the removal of heavy metals (Bano et al. 2018).

24.5.3 Bioremediation of Cadmium by *Penicillium chrysogenum* XJ-1

Xu et al. (2015) conducted interesting studies using the ability of cadmium bioremediation by *Penicillium chrysogenum* XJ-1. Using physicochemical and biochemical methods, they investigated the morphological and antioxidative response of the fungus to different concentrations of Cd (1–10 mm). They found that with the increase in Cd concentration, malondialdehyde level was increased by 14.82–94.67 times and Cd was mainly bound to the cell wall of the fungus. The results also reported that the biosorption, cellular sequestration, and antioxidant defense were involved in Cd detoxification. At 1 mM Cd concentration, superoxide dismutase (SOD), glutathione reductase (GR), and glucose-6-phosphate dehydrogenase (G6PDH) levels were increased and at 5 mM concentration, catalase level was peaked. The glutathione/oxidized glutathione ratio was also increased on exposure to Cd. The group also studied in situ application of the XJ-1 fungus where they sowed pak choi plant seeds in the Cd polluted soil (5–50 mg/kg) and found that plant yield was increased through reduced Cd bioavailability (Xu et al. 2015). The in situ results obtained signify the potential importance of the fungus to remediate Cd polluted sites as a promising candidate (Table 24.1).

24.6 Radio Tolerant Species

24.6.1 Uranium Tolerant *Saccharomyces*

Strandberg et al. (1981) studied the uranium accumulation ability of *Saccharomyces cerevisiae* and *Pseudomonas aeruginosa*. *S. cerevisiae* accumulates the element on extracellular surface while *Pseudomonas aeruginosa* accumulates it intracellularly. Additionally, it was found superior to *S. cerevisiae* in terms of accumulation rate. Environmental parameters such as pH, temperature, and interference by certain anions and cations affected the rate and accumulation ability of *S. cerevisiae*. *Pseudomonas aeruginosa* observed not giving any response to environmental parameters. Both the organisms work irrespective of metabolism for metal uptake. Formaldehyde and $HgCl_2$ pretreatments increased rate of uranium uptake by *S. cerevisiae* cells but it did not affect the other. The solution of the pH and temperature both considerably affected the metal uptake by *S. cerevisiae*. The accumulated uranium could be removed chemically from *S. cerevisiae* cells, and the cells could then be reused as a biosorbent (Strandberg et al. 1981).

24.6.2 Uranium and Thorium Biosorption by *Rhizopus arrhizus*

Tsezos and Volesky (1981) from Canada studied biosorption of uranium and thorium by various living beings and materials. They found that *Rhizopus arrhizus* at pH 4 exhibited the highest biosorption capacity for both the elements in excess of 180 mg/g. It was also found that *R. arrhizus* removed approximately 2.5 and 3.3

Table 24.1 Metalophilic fungal species

S. no.	Fungal sp.	Heavy metal	Reference
1	*Penicillium* sp.	Pb, Cu, Cd	Nazareth and Marbaniang (2008)
2	*A. terreus*, *C. clodosporioides*, *F. oxysporum*, *G. roseum*, *Penicillium* spp., *T. helices*, *T. koningii*	Cd	Massaccesi et al. (2002)
3	*A. flavus*, *A. gracilis*, *A. penicillioides*, *A. restrictus*, *S. halophilus*	Cd, Cu, Fe, Mn, Pb, Zn	Bano et al. (2018)
4	*P. simplicissimum*	Cd and Zn	Fan et al. (2008)
5	*P. chrysogenum*	Cd	Holan and Volesky (1995)
6	*P. canescens*	Cd, Pb, As, Hg	Say et al. (2003)
7	*P. purpurogenum*	Cr	Say et al. (2004)
8	*P. cyclopium*	Cu	Ianis et al. (2006)
9	*P. italicum*	Mn, Fe, Ni, Co	Mendil et al. (2008)

times more uranium than the ion exchange resin and activated carbon, respectively when uranium was placed at the equilibrium concentration of 30 mg/L. Talking about thorium, under the same conditions, *R. arrhizus* removed 20 times more thorium than the ion exchange resin and 2.3 times more than the activated carbon. *R. arrhizus* showed higher uptake and a generally more favorable isotherm for both uranium and thorium than all other biomass types examined in the studies. Other biomass used included *Aspergillus niger*, *Aspergillus terreus*, *Penicillium chrysogenum*, *Pseudomonas fluorescens*, *Streptomyces niveus* (Tsezos and Volesky 1981) (Table 24.2).

24.7 Hydrocarbon Degradation

24.7.1 n-Alkanes Degradation

Barnes et al. (2018) conducted a study to identify potential hydrocarbon degrading fungal species from marine niches in India. In their study, they identified 10 potential isolates using ITS rDNA sequencing. Out of ten, six are *Aspergillus* spp., two are *Fusarium*, and one each of *Penicillium* and *Acremonium* spp. The sources included mangrove sediments, Arabian Sea sediments, and tarballs. *Penicillium citrinum* (#NIOSN-M126) showed the highest efficiency of 77% reduction in crude oil content. For the identification, they used a mineral salt medium with 5% petrol as a sole carbon source. The isolates that were able to grow in the medium and showing increased biomass were selected. The 10 isolates were coded as IOSNM113, NIOSN-M126, NIOSN-M142, NIOSN-M109, NIOSN-SK56S57, NIOSN-

Table 24.2 Uranium and thorium tolerant fungi

S. no.	Organism	Radioactive element	Reference
1	*S. cerevisiae*, *P. aeruginosa*	Uranium	Strandberg et al. (1981)
2	*R. arrhizus*	Uranium and thorium	Tsezos and Volesky (1981)

SK56S32, NIOSN-SK56S22, NIOSN-SK56C42, NIOSN-T5, and NIOSN-T4. Four of them obtained from mangrove environments (NIOSN-M113, NIOSN-M126, NIOSN-M142, and NIOSN-M127), four from marine environments (NIOSN-SK56S57, NIOSNSK56S32, NIOSN-SK56S22, and NIOSN-SK56C42), and two from tarballs (NIOSN-T5 and NIOSN-T4). For qualitative analysis, gas chromatography was performed to detect n-alkanes in the incubated medium. *Penicillium citrinum* NIOSN-M126 showed the highest efficiency of degradation of the long-chain *n*-alkanes (C29–C20), while *Aspergillus sydowii* NIOSN-SK56C42 was least efficient (Barnes et al. 2018). Ferrari et al. (2011) from Australia recovered 91 fungal species from contaminated and non-contaminated soils from sub-Antarctic Macquarie Island and evaluated their potential for hydrocarbon degradation. In their studies, they used both of traditional high nutrient and a low nutrient broth approach to recover greater fungal species diversity from both pristine and SAB (special Antarctic blend) diesel fuel contaminated soil. The species were identified using standard molecular techniques targeting the highly variable ITS region. The total of 91 recovered fungi includes 63 unidentified fungal species. Few of the identified species include *Trichophyton* sp., *Antarctomyces psychrotrophicus*, *Pseudeurotium bakeri*, *Aspergillus fumigatus*, *Penicillium* sp., etc. Few of the recovered species (Cladophialophora and Exophiala genera) have also been reported for their potential to breakdown aromatic hydrocarbons (Ferrari et al. 2011). Borowik et al. (2017) studied fungal diversity in diesel contaminated sites. In their 270-day study, it was found that the fungal diversity was altered than that of the unpolluted site. In diesel oil polluted soil, the colony development index was significantly increased but the fugal diversity index was decreased. The polluted soil was characterized by higher activity of oxidoreductases and a higher number of fungi as compared to unpolluted soil but the lasting effect of diesel oil led to a decrease in functional diversity of fungal communities. The studied soil was not subjected to remediation activities. After 270-day study, 64% of four-ringed, 28% of five-ringed, 21% of 2–3 ringed, and 16% of six-ringed PAHs underwent degradation. The polluted soil included mainly *Fusarium* (37.9%), *Candida* (13.8%), *Microsporum* (13.8%), *Penicillium* (13.8%) genera, and the unpolluted soil showed the dominance of *Penicillium* (26.5%), *Microsporum* (21.1%), *Fusarium* (21.1%), and *Candida* (15.8%). It is possible to reduce the hydrocarbons considerably using identified fungal species (Borowik et al. 2017).

24.7.2 High Molecular Weight PAHs Degradation by Bacteria–Fungi Coculture

An Australian research group investigated the biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons (PAHs) in liquid media and soil. They used bacterial *Stenotrophomonas maltophilia* (VUN 10,009 and 10,010) and a fungus *Penicillium janthinellum* (VUO 10,201) which were isolated from separate creosote and manufactured-gas plant-contaminated soils. The bacteria used pyrene as its sole carbon and energy source in a basal salt medium (BSM) and successfully mineralized significant amount of benzo[*a*]pyreneco metabolically when pyrene was also present in BSM. *P. janthinellum* (VUO 10,201) alone failed to utilize any high molecular weight PAH as the sole carbon and energy source. But when cultured in nutrient broth, it partially degraded them. The axenic cultures do not grow significantly though they support degradation of small amounts of chrysene, benz[*a*]anthracene, benzo[*a*]pyrene, and dibenz[*a*,*h*]anthracene in BSM containing a single PAH. However, when either bacterial consortium VUN 10,009 or VUN 10,010 cocultivated with *P. janthinellum* VUO 10,201 (fungal–bacterial cocultures), significant degradation along with microbial growth was achieved. It was achieved on pyrene, chrysene, benz[*a*]anthracene, benzo[*a*]pyrene, and dibenz[*a*,*h*]anthracene, each as a single PAH in BSM. The cocultures mineralized about 25% of the benzo[*a*]pyrene to CO_2 over 49 days, accompanied by transient accumulation and disappearance of intermediates detected by high-pressure liquid chromatography. When these fungal-bacterial cocultures were inoculated into PAH-contaminated soil, it significantly improved degradation of high-molecular-weight PAHs, benzo[*a*]pyrene mineralization (53% of added [^{14}C]benzo[*a*]pyrene was recovered as $^{14}CO_2$ in 100 days), and reduction in the mutagenicity of organic soil extracts, compared with the indigenous microorganisms and soil amended with only axenic inocula (Boonchan et al. 2000).

24.7.3 Heavy Hydrocarbon Degradation by Bioaugmentation of Native Fungi

Medaura et al. (2021) applied bioaugmentation of fungal species to cure aged industrially polluted soil containing heavy hydrocarbons. They applied defined consortium of six potentially hydrocarbonoclastic fungi belonging to the genera *Penicillium*, *Ulocladium*, *Aspergillus*, and *Fusarium* in the microcosm assays for bioaugmentation and biostimulation. All the fungi were previously isolated from polluted sites. The bioaugmentation applied degradation performance was compared with biostimulation (water and nutrient addition) as well as with untreated soil as a control. The results obtained showed that fungal bioaugmentation degraded more efficiently than biostimulation. In bioaugmentation microcosm, total petroleum hydrocarbons (TPH) C14–C35 decreased by 39.90 $\pm$ 1.99% while biostimulated microcosms resulted in a 24.17 $\pm$ 1.31% reduction in TPH (C14–C35). Similarly, the effect on high molecular weight polycyclic aromatic hydrocarbons

(HMW-PAHs) was also studied. In bioaugmentation microcosm, it was found that the five-ringed benzo(a)fluoranthene and benzo(a)pyrene were reduced by 36% and 46%, respectively, while the six-ringed benzoperylene decreased by 28%, after 120 days of treatment. On the other hand, biostimulation microcosm showed a reduction of five- and six-ringed PAHs only 8% and 5% respectively. Further studies revealed that bioaugmentation promoted the growth of autochthonous active hydrocarbon-degrading bacteria (Medaura et al. 2021).

24.7.4 Phenanthrene Removal by *Penicillium frequentans*

The study conducted by Meléndez-Estrada et al. (2006) with *Penicillium frequentans* showed that oxygen concentration has a significant effect on phenanthrene removal. *P. frequentans* was grown on sugarcane bagasse pith mixed with soil spiked supplemented with 200 mg/L of phenanthrene to obtain a final bagasse/soil ratio of 1:16.The C/N ratio was adjusted to 60 and moisture content adjusted to 40%. Various oxygen concentrations were adjusted to 20%, 10%, 5%, and 2% and almost 0% in the soil gas phage for each treatment. A high removal rate was obtained for the higher oxygen concentrations, reaching 52% removal after 17 days with 20% oxygen. An opposite phenanthrene removal trend was found under low oxygen concentrations, reaching only 13% at close to 0% oxygen after 17 days of incubation. An explanation was presented based on the adsorption of soil components due to the higher content in organic matter and clay (Meléndez-Estrada et al. 2006).

24.7.5 Fluorene Degradation by *Absidia cylindrospora*

Garon et al. (2002) studied fluorine degradation in soil slurry using various fungal isolates and with reference to bioaugmentation. Totally, 47 fungal species were isolated from a contaminated site and amongst them, *Absidia cylindrospora* was found to be the most potent one. The strain was further used for bioaugmentation process where more than 90% degradation rate was achieved. In the absence of bioaugmentation process, it took 576 h to achieve the same rate and the bioaugmentation process reduced the required time to 288 h. Malstosyl-cyclodextrin improves the solubility of fluorine by forming inclusion complexes. This helps to enhance the bioavailability of fluorine in the soil slurry. The study helps to utilize *Absidia cylindrospora* as a potent tool for fluorine degradation in soil through bioremediation process (Garon et al. 2004). In their prior studies, Garon et al. (2002) also found that the addition of surfactant Tween 80 enhances the biodegradation of fluorine by *Doratomyces stemonitis* and *Penicillium chrysogenum*. In the case of *Doratomyces stemonitis*, Tween 80 concentration of 0.324 mM resulted change in biodegradation from 46% to 62% in 2 days. Similarly, the change for *Penicillium chrysogenum* with the same Tween 80 concentration within the same time period is from 28% to 61%.

24.8 Degradation of Phenol and Its Derivatives

24.8.1 Biodegradation of Phenols at Low Temperatures

Phenol is toxic even at low concentrations to many living organisms. This retards the growth of many biodegradative agents at the contaminated sites and affects the process of bioremediation. The reporting of cold adapted microorganisms tolerant to phenol is a hope for phenol degradation at low temperature sites. Margesin et al. (2005) isolated two phenol degrading psychrophilic fungal species, both from *Rhodotorula* species: *Rhodotorula psychrophenolica* sp. nov. (type strain AG21T 5CBS 10,438T 5DSM 18,767T) isolated from mud at the foot of a glacier (thawing zone) and *Rhodotorula glacialis* sp. nov. (type strain A19T 5CBS 10,436T 5DSM 18,766T) isolated from a glacier cryoconite. These species utilized phenol concentration up to 12.5 mM and 5 mM as a sole carbon source at 10 °C respectively (Margesin et al. 2007). Another fungal species *Trichosporondulcitum* and *Urediniomycetes* isolated from hydrocarbon contaminated alpine soils showed the potential of degrading phenol. The optimum temperature for *Trichosporondulcitum* was found at 20 °C while for *Urediniomycetes* strain it is 10 °C. From the same soil sample, two bacterial species of *Rhodococcus* spp. were isolated. The phenol degrading activity of *Urediniomycetes* strain at 1 °C was found faster than that of *Rhodococcus* spp. at 10 °C. Under fed-batch systems provided with mineral medium and phenol as sole carbon source, both the bacteria and fungi were assessed for phenol degradation at 10 °C. The yeast strains degraded phenol concentration up to 15 mM while both bacteria degraded up to 12.5 mM of phenol under provided environment (Margesin et al. 2005). Another group isolated a fungus *Aureobasidium pullulans* FE13 from stainless steel effluents. It was examined for phenol degradation through immobilization and as free cells. The rate of degradation with free cells was found to be 18.35 mg/L/h while alginate immobilized cells showed the degradation rate of 20.45 mg/L/h. However, the significant information is that immobilized cells showed longer viability compared to free cells and thus increasing phenol degradation efficiency (dos Santos et al. 2009). *Candida tropicalis* HP 15 showed the phenol degradation up to 2.5 g/L through beta-ketoadipate pathway (Krug et al. 1985).

24.8.2 Halophilic Fungi for Phenol Degradation

Jiang et al. (2016) isolated *Debaryomyces* sp. on the basis of phenol tolerance from activated sludge of a pharmaceutical factory in Wuhan, China. The isolation medium was supplied with 5% NaCl. The species showed close resemblance with *Debaryomyces hansenii* and *Debaryomyces subglobosus*. To examine the phenol degradation potential of the fungus, the growth medium was supplemented with 100–1300 mg/L of phenol. The growth temperature provided was 30 °C at 160 rpm. It was observed that increasing phenol concentration was associated with a decrease in cellular biomass. In the results, it was found that phenol concentrations up to

500 mg/L degraded in a short time (around 32 h) as compared to the higher concentrations. Concentrations above 900 mg/L required more time (96 h and more) for complete degradation of phenol in the media. Other parameters checked included salt and metal tolerance, optimum pH, and agitation speed. At NaCl concentration of 1%, maximum fungal growth and phenol degradation were achieved. The phenol degradation was affected by Co and Ni and not by Mn and Zn. The fungus growth was maximum at neutral pH while the optimum pH value for phenol degradation was 6.0. The agitation speed at 200 rpm helped to degrade phenol with higher efficiency (Jiang et al. 2016).

24.8.3 Bioremediation by Specific Extremofungal Species

24.8.3.1 By Halotolerant Trichoderma

Divya et al. (2014) from Kerala studied the ability of halotolerant *Trichoderma viridae* strain having the ability to remediate the phenol polluted sites. The group found that the isolated halotolerant strain *Trichoderma viridae* Pers. NFCCI-2745 is also phenol-tolerant. It produces laccase enzyme and the ability of laccase to oxidize phenol is very well known. The source of the strain is a phenol contaminated saline environment. Additionally, at a 5–10 ppm saline environment, enhanced laccase production was found. The organism is resistant to even 30 ppm salinity but it results in reduced biomass and reduced laccase production. Even, the enzyme secretion was also found in response to phenol concentration. Different phenolic compounds were used in this study and their optimum concentration ranges between 20 and 80 mg/L. Beyond 200 mg/L concentration of phenolic compounds, the enzyme activity was decreased and completely stopped at 800 mg/L. The ability of the strain can also be utilized to remove the sites which are polluted by industrial effluents containing phenolic compounds (Divya et al. 2014).

24.8.3.2 By *Candida tropicalis*

Chang et al. (1998) produced fusant of *Candida tropicalis* using protoplast fusion as a selective technique. Then they studied the comparative of phenol degradation abilities of *Candida tropicalis* and the produced fusant. The studies were carried out under batch and high concentration conditions. They found that the oxygen uptake activities of both yeast and fusant peak at pH 7.0 and 32 °C. But surprisingly the fusant was more active than the control strain. The fusant showed less susceptibility to phenol inhibition than that of the control strain. The fusant also showed better phenol degradation than the control. It was observed that when the phenol concentration is $\geq$3300 mg/L, the ability of *C. tropicalis* was completely inhibited, whereas for the *C. tropicalis* fusant complete inhibition is absent until the phenol concentration is $\geq$4000 mg/L. However, they were virtually identical and remain fairly constant at approximately 0.5 mg MLVSS/mg C6H5OH (MLVSS: mixed liquor volatile suspended solids) for both the strains (Chang et al. 1998).

24.8.3.3 By Halotolerant Penicillium

A research group isolated a fungus from a salt mine in Portugal. The isolate was enriched in the presence of phenol and high salt concentration. Further, it was identified as a halotolerant strain of *Penicillium chrysogenum* and was able to tolerate up to 5.8% NaCl in the medium. Further, it was found to utilize at least 300 mg/L of phenol as a sole carbon and energy source and did not accumulate any intermediates. The fungus reduced the phenol toxicity under saline conditions. At 5.9% NaCl, *P. chrysogenum* CLONA2 was able to rapidly detoxify phenol. Degradation of phenol in the presence of glucose suggested that it could proceed via hydroquinone. Based on the results achieved, the strain can possibly be used in the biological treatment of phenol-containing wastewaters like that of petrochemical effluents (Leitão et al. 2007).

24.8.3.4 By *Penicillium frequentans*

Hofrichter et al. (1994) studied a soil isolated fungus *Penicillium frequentans* Bi 7/2 strain for its activity to degrade halogenated phenols. These halogenated phenols are frequently released as major pollutants from chemical industries. The fungus was isolated from a soil contaminated with polyaromatic hydrocarbons. The crude extract made of the fungus was found capable of degrading various monohalogenated phenols. The fungus also metabolized phenol derivatives like 3,4-dichlorophenol, 2,4-dichlorophenol in the presence of phenol as a cosubstrate. The degradation process occurs through many reactions and mainly occurs because of unspecific intracellular enzymes like phenol hydroxylase, catechol-1,2-dioxygenase, and muconatecycloisomerase I. The authors have proposed a pathway for the degradation process of both halogenated phenols and dichlorophenols (Hofrichter et al. 1994).

24.9 Bioremediation at Low Temperature

Cold regions like Arctic, Alpine, and Antarctic environments are being studied for various objectives involving human activities. They are being under focus for petroleum exploration, production, transport, etc. Such kind of activities increases the risk of accidental oil release in these areas (Buzzini and Margesin 2014). Various hydrocarbon-degrading microorganisms have also been described in cold environments. When some contaminant is introduced to their natural habitat, they may adapt to the contamination in order to survive. This has been reported where the introduction of pollutant induced a number of degradative microorganisms in particular environments (Aislabie et al. 2001; Margesin and Schinner 2001; Bej et al. 2010; Greer et al. 2010). Bioremediation in hydrocarbon contaminated cold environments like aquatic and terrestrial sites has been reported (Brakstad 2008; Filler et al. 2008; Bej et al. 2010; Greer et al. 2010). Along with this, successful implementation of different remediation schemes like biopiles, land farming, and engineered bioremediation have been achieved (Filler et al. 2008). Cold adapted yeasts can be utilized for bioremediation of contaminated sites containing various hydrocarbons like petroleum products and phenolic compounds. For soil

bioremediation, biostimulation and bioaugmentation approaches can be applied. Growing in natural conditions imparts many limitations over the growth of microorganisms like nutrient deprivation, pH adversity, temperature, and oxygen conditions. These limiting conditions reduce the rate of bioremediation. When the nutrients and optimum conditions are provided to the indigenous microorganisms, it stimulates their growth and speeds up the bioremediation rate. This approach is referred to as biostimulation. In bioaugmentation, external microorganisms are added to the contaminated sites to increase the rate of the biodegradation process.

24.10 Salt Crystallization Cleanup by Halophilic Fungi

Mansour (2017) studied the cleanup of salt crystals formed on sandstones on the site of Nile river in Egypt. Various halophilic fungi like *Cladosporium sphaerospermum*, *Wallemia sebi*, *Aureobasidium pullulans*, and *Aspergillus nidulans* were evaluated for their ability to clean up halites formed on the surface of sandstones. Analytical studies like X-ray diffraction (XRD), scanning electron microscopy (SEM), and total dilation salt (TDS) confirmed that the stones treated with fungi showed lowered concentrations of salt as compared to control. Further, *W. sebi*, *A. pullulans*, and *A. nidulans* were grown on NaCl concentration ranging from 0 up to 25% to check growth stimulation. At 5% NaCl, maximum mycelial growth was achieved. However, *C. sphaerospermum* showed best growth without NaCl and *W. sebi* was able to grow even at 20% NaCl. In conclusion, it was found that *W. sebi* consumed maximum halites while *A. nidulans* gave the worst results showing the change in coloration of sandstone. Furthermore, *W. sebi* and *A. pullulans* are suggested for desalination of archaeological stone monuments (Mansour 2017).

24.11 Acidophiles in Bioremediation

Fungal acidophiles like *Acidomyces acidophilus* and *A. richmondensis* are described for their bioremediation potential of contaminated sites where extreme conditions like that of pH and toxic compounds are present. Especially *Acidomyces acidophilus* has a great potential in bioremediation. It is a pigmented ascomycete capable of growing in extremely acidic conditions in the pH range of 1–3 (Chan et al. 2019). The ability of the fungus to survive in adverse environmental conditions such as extreme pH, temperature, and toxins is by the virtue of its melanin-containing cell wall (Hujslová et al. 2013). The intracellular enzymatic regulation mechanisms of *A. acidophilus* at low pHs, reactive oxygen species, and extreme temperatures are now widely considered as valuable resources for their exploitation in novel biotechnological applications (such as cleaning of contaminated soils or water) and in the field of biocatalysis (Chan et al. 2019). The first report of *Acidomyces acidophilus* is from a highly acidic, sulfate-containing industrial water. Subsequently, more *Acidomyces* species were isolated and identified successfully from various extreme

environments (Chan et al. 2019). Another species isolated by Baker et al. (2004), *Acidomyces richmondens*, is from sulfuric acid ore mine drainage in Richmond, USA, even at pH 0.5 and 0.9 and thermophilic temperature in the range of 35–57 °C (Baker et al. 2004). Along with being acidophilic, *A. acidophilus* has developed tolerance toward high concentration of toxic metals and metalloids stresses such as Al, As, Cu, Fe, or U (Chan et al. 2018). Jambon et al. (2019) reported biodegradation of chlorendic acid by fungi isolated from polluted sites in Belgium. Chlorendic acid is a highly chlorinated and recalcitrant organic pollutant. The soil samples were collected from around the roots of the plants common bent (*Agrostis capillaris*) and a hybrid poplar [*Populus deltoides* × (*Populus trichocarpa* × *P. deltoids*) cv. Grimminge]. Both plants are able to grow on chlorendic acid contaminated sites in Belgium. Out of the 75 fungi isolated from the samples, 16 degraded chlorendic acid between 10 and 20% in liquid media and 4 removed 20–50%. *Chlonostachys* sp. 2C-2c degraded up to 71% in the liquid phase. The fungal isolates were observed producing higher levels of hydroxyl radicals on exposure to chlorendic acid indicating oxidative degradation of the pollutant through the production of Fenton-mediated hydroxyl radicals. One of the ascomycetes isolate *Penicillium* sp. 1D-2a was found to degrade 58% chlorendic acid in the soil after 28 days. This study gives a new perspective for in situ degradation of the recalcitrant compound chlorendic acid (Jambon et al. 2019). Tkavc et al. (2018) have characterized an acidophilic fungi *Rhodotorula taiwanensis* MD1149. The group studied 27 fungal species to find a suitable candidate for bioremediation of acidic radioactive environmental sites. They were having characteristics for resistance to ionizing radiation (chronic and acute), heavy metals, pH minima, temperature maxima and optima, and their ability to form biofilms. Remarkably, many yeasts are extremely resistant to ionizing radiation and heavy metals. The fungal species included 16 ascomycetous and 11 basidiomycetous yeasts isolated from diverse environments including arctic ice, acid mine drainage, red wine, and apple juice, as well as dry environments with elevated temperatures. However, the studies identified only one potential fungus strain, i.e., *Rhodotorula taiwanensis* MD1149 to fulfill the expectations. The strain was isolated from a sediment sample from an abandoned acid mine drainage in Maryland, USA. The fungus is a red pigmented, unicellular, non-sporulating, ovoidal, obligately aerobic, budding yeast. MD 1149 showed resistance to gamma radiations. It was found capable of growing under 66 Gy/h at 2.3 pH value and in the presence of high concentrations of heavy metal compounds of mercury and chromium. The strain was found forming biofilms under high-level cgronic radiation and low pH. The strain has undergone whole genome sequencing and presents a strong potential role in the bioremediation of acidic radioactive sites (Tkavc et al. 2018).

24.12 Conclusions

Extremophiles of different kinds are being studied for their novel characteristic to survive in extreme conditions of pH, temperature, salt concentration, hydrocarbon contamination, etc. The approach finds applications in industrial and environmental

issues. An important source of biochemicals like enzymes and proteins will help in industrial catalysis. Fungi have a wide range of metabolism. Especially, extremophilic fungi can help in the degradation or inactivation of various environmental contaminants at the sites where normal microorganisms cannot help. They have been isolated from diverse sources like low and high temperatures, acidic conditions and high concentration of heavy metals, etc. Many of the isolated species show a greater potential in bioremediation of such contaminated sites. Further studies will help to identify new species and help them to perform the activity with enhanced efficiency.

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Extremophilic Fungi: Potential Applications in Sustainable Agriculture

25

Sanjay Sahay

Abstract

Food is the most basic requirement of life which has been hard hit due to climate change effect. Unfortunately, unsustainable agricultural practices pursued for many decades to produce more have left behind a substantial proportion of polluted and depleted water sources and highly affected soil concerning its structure, and chemical and microbiological composition. To ensure continued supply of food to billions of stomachs across the world, remediation of the so-called problem soils and sustainable use of soil in general have now become imperative. Fungi constitute an important part of soil microbiome which by their efficient secretomes and plant growth-promoting activities play a key role in soil structure and fertility maintenance. Extremophilic fungi with their capacity to function under various stress conditions and to confer upon plants' tolerance to various abiotic and biotic stresses hold a key to bioremediate problem soils, restore soil fertility, and augment stress tolerance to the plants.

Keywords

Extreme-fungi in soil management · Extreme-fungi in crop improvement · Extreme-fungi and photosynthesis · Extreme-fungi as biocontrol · Extreme-fungi and abiotic stress

S. Sahay (✉)
Sarojini Naidu Government Postgraduate Girl's (Autonomous) College, Bhopal, Madhya Pradesh, India

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25.1 Introduction

Fungi have been serving humanity since very early in civilization by providing fermented food and beverages. Advancement in the genetics and biochemistry of fungi has opened up the possibility for their diverse applications in industrial and environmental fields. Fungi as saprotrophs, phytopathogens, endophytes, and Arbuscular mycorrhizal forms perform varied services to agriculture including structural and fertility management of soil and conferring tolerance to various stresses upon plants. As decomposer, the importance of fungi in maintaining the physicochemical nature of the soil is well known.

Fungi as part of decomposers perform an important function in unlocking simpler organic matters from dead animals and plants and their detached parts thus adding organic matters to the soil (Aitken 1993). Fungi also produce organic substances such as glomalin that improve soil structure (Spurgeon et al. 2013). Fungi also improve the availability of plant nutrients, for example, by transforming complex insoluble and unusable phosphate into a soluble and usable form, carrying immobile metal nutrients close to root hair, and producing plant growth-promoting and phytopathogen inhibitory compounds. In soil, fungi vis-à-vis bacteria like mild acidic conditions, not many disturbed soils, perennial plant community, nutrients supply from nearby plants and higher carbon to nitrogen (C:N) content (Lavelle and Spain 2005). The mycelial structure of fungi enables them to grow deeper in the soil and connect roots to more distant nutrient sources (Lowenfels and Lewis 2006). Unfortunately unsustainable practices in agriculture such as the unjudicious application of synthetic fertilizers and pesticides have apart from polluting soil and water also altered the soil microbiome including soil fungi.

Fungi in the extreme climate have evolved novel ability to tolerate various abiotic stresses such as tolerance to extreme climates (high and low temperatures), extreme pH conditions (alkaline and acidic), extreme salt (sodic), and higher metal concentrations. During the last few decades, surveys in such extremophilic climates have revealed the existence of strains and species of extremophilic fungi that can not only perform their normal functions under extreme conditions but also exhibit various ameliorative activities. Further physiological and genetical studies on them have led to the discovery of mechanisms of tolerance to specific extreme conditions. All these developments have opened up vistas in applying extremophilic fungi and their biomolecules to bioremediate problem soils, maintain decomposition activity under extreme conditions, restore fertility in problem soils, manage soil phytopathogens, and ensure beneficial activities of endophytes and AMF under extreme conditions.

25.2 Agricultural Constraints and Fungal Intervention

An ideal agriculture system requires at least fertile soil, a proper supply of water and nutrients, and suitable crop variety. However, global climate change and non-judicious agricultural practices to produce more have led to such problems as

depletion of water sources and highly degraded soil concerning its structure, and chemical and microbiological compositions in many parts of the world. The current focus to restrict the use of chemical fertilizers and pest controls has also enhanced nutritional and biotic stress respectively for the crop. Agriculture under these constraints put plants in mainly three abiotic stresses viz., water, temperature (low or high), and salt stresses (Egamberdieva et al. 2008; Egamberdieva and Lugtenberg 2014). The problems may be tackled at the level of the soil (by amending soil) and plant (by conferring stress tolerance). Plant growth-promoting and pathogen inhibiting activities of microbes especially fungi may be useful. Mainly three categories of fungi viz., open soil dweller, Arbuscular mycorrhizic fungi (AMF), and endophytic fungi (EPF) have been studied concerning their role in promoting plant growth and yield under normal and various stress conditions. The three categories of extremophilic fungi on the other hand are more potential to provide these benefits as they are acclimatized to carry out their functions under stressful conditions with variously upgraded tools (e.g., enzymes).

25.2.1 Soil Management

25.2.1.1 Soil Structure Improvement

Many fungi produce coagulating substances, of which glomalin is a highly studied one. These substances play a very effective role in aggregating the soil particles into microaggregates (Wright and Upadhyaya 1998). Several studies have demonstrated the direct relationship between glomalin-producing fungi and aggregate stability (Spurgeon et al. 2013; Wright and Upadhyaya 1998). As in the case of natural grassland and forests as well as agricultural fields, soil receives green litter either as fallen plant parts due to senescence (Girisha et al. 2003) or plant residues left after harvesting (Cookson et al. 2008). The litter in abundant quantity is also received from thinned trees during the process of thinning where it is in practice (Tian et al. 2010). Thus litter in different amount and composition is incorporated in various categories of forests which in turn determine the soil microbial community according to their food preference/enzymatic capacity. Among microbes, fungi play a crucial role in the decomposition of lignoellulosic mass and contribution of thus formed organic matter to the soil. The organic matter in the soil helps in forming favorable soil structure (microaggregate formation) and enhances the nutritional status and water retention capacity of the soil (Jiménez-Morillo et al. 2016). Soil organic matter does play an effective role in maintaining soil resistance and stability, though a scientific explanation of this relationship is not available. Deforestation-led-degraded soil that may exhibit poor fungal diversity and soil functional stability partially explains fungal diversity and soil functional stability relationship (Chaer et al. 2009). The extremophilic fungi have a potential to carry out this function under extreme climatic or soil conditions.

25.2.1.2 Soil Amendments

There are the so-called problem soils such as saline and sodic, acidic, and acid sulfate soils which constitute a considerable proportion of arable land globally. For example, India has approximately 60 million hectares of such problem soils out of about 140 million hectares of net cultivable area (Goswami 1982). Of these problem soils, saline and sodic soils are the most problematic ones. Accumulation of higher concentration of salts in arid and semi-arid soil as a result of natural causes such as rainfall and evapotranspiration dynamics (Zhu 2001; Zheng et al. 2009), and man-made practices such as assisted irrigation (Allbed and Kumar 2013) are responsible for the development of these types of soil. They have Na^+ and Cl^- in the toxic concentrations that cause, in plants, disruption of enzymes' structure and damages to cell organelles, and negatively affect metabolic activities such as protein biosynthesis. Soil exhibits waterlogging and poor aeration. Plants in saline soil are exposed to osmotic, ionic, and oxidative stress, and thus exhibit highly reduced viability and productivity (Saxena et al. 2017). The saline-sodic soils are also characterized by a paucity of fungal diversity. The problem soils are recognized as the main constraints in the way of food security (FAO 2009).

Various types of extremophilic fungi may be used to augment such soils to amend them. For example, the haloalkaliphilic fungi can ameliorate such soils by secreting organic acids, absorbing salt ions, and adding biomolecules such as cellulases that have a positive impact on soil physical properties, fertility, and even health (Wei and Zhang 2019). The potential alkaliphilic/alkalitolerant fungi have been reported from various studies. In one study, taxa belonging to Emericellopsis lineage (Hypocreales and Hypocreomycetidae) were found to dominate followed by the members of families Plectosphaerellaceae (Hypocreomycetidae), Pleosporaceae (Dothideomycetes), and Chaetomiaceae (Sordariomycetidae) in alkaline/saline soil. The fungal species belonging to these families such as *Sodiomyces* species (Plectosphaerellaceae), *Acrostalagmus luteoalbus* (Plectosphaerellaceae), *Emericellopsis alkaline* (Hypocreales), *Thielavia* spp. (Chaetomiaceae), and *Alternaria* sp. were especially found to dominate the scene. Members of *Scopulariopsis* (Microascales) and species of *Fusarium*, *Cladosporium*, and *Acremonium*-like fungus from Bionectriaceae were those found as moderate alkaliphilic ones (Grum-Grzhimaylo et al. 2016). In another study, the species of fungi reported in moderate saline soil were *Trichoderma* spp., *Penicillium* spp., *Fusarium* spp., *Geocladium* spp., and *Paceliomyces* and in hypersaline soil *Eurotium* spp., *Wallemia* spp., and *Phaeotheca* spp. (Bronicka et al. 2007). Fungi isolated from salterns are represented by black yeasts (*Hortaea werneckii*, *Phaeotheca triangularis*, *Aureobasidium pullulans*, and *Trimmatostroma salinum*), *Cladosporium* spp., *Aspergillus* spp., and *Penicillium* spp. (Chung et al. 2019). Apart from these, chaotolerant fungi such as *Hortaea werneckii* and *Wallemia ichthyophaga* that can thrive in 1.8 M $MgCl_2$ concentrations (Zajc et al. 2014) are available to be used in the case of chaotrope (e.g., $MgCl_2$, $CaCl_2$, urea, NH_4NO_3, phenol, and NaBr)-polluted soils (Hallsworth et al. 2003).

25.2.1.3 Soil Bioremediation

Heavily contaminated soils with hazardous chemicals such as heavy metals (due to atmospheric deposition), complex chemicals (via use of pesticides), or residual organic compounds (via operations like wastewater and sludge treatment and compost landfilling) need to be remediated. The available main processes are bioslurry reactor, biopile, and land farming which are compatible with the application of fungal cultures or enzymes (Mougin et al. 2009). The ligninolytic white rot fungi have been in the center for any of such applications because of their capability of producing required enzymes in higher amounts (Aitken 1993; Barr and Aust 1994). Fungal oxidases, laccases, and peroxidases are the main enzymes used in soil bioremediation of soil. Among the three important remediation technologies, bioslurry has been finding more attention as this ensures large-scale operation, rapid enzyme/fungi-contaminant contact, and degradation of pollutant efficiently (for details, see Mougin et al. 2009). Extremophilic fungi that thrive under harsh conditions produce variously adapted enzymes such as metal tolerant, thermophilic, and psychrophilic. These enzymes are expected to be of immense potential for applying for soil bioremediation purposes. Extremophilic laccases (Sahay et al. 2019), peroxidases (Chandra et al. 2017), cellulases (Dutta et al. 2008), etc. have been characterized from extremophilic fungi. Extremophilic fungi have also been evaluated for bioremediation purposes (Baker et al. 2004; Bano et al. 2018; Bej et al. 2010).

25.2.1.4 Soil Fertility Restoration/Biofertilizer

Filamentous fungus, for example, *Mortierella*, exists in diverse types of environments including extreme ones and serves as an efficient decomposer, and plant growth-promoting fungus (PGPF) by improving the availability of P and Fe, synthesizing phytohormones and 1-aminocyclopropane-1-carboxylate (ACC) deaminase, and antagonizing pathogenic fungi in soil. The strains of this fungus are found in bulk soil, rhizosphere, and plant tissues under extreme conditions (Ozimek and Hanaka 2021).

Arbuscular mycorrhizal fungi (AMF) constitute an important group of fungi that help in maintaining soil fertility in agricultural, forest, and horticultural soils (Smith and Read 1997). Several experiments proved the beneficial role of AMF in increasing crop yield following AMF inoculation of crop plants (Bagyaraj and Ashwin 2017). The AMF symbiosis has been reported to exhibit a positive impact on soil structure, nutrient cycling, plant root formation and establishment, plant tolerance to stresses, plant community diversity, and plant's ability to take up low mobility ions (Azcón-Aguilar and Barea 1997). AMF in combination with plant growth-promoting rhizobacteria (PGPR) act synergistically to improve soil structure and fertility (Bagyaraj and Ashwin 2017). In forest soil, ectomycorrhizae on the roots produce extractable dissolved organic carbon that stimulates the growth and proliferation by other microbes (Högberg and Högberg 2002) and also control the water retaining capacity of the soil (Jiménez-Morillo et al. 2016). AMF increase nutrient uptake for the plants, particularly immobile nutrients such as phosphorus (P), copper (Cu), and zinc (Zn) in soil which are not accessible to plant roots in normal condition

due to slow immobility (Marschner 2012; Ortas 2003). Additionally, AMF assist the plants to tolerate various environmental stresses such as salinity, drought, heat, and pollutants in the rhizosphere (Aranda et al. 2013; Bowles et al. 2016; Chandrasekaran et al. 2016; Maya et al. 2014). The mycorrhizal application also reduces the quantitative use of chemical fertilizer input especially phosphate fertilizer (Charron et al. 2001; Ortas 2012). The effect of nutrient- and water-deficient conditions in the field can be reduced in a well-managed rhizosphere with increased microbial community and nutrient availability which benefit plant-soil quality (Barea et al. 2005).

In the Arctic ecosystem where low water and nutrient content are major constraints, the role of mycorrhizal fungi becomes very important (Timling and Taylor 2012). It has been reported that as much as 88% of nitrogen is provided by mycorrhizae especially ectomycorrhizae (ECM) (Hobbie and Hobbie 2006). A large number of ECM fungi have been reported from the Arctic ecosystems (Bjorbaekmo et al. 2010; Deslippe et al. 2011; Geml et al. 2012) such as *Thelephora*, *Sebacina*, and *Clavulina*. ECM isolated and reported from root tips and soil clones in the Arctic showed higher species richness (Bjorbaekmo et al. 2010; Walker et al. 2011; Geml et al. 2012). They are highly potential bioresources to provide growth-promoting factors to the plants in novel cold areas.

The psychrotrophic yeast strains isolated from *Nothofagus* spp. and *Vaccinium* spp. in Northwest-Patagonia have been found to exhibit various plant growth-promoting activities. The yeast *Aureobasidium pullullans*, for example, was identified as a producer of auxin-like and siderophores compounds while yeasts *Holtermaniella takashimae* and *Candida maritime* as phosphate solubilizers (Mestre et al. 2016).

25.2.2 Crop's Stress Management

25.2.2.1 Abiotic Stress

Plants in agriculture systems face mainly three abiotic stresses viz., water, temperature (low or high), and salt stresses (Egamberdieva et al. 2008; Egamberdieva and Lugtenberg 2014) that can also be handled at plant level by conferring various stress tolerance upon plants. There are at least three physiological targets whose improvement has been considered to bring about overall tolerance to various abiotic stresses by the fungi of various types. Apart from these, other mechanisms imparting tolerance to various abiotic stresses have also been reported (Fig. 25.1) (Malhi et al. 2021).

Water Relations

Water status is very important in plant growth and physiology. The contribution of fungi to improve plants' water relations is mainly credited to AMF. AM plants (plants with associated AMF) exhibit in many cases a better water status under water stress conditions as compared to non-AM plants (Augé 2001). Rather improved water-use efficiency for the plant has been reported by AMF application (Bowles

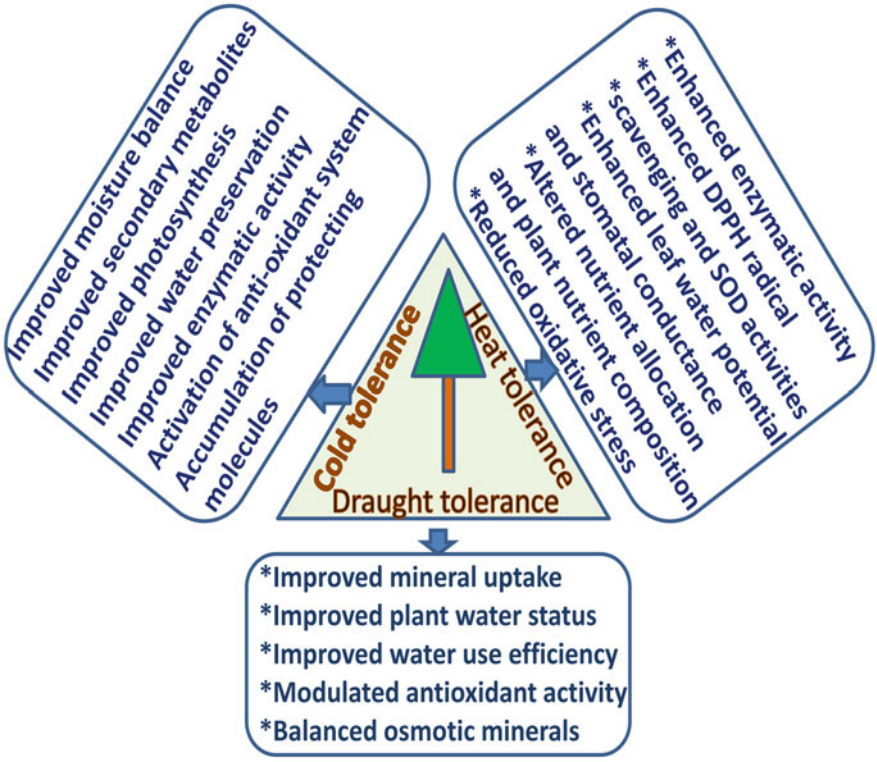

Fig. 25.1 Schematic representation of mechanisms imparting tolerance to various abiotic stresses (Malhi et al. 2021)

et al. 2016). For instances, AM maize has been shown to exhibit higher water holding capacity and relative water content under low- and high-temperature stress (Zhu et al. 2010a, 2011; Liu et al. 2014b) and AM bean (*Phaseolus vulgaris*) has been found to have higher leaf water potential under low-temperature stress (El Tohamy et al. 1999). In contrast to these reports, under low-temperature stress conditions, AM plants have also been found to have the same water content as non-AM plants (Aroca et al. 2007; Liu et al. 2014a). Uptake of water by roots depends upon root hydraulic conductivity, stomatal movement, transpiration rate, and the activity of aquaporin belonging to the intrinsic membrane protein family (Luu and Maurel 2005). AM plants' better water status has been ascribed to the increased water extraction by the external hyphae of AMF (Faber et al. 1991), higher hydraulic conductivity of AM plant roots (Augé and Stodola 1990), higher stomatal conductance, and transpiration rate of AM maize (Zhu et al. 2010a, 2011).

Aquaporins that facilitate the passive water flow through membranes (Kruse et al. 2006) have been found to exhibit enhanced activity in AM plants (Liu et al. 2014b). AMF can regulate in addition to their host plants aquaporin genes (*PvPIP1;3*) thus improving the water status of the host (Liu et al. 2014b). AM plants exhibit under low-temperature stress higher expression of *PvPIP1;3* gene and higher abundance of

PIP protein (Aroca et al. 2007). Upregulation of other membrane intrinsic protein genes (*PIP1;1*, *PIP1;3*, *PIP2;1*, and *PIP2;5*) has also been reported in AM rice (*Oryza sativa*) plants (Liu et al. 2014b).

Ectomycorrhizal fungi or ECM occupy the surface of lateral roots superficially near root tips forming three distinct regions related to water transport viz., the extraradical network of mycelia, mantle sheath, and Hartig net (Fig. 25.2). The growing mycelial tips take up water and nutrient actively. A part of hyphae is grouped together and differentiated into rope-like rhizomorphs (Agerer 2001). Water and nutrients flowing along rhizomorphs may follow two paths at hyphae–root interface—the hyphal mantle sheath occupying the surface of root tip mainly meant for storage, and the intercellular hypha Hartig net in the plant epidermis and outer cortex meant for water-nutrient exchange between hyphae and plant cells (Smith and Read 1997).

Fungal aquaporin is believed to enhance hydration at the hyphal-root cell interface and consequent apoplastic water transport. A direct relationship between overexpression of aquaporin gene in mycorrhiza *Laccaria bicolor* and enhancement of root hydraulic conductance of its host *Picea glauca* has proved fungal aquaporin's ability to regulate host plant water status (Xu 2015).

Photosynthesis

Photosynthesis is a crucial physiological process in plants; any alteration in its rate has direct implications in stress tolerance ability including temperature-related stress (Wahid et al. 2007). AM plants under temperature stress have been reported to exhibit a higher net photosynthetic rate than their non-AM counterparts (Ruotsalainen and Kytöviita 2004; Zhu et al. 2010a, 2011, 2015). Temperature stress suppresses chlorophyll biosynthesis or promotes chlorophyll degradation resulting in the reduction of chlorophyll concentration in the plants. AM plants have been reported to exhibit higher chlorophyll concentration as compared to their non-AM counterparts under temperatures stress (Paradis et al. 1995; Zhu et al. 2010a, 2011). These findings imply that AMF inoculation alleviates temperature stress-related reduction in chlorophyll synthesis or degradation of chloroplasts, thereby maintaining the photosynthetic rate (Evelin et al. 2009). In another report AM maize has been found to have higher carotenoid concentration compared to non-AM maize (Zhu et al. 2011). Carotenoid is known to add light-harvesting during photosynthesis and also helps photoprotection of chloroplast (Young 1991). AMF inoculation in maize thus adds to stabilization of membrane lipid of the thylakoid and photoprotection of chloroplast (Karim et al. 1999; Wahid et al. 2007). Another target of temperature stress injury in chloroplast has been reported to be the oxygen-evolving complex of photosystem II (Allakhverdiev et al. 2008). Two indicators viz., the ratio of Fv/maximum fluorescence (Fm) (maximum quantum efficiency of PSII primary photochemistry) and of Fv/primary fluorescence (Fo) (potential photochemical efficiency of PSII) have been used to evaluate the damage caused by environmental stresses (Krause and Weis 1991; Maxwell and Johnson 2000). AM maize has been reported to exhibit higher Fv/Fm and Fv/Fo as compared to non-AM maize plants under temperature stresses (Zhu et al. 2010a, 2011). The finding

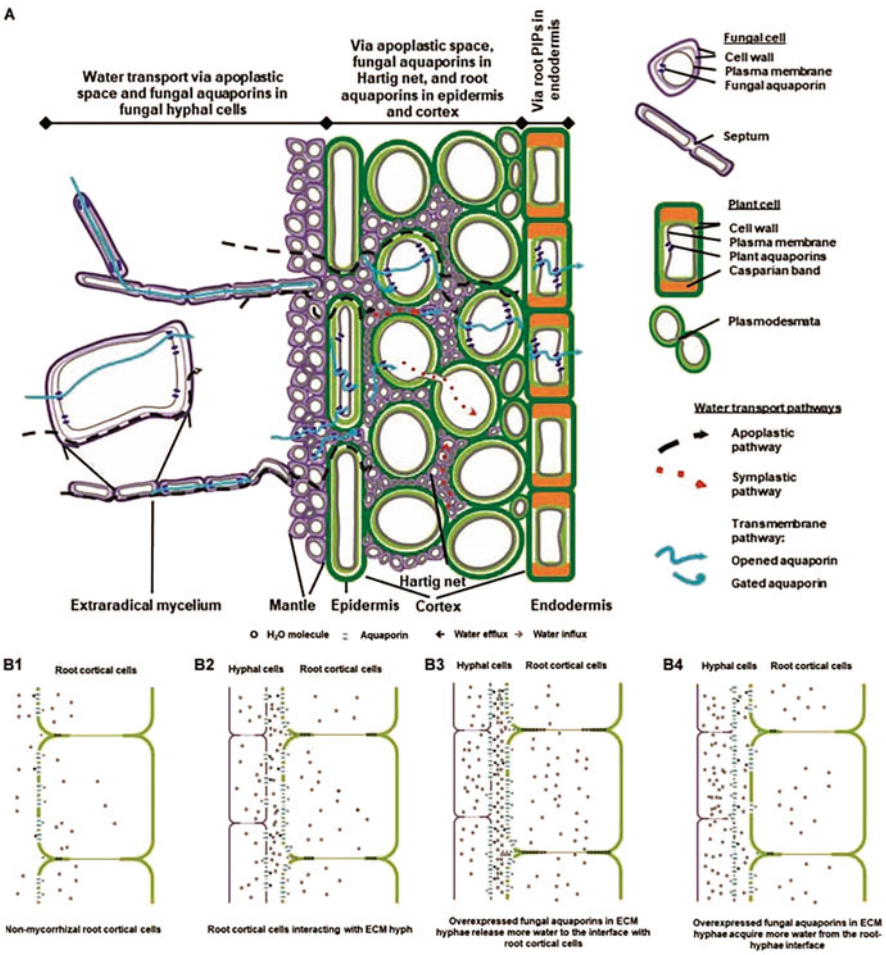

Fig. 25.2 Schematic representation of water transport pathways and aquaporin participation in ECM associated plant (Xu and Zwiazek 2020). (**a**) ECM–root interaction as originally given earlier (Steudle and Peterson 1998; Lehto and Zwiazek 2011). (**b**) Fungal and plant aquaporins at the hypha–root interface participating in water transport activity. (B1) Water transport pathway involving apoplast and cell–cell route in a non-ECM associated root; (B2) Water transport in ECM associated root highlighting changes in normal water transport pathway and enhancement in rate of hydration due to involvement of mycorrhizal hyphae and aquaporins; (B3) Enhancement of hydration rate as a result of upregulation of fungal aquaporins; (B4) Upregulated fungal aquaporins may alternatively lead to acquisition of more water by fungal hyphae. (Reproduced with permission from author)

suggests that AMF protects the PSII reaction center and the chloroplast from the damaging impact of temperature stress. Temperature stress may thus affect the reaction center of PSII, the number of open PSII units, PSII photochemical reaction, and electron transport in chloroplast negatively (Camejo et al. 2005; Baker 2008).

AM symbiosis can mitigate all these unfavorable effects of temperature stress on the PSII reaction center which in turn improves the efficiency of PSII photochemistry and photosynthetic ability of chloroplast. Contrasting reports, however, show that in wheat (*Triticum aestivum*) AM symbiosis has no impact on electron transfer, the quantum yield, and non-photochemical quenching under high-temperature condition (Cabral et al. 2016). Similarly, AMF inoculation could not cause any impact on the photosynthetic rate in the citrus plants (Wu and Zou 2010) under low-temperature stress. Charest et al. (1993) also found that the chlorophyll concentration of AM maize plants was lower than the non-AM plants.

Salt Stress Tolerance

Salt tolerance is carried out via modulation of several physiological activities such as reduction in lipid peroxidation, enhancement of proline accumulation, NHX1 antiporter gene expression, and ecophysiological performance. AMF inoculation in maize plants with three native AMF was reported to cause photosystem II enhancement and stomatal conductance, reduce ROS production, the oxidative damage to lipids, and the membrane electrolyte leakage (Estrada et al. 2013), and also reduce malondialdehyde (MDA)/electrolyte leakage in leaves (Wang et al. 2020).

AMF and Abiotic Stress

In saline–sodic soil the density of AMF was generally found low and represented by *Glomus*, *Scutellospora*, *Acaulospora*, *Sclerocystis*, and *Gigaspora*, *Acaulospora* and *Glomus* in the rhizosphere of maize, *Scutellospora* and *Glomus* in the rhizosphere of tulsi, onion, and rice, *Glomus*, and *Sclerocystis* in the rhizosphere of guava, and *Gigaspora* and *Glomus* in the rhizosphere of bamboo (Srimathi Priya et al. 2014). AMF were found to be low in diversity in saline–alkaline soil of Yellow River Delta, species richness being even lower in winter (Wang and Liu 2001). Salinity has been found to be inhibitory to AMF (Aliasgharzad et al. 2001). AMF have also been found in very low density in the upper Arctic (Kytöviita 2005) and Antarctica soils (Barbosa et al. 2017). Thus although AMF confer stress tolerance on symbiont plants, they are less tolerant to various abiotic stresses.

AMF are known to impart tolerance to temperature stresses in plants (Duhamel and Vandenkoornhuyse 2013). They help alter plant physiology toward withstanding stress conditions (Miransari et al. 2008). Thus under high- and low-temperature stress, AM plants perform better than non-AM plants showing higher shoot and root dry weights (Zhu et al. 2010b; Latef and Chaoxing 2011; Liu et al. 2014a). AMF have been reported to enhance leaf and root number, crown diameter, and leaf area in strawberry (*Fragaria ananassa*) (Matsubara et al. 2004) and root length and root diameter in *Dichanthelium lanuginosum* (Bunn et al. 2009). Similarly, a 1.53 °C increase in temperature enhances seed number and plant biomass in *Medicago truncatula* colonized by *Rhizophagus irregularis* (Hu et al. 2015). The ability to perform better under temperature stresses has partially been attributed to AMF's contribution to enhancing hosts' photosynthesis and nutrient uptake (especially phosphate).

Contrasting reports are also available that show that AM symbiosis causes no or negative growth enhancement in host plants under temperature stress. For example, AM maize showed similar shoot and root dry weights to non-AM maize under temperature stress (Zhu et al. 2015), while under high-temperature stress AM cyclamen (*Cyclamen persicum*) showed lower root and tuber dry weights as compared to non-AM plants (Maya and Matsubara 2013). The reasons ascribed to these discrepancies are the possible underperformance of AMF in nutrition mobilization and/or the high carbon cost–benefit ratio of AMF with the host plants (Martin and Stutz 2004; Chen et al. 2014).

Endophytes and Abiotic Stress

Endophytic fungi occupy the inner tissue of plants deriving food and protection from the host and in return bestow upon the host an enhanced ability to resist various biotic and abiotic stresses (Saikkonen et al. 1998). They may spend the whole or part of their lifecycle in the host without causing any infection (Tan and Zou 2001).

The endophytic fungus *Curvularia crepinii* isolated from the roots of *Hedyotis diffusa* growing in the geothermal ecosystems of Southwest China has been reported to improve thermotolerance of host plants significantly under laboratory conditions. The endophyte-treated plant showed a lower death rate as compared to non-treated plants under thermal stress conditions (Zhou et al. 2015). *Thermomyces* sp., a thermophilic endophytic (CpE) fungus, isolated from *Cullen plicata* (a desert plant) roots was found to assist cucumber plants in heat stress tolerance grown in the summer season (Ali et al. 2018). The fungal inoculants could alleviate heat stress-related adverse effects in this plant by avoiding alteration in the PSII quantum efficiency, photosynthesis rate, water use efficiency, and root length vis-à-vis noninoculated plants. The inoculated plant also showed a higher amount of total sugars, flavonoids, saponins, soluble proteins, and antioxidant enzyme activities (Ali et al. 2018).

The role of introduced salt-tolerant Antarctica microorganisms (endosymbiotic fungus) has been tested in causing overall growth promotion of four plants under salt stress viz., tomato, onion, cayenne, and lettuce (Acuña-Rodríguez et al. 2019). The following four types of treatments were used: (a) uninoculated plants without saline stress (control), (b) uninoculated plants with saline stress (200 mM NaCl) (control), (c) plants inoculated with the microorganism consortium with no saline stress, and (d) inoculated plants subjected to saline stress. The inoculated plants were found in general to show improved survival in presence of salt stress as compared to uninoculated plants. Although saline stress caused a negative impact on all the selected measurable traits (lipid peroxidation, proline accumulation, expression of NHX1 antiporter gene, and ecophysiological performance) of inoculated and uninoculated plants, impact was significantly less in inoculated plants. In salt stress conditions, the ecophysiological performance, proline accumulation, and NHX1 expression were found to higher while lipid peroxidation to be lower in inoculated plants vis-à-vis uninoculated ones. Overall, the biomass of inoculated plants under normal and salt stress conditions was found to be the same indicating the salt stress alleviating effect of inoculants (Acuña-Rodríguez et al. 2019). The two fungal endophytes from

Antarctica viz., *Penicillium brevicompactum* and *P. chrysogenum* have been reported to improve sequestration of Na^+ in vacuoles in case of tomato and lettuce plants by upregulating the expression of vacuolar NHX1 Na^+/H^+ antiporters apart from causing a positive impact on nutrients availability, net photosynthesis, water use efficiency, yield, and survival under salt stress conditions (Molina-Montenegro et al. 2020).

Endophytic fungi have also been reported to impart UV protection in the Antarctica plant *Colobanthus quitensis* (Barrera et al. 2020). The fungal inoculant reduced lipid peroxidation and improved photosynthesis efficiency in the presence of high UV-B radiation conditions. Analysis at the molecular level revealed that the inoculants reduced *CqUVR8*, *CqHY5*, and *CqFLS* transcript levels, and enhanced (eightfold) the content of quercetin, a ROS-scavenger flavonoid, in leaves under high UV-B after 48 h of treatment (Barrera et al. 2020).

Endophytic fungi from the plants of desert areas have also been found to help symbiont tolerating abiotic stress, such as heat and drought. For example, thermotolerant endophytic fungal species *Thermomyces lanuginosus* has been reported to equip the desert plant *Cullen plicata* Delile with the ability to show a higher growth rate and resistance to drought and heat stress (Ali et al. 2019). The fungus *Piriformospora indica*, originally isolated from the spore of *Glomus mosseae* in the rhizosphere xerophytic plants (Verma et al. 1998), can colonize root of broad host plants belonging to monocotyledons and dicotyledons and confer tolerance to abiotic (drought, low and high temperatures, salinity, and heavy metals) and biotic (root and foliar pathogens) stresses (Johnson et al. 2014).

Some reports tell that plants in various extreme climates harbor fungal endophytes capable of conferring tolerance to such climates related stresses (Rodriguez and Redman 2008). Thus grass species *Leymus mollis* (dunegrass) from coastal habitat is home to symbiotic fungal endophytes *F. culmorum* that can confer salinity tolerance to the host plants (Rodriguez and Redman 2008). The endophytic fungi from halotolerant *Ipomeapes-caprae* LR Br have been found to impart salinity tolerance in *Oryza sativa* L. (Manasa et al. 2020). The grass, *Dichanthelium lanuginosum*, from geothermal soils in Yellowstone National Park harbors endophyte, *Curvularia protuberate*, which provides heat tolerance to the host plant (Redman et al. 2002). In this case, it was later found that the heat tolerance was indeed provided by the virus inhabited by the fungus (Marquez et al. 2007). The habitat-specific endophytes were found to exhibit a broad host range and thus very important for managing plants under stressful climates. The habitat-specific fungal endophytes (*F. culmorum* and *Cu. protuberata*) have been used to inoculate two commercial rice varieties to inculcate salt and drought tolerance in them (Redman et al. 2011). The inoculated plants were found to develop salt and drought tolerance, exhibit water consumption reduced by 20–30%, and growth and yield increased significantly (Redman et al. 2011).

25.2.2.2 Biotic Stress

Fungi also regulate plant pathogens in the soil (Frąc et al. 2015). Mycoparasitic fungi that can derive nutrition from other live fungi are considered to be important for such

regulation. Mycoparasitic fungi may be contact necrotroph [when there is no penetration of host mycelium but host mycelium may lyse], invasive nectrotroph [when host mycelium is penetrated followed by degeneration of cytoplasm e.g., Chytrids and members of the Oomycota, including *Spizellomyces* and *Pythium*-like fungi, and, *Fusarium* spp., *Gliomastix* spp. (both Ascomycota) and *Mortierella ramanniana* (Mortierellomycotina) which have been reported from dead and dying spores of *AMF*], intracellular biotroph [when complete mycoparasite mycelium is present in the host and host cytoplasm remains healthy], houstorial biotroph [when only haustoria are sent inside host mycelium and host cytoplasm remains healthy e.g., parasitic species of *Piptocephalis* (Zoopagomycotina) and *Dimargaris* (Kickxellomycotina) growing on living saprotrophic hosts such as *Pilobolus*, *Pilaira* and *Phycomyces (Mucoromycotina)*], and fusion biotroph [when mycoparasite and host mycelium are closely appressed often former's hyphae coil around latter's one and cytoplasmic contact is ensured via micropore(s) or short penetrative hyphal branch] (Jeffries 1995). *Nematode trapping fungi such as Arthrobotrys* has been found to attack not only nematodes but also fungus (Nordbring-Hertz 2004). The species of *Trichoderma* have special significance in their role as competitive saprotrophs, opportunistic mycoparasite, and possibly plants' symbionts (Harman et al. 2004). *Trichoderma viride,* for example, attacks *Rhizoctonia solani* (damping off fungus) and *Armillaria* and *Armillariella* (trees' pathogen). There are strains of *Trichoderma* and *Gliocladium* developed to serve as biocontrol for crop pathogens. Mycoparasitic fungi such as *Trichoderma* sp. play a special role in controlling pathogenic fungi in the soil (Dawidziuk et al. 2016). Soil health is crucial for sustainable agriculture (Cardoso et al. 2013). The antagonistic fungi (*Glomus* spp. and *Trichoderma* spp.) are recommended to be used to inhibit fungal pathogens (Dawidziuk et al. 2016). The *Trichoderma* species (*T. asperellum, T. atroviride, T. harzianum, T. virens*, and *T. viride*), for example, are recommended as biocontrol in horticulture (López-Bucio et al. 2015). The species of *Trichoderma* have been reported from various cold areas (Sahay 2021) and Mediterranean sponges (Gal-Hemed et al. 2011). Apart from these psychrotolerant and halotolerant strains, thermo cum halotolerant strains *T. asperellum*, TaDOR673 and *T. asperellum*, TaDOR7316 have also been isolated (Poosapati et al. 2014) implying their applicability as a biocontrol in extremophilic areas.

The role of endophytes and their volatile products in controlling plant pathogens have especially been studied. The fungal endophyte, for example, *Epicoccum nigrum* from sugarcane and *Colletotrichum gloeosporioides* and *Clonostachys rosea* from cacao plant (*Theobroma cacao*) antagonize several pathogens (de Lima Favaro et al. 2012; Mejia et al. 2008). The inoculation of *Pi. indica* has been demonstrated to confer resistance to various root diseases in the case of barley (Waller et al. 2005), maize (Kumar et al. 2009), tomato (Fakhro et al. 2010), and wheat (Rabiey et al. 2015). The fungus has been reported to repress even viral (Pepino Mosaic Virus) infection (Fakhro et al. 2010) (Table 25.1).

The secondary metabolites from sugarcane endophyte *E. nigrum* (Brown et al. 1987) such as epicorazines A–B (Baute et al. 1978) and flavipin (Bamford et al. 1961) have been associated with *E. nigrum* biocontrol activity (Brown et al. 1987;

Table 25.1 Antimicrobial compounds and their targets obtained from endophytic fungi

Endophyte	Source plant	Bioactive compound	Target pathogen	Host	Reference
Piriformospora indica	*Glomus mosseae* (spore)	–	*Fusarium culmorum*	Barley	Waller et al. (2005)
				Wheat	Serfling et al. (2007)
			F. graminearum	Barley	Deshmukh and Kogel (2007)
			F. verticillioides	Maize	Kumar et al. (2009)
			F. oxysporum	Tomato	Qiang et al. (2012)
			Verticillium dahliae	Tomato	Fakhro et al. (2010)
			Rhizoctonia solani	Barley	Qiang et al. (2012)
			Blumeria graminis f. sp. hordei	Barley	Waller et al. (2005)
			B. graminis f. sp. tritici	Wheat	Serfling et al. (2007)
			Pepino mosaic virus	Tomato	Fakhro et al. (2010)
Epicoccum nigrum	Sugarcane	Flavipin Epicorazines A–B	*Sclerotinia sclerotiorum*	Sunflower	
			Pythium	Cotton	
			Phytoplasma	Apple	
			Monilinia spp.	Peaches	de Lima Favaro et al. (2012)
				Nectarines	Brown et al. (1987)
P. brevicompactum	Barley		*Cochiolobus*, *fusarium*	Barley	
			Pyrenophora and *Rhynchosporium*		Murphy et al. (2015)
Colletotrichum sp.,	*Artemisia annua*	Colletonoic acid	Bacteria and fungi		Hussain et al. (2014) and Bills et al. (2002)
Co. gloeosporioides	*Theobroma cacao*	–	*Phytophthora palmivora*	Cacao	Mejia et al. (2008)

Do	*A. mongolica*	Colletotric acid	Bacteria and fungi		
			Helminthosporium sativum		Zou et al. (2000)
Clonostachys rosea	Do		*Moniliophthora roreri*	Do	Do
Pestalatiopsis jester	–	Jesterone	Anti-oomycete activity		Li and Strobel (2001)
Pe. microspora	*Terminalia morobensis*		Anti-microbial activity		Strobel et al. (2002) and Strobel and Daisy (2003)
Pe. microspora	Rain forest's tree	Ambuic acid	*Fusarium* spp. and *Pythium ultimum*		Li et al. (2001)
Cordyceps dipterigena		Cordycepsidone A	*Gibberella fujikuroi*		Varughese et al. (2012)
Cryptosporiopsis quercina, hardwood		Cryptocandin	*Sclerotinia sclerotiorum, Botrytis cinerea*		Strobel et al. (1999)
Do	Do	Cryptocin	*Pyricularia oryzae*		
			Fusarium oxysporum		
			Geotrichum candidum,		
			Rhizoctonia solarium		
			S. sclerotiorum, *Py. ultimum*,		
			Phytophthora cinnamon and *Ph. citrophthora*		Li et al. (2000)
Phomopsis sp.		Phomopsichalasin	*Bacillus subtilis*, *Salmonella enterica*, and *Staphylococcus aureus*		Horn et al. (1995)
Muscodor albus	*Cinnamomum zeylanicum*	28 volatile compounds	Bacteria and fungi		Worapong et al. (2001) and Strobel et al. (2001)
Muscodor crispans	*Ananas ananassoides*	Mixed volatile compounds	Bacteria and fungi		
			Pythium ultimum		

(continued)

Table 25.1 (continued)

Endophyte	Source plant	Bioactive compound	Target pathogen	Host	Reference
			Alternaria helianthi,		
			Botrytis cinerea,		
			Fusarium culmorum,		
			F. oxysporum,		
			Phytophtora cinnamomi,		
			Ph. palmivora,		
			Rhizoctonia solani,		
			Sc. Sclerotiorum, and		
			Verticillium dahlia		
			Xanthomonas axonopodis pv. *citri*		Mitchell et al. (2010)
Daldinia concentrica	Olive tree	27 volatiles	*Aspergillus niger*	Peanuts	
			Penicillium digitatum		
			Botrytis cinerea, etc. 12 fungi belonging to asco, Basiodio, and oomycetes and post-harvest fungi of plums, apricot, and resin		Liarzi et al. (2016)

Table 25.2 Insecticidal compounds /insect repellants from endophytes and their target insects

Endophyte	Source plant	Bioactive compound	Target insect	Reference
Epichloë endophytes	Ryegrass	Peramine	Argentine stem weevil	Johnson et al. (2013)
E. endophytes R-37		Epoxy-janthitrems	Broad pesticidal effect	Finch et al. (2012)
Beauveria bassiana			Coffee berry borer	Vega et al. (2010)
Clonostachys rosea	Do			
Nodulisporium sp.	*Bontia daphnoides*	Nodulisporic acids	Larvae of the blowfly	Demain (2000)
Muscodor vitigenus	Lianas	Naphthalene	General insect repellent *Cephus cintus*	Daisy et al. (2002)
Be. bassiana				
Clonostachys rosea			Coffee berry borer	Vega et al. (2010)
Curvularia lunata	*Melia azedaracht*		Instar larvae of the cotton leaf worm	Saad et al. (2019)

Madrigal et al. 1991; Madrigal and Melgarejo 1995). The secondary metabolite ambuic acid from the endophytic fungus *Pestalotiopsis microspora* shows inhibitory activity against *Fusarium* and *Pythium ultimum* (Li et al. 2001) and Colletonoic acid (Bills et al. 2002) from *Colletotrichum* sp., against many bacteria, fungi, and algae activities (Hussain et al. 2014). Other important secondary metabolites from endofungus *Cryptosporiopsis quercina* such as Cryptocandin inhibits many phytopathogenic fungi, e.g., *Sclerotinia sclerotiorum* and *Botrytis cinerea* (Strobel et al. 1999) and Cryptocin (Li et al. 2000) antagonizes phytogens *Pyricularia oryzae*, *F. oxysporum*, *Geotrichum candidum*, *Rhizoctonia solarum*, *S. sclerotiorum*, *Pythium ultimum*, *Phytophthora cinnamon*, and *Ph. citrophthora* (Li et al. 2000) (Table 25.1).

Furthermore, volatile compounds (chemically alcohols, esters, ketones, acids, and lipids) from the endophytic fungus *Muscodor albus* isolated from cinnamon (*Cinnamomum zeylanicum*) plant (Worapong et al. 2001), *M. crispans* isolated from pineapple (*Ananas ananassoides*), and *Daldinia concentric* from olive tree are inhibitory to many of the phytopathogenic fungi. The antimicrobial volatile compounds from *M. crispans* have additional advantages, i.e., they are harmless and can be used as safe antimicrobials in food and agriculture (Mitchell et al. 2010) (Table 25.1).

Endophytes also generate compounds that are insecticidal or insect repellant (Table 25.2). Apart from these, entomopathogenic fungal endophytes have also been reported to have potential activity against insect pests of important crops reducing their survival and/or reproduction (Table 25.3).

Losses during storage of fruits, vegetables, and grains as caused by fungal pathogens such as *Alternaria*, *Aspergillus*, *Botrytis*, *Fusarium*, *Geotrichum*,

Table 25.3 Entomopathogenic fungal endophytes as potential biocontrol against insect pests of important crops (adapted from Mantzoukas and Eliopoulos 2020)

Endophytic species	Crop	Target pest	Activities	Reference
Beauveria. bassiana	Coffee	*Hypothenemus hampei*	Pathogenize	Vega et al. (2008)
	Maize	*Ostrinia nubilalis*	Reduce pest population	Bing and Lewis (1991)
		Sesamia calamistis	Reduce larval tunneling	Cherry et al. (2004)
	Banana	*Cosmopolites sordidus*	Reduce larval survival	Akello et al. (2008)
	Tomato	*Helicoverpa zea*	Hardly affect larval mortality	Powell et al. (2009)
		Helicoverpa armigera	Reduce infestation	Qayyum et al. (2015)
	Sorghum	*Chilo partellus*	Reduce larval tunneling	Reddy et al. (2009)
		Sesamia nonagrioides	Reduce infestation	Mantzoukas et al. (2015) and Mantzoukas and Grammatikopoulos (2019)
	Opium poppy	*Iraella luteipes*	Reduce larval survival	Quesada-Moraga et al. (2009)
	Cotton	*Aphis gossypii*	Reduce reproduction	Castillo-Lopez et al. (2014)
		Chortoicetes terminifera	Reduce growth rate	Gurulingappa et al. (2010)
		Rachiplusia nu	Reduce larval feeding	Russo et al. (2019)
	Melon	*Aphis gossypii*	Reduce reproduction	
			No effect on natural enemies	González-Mas et al. (2019)
	Fava bean	*Helicoverpa armigera*	Reduce larval survival	Vidal and Jaber (2015)
		Liriomyza huidobrensis	Reduce pest population	Akutse et al. (2013)
		Acyrthosiphon pisum	Reduce pest population	Akello and Sikora (2012)

(continued)

Table 25.3 (continued)

Endophytic species	Crop	Target pest	Activities	Reference
	Common bean	*Helicoverpa armigera*	Reduce larval survival	Vidal and Jaber (2015)
		Liriomyza huidobrensis	Reduce pest population	Akutse et al. (2013)
	White jute	*Apion corchori*	Reduce infestation	Biswas et al. (2013)
	Soybean	*Aphis glycines*	Hardly affect pest population	Clifton et al. (2018)
		Helicoverpa gelotopoeon	Decrease larval feeding	Russo et al. (2018)
	Grapevine	*Planococcus ficus*	Reduce infestation	Rondot and Reineke (2018)
		Empoasca vitis	Reduce infestation	Do
	Pepper	*Myzus persicae*	Increase pest mortality	Mantzoukas and Lagogiannis (2019)
			Reduce development and fecundity	Jaber and Araj (2018)
	Strawberry	*Myzus persicae*	Reduce feeding	Manoussopoulos et al. (2019)
	Cauliflower	*Bemisia tabaci*	Reduce pest survival	Jaber et al. (2018)
	Pecan	*Melanocallis caryaefoliae*	Reduce pest population	Ramakuwela et al. (2020)
		Monellia caryella	Do	
	Lemon	*Diaphorina citri*	Reduce reproduction and survival	Peña-Peña et al. (2015)
Lecanicillium lecanii	Cotton	*Aphis gossypii*	Reduce reproduction	Gurulingappa et al. (2010)
Lecanicillium muscarium	Cauliflower	*Plutella xylostella*	Increase larval mortality	Kuchár et al. (2019)
Aspergillus parasiticus	Cotton	*Chortoicetes terminifera*	Reduce growth rate	Gurulingappa et al. (2010)
Metarhizium anisopliae	Fava bean	*Acyrthosiphon pisum*	Hardly pest population	Akello and Sikora (2012)
	Pepper	*Myzus persicae*	Increase pest mortality	Mantzoukas and Lagogiannis (2019)

(continued)

Table 25.3 (continued)

Endophytic species	Crop	Target pest	Activities	Reference
	Rapeseed	*Aphis fabae*	Reduce larval survival	Batta (2013)
		Plutella xylostella	Reduce larval survival	Batta (2013)
	Strawberry	*Myzus persicae*	Reduce feeding	Manoussopoulos et al. (2019)
Metarhizium brunneum	Soybean	*Aphis glycines*	Increase pest population	Clifton et al. (2018)
	Cauliflower	*Bemisia tabaci*	Reduce pest survival	Jaber et al. (2018)
	Melon	*Aphis gossypii*	Reduce reproduction & survival	González-Mas et al. (2019)
Metarhizium robertsii	Sorghum	*Sesamia nonagrioides*	Reduce infestation	Mantzoukas and Grammatikopoulos (2019)
Clonostachys rosea	Coffee	*Hypothenemus hampei*	Pathogenize	Vega et al. (2008)
Purpureocillium lilacinum	Cotton	*Aphis gossypii*	Reduce reproduction	Castillo-Lopez et al. (2014)
Isaria fumosorosea	Sorghum	*Sesamia nonagrioides*	Reduce infestation	Mantzoukas and Grammatikopoulos (2019)
	Pepper	*Myzus persicae*	Increase pest mortality	Mantzoukas and Lagogiannis (2019)
	Lemon	*Diaphorina citri*	Reduce reproduction and survival	Peña-Peña et al. (2015)

Gloeosporium, *Mucor*, *Monilinia*, *Penicillium*, *Rhizopus*, and other genera called postharvest loss are significant. There is a worldwide concern to reduce it. Some of the recommended practices to control postharvest losses include avoiding injury during harvesting and handling, sanitation practices, and application of fungicides. But all these are either insufficient or unsustainable (Pimenta et al. 2009). One of the promising alternatives proposed is to use antagonistic microbes such as bacteria, fungi, and yeasts (Schisler et al. 2011). Among these microbes yeasts have been found more suitable for their inherent features such as ability to establish on the wounds, fast scavenge sugary exudates making the wound unsuitable for forthcoming pathogens, survive at a low temperature, acidic pH, and conditions of osmotic stress, resist desiccation, and tolerate to chemicals. Moreover, the ease of their cultivation, fast-growth, and low input requirements make them attractive for biocontrol formulations (Schisler et al. 2011). Yeasts that could reduce postharvest

pathogens of various crops have been reported as follows: *Au. pullulans* in cherry fruit, *Au. pullulans* and *Rhodotorula glutinis* in apple fruit, *Candida sake* in strawberry fruit, *Cryptococcus albidus* in pear fruit, *Cr. laurentii*, *Rh. glutinis* in mango fruit, etc. (Schisler et al. 2011).

The psychrotrophic yeast strains isolated from *Nothofagus* sp. and *Vaccinium* sp. in Northwest-Patagonia have been found to exhibit an inhibitory effect on some phytopathogen like *Verticillium dahliae* PPRI5569 and *Pythium aphanidermatum* PPRI 9009, but could not affect the growth of *F. oxysporum* PPRI5457 (Mestre et al. 2016). Marine yeasts (*Debaryomyces hansenii* and *Rhodotorula minuta*) in combination with ClO_2 were found to control postharvest anthracnose (*Co. gloeosporioides*) of mango fruits (Reyes-Perez et al. 2019).

Chitinase as Biocontrol

The enzyme chitinase has also been studied as a potential tool to manage pathogenic fungi. Of the seven chitinases purified from the thermophilic fungus, *Thermomyces lanuginosus* SSBP (Chit1), six have been found to inhibit *Aspergillus niger*, *A. flavus*, *A. alliaceus*, *A. ochraceus*, *F. verticillioides*, and *Mucor* sp. (Okongo et al. 2019). An exochitinase (MtChit) purified from thermophilic mold *Myceliopthora thermophila* was found to inhibit the growth of plant pathogenic fungi such as *F. oxysporum* and *Cu. lunata*. The enzyme is acid and organic acid solvent tolerant and thermostable that could be produced in heterologous system *Pichia pastoris* in high titers expressed from GAP promoter (Dua et al. 2017).

Cold active chitinases from Antarctica fungus *Lecanicillium muscarium* CCFEE 5003 have also been found to be very effective antimycotic molecules that could be used to control pathogenic fungi including oomycetous one in a cold environment. Production of this enzyme has been optimized at the bench-top bioreactor level (Fenice 2016).

Antifungal Protein (AFuP) as Biocontrol

AFuPs are small, cysteine-rich, and cationic proteins that inhibit many of the species of fungi (Lee et al. 1999). Genome mining has revealed that fungi harbor many AFuP-like sequences falling in four major classes viz., A, B, C, and the newer ones (Tóth et al. 2016; Garrigues et al. 2018). Various fungi produce more than one type of any or many of these classes of AFuPs. The genome of fungus *P. chrysogenum*, for example, codes for one AFuP of each of the three classes (classes A, B, and C) while that of *Penicillium digitatum* only one (class B) (Garrigues et al. 2017) and *Neosartorya fischeri* one each of two AFuPs (classes A and C).

The fungal species *P. chrysogenum* (Khan et al. 2020; Leitão et al. 2012), *P. brevicompactum* (Zhang et al. 2018), and *Monascus pilosus* (Zheng et al. 2021) which have extremophilic strains produce AFuPs. The representatives of class B AFuP is produced by *P. chrysogenum* (Huber et al. 2018) and *Mo. pilosus* (Tu et al. 2016), and that of class C, i.e., the BP protein, is produced by *P. brevicompactum* (Seibold et al. 2011) and the Pc-Arctin is produced by *P. chrysogenum* (Chen et al. 2013). AFuPs such as PAF and PAFB from *P. chrysogenum* were also found to be active against human pathogenic yeasts and molds such as *A. fumigates*, *A. terreus*,

and *Trichophyton rubrum* (Huber et al. 2018). *P. expansum* codes for A, B, and C AfuP of which A type one (PeAFPs) was found to have efficient activity against fungal infections caused by *Botrytis cinerea* in tomato leaves and *P. digitatum* in oranges (Garrigues et al. 2018).

The psychrotolerant and osmotolerant yeast Starmerella bacillaris (Nadai et al. 2018) isolated from overripe and botrytized grape berries have been reported to exhibit antifungal activity against *Botrytis cinerea*, a wine degrader and *P. expansum* causing postharvest rot in apples (Lemos et al. 2016).

25.2.3 Crop Improvement

25.2.3.1 AFP Coding Gene for Crop Improvement

Antifreeze protein (AFP) and glycoprotein (AFGP) are stress proteins that protect the organisms from freezing injury. They do so by reducing the freezing point by the adsorption–prevention process. They have been isolated from bacteria, fungi, polar fishes, insects, and plants. AFPs bind the growing ice surface, give it a specific shape, and discourage its further enlargement. Besides, AFPs stop the fusion of ice crystals forming large aggregates that may be fatal to cells. AFPs lower the freezing point but not the melting point, the process being called thermal hysteresis or TH. TH exhibited by plants is mild TH (0.1–0.5 °C), by fishes is moderate TH (1–5 °C), and by insects is high (5 °C). TH of yeast *Glaciozyma* sp. *AY30* is ~0.42 °C falling in the mild range (Chakraborty and Jana 2019).

Both AFP and AFGP have been reported to be useful in imparting cold tolerance to plants (e.g., tobacco, tomato, and potato) (Hightower et al. 1991; Fan et al. 2002; Gupta and Deswal 2014). Thus obtained transgenic plants exhibit different psychrotolerant phenotypes. For example, transgenic tobacco carrying carrot AFP gene *DcAFP* exhibited retardation in ice recrystallization, enhancement of TH from 0.35 to 0.56 °C, reduction in ion leakage, and faster recovery from cold stress (Fan et al. 2002). Likewise, AFP transgene from *Lolium perenne* in transgenic tomato showed three times more relative water content and 2.6 times less electrolyte leakage (cf wild type tomato plant) (Balamurugan et al. 2018) and in transgenic *Arabidopsis* and *E. coli* enhanced cold hardiness (Zhang et al. 2010). In another report, *Arabidopsis* plants carrying transgene LpIRI-a/b exhibited a survival rate of 85–100% (cf 73% shown by wild type) when exposed to cold at −4 °C for 7 days (Zhang et al. 2010). AFP transgenic plants have been found in general to exhibit retardation in ice recrystallization, production of TH (Griffith and Yaish 2004), and enhancement of general cold hardiness (Gupta and Deswal 2014). In one report chimeric AFP gene (spa-afa-5) has been found to exhibit 10 times more effect than the original AFP gene (afa-3) (Hightower et al. 1991) in a transgenic tomato plant, afa-3 being the AFP III from winter flounder and spa being staphylococcal protein-A.

Ice binding proteins (AFP) have been isolated and studied from psychrophilic fungi *Antarctomyces pellizariae* and *A. psychrotrophicus* (Thelebolales) of Ascomycetes (Batista et al. 2020) and *Typhula ishikariensis* (Kondo et al. 2012),

yeasts *Goffeauzyma gastric* (Villarreal et al. 2018), *Leucosporidium* sp. (Lee et al. 2012), and *Glaciozyma antarctica* (Hashim et al. 2013). Although THs of plant and yeast AFPs are almost parallel, plant AFPs have been used to test their ability to confer cold-hardiness in mesophilic plants (Gupta and Deswal 2014). Yeast/fungal AFP is yet to be tested and applied to impart cold tolerance to plants.

25.2.3.2 Chitinase Coding Genes and Crop Improvement

The transgenic potato and tobacco-containing endochitinase transgene from *T. harzianum* introduced by *Agrobacterium tumefacience* mediated transformation have been found to exhibit tolerance to complete resistance to many phytogenic fungi such as *Alternaria alternate*, *A. solani*, *B. cinerea*, and *R. solani* (Lorito et al. 1998). Endochitinase gene from *T. virens* has also been found to impart resistance in transgenic cotton and tobacco against soil-borne fungus *Rhizoctonia solani* and a foliar pathogen, *Alternaria alternate* (Emani et al. 2003).

25.2.3.3 AFuP Coding Gene for Crop Improvement

Antifungal protein-coding genes from *A. giganteus* have been expressed from the promoters of three maize pathogenesis-related (PR) genes ZmPR4, mpi, and, PRms in transgenic rice, and their impact was assessed against the most devastating fungal pathogen *Magnaporthe grisea* causing rice blast disease. The transgenic rice plants showed resistance to *M. grisea* at various levels indicating the effectiveness of the strategy (Moreno et al. 2005). Recently, the antifungal protein gene PeafpA from *P. expansum* has been cloned and expressed using *P. chrysogenum* based expression cassette pSK275 and its antifungal activities have been tested (Garrigues et al. 2018). Both *P. chrysogenum* and *P. expansum* are found in Antarctica soil (Sahay 2021). This result shows potential for its use to engineer phytopathogenic fungal-resistant transgenic crops.

Although the PeAFPA has not shown hemolytic activity, it has to face other biosafety and public acceptance tests.

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Extremophilic Fungi for the Synthesis of Nanomolecules

26

Harshita Shukla and Shyamji Shukla

Abstract

Since the past few decades, metal nanoparticles have attracted enormous attention in the scientific world as a result of their anti-cancer, catalytic, optical, electronic, and magnetic properties. Conventionally, the synthesis of metal nanoparticles is carried out by diverse physical and chemical methods. But these conventional methods are accompanied with a number of drawbacks, viz. elevated energy consumption, increased cost, and the use of toxic chemical substances. The application of various microbial species for biological synthesis of nanoparticles has recently presented an alternative synthetic platform in an eco-friendly and cost-effective way. Extremophiles are one of the most unexplored groups of organisms. Even though environment has provided enormous strength to all living organisms for their survival, sometimes under extreme environmental conditions, viz. high or coldness, salinity, pH, pressure, radiation, chemical extremes, lack of nutrition, osmotic barriers, geological scale/barriers, or polyextremity, etc. normal survival may not be achievable. However certain organisms possess unusual characteristics, which make them capable of thriving in such extreme habitats. The present chapter discusses the role of a variety of microorganisms in the synthesis of different metal nanoparticles. Such biologically synthesized nanoparticles by microbes are safe, eco-friendly and have numerous applications in the fields like agriculture, textile, medicine, drug delivery, biochemical sensors, and allied areas. Although several researchers have exploited microorganisms for the biosynthesis of metallic nanoparticles

H. Shukla
Department of Biotechnology, Sri Guru Tegh Bahadur Khalsa College, Jabalpur, Madhya Pradesh, India

S. Shukla (✉)
Department of Biotechnology, Mata Gujri Mahila Mahavidyalaya (Autonomous), Jabalpur, Madhya Pradesh, India

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but future works need to be carried out for large-scale production, increased stability, reduced time to get desirable shape and size and their probable applications in a number of fields. This chapter gives an outline of the promising use of fungi in the field of metal nanoparticles synthesis, its potential mechanisms and efficient applications in diverse areas of research.

Keywords

Nanoparticles · Extremophiles · Biosynthesis and eco-friendly

26.1 Introduction

Nanotechnology has emerged as an extremely significant field as a result of its widespread applications in diverse sectors of science and technology. It is gradually taking a center stage in the fields of catalysis, optics, biomedical sciences, mechanics, magnetics, and energy science. Nanotechnology is the science which deals with the design, synthesis, characterization, and applications of particles having diameter less than 100 nm known as nanoparticles. These nanoparticles are considered as tiny entity that behaves like a complete unit in terms of its transport and properties. The word "nano" is derived from Greek word meaning dwarf or extremely small (Rai et al. 2008). When used as a prefix, it implies 10^{-9}. A nanometer (nm) is one-billionth of a meter and is equal to the length of three atoms side by side. Several biomolecules inside the body of living organisms lie in the range of nanometer scale including a DNA molecule (2.5 nm wide), a protein molecule (50 nm), a flu virus (100 nm), and thickness of hair (10,000 nm). The average diameter of nanoparticles usually ranges between 0.1 and 100 nm and is generally synthesized via two conventional methods, viz. top-down and bottom-up approach (Fendler 1998; Thakkar et al. 2010). In top-down approach, the bulk materials are step by step decreased to nanosized materials, whereas in bottom-up approach, atoms or molecules are collected to form molecular structures in nanometer range. The chemical and biological synthetic methods of nanoparticles often employ the bottom-up approach (Fendler 1998).

Nanoparticles exhibit exclusive properties like physical, chemical, electronic, electrical, mechanical, magnetic, thermal, dielectric, optical, and biological properties (Schmid 1992). The physical properties of nanoparticles greatly differ from most of the other materials because of their decreasing dimensions. These physical properties are credited to their large surface areas to volume ratio, large surface energy, spatial confinement, and reduced imperfections. Nanoparticles are beneficial in comparison to the regular sized bulk materials as a consequence of their extraordinary properties like phenomenon of surface plasmon resonance (SPR), increased Rayleigh scattering, and surface-enhanced Raman scattering (SERS). Nanoparticles are hence considered as building blocks of the subsequent generation of optoelectronics, electronics, various chemical and biochemical sensors (Wong and Schwaneberg 2003). The synthesis of nanoparticles possessing a variety of

chemical compositions, sizes, shapes, and controlled dispersities is the most significant area of research in nanotechnology. The development of reliable, cost-effective, and eco-friendly experimental procedure for the synthesis of nanomaterials is one of the challenges in nanotechnology. A number of manufacturing techniques employing atomistic, molecular, and particulate processing during a vacuum or in a very liquid medium are currently been applied for nanoparticles synthesis (Daniel and Astruc 2004). But majority of the approaches are capital intensive and moreover are incapable in efficient production.

Several methods including physical, chemical, biological, and hybrid approaches are present to synthesize different types of nanoparticles (Mohanpuria et al. 2008; Liu et al. 2011). Even though a variety of physical and chemical techniques are comprehensively used for the synthesis of nanoparticles, the involvement of toxic chemicals in these procedures is a matter of utmost importance. Besides this these techniques also include the application of toxic chemicals on the surface of nanoparticles and non-polar solvents which restricts their applications in biotechnological and clinical fields. Therefore, to overcome these drawbacks accompanied with the physical and chemical methods it is necessary to develop novel, biocompatible, non-toxic, and eco-friendly methods for nanoparticles synthesis. Accordingly, researchers working in the field of nanoparticle synthesis and assembly have inclined their attention toward various biological sources for nanoparticles fabrication. Numerous biological systems including plants (Shankar et al. 2004), fungi (Sastry et al. 2003), bacteria (Joerger et al. 2001), actinomycetes (Sastry et al. 2003), along with some other organisms have the potential of nanoparticles synthesis.

The applications of biological agents for the synthesis of nanoparticles have led to the emergence of an inherent, clean, non-toxic, and environment friendly field of science known as nanobiotechnology. Applications of microbial interactions with metals in various fields including bioremediation, biomineralization, bioleaching, biocorrosion, and biosynthesis of nanoparticles have been the focus of researchers around the world (Klaus et al. 2009). With the advent of these applications nanobiotechnology has developed as a potential research field. This field actually represents an interaction of field of biotechnology and nanotechnology.

Biosynthesized nanoparticles produced by a biogenic enzymatic method are much better, in several ways, as compared to those synthesized via chemical and physical approach. Although physicochemical methods are capable to fabricate huge amount of nanoparticles with a defined size and shape in a tremendously small period of time, they are complex, old-fashioned, expensive, and ineffective. Moreover they release a number of hazardous toxic wastes that are dangerous to the human health as well as the environment. With the development of biological methods, the involvement of costly chemicals is restricted besides it is not as energy intensive as the chemical methods and is additionally eco-friendly.

The biosynthetic method is further facilitated by the property of most of the microorganisms to survive in varying temperature, pH, and pressure conditions. The biosynthesized nanoparticles possess greater catalytic reactivity, larger specific area, and enhanced contact between the enzyme and metal salt because of the microbial carrier matrix (Bhattacharya and Mukherjee 2008). In the microbial synthesis of

nanoparticles, microorganisms attach the target ions on their cell surface from the surrounding environment and reduce the metal ions into the element metal by secreting enzymes produced by cellular activities. This approach is divided into two types: intracellular and extracellular synthesis depending upon the vicinity where nanoparticles are synthesized (Mann 2001). During the intracellular method ions are transported inside the microbial cell where they are reduced to corresponding metal nanoparticles by the enzymes secreted in the cell. Whereas the metal ions are trapped on the cellular surface of microorganisms during the extracellular synthesis and reduced thereafter into metal nanoparticles by enzymes present inside the cells (Zhang et al. 2011a, b).

26.2 Extremophiles

Different groups of extremophilic organisms grow under different optimal conditions like acidophilic organisms grow best between pH 1 and pH 5, however, alkaliphilic organisms grow at pH above 9; halophilic organisms require high salt concentrations for optimum growth; thermophilic grows at temperatures ranging from 60 to 80 °C; hyperthermophilic organisms grow above 80 °C; psychrophilic organisms grow at 15 °C or lower temperature, showing a maximum growth at 20 °C and minimal growth at or below 0 °C; piezophilic or barophilic organisms exhibit optimum growth at high hydrostatic pressure; oligotrophic grows well in nutritionally limited environments; endolithic organisms dwell within rock or pores of mineral grains; and xerophilic organisms grow under dry conditions, in the presence of tide (Prasanti et al. 2015).

Conversely, some extremophilic organisms are able to survive simultaneously under multiple stress conditions. Such organisms are called polyextremophiles. Few common examples of these include thermoacidophilic and haloalkaliphilic organisms. Extremophilic organisms capable of bearing multiple extreme conditions are called as polyextremophiles. For example, organisms inhabiting the hot rocks under the deep Earth's surface are also thermophilic and barophilic like *Thermococcus barophilus*.

Similarly polyextremophile which inhabits high mountains present in the desert is a radioresistant xerophile, a psychrophile, and an oligotroph. Polyextremophiles are recognized for their ability to endure high as well as low pH levels. The exploitation of extremophilic organisms and their biodiversity makes them useful for developing different techniques and products for human welfare (MacElroy 1974; Rothschild 2007; Tiquia and Mormile 2010; Tiquia-Arashiro and Mormile 2013; Tiquia-Arashiro 2014a, b). Extremophilic organisms produce enzymes called extremozymes that are active under extreme environmental conditions. These enzymes have great biotechnological potential. Extremozymes are known for their widespread applications in industrial production procedures and research applications as a result of their capability to stay active even under the severe conditions (e.g., heat, pressure, and pH) usually employed in these processes. As reported by several researchers the identification of the extremophiles provided

opportunities for industrial, biotechnological, and medical use (Adams and Kelly 1998; Niehaus et al. 1999; Tiquia and Mormile 2010; Oren 2012; Gabani and Singh 2013; Pomaranski and Tiquia-Arashiro 2016).

26.3 Metallic Nanoparticles

Metallic nanoparticles have fascinated scientists around the world and are now profoundly applied in the fields of biomedical sciences and engineering. Due to their varied applications in different fields, viz. electronics, cosmetics, coatings, packaging, optics and biotechnology metallic nanoparticles have gained great interest of the scientific community. For example, improved and easy-to-create coatings for electronics applications are formed by merging nanoparticles into a solid at comparatively lower temperatures, without melting. In general, nanoparticles have a wavelength below the critical wavelength of sunshine which makes them transparent and subsequently useful for applications in cosmetics, coatings, and packaging. Metallic nanoparticles and single strands of DNA are usually attached together nondestructively which eventually opens prospects for applications in the field of medical diagnostics. Nanoparticles can pass through the vasculature and focus on any organ. This property can lead to novel therapeutic, imaging, and biomedical applications. Nowadays these nanoparticles after synthesis are modified with a variety of chemical functional groups which allow them to be conjugated with antibodies, ligands, and medicines of interest. This could pave the way for large array of potential applications in biotechnology, magnetic separation, pre-concentration of target analytes, targeted drug delivery, and vehicles for gene and drug delivery and more importantly diagnostic imaging (Mody et al. 2010).

26.4 Biosynthesis of Nanoparticles

Metallic NPs are usually synthesized by the conventional physical and chemical methods out of which the wet-chemical approach is the most commonly used. During this process nanoparticles are grown in an extremely liquid medium containing various reactants, specifically reducing agents like sodium borohydride (Kim et al. 2007), potassium bitartrate (Tan et al. 2003), and methoxypolyethylene glycol (Mallick et al. 2004). Besides these chemicals certain stabilizing agents like sodium dodecyl benzyl sulfate (Li et al. 1999) or polyvinyl pyrrolidone (Tan et al. 2003) are mixed to the reaction mixture for preventing the agglomeration of metallic nanoparticles. In general, the chemical methods are cheaper for prime volume; though, their disadvantages include contamination from precursor chemicals, use of toxic solvents, and generation of hazardous by-products. Consequently, the biological approach for synthesis of nanoparticles has become significant. Moreover biologically synthesized nanoparticles involving an enzymatic process are much better, in many ways, in comparison to those particles fabricated by using chemical methods.

One of the issues in the biosynthesis of nanoparticle is the use of suitable stabilizing agent to achieve stability and homogeneity of nanomaterials. There are a number of physicochemical approaches applied for the stabilization of nanomaterials which function on the basis of any one of these principles, viz. electrostatic, steric, electrosteric stabilization, and stabilization by a ligand or a solvent (Roucoux et al. 2002). However, these physicochemical stabilization procedures possess severe drawbacks, including high capital investment and use of dangerous chemicals and practices which are in turn harmful for the environment. Conversely, the biological agents involved in the synthesis of nanoparticles comprise an additional benefit of providing an outstanding stability to the synthesized nanoparticles. Regardless of the actual fact that the equipment and also the mechanisms for the synthesis of nanomaterials are comprehensively analyzed by scientists, the stabilizing agents involved during the nanomaterial synthesis procedures still remain unexplored.

26.5 Microbial Synthesis of Nanoparticles

Synthesis of nanoparticles using microorganisms involves catching the target ions from their surrounding and then converting them into the metallic nanoparticles by enzymatic process. Depending on the position of metal ions corresponding to microbial cell this method is divided into two types, namely intracellular and extracellular synthesis (Mann 2001). The intracellular method involves synthesis of nanoparticles inside the microbial cells by reducing enzymes, whereas the extracellular synthesis of nanoparticles takes place outside the microbial cells on their surface (Zhang et al. 2011a, b). Although microorganisms are well-known as cell factory synthesizing a variety of biomolecules in cost-effective and eco-friendly ways, the biosynthesis of nanoparticles has some restrictions like (1) the probable toxicity of reactants and products against the microorganisms used, (2) expensive cultivation of microbial cells in sterile conditions, (3) low productivity, and (4) other economic aspects involved in purification and characterization of biosynthesized nanoparticles. To overcome these drawbacks, extremophilic microorganisms possessing ability to grow under extreme environmental conditions, viz. very high or very low temperature, pH, salt concentration, etc. are being used for the biosynthesis of nanoparticles.

26.6 Nanoparticle Synthesis by Extremophilic Microbes

The extremophilic microbes offer very potential solution to various environmental challenges by exhibiting remarkable tolerance ability toward extreme conditions. This property makes them perfect biological agents for the biosynthesis of nanoparticles. These microorganisms can be cultivated even under non-sterile conditions by using low-cost media resulting in the synthesis of extremely expensive biomolecules, biochemicals, bioanalytics, and enzymes having different

bioactivities. Extremophilic enzymes possess immense potential in several fields' particularly industrial biotechnology, since they can be used under harsh conditions, which can result in substrate transformations that are not possible with normal enzymes (Colombo et al. 1995). In comparison to chemical catalysis, enzyme-mediated reactions provide more specific stereo-selectivity in organic synthesis (Koeller and Wong 2001). The most important limitations accompanied by the use of these enzymes are their stability for long time and recyclability (Schmid et al. 2011). Lately, a number of nanoparticles are in use to improve conventional enzyme immobilization methods so as to strengthen activity and stability of enzymes which in turn will minimize the costs in industrial biotechnology (Abad et al. 2005; Yiu and Keane 2012).

26.7 Nanoparticle Synthesis by Halophilic Fungi

Majority of metal tolerant microbes inhabit the marine environments particularly the underside of the ocean and play a significant role in the biogeochemical cycling of inorganic elements. Hence marine environments present a potential source of microbes with metal tolerance capacity. Moreover, the marine ecosystem is constantly exposed to metallic pollution as a result of volcanic eruptions, natural weathering of the rocks, anthropogenic activities like mining, combustion of fuels, industrial, and concrete sewage. However extensive concentrations of metals are found in estuaries and solar salterns since they serve as efficient traps for river borne metals (Chapman and Wang 2001). Therefore halophiles residing in these marine ecosystems are continuously exposed to metals which makes them an effective source for nanoparticle synthesis. Various research works have reported nanoparticles biosynthesis by halophiles like bacteria, archaea, fungi, and algae.

Some common species of halophilic yeasts and fungi that are known to synthesize nanomaterials include *Pichia capsulata*, which is halophilic yeast isolated from mangrove. It performs extracellular synthesis of silver nanoparticles (Manivannan et al. 2010). *P. capsulata* utilizes a rapid process to synthesize silver nanoparticles under optimum conditions, viz. pH of about 6.0, 0:3% NaCl concentration, and a temperature of 5 °C. During further study it was revealed that the whole reduction process was catalyzed by an NADH-dependent (NADH: nicotinamide adenine dinucleotide) nitrate reductase enzyme which was later partially purified.

In a similar study a metal tolerant yeast *Yarrowia lipolytica* isolated from polluted areas containing toxic and unsafe metals is applied for the biotransformation of organic compounds, production of novel enzymes, cloning and expression of heterologous proteins, bioremediation of hydrophobic substrate contaminated environments, and the treatment or upgradation of wastes. The metal tolerance observed in this yeast was attributed to the presence of SOD (a copper tolerating protein), reductases, CRF1, metallothioneins, efflux mechanisms, and melanin (Bankar et al. 2009). Likewise a tropical marine isolate of *Y. lipolytica* (NCIM 3589) obtained from oil-polluted seawater near Mumbai, India was found to synthesize gold nanoparticles. This synthetic process occurs at 30 °C within 72 h. TEM

analysis confirmed the cell wall associated synthesis of nanoparticles. The size of the nanoparticles was observed to be affected by the pH. According to study at pH 2.0, gold nucleation was observed within 15 min. With increasing period of time, these developed into large triangular and hexagonal plates. Conversely the scale of the nanostructures at pH 7.0 and at 9.0 was around 15 nm (Agnihotri et al. 2009). This principle was employed in the custom designing of gold nanoparticles with specific sizes.

In a study the size of nanoparticle decreased with increase in cell numbers and same gold salt concentrations. Whereas it was observed that the size of nanoparticles increased with increasing concentration of the gold salt and the same cell numbers. The isolation of cell-associated gold nanoparticles can be done by incubation in a medium at 20 °C (Pimprikar et al. 2009). A dark-colored pigment called melanin was isolated from this yeast which was later proved to be one of the major factors responsible for nanoparticle synthesis. The synthesis of gold nanostructures was observed to be facilitated by both the cell-extracted and induced melanin (obtained by incubating resting cells with L-3,4-dihydroxyphenylalanine (L-DOPA)). Synthesis of gold nanostructures has been reported by another cold-adapted marine strain of *Y. lipolytica* (NCIM 3590) also. As the natural amount of melanin in this organism was low, a precursor L-DOPA was incubated with the yeast to induce the overproduction of melanin. This melanin also aided the rapid fabrication of silver and gold nanoparticles. Certain functional groups on the cell surface were thought to play a role in the reductive and stabilization processes (Pawar et al. 2012). The synthetic ability during this case was also related to the dark-colored pigment, melanin. These nanoparticles displayed antibiofilm activity against pathogenic bacteria.

One more fungus, *Penicillium fellutanum* isolated from the rhizosphere of an Indian endemic mangrove plant (*Rhizophora annamala*) was examined for silver nanoparticles synthesis. It was observed that highest biosynthesis of nanoparticles occurred in the culture filtrate containing 1.0 mM $AgNO_3$, 0.3% NaCl at pH 6.0 and temperature 5 °C for 24 h. The nanoparticles were synthesized extracellularly having spherical shape with size ranging from 5 to 25 nm. During the analysis of cell-free supernatant a protein of about 70 kDa was reported which was considered to be responsible for converting the metal ions to their zero valence state (Kathiresan et al. 2009). Preliminary presence of silver nanoparticles within the culture filtrate was confirmed by absorption peak at 430 nm, through UV-Vis spectroscopic analysis which was further confirmed by the use of transmission electron microscopy.

Aspergillus species a yet another halophilic fungus is able to survive mostly under all types of weather conditions found around the world. This fungus is generally associated with environments possessing high starchy concentrations. As mangrove ecosystems are made up of debris from leaves, inflorescence, and stems, they are rich in starch content and therefore *Aspergillus sp.* inhabit these ecosystems in great numbers (Seelan et al. 2009). Spherical silver nanoparticles having diameter of 5–35 nm were synthesized by a strain of *A. niger* (AUCAS 237) isolated from a mangrove sediment of Vellar estuary, India. These nanoparticles exhibited efficient antimicrobial activity against various clinically pathogenic microorganisms including Gram-negative bacteria and a few fungi. The activity of nanoparticles was

increased when they were stabilized with polyvinyl alcohol. FTIR analysis also reported the probability of presence of proteins acting as promising capping and stabilizing agents (Kathiresan et al. 2010).

In a study silver nanoparticles were synthesized by using a different strain of *A. niger* isolated from the Gulf of Cambay, India. These nanoparticles exhibited potential optical properties and were spherical in shape with a diameter of 5–26 nm (Vala et al. 2012). Symbiotic association between microorganisms and various marine animals like sponges are well-known. One such symbiotically associates strain of *A. terreus* (MP1) was isolated from a marine sponge and its mycelial extract was utilized for the synthesis of silver nanoparticles. These particles also showed potential inhibitory activity against the pathogenic bacterial strains including *Staphylococcus aureus*, *Klebsiella pneumoniae,* and *Salmonella sp.* (Meenupriya et al. 2011).

In similar study marine yeast called *Rhodospiridium diobovatum* was reported to synthesize lead sulfide (PbS) nanoparticles intracellulary with the help of nonprotein thiols (Seshadri et al. 2012). The characterization of PbS nanoparticles was performed by UV-visible absorption spectroscopy, diffraction (XRD), and energy dispersive atomic spectroscopy (EDAX). During UV-visible absorption spectroscopy, a peak at 320 nm was obtained indicating the presence of nanosized particles. Further XRD analysis established the presence of PbS nanoparticles of cubic shape. However, the size of these crystalline particles was determined to be in the range of 2–5 nm. However, elemental analysis by EDAX discovered the existence of particles made up of lead and sulfur in the ratio of 1:2. These observations confirmed capping of PbS nanoparticles by a sulfur-rich peptide. The yeast grown in the presence of lead exhibited a striking increase (280 overcome the control) in nonprotein thiols during the stationary phase. A capping agent consisting of sulfur-rich peptide was also observed. These were probably concerned in fabrication of the nanoparticles (Seshadri et al. 2012).

26.8 Nanoparticle Synthesis by Thermophilic Fungi

Thermophiles are organisms which require heat for their growth and development (thermophile), they can even withstand high temperatures (thermotolerant). A few thermophiles are also able to tolerate extreme stress conditions like toxicity of heavy metal ions or metals. Most of them are therefore capable to survive and grow under high metal ion concentrations and even have probability of binding huge quantities of metallic cations. Additionally, many of these microorganisms are efficient enough to synthesize nanoparticles. This outstanding ability of such microorganisms to survive in the presence of heavy metal ions makes them most appropriate candidates for the synthesis of nanoparticle. Thermophiles inhabit both natural and man-made habitats. Natural habitats harbored by such a type of thermophilic microorganisms include terrestrial geothermal, volcanic areas, and deep-sea hydrothermal vents (Mehta and Satyanarayana 2013).

Both culture-dependent and culture-independent approaches have been applied so far for the isolation and identification of extreme thermophiles and hyperthermophiles from their natural habitats. Geothermal and volcanic areas mainly include habitats like terrestrial fumaroles (e.g., solfataras), terrestrial hot springs, and geysers. Whereas some other natural habitats consist of geothermally heated oil and petroleum reservoirs and sun-heated soils/sediments (Greene et al. 1997; Engle et al. 1995; Ward et al. 1987; Zillig et al. 1980; Mehta and Satyanarayana 2013). The temperature of these habitats is comparatively lower as that of other natural habitats and is therefore appropriate for the isolation of moderate and extreme thermophiles containing enzymes functional at high temperatures and also aid in their survival.

According to a study by Syed et al. (2013) the biosynthesis of silver nanoparticles was performed by the thermophilic fungus *Humicola sp.* This fungus extracellularly reduces the Ag + ions present in the reaction mixture resulting in the synthesis of silver nanoparticles. During this study the researchers demonstrated that dimensions of these nanoparticles could be controlled within the size range of 5–25 nm, so that they can be applied in the field of biomedicine. Formation of silver nanoparticles was reported by the change in color of reaction mixture from pale yellow to brown. It was further confirmed by the application of advanced characterization techniques.

The biosynthesized silver nanoparticles are found to be non-toxic toward cancerous and normal cells up to concentrations of 50 μg/ml. Therefore, they find a variety of applications in targeted drug delivery systems (Syed et al. 2013). Likewise biosynthesis of biomedically important cerium oxide (CeO_2) nanoparticles using the thermophilic fungus *Humicola* sp. was reported. When this fungus was exposed to aqueous solutions of oxide precursor cerium (III) nitrate hexahydrate ($CeN_3O_96H_2O$) extracellular formation of CeO_2 nanoparticles containing Ce (III) and Ce (IV) in mixed oxidation states was confirmed by X-ray photoemission spectroscopy (XPS). These biosynthesized nanoparticles were found to be naturally capped by proteins secreted by the fungus which prevents them to agglomerate. A thermostable enzyme or protein is the one when a high defined unfolding (transition) temperature (Tm) or an extended half-life at a particular temperature is observed. A hot temperature for growth is generally above the thermophile boundary that is greater than 55 °C. Except for a few of them most of the proteins from thermophiles are thermostable.

26.9 Nanoparticles by Psychrophilic Fungi

Psychrophiles are organisms, which need low temperatures for their growth and survival and can even tolerate very low temperature conditions (psychrotolerant). They are also divided into two classes, namely stenopsychrophiles and eurypsychrophiles. Stenopsychrophiles, also known as obligate psychrophiles, can only stay alive only at temperatures below 15 °C; however, eurypsychrophilic also known as mesotolerant organisms cultivate best below 15 °C but can also withstand and grow at higher temperatures. Thus several studies have suggested that

psychrophiles have an optimum growth temperature below 15 °C but cannot grow at temperature greater than 20 °C. However, psychrotolerant microorganisms, also known as psychrotrophs are those which might tolerate cold conditions but have an optimum growing temperature of about 20 °C. Numerous organisms remain metabolically active at temperatures below freezing conditions (Koshima 1984). A number of psychrophiles have established the potential to tolerate extreme stress conditions, viz. toxicity of heavy metal ions or metals. Besides this they have also exhibited the potential of nanoparticles synthesis.

Psychrophilic fungus *Humicola marvinii* was found to be able to metabolize and reproduce best at temperatures below 15 °C, while they are reported to survive even with greatly reduced metabolism all the way down at a temp of about −20 °C (Von Klein et al. 2002; Auman et al. 2006; Kumar et al. 2007; Weinstein et al. 1997). These cold-loving microorganisms are generally present in Polar region, deep sea, mountains, glaciers, fresh and marine waters, polar and high alpine soils. These fungi face many problems under such environments including membrane rigidity, protein misfolding, and slower reaction rates.

26.10 Nanoparticles by Alkaliphilic and Acidophilic Fungi

Acidophiles and alkaliphiles are another group of microorganisms exploited for nanoparticles biosynthesis. The biomolecules present in these fungi are not only involved in the biosynthesis of nanoparticles but they also provide excellent stability to the biosynthesized nanomaterial. These biomolecules including proteins, peptides, and a special class of metal-binding molecules are called as phytochelatins that are used for the in vitro stabilization of synthesized nanomaterials. Organisms that grow at the extreme pH conditions are divided into two classes, viz. acidophiles, showing maximum growth below pH 3, and alkaliphiles, exhibiting optimum growth at pH greater than 9 (Rothschild and Mancinelli 2001; Wiegel 2011).

Fungi can collect metals by different mechanisms including physicochemical and biological mechanisms. It involves binding of metabolites and polymers, to specific polypeptides extracellularly and metabolism dependent accumulation. Mukherjee et al. (2001) reported the formation of gold nanoparticles having diameter of 20 nm when the acidophilic fungus, *Verticillium sp.* (AAT-TS-4), was exposed to aqueous $AuCl^{4-}$ ions resulting in the reduction of the metal ions. The synthesis of gold nanoparticles occurred both extracellularly that is on the surface and intracellularly that is within the fungal cells with slight reduction of the metal ions in solution.

The presence of diffuse rings with lattice spacings was confirmed by the application of selected area diffraction analysis of one gold particle. Bulk of the gold nanoparticles were found on the membrane surface which is surprising because usually no more than 10 ± 15 nanoparticles in one cell of bacteria are reported (Klaus et al. 1999). Further it has also been demonstrated that the scale of distribution of gold nanoparticles produced using *Verticillium* sp. is way narrower in comparison to that observed for silver nanoparticles synthesized by bacteria (Klaus et al. 1999). Mostly spherical AuNPs were observed on the cytoplasmic

membrane along with a few triangular and hexagonal particles as well. A big, quasi-hexagonal gold particle was reported within the cytoplasm (Mukherjee et al. 2001).

The acidophilic fungus *Verticillium* sp. when exposed to silver nitrate solution reduction and accumulation as silver nanoparticles within the fungal cell was observed (Sastry et al. 2003). The change in the color of fungal biomass to dark brown after reaction with Ag^+ ions indicated the reduction of the metal ions and synthesis of silver nanoparticles inside the fungal cell. In case of this fungus the formation of silver nanoparticles was observed only inside the fungal cell and not outside. The absorption maxima of normally harvested fungal mycelium was not found at 400–800 nm, whereas that of the fungal cells exposed to Ag + ions was found at 450 nm which was also used as indicator of reactivity.

The occurrence of the broad resonance revealed an aggregated structure of the silver nanoparticles within the film. The extracellular reduction of the Ag^+ ions in solution followed by their precipitation onto the cells could be a probable mechanism for detecting the presence of silver nanoparticles within the fungal biomass. The exact mechanism involved in the intracellular formation of silver nanoparticles is not yet completely described. The mechanism revealed by Sastry et al. (2003) demonstrated that the nanoparticles are formed on the surface of the mycelia and not in solution, the chief step includes trapping of the Ag + ions on the surface of the fungal cells perhaps via electrostatic interaction between the Ag + and charged carboxylate groups in enzymes present within the semipermeable membrane of the mycelia. It is followed by the reduction of the silver ions by enzymes present inside the cytomembrane resulting in the formation of silver nanoparticles. However few Ag + ions disperse through the cytomembrane and are reduced by enzymes present on the cytoplasmic membrane and within the cytoplasm. Further possibility of diffusion of smaller silver nanoparticles across the cytomembrane and entrapment within the cytoplasm was declared by several researchers (Sastry et al. 2003).

26.11 *Bipolaris nodulosa*

Examination of mycelia free media (MFM) of *Bipolaris nodulosa* indicated their potential to synthesize anisotropic silver nanoparticles (Saha et al. 2010). After exposure to aqueous silver nitrate solution (1 mM), the mycelia free media exhibited a steady change in color with time from yellowish to light pink, brown, and finally to dark brown within 24 h. The formation of silver nanoparticles was further confirmed by using the UV–Vis spectroscopic analysis during which an absorbtion peak at 420 nm characteristic for silver nanoparticles was obtained. These nanoparticles were found to be monodisperse in nature having dimensions ranging from 10 to 60 nm via laser diffraction analysis. Moreover antimicrobial potential of these nanoparticles was examined against *Bacillus*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Escherichia,* and *Micrococcus luteus* wherein they showed potential antimicrobial activity at a concentration of 100 μg/ml (Saha et al. 2010).

26.12 *Fusarium* sp.

Fusarium oxysporum is the most highly explored and exploited for the formation of silver nanoparticles out of all types of fungi. Extracellular synthesis of numerous nanoparticles, viz. gold, silver, bimetallic Au–Ag alloy, silica, titania, zirconia, quantum dots, magnetite, strontianite, Bi_2O_3 and barium titanate has been reported. In 2002, the synthesis of spherical and triangular gold nanoparticles ranging from 20 to 40 nm was revealed by Mukherjee et al. The FTIR spectrum indicated the presence of amide (I) and (II) bands from carbonyl and amine stretch vibrations in proteins, respectively. In addition presence of a protein having molecular mass between 66 and 10 kDa responsible for nanoparticles stabilization was also observed, via. electrophoresis. In another study synthesis of Zirconia nanoparticles was demonstrated when cationic proteins produced by *F. oxysporum* were incubated with ZrF6-2 anions. The protein having molecular mass of about 24–28 kDa was declared to be involved in the formation of zirconia nanoparticles (Bansal et al. 2004). These nanoparticles were in particular quasi-spherical in shape having size in between 3 and 11 nm.

F. oxysporum exhibited the extracellular synthesis of silver nanoparticles and bimetallic gold–silver (Au–Ag) alloy nanoparticles. Biomedical applications of Au–Ag alloy nanoparticles have also been demonstrated. Likewise, production of silica and titania nanoparticles with SiF_6^{-2} and TiF_6^{-2} anionic complexes results in the synthesis of crystalline titania and calcinations at temperature of 300 °C (Bansal et al. 2005). Moreover, ternary oxide, barium titanate nanoparticles ($BaTiO_3$) of irregular quasi-spherical morphology with an average size of 4 ± 1 nm were also synthesized. After the mixing of salts $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$ and incubating for 24 h, it resulted in the formation of crystalline magnetite nanoparticles with single-domain characteristics. The size of these particles was 20–50 nm and they were quasi-spherical in shape (Bharde et al. 2006).

Lately, extracellular production of optoelectronic material Bi_2O_3 nanocrystals of size between 5 and 8 nm with quasi-spherical morphology and good tunable properties by *F. oxysporum* was reported. On addition of bismuth nitrate as a precursor, nanocrystal synthesized were in monoclinic and tetragonal phases (Uddin et al. 2008). Similarly Kumar et al. (2007) have revealed the enzymatic synthesis of silver nanoparticles using NADPH-dependent nitrate reductase purified from *F. oxysporum*. It was later confirmed by protein assays that an NADH-dependent reductase is that the chief factor involved in biosynthesis processes. Besides this the enzymatic method of in vitro synthesis of silver nanoparticles by NADPH-dependent nitrate reductase isolated from *F. oxysporum* with capping peptide, phytochelatin was reported in a recent study (Durán et al. 2007).

26.13 *Aspergillus* sp.

Kumar et al. in the year 2008 demonstrated the biosynthesis of silver nanoparticles by using *Aspergillus niger* isolated from soil. Cell-free filtrate of A. niger was exposed to 1 mmol/L nitrate and rotated on a rotary shaker at 120 rpm and 25 °C. After 72 h of treatment with nitrate solution *Aspergillus flavus* started gathering silver nanoparticles on their cell surface. The average size of these silver nanoparticles (Ag NPs) was observed to be around 8.92 nm. Further these Ag NPs were reported to exhibit a characteristic absorption peak at 420 nm and emission peak at 553 nm (Vigneshwaran et al. 2006). In a similar study mycosynthesis of silver nanoparticles by *Aspergillus clavatus* was demonstrated extracellularly (Saravanan and Nanda 2010; Verma et al. 2010). During another research work reduction of aqueous Ag ion was done by using culture supernatants of *Aspergillus terreus* resulting in the formation of silver nanoparticles. These mycosynthesized silver nanoparticles were found to be polydispersed spherical particles ranging in size from 1 to 20 nm and also showed potential inhibitory activity against most of the plant pathogenic fungi and bacteria. Disc-diffusion technique was applied to examine the antibacterial action of Ag NPs against pathogenic bacteria, viz. *E. coli, Candida albicans, and Pseudomonas fluorescence.* Likewise, antimicrobial activity against pathogenic fungal and bacterial strains of silver nanoparticles was reported in a recent study (Jaidev and Narasimha 2010).

26.14 Mechanism of Mycogenesis of Metal Nanoparticles

Nature is a great reservoir of microorganisms which act as biofactories for the synthesis of metal nanoparticles having potential bio-prospective applications (Azizi et al. 2017). Recently, the novel biosynthetic pathway and varied applications of nanobiotechnology have drawn the attention of researchers to work on this field. Microbial synthesis of nanoparticles has great benefits and huge potential to humans, because they eradicate the use of toxic chemicals and involve cost-effective synthetic procedure. One of the major advantages of biosynthesis of metal nanoparticles is their enormous role in the environment protection which makes it an eco-friendly approach and a significant approach toward green technologies (Azizi et al. 2017; Ottoni et al. 2017). Lately, mycosynthesis of nanoparticles has attracted majority of researchers; however, the correct mechanism linked to the synthesis has not been explored yet.

Fungal kingdom comprises multicellular eukaryotic organisms that are particularly heterotrophic and play vital role in nutrient cycling in an ecosystem. Fungi maintain symbiotic associations with plant and bacteria and reproduce both sexually and asexually. Major groups of fungi include molds, mildews, yeasts, rusts, and mushrooms. Fungi secreted a variety of enzymes in comparison to bacteria and therefore synthesize huge number of nanoparticles. They also generate different biomolecules which are involved in the bioreduction and stabilization process of nanoparticles. Out of all fungal enzymes especially the reductase enzymes are

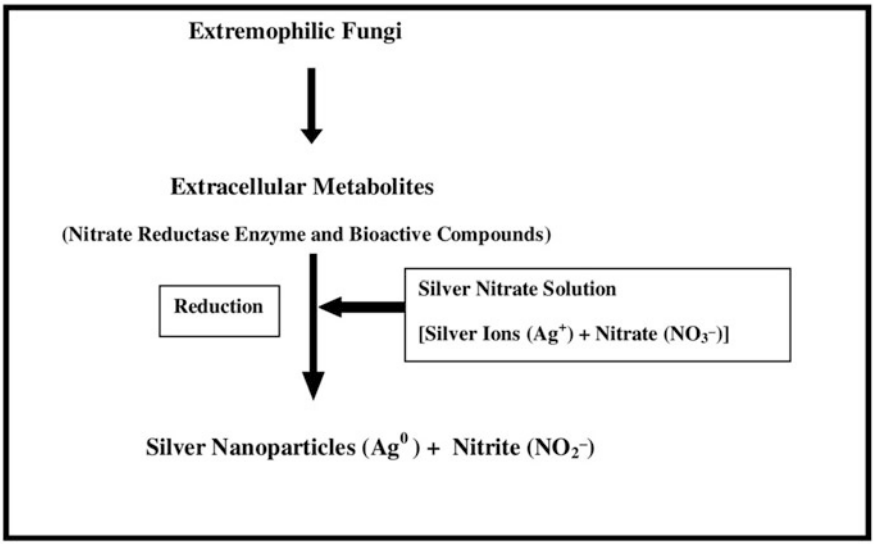

Fig. 26.1 Mechanism of nanoparticles synthesis by extremophilic fungi

probably responsible for the nanoparticle fabrication and stabilization (Fig. 26.1). Since the fungal cell surface possesses negative charge and contains sticky substances, the metal ions adhere on the cell surface via electrostatic interaction. It is well-known fact that fungi performs both extracellular and intracellular synthesis of nanoparticles. Intracellular synthesis approach is more appropriate for synthesis of composite films. However, during extracellular synthesis, the metal ions are immobilized in a suitable carrier or support (Duhan et al. 2017). The metabolites and enzymes secreted by the fungi are the chief components and play important role in conversion of toxic materials into non-toxic materials; these compounds may also be also involved in the synthetic mechanism of nanoparticles (Owaid and Ibraheem 2017).

Reports confirmed the secretion of proteins and enzymes by a fungus named *Trichoderma reesei* outside the hyphae which are known to play a key role in silver nanoparticles synthesis. Usually the nanoparticles synthesized by fungi are monodispersed with definite size and shape having diverse chemical compositions. Since they secrete diversity of enzymes inside and outside of the cells, fungi become most suitable choice over other microbes for nanoparticles synthesis (Velhal et al. 2016). Moreover the research work carried out in the field of mycogenesis of nanoparticle has led to emergence of a new field of science called nanobiotechnology with major potential due to wide range diversity and availability of fungi. In comparison to bacteria, fungi possess greater metal tolerance and metal-binding capability which aids in their uptake capacity, hence fungal biomass is an attractive biological agent for industrial production of nanoparticles. Moreover the downstream processing involved with fungal biomass is simpler which makes their

employment efficient and cost-effective in the synthesis of metal nanoparticles (Yadav et al. 2015). A variety of fungal species are already known for the synthesis of different metal nanoparticles, viz. Au, Ag, Pd, Cu, Zn, Ti, Fe, and Pt. Fungi exhibit slower growth kinetics, as a consequence they provide enhanced regulation of shape, size, and long term stability of nanoparticles (Zhao et al. 2018).

Studies conducted on fungus-mediated synthesis of nanoparticles mainly focus on their possible mechanism, action of nitrate reductase, electron shuttle quinones. These studies suggest that mainly nitrate reductase and α-NADPH-dependent reductases are responsible for nanoparticle synthesis in both bacteria and fungi. Extracellularly synthesized nanoparticles are stabilized by the proteins and enzymes secreted by the fungal cell. During a research, it was revealed that minimum four proteins including NADH-dependent reductase having relative molecular mass are involved in nanoparticles synthesis. The analysis of fluorescence spectra of these enzymes demonstrates the presence of native type of these proteins within the aqueous extract or solution similar to those binded on the surface of nanoparticles (Shah et al. 2015).

Further research work indicates that the metal ions reduction and the surface binding molecule to the nanoparticles comprise protein molecules. Thus it could be concluded from the studies that fungus contains polypeptides and enzymes which act as a reducer as well as stabilizing agents. As a result of the cellular complexity of fungi, further more research is needed to explore the detailed mechanism of synthesis. Moreover extensive research works are required to produce nanoparticles of definite shape and size and also to increase the speed of synthesis of nanoparticles. With the advent of recent techniques much information has been generated which has enhanced the applications of nanoparticle biosynthesis in various fields. However, the awareness about concrete mechanism of the mycosynthesis of nanoparticles requires more study and experimental trials.

26.15 Characterization Techniques for Nanoparticles

Characterization studies of nanoparticles include the analysis of shape, size, and structure of different types of nanoparticles which is usually significant for material research. According to Liu et al. (2010) nanomaterials are found in unique structure and size and are estimated to possess diverse bioactivities. New findings in the field of materials research, processes, and phenomenon at the nano-scale would offer possible applications for fabrication of novel nanosystems and nanostructures. A variety of characterization techniques, namely UV–visible spectroscopy, diffraction technique (XRD), Fourier transform infrared spectroscopy (FTIR), atomic force microscopy (AFM), scanning electron microscopy (SEM), transmission electron microscopy (TEM), energy dispersive X-ray spectroscopy (EDX), etc. are involved in the characterization of metal nanoparticles. Details of these techniques are discussed below.

26.16 UV–Visible Spectroscopy

UV–visible spectroscopy is most consistent and efficient technique for the preliminary characterization of synthesized nanoparticles which is designed not only to monitor the synthesis but also to analyze the stability of nanoparticles. This technique is a potential approach to characterize metal nanoparticles since they require brilliant color which can be visualize by oculus. Metal nanoparticles have elevated extinction coefficient and their surface plasmon resonance is dependent on the size and shape. Therefore, this method is the most suitable for the qualitative assessment of the nanoparticles. Absorption of the suspended nanoparticles is particularly calculated by the Beers law wherein absorption value (A) mainly depends on concentration of nanoparticles, path length (l) of measuring cell, and extinction coefficient of nanoparticles which is depicted by the following formula:

$$A = \varepsilon cl$$

Particle size, shape, morphology, nature of stabilizing agent, pH, temperature, type of adsorbate that are present on the surface of nanoparticles and nature of the encompassing medium affect the position of the optical phenomenon. The width of optical phenomenon increases with decreasing size of nanoparticle within the intrinsic size region and also increases with increase within the extrinsic size region (Sudha et al. 2013; Aziz et al. 2015). A Doppler effect within the extrinsic region in the SPR is visible as the size of the nanoparticles increases. As the size of the gold nanoparticles increases it exhibits change in color from ruby red to purple and finally blue. It was revealed particularly that when the space between the particles becomes equal to their average diameter, the particles get aggregated; consequently, the plasmon resonance of each and every particle couples and their absorbance is red shifted. These optical characteristics of metal nanoparticles are employed for making sensors. In addition to this, UV–Vis spectroscopy is fast, easy, simple, and sensitive for numerous types of nanoparticles. It requires only a relatively short period for quantification and therefore a calibration is not needed for characterization of nanoparticles present in the colloidal suspensions (Tomaszewska et al. 2013).

26.17 Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared spectroscopy is applied for the identification of specific type of chemical bonds or functional groups depending on their definite unique absorption patterns. It works by measuring the stretching of chemical bond and bending patterns by absorption of energy through infrared spectroscopy. This energy lies within the infrared (IR) region of electromagnetic spectrum. FTIR is a significant technique as it possesses various advantages over the conventional IR spectroscopy. FTIR therefore provides the information about surface chemistry of nanoparticles via identification of the specific functional groups attached to the surface of metal

nanoparticles since it depicts different absorption patterns as compared to those of respective free groups.

26.18 X-Ray Diffraction Technique (XRD)

X-ray diffraction technique is a non-destructive technique employed for the identification of the crystalline phase of nanoparticles. For analysis through this method the crystalline or powdered sample is positioned over a sample holder and X-rays of a set wavelength are allowed to pass through them, thereby illustrating their structural characteristics. Thereafter the intensity of the reflected radiation is recorded by using a goniometer (Wang et al. 2002). Data obtained are then analyzed by Bragg's equation, to compute inter atomic spacing for the reflection angle. Following equation is applied:

$$n\lambda = 2d \sin\theta$$

where n is an integer, λ is wavelength, d is the distance between atomic planes, and θ is the angle of incidence of the X-ray beam and therefore the atomic planes. Broad peaks of metal nanoparticles are observed on XRD. The broadening provides information about crystalline nature and size by Debye–Scherrer equation:

$$d = \kappa\lambda/\beta\cos\theta$$

In this equation, κ is the shape factor, λ is X-ray wavelength, β is the line broadening at half the utmost intensity (FWHM) in radius, and θ is the Bragg's angle.

26.19 Atomic Force Microscopy (AFM)

Atomic force microscopy is a significant tool used for the analysis of the surface morphology and phase via generation of a 3D map of the sample surface. The vertical resolution of this instrument is less than 0.1 nm, while the lateral resolution is of around 1 nm. A cantilever having a fine pointed tip which is fixed perpendicular to the longitudinal direction of the cantilever is mounted on the sample. From the back of the cantilever a laser beam is reflected into a spot sensitive to photodiode and the deflection of the cantilever caused by Vander Waal's force acting between the sample and tip is recorded. As a result of these interactions signals are generated. These signals in turn are further processed to produce the topographical information of the nanoparticles surface.

26.20 Transmission Electron Microscopy (TEM)

Transmission electron microscopy is applied for the study of the shape, size, and dispersity of the metal nanoparticles. Smaller molecules particularly having size less than 1 μm generate diffraction effects and hence cannot be observed by optical microscopy. Monograph related information depends on the resolution applied, whereas the resolution is chiefly dependent on the wavelength of the radiation beam chosen for the imaging. Application of a short wavelength beam results in higher resolution. TEM is most extensively used technique for the characterization of the metal nanoparticles. This instrument is similar to a slide projector except that it uses an electron beam of around 100–300 kV and this transmitted beam is further projected on to a phosphor screen resulting in the formation of the final image. It usually gives information about characters like topography, monodispersity, composition, and crystallinity of the synthesized nanoparticles.

26.21 Scanning Electron Microscopy (SEM)

Currently, very advanced high resolution microscopic techniques are being applied in the field of nanoscience and nanotechnology. It has aided in the analysis and illustration of nanomaterials very deeply by using a ray of high-energy electrons to probe. SEM is mainly employed for the study of the morphology and topology of the metal nanoparticles. In SEM, the surface of specimen is scanned by passing the electron beam of accelerated voltage through the sample surface. The pictures or images of the surface are thereafter obtained by collection of backscattered and the secondary electrons via the detector followed by their analysis. It is principally a surface imaging technique, which is fully competent of resolving different sized nanoparticles, their distributions, shapes, and the surface topology (Wang et al. 2002). The only restriction of SEM is that it can only generate useful information related to the purity and the degree of particle aggregation but it is not able to determine their internal structure. This technique is particularly appropriate for the morphological identification of nanoparticles having dimensions below10 nm.

26.22 Electron Energy Loss Spectroscopy (EELS)

Electron energy loss spectroscopy is a technique applied for the examination of the component elements of nanomaterials. During this process the energy transferred via such an interaction is in direct relation to the ionization potential of the atom and hence, the spectrum obtained can be compared to that of known samples or standard samples. A nanomaterial is initially subjected to a ray of kinetic energies because of which some of the electrons lose energy by inelastic scattering, which is first and foremost an interaction of the sample. The inelastic scattering consequence is both an energy loss and a momentum change. Various interactions including plasmon

excitations or inner shell ionization, phonon excitation, and inter- or intra-band transitions are observed (Wang et al. 2002).

26.23 Energy Dispersive X-Ray Spectroscopy (EDS or EDX)

Energy dispersive X-ray spectroscopy (EDS or EDX) is a potential investigating tool that is specifically used for chemical characterization of the nanoparticles. The basic principle of this technique is that each element of the periodic table has a unique electronic structure representing the fundamental properties of the elements. Therefore, the response of every element toward electromagnetic waves is unique. This type of examination depends on the interaction between matter and light performed via X-rays penetration through the sample.

26.24 Thermo-Gravimetric/Differential Thermal Analyzer (TG/DTA)

The TG/DTA is an instrument formed by combination of TG, which measures continuously utilizing a horizontal differential type of balance beam, with the extremely flexible feature of DTA. Through this instrument mostly reaction velocity is measured, acceleration degradation tests are performed, as well as water contamination is analyzed, detection of heavy metal and measurement of ash content in reaction velocity and acceleration decomposition are carried out (Fulias et al. 2013). The light weight organization of the balance beam enhances the stability with respect to fluctuations in temperature and extremely susceptible balance and also stability of the differential balance to deal with disturbance like oscillation. After carrying out all measurements, the instrument is automatically cooled to a set temperature, by the use of a cooling unit which further increases the efficiency of measurements.

26.25 Optimization of Silver Nanoparticles Synthesis

Even though the biosynthesis of silver nanoparticles using fungi is uncomplicated and cost-effective, the factors used in this method should be optimized so as to attain good quality monodispersity, stability, and biocompatibility of the nanoparticles (Balakumaran et al. 2015). From the different research works it could be inferred that a vast diversity of fungi have potential for use in the biosynthesis of nanoparticles. Besides this, it is also significant to optimize the synthesis conditions according to their individual characteristics (Ottoni et al. 2017). To achieve the preferred nanoparticle yield and characteristics, parameters including agitation, temperature, light, and culture and time of synthesis need to be adjusted since they vary depending on the type of fungus used. Factors involved for both fungal cultivation and process of synthesis are adjusted for regulating the size and shape of nanoparticles (Birla et al. 2013). Recent studies have revealed that modifications in temperature, meal

concentration, pH, culture medium, and amount of biomass can be used to obtain nanoparticles possessing diverse physicochemical properties (Birla et al. 2013; Rajput et al. 2016; Saxena et al. 2016; Liang et al. 2017).

26.26 Effect of Temperature

The temperature used during the mycosynthesis of silver nanoparticles can influence parameters such as the synthesis speed and also the size and stability of the nanoparticles (Elamawi et al. 2018). In a similar study synthesis of nanoparticles was conducted by using the cell-free filtrate of *Trichoderma harzianum.* Ahluwalia et al. (2014) reported that the rate of synthesis increased with the increase in temperature up to 40 °C, which in turn was declared as the optimum temperature. In a similar work the filtrate of *Fusarium oxysporum* was used to obtain huge protein secretion by the fungal biomass at temperatures ranging between 60 and 80 °C. It was observed that with gradual increase of the temperature, the synthesis rate and surface plasmon absorbance also increased (Birla et al. 2013). In another study during the synthesis using the endophytic fungus *Colletotrichum sp.* ALF2-6, it was revealed that the reaction rate increased at higher temperatures and synthesis completed within 20 min at temperatures above 50 °C (Azmath et al. 2016). Likewise Phanjom and Ahmed (2017) used *Aspergillus oryzae* (MTCC ## 1846) and found that temperature increased the speed of synthesis. At different temperatures of 30, 50, 70, and 90 °C the synthesis was completed in 6 h, 1 h, 45 min, and 20 min, respectively, whereas no synthesis occurred at temperature of 10 °C.

During a research work conducted by AbdelRahim et al. (2017), no synthesis of silver nanoparticles was observed when the filtrate of leak fungus at 80 or 10 °C was employed. This result was considered due to denaturation or inactivation of enzymes and other molecules. Even though most of the researches have revealed rapid rates of synthesis at higher temperatures, it is important to realize the need for standardization of these parameters during nanoparticle synthesis. In addition to affect the synthesis rate, the temperature also produces profound affects on the size and stability of nanoparticles. AbdelRahim et al. (2017) synthesized nanoparticles of different sizes, viz. 86, 25.89, and 48.43 nm, at temperatures of 40, 20, and 60 °C, respectively, with the smallest size obtained at the intermediate temperature. In a similar work Shahzad et al. (2019) fabricated nanoparticles of 322.8 nm size at 25 °C using the fungus *Aspergillus fumigatus* BTCB10. Increasing in size with increasing temperature was observed with the biggest size reaching 1073.45 nm at 55 °C. In another study the fungus *Fusarium oxysporum* was employed for nanoparticles synthesis, wherein the size of biosynthesized nanoparticles decreased to 30.24 nm with the temperature increasing up to 50 °C (Husseiny et al. 2015).

These diverse results point toward the fact that the effect of temperature on the dimensions and stability of the biosynthesized nanoparticles varies depending upon the fungus species used. Banu and Balasubramanian (2014) declared 30 °C to be the optimum temperature for synthesis of highly stable silver nanoparticles by using

Isaria fumosorosea. Similarly, Balakumaran et al. (2015) also reported the same optimum temperature for the synthesis of nanoparticles using the fungus *Guignardia mangifera*. These variations in the effects of temperature on the nanoparticles synthesis take place even within the identical genus of fungus. *Trichoderma longibrachiatum* synthesized nanoparticles at a temperature of 28 °C, while no production of nanoparticles was seen at 23 or 33 °C (Elamawi et al. 2018), however, *Trichoderma viride* exhibited potential synthesis at temperatures of 10, 27, and 40 °C (Fayaz et al. 2010).

The biosynthesis of nanoparticles by few fungal species at higher temperatures corresponds to the fact that electron transfer occurs from free amino acids to silver ions. Conversely, very high temperatures, between 80 and 100 °C, lead to the denaturation of proteins that create the nanoparticle capping. This denaturation modifies the nucleation of Ag^{+} ions, followed by the nanoparticles aggregation and increasing size (Birla et al. 2013). Husseiny et al. (2015) reported that increased nanoparticle size and loss of stability occur at unsuitable temperatures, as a consequence of the low enzymatic activity concerned within the synthesis.

26.27 Effect of pH

Alteration of the pH of nanoparticles biosynthesis process is usually carried out to regulate some specific features of the metal nanoparticles. Nayak et al. (2011) observed that changes are induced in the conformation of nitrate reductase enzymes so as to maintain the concentration of protons in the reaction medium, which in turn produces a characteristic alteration in the morphology and size of the nanoparticles. Further it is reported that since there is a big competition between protons and metal ions for forming bonds with charged regions at higher pH; therefore, alkaline pH results in higher synthesis of nanoparticles (Sintubin et al. 2009). It was also observed that a higher alkaline pH led to the synthesis of smaller nanoparticles during a very lesser time with low polydispersity index values. These specific features point toward enhanced stability, because of the electrostatic repulsion between the anions present within the dispersion (Gurunathan et al. 2009). In another study Colletotrichum sp. ALF2-6 was used for biosynthesis of nanoparticles employing alkaline pH. It was observed that synthesis was faster at alkaline pH and at temperature of 50 °C as compared to that at lower pH. The whole process was accomplished in around 20 min (Azmath et al. 2016). Similarly, in a research work performed by Birla et al. (2013), utilizing *Fusarium oxysporum*, highest nanoparticle synthesis was obtained between pH 9 and 11, whereas lower synthesis occurred at pH 7 including the aggregates formation between pH 3 and 5.

Conversely, Husseiny et al. (2015), during their study with the same fungus, observed that the rate of nanoparticle synthesis decreased with the increase in the pH because of the lower activity of the reductases enzyme involved in the synthesis at higher pH. Additionally few other studies have revealed appreciable synthesis of nanoparticles at neutral or slightly alkaline pH. Banu and Balasubramanian (2014) reported nanoparticles synthesis using *Isaria fumosorosea* at pH 8.5 with better

physicochemical characteristics, in comparison to nanoparticles synthesized at pH 4.5 and 6.5. Likewise during a synthetic procedure using *Guignardia mangiferae*, change in color was not observed between pH 1 and 4; however, color change began to appear at pH 5 and 6. With the increase in pH, the amount of the dispersion increased, with the nanoparticles possessing higher monodispersion and stability at pH 7 (Balakumaran et al. 2015).

26.28 Effect of Silver Nitrate ($AgNO_3$) Concentration

Most of the studies have examined the effect of silver nitrate ($AgNO_3$) concentration on the extracellular synthesis of silver nanoparticles employing fungi particularly at a concentration of 1 mM (Saxena et al. 2016; Xue et al. 2016). In a few cases, a lesser metal precursor concentration led to the synthesis of smaller sized nanoparticles and an enhanced dispersion (Kaviya et al. 2011; Phanjom and Ahmed 2017). AbdelRahim et al. (2017), while using the fungus *Rhizopus stolonifer*, produced the nanoparticles of smallest size (2.86 nm) at 10 mM concentration of $AgNO_3$. Further nanoparticles of 54.67 and 14.23 nm size were synthesized at 100 and 1 mM, respectively. Same conclusions were recorded by Husseiny et al. (2015) while using the fungus *Fusarium oxysporum*. Phanjom and Ahmed (2017) studied the synthesis of nanoparticles using *Aspergillus oryzae* and different $AgNO_3$ concentrations between 1 and 10 mM.

It was observed that at $AgNO_3$ concentrations up to 8 mM, the nanoparticles presented sizes between 7.22 and 17.06 nm, while the size increased to 45.93 and 62.12 nm at $AgNO_3$ concentrations of 9 and 10 mM, respectively. This effect was attributed to the lack of functional groups available for the reaction when the metal precursor concentration was increased. In a study employing *Fusarium oxysporum*, it was found that the quantity of nanoparticles increased as the precursor concentration was increased between 0.1 and 1.5 mM, while no differences were observed at higher concentrations (Birla et al. 2013). These findings suggest that there is a limit to the concentration of $AgNO_3$ used, in order to obtain nanoparticles with satisfactory physicochemical characteristics. The addition of excess amounts of metal ions results in very large nanoparticles with irregular morphology (AbdelRahim et al. 2017), due to competition between the silver ions and functional groups from the fungus filtrate (Shahzad et al. 2019). As the concentration of the metal precursor increases, so also does the intensity of color of the dispersion (Ahluwalia et al. 2014; Phanjom and Ahmed 2017).

26.29 Effect of the Culture Medium

It is well-known that microorganisms respond in different ways, depending upon the culture medium and the conditions of cultivation. Adjustments in these conditions cause the production of various metabolites and proteins (Costa Silva et al. 2017). During the synthesis of nanoparticles by fungi, substrate specific for the enzymes is

added in the medium which in turn induces their production and release by the fungus. Thus they enhance the reduction of silver and resulting in the formation of silver nanoparticles (Husseiny et al. 2015). Similarly Hamedi et al. (2017), during their work cultivated *Fusarium oxysporum* on a medium manipulated to enhance the nitrate reductase enzyme activity. The nanoparticle suspension formed by employing the fungal filtrate of the fungus grown in the enzyme inducing medium depicted higher concentrations and particularly smaller sized nanoparticles. This change was credited to the enhancement of the enzymatic activity through the modification of nitrogen source in the medium, thereby elevating the rate of nanoparticle synthesis. Different responses were recorded in studies involving the use of different media for the fungal cultivation. In a similar study Saxena et al. (2016) performed synthesis of silver nanoparticles, via cultivated *Sclerotinia sclerotiorum*, in various broths resulting in the highest production of nanoparticles within the potato dextrose medium. The chitin supplemented filtrate was found to contain around three times more protein and depicted increased production of nanoparticles. Likewise Birla et al. (2013) examined ten different media for cultivation of *Fusarium oxysporum*, thereby observing huge production of silver nanoparticles by utilizing the filtrate from the fungus cultured in MGYP medium.

26.30 Effect of the Quantity of Biomass

The quantity of biomass employed has an effect on the silver nanoparticles synthesis and characteristics. On the one hand where few studies have indicated increased nanoparticle synthesis in the presence of lower biomass concentrations, on the other hand many have found elevated synthesis rates in the presence of higher biomass concentrations (Birla et al. 2013; Korbekandi et al. 2013; Balakumaran et al. 2015; Elamawi et al. 2018). In one such study Balakumaran et al. (2015) suspended 10, 20, and 30 g quantities of *Guignardia mangiferae* biomass in 100 mL of water and obtained their filtrates, respectively. These filtrates were separately subjected to silver nanoparticles synthetic procedures resulting in the synthesis of silver nanoparticles with better physicochemical characteristics in the filtrate of lower biomass concentration. Similarly Shahzad et al. (2019) utilized filtrates of 1, 4, 7, and 10 g biomass of *Aspergillus fumigatus* BTCB10 observing larger production of silver nanoparticles with smaller size and better dispersion in the filtrate obtained from mixture containing 7 g of biomass. During the research it was recorded that while using a higher biomass concentration of *Penicillium oxalicum*, increased nanoparticle production was obtained which was considered due to better release of the nitrate reductase enzyme by the mycelium. In a similar study Saxena et al. (2016) obtained higher silver nanoparticle production in the presence of increased *Sclerotinia sclerotiorum* biomass. Correlation between the biomass and the release of biomolecules responsible for the synthesis nanoparticles has been reported by Birla et al. (2013).

26.31 Applications of Biosynthesized Nanomolecules

Nanoparticles synthesized using fungi particularly the silver nanoparticles have a variety of potential applications in the areas of medicine, agriculture, optics, electronics, etc. As a consequence of large amount of metabolites produced from fungi, they present to be an attractive source for nanoparticle synthesis. An additional factor under consideration is the capability of fungi to produce antibiotics which remain present within the capping, thereby playing a significant role in the nanoparticle stabilization. Several studies related to biological synthesis of nanoparticles employing fungi have revealed outcomes that show promising applications in controlling pathogenic fungi and bacteria, combating cancer cells and viruses, and providing larvicidal and insecticidal activities.

26.32 Health Applications

A number of studies have demonstrated the applications of biogenic silver nanoparticles in the sectors of health, especially the control of pathogenic bacteria and fungi. The growth of bacterial cells is directly inhibited by the nanoparticles, which make contact with the cytomembrane and lead to stepwise metabolic responses, resulting in the assembly of reactive oxygen species (Gudikandula et al. 2017). The size of nanoparticles directly affects their antimicrobial potential, because smaller nanoparticles produce larger effects (Lu et al. 2013). Small sized nanoparticles can pass through the semipermeable membrane of bacterial cells and harm the respiratory chain, modify permeability, cause DNA and RNA damage, affect biological process, and ultimately result in necrobiosis (Morones et al. 2005; Rai et al. 2009).

Antifungal compounds (such as biogenic silver nanoparticles) that are synthesized from sustainable biological sources are often cost-effective and secure alternative for the systemic treatment of external and internal fungal infections, thus enabling the control of resistant fungi (Ashajyothi et al. 2016). The poisonous ions attach to sulfur containing proteins, disturbing the permeability of cell, thereby leading to modifications of the DNA replication mechanism. Additionally the inhibition of enzymatic activity is because of the binding of nanoparticles with thiol groups. As a result of this inactivation an oxidative stress develops, which in turn affects electron transport and protein oxidation (Rai et al. 2012). The potential activity of silver nanoparticles synthesized using the fungus *Guignardia mangiferae* against gram-negative bacteria was reported by Balakumaran et al. (2015). The adverse effects produced by nanoparticles included increased permeability, alteration of membrane transport, and release of nucleic acids.

Sometimes the peptidoglycans constituting the cytomembrane act as an obstacle and inhibit the internalization of nanoparticles, thereby lowering their side-effects toward gram-positive bacteria. While analyzing the antimicrobial activity of silver nanoparticles synthesized using *Aspergillus niger*, Gade et al. (2008) recorded efficient inhibitory activity toward the bacteria *E. coli* and *S. aureus* similar to that

observed in case of antibiotic gentamicin. Use of silver nanoparticles in combination with antibiotics and antifungal substances also represents a probable solution to the emerging resistance toward these drugs. Biosynthesized silver nanoparticles using candida were tested for their inhibitory activity along with the antibiotic ciprofloxacin against *Staphylococcus aureus, Escherichia coli, Bacillus cereus, Vibrio cholerae, and Proteus vulgaris*. It was observed that the combination of antibiotic improved the activity of nanoparticles, as compared to that when used alone.

Similarly it was examined the antimicrobial and antifungal potential of silver nanoparticles biosynthesized by using the filtrate of *Aspergillus flavus*. These mycosynthesized nanoparticles were efficient in inhibiting the activity of bacteria *Bacillus cereus, Bacillus globigii, Enterobacter aerogenes, escherichia, and Staphylococcus aureus.* Out of these the highest activity was exhibited against *B. subtilis and E. coli*. It was also observed that the activity was dependent on concentration, with optimum outcomes obtained while employing the nanoparticles in combination with the antibiotic tetracycline, in spite of using alone. Likewise concentration dependent activity of the nanoparticles was also obtained against fungi *Aspergillus niger* and *Trichoderma harzianum*. Silver nanoparticles synthesized by using the fungi *Trametes ljubarsky* and *Ganoderma enigmaticum* for the control of gram-positive and gram-negative bacteria, namely *Bacillus subtilis, Staphylococcus aureus, Micrococcus luteus, Bacillus cereus, Bacillus megaterium, E. coli, Enterobacter aerogenes, Klebsiella pneumoniae, Salmonella typhimurium, Proteus vulgaris, Pseudomonas aeruginosa, and Salmonella paratyphi* Gudikandula et al. (2017).

Both types of nanoparticle possessed potential antibacterial activity. Moreover biologically synthesized silver nanoparticles have depicted effective activity even against the multidrug resistant microorganisms. For example, an optimized process for the synthesis of silver nanoparticles wherein *Penicillium sp*. was used for the synthesis of silver nanoparticles having potential activity against the multidrug resistant bacterial species, viz. *E. coli* and *S. aureus* was developed. Besides their antimicrobial activity, biosynthesized silver nanoparticles are also reported to induce profound effects on tumor cells. In a similar study Husseiny et al. (2015) recorded the antibacterial and antitumor activity of silver nanoparticles synthesized using *Fusarium oxysporum*. These nanoparticles were efficient in inhibiting the growth of *E. coli* and *S. aureus*, as well as a tumor cell line. After the exposure of tumor cells to the nanoparticles, the coffee IC50 value (121.23 μg cm^3) for MCF-7 cells (human breast adenocarcinoma) was obtained which revealed elevated cytotoxicity and also the potential tumor control activity of biosynthesized nanoparticles. These activities were attributed to the participation of the silver nanoparticles in disruption of the mitochondrial respiratory chain, which in turn causes the congregation of reactive oxygen species, thereby leading to the hindrance of the nucleotide (ATP) synthesis, following the damage of the nucleic acids (Husseiny et al. 2015).

26.33 Agriculture and Pest Control Applications

Several research works have been carried out to assess the potential of biosynthesized silver nanoparticles for the regulation of and other pests harmful for agriculture. Silver nanoparticles synthesized using the fungus *Aspergillus versicolor* exhibited inhibitory effects against the phytopathogenic fungi *Sclerotinia sclerotiorum* and *Botrytis cinerea* causing disease in strawberry plants. These nanoparticles demonstrated concentration dependent activity against both pests, with the highest activity against *B. cinerea* (Elgorban et al. 2016). In a similar study Qian et al. (2013) mycosynthesized silver nanoparticles utilizing the fungus *Epicoccum nigrum* and recorded their activity against some pathogenic fungi, namely *C. albicans, Fusarium solani, Sporothrix schenckii, Cryptococcus neoformans, Aspergillus flavus*. Recent studies have investigated the potential activities of mixture including biogenic nanoparticles and conventional biopesticides. Likewise the potential activity of mycosynthesized silver nanoparticles by the fungus *Alternaria alternata* combined with the antifungal compound fluconazole, against the deadly phytopathogenic fungi *Phoma glomerata*, *Phoma herbarum*, and *Fusarium semitectum* was recorded.

26.34 Environmental Applications

Besides a number of medical applications biosynthesized nanoparticles (BNPs) have also been investigated by many researchers for their application in the sector of environmental remediation (Njagi et al. 2011; Hussain et al. 2016). Recent researches illustrate two major ways for clean-up and rejuvenation of contaminated sites by employing BNPs, the first one includes adsorption of the pollutant and the second one involves contaminant degradation or dehalogenation. Adsorption is also a dreadfully attractive approach to stimulate strong contaminants, since it is characteristically extremely competent, leading to the decrease in chemical sludge concentrations without the requirement of any strong technological knowledge. However researchers were able to eliminate heavy metals using cellulose BNPs and nanoparticles synthesized from microbial biomass. Lately bacterial cellulose nanofibers (BCF), researchers have drawn attention of most of the researchers because of their unique structure and properties. This BNP consists of a nanofibrillar structure, possessing elevated mechanical strength, increased surface area to volume ratio, inherent environmental inertness, and will be easily functionalized by incorporating chemical moieties which is able to increase the binding efficiency of pollutants.

These properties are favorable for adsorbents used in the remediation of environmental contaminants (Shah and Brown Jr 2005). Carboxylation is the conventionally employed method to increase the adsorption capacity of cellulose. Srivastava and collaborators depicted one more case of successful carboxylation which caused enhanced heavy metal removal. It was indicated further that the capacity of removal of heavy metals, viz. Ni^{2+}, Cr^{3+}, Cd^{2+}, and Pb^{2+} after incorporation of carboxylic

groups into cellulose was increased by 3–10 times as compared to that of the unmodified NPs (Srivastava et al. 2012). Similarly, it was observed that the presence of carboxylate groups within the cellulose generates a material capable of attaining 2–3 times greater removal capacity of UO_2^{2+} than conventional adsorbents (i.e. silica particles, hydrogels, polymer particles, and montmorillonite). Conversely, researchers also revealed that the integration of cysteine, containing thiol groups, was efficient for the removal of Cr (VI) and Pb (II) (Yang et al. 2014).

Some studies have also depicted the removal of pollutants from aqueous environments by employing non-active (dead) microbial mass for biosorption (Gupta and Suhas 2009; Ngah et al. 2011). The exceptionally high surface area to volume ratio of BNPs and a number of functional groups (carboxyl, hydroxyl, sulfate, phosphate, and others) within these nanoparticles having high affinity for these pollutants are being considered favorable properties behind their potential activity. In addition to adsorption, researchers have also demonstrated the application of BNPs for the successful removal of contaminants from the water sources, soils, and sediments.

In a recent research BNPs of Pd have been investigated for remediation purposes as a consequence of widespread application of Pd as a catalysts in chemistry for processes like dehalogenation, reduction, etc. (Mabbett et al. 2004; De Windt et al. 2006). The majority research works have aimed on commercial use of biosynthesized Pd nanoparticles for the dehalogenation of contaminants from wastewater, groundwater including soil remediation.

26.35 Concluding Remarks and Future Challenges

Microbes possess an outstanding capacity for the synthesis of metal nanoparticles, having widespread applications in different areas. Therefore in the present scenario, mycogenesis of nanoparticles has become a center of attraction as a biological source of various metal nanoparticles. Mycosynthesized nanoparticles have exhibited efficient and promising potential applications in a number of fields, namely agriculture, food, textile, medicine, cosmetics, optical, and electronics. The advantages of fungi mediated synthesis of nanoparticles include easy handling and downstream processing. Recently, nanoparticles have been designed using metals, some metal sulfides, and only a few oxides. This has led to the emergence of the need to develop procedures for noble synthesis of nanostructures using other metal oxides, nitrides, carbides, etc. Biosynthesis of nanoparticles utilizing different biological agents presents a secure, comparatively cost-effective, and eco-friendly approach. Among all the biological agents fungi are reported to be considerably good medium for the synthesis of nanoparticles applicable in several fields. Moreover development of smart drug delivery system to be delivered to a particular site has proven to be helpful in the field of disease diagnosis. Construction of smart biosensors and detection systems is indeed advantageous to protect agricultural crops from the harmful effects of insects and pathogens.

Precisely it can be concluded that myconanotechnology is still a developing field. The applications of nanoparticles are continuously increasing but studies on their toxic effects, collection within the environment and their effect on human health and animals need to be carried out in near future. In addition the biosynthesized nanoparticles can also be applied for the treatment of deadly diseases which will pave the ways for the new opportunities in the field of biomedicine. Also, these metal nanoparticles would provide a probable answer for the prevailing energy crisis by presenting themselves as efficient energy-driven devices. A review of past researches have also revealed that a significant work has been done for testing the in vitro applications of nanoparticles but considerably less data is available on in vivo applications. Further, a detailed study is still required to clarify and intricate the knowledge and functions of nanomaterials to achieve various landmarks in the fields of medicines, agriculture, cosmetics, electronics, environment, etc. In spite of a vast range of advantages associated with fungal-mediated synthesis of metal nanoparticles, there are certain variety of limitations and challenges also. Conversely more research is required to achieve optimized reaction conditions to obtain nanoparticles of better size, shape, and monodispersity.

Therefore according to the reports available from several research works carried out it could be inferred that the biogenic synthesis of silver nanoparticles using fungi offers several advantages and has promising applications in number of areas including health and agriculture. Moreover these nanoparticles possess extraordinary stability because of the bioactive compounds present as capping agents derived from the fungal biomass. Hence fungal systems present a cost-effective, eco-friendly, and green synthetic approach for the synthesis of variety of metal nanoparticles. But at the end of the day extensive research works need to be performed to explore more information about the vast biodiversity of fungal flora and the mechanism by which they synthesize nanoparticles.

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Fungal Extremozymes: A Potential Bioresource for Green Chemistry

27

Imran Mohsin and Anastassios C. Papageorgiou

Abstract

Green chemistry is a rapidly evolving area that aims to reduce the hazards released to the environment during various chemical processes and improve the efficiency of bioconversion. Application of green chemistry could therefore lead to less environmental pollution and better economic outcomes. Enzymes, as biocatalysts, are expected to play a central role in green chemistry owing to their reusability, catalytic efficiency, and specificity. Besides, biocatalytic reactions result in no toxic waste in contrast to chemical processes that require careful disposal. However, the use of enzymes in chemical reactions presents various challenges, including stability and unwanted side-reactions. Fungi have drawn significant attention in recent years as a new source of enzymes that could be used in harsh conditions to improve various industrial processes, such as biofuel production and biomass conversion. Combined with modern bioengineering techniques, fungal extremozymes have emerged as promising tools in future applications. Also, structural information has provided new insights into the function and stability of various fungal extremozymes. This review is focused on latest progress in fungal extremozymes, in particular their structural features as well as the current research efforts to improve their properties for better use in green chemistry applications.

Keywords

Fungus · Biocatalysis · Crystal structure · Biomass degradation · Enzyme improvement · Enzyme stability

I. Mohsin · A. C. Papageorgiou (✉)
Turku Bioscience Centre, University of Turku and Åbo Akademi University, Turku, Finland
e-mail: anapap@utu.fi

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27.1 Introduction

Green chemistry has become a worldwide approach toward sustainable growth. New processes using materials which do not emit pollutants and toxic waste to the environment are being pursued. The development, however, of new safer methods that produce less harmful products is not an easy task and presents many challenges. Enzymes have become a great tool for scientists in the field of green chemistry (Table 27.1) (Anna Calarco 2015). As enzymes act as biocatalysts, they require mild conditions to function; thus, they can save key resources such as energy or water (Sheldon and Woodley 2018). Also, enzymes are attractive alternatives owing to their minimal impact on the environment and low costs. Taking into account the benefits of green chemistry, enzyme biocatalysis is now found in various traditional chemical processes in several fields (Cipolatti et al. 2019). This change is going to expand to even more areas owing to new emerging technologies in enzyme engineering.

Fungi have received significant attention in recent years (de Cassia Pereira et al. 2015; Hyde et al. 2019), especially as a reservoir of extremozymes for use in many biotechnological applications (Table 27.2). Thermophilic fungi, in particular, have drawn considerable interest owing to their ability to grow at a temperature of 50 °C or above and at a minimum of 20 °C or above (Maheshwari et al. 2000). Various thermophilic fungi have been isolated in recent years and the enzymes they produce have been investigated at functional and structural level. In this review, an up-to-date information on structure–function aspects of fungal extremozymes is presented and the current research efforts to improve their properties for better use in green chemistry applications are discussed.

27.2 Cellulases

The enzymatic hydrolysis of cellulose to its constituent monosaccharides for the low-cost production of food and biofuels has attracted considerable attention in recent years. Cellulose is the most abundant and renewable non-fossil carbon source on Earth and accounts for 20–50% in dry weight of the plant cell wall material. Compared to current industrial procedures such as heat, mechanical, and acid treatment of cellulose, cellulose degradation by enzymes is considered a more environment-friendly process (Wilson 2009). However, cellulose is the most refractory carbohydrate polymer to enzymatic degradation among all polysaccharides of the plant cell wall. Thus, efficient enzymatic degradation of cellulose to glucose requires the synergistic action of endocellulases (E.C.3.1.1.4), exocellulases (cellobiohydrolases, CBH, E.C.3.2.1.91), and β-glucosidases (E.C.3.2.1.21). Endocellulases initiate hydrolysis by cutting internal glycosidic linkages in a random fashion, resulting in a swift decrease of polymer length and a gradual increase in the reducing sugar concentration. Exocellulases act upon either the reducing or the non-reducing ends to release cello-oligosaccharides and cellobiose units. In the

Table 27.1 Enzymes with potential applications in green chemistry

EC number	Enzyme class	Catalyzed reactions	Representative enzymes	Polymer synthesis	Polymer modification
1	Oxidoreductases	Redox reactions by electron transfer	Peroxidase, laccase, tyrosinase, glucose oxidase	Yes	Yes
2	Transferases	Transfer of a functional group from one compound (donor) to another compound (acceptor)	Phosphorylase, glycosyltransferase, acyltransferase	Yes	Yes
3	Hydrolases	Hydrolysis of various bonds	Glycosidase (cellulase, amylase, xylanase, chitinase, hyaluronidase), lipase, protease, peptidase, feruloyl esterase, nitrilase	Yes	Yes
4	Lyases	Cleavage of C–C, C–O, C–N, and other bonds by using other than hydrolysis or oxidation	Decarboxylase, aldolase, dehydratase	No	No
5	Isomerases	Either racemization or epimerization of chiral centers; isomerases are subdivided according to their substrate specificity	Racemase, epimerase, isomerase	No	Yes
6	Ligases	Linking of two molecules with concurrent hydrolysis of the diphosphate-bond in ATP or a similar triphosphate	Ligase, synthase, acyl CoA synthase	No	No

Table 27.2 Fungal enzymes with industrial importance

Enzyme	Preferred Properties	Representative fungi	Applications
Cellulases	Thermostable	*Melanocarpus albomyces, Chaetomium thermophilum, Humicola insolens*	Biofuel, biorefinery, paper and pulp industry
Xylanases	Alkaliphilic, thermophilic	*Humicola insolens, Melanocarpus albomyces, Sporotrichum thermophile*	Fuels, chemicals, paper, brewing industry
Feruloyl esterases	Thermophilic	*Talaromyces cellulolyticus, Sporotrichum thermophile*	Food, cosmetics, chemical synthesis
Laccases	Thermophilic, alkaliphilic	*Sporotrichum thermophile, M. albomyces, C. thermophilum, T. versicolor*	Bioenergy, pulp and denim treatment, food and beverages, wastewater, bioenergy
Lipases	Thermotolerant	*Candida* sp., *Penicillium* sp., *Aspergillus* sp.	Detergents, therapeutics, food supplements
Nitrilases	Thermostable	*Ascomycota*	Organic synthesis, bioremediation
Transaminases	Thermostable	*Thermomyces stellatus*	Organic synthesis
Tyrosinases	Thermostable	*Agaricus bisporus*, *Neurospora crassa*, *Aspergillus oryzae, Aspergillus niger*	Organic synthesis, bioremediation
Keratinases	Thermotolerant, alkaliphilic	*Aspergillus fumigatus*, *Myceliophthora thermophila*	Animal feed, medicine, detergents

last step, β-glucosidases cleave cellobiose to release glucose molecules (Vlasenko et al. 2010).

Owing to the difficulties for its breakdown, cellulose is subjected to higher temperatures to swell and become more susceptible to breaking. Besides, the use of higher temperatures in industrial processes offers additional advantages such as substrate and product solubility, reduced hydrolysis time, and minimum risk of microbial contamination. Enzymatic hydrolysis by thermophilic cellulases has therefore become a key step for efficient biomass degradation (Atalah et al. 2019).

Cellulases are classified into 13 glycoside hydrolase (GH) families (http://www.cazy.org): 1, 3, 5, 6, 7, 8, 9, 12, 26, 44, 45, 48, and 61. Thermophilic fungal cellulases are found in families 1, 3, 5, 6, 7, 12, 45, and 61 (Li and Papageorgiou 2019). GH61 family members are now identified as Cu(II) ion-dependent lytic polysaccharide monooxygenases (LPMOs) and have been included in the auxiliary activity families of the CAZy database (Busk and Lange 2015).

27.2.1 Cellulase Production and Characterization

Heterologous host organisms, such as *E. coli*, yeast, and filamentous fungi have been used for the expression of cloned cellulase genes of thermophilic fungi (Li and Papageorgiou 2019). The majority of the recombinant cellulases expressed in yeast and filamentous fungi are glycosylated (Li et al. 2009; Takashima et al. 1999). Glycosylation could be a contributing factor to the thermostability improvement of cellulases according to previous reports (Meldgaard and Svendsen 1994). The mechanism, however, is still unknown. It has been reported that N-glycosylation could increase solubility and reduce aggregation (Ioannou et al. 1998; Kayser et al. 2011). Also, analysis of protein structures deposited in the Protein Data Bank has also indicated a decrease in protein dynamics upon N-glycosylation without significant global or local structural changes (Lee et al. 2015).

Thermophilic fungal cellulases are usually single polypeptides although some β-glucosidases exist as dimers (Gudmundsson et al. 2016; Mamma et al. 2004). The molecular weight of thermophilic fungal cellulases has a wide range (30–250 kDa) with different carbohydrate contents (2–50%). The majority of the purified cellulases from thermophilic fungi exhibit similar optimal pH and temperature. Indeed, thermophilic fungal cellulases are found active in the pH range 4.0–7.0 and display a high-temperature maximum at 50–80 °C for activity (Li and Papageorgiou 2019). Also, they exhibit remarkable thermal stability and are stable at 60 °C with longer half-life at 70, 80, and 90 °C than those from mesophilic fungi.

27.2.2 Primary and Three-Dimensional (3D) Structure

27.2.2.1 Primary Structure

Most cellulases are characterized by a modular structure. Typically, endocellulases and cellobiohydrolases consist of four modules: a signal peptide that facilitates secretion, a cellulose-binding domain (CBD) for the enzyme's attachment to the substrate, a catalytic domain (CD) used for the hydrolysis of the substrate, and a hinge region (linker) which is usually post-translationally glycosylated and rich in Ser, Thr, and Pro residues.

CBDs consist of less than 40 amino acids and interact with cellulose through a flat platform-like hydrophobic binding site that is thought to be complementary to the flat surfaces presented by cellulose crystals (Shoseyov et al. 2006). Studies have shown that deletion of the CBDs present in *T. reesei* Cel7A and Cel6A and *Humicola grisea* CBH1 reduces significantly enzymatic activity toward crystalline cellulose (Takashima et al. 1998), suggesting that the efficient hydrolysis of crystalline cellulose requires tight interactions to cellulose through the CBDs. Aromatic residues in CBDs have been suggested to affect the cellulose-binding ability and enzymatic activity (Takashima et al. 2007).

Variations in the primary structure have been identified. *Talaromyces emersonii* CBHII, for instance, has a modular structure (Murray et al. 2003), whereas *T. emersonii* CBH1 consists solely of a catalytic domain (Grassick et al. 2004).

Similar variations are found in *Chaetomium thermophilum* CBHs (CBH1, CBH2, and CBH3) where CBH1 and CBH2 consist of a typical CBD, a linker, and a CD, whereas CBH3 only comprises a catalytic domain and lacks a CBD and a hinge region (Li et al. 2009). However, cellulases without CBDs can still be efficiently used (Le Costaouëc et al. 2013; Pakarinen et al. 2014).

27.2.2.2 Three-Dimensional (3D) Structural Details

3D structures of thermophilic fungal cellulases from families 1, 5, 6, 7, 12, and 45 have been reported. Details have been recently reviewed (Li and Papageorgiou 2019).

27.2.3 Improvement of Thermophilic Fungal Cellulases

Two main research approaches are presently in use for improvement and modification of enzyme function: structure-based rational site-directed mutagenesis and random mutagenesis through directed evolution. Detailed knowledge of the 3D structure of a protein is required for site-directed mutagenesis. In contrast, the directed evolution approach is not limited by the absence of structural details but requires an efficient method for high-throughput screening (Labrou 2010).

27.2.3.1 Thermostability Improvement

Generally, the mechanism of protein thermostability has been studied more extensively in thermophilic bacteria and hyperthermophilic archaea (Pack and Yoo 2004; Trivedi et al. 2006). However, a common thermostability mechanism has not yet been established. Several contributing factors to protein thermostability have been proposed. An increase in ion pairs on the protein surface and a more hydrophobic interior have been put forward as the major factors of improved protein thermostability (Taylor and Vaisman 2010). Nevertheless, compared with thermophilic proteins from thermophilic bacteria and hyperthermophilic archaea, the understanding of the nature and mechanism of thermostability in proteins from thermophilic fungi is relatively poor and additional studies are needed.

Despite that cellulases from thermophilic fungi are already thermostable, additional increase of their thermostability is desirable for industrial applications. Use of error-prone PCR, for example, in *Melanocarpus albomyces* Cel7B resulted in the improvement of the enzyme and the identification of two positive thermostable mutants (Voutilainen et al. 2007). Also, introduction of extra disulfide bridges to the catalytic module of *Talaromyces emersonii* Cel7A gave rise to three mutants with improved thermostability as revealed by Avicel hydrolysis efficiency at 75 °C (Voutilainen et al. 2010).

Mutants of three Cys residues of the thermostable *Humicola grisea* Cel12A were found to affect the stability of the enzyme (Sandgren et al. 2005). A report of fold-specific thermostability through variations in amino acid compositions of endoglucanases has provided new strategies for thermostability improvement (Yennamalli et al. 2011).

Random mutagenesis and recombination of beneficial mutations were employed for the construction of a chimeric Cel6A cellobiohydrolase (Wu and Arnold 2013). Consequently, increased hydrophobic interactions and reduced loop flexibility by introduction of Pro residues were found to improve thermostability.

A computational approach, namely SCHEMA, which uses protein structure data to generate new sequences with minimal structure disruption when they are introduced in chimeric proteins has been employed to create thermostable fungal cellulases (Heinzelman et al. 2009). Application of SCHEMA using the high-resolution structure of *Humicola insolens* CBHII as a template resulted in a collection of CBHII chimeras with high thermostability (Varrot et al. 2003).

Improvement of cellulase stability in detergent solutions has also been pursued (Otzen et al. 1999). The anionic surfactant C12-LAS can inactivate *H. insolens* Cel45 endoglucanase because of the positive charges at the surface of the enzyme (Otzen et al. 1999). Mutation of surface residues R158E/R196E was found to improve stability, most likely by preventing C12-LAS from binding to the protein.

27.2.3.2 Activity Improvement

Improvement of cellulase activity has been pursued in recent years by using site-directed mutagenesis and directed evolution. However, the lack of general rules for site-directed mutagenesis and the limitations of screening methods have resulted in only a few successful examples of cellulase mutants with considerably higher activity than the wild-type enzymes (Percival Zhang et al. 2006). Directed evolution of *Chaetomium thermophilum* CBHII produced mutants that were able to retain more than 50% of their activity at 80 °C for 1 h, while the wild type abolished all of its activity under the same conditions (Wang et al. 2012).

Addition or replacement of a CBD to alter the enzyme characteristics and to improve hydrolytic activity has also been tried (Limon et al. 2001; Shoseyov et al. 2006; Szijarto et al. 2008; Takashima et al. 1999).

27.2.4 Glycosidic Bond Synthesis

Glycosynthases are engineered enzymes able to catalyze the synthesis of glycosidic bonds (Hayes and Pietruszka 2017). They are best described as retaining GH mutants in which the catalytic nucleophile (Asp, Glu) has been replaced by a non-nucleophilic residue, usually a smaller uncharged amino acid. Cellulase engineering to produce glycosynthases by site-directed mutagenesis has been actively pursued (Shaikh and Withers 2008). The first glycosynthase from thermophilic fungus was derived from *Humicola insolens* Cel7B following mutation of catalytic residue Glu197 to Ala. The resultant Cel7B E197A glycosynthase was able to catalyze regio- and stereo-selective glycosylation in high yield (Fort et al. 2000). Three mutants of the *H. insolens* Cel7B E197A glycosynthase, namely E197A/H209A and E197A/H209G double mutants, and Cel7B E197A/H209A/A211T triple mutant, were subsequently produced and characterized (Blanchard et al. 2007). The results suggested that appropriate active site mutations could modulate

the regioselectivity of the glycosylation reaction. Apart from glycosynthases, use of β-glucosidases for the synthesis of various glycoconjugates, such as alkyl glucosides and aminoglycosides, has also been pursued in recent years. A GH3 β-glucosidase from the thermophilic fungus *Myceliophthora thermophila* was found to act as an efficient biocatalyst in alkyl glycoside synthesis (Karnaouri et al. 2013).

27.3 Xylanases

Xylanases (EC 3.2.1.8; endo-β-1,4-xylanases) are the main enzymes that hydrolyze internal bonds in xylan, a particularly resistant to degradation component of plant cell walls and the second most abundant polysaccharide in nature after cellulose (Chadha et al. 2019). Apart from their use in the conversion of lignocellulose biomass into fermentable sugars, xylanases have also been successfully employed in the saccharification of agrowaste, such as wheat straw, corn cobs, birch, and spruce biomass.

Xylanases are found in GH families 3, 5, 8, 9, 10, 11, 12, 16, 26, 30, 43, 44, 51, 62, 98, and 141. Their majority, however, belongs to the GH10 and GH11 families based on sequence considerations. GH11 xylanases are more specific than GH10 xylanases as the former do not act on substituted forms of xylans.

Sporotrichum thermophile (syn *Myceliophthora thermophila*), a major thermophilic fungus isolated from soil in eastern Russia, is a powerful cellulolytic organism that produces a variety of enzymes with immense industrial potential (Karnaouri et al. 2014). *S. thermophile* synthesizes a complete set of enzymes, including GH10 and GH11 xylanases (Bala and Singh 2016). Characterization of *S. thermophile* GH10 and GH11 xylanases has been reported (Basit et al. 2018).

A xylanase from the thermophilic and thermotolerant fungus *Myceliophthora heterothallica* F.2.1.4. has been purified and characterized (de Oliveira Simões et al. 2019). The enzyme has 27 kDa MW and displays maximum activity at pH 4.5 and 65–70 °C. It maintains more than 80% of its residual activity when exposed to (1) temperatures between 30 and 60 °C for 1 h and (2) pH 5–10 for 24 h at 4 and 25 °C.

A GH10 xylanase PspXyn10 produced by the mesophilic fungus *Penicillium* sp. has been characterized and found to exhibit thermostability (Shibata et al. 2017). The molecular weight of PspXyn10 was estimated to be 55 kDa and found to contain a CBM. Its optimal temperature and pH for xylanase activity were 75 °C and pH 4.5, respectively. PspXyn10 retained more than 80% of its xylanase activity after incubation at 65 °C for 10 min.

A purified xylanase produced by *T. aurantiacus* M-2 was found to be acidophilic and thermostable (Ping et al. 2018). Its relative molecular mass was approximately 31.0 kDa. The purified xylanase exhibited maximum activity at 75 °C and pH 5.0, and it was stable after treatment at a pH range from 2.0 to 10.0 or a temperature range from 30 to 80 °C for 2 h. A GH10 xylanase from *Aspergillus fumigatus* var. *niveus* (AFUMN-GH10) contains no carbohydrate-binding module. The enzyme was able

to retain its activity in a pH range from 4.5 to 7.0, with an optimal temperature at 60 °C (Velasco et al. 2019).

27.3.1 Structural Details

Homology modeling studies of GH10 and GH11 xylanases from S. *thermophile* suggested structural similarities and only minor differences with other fungal xylanases (Basit et al. 2018). The crystal structure of a GH10 xylanase from the fungus *Fusarium oxysporum* (PDB id 3u7b) has been reported (Dimarogona et al. 2012). The structure is similar to that of other GH10 xylanases and is characterized by a $(\beta/\alpha)_8$-barrel fold. Differences have been identified in the loop regions. Sequence alignment and homology modeling suggested the presence of a long loop between strand β6b and helix α6 that may play a role in the catalytic efficiency of the enzyme.

The GH11 xylanases exhibit a β-jelly-roll fold. The structure of xylanase XynCDBFV, a GH11 xylanase from ruminal fungus *Neocallimastix patriciarum* has been determined and the catalytic residues Glu109 and Glu202 were identified (Cheng et al. 2014). The structure of a GH11 xylanases from *Fusarium oxysporum* has been elucidated to 1.56-Å resolution and deposited to the PDB (pdb ID 5jrm; Gomez S, Payne AM, Savko M, Fox GC, Shepard WE, Fernandez FJ, Vega MC, unpublished). The structure of an acidophilic GH11 xylanase (XynC) from *Aspergillus kawachii*, a filamentous fungus used for brewing the Japanese distilled spirit shochu, has been determined to 2.0 Å resolution (Fig. 27.1). The structure is characterized by a negatively charged surface, which was postulated to be responsible for the stability of the enzyme in acidic environments (Fushinobu et al. 1998).

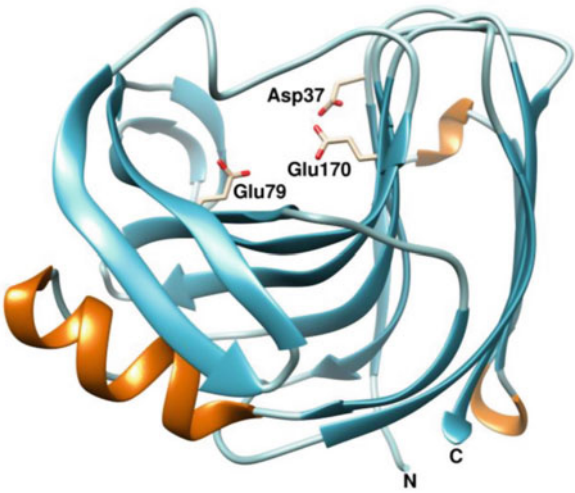

Fig. 27.1 Crystal structure of *Aspergillus kawachii* XynC (PDB id 1bk1). The catalytic residues Glu79 (nucleophile), Glu170 (acid-base catalyst), and Asp37 are shown as sticks and labeled. N- and C-termini are depicted. Figures of crystal structures were created with Chimera (Pettersen et al. 2004)

27.3.2 Xylanase Improvement

The GH10 xylanase Xyn10A_ASPNG from *Aspergillus niger* (Song et al. 2015) was subjected to iterative saturation mutagenesis (ISM). After four rounds of ISM, a quintuple mutant 4S1 (R25W/V29A/I31L/L43F/T58I) was generated with improved thermostability compared to the wild type. The 4S1 mutant retained 30% of its initial activity after 15 min heating at 65 °C and its melting temperature T_m increased by 17.4 °C compared to the wild type. For comparison, the wild-type enzyme retained 0.2% of its initial activity after heat treatment for 10 min at 60 °C and was completely inactivated after 2 min at 65 °C. Although each of the five mutations in 4S1 was found to contribute to thermoresistance, their synergistic action was suggested to be responsible for the dramatic improvement of the 4S1 enzyme thermotolerance.

An N-terminal region (NTR) has been identified in XynCDBFV from the ruminal fungus *Neocallimastix patriciarum* (Cheng et al. 2014). In the truncated mutant, it was clearly shown that the NTR plays a role in the catalytic activity of XynCDBFV and is required for the thermophilic functions of the enzyme. Removal of NTR resulted in a truncated mutant that retained 61.5% and 19.5% enzymatic activity at 55 and 75 °C, respectively, compared with the wild-type enzyme. Elimination of a disulfide bond in the C4A/C172A mutant resulted in 23.3% activity. These results suggested that NTR plays a role in XynCDBFV thermostability, and the Cys-4/Cys-172 disulfide bond is critical to the NTR-mediated interactions. Further modifications included four single mutants by substituting residues from 87 to 90 by site-directed mutagenesis (Han et al. 2019). Temperature stability measurements showed promising enhancement of thermostability for all four single mutants. The mutants retained 50% of their activities after incubation at the optimal temperature 60 °C for 1 h, while the retained activity for wild-type XynCDBFV was only 20.94% at the same condition. The increase in thermostability was attributed to a novel hydrogen bonding interaction. However, the enzyme activity of the single mutants was compromised with their thermostability. Combined mutations displayed an antagonistic effect due to the closed contact of the mutated residues.

A novel GH11 endoxylanase (Liu et al. 2019) was constructed via DNA shuffling by using the catalytic domains of two xylanases as parent sequences: *Bacillus amyloliquefaciens* xylanase A (BaxA), which is mesophilic and xylanase A (TfxA) from *Thermomonospora fusca*, a thermophilic soil bacterium. Notably, TfxA, one of the most thermostable xylanases, was able to retain 96% of its catalytic activity after incubation at 75 °C for 18 h.

A thermostable GH11 xylanase TlXynA from *Thermomyces lanuginosus* was mutated to improve its pH-tolerance using a rational structure-based approach (Wu et al. 2020).

A thermostable Xyn10A of *A. fumigatus* Z5, which belongs to GH10 family and has a CBM1 domain linked to its C-terminus by a Ser/Thr-rich linker was studied (Miao et al. 2018). Removal of the CBM1 domain had little effect on the thermostability but further truncation of the linker region significantly decreased its stability at high temperatures.

Protein engineering of a GH11 xylanase from *Aspergillus fumigatus* RT-1 was performed near the active site and at the N-terminal region to improve the catalytic efficiency of the enzyme toward pretreated kenaf (Damis et al. 2019). A 13.9-fold increase in catalytic efficiency for a double mutant showed the most effective hydrolysis reaction. The enhanced catalytic efficiency resulted in an increase in sugar yield of up to 28% from pretreated kenaf. In addition, another mutant showed improved thermostability and acid stability. Notably, these mutations were located at distances less than 15 Å from the active site and at putative secondary binding sites away from the active site.

Lytic polysaccharide monooxygenases (LPMOs) are capable of breaking down xylans. A xylan-active LPMO from *Pycnoporus coccineus Pc*AA14B LPMO and a GH30_7 family xylanase (*Tt*Xyn30A) from *Thermothelomyces thermophila* were found to act synergistically with a family GH11 endoxylanase (*An*Xyn11) in the degradation of xylan-containing substrates, resulting in an increase of the released total oligosaccharides (Zerva et al. 2020).

The replacement of an N-terminal segment in *Ao*Xyn11, a mesophilic family 11 xylanase from *Aspergillus oryzae*, by the corresponding N-terminal of *Ev*Xyn11^{TS}, a hyperthermotolerant family 11 xylanase, led to a hybrid xylanase with improved thermostability (Yin et al. 2013). The new xylanase, NhXyn11^{57}, was overexpressed in *Pichia pastoris* and its temperature optimum was 75 °C, much higher than that of AoXyn11. AoXyn11 and NhXyn11^{57} were thermostable at 40 and 65 °C, respectively. A poly-threonine helix from the thermostable GH10 family xylanase XynAF0 from the thermophilic composting fungus *Aspergillus fumigatus* Z5 was introduced to the C-terminal of another GH10 xylanase, improving its thermostability (Li et al. 2019). Thus, the creation of hybrid xylanases can be another strategy for thermostability modifications in this family of enzymes.

Aspergillus kawachii produces, apart from XynC, a second GH11 xylanase, namely XynB, which is neutrophilic. Mutants to adjust the pH optimum of xylanases have been studied. In acidophilic GH11 xylanases, the residue adjacent to the acid/base catalyst is Asp, whereas in neutrophilic and alkaliphilic GH11 xylanases is Asn. A D37N mutation in *Aspergillus kawachii* XynC GH11 xylanase (Fig. 27.1) raised the pH optimum of XynC from 2.8 to 5.5, whereas an N43D mutation in *A. kawachii* XynB GH11 lowered the pH optimum of XynB from 4.2 to 3.6 (Fushinobu et al. 2011).

27.4 Feruloyl Esterases

Feruloyl esterases (FAEs; EC 3.1.2.72) are enzymes that catalyze the hydrolysis of ester bonds between ferulic (hydroxycinnamic) acid (FA) and plant cell wall polysaccharides (Dilokpimol et al. 2016). They act as accessory enzymes of plant biomass degradation to facilitate the access of other enzymes, such as xylanases, xylosidases, and arabinofuranosidases, to sites of action during biomass conversion. FAEs act synergistically with xylanases to release FA from cell wall material. Based on their catalytic properties, FAEs have also attracted a great deal of attention in

recent years for use in the food, pharmaceutical, and cosmetics industries as synthetic tools of novel hydroxycinnamates with enhanced antioxidant activity and custom-made lipophilicity (Faulds 2010; Koseki et al. 2009). Recent amino acid based sequence analysis has classified fungal FAEs in 13 subfamilies (SFs) (Dilokpimol et al. 2016) as a replacement of the old classification system of ABCD, which was based on substrate specificity (Crepin et al. 2004). Based on the analysis of 1000 fungal FAEs, SF5 and SF7 FAEs were found suitable for biorefinery applications, such as the production of biofuels, where a complete degradation of the plant cell wall is desired (Underlin et al. 2020). In contrast, SF6 FAEs are promising enzymes for industrial applications that require a high release of only FA and *p*-coumaric acid, which are needed as precursors for the production of biochemicals. Finally, FAEs of SF1, 9, and 13 display an overall low release of hydroxycinnamates from plant cell wall-derived and natural substrates. A feruloyl esterase from *Aspergillus terreus* (Mäkelä et al. 2018) was used to expand the carbohydrate esterase 1 (CE1) family of the CAZy database. Phylogenetic analysis showed that the CE1 family can be subdivided into five groups to include more members of fungal FAEs.

An FAE from the thermophilic fungus *Sporotrichum thermophile* has shown stability at the pH range 5.0–7.0 and retained 70% of its activity after 7 h at 50 °C while it lost 50% of its activity after 45 min at 55 °C and after 12 min at 60 °C (Topakas et al. 2004). Characterization of an FAE (*Tc*FaeB) from *Talaromyces cellulolyticus*, a high cellulolytic-enzyme producing fungus, has been reported (Watanabe et al. 2015). Thermal stability measurement using differential scanning calorimetry showed that *Tc*FaeB has a *Tm* value of 70 °C and optimum temperature of the enzyme was estimated to be 65 °C at pH 4.5–6.5, suggesting that this enzyme may be applicable for biomass saccharification processes.

27.4.1 Structural Features of FAEs

FAEs exhibit an α/β hydrolase fold with a Ser-His-Asp catalytic triad (Hermoso et al. 2004). The FA has been found to bind to a shallow surface pocket able to accommodate the methoxy and hydroxyl moieties of the substrate owing to the presence of hydrophobic and hydrogen bonding specificity determinants (Prates et al. 2001). Although fungal FAEs show structural similarity to lipases, their catalytic sites are different with more hydrophobic residues present in the active site of lipases (McAuley et al. 2004). Fungal and bacterial FAEs show different topology of secondary structure elements, leading to suggestion that bacterial FAEs diverged earlier than the fungal FAEs and the lipases from a common ancestor.

The crystal structure of an FAE from *Fusarium oxysporum* has been reported (Dimarogona et al. 2020). Similar to other FAEs, the structure revealed a large lid domain covering the active site with a potential regulatory role (Fig. 27.2). This lid domain, however, is absent in *A. niger* FAE (McAuley et al. 2004) and more fungal structures should be determined to understand its exact role. A disulfide bond brings together the serine and histidine residues of the catalytic triad. Several differences

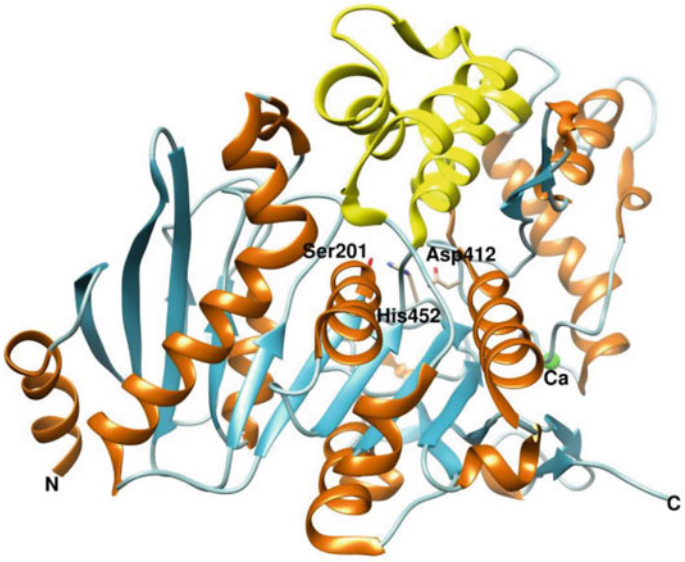

Fig. 27.2 Crystal structure of an *F. oxysporum* FAE (PDB id 6fat). The catalytic triad is shown. The characteristic lid is colored in yellow. A bound Ca ion is depicted as a green sphere

were identified, mainly in the metal coordination site and the substrate binding pocket.

27.4.2 Improvement Efforts of FAEs

Directed evolution studies have been reported in the filamentous fungus *Aspergillus niger* FAE (*An*Fae) (Zhang et al. 2012). The resultant mutant exhibited 80% residual activity after heat treatment at 90 °C for 15 min and an increase in half-life from 15 min to >4000 min at 55 °C. The thermostable mutant displayed significantly enhanced performance compared to the parental *An*FaeA, suggesting it could be useful in biotechnological applications. Directed evolution has also been applied to EstF27 identified from a soil metagenomic library (Cao et al. 2015). Structural analysis showed that a new disulfide bond and hydrophobic interactions formed by the mutations may play an important role in stabilizing the protein.

The effect of glycosylation has been studied in feruloyl esterase 1a from *Myceliophthora thermophila* (Bonzom et al. 2019). Heterologous expression of the enzyme in three different hosts (*M. thermophila*, *P. pastoris*, *E. coli*) revealed that the enzyme produced in *E. coli* had the lowest catalytic efficiency compared to its glycosylated form. Moreover, differences were found depending on the degree of glycosylation, suggesting that careful choice of the expression host should be considered for enzyme optimization depending on the specific biotechnological application.

Examples of chimeric enzymes have been reported. *An*FAEA and a dockerin from *Clostridium thermocellum* were connected and the resultant protein was expressed in *A. niger* and characterized (Levasseur et al. 2004), showing higher enzymatic activity than the parental *An*FAEA. A type A FAE from *A. awamori* (*Aw*FaeA) and family 42 carbohydrate-binding module (AkCBM42) from glycoside hydrolase family 54 α-L-arabinofuranosidase of *A. kawachii* have been used to create a chimeric enzyme (Koseki et al. 2010). The new enzyme was found to be more thermostable than *Aw*FaeA.

27.5 Laccases

Laccases (EC 1.10.3.2; *p*-diphenol:dioxygen oxidoreductases) belong to the family of multi-copper oxidases and can oxidize a wide range of aromatic and non-aromatic compounds, such as substituted phenols, non-phenolic compounds, and some inorganic ions (Agrawal et al. 2018). Owing to their ability to degrade and detoxify a wide range of phenolic and non-phenolic compounds, including lignin, laccases have received broad attention for use in several industrial and biotechnological applications, including eco-friendly synthesis of fine chemicals and the gentle derivatization of biologically active compounds (Mikolasch and Schauer 2009). Fungal laccases are characterized by higher redox potential compared to the bacterial ones; thus, they are more suitable for use in a wide range of substrates and act as "green biocatalysts" in various areas of biotechnology, including bioremediation (Mäkelä et al. 2020) and bio-bleaching (Baldrian 2006). Fungal laccases have been purified from wood-rotting white-rot basidiomycetes and to a lesser extent from other groups of fungi (e.g., other groups of basidiomycetes, ascomycetes, and imperfect fungi) (Baldrian 2006). Glycosylation (typically between 10 and 25%) has been detected in fungal laccases and it was suggested that it protects the enzymes from protease degradation in addition to the structural role it plays (Maestre-Reyna et al. 2015; Yoshitake et al. 1993).

Fungal laccases require only molecular oxygen as co-substrate, resulting in significant cost savings and reduced protein inactivation compared to the use of H_2O_2 as co-substrate in peroxidases. Moreover, the biocatalytic process products are water and a corresponding radical derived from the reducing substrate which afterwards evolves to the formation of insoluble polymers that are easily removable by filtration or other conventional technologies (Songulashvili et al. 2016).

A laccase from *Sporotrichum thermophile* exhibits temperature and pH optima of 60 °C and 3.0, respectively. The enzyme was found stable in organic solvents, such as DMSO and ethanol, and has been used in decolorization of six synthetic dyes (Kunamneni et al. 2008). In the field of bioelectrocatalysis, fungal laccases, for example, *Trametes versicolor* laccase (*Tv*L), have been intensively studied because of their high redox potential compared to the bacterial ones that makes them suitable for use in enzymatic biofuel cells (EBFCs) (Arregui et al. 2019).

Municipal wastewater is characterized by high alkalinity and high concentration of metal ions. Thus, laccases from alkaliphilic fungi have been studied for use in

such environments (Prakash et al. 2019). Metagenomic studies of Soda Lake have led to the identification of various fungi with laccase-like oxidase activity suitable for degradation of phenolic compounds.

Thermophilic fungi have been also a source of laccases with thermostability properties (Hildén et al. 2009). Thermostable laccases have found use in various industrial applications, such as pulp and denim bleaching, and in the food and beverage industry as stabilizers (Osma et al. 2010). Among ascomycetes, significant thermostability has been detected in a laccase from the thermophilic fungus *Melanocarpus albomyces* which can retain its activity for 24 h at 50 °C and for 2 h at 60 °C (Kiiskinen et al. 2002). A laccase produced *by C. thermophilum* has been characterized and sequenced (Chefetz et al. 1998). The enzyme retains activity for 1 h at 70 °C and has half-lives of 24 and 12 h at 40 and 50 °C, respectively. It is also stable at a wide pH range, from pH 5 to 10.

27.5.1 Structural Details of Laccases

Fungal laccases are enzymes with 520–550 amino acids and a molecular weight of 60–70 kDa in their glycosylated form. They exist mainly as monomers although there are cases of homodimeric, heterodimeric, and multimeric laccases. The three-dimensional structure of several fungal laccases has been reported. A characteristic of fungal laccases is the presence of 10 histidines with 8 of them belonging to four HXH motifs (Sitarz et al. 2016).

Laccases are characterized by an active center that contains four copper ions, each identified based on its spectroscopic properties. The T1 copper ("blue" copper) exhibits a strong absorption around 600 nm and is paramagnetic, T2 ("non-blue" copper) is also paramagnetic with an absorbance at 610 nm, and T3 contains a diamagnetic spin-coupled copper–copper pair with an absorbance at 330 nm. The T1 copper site and the T2/T3 trinuclear copper cluster are connected to each other through a strongly conserved internal electron transfer pathway. The substrates are oxidized by the T1 copper and the extracted electrons are transferred, probably through a highly conserved His-Cys-His tripeptide motif, to the T2/T3 site, where molecular oxygen is reduced to water (Mehra et al. 2018).

The crystal structure of a laccase produced by *M. albomyces* has been determined (Hakulinen et al. 2002). The molecule is divided into three domains (Fig. 27.3). Domain 1 includes residues involved in the binding of coppers at the trinuclear site. Domain 2 includes residues that are involved in the binding of reducing substrates. Domain 3 contains residues that participate in the binding of coppers at the mononuclear and trinuclear sites as well as in substrate binding. Electron-donating organic substrate molecules are bound in a hydrophobic pocket of domain 2.

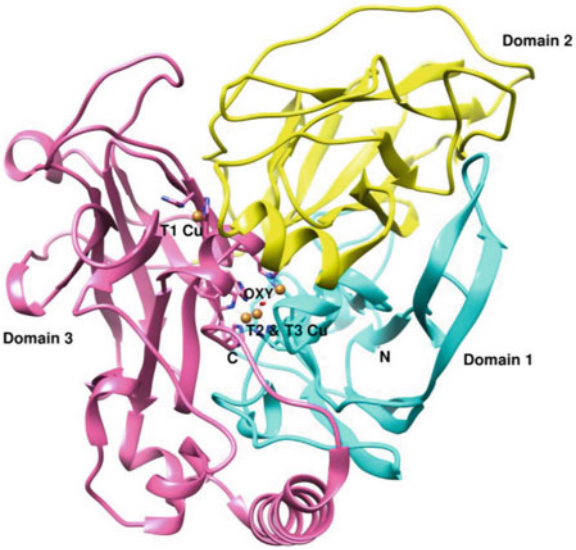

Fig. 27.3 Crystal structure of *M. albomyces* laccase (PDB id 1gw0). The three domains are shown in different colors. Copper ions are depicted as brown spheres. The oxygen atom is colored in red

27.5.2 Improvement of Fungal Laccases

Thermostable fungal laccase chimeras have been generated by the SCHEMA-RASPP computational approach (Mateljak et al. 2019). The most thermostable variant showed a five-fold increase (up to 108 min) of its thermal inactivation half-life at 70 °C. Interestingly, ancient laccases have recently emerged as a promising source of novel laccases (Gomez-Fernandez et al. 2020). A fungal Mesozoic laccase (dated back 250–500 million years) was resurrected and showed strikingly high heterologous expression and pH stability. Directed evolution, rational, and semi-rational approaches to improve laccase function have been reported (Mate and Alcalde 2015). N-glycosylation sites are found mostly conserved in fungal laccases and they have been suggested as potential modulators of the laccase properties (Ernst et al. 2018).

27.6 Lipases

Lipases (EC 3.1.1.3) catalyze the hydrolysis and synthesis of acylglycerol and other water-insoluble esters. They are important industrial enzymes that have found use in food, chemical, drug, biodiesel, and detergent industries. As flavor and fragrance compounds are esters formed by short-chain carboxylic acids and alcohols, the use of lipases for the enzymatic synthesis of flavor esters offers a more efficient, economically benign, and promising alternative approach compared to the traditional methods of chemical synthesis or extraction from natural sources (Dhake et al. 2013; Verma 2019). Biodiesel consists of fatty acid alkyl esters (FAAE) derived from triglycerides (TGs) by transesterification with alcohols. Thus, fungal lipases

have been used in biodiesel production either free and immobilized or as whole cells and fermented solids (Aguieiras et al. 2015).

Thermophilic fungi have been a promising source of new thermostable lipases. Lipases from thermophiles are mostly used in wastewater treatments. Using solid-state fermentation, a thermostable lipase from *Thermomyces lanuginosus* was obtained (Avila-Cisneros et al. 2014). The enzyme exhibited thermostability and it has been used in various applications, from chemical synthesis and biodiesel production to transesterification reactions.

Thermophilic lipases are also tools in the pulp industry as the processing of lignocellulosic material leads to the formation of pitch, a substance rich in esters able to clog machines. A lipase from *Aspergillus oryzae* has been used to control pitch formation (Gutiérrez et al. 2009).

Psychrophilic and alkaliphilic lipases are usually added to the detergent formulation as polymer-degrading agents (Joseph et al. 2008). *Talaromyces thermophilus* produces a thermoactive and alkaline lipase which retains activity at pH 9.5 (Romdhane et al. 2010). The enzyme is stable at 60 °C and retains 65% of its enzyme activity after 30 min incubation at 70 °C. Its half-activity is retained after incubation for 40 min at 80 °C. The optimum pH for the enzyme activity was 9.0 and the lipase was stable from pH 8.0 to 12.0. Higher frequency of hydrophobic amino acids, such as Ala, Val, Leu, and Gly in thermostable lipases from *T. lanuginosus* has been suggested for increased thermal stability (Zheng et al. 2011).

A lipase from the biotrophic fungus *Sporisorium reilianum* SRZ2 (SRL) with 73% amino acid sequence identity to *Candida antarctica* lipase B (CALB) was cloned and overexpressed in *Pichia pastoris* retained 75% of its activity at pH 3–11 for 72 h and it has been suggested to act as a thermophilic fungal lipase (Shen et al. 2020). Moreover, LipG7 from the Antarctic filamentous fungus *Geomyces* sp. P7 retained 100% of its initial activity after 1 h of incubation at 100 °C (Florczak et al. 2013). An acidic and thermostable lipase with a preference for the medium-chain length *p*-nitrophenyl esters (C12) rather than short and long-chain length substrates has been characterized from the thermophilic fungus *Neosartorya fischeri* P1 (Sun et al. 2016).

27.6.1 Structural Details of Lipases

Lipases have a common α/β-hydrolase fold, a catalytic triad (Ser-His-Asp/Glu) similar to that found in serine proteases and a lid covering the active site (Holmquist 2000). The lid is displaced upon activation and opens up the binding pocket, thus making the active site accessible to the substrate (Stauch et al. 2015) (Fig. 27.4). The ester hydrolysis mechanism of lipases is similar to that of carboxyl esterases and serine proteases. It involves a nucleophilic attack on the carbonyl carbon of the ester bond by the catalytic triad, leading to the formation of an acyl-enzyme intermediate and alcohol. This acyl-enzyme intermediate in second nucleophilic attack is hydrolyzed by water and yields carboxylic acid.

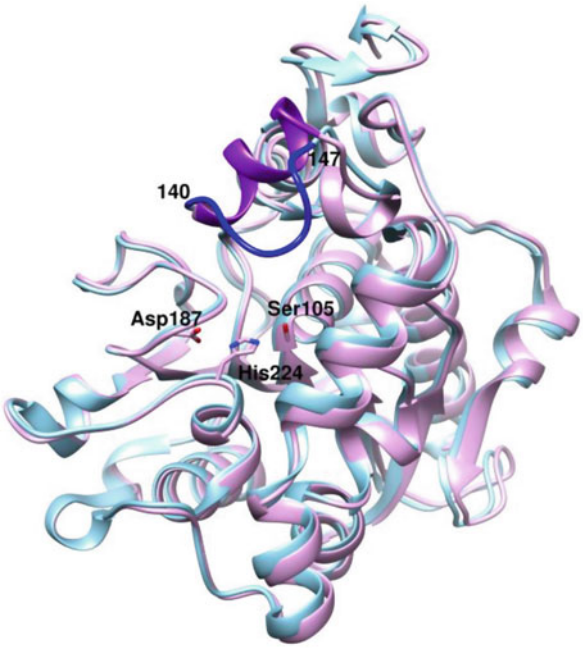

Fig. 27.4 Lid displacement in CALB. The conformational change of residues 140–147 is shown after superposition of the closed conformation structure (PDB id 5a71; cyan) onto the open conformation structure (PDB id 1tca; pink). The residues of the catalytic triad are shown as sticks

27.6.2 Improvement of Catalytic Efficiency in Lipases

There have been enormous efforts to increase the catalytic efficiency and thermal properties of the lipases. The Lipase Engineering Database (LED; http://www.led.uni-stuttgart.de) provides up-to-date information about the updated and engineered lipases. Lipases are synthetically designed, genetically engineered, cloned, and expressed using suitable expression systems in recombinant organisms. The methods employed (Bornscheuer et al. 2002) for this purpose are:

- Physicochemical methods (e.g., immobilization and solubilization).
- Reaction engineering methods (e.g., use of acyl donors and ionic liquids).
- Molecular biology methods (e.g., rational protein design and directed evolution).

Notably, the conversion through directed evolution of the catalytic Ser to Cys in CALB along with four mutations resulted in 40-fold higher activity lipases (Cen et al. 2019), suggesting that similar approaches could be useful for the fungal lipases.

27.7 Nitrilases

Nitrilases (NLases; EC 3.5.5.-) catalyze the hydrolysis of different nitriles to corresponding amides and acids. They can be used in organic synthesis to improve industrial biocatalysis (Gong et al. 2017). Bacterial NLases have been characterized

in detail. According to their substrate specificity, they are classified as aromatic NLases, aliphatic NLases, and arylacetoNLases. In contrast, studies of fungal nitrilases have been slow (Martínková 2019). Fungal nitrilases belong to aromatic nitrilases and arylacetonitrilases. A recent bioinformatics search identified various nitrilases in fungi (Rucká et al. 2020). In general, fungal nitrilases are considered advantageous over the bacterial ones regarding activity, thermostability, and selectivity (Wu et al. 2013).

27.7.1 Structural Details of Nitrilases

No structure of a fungal nitrilase is currently available. Insights into the catalytic mechanism have been provided by an archaeal nitrilase (Raczynska et al. 2011). A two-fold dimer symmetry has been identified with each subunit of the dimer characterized by an αββα sandwich fold, resulting in a super-sandwich αββααββα structure formed by the association of the two subunits. Dimerization is achieved through multiple approaches, including interactions of the extended C-terminal of each subunit and interactions between arginine and glutamate residues that form salt bridges. The binding pocket lies close to the inter-subunit interface, while a binding loop assists the binding of the substrate. A Lys residue was suggested as the acid in the catalytic reaction.

27.7.2 Improvement

Site-directed mutagenesis of a fungal nitrilase from *Gibberella intermedia* resulted in mutants with higher catalytic activity and increased stability (Gong et al. 2016). Notably, point mutations near the active site of an *A. niger* nitrilase were able to change the enantioselectivity of the enzyme (Petříčková et al. 2012). Mutagenesis studies on bacterial nitrilases to improve their thermostability have been carried out and may apply to their fungal counterparts as well (Xu et al. 2018).

27.8 Transaminases

Transaminases (TAs), or aminotransferases, are enzymes that catalyze the transfer of an amino group from an amino donor to an acceptor for chiral amino acid or amine synthesis (Guo and Berglund 2017). Owing to their excellent enantioselectivity, environmental friendliness, and compatibility with other enzymatic or chemical systems, TAs have drawn attention in the area of biocatalysis. The most known example of use of a TA is in the synthesis of the antidiabetic drug sitagliptin (Savile et al. 2010). An in silico strategy for sequence-based prediction of substrate specificity and enantiopreference revealed 17 novel (R)-selective TAs, many of them from fungi (Höhne et al. 2010).

27.8.1 Structural Details of TAs

The structure of a pyruvate TA from the fungus *Nectria haematococca* (Sayer et al. 2014) has been determined and provided initial insights into the R-enantioselectivity of the TAs. Also, the structure of an (R)-selective amine TA from *A. fumigatus* has been elucidated (Thomsen et al. 2014). The enzyme has the typical fold found in class IV of PLP-dependent enzymes and its overall structure is similar to that of a branched-chain amino acid aminotransferase from *T. thermophilus* and D-amino acid aminotransferase from *Bacillus* sp. YM-1 (Fig. 27.5). An N-terminal α-helical extension has been found that was suggested to play a role in the enzyme's stability. The structure of an ω-transaminase from *Aspergillus terreus* has been solved and found to exhibit also the class IV fold (Łyskowski et al. 2014).

27.8.2 TA Improvement

A thermostable (R)-TA (Huang et al. 2017) from *A. terreus* (*At*RTA) has been characterized. A homolog of *At*RTA from the thermotolerant fungus *Thermomyces stellatus* (*Ts*RTA) has been reported (Heckmann et al. 2020). The thermostability of *Ts*RTA (40% retained activity after 7 days at 40 °C) was initially attributed to its tetrameric form in solution. However, subsequent studies of *At*RTA revealed that the enzyme also exists predominantly as a tetramer but, in contrast to *Ts*RTA, it is inactivated within 48 h at 40 °C. The engineering of a cysteine residue to promote

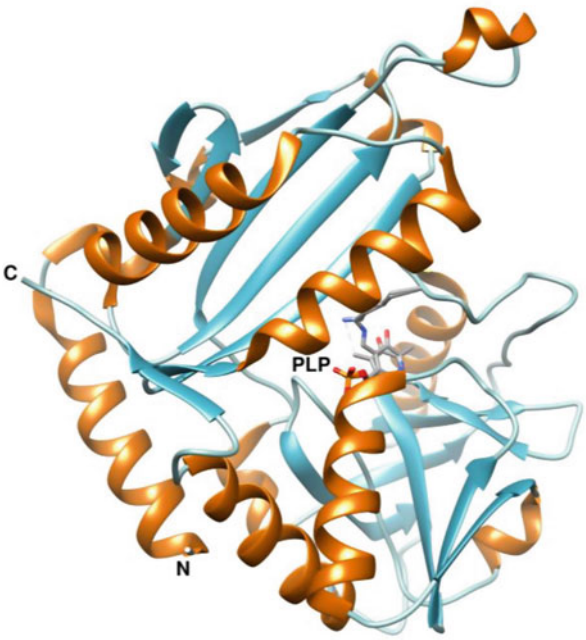

Fig. 27.5 Crystal structure of *A. fumigatus* (R)-selective amine TA (PDB id 4chi). The PLP cofactor is depicted in sticks

disulfide bond formation across the dimer–dimer interface stabilized both enzymes, with *Ts*RTA_G205C mutant retaining almost full activity after incubation at 50 °C for 7 days.

27.9 Tyrosinases

Tyrosinases (EC 1.14.18.1, monophenol, *o*-diphenol:oxygen oxidoreductases) together with laccases (EC 1.10.3.2) form two subgroups of phenoloxidases. As in laccases, tyrosinases are also copper-containing enzymes. In tyrosinases, the copper ions are known as CuA and CuB (Ba and Vinoth Kumar 2017).

Tyrosinases are found in bacteria, fungi, and plants. Fungal tyrosinases from *Agaricus bisporus*, *Neurospora crassa*, *Aspergillus oryzae,* and *Aspergillus niger* have been largely explored (Agarwal et al. 2017). Detailed characterization of a tyrosinase from *T. reesei* has also been reported (Selinheimo et al. 2006). Tyrosinases have been used either in a free or immobilized form for the removal of micro-pollutants in the environment (Ba and Vinoth Kumar 2017). Besides, tyrosinases are employed in L-DOPA synthesis and biosensor development (Min et al. 2019).

27.9.1 Structural Details of Tyrosinases

Crystal structures of an *Aspergillus oryzae* fungal tyrosinase are available (Fujieda et al. 2020) and have provided information into the copper movements during the catalytic reaction (Fig. 27.6). No structures are currently available for tyrosinases from extremophilic fungi.

27.9.2 Tyrosinase Improvement

The use of D-DOPA in Parkinson's disease has been proposed as more effective than the use of L-DOPA. As fungal tyrosinases have lower affinity for D-tyrosine, improvement of their catalytic activity against D-tyrosine has been pursued (Ali et al. 2020). Thermostable tyrosinases have been proposed for the removal and bioconversion of phenol from wastewater (Lee et al. 1996). Immobilization of the enzyme appears to have beneficial effects. Indeed, an immobilized mushroom tyrosinase has been found to work at high temperatures (100 °C) and in organic solvents (Wu et al. 2018). Also, immobilized *Aspergillus niger* tyrosinase has shown an increased thermal and pH stability (Agarwal et al. 2016). A computational approach which has been carried out to improve the thermostability of a bacterial tyrosinase (Guo et al. 2015) may be applicable for fungal enzymes as well.

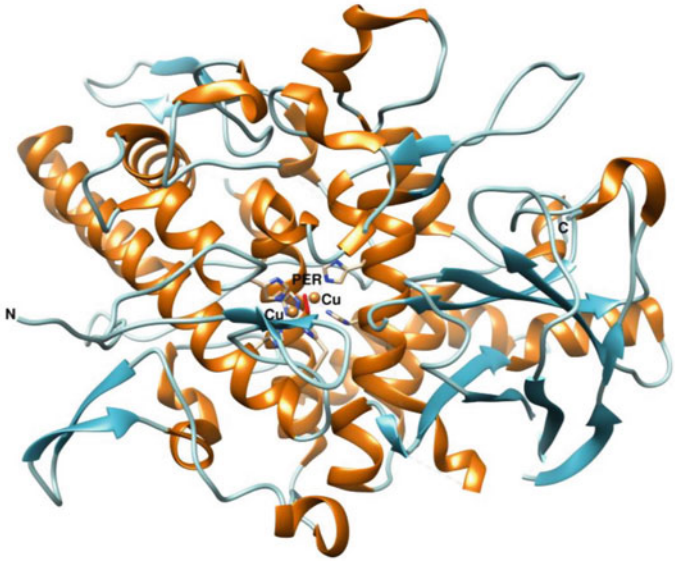

Fig. 27.6 Crystal structure of *A. oryzae* C92A tyrosinase mutant in the presence of peroxide (PDB id 6jub). Cu atoms are shown as brown spheres and peroxide is colored in red

27.10 Keratinases

Keratinases are key enzymes used for the degradation of agricultural and industrial keratin waste. Keratin is the most abundant insoluble structural fibrous protein and a major constituent of hair, nails, horn, wool, skin, and feather. Chemical and physical treatment of keratin are currently unfriendly to the environment, leading to a product with poor digestibility, variable nutrient quality, and destruction of several amino acids (Mabrouk 2008). Keratinases have been identified in a wide variety of microorganisms (Brandelli et al. 2010). A crude enzyme preparation from *Aspergillus fumigatus* (Santos et al. 1996) showed remarkable thermostability by retaining ~90% of its original activity at 70 °C for 1.5 h. Besides, a novel thermophilic *M. thermophila* strain GZUIFR-H49-1 with potential applications for production of thermostable keratinase has been identified (Liang et al. 2011). Also, screening of almost 300 fungi species revealed that *Aspergillus flavus* was the most productive fungus in keratinolytic enzymes (Friedrich et al. 1999).

27.10.1 Structural Details of Keratinases

Structural details of keratinases have been obtained from bacterial sources and the crystal structures of five keratinolytic enzymes are known (Qiu et al. 2020). Phylogenetic and structure-based analysis has shown the presence of other keratinases in

various extremophiles, including fungi. The structure of the keratinase fervidolysin has suggested a distant relationship with pro-subtilisin E using structural alignment (Kim et al. 2004; Kluskens et al. 2002). The structure also suggested a functional relationship of fervidolysin to the fibronectin-like domains of the human promatrix metalloprotease-2 that degrades the fibrous polymeric substrate gelatin. The structure of a novel heat-stable keratinase (Wu et al. 2017) from the feather-degrading thermophilic bacterium *Meiothermus taiwanensis* WR-220 has shown similarities with the overall fold of the catalytic domain of fervidolysin.

27.10.2 Improvement

Computational techniques to improve the thermostability of bacterial keratinases have been applied (Liu et al. 2013) and they may be extended to other keratinases of industrial interest. A synergistic action of keratinases with LPMOs has been proposed (Lange et al. 2016), thus offering additional strategies to improve keratinase performance.

27.11 Conclusions

Fungal extremozymes are promising alternatives for use in green chemistry. However, a systematic functional and structural characterization is necessary to understand better their stability, behavior in extreme environments, catalytic mechanism, synergism, and evolutionary relationships. Currently, the two most preferable approaches for producing novel enzyme variants are site-directed mutagenesis and directed evolution. Further improvement of fungal extremozymes will help in developing better and more versatile enzymes for their use either alone or in mixtures with other enzymes for green chemistry applications.

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Fungal Extremozymes in Green Chemistry 28

Ajay Nair, Archana S. Rao, K. Nivetha, Prakruthi Acharya, Aneesa Fasim, Veena S. More, K. S. Anantharaju, and Sunil S. More

Abstract

An endeavor to accommodate sustainability within chemical manufacturing led to the recently popularized green chemistry. For a chemical process to be green, it must meet the following criteria: avoiding usage of non-biodegradable materials, all industrial processing transformations should lead to minimal waste accumulation, the processes must be cost-effective, both energetically and economically, etc. This is why biocatalysis using enzymes brought about a monumental shift toward a greener chemical process. Since its initial introduction, enzymes have mediated almost all industrial sectors, from pharmaceutics to food industry, pulp, textile, agro-waste management, bioremediation, etc. Recently, metagenomic approaches have led to the discovery of enzymes from extremophilic organisms that thrive under conditions considered optimal for most life on earth. Most of these extreme-loving organisms are bacterial or fungal in origin. Regardless of their origin, it has been demonstrated that extremozymes produced by them are far more efficient and resilient under the harsh industrial conditions. This makes them preferred candidates to be used as biocatalysts. Recently, several extremozymes of fungal origin have gained interest as potential industrial biotransformants. This chapter discusses the applications of fungal extremozymes, improves on the synthesis, bioconversions, and bioremediation processes.

A. Nair · A. S. Rao · K. Nivetha · P. Acharya · A. Fasim · S. S. More (✉)
School of Basic and Applied Sciences, Dayananda Sagar University, Bangalore, Karnataka, India

V. S. More
Department of Biotechnology, Sapthagiri College of Engineering, Bangalore, Karnataka, India

K. S. Anantharaju
Department of Chemistry, Dayananda Sagar College of Engineering, Bangalore, Karnataka, India

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Keywords

Bioremediation · Fungal extremozymes · Biocatalysis · Extremophiles

28.1 Introduction

Microorganisms are by far the most prevalent species inhabiting our planet. To understand them is to understand the evolutionary timeline of biological causation; to understand them is to realize the boundary conditions that define life. Now there are microorganisms (such as mesophiles) that thrive within the prescribed life-promoting parameters. But there are others (such as extremophiles) which thrive under conditions that are usually considered optimal. Of the several strategies employed for survival under extreme conditions, the production of specialized enzymes called extremozymes is ranked very high. In recent times, it has therefore been a persistent endeavor to explore extremophiles toward the functional characterization of more and more extremozymes. The largely unexplored variety of microbes- the extremophiles and the mechanisms involved in their production of extremozymes under extreme conditions supporting their survival.

Enzymes play a significant role commercially as an ecofriendly alternative to synthetic chemicals, in a wide variety of industries such as pharmaceuticals, food and beverage production, biofuels, and chemical production. The inherent effects of the microbial enzymes produced by mesophiles (moderate temperature-dwelling) are found to be of profound importance in several processes such as antibiotic production, organic acid production, flavor enhancement, chiral compound production, as emulsifiers, and many more. Likewise, there are several industrial protocols involving harsh conditions which can be endured by specific enzymes such as **extremozymes** that are found to be produced by the extremophiles. Several properties of the extremozymes such as reproducibility, consistency, and high yields must be explored to understand their wide range of applications in several industries (Sarmiento et al. 2015). Such extremozymes demonstrated a wide range of activity, efficiency, and stability under extreme conditions of temperature, pH, salt concentration, etc. (Fig. 28.1).

The industrial processes involve reactions carried out under extreme conditions of temperature, pressure, pH, etc. Enzyme catalysis, in the recent past, has been preferred over chemical reagents as far as bioprocessing is concerned. This is due to the ability of enzymes to carry out processes, which are far greener and more cost-effective. However, the enzymes secreted by microorganisms thriving within the realms of normal growth parameters fail to withstand the harsh industrial conditions. This is why chemical processes are preferred over enzyme catalysis. But using synthetic chemicals would have inclusions such as high cost, toxicity, environmental pollution from industrial effluents, etc. Therefore, a desperate need to make industrial processes more sustainable, green, without compromising on the efficacy of the process led to the discovery of extremozymes.

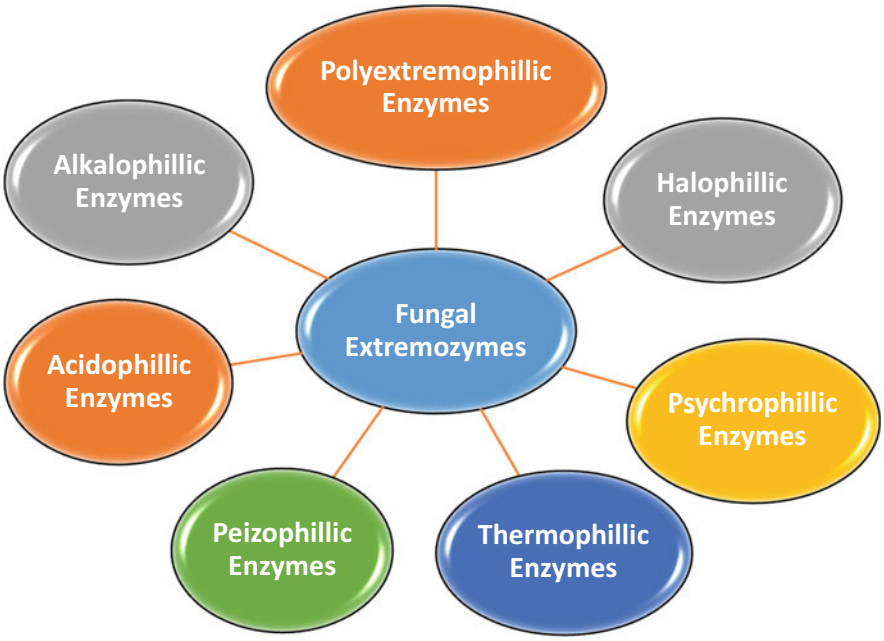

Fig. 28.1 Fungal enzymes from different extremophilic conditions

Several extremozymes have been extensively studied and employed for various reactions. Most of the extremozymes known are bacterial in origin. There are again a large number of fungal extremophiles which can be explored more to employ them successfully for green chemistry. Many researches carried out to explore fungal extremophiles indicate the production of extremozymes of various factors, which are beneficial for chemical conversions and hence used in different industries. The table below summarizes the use of various extremozymes fungal in origin for different industrial processes (Table 28.1).

Since their discovery, extremozymes have created a monumental shift in the area of bioprocessing using ecofriendly alternatives. Not only do these extreme enzymes are more efficient catalytically than their mesophilic counterparts (optimal enzymatic activity for a given condition) but also each extremozyme also seems to possess an innate plasticity that allows it to be functional at varying physicochemical parameters. Therefore, it is worthwhile to continue the pursuit of characterizing more and more extremozymes which can be further added to the list of industrially important enzymes. The following sections discuss the applications of fungal extremozymes in different reactions involved in various industrial processes.

Table 28.1 Applications of fungal extremozymes

Fungal extremozymes	Producer	Extreme condition	Applications	Reference
Amylases and cellulases	*Cystofilobasidium capitatum*, *Mrakia blollopis, Rhodotorula glacialis*, *Tetracladium* sp., *Saccharomyces cerevisiae*	Low temperature	Bioethanol industries Wine production Food industries	Tiquia-Arashiro and Grube (2019)
Cell wall-degrading enzymes, antifungal metabolites	*Aureobasidium pullulans* and *Rhodotorula mucilaginosa, Papiliotrema laurentii*	Low temperature	Cold preservation of fruits and vegetables	Tiquia-Arashiro and Grube (2019)
Conversion of albendazole (a prodrug) to albendazole sulfoxide	*Rhizomucor pusillus*	High temperature	Pharmaceutical industries	Tiquia-Arashiro and Grube (2019)
Ferulic acid to guaiacol	*Sporotrichum thermophile*	High temperature	Pharmaceutical industries	Tiquia-Arashiro and Grube (2019)
Biotransformation of steroids, steroid production	*Acremonium alabamensis* and *Talaromyces emersonii*	High temperature	Pharmaceutical industries	Tiquia-Arashiro and Grube (2019)
Cellulases	*Myceliophthora thermophile* and *Thielavia terrestris*	High temperature	Biofuel, food industries	Tiquia-Arashiro and Grube (2019)
Xylanases	*S. thermophile*	High temperature	Biomass degradation, biofuels, paper, pulp	Tiquia-Arashiro and Grube (2019)
Cellulases, xylanases, esterases, glucosidases, mannanases, and phytases	*Chaetomium thermophilum*, *Malbranchea cinnamomea*, *Paecilomyces thermophile*, *Thermoascus aurantiacus,* and *Thermomyces lanuginosus*	High temperature	Bioconversions	Tiquia-Arashiro and Grube (2019)
Proteases	*Aspergillus flavus*	High salt concentration	Food, textile, paper and pulp industries	Tiquia-Arashiro and Grube (2019)

(continued)

Table 28.1 (continued)

Fungal extremozymes	Producer	Extreme condition	Applications	Reference
Amylases	*Engyodontium album, A. gracilis, A. penicillioides*	High salt concentration	Food, textile, paper and pulp industries	Tiquia-Arashiro and Grube (2019)
Cellulases	*A. terreus, Penicillium* sp.	High salt concentration	Food, textile, paper and pulp industries	Tiquia-Arashiro and Grube (2019)
Beta mannanases	*Penicillium oxalicum* GZ-2, *Aureobasidium pullulans, Bispora antennata*	Acidic	Food, feed, and detergent industries, paper	Liao et al. (2014), Li et al. (1993) and Liao et al. (2012)
Hemicellulase	*Teratospaeria acidotherma, A. niger*	Acidic	Food, feed and detergent industries, paper	Hatzinikolaou et al. (2005) and Isobe et al. (2013)
α-Amylases	*Thermomyces lanuginosus, Saitozyma*	Acidic	Industrial processes	Kunamneni et al. (2005)
Laccases	*Trametes hirsuta* Bm-2	Acidic	Pulp and paper industry, biosensors, fuel cells, stabilizers	Ghindilis (2000), Ben Younes et al. (2007) and Zapatae Castillo et al. (2012)
Proteases	*A. jilinensis*	Alkaline	Food and beverages	Wu et al. (2006)
Cellulase and hemicellulase	*P. citrinum*	Alkaline	Paper, pulp, polymer industries	Raghukumar et al. (2004)

28.2 Fungal Extremozymes in Green Chemistry

28.2.1 Psychrophilic Enzymes

Psychrophilic fungi are microorganisms that are known to efficiently adapt at very cold temperatures. These microorganisms are able to grow from −20 to 10 °C and are termed as Psychrophiles. However, they cannot grow above 15 °C, and psychrotolerants grow around 20–25 °C, but have been shown to be metabolically active even at 0 °C. Among the psychrophilic fungal species, certain varieties of lichenised fungi *Lecidia acarospora* and *Umbilicaria* and other cold-adapted yeasts such as *Debaryomyces hansenii, Cryptococcus* sp. and *Rhodotorula mucilaginosa, Crystobasidium larynges, Vishniacozyma victoriae,* and *Papiliotrema laurentii* on a major portion and on a minor portion *Candida, Dioszegia, Makia,* and *Naganishia*,

have been reported for study and to understand their extremophilic mechanisms responsible for them to strive in a very cold environments (Tiquia-Arashiro and Grube 2019). Metagenomics since its inception has offered a culture-free platform to characterize and isolate microorganisms that were once not cultivable. This helps in identifying specific extremophilic fungal species that are of great importance from an industrial point of view. Metagenomic analysis in extremely cold regions has identified less than 10% of microbial eukaryotes (predominantly occupied by the genera Ascomycetes and Basidiomycetes fungal species) in comparison with the prokaryotes in most of the extremophilic regions around the world. Meta-barcoding, by utilizing internal transcribed spacers (ITS) and D1/D2 domain of large rDNA as barcode sequences, is said to be a significant molecular technique in successfully identifying fungal species from Antarctic regions (Tiquia-Arashiro and Grube 2019).

The cold-adapted enzymes are involved in many chemical transformations that need to be made at lower temperatures. Low temperature bioprocesses are far more economical as they cut down on the energy cost. The low temperature processes also avoid the growth of unwanted mesophilic microbes.

The hydrolytic enzymes of the psychrophilic yeasts such as amylases, lipases, and proteases are found to play a potential role in several industrial processes. Amylases and cellulases produced by psychrophiles such as *Cystofilobasidium capitatum*, *Mrakia blollopis, Rhodotorula glacialis*, *Tetracladium* sp. are found to play a significant role in bioethanol industries. This is mainly due to the ability of these enzymes to remain highly active at very low temperatures, which helps in making the process cost-effective. Psychrophilic fungi are of major importance as they can be potentially used during fermentation processes at very low temperatures, which helps in improving the flavor of fermented foods. They can be also used in the production of alcoholic beverages, bread, and dairy products. There is an increasing demand in recent times for alcoholic beverages with lower alcoholic content as they are found to have lesser negative impacts on the aroma and flavor. Although this could be done by several physical and chemical techniques, yet an organic approach to reduce the alcohol content in wines is to use psychrophilic *Saccharomyces* and other yeasts, which are potentially used in the process of must fermentation at decreased temperatures (Tiquia-Arashiro and Grube 2019).

Spanish wineries were found to use this technique by using psychrotolerant yeast *Candida sake* to ferment concentrated natural must at 12 °C. Further, it was noticed that this process had avoided the typical lag phase of the psychrophilic *S. cerevisiae* during the fermentation process, thereby reducing the ethanol and glycerol content in the wines. Also, the use of psychrophilic fungi in fermentation processes not only improved the flavor but has also proved to avoid microbial contamination. This was proved by observing successful continuous fermentation at 5–15 °C, while using a psychrophilic strain *S. cerevisiae* being immobilized onto apple cuts. *S. cerevisiae* (AXAZ-1) is also used in beer production as it is known to improve the quality of beer, as it is responsible to provide acceptable quantities of volatile acids and decreased diacetyl and polyphenol contents in the beer, when compared to other commercial beers (Tiquia-Arashiro and Grube 2019).

Psychrophilic fungi also play a significant role in degrading a wide range of hydrocarbons. Psychrophilic yeasts such as *Cryptococcus terreus* and *Rhodotorula* sp. identified from Alps regions are found to degrade phenol and phenol-related mono-aromatic compounds. This is also done by utilizing immobilized yeast cells at low temperatures, around 10 °C. *Mrakia blollopis*, an Antarctic yeast is used in the treatment of contaminant materials as it is known to possess high biological oxygen demand (BOD) removal rate in wastewaters at low temperatures. *M. blollopis* is also known to ferment cellulosic biomass, in fermentation industries. They also may play a role in the bioremediation of contaminated water and soils (Tiquia-Arashiro and Grube 2019).

Different psychrophilic yeast species have been potentially used for the biological control of fungal phytopathogens, due to various control mechanisms offered by the yeasts. They mainly include induction of host resistance, exhibition of mycoparasitism, microbial competition for space and nutrients and production of antifungal compounds and cell wall-degrading enzymes. Psychrophilic yeasts also play a role in post-harvest biocontrol from various phytopathogens. *Aureobasidium pullulans* and *Rhodotorula mucilaginosa* are known to thrive in extremely cold temperatures. Antifungal mechanisms of these psychrophilic fungal species help them to reduce fruit decay to about 33% produced by *Penicillium expansum* in refrigerated packages. Other psychrophiles such as *R. mucilaginosa* and *Cryptococcus laurentii* (now *Papiliotrema laurentii*) are found to be promising isolates in reducing the decay of fruits and vegetables, especially cherry tomatoes, by acting against *Botrytis cinerea* and *Penicillium expansum*, which are the common fungal contaminants (Tiquia-Arashiro and Grube 2019).

Psychrophilic oleaginous yeasts isolated from Tibetan Plateau are known as potential biodiesel sources as they are capable of producing lipids from several economical substrates by the process of fermentation under aerobic conditions and decreased temperatures. Some of these psychrophiles include *Yarrowia lipolytica, Cryptococcus* species (accumulates over 30% of lipid content), *Rhodotorula glacialis* (with 68% of lipid biomass) *R. glutinis* and *R. glacialis* (Tiquia-Arashiro and Grube 2019).

Candida albicans produced lipase with optimal temperature of 15 °C (Lan et al. 2011). Another yeast *Glaciozyma antartica* living in temperature range of 4–200 °C produces many cold-adapted enzymes, viz., chitinase, protease, etc., and antifreeze proteins (Boo et al. 2013). Yeasts like fungus *Guehomyces pullulans* produce β-D-galactosidases active at cold temperatures.

Several fungal species (such as *Penicillium, Alternaria, Phoma,* etc.) isolated from the Antarctic have also shown, though minimally, to produce psychrophilic xylanases (Bradner et al. 1999). Some of the characteristic features underlying psychrophilic xylanases include: a low temperature optimum with improved catalytic efficiency, increased flexibility, etc. (Collins et al. 2003). Protein quenching studies demonstrated conclusively that the cold-adapted xylanases have increased flexibility and increased low temperature activity. The study therefore proposed that perhaps xylanase activity under psychrophilicity requires a tradeoff between enzymatic flexibility and stability of the molecular structure. This is achieved by reducing

the number of salt bridges and enhanced exposure of the hydrophobic residues (Van Petegem et al. 2002).

Hence, exploring, identifying, and understanding the metabolic potential of psychrophilic fungal communities aid in discovering several species and their novel genes that are required in wide varieties of applications.

28.2.2 Thermophilic Enzymes

Thermophilic fungi belong to a small group of mycota that are capable of growing at an increased temperature of at or beyond 50 °C, while thermotolerant fungi grow at an optimum of 20–55 °C. Thermophily in these fungi is comparatively less extreme when compared to other archaeal and eubacterial species that survive at areas where the temperature goes beyond 100 °C, such as hot springs, hydrothermal vents, and solfatara fields. Thermophilic fungi grow in various natural habitats such as animal/municipal refuse, plant/ mushroom compost, bird nest materials storage products, desert soils, alkalescent thermal springs, and other organic matter accumulations/piles of agricultural and forestry products. Thermophilic fungi are capable of surviving under extreme stress conditions such as desiccation and oxygen levels. They are the primary components of microflora in these diverse range of locations, as they provide an aerobic, warm, and a humid environment that are found to be ideal for fungal growth (Tiquia-Arashiro and Grube 2019).

Saprophytic mesophiles exhibit exothermic reactions, which increase the temperature of the substratum to about 40 °C. This elevated temperature provides a favorable environment for the germination of spores of the thermophilic fungi. Consequently, it is observed that these thermophiles to outgrow the mesophilic microflora. The earliest known thermophilic fungus that was identified on bread was found to be *Mucor pusillus*. Further, *Thermomyces lanuginosus*, another thermophilic fungus was isolated from potato. Later, a wide range of thermophilic fungi were isolated and were found to belong to a wide variety of genera such as Ascomycotina, Deuteromycotina, and Mastigomycotina (Tiquia-Arashiro and Grube 2019).

Cellulose is the most abundant non-fossil carbon source available on earth. Its primary constituents are of great importance in biofuel production and food industries. Although several options such as mechanical treatment, heat treatment, or acid treatment are industrially available for the breakdown of cellulose into its principal constituents, yet enzymatic breakdown is better favored on a large-scale, since it is an ecofriendly process. Several microbial cellulases are capable of breaking down cellulose, yet thermophilic enzymes from thermophilic fungi hold a better advantage over the other enzymes. This is because, during the breakdown process as the temperature increases, cellulose tends to swell up. Cellulose breakdown is much easier than when performed at a lower temperature. Hence, the thermophilic enzymes are favorable for cellulose breakdown, whose activity is high at higher temperatures. Thus, several thermophilic fungi have been isolated from various locations and their cellulases are characterized at structural and

functional levels. Studies suggest that the genomes of a few thermophilic fungi (*Myceliophthora thermophile* and *Thielavia terrestris*) are sequenced and their enzymes were found to be highly efficient in hydrolyzing several major polysaccharides (Tiquia-Arashiro and Grube 2019).

Thermostable enzymes are highly desirable commodities and are well exploited in industrial bioprocesses due to several advantages. Some of the commonly explored thermostable enzymes are produced from thermophilic fungi such as *Sporotrichum thermophile, Scytalidium*, *Thermotoga,* and *Thermoascus*. Several strains of *S. thermophile* (thermophilic mold) are known to produce highly active xylanases that are capable of catalyzing the hydrolysis of several types of substituted and non-substituted xylans. Xylanases from thermophilic fungi are of great demand in the industrial processes such as biofuel, food, paper and pulp production processes. This is due to its thermostability at higher temperatures, during which the time taken for hydrolysis is relatively reduced. Also, at higher temperatures, the viscosity of the medium decreases, which favors the process of hydrolysis. Thermostable xylanases are potentially used for the biomass conversion of lignocellulosics into fermentable sugars that are useful for several bioprocesses and for the saccharification of agrowastes such as corn, cobs, bruce, straw, spruce biomass, etc. Several other examples of thermophilic fungi that are capable of producing industrially important enzymes such as cellulases, xylanases, esterases, glucosidases, mannanases, and phytases include *Chaetomium thermophilum*, *Malbranchea cinnamomea*, *Paecilomyces thermophile*, *Thermoascus aurantiacus,* and *Thermomyces lanuginosus*(Tiquia-Arashiro and Grube 2019).

Much of that we understand about Xylanases has come from fungi and bacteria that are majorly found in and around the mesophilic ranges of life; be it temperature or pH (Subramaniyan and Prema 2002). However recently, the overall effort to characterize extremophiles has produced a range of extremophilic xylanases that are far more resilient and efficient than their mesophilic counterparts. Xylanases isolated from thermophilic molds have been shown to possess amplified kinetic efficiency and stability at high temperatures, with complementary activity profiles (Ghatora et al. 2007). A recent study suggested that using a combinatorial approach involving mannanolytic enzymes, galactosidases, acetyl glucomannan esterase, mannosidase, glucosidase for a comprehensive hydrolysis of sugars (Luonteri et al. 1998). Investigations looking at the structural conformations, sequence alignments between extremophilic and mesophilic xylanases revealed that there is a lot of similarity between the two except for a few differences. Xylanases isolated from thermophilic fungi have been shown to improve thermostability by undergoing few necessary structural modifications. These include: alterations to the number of salt bridges and hydrogen bonds, increase in the number of charged surface residues, presence of tandem repeats, etc. (Turunen et al. 2002).

Hence, thermophilic fungi can be well-exploited commercially in pharmaceutical industry for various applications, especially in an ecofriendly and an economic point of view.

28.2.3 Halophilic Enzymes

The halophiles are microorganisms which can thrive in areas of extremely high salt concentrations such as oceans and brackish salt lakes. Halophiles and halotolerants inhabit such regions of high salt concentrations (3–5% NaCl). They also play a major role in participating in biogeochemical cycles such as carbon, nitrogen, phosphorus, sulfur at areas under extreme conditions. Industrially, salted foods are produced with the addition of increased amount of salts, mainly for the flavor and preservation of the food products. To these industries, halophilic microorganisms appear to be of great importance especially due to their ability to survive under these extreme hypersaline conditions and to produce metabolites that are useful for various biotechnological applications. *Hortaea werneckii* (halotolerant fungi), *Sulfolobus solfataricus, Haloarcula* sp., *Cladosporium* sp., *Eurotium*, *Emerciella, Aspergillus, Penicillium, Wallemia ichthyophaga* (obligate halophile), and other melanized and non-melanized fungal species are some of the common halophilic fungal species available in brackish hypersaline environments and some of their genomes have been explored for a variety of industrial applications. The proteomic analysis of halophilic fungi indicates lower hydrophobicity, lower number of cytosine residues, and increased repetition of acidic residues (Tiquia-Arashiro and Grube 2019).

The survival mechanism of prokaryotes in high saline conditions is mainly due to hyperaccumulation of potassium ions in hypersaline environments. On the other hand, halophilic fungi are incapable of tolerating high intracellular ion concentrations, and hence their survival mechanism varies from that of halophilic prokaryotes. They tend to produce high amounts of organic solutes, glycerol, and trehalose, which helps them to maintain positive turgor pressure at increased salinity. Also, the fungal metabolites are extracellular unlike that of halophilic prokaryotes that exhibit better performance in both quantity and quality. Thus they are better preferred for several industrial applications. There are several parameters based on which the growth and viability of halophilic fungi are assessed when used in industries that include sampling time, geographical distribution/location, dissolved oxygen levels in the surrounding, availability of the water activity along with organic and inorganic nutrients essential for its survival (Tiquia-Arashiro and Grube 2019).

Studies suggest that halophilic fungal species produce highly specific bioactive metabolites with biological activities of industrial importance, such as antibacterial potential, hemolysis, etc. It was observed that decreased temperature and lower water activity improved hemolytic activity of halophilic fungi, thereby improving its stress response. Similarly, the extracellular metabolites produced by halophilic fungi are found to be antibacterial in nature, which when used in industries helps in avoiding bacterial contamination. Some of the examples of halophilic fungi with antibacterial potential include *Aspergillus flavus*, *A. gracilis*, and *A. penicillioides.* Increase in salt concentrations causes increase in the antioxidant capacity of halophilic fungi and this is assessed by performing various antioxidant enzyme assays such as catalase assay, superoxide dismutase, glutathione S transferase assay, and guaiacol peroxidase assay, followed by thin layer chromatography and total phenolic content assay. Hence, they can contribute to the production of antioxidants with

commercial viability when subjected to high concentrations of salt levels (Tiquia-Arashiro and Grube 2019).

Fructooligosaccharide possesses a number of commercial applications by their use as artificial sweeteners and prebiotics, by reducing high cholesterol levels, by curing traveler's diarrhea and constipation. Fructooligosaccharide can be produced by certain halophilic fungi (*Cladosporium cladosporioides*) from sucrose. Studies suggest that several species of thermohalophilic fungi are capable of producing a number of industrially important enzymes (both themostable and halostable) in an economic and an ecofriendly manner, since they are capable of being highly active at high temperature and increased salt concentrations. Some of these enzymes include proteases (*Aspergillus flavus*), amylases (*Engyodontium album, A. gracilis, A. penicillioides*), and celluloses (*A. terreus, Penicillium* sp.). These are of great importance in food, textile, paper and pulp industries. Certain halophilic fungi such as *Aspergillus* sp. nov. F1 have been found to produce secondary metabolites that act as cytotoxic compounds, which when extracted and purified exhibited anti-cancerous properties. This shows the importance of halophilic fungi in pharmaceutical industries (Tiquia-Arashiro and Grube 2019).

Several other applications of halophilic fungi include

1. phenol degradation (*Debaryomyces* sp.)
2. bioremediation of halite on sandstones,
3. treatment of salt damage on building materials due to dampness (*Cladosporium sphaerospermum, Aureobasidium pullulans, Aspergillus nidulans,* and *Wallemia sebi*).
4. heavy metal removal by obligate halophilic fungal species (*A. flavus, A. gracilis, A. penicillioides, A. restrictus, and Sterigmatomyces halophilus*).

Hence, exploring several halophilic fungi from diverse locations helps in understanding their potential in industrial applications and thereby helps in promoting research in this field (Tiquia-Arashiro and Grube 2019).

28.2.4 Acidophilic Enzymes

Acidophilic fungi are very important in the field of biotechnology. The fungi inhabiting acidic niches are a source for acid stable enzymes which can be employed in many industrial processes and products. Acidophilic environments are created on earth naturally and also by anthropogenic activities. The microorganisms living in such regions have adapted and evolved naturally to dwell in high acidic conditions. The enzymes produced by acidophiles are exploited for many industrial processes.

Acidophilic fungi are very important for the field of biotechnology. The fungi inhabiting the acidic niches are the source for acid stable enzymes which can be employed in many industrial processes and products. Acidophilic environments are created on earth naturally and also by anthropogenic activities. The microorganisms living in such regions have adapted and evolved naturally to dwell in high acidic

conditions. The enzymes produced by acidophiles are exploited for many industrial processes.

Mannan is the main constituent of hemicellulose in plants with β-1,4 backbone of mannose and glucose residues. Beta mannanases are the enzymes which help in cleaving this linkage and are applicable in many industries like paper, pulp, animal feed, food, and oil industries. Most of the beta mannanases produced from fungi are acidic in nature which has optimal pH range of 2–3. Fungi like *Penicillium* sp. 40 (Kimura et al. 2000), *Aureobasidium pullulans* (Li et al. 1993), *Penicillium oxalicum* GZ-2 (Liao et al. 2014), *Bispora antennata* (Liao et al. 2012), etc. Mannanases with xylanases in combination are effective bleaching agents for pulp processing (Woodcock et al. 1989).

Xylan is the second most important constituent of hemicellulose of monocots and woody plants. Xylan degrading enzymes are hence very useful in industries like paper, pulp, textile, and agriculture. Xylanase can break down 1–4 glycosidic linkage between the units of xylopyranol. The fungal xylanases from extreme environments are having high economical value for such industrial processes. In pulp industry for pre-bleaching protocols, in bakeries to process dough, in feed processing, etc., the enzyme is useful. Li et al. (1993) reported xylanases with activity at pH 2–5 from *Penicillium* sp., and with activity at 4.8 from *Aureobasidium pullulans*.

Hemicellulases are involved in bioconversion of lignocellulosic biomass in food, feed, and detergent industries. The use of hemicellulase in paper industries enhances the quality of paper. Fungal mannanases are acidic in nature with optimal pH range of 2–6 and hence becomes industrially important. Filamentous fungi like *Aspergillus, Penicillium,* and *Trichoderma* are promising sources of mannanases (Morreira and Filho 2008).

D-galactosidases, (lactase) which hydrolyzes lactose into its monomers, are required by food and dairy industries for whey processing, to produce food supplements for lactose intolerant individuals, for low lactose food milk products, cheese, sweet yoghurt production, etc. (Wang et al. 2009; Baumgartner and Hinrichs 2000). *A. niger* with high thermal and low pH stability, *Teratospaeria acidotherma* AIUBGA-1 with optimum pH of 4 are reported (Hatzinikolaou et al. 2005; Isobe et al. 2013). β-galactosidases are used as digestive supplements in many products, which need to be active at low pH of stomach. Hence the enzyme with optimum pH of acidic range is highly useful (Lin et al. 1993).

Laccases degrade lignin and hence are used for the delignification in paper and pulp industries and also used for synthesis of biosensors and fuel cells (Ghindilis 2000). Also used in beer as stabilizers, in dye and effluent treatment from industries (Ben Younes et al. 2007). Laccases from fungi with optimum pH in acidic range are hence useful. *Trametes hirsute* Bm-2 produced laccase with optimum pH of 4–4.5 (Zapatae Castillo et al. 2012), *T. versicolor* with laccase of pH 4–5 (Minussi et al. 2007), *Ganoderma lucidum* with optimum pH 3.5 (Ko et al. 2001), etc.

Therefore, fungi producing enzymes which are active under low pH can be explored more for the industrial uses.

28.2.5 Alkaliphilic Enzymes

Industrial bioprocessing requires several chemical transformations and most of these transformations take place under alkaline conditions. pH-tolerant enzymes produced by mesophiles have been an ecofriendly bedrock for these chemical processes majorly. However, due to limitations in catalytic efficiency it became prudent to look for extremozymes with optimum biocatalysis at alkaline pH.

Proteases are specialized enzymes produced by the salt-loving Haloalkaliphiles which thrive under high salt conditions. Microbial proteases in general are among the most extensively studied hydrolytic enzymes. With a biotechnological potential that extends into several industrial processes, from detergent, leather, textile, dairy, etc., this group of enzymes represent roughly 60% of the net enzyme sales in the world (Zambare et al. 2011). Considering fungi can grow on cheaper substrates, without compromising on the enzyme yield, in recent times, fungal proteases have grown as potential source for industrial proteases (Anitha and Palanivelu 2013).

Proteases secreted by alkaliphilic Aspergillus species (*A. jilinensis*) have been extensively studied because of their innate ability to produce the enzyme in huge amounts. These proteases find special purpose in food and beverage industry (Wu et al. 2006). Lately, fungal proteases are being used as laundry detergent additives, providing for almost 25% of the world's net requirement (Demain and Adrio 2008). Incorporation of these extremozymes has improved the detergent's ability to remove tough proteinaceous stains. Alkaline proteases from *A. flavus* and *C. coronatus* are also being used religiously as ecofriendlier alternative to using hazardous chemicals during the leather tanning process (Laxman et al. 2005). Fungal proteases have found their way into pharmaceutical industries, where they are used in the elimination of keratin in acne, elimination of callus, and also improving drug delivery (Brandelli et al. 2010).

Almost all of the industrially used cellulases and hemicellulases are primarily purified from well-studied non-extremophilic bacteria and fungi. But recent studies have shown alkaline thermophilic fungi to be much preferable sources of cellulases in textile and detergent industries (Ito 1997). Cellulases and hemicellulases extracted from *P. citrinum* have shown to be functionally efficient at extreme alkaline conditions (Raghukumar et al. 2004). Evaluation of several thermophilic fungal species, such as Thermomyces, Myceliophthora, Aspergillus for their ability to produce cellulolytic and hemicellulolytic enzymes, demonstrated superior cellulolytic and xylanolytic activity. The α-arabinosidase activity in these enzymes was particularly high. Also, the capacity to hydrolyze lignocellulose in the substrate with an improved thermal stability has further added to its potential as enzymes highly sought after in polymer industries (Cantarel et al. 2009).

Although most of the natural environments on this planet remain predominantly neutral in pH, habitats with varying degrees of pH (alkaline or acidic) are not that uncommon. Geothermal regions, soils saturated with carbonate, soda deserts and lakes, etc. have been shown to exhibit extreme ranges of pH (Kimura et al. 2000). Alkaliphilic xylanases, especially from *Penicillium* sp., and *Aspergillus* sp. are hugely dependent on pKa of the catalytic residues that are in turn dependent on

the immediate ambient conditions. Interestingly it was noted that some acidophilic xylanases have an aspartate residue hydrogen bonded to a general acid/base catalyst which is flexible and can be substituted with an asparagine when the enzyme is exposed to alkaline conditions. With this inherent plasticity to work efficiently under varying pH, it was observed that stability of the extremozymes at extreme pH is accounted for by the biased distribution of charged residues (Fushinobu et al. 1998).

Xylanases have application in several industrial processes, covering every sector of commercial enzyme market (Bhat 2000). From baking, to textile, to pulp, the industrial applications are boundless. Some of the other less studied applications include: in brewing, coffee extraction, in protoplastation of plant cell, synthesis of compounds that are potentially pharmacologically active (Christakopoulos et al. 2003).

28.3 Fungal Extremozymes in Specific Green Industrial Processes

28.3.1 Food Processing

Fungal extremozymes are being rigorously used in the food industry, due to their ability to remain functionally versatile under extreme processing conditions. Extremophilic proteases, lipases, and amylases have shown to biocatalyze with minimal energy and more endurance. As a result, they found their way into industries on confectionery and dairy. Viticulture and processing of juice primarily depends on laccases belonging to the Basidiomycetes and Ascomycetes. Special class of laccases are applied to bottle corks to minimize tart flavor during extended wine storage. Laccases also play hugely in improving the baking structure by stabilizing gluten in the dough. Additionally, by reducing the viscosity of the dough, it improves on the workability. Lipases extracted from filamentous fungi such as *Aspergillus*, *Mucor* have been actively used to catalyze the synthesis esters that impart flavor and fragrance. These lipases significantly enhance flavor in Italian cheeses by catalyzing mild lipolysis to help them mature aster (Jooyandeh et al. 2009).

Another biocatalyst that falls under the category of hydrolases is Pectinase that has been lately sought after to allow fruit juice pressing, in particular, during the production of apple juice. Not only this, but also extremophilic pectinases help to minimize the turbidity in wines and remove mucous coating from coffee beans during its fermentation process (Chandra and Enespa Singh 2020).

Microbial proteases in general are among the most extensively studied hydrolytic enzymes. With a biotechnological potential that extends into several industrial processes, from detergent, leather, textile, dairy, etc., this group of enzymes represents roughly 60% of the net enzyme sales in the world (Zambare et al. 2011). Proteases secreted by alkaliphilic *Aspergillus* species (*A. jilinensis*) have been extensively studied because of their innate ability to produce the enzyme in huge amounts. These proteases find special purpose in food and beverage industry

(Wu et al. 2006). Fungal extremophilic proteases are being actively used for the production of milk substitutes. In the past, use of proteases in dairy industry would fail to correct the bitter taste in milk. However, fungal proteases when used appropriately help with the rectification of this unwanted trait. Precipitation of casein protein has been greatly improved with the use of acid proteases from the *Mucor* species. Additionally, for the production of soy sauce which involves the hydrolysis of soy proteins has been catalyzed using proteases extracted from the *A. niger* and *A. oryzae* (de Souza et al. 2015).

28.3.2 Textile and Pulp Industry

In textile industry, bleaching of cotton and bio-stoning process are key to the production of cotton. Fungal laccases help improve whiteness in cotton during its conventional production process. Also, during the bleaching of textile dyes, the use of laccases instead of physical and chemical methods leads to the proper degradation of dyes with next to no entering of the synthetic in the industrial waste and later to the environment. This is because synthetic dyes are resistant to fading under exposure to light, water, and other chemicals. However, laccases catalyze the complete elimination of dyes from fabrics during the bleaching process. Dyeing fabric with deeper colors has been found to be economically unprofitable. But by using biocatalysts such as laccases which enhance the fixation of the dye, one might not need to apply the dye in excess. Alternatively, to prevent wool from shrinking, the traditional method involves chlorination. But doing so impacts the environment negatively. Instead, treatment of wool with proteinases hugely reduces the deleterious environmental impact, in addition to preventing the wool from shrinking.

Deinking is a process in paper industries for reducing the brightness of the paper. The process is performed by chemical modes using hazardous chemicals like surfactants, chelating agents, sodium hydroxide, hydrogen peroxide, sodium carbonate, sodium silicate, etc. These can be replaced by microbial enzymes which are ecofriendly. White rot fungi are best source for hydrolytic enzymes like manganese peroxide, laccase, and xylanase for degradation of lignin and hemicellulose. Bio-bleaching involves cellulose hydrolysis facilitating ink detachment. Increased brightness and dirt removal are the advantages of using enzymes. Extremozymes like alkaliphilic xylanase and mannase increase paper quality and hence used in bio-bleaching protocols. Recycling of the old paper involves deinking of newspaper pulp, where combination of xylanase and laccase was found to be performing better in enhancing the paper quality and decreasing the chemical consumption. Laccase helps in removing the lignin content and hence used in lignin-rich pulp. Either lipases or laccase and hemicellulase together can be used in deinking for the old newspapers. The enzymes like amylase, cellulase, lipase, pectinase, and xylanase are important for pulp processing at different stages like deinking, draining, fiber modification, debarking, bleaching, etc. As all these processes involve many harsh conditions like alkaline pH, high salt concentration, temperatures, the extremozymes are very important.

28.3.3 Pharmaceutical Industry

Enzymes for hot temperatures are important for many industrial processes. Industrially, hot extremozymes bear a wide range of applications in industries such as pharmaceuticals, pulp and paper, food and beverages, and many more. These extremozymes benefit in various biotechnological processes as they can

1. undergo separation from other heat-labile enzymes during production steps,
2. shorten fermentation processes due to high growth rate at increased temperatures and low concentration at decreased nutrient content,
3. lower energy consumption owing to lower viscosity of the liquids at high temperature,
4. retain high efficiency at extreme conditions,
5. alleviate the constraints of industrial processes.
   (Sarmiento et al. 2015; Pabulo and Rampelotto 2016).

Apart from a wide range of biotechnological applications, thermophilic fungi are found to be useful in pharmaceutical industries, especially in the process of biotransformation of organic compounds for bioactive compound synthesis. The use of thermophilic fungi makes the process hazard-free that helps to overcome the disadvantages of other chemical processes such as minimizing the complications of isomerization, racemization, and epimerization. Thermophilic enzymes display thermostability that offers better advantages than the enzymes from mesophiles during industrial processes. It is found that thermophilic enzymes cloned and expressed in mesophilic hosts can easily be purified by heat treatment and are capable of withstanding high amounts of chemical solvents. Such reactions are favorable in pharmaceutical industries since there exists a high demand for enantiomerically pure compounds. Thermophiles during biocatalytic reactions help in improving transfer rates and substrate solubility, decrease the viscosity of the medium, and avoid the risk of mesophilic microbial contamination. Thermophilic fungi are well-known to resist denaturants present in extreme alkaline and acidic conditions (Tiquia-Arashiro and Grube 2019).

Some of the biotransformation reactions performed in pharmaceutical industries using thermophilic fungi includes:

(a) albendazole (a prodrug) to albendazole sulfoxide by *Rhizomucor pusillus.* This when performed chemically is a difficult process as it is a site-specific reaction that may also lead to environmental pollution. This process with thermophilic fungi offers a better yield at a high temperature when compared to mesophilic fungi.
(b) ferulic acid to guaiacol by *Sporotrichum thermophile*.
(c) biotransformation of steroids by thermophilic fungi is said to produce effective steroids in an ecofriendly and economic manner. *Acremonium alabamensis* and *Talaromyces emersonii* are commonly used thermophilic fungi for steroid production in pharmaceutical industries (Tiquia-Arashiro and Grube 2019).

Thermophilic fungi are also known to help in predicting mammalian drug metabolism and metabolite toxicity, as microbial models. This is a very crucial step during drug innovation as well as to establish the safety and efficacy of the drug prior to human consumption. Using thermophilic fungi offers several advantages such as ease of handling, cost-effective process, potential to decrease animal use for the same and to improve scale-up capacity. Some of them that are commonly used for this approach are *Rhizomucor pusillus* NRRL 28626, *Rhizomucor pusillus* NRRL 28626, and *Thermomyces lanuginosus* NCIM-1934. Thermophilic fungi have also been proven to be potential in the production of novel value-added metabolites, such as Losartan as Human Peroxisome Proliferator Activated Receptor-Gamma (PPAR-γ) and Human angiotensin Receptor (AT1R) Binders, Hepatitis C Virus RNA-Dependent RNA Polymerase NS5B inhibition potentials of albendazole and its biotransformed metabolites. Thermophilic fungi also aid in antibiotic production, e.g. Myriocin, a new crystalline antifungal compound that acts against *Candida* sp., *Trichophyton granulosum* and *Microsporum gypseum*. Other antibiotics such as thermozymocidin penicillin G, 6-aminopenicillanic acid, sillucin, miehein, and vioxanthin are known to be produced by thermophilic fungi (Tiquia-Arashiro and Grube 2019).

Industrial bioprocessing requires several chemical transformations and most of these transformations take place under alkaline conditions. pH tolerant enzymes produced by neutrophils have been an ecofriendly bedrock for these chemical processes majorly. However, due to limitations in catalytic efficiency it became prudent to look for extremozymes with optimum biocatalysis at alkaline pH. Certain halophilic fungi such as *Aspergillus* sp. nov. F1 have been found to produce secondary metabolites that act as cytotoxic compounds, which when extracted and purified exhibited anti-cancerous properties. This shows the importance of halophilic fungi in pharmaceutical industries (Tiquia-Arashiro and Grube 2019).

28.3.4 Bioconversion

Fungi, among all microorganisms, have been identified as key mediators during biofuel production and other bioconversion processes. The supremacy of fungi in all areas of bioconversion processes is attributable to their ability to specifically degrade lignocellulosic materials with enzymes possessing superior redox potentials and themostability. Lignin biodegradation is one of the key steps involved in biofuel production and during the conversion of other biorefinery products. Lignin modifying enzymes (LMEs) comprise a wide collection of enzymes ranging from peroxidases, to laccases, to oxidases that are capable of disrupting lignin components, or oxidizing various phenolic compounds. Most the LMEs work synergistically during the lignification and depolymerization of lignin polymers. Thermostable cellulases are being preferred lately as suitable candidates for bioprocessing industries because of their ability to efficiently hydrolyze lignocellulosic substrates by improving on the reaction rate, rate at which organic compounds become bioavailable, while lowering the viscosity and economic cost. In the recent

times, adopting an enzyme-assisted technology has increased the economic viability during the production of biofuels. Cellulases hold a key role in the hydrolysis of cellulosic polymers toward the secretion of fermentable sugars, which eventually lead to the production of biofuels.

Biofuels made from lignocellulosic non-food waste biomass represent second-generation biofuels. Fungal lignocellulosic biomass offers a renewable source of carbon. It can be also converted into several value-added products. At the same time, the waste biomass contributes hugely to the fermentation process, wherein the waste produced can also be used as animal feed with high nutritional quality (Srivastava et al. 2018).

28.3.5 Bioremediation

The industrial revolution has transformed the socioeconomic status of our society. It has impacted every facet of human growth from food to energy, production, sanitation, and manufacturing technologies. While it led to prosperity and improved quality of life, it has adversely affected the ecosystem and the environment posing serious threats to humankind and its survival. The earlier section of this chapter discussed the applications of fungal extremozymes for industrial processes. The same array of extremozymes is also being employed for the bioremediation of industrial wastes as discussed below (Fig. 28.2).

Increased industrialization has led to the depletion of natural resources, global warming, and the accumulation of inorganic and organic pollutants in soil, air, and

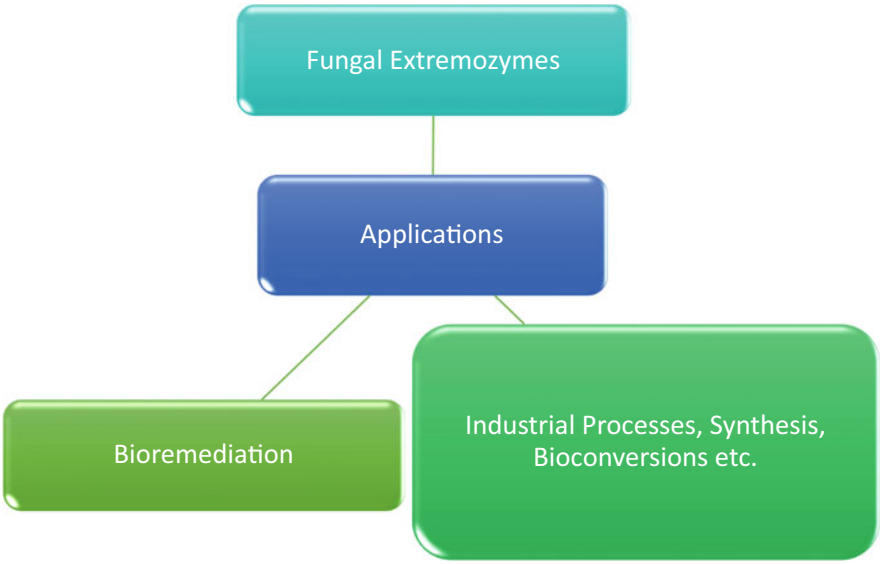

Fig. 28.2 Applications of fungal extremozymes for green chemistry

water (Singh et al. 2020). Solid and chemical waste management has become the foremost concern to conserve the environment. Pollutants like polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs), and heavy metals have contaminated the soil causing the quality of soil to decrease drastically. Many physical and chemical treatments were introduced to remove these pollutants; however, large-scale application of these treatments was not achievable (Deshmukh et al. 2016). Bioremediation is recognized as an effective alternative to combat these issues. It is a procedure that converts hazardous compounds into non/less-hazardous compounds by the action of microbes like fungi, bacteria, algae (Singh et al. 2020). It is an environmentally friendly, economical, and efficient method in the disposal of these toxic chemicals. Biostimulation, bioaugmentation, and natural attenuation are certain major techniques that can be followed to carry out the technique of bioremediation (Deshmukh et al. 2016).

28.3.6 Mycoremediation

The application of fungi in bioremediation is termed as "mycoremediation." Certain fungi can grow and survive in polluted environments as they are adapted to metabolize and utilize the chemicals, as a nutritional resource. They produce hydrolytic enzymes such as protease, lipase, nuclease, amylase, etc. that degrade complex chemicals into simple less toxic compounds or transform metals, metalloids, and other organic materials into harmless ions via redox reactions. Fungi can also immobilize and store the metalloids or metals in mycosphere or various other parts of the cell. Another distinct characteristic of fungi is that they can degrade compounds that are not intoxicated by bacteria (Singh et al. 2020). Extremophilic fungi have been isolated from highly contaminated environments such as acidic mining wastewaters, hot volcanic geothermal regions, etc. (Chan et al. 2019). However, bioremediation is a recent development and there is limited data available on the use of extremophilic fungi in this process. Hence this chapter concentrates on the diverse role of extremophilic fungal groups in examining the technique of bioremediation.

Extremophilic organisms, counting marine fungi, are imperative resources of steady and treasured enzymes defined as "extremozymes ." Numerous marine extremozymes have been employed for biotechnological studies. However, only a few extremozymes are presently being produced and utilized at the industrial level. Therefore, additional scientific trials are needed to understand the possible applications of extremozymes (Barone et al. 2019).

28.3.6.1 Marine Extremophilic Fungi in Mycobioremediation

Salinity predominates oceanic environment. The adaptive strategies employed by extremophiles for a successful halotolerance make them ideal candidates to be employed in the bioremediation process.

Salt pans are a significant resource for extremophilic microorganisms. However, not many fungi species can survive in such extreme saline conditions as it adversely affects the protein structure and hampers the enzyme function. Few fungi, such as black yeasts, *Hortaea werneckii, Phaeotheca triangularis, Aureobasidium pullulans,* and *Trimatostroma salinum* have been isolated from salt pans having 15–30% salinity is reported. *Phaeotheca triangularis* and black yeasts are described as obligate halophiles, whereas other species are facultative halotolerants (Damare et al. 2012). Marine fungi have been found to tolerate high concentrations of heavy metals such as lead and copper and their interaction with metal ions is one such property that can be used in bioremediation.

Trichoderma viride Pers NFCCI-2745 was isolated from an estuary polluted with phenolics and it was reported to produce a halotolerant laccase enzyme. This enzyme was utilized in the bioremediation and removal of phenolics. Related applications of enzyme facilitated bioremediation were verified for decolorizing remazol brilliant Blue-R dye. Three basidiomycetes isolated from marine sponges were used. Similarly, *C. unicolor, marine white rot basidiomycete* was employed to degrade anthraquinone reactive blue 4 dye. Reports also suggest that marine fungi belonging to the genus *Penicillium* and *Trichoderma harzianum* aid in the biotransformation of persistent organic pollutants like PCB 118 and pentachlorophenol, respectively. Additional marine-derived fungi including *Mucor*, *Aspergillus*, and slime mold confirmed bioremediation possibility for water-soluble crude oil fractions. However, higher concentrations caused toxicity to the microbes (Singh et al. 2020).

Marine species of *Aspergillus sclerotiorum CBMAI 849* (isolated from cnidarians) were proficient in reducing 99.7% of pyrene and 76.6% benzo[a] of pyrene after 8 and 16 days, respectively. They also reported considerable quantities of benzo[a]pyrene (>50.0%) reduction by a *Mucor racemosus CBMAI 847*. The mechanism of hydroxylation was studied by coupling with sulfate ions which are reduced by a cytochrome P-450 monooxygenase enzyme produced by the marine extremophilic fungi. Another study reported the use of a marine fungus *Aspergillus candidus* that could grow in the presence of arsenic (25 and 50 mg/L) and decreased the volume of the metal by the bioaccumulation process (Damare et al. 2012).

28.3.6.2 Other Extremophilic Fungi in Mycobioremediation

Numerous extremophile fungi such as *Coniochaeta fodinicola, Teratosphaeria acidotherma, Hortaea acidophilia, and Acidomyces acidophilus* were known to produce metabolites and novel enzymes to endure severe environments. These fungi are utilized as bioremediation agents to remove toxic metalloids from water and soil.

A. acidophilus WKC-1 was tested for biosorption of arsenic and antimony. It was observed that PO_4-,SO_3,–OH, –NH, –CH are few of the functional groups that are recognized as the significant biosorption binding sites for As^{5+} and Sb^{5+}. The isolate WKC-1 showed a high percentage of As^{5+} removal (around 70.30%). The ability of the fungi to tolerate low pH and high arsenic concentration together makes it a potential candidate to be used in the bioremediation of arsenic (Chan et al. 2019).

A psychrophilic fungus, *Cryptococcus* sp. was isolated from deep-sea sediments. It displayed tolerance and development in the existence of high levels of heavy metals (up to 100 mg/L) $ZnSO_4$, $CdCl_2$, $CuSO_4$, $Pb(CH_3COO)_2$. This conveyed that *Cryptococcus* sp. can be used in the bioremediation process under extreme conditions with good quality of its activity being exhibited (Deshmukh et al. 2016).

A study was conducted by Bano et al. (2018) to examine the biosorption of copper, lead, zinc, cadmium, ferrous, and manganese by testing with the following fungi species: *Aspergillus flavus, A. gracilis, A. penicillioides, A. penicillioides, A. restrictus,* and *Sterigmatomyces. A. flavus* and *Sterigmatomyces halophilus* showed a potential for heavy metal removal with a value of 85% and 83%, respectively.

28.3.7 Extremozymatic Bioremediation

The extremophiles and their extremozymes are one of the most desirable bioremediation tools.

28.3.7.1 Laccases

Cladosporium oxysporum, Curvularia lonarensis, Aspergillus niger, Fusarium equiseti, Cladosporium funiculosum, Cladosporium halotolerans were isolated and identified from a hypersaline lake in Maharashtra. The fungi were highly tolerant to alkaline conditions and employed to treat the municipal wastewater treatment plants and sewages having high concentration of metal ions and alkaline pH. Studies also describe that *Cerrena unicolor* MTCC 5159 produces halotolerant laccase that assists in the degradation process of alkaline raw textile mill effluents.

Laccases not only oxidize phenolic and methoxyphenolic acids, but also decarboxylate and attack their methoxy groups (demethylation). These enzymes are involved in the depolymerization of lignin (a constituent of biomass) (Gangola et al. 2019). It comprises phenylpropanoid units linked by C–C and C–O bonds. Since fewer organisms produce laccases extracellularly, fungal laccase plays a potential role in lignin depolymerization. Hence extremophilic fungal laccase works as an outstanding bioinoculant for bioremediation (Prakash et al. 2019).

28.3.7.2 Peroxidases

Lignin peroxidase (LiP) and manganese peroxidase (MnP) were exposed in the mid-1980s in *Penicillium chrysosporium* and defined as true ligninases since they display high redox potential. LiP and MnP catalyze the oxidation of lignin units by H_2O_2 LiP which degrades non-phenolic lignin, whereas MnP generates Mn^{3+}, which acts as a diffusible oxidizer on phenolic or non-phenolic lignin units via lipid peroxidation reactions. The use of peroxidases for soil cleaning has been considered, precisely for soils contaminated with aromatic hydrocarbons and detoxified by autochthonous fungi producing peroxidases (Mougin et al. 2009). Peroxidases are operative for the biodegradation of oil spills, carbamate insecticides, and organophosphates. The key benefits of this enzyme are its ready availability,

tolerance to water-miscible solvents, and lack of cofactor stereoselectivity that make them suitable enzymes for biotechnological research (Gangola et al. 2019).

28.3.7.3 Catalases

Accumulation of reactive oxygen species (ROS) results in damage to cellular macromolecules, which is toxic for cellular integrity. The chief defense mechanism to ROS in fungi involves catalases and peroxidase. Heavy metals such as copper (Cu), lead (Pb), cadmium (Cd), and zinc (Zn) have been reported to be among the major reasons for ROS induction in microbial cells. To date, all studies indicate an increase in anti-oxidative activity of enzymes in the presence of heavy metals and ROS. A study reported higher catalase activity when Cu^{2+}, Pb^{2+} 50 (mg/L) were added in combination or individually to the fungal consortia comprising of *A. niger, Rhizopus* sp., and *Penicillium* sp. Hence catalase activity can be employed as a screening technique to check the effectiveness of bioremediation in oil-contaminated soil. Hence, engineering this fungal enzyme can be favorable for bioremediation of metal-polluted spots (Deshmukh et al. 2016).

Studies on the effects of extremozymes in bioremediation are less understood. The limited studies on extremophiles are due to their distinct nutritional supplies and stimulating growth conditions (Jin et al. 2019). Extremozymes from fungal sources are an important arsenal in the bioremediation process and many techniques and extreme environments are explored to screen and isolate extremozymes. The biodiversity of extremophiles and their adaptations to harsh conditions is one of the important means that can help reverse the damage and destruction caused by this environment.

28.4 Conclusion

The endeavor to blend industrial productivity with sustainability has been a constant source of inspiration to seek newer alternatives. With the addition of biocatalysis in industrial chemical transformations, the concept of green chemistry has been very closely bound to enzyme catalysis. This in essence has been seen as an alternative to the classic chemical catalysis which has predominated majorly since the industrial revolution. In the recent times, biocatalysis has evolved toward more specialized extremophilic enzymes, extremozymes as they are termed, which have the ability to withstand an array of harsh conditions, one that would rival the efficiencies of most enzymes produced within the mesophilic realm, be it properties of extreme halotolerance, thermal stability or enduring freezing temperatures. But very little is known about the manner in which these specialized enzymes work, or the molecular strategies they adopt to optimally function. Therefore, any further strides made, either from a bioprocessing point of view or be it the use of these enzymes in the utilization of industrial wastes or for bioremediation, would require a more comprehensive understanding of these enzymes.

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Phylogenomics, Microbiome and Morphological Insights of Truffles: The Tale of a Sensory Stimulating Ectomycorrhizal Filamentous Fungus

29

Mohan Das, Ananya Pal, Subhodeep Banerjee, Subhara Dey, and Rintu Banerjee

Abstract

Truffles are generally considered as subterraneous ascomycete, capable of forming ectomycorrhizas with plant roots via symbiosis. The fruiting body of truffles are widely appreciated around the world for its distinctive aroma. Although the plantation system for truffles is established, it still faces an array of challenges. The queries are very unique on its own and yet to be resolved. The taxonomic classification seems to be highly complicated and needs proper sampling to achieve progress using advanced technologies. The truffles being an extremophile need precise soil and weather conditions for its growth. The unique feature of truffle is its extreme slow growth rate for rejuvenation under optimum cultivation conditions. While positioning of truffles is a very crucial criterion to understand the type of symbiosis it maintains with different plants. Together with this it is apprehended that it is the plant and the rhizospheric soil which partially determines the microbial community within truffles. This in turn partially contributes in producing the distinctive aroma of truffles. Nevertheless, it is worth mentioning that a truffle renders several pharmacogenetic effects on human system, which need more investigation to unravel this exorbitant creation of nature.

M. Das · R. Banerjee (✉)
Agricultural and Food Engineering Department, Indian Institute of Technology, Kharagpur, West Bengal, India
e-mail: rb@iitkgp.ac.in

A. Pal · S. Dey
P. K. Sinha Centre for Bioenergy and Renewables, Indian Institute of Technology, Kharagpur, West Bengal, India

S. Banerjee
Advanced Technology Development Centre, Indian Institute of Technology, Kharagpur, West Bengal, India

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Keywords

Truffles · Phylogeny · Microbiome · Molecular characterization · Health benefits

29.1 Introduction

Apicius' Legendary Banquet considers truffles as an Epicurean delicacy honoured by Greeks and Romans. The first article on the nature of "Truffles" was published by Ciccarelli in 1564. Hervé This, the discoverer of molecular gastronomy, named truffles as 'diamonds' or 'the best food'. In true sense, truffles are actually hypogeous edible fungus, which possibly undergoes a complicated life cycle, a time period when a symbiotic relationship between the mycelium and the roots of trees (e.g. Oak, hazel, willow, poplar) and shrubs (e.g. *Cistus*) gets established. The truffle or the fruiting body finally develops due to the aggregation of the hyphae. Generally, species belonging to the genus *Tuber* are regarded as "true truffles" and are also considered as one of the very few ectomycorrhizal Ascomycetes. Although according to the classical classification system truffles were in the order *Tuberales*, which includes hypogeous ascomycetes, today they are under the order of *Pezizales* (including both hypogeous and epigeous fungi) and are irrespective of their saprotrophic or symbiotic nature, since they are all attached to each other phylogenetically (Antonietta et al. 2006).

Tuber magnatum or the "white truffle" and *Tuber melanosporum* Vittad or the "black truffle" are in high demand due to their characteristic taste and aroma, which actually results from a concocted blend of volatile compounds. The formation of ascocarp and truffle life is influenced both by biotic (bacteria, fungi, yeasts, mesofauna) and abiotic (composition of soil, rain, temperature and sunshine). Most of the truffle species share common features and therefore need calcareous soil (pH 7–8), with exceptions for *Tuber borchii* since it needs acidic soils. The demographic locations define the extreme criteria and conditions of truffle growth and availability. The entire Europe is mostly rewarded with *Tuber borchii* and *Tuber maculatum* whereas Western and Southern Europe (e.g. Spain, Italy, France) are especially prized with *Tuber melanosporum* and handsome collection of *Tuber magnatum* is mostly from Italy and in limited quantity from Eastern Europe (e.g. Croatia, Slovenia, Hungary) (Antonietta et al. 2006; Das et al. 2020).

The report on *Tuber melanosporum* by Zampieri et al. (2011) emphasizes transcriptional profiling for expressing the specific gene of different traits in different climatic regions indicates phenotypic characters and specifically mentioned the profiling in colder climatic conditions (4 °C) with other temperature variations. The validation of experiment has been carried out using standard protocol of RT-PCR where the genes show better expression in case of heat shock protein, cell wall production and lipid accumulation profiling of *T. melanosporum*. Thus, it is a real challenge to the mycologist to grow truffle ascomata in low temperature and environmental conditions. As mentioned earlier, *T. melanosporum* has an interesting life cycle, where root colonization is mainly taken place in winter that establishes a

symbiotic relationship with the host that can withstand the harsher climatic conditions (Martin et al. 2010). Similarly for the growth and maturation of *Tuber borchii*, dehydrin-like proteins play a significant role that code the high expression of the protein under colder condition and osmotic stresses that are needed for proper growth of *Tuber* spp. Thomas and Büntgen (2019) reported that summer temperature and precipitation are the two important factors for reduced growth of truffles as it has been reported that under low temperature condition and optimum soil and moisture ratio the growth of truffle can be accelerated. Thus, the environmental issues for truffle growth are a real challenge to the scientific community due to its extreme low growth profile. Whereas, the dessert truffles cultivation occurs in semiarid conditions of the Algerian deserts of the Sahara and are thus extremely well adapted to less rainfall and harsh climatic conditions such as dry and hot environment, i.e. contrast to the cold loving truffles as discussed (Bradai et al. 2015).

Truffles are mostly hunted with the aid of trained pigs and dogs. The huge demand for truffles on one side and dropping productivity on the other have enforced the scientists to understand the extreme culturable conditions and mimic the system and for this purpose advanced strategies and approaches have been adopted to understand the bottlenecks of the system, its environmental microbiology, its molecular definition and phylogenetic lineages to illustrate, explore and search possible chances to overcome the hurdles of truffle production (Antonietta et al. 2006; Das et al. 2020; Dash et al. 2016).

29.2 Morphological Diversification of Truffles Through Evolutionary Approach

The truffles which possess sac-like spore-producing structures are classified as the morals and cup fungi in the class Ascomycetes. Ascomycetes are characterized by sexual spores called ascospores, formed in sac-like structures called asci, millions of which form the fruiting body called ascoma (plural: ascomata) with tightly interwoven hyphae. In evolutionary sequence, truffles are mostly similar to the members of the order *Pezizales* which includes family like *Pezizaceae*, *Sarcosomataceae* and *Otideaceae*. During the course of evolution, family members of *Otideaceae* show gradual changes to sequestrate (their fruit-body does not liberate spores at maturity) or become hypogenous (they produce their ascomata underground). Under this family, *Genea* produces closed but hollow ascomata, while *Geopora* has much less air space inside the fruit-body and is hypogenous. Although a solid truffle of the genus *Tuber*, family *Tuberaceae* which is evolved by the elimination of airspace altogether, possesses sequestrated, hypogenous and solid (no air spaces anymore) ascomata. The ascospores with highly ornamented walls are found within rounded and thin walled asciin, a highly convoluted hymenium. Their hypogenous nature has necessitated a new method for passive spore dispersal. The odours released from mature ascospores lead many mammals to unearth and consume them subsequently depositing the still viable spores elsewhere. Thus, forcible spore discharge mechanism is replaced by spore dispersal via animal mycophagy (Kendrick 1985).

True truffles, belonging to the genus *Tuber*, are characterized by a pattern of sterile and fertile veins filling the gleba, which darken as the fertile tissue matures and vary in colour by species. Their stereothecia has a distinct, simple to layered peridium enclosing a gleba of fertile tissue marbled with sterile, hypha-stuffed veins that tend to open through the peridium. Ascomata with smooth pubescent surface or with rounded or angular warts may be white, grey, yellow, olive, brown, reddish brown, dark brown or black in colour. The gross morphological features that help to distinguish *Tuber* species are the texture of the peridium and microscopic characteristics of the asci and spores. The peridium may bear large warts as in *T. aestivum* or it could be rough, scaly, pubescent or glabrous as in *T. oregonense*. Asci may be globose or sub-globose or more flask-shaped and bear a stem as in *T. lyonii*. Finally, spores may be alveolate-reticulate or spiny, as in *T. maculatum* and *T. melanosporum*, respectively (Trappe et al. 2008, 2010). The species *Tuber gennadii* is characterized by having locules lined by a palisade of asci and was hypothesized to be an intermediate form of epigeous and hypogeous fruiting behaviour.

Besides the members of ascomycetes, some truffle-like members are in the primitive fungal class Zygomycetes and within Basidiomycetes, named as false truffles. The false truffles of Basidiomycetes produce underground truffle-like basidiocarps, evolved by extensive morphological divergence. Being intermediate species between truffles and mushrooms, they have a vestigial stem, unable to lift them out of the ground; while the cap remains closed with crowded gills which are distorted or even replaced by chambers. Such fruit-bodies are named as 'secotioid' in reference to genus *Secotium*. Finally, the losing of vestigial stem or columella occurs to improve. Selection for animal dispersal (instead of forcible spore dispersal) and water loss reduction forces the morphological evolution of the false truffle *Rhizopogon* from a suilloid ancestor (Trappe et al. 2008).

Most false truffles have a gleba (the internal, spore-producing tissue) that is divided up into small chambers called locules. Many false truffles also have a short stipe at the base or a stipe-columella that extends into the gleba as a branching structure. Aside from the stipe-columella, the gleba of false truffles is fairly regular in appearance. This easily separates them from true truffles, which appear marbled when sliced in half. Some examples of false truffles are *Rhizopogon luteolus* (yellow false truffles), *Alpova trappei*, *Gastroboletus turbinatus*, *Hymenogaster boozeri*, etc. (Trappe et al. 2010).

Genus *Rhizopogon* has white to yellow, salmon, red or brown peridium with wine-like, cheesy or spicy-pungent odours. Gleba varies from white to yellow in youth, becomes olive, olive-grey, olive-brown, orange-brown or blackish brown in maturity. Genus *Alpova* with yellow to reddish brown peridium has yellow to ochraceous (in youth) gleba which is gelatinous and sticky to touch. It stains reddish brown when exposed. The chambers with spores in a gelatinous matrix are walled off by meandering veins. Genus *Endogone* under Phylum Zygomycota has solid grey to bright yellow to brown gleba with a mass of spores and mycelium without an organized structure. Peridium if present varies from white to bright yellow to brown in colour with smooth to cottony texture (Trappe et al. 2008).

29.3 Truffle Microbiome

29.3.1 Bacterial Communities

While investigating the microbial diversity in truffles it has been observed that bacterial communities tend to colonize in million to billion cells/gram within the inner region and outer parts of fruiting bodies. Compositional analysis of bacterial community has also been investigated in context to its life cycle (fruiting body v/s mycorrhizas), maturation state of fruiting body and the peridium (outer layer) v/s the gleba (inner layer). Based on both culture-independent and culture-dependent methodologies, it has been reported that different species of truffles contain complex communities of bacteria including mostly *Actinobacteria*, *Bacteroides*, *Firmicutes* and *Proteobacteria*. There exist considerable differences between bacterial composition of *Tuber borchii* with *Tuber melanosporum* and *Tuber magnatum*, since it has been observed that the microbiota of *Tuberborchii* is dominated by *Betaproteobacteria*, *Bacteroidetes* and *Gammaproteobacteria*. It has been reported that a *Bacteroidetes* strain may exist under axenic conditions within the mycelium of *T. borchii*, which suggests strong association of truffles with bacteria. Maturation of fruiting bodies occurs after melanization of the gleba, which is mainly due to the formation of spores occurring within the fungal asci. The uniqueness in the aroma of truffles is mostly dictated by the metabolic activities played by the microbes present within them. For truffles like *T. borchii*, *T. magnatum* and *T. melanosporum*, which are mostly harvested in Europe, the process of melanization extends for a few months and occurs during the season of winter or late autumn. With increasing maturity a significant decrease in bacterial count has been observed for the aforementioned varieties, as proved by fluorescence in situ hybridization (FISH). Nevertheless, it is worth mentioning that there did not exist any considerable differences in composition of the community, especially for *Actinobacteria*, *Alphaproteobacteria*, *Bacteroidetes*, *Betaproteobacteria*, *Gammaproteobacteria* and *Firmicutes* (Vahdatzadeh et al. 2015). Therefore, it is apprehended that it is the ratio of different microbes and their metabolic system which is actually determining and dictating for the unique aroma of truffles.

29.3.2 Yeast Communities

While assessing the presence of yeast community within fruiting bodies of *T. magnatum*, *T. aestivum*, *T. melanosporum*, soil of truffle orchard and ectomycorrhizas, it has been observed that fruiting bodies and truffle ectomycorrhizas are highly colonized by yeasts, reaching 3×10^7 CFU/g of dry fruiting bodies. The results are based on culturable techniques, which although provide insights about the system but may overlook the detailed diversity of the system. The diversity is mostly dominated by *Cryptococcus albidus*, *Debaryomyces hansenii*, *Cryptococcus humicola*, *Saccharomyces paradoxus* and *Rhodotorula mucilaginosa*. The density and types of species may vary between gleba and

paridium as well as with change of distinct species. Indeed, isolated yeasts were detected by culturable methods from the peridium of *T. melanosporum* and *T. aestivum* but not from intact truffles gleba (Vahdatzadeh et al. 2015).

29.3.3 Fungal Communities

Truffles are mostly colonized by guest filamentous fungi, as isolated from *Tuber rufum*, *Tuber aestivum*, *Tuber nitidum*, *Tuber brumale*, *Tuber magnatum*, *Tuber melanosporum*, *Tuber excavatum* and *Tuber puberulum*. The loose association between truffles and fungi was depicted from the observation that only 26% of truffles have guest fungi. Filamentous fungi mostly colonize within the peridium of truffles, having a density of 10^2 CFU/g of truffle fruiting bodies, whereas they seem to be mostly absent in the gleba of truffles (Vahdatzadeh et al. 2015).

29.4 Taxon Sampling and Phylogenetic Analyses

The phylogenetic position of hypogeous taxa inside *Pezizales* is determined by LSU rDNA (large subunit ribosomal deoxyribonucleic acid) sequences of 134 epigeous pezizalean species (out of 141 specimens) and 48 hypogeous species (out of 55 specimens) by Laessoe and Hansen (2007). Considering the entire of *Pezizales*, three subsets representing three distinct lineages of *Pezizales* were constructed. The taxa representing the family *Pezizaceae* for lineage A, *Caloscyphaceae*, *Tuberaceae*, *Discinaceae*, *Rhizinaceae*, *Morchellaceae* and *Helvellaceae* for lineage B and *Ascodesmidaceae* and *Pyronemataceae* for lineage C were assessed in the three alignments.

29.4.1 Phylogenetic Relationship Within Lineage A

Within lineage A, parsimony analysis yielding three MPTs considering 973 characters revealed *Pezizaceae* as monophyletic having a sister group named *Ascobolaceae*. The consensus tree is although resolved but deep root relationships are yet to be well understood and supported. Out of 14 lineages, five or six housed 17 species of truffles, covering 11 genera. Although during analysis by maximum parsimony (MP) *Eremiomycese chinulatus* has been resolved and attached with *Peziza vacini* but after maximum likelihood (ML) and Bayesian analyses *E. echinulatus* is positioned with *Plicaria–Hapsidomyces* lineage together with *Peziza phyllogena*. The genus *Pachyphloeus*, apothecial *Scabropezia* and anamorph *Glischroderma* form a highly supported clade, to which *Amylacustasmanicus* forms a sister taxon. It has been reported that *Sarcosphaera* forms a group with two species of *Hydnotryopsis* genera. Within all the MP trees, although *Mattirolomyces* has been nested inside *Peziza s. str.* lineage but after ML and Bayesian analyses *Mattirolomyces* group with *Iodophanus*, being a sister group of *Peziza s. str.* lineage.

It has been suggested that *Iodowynnea* has close relation with *Kalihariturber*. The species of *Peziza*, *P. whitei* and *P. ellipsosora* along with the genera *Tirmania*, *Ruhlandiella*, *Cazia* and *Terfezia* are found to resolve apothecia forming species of *Peziza* within the *P. depressa–Ruhlandiella* lineage (Laessoe and Hansen 2007).

29.4.2 Phylogenetic Relationship Within Lineage B

For determining phylogenetic relationship within lineage B, parsimony analyses yielded three MPTs considering 699 characters. It has been reported that the consensus tree is resolved but except *Tuberaceae* there is a lack of support for other families. On the basis of Bayesian analyses, *Underwoodia columnaris* has been excluded since it is unresolved but *Helvellaceae* is highly supported. But maximum parsimony (MP) and maximum likelihood (ML) analyses resolved a *Helvellaceae–Tuberaceae* and *Morchellaceae–Discinaceae* lineage. Although the exact position is yet to be determined, *Fischerula subcaulis* and *Leucangium* are variedly positioned inside *Morchellaceae–Discinaceae* lineage. In all the analyses, a monophyletic group has been formed by two *Hydnotyra* species, named *H. cubispora* and *H. cerebriformis* which is placed inside *Discinaceae*. A sister group of a clade of *Wynella silvicola* and species of *Helvella* has been formed by *Balsamia oregonensis* and *Balsamia magnata*. A clade composed of four additional genera of truffles, namely *Choiromyces s. Str.*, *Dingleya*, *Labyrinthomyces* and *Reddellomyces* included 11 *Tuber* species as a sister group (Laessoe and Hansen 2007).

29.4.3 Phylogenetic Relationship Within Lineage C

For establishing phylogenetic relationship within lineage C, parsimony analyses have been performed which yielded three MPTs (Most Parsimonious Trees) considering a total of 894 characters. Based on the studies, it has been reported that the consensus tree is almost totally resolved but the three MPTs were not capable enough to explain deep root relationships. It has been suggested that *Pyronemataceae* are paraphyletic since *Ascodesmidaceae* are placed inside it, which is mostly accepted as monophyletic. After performing the analyses, the pyronemataceous taxa were recovered within 12 clades, where nine of the species of truffles included were nested inside three supported clades. A monophyletic group was formed by five species of hypogeous *Genea*, which is actually acting as a sister group to *Humaria hemisphaerica*; whereas taking five epigeous *Geopora* species, a monophyletic group was formed by *Geopora cooperi*. It has been suggested that *Geopora* is actually sister group, where *Miladina*, *Scutellinia* and *Ramsbottomia* are in the clade. *Stephensia*is monophyletic and so *Stephensi abombycina* forms group with *Paurocotylispila*, *Geopyxis carbonaria* and *Stephensias hanorii* (Laessoe and Hansen 2007).

29.5 Life Cycle and Molecular Characterization

With the advancement in different techniques and understanding the reproductive biology of *Tuber* spp. at a molecular level, it was hypothesized that these are selfing organisms. *Tuber melanosporum* popularly known as black truffles is considered as the model for the study of the biology of *Tuber* spp. because till date only the whole genome of *Tuber melanosporum* has been sequenced. The existence pattern of *Tuber* spp. is partitioned into stages, which starts with the development of filamentous vegetative mycelia and ends with the association of the fungal hyphae with the host root to build up a fungal mantle and the Hartig net.

29.5.1 Life Cycle of *Tuber* sp.

Truffle has been considered as a highly esteemed food since the eighteenth century. During this period the experts in this field have made various endeavours to comprehend the riddle behind Truffle's life cycle. Most recent contemplates made based upon genetic and genomic approaches have helped mycologists to take a rise towards a better understanding of the life cycle and sexual mode of *Tuber* spp. To gain more knowledge about the *Tuber* sp. scientists considered *T. melanosporum* as the model organism, since the whole-genome sequencing of this species has been conducted and the information related to the genome sequence was available to intricate studies. After numerous in-depth molecular studies performed by the researchers, an overview regarding the life cycle of *Tuber* sp. was made for better understanding. In general, the life cycle is divided into (1) the formation of the underground ascocarp, i.e. the fruiting body (Truffles) (2) the formation of ascospores which are released into the surrounding environment and (3) the infection and establishment of a symbiotic relationship made by the ascospores in the roots of a nearby host plant.

It has been observed that throughout the life cycle of *T. melanosporum* the haploid phase predominates as compared to the dikaryotic phase. The ascospores which are released from the mature ascocarps have been observed to undergo a symbiotic relationship with the nearby host plant. For the establishment of a symbiotic relationship between the fungi and the host plant root it was found that the production of indole-3-acetic acid (IAA) and ethylene which are considered to be plant hormones should be secreted by the mycelia first, so that the induction of shortening of the main root and the sideways root formation can occur. An extra radical pseudo-parenchymatous mantle formation by hyphae around small and growth-restricted lateral secondary roots takes place, which is followed by the colonization of hyphae which grows between rhizodermis and cortical cells, which further leads to the formation of Hartig net. An immature fruiting body is formed due to the fertilization of uniparental maternal hyphae and dikaryotic hyphae, after the successful establishment of symbiosis with the host plant. The karyogamy and meiosis in the ascus mother cells lead to the formation of mature asci containing haploid ascospores (Rubini et al. 2014). Therefore, it has been noticed that a mature

fruiting body (truffle) is composed of gleba and based on the data available, it has been observed that the structure of gleba in the fruiting body is from a haploid hyphae of uniparental (maternal) source, where the rate of outcrossing is either potential need or else necessary. Kües and Martin (2011) through their studies showed that the ascospores which are discharged in the soil from a mature ascocarp germinates and turns out to be either *MAT(+)* or *MAT(−)* primary haploid mycelia. It was observed that a single lateral root was not coexisted by different homokaryotic mycelia rather only a single genotype dominates. The reason behind the dominance of a single genotype was found to be due to the competition among completely different strains of mycorrhiza that share similar mating type. Under favourable surroundings and in the presence of other factors, *MAT(+)* and/or *MAT(−)* mycelia establishes a successful mycorrhiza on the root of the host plant and follows a symbiotic relationship whereas some get dispersed in the soil and grow nonsymbiotically. Contact between the mycelia of opposite *MAT* locus, which originates from ascospores or mycorrhizas from adjacent host plants is vital for fertilization to occur. Fertilization leads to the formation of fruiting bodies/ truffles.

The life cycles of Tuber spp. are still poorly understood and numerous questions are yet to be answered. Research is still going on with different tools to gather and learn more about the life cycle of *Tuber* spp. In the following section, we will learn more about the *MAT* locus and also the major role played by it during a particular segment of the life cycle.

29.5.2 *MAT* Locus of *T. melanosporum*

With the aid of advanced molecular techniques, it has been discovered that a single locus which is found in small regions of the genome is responsible for controlling the sexual reproduction in filamentous ascomycetes. These small sections of the genome are now known as the mating type (*MAT*) loci. It was found that a *MAT* loci with two major regulators were responsible for coordinating the sexual reproduction in ectomycorrhizal fungi. Through transcriptional analysis, it was found that the *MAT1–1-1 and* the *MAT1–2-1* sequence were responsible for encoding an α-box domain protein and a high-mobility group (HMG) domain macromolecule, respectively. The ascomycetes generally follow two types of reproductive mode, i.e. heterothallism and homothallism. In the heterothallic mode, two *MAT* sequences happen to occur in different strains and are self-sterile, thus crossing among strains of opposite mating type is necessary. Interestingly, both the versions of the *MAT* locus in ectomycorrhizal fungi are not the allelic versions and are known as idiomorphs. In the case of homothallic mode, both the *MAT* genes were found harbouring in a single strain making them capable of undergoing selfing or crossing with any other of the alike species, as they don't have any separate sexual identity (Rubini et al. 2012, 2014).

The whole-genome sequencing of *T. melanosporum* has already been conducted and it was found that the *MAT1–2* and *MAT1–1* segment were approximately 7430 bp and 5550 bp, respectively (Rubini et al. 2014). The *MAT* locus is flanked

with genomic regions that are found conserved among ascomycetes. Some of the genes like *APN2* and *SLA2* were found to be linked to the *MAT* locus in *Pezizomycotina* and some *Saccharomycotina* species and are considered as an indication of a preserved evolutionary source of the *MAT* locus (Rubini et al. 2012).

For the scientific community, the arrangement of *MAT* locus in the *T. melanosporum* genome offered numerous definitive proof regarding the molecular basis of sexual reproduction in ectomycorrhizal fungal species and also the knowledge obtained from genome sequencing of *T. melanosporum* genome has provided clues for further understanding and categorization of potential sexual reproduction strategies in use by the other *Tuber* sp.

29.5.3 Molecular Tools for the Identification of *Tuber* sp.

Over the years crucial knowledge regarding the diversity and organization of ectomycorrhizal fungi has been gained on the basis of data obtained from a variety of techniques such as enzymatic polymorphism analysis, immunological techniques, etc. Urbanelli et al. (1998) showed in his studies that a specific host plant can be mycorrhized by various taxa on a very small and precise root area. For the field studies, work with a single root tip is very important, but the aforementioned techniques were not efficient enough to provide reliable data from a small number of samples. With time flow it became obvious that studies using these techniques aren't enough to explore more about the *Tuber* spp. Furthermore, the formation of fruit-bodies is also reliant upon ecological factors and also knowing the fact that these organisms don't deliver fruit-bodies each year. Subsequently, while depicting ectomycorrhizal fungal diversity, it is obvious that the ectomycorrhizas itself must be studied to decide the fungi that are well connected with the host plant's root.

Morphological studies of ectomycorrhizal roots have provided valuable inputs for distinguishing the fungi; however, most species have not been depicted by this technique. Through techniques like sporocarp studies and morphological studies the foundation for understanding truffles has been established, but the utility of advanced molecular techniques like PCR has helped to understand the ectomycorrhizal fungal diversity and ecological factors in a bigger picture. Techniques like morphological techniques have advantages as well as disadvantages; for example, it was observed that morphological grouping is not exact enough to precisely portray ectomycorrhizal fungal communities, as morphological studies are too time-consuming to learn and might be reliable among laboratories.

In 1990, White along with his co-scientist first developed the universal primer for PCR to amplify the nuclear ITS segment. Bruns and Gardes (1993) designed (ITS1-F) and (ITS4-B) primers which were expected to be explicit for organisms and basidiomycetes, respectively. The rRNA genes like internal transcribed spacer (ITS) or intergenic spacer (lGS) which are non-coding genes provide exceedingly variable target amongst morphologically diversified mycorrhizal fungal strains. Grades et al. in 1991 showed that intraspecific variation in the ITS regions is low. The ITS region

in the genome was reported to be 600 bp–800 bp which was appropriate for amplification and also suitable for amplification with universal primers (White et al. 1990) in PCR method and rDNA, with the ITS region blanketed, is present within the genome as a pair of copies, allowing amplification from very small as well as less concentrated samples. Interest in the identification of ectomycorrhizal fungal community on the roots increased because of the sole reason, i.e. the evaluation based on ITS location turned into particularly less complicated. A major drawback in the ITS segment is that the level of intraspecific variation isn't uniform among different species and error in identification of the symbiont may occur due to contaminant DNA of other origins. On the other hand, the ITS segment is in widespread sufficiently variable to permit clear discrimination among distantly related species or genera and consequently allowed the construction of the phylogenetic tree of *Tuber* spp. Different molecular markers were used to identify ectomycorrhizal fungal species, like in restriction fragment length polymorphism (RFLPs) restriction enzyme digested ITS regions were used for the identification. PCR technique has been widely used to identify the ectomycorrhizal fungal species from root tip samples.

Development in the PCR-based method was coming up with the use of random oligonucleotide primers to spot the bacterial strains. In 1990, Williams and his research team got up with a similar idea that makes use of autoradiography in preference to radioactivity in visualizing the amplification effects and thus were mostly used because of its ease. This method in present days is popularly referred to as random amplified polymorphic DNA (RAPD). The important elements of the RAPD technique include: firstly no prior knowledge about the priming sites in the unknown target genome is needed and secondly only a single primer is needed to amplify the region located between the priming sites. The advantage of RAPD markers has made it possible to recognize the strains or isolates of non-sporulating root with great resolution. It is practically cost-effective, fast and maybe beneficial in any laboratory geared up for PCR strategies. The bottleneck of this method is reproducibility because of which it cannot be used for the study of the symbiotic stage as the random primer applied fails to differentiate and thus amplifies the DNA from the symbiotic partner (plant and fungus) and sometimes from accidental contaminants which inhabit the rhizosphere, which led to a generation of complex DNA fingerprints which is difficult to analyse.

Research community which is dedicated to exploring more about *Tuber* spp. is using another PCR-based technique other than RFLP and RAPD, i.e. real-time PCR technique (RT-PCR). The principle of this method is based upon the quantification of the fluorescent signals which are generated from a fluorescently labelled sequence-specific probe or an intercalate dye. The strength of the signal is directly proportional to the amplified PCR product in a reaction mixture. Thus, it became possible to observe the PCR during the exponential period by recording the amount of fluorescence emission per cycle. This method is widely used for quantifying fungal DNA. This method has wide applications for quantification of mycelia biomass and extra radical hyphal biomass. Suz et al. (2008) and Rizzello et al. (2012) used RT-PCR with SYBR Green dye for staining nucleic acid and evaluate

the amount of soil mycelium in productive against non-productive truffle ranch. These aforementioned techniques proved to be very beneficial in learning more about truffles but a lot more study is yet to be done.

29.6 Overcoming the Bottlenecks of Truffle Production

A gourmet food has been the tagline for truffles for decades, priced not only for the flavour but also for the rarity. The reproductive system of truffles is still not fully understood and still eludes the biologist to a large scale. The bottleneck for truffle cultivation remains the inability of humans to make them mate under controlled conditions. Researchers are now looking for molecular markers to forecast the population genetics of truffles and thus shade some light into the cultivation of truffles to type cast the species with morphological similarity (Henrion et al. 1994). Genetic variability has been studied in species that have an economic interest to cultivate *T. magnatum* and *T. melanosporum*, the species for white and black truffles extensively used in gourmet foods. *T. melanosporum* population had a strong bottleneck due to phylogeographic signal absence during last glaciations (Bertault et al. 2001). The findings always tend to implicate that a huge environmental factors are responsible for the difference in taste and morphology for the truffles species found across different geographical regions. *Tuber* spp. is proposed to have a self-reproductive system. This finding has been a great influence on studies of genetics for *T. melanosporum* and *T. magnatum* for sampling and mating model strategies (Frizzi et al. 2001; Murat et al. 2004; Rubini et al. 2004). The research has led us to differentiate their population genetics and track the speciation for better cultivation and maintaining the symbiosis of the species with the environment. It has been found that some *Tuber* species like *T. aestivum* and *T. uncinatum* outcross following the studies on *T. magnatum* (Wedén 2004; Wedén et al. 2004). The genes for mating type have not been isolated from the fungi in spite of several attempts. More and more population genetics studies are required to get an insight into the life cycle of these species. Sampling has become a hurdle for these experiments due to its low availability throughout a region. The morphology of the fertilization process is still a great mystery, only hypothesis is made for the fact that absence of male hyphae may be fulfilled by some detached cells such as ascospores (Urban et al. 2004). Truffle requires a unique set of ecological parameters unmatched to any other cultivars and thus the artificial plantations are also required to be set in natural conditions to get advantage of the climate factors and soil health (Hall et al. 2003). There is thus a need for representation of local truffle biodiversity as one of the conditions for improvement of artificial plantations to succeed (Rubini et al. 2004). This includes the plants that grow and feed on.

The recent draughts have induced a decline in the harvest of Mediterranean truffle and thus climate change and seasons change in precipitation and summer arids are one of the factors responsible for change in decline in truffle production which has led to huge loss in economics for black truffle sales and rise in prices (Büntgen et al. 2012). The change in smell of the truffles due to different conditions has misled the

pigs and dogs which are used to hunt truffles underground the soils which favour truffles having dimethyl sulphide compounds (Talou et al. 1990). Truffles require a strict soil property, for example *T. melanosporum* requires a soil with C/N ratio of 10.

29.7 Biological Importance of Secondary Metabolites

Like other fungal metabolites, i.e. mycotoxin, phytotoxin or aromatic compounds, truffle metabolites possess an important role in the ecosystem as well as in its own life cycle. Being ectomycorrhizas in nature, truffle volatiles have an immense concern in mycophagal mode of spore dispersal. Besides odorant signals for mammals and insects, they also participate in microbes and plant interaction to regulate a complex molecular interrelation among soil fauna and flora. More than 200 volatile organic compounds (VOC) and many non-volatile compounds, associated with the fruit-body, free-living mycelium and mycorrhiza of truffles are responsible for the interactions with the host plants and non-host plants (the so-called brunt, a zone with scarce herbaceous cover). Some common VOCs, found in fruit-body of all truffles are 1-octen-3-ol, 3-methyl-1-butanol, 2-methyl-1-butanol, dimethyl disulphide, etc. Structurally they are alcohols, aldehydes, ketones, esters, aromatic groups and sulphur compounds. Most characterized VOCs can be classified as fatty acid derived VOCs, terpenoids, sulphur-containing compounds and aromatic compounds. VOCs like 3-octanone and 1-octen-3-ol derived from the fatty acid metabolism are responsible for the strong fungal smell typical of *T. borchii* and of some other fungi (Abraham and Berger 1994; Chiron and Michelot 2005; Venkateshwarlu et al. 1999; Wnouk et al. 1983). Compounds like 3-methyl-1-butanol, 2-methyl-1-butanol and their respective aldehydes, all are derived from fatty acid catabolism. These compounds along with dimethyl sulphide are considered as the key contributors to the absolute aroma of *T. melanosporum*. Although terpenoids are the minor part of truffles VOCs, they might have ecological importance. Some monoterpenes and sesquiterpenes, which could be involved in microbial defence, are identified in *T. borchii* (Zeppa et al. 2004) and *T. brumale* (Mauriello et al. 2004) at different maturation stages. Aromadendrene is rendered as a good marker for fruit-body maturity in *T. borchii* as it has limited quantity in every immature fruit-body of *T. borchii.* Sulphur-containing compounds like thiols, sulphides, thioesters, thiophenones and thioalcohols are characteristic of most truffle species. They might act as fumigants against microbes (Bending and Lincoln 1999) and as repellents against amphipods (Schnitzler et al. 1998). One sulphur-containing compound, dimethyl trisulphide has also been detected in pure mycelial cultures of *T. borchii* (Tirillini et al. 2000). Among non-VOCs quinonoid and polyphenolic biopolymers are reported as the major constituents of *T. melanosporum*'s melanin (De Angelis et al. 1996). Some aromatic compounds found in truffles are 2-phenylethanol, benzaldehyde and 1-methoxy-3-methylbenzene.

VOCs produced during ectomycorrhiza formation of *Tuber borchii* with *Tilia americana* were investigated by several research groups (Menotta et al. 2004;

Gioacchini et al. 2002). During their growth, the pre-mycorrhizal stage is reported to have specific VOCs as hydrocarbons, alcohols, a brominated cholesterol derivative, ketones and terpenoids including sesquiterpene Germacrene D, as well as dehydroaromadendrene, longicyclene and β-cubebene which might be responsible for chemotropism of hyphae towards roots of the host (Menotta et al. 2004).

Truffle metabolites have great impact on the interaction with plants and insects. Some metabolites are reported to affect the root architecture of plant under laboratory conditions resulting in primary root shortening and their elimination (Splivallo et al. 2007). White truffle mycelia are reported to produce hormone ethylene (through the KMBA pathway) which was detected to play role in root hair elongation of the non-host plant. Some truffles like *T. melanosporum* can form a brunt, a peculiar zone surrounding the host plan where the herbaceous cover is scarce. Due to having truffle mycelium in the soil, ethylene or IAA released by truffles might explain the reason for dying out the herbaceous plants in the brunt. On the other side, 1-octen-3-ol extruded by both truffle mycelium and fruiting bodies generally exerts toxic effects on plants including shortening the primary root, loss of chlorophyll through oxidative stress (Splivallo et al. 2007). Truffles attract mammals ranging from squirrel to pig to consume the fruiting bodies for spore dispersal. Truffle hunter traditionally uses pig and trained dogs to locate the truffle underground. A steroidal pheromone, 5-α-androstenol secreted by black truffles was reported to be responsible for attracting pig (Claus et al. 1981).

29.8 Aroma of Truffles

Truffles are admired for their unique smells worldwide, making them one of the top listed luxury food items beside their rarity, limited seasonal availability and high price. Species variability, maturity of the fruiting body, microbial flora along with geographical origin are some important factors determining truffle aroma (Zeppa et al. 2004; Gioacchini et al. 2002). Being one of the most aromatic species, *Tuber melanosporum* with complex aroma is commonly known as '*Black Diamond of Cuisine'*. Another black truffle, *Tuber brumale* has characteristic musky odour with 'earthy notes'. The most expensive truffle, *T. magnatum* with complex aroma of garlic and cheese is considered as the finest species. The aromatic profile of a single species usually contains more than 50 volatile constituents (Bellesia et al. 1998; Mauriello et al. 2004). Beside some common truffle volatiles, there are some species-specific compounds. For example, thiophene derivatives 2-methyl-4, 5-dihydrothiophene and 3-methyl-4,5-dihydrothiophene are specific for *Tuber borchii* while other sulphur volatile 2,4-dithiapentane is a dominant constitute of *T. magnatum* (Splivallo et al. 2011). 1-methoxy-3-methylbenzene is specific for the Black truffle species like *T. aestivum*, *T. melanosporum*, *T. brumale*, etc. (Mauriello et al. 2004). During the truffle formation numerous microbes might end up trapped inside fruiting bodies. As a matter of fact, this microbial diversity also plays a speculative role in truffle aroma formation (Tirillini et al. 2000).

29.8.1 Biosynthesis

The hypothetical biosynthetic routes of the precursor metabolites for major and characteristic components of truffle aroma have documented from the sequencing of the black truffle (*T. melanosporum*) genome (Martin et al. 2010). Two main sulphur-containing VOC (S-VOC) biosynthetic pathways executed through L-methionine catabolism have been found in some bacteria and in some ascomycetous yeasts, leading to form methane thiol (MTL), a bad smelling thiol compound detected in truffles at low concentration (Arfi et al. 2006; Liu et al. 2008). When transcript abundance profiles were emphasized for fruit-bodies vs. mycelia, it was found that during maturation of the fruiting body of *T. melanosporum*, the relative concentration of methionine tends to remain constant, as sulphur assimilation and metabolism are particularly sustained in fruiting bodies (Martin et al. 2010). Beside methionine C-lyase, cystathionine C-S lyase, which uses cystathionine as substrate for MTL production has been found overexpressed in *T. melanosporum* fruiting body (Harki et al. 2006). Ultimately the complete balanced amount of diverse compounds creates the specific truffle flavour. It is possible because of the highly tuned regulation of the genes coding for various flavour biosynthetic enzymes during the truffle development and maturation. Some of these volatile are derived from free amino acid catabolism through Ehrlich pathways (Singh et al. 2019). A candidate gene for the carboxylation and cancellation process of this pathway has been proposed for *T. melanosporum* (Martin et al. 2010). Besides these genes for isoprenoid pathway have been characterized in *T. borchii* and all genes for mevalonate pathway have been identified within the genome of black truffle (Guidi et al. 2006). Nevertheless, it is worth mentioning that via these entire pathways truffle can potentially synthesize members of the most diverse family of natural products.

29.8.2 Potential Health Benefits

Truffles mostly consist of nitrogenous products of which proteins and mineral salts particularly that are rich in potassium and phosphorus are available. Comparing other forms of fungus truffle is 75% water rather than 90% water in other fungi that are cultivated. For example, truffle aphasia possesses 48 g of carbohydrate, 2 g of protein and 3.2 g of fat per 100 g of dry weight. There are many studies which indicate that truffles are easily digested and the value of truffles is due to its unique fragrance. The aromatic fragrance mainly consists of organic sulphur compounds. This noted component of truffles has made it an integral part of delicate foods blended with other components of Mediterranean and French cuisines that the cooks are very proud to offer. True truffles are mostly cultivated or collected mainly due to this extraordinary nature but they also offer a valuable medicinal component that can prove to be having a dual role to both tongue and health.

29.8.2.1 Antioxidant Properties

Truffles are great source of vitamin A, β-carotene, vitamin C and other phenolic components, which have a great role to play in scavenging the free radicals and chelate ferric ions by reducing the reactive oxygen species roaming through our bodies (Al-Laith 2010; Biswas et al. 2020; Kundu et al. 2020). Methanolic extract of different truffles species of the *Tuber* genera states that *T. aestivum* comprises high phenolic and ergostrerol compounds that directly correlate to great antioxidant properties (Das et al. 2020).

Desert truffles are also considered a source of higher phenolic content and thus have a greater antioxidant potential. According to studies by Hamza et al. in 2016, ascorbic acid, carotenoids and anthocyanins content of 10.63 mg, 1.17 mg and 29.1 mg, respectively, per 100 g in *T. nivea* are facts supporting its antioxidant properties. Catechin present in *T. boudieri* is responsible for the high radical scavenging activity, being the principal phenolic component of the truffle (Doğan and Aydın 2013) along with ferulic acid, *p*-coumaric acid and cinnamic acid.

Chen et al. (2016) demonstrated that Chinese truffles have high antioxidant properties like the DPPH radical scavenging activity of *T. huidongense* polysaccharides. *T. latisporum*, *T. subglobosum* and *T. pseudohimalayense* possessed antioxidant properties, in particular, *T. latisporum* and *T. pseudohimalayense* had predominant amount of phenolic compounds and thus possessed more scavenging activities than *T. subglobosum* (Yan et al. 2017).

29.8.2.2 Anti-Inflammatory Properties

The phenolics present in truffles react with free radicals and mono oxygen species and serve as antioxidants (Sies and Stahl 1995). Luo et al. (2011) experimented that *T. indicum* polysaccharides have proven to have a cytoprotective effect on the PC12 (rat adrenal medulla pheochromocytoma) cells during exposure to hydrogen peroxide stressors. The antioxidant properties with all the phenolics, terpenoids and polysaccharides attribute to the fact that they have also anti-inflammatory properties (Friedman 2016). Zhang et al. (2018) experimented on hyperglycaemic rats with aqueous extract of *T. melanosporum*, which showed reduced glucose levels like other standard diabetic rat treated with antidiabetic drug, glibenclamide. This glucose reducing effect of rats can be attributed to the correlation with Nrf2 and NF-kB pathways and variation in superoxide dismutase, catalase and vitamin C metabolism and regulatory changes. In addition a hepatoprotective effect of *T. claveryi* extract was also noticed by Janakat and Nassar (2010). Inhibition of COX-1 and 12-LOX pathways, 12(S)-hydroxy-(5Z, 8E, 10E)-heptadecatrienoic acid (12-HHT), thromboxane B2 (TXB2) and 12(S)-hydroxy-(5Z, 8Z, 10E, 14Z)-eicosatetraenoic acid (12-HETE), the ones which are usually overexpressed in different inflammatory diseases was observed by inducing Truffle extract treatment from *T. magnatum* (Beara et al. 2014).

29.8.2.3 Antitumour Activity

Currently, many research works are going on in finding out the anticancer properties of truffles. Methanolic extracts of various truffles species *T. aestivum* and *T. magnatum* have shown significant in vitro cytotoxic effects on cancer cell lines (HeLa, MCF-7 and HT-29). Aqueous extract of these truffles has also shown prominent anticancer activity against breast adenocarcinoma MCF-7. Silver nanoparticles were synthesized from aqueous extract of truffles that are rich in flavonoids and amino acids (Khadri et al. 2017); during colorimetric assay with the help of sulforhodamine B they showed significant cytotoxic effects against MCF-7 cells with IC50 value of 10 mg/mL. *T. claveryi* extracts were used in doing MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide) assay on defined cancer cell lines and human brain carcinoma cell lines (U-87MG) with IC50 values of 50.3 μg/mL of dry weight (Dahham et al. 2016). The anticancer activities of extracts from truffles can be correlated with the use of different bioactive compounds such as stigmasterol, phytosterol, squalene and lupeol (Dahham et al. 2016). These compounds play a vital role in inhibiting the growth of mutagenesis together with the stimulation of cell apoptosis (Woyengo et al. 2009), downregulation of lipid peroxidation and increase in the levels of glutathione peroxide dismutase which directly causes decrease in tumour size and decrease in survival rate of carcinoma in mice (Ghosh et al. 2011; Das et al. 2018).

Truffle also contains different polysaccharides that have anticancer activity such as β-glucan polymers (Friedman 2016). The polysaccharides play a vital role in cell to cell communication (Zong et al. 2012) and signal recognition, as it has been reported that derivatives of these play a major role in preventing metastasis including raising the immunity through creation of cytokines, interferons and immunoglobulins to act against cancer antigens (Li et al. 2018; Moradali et al. 2007; Wasser 2003). 52 polysaccharides have been isolated from different truffle species, viz. *T. aestivum*, *T. indicum*, *T. melanosporum and T. sinense* which showed antitumour activity against A549, HCT-116, HepG2, HL-60 and SK-BR-3 cell lines (Zhao et al. 2014). Oleic acid content in truffles has the ability to supress the overexpression of HER2 oncogene; they also induce caspase 3 activity and consequently cancer cell death (Carrillo et al. 2012; Menendez et al. 2006). Aqueous extract of the *T. boudieri* showed increasing TH1 cytokines and decrease in TH2 cytokines that can stimulate lymphocyte proliferation and induce phagocytosis (Al Obaydi et al. 2020). Further work is going on in assessing truffles activities as antitumour agents.

29.8.2.4 Anti-Microbial Properties

Desert truffle has been reported to possess antimicrobial activity for a few decades now. Aqueous extract of *T. claveryi* showed growth inhibition of *Staphylococcus aureus* by 66.4% and *Pseudomonas aeruginosa* by 40% (Janakat et al. 2004; Casarica et al. 2016). Owaid et al. (2018) demonstrated silver nanoparticles synthesized from *Tirmania* spp. truffle possess antibacterial action against gram negative and gram positive species mainly of the eyes, *Pseudomonas aeruginosa*. Doğan and Aydın (2013) reported about antibacterial action of aqueous extract of

T. boudteri specifically against *Candida albicans*. Truffle lectins possess the ability to recognize bacterial exopolysaccharides and destroy them (Elsayed et al. 2014). *Salmonella typhimurium*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermis and Bacillus subtilis* species of bacteria were subjected to treatment of *T. nivea* extract and exhibited inhibitory activities (Hamza et al. 2016; Das et al. 2017). Ethyl acetate extract of *T. pinoyi* containing pyrazines and derivatives proved to be effective against *Bacillus subtilis* and *Staphylococcus aureus* (Dib-Bellahouel and Fortas 2011). The methanolic extract of the same fungus has shown inhibiting *Staphylococcus* infection in chicken soup in refrigerator (Stojković et al. 2013). Nadim et al. (2015) proposed that laccase from *T. magnatum* catalyses the oxidized phenols releasing superoxide anion radicals leading to inhibiting pathogenic bacteria.

29.9 Conclusion

Based on the research progress reported till date, it is worth mentioning that truffles are nature's artistic creation which needs more engagement of scientific researchers to step forward for understanding the taxonomic classification and their phylogenetic relationship, chemical component profile of each edible truffle and its pharmaceutical importance, artificial cultivation in green houses and detailed structural studies. Truffles are a kind of aromatic bomb, therefore extraction of such compounds as well as their importance to chemical ecology needs to be studied in detail. While considering the pharmacogenic potential of truffles, it has been observed that they are effectively capable of curing several disorders. But there is a need that different species and their potential role in curing diseases are to be investigated, so that by simply feeding this exquisite cuisine one can get rid of diseases.

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