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Extremophilic Microbial Processing of Lignocellulosic Feedstocks to Biofuels, Value-Added Products, and Usable Power

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Preface

Microorganisms have been an essential part of our lives. They are closely associated with the life of other organisms including plants and animals. Though initially the microorganisms were believed to the causative agents of disease, later the enormous benefits of microorganisms for use in fermentation were realized. The microorganisms are indispensable to day-to-day activities. They closely interact with other higher organisms and their interactions drive the ecosystem. Any interruptions to ecological balance will lead to irreversible damage to the environment. Microorganisms have been used for a wide range of applications including agricultural, industrial, environmental bioremediation, biomining, biofuels, and even for space biology applications. With advances in research in microbiology, microorganisms have been realized as promising candidates for mediating catalysis at enhanced rates and at an economical manner for industrially processes. Slowly, the concept of biochemical was evolved with the aim of replacing the chemical catalysts with microbial catalysts for industrial biotechnology applications. Unlike the chemical catalysts, the microorganisms can mediate catalysis at the ambient conditions of temperature and pressure. The microorganisms can thrive and operate at a broader range of operating conditions making the microbial process more economical for industrial applications. The use of enzymes has advantages such as sensitivity and selectivity, but they have a narrow range of operating conditions, and they are sensitive to higher/lower conditions such as temperature and pH. Microorganisms, on the other hand, catalyze the substrates using a complex set of enzymatic machinery. Although the normal microorganisms can operate at much broader range of operating conditions than enzymes, still they have limitations that they cannot operate under extreme conditions. This limitation can be circumvented by the use of extremophiles, which can be operated at extreme conditions of temperature, pH, pressure, salinity, etc.

This text focuses on the various extremophilic bioprocesses for the conversion of lignocellulosic biomass and gaseous wastes to biofuels, value-added products, and biopower. Lignocellulosic biomass is the most abundant feedstock on earth, and it comes from domestic, industrial, and agricultural sources. As per the Renewable Chemicals Factsheet of NNFCC consultancy company, it has been estimated that

lignin alone constitutes about 50 million tons produced annually from pulp and paper industries. Lignin acts as the cement that rigidly holds the hemicellulose and cellulose in plant cell wall and hinders the biodegradation process because of its heterogeneity. Lignin confers recalcitrant nature to the lignocellulosic biomass. Several physical and chemical methods have been documented in the literature for the disposal/pretreatment of lignocellulosic biomass. However, most of these methods suffer from high cost, need for sophisticated facility, environmental issues, and difficulties in practical implementation. Burning of these biomasses has been prevalent in developing countries. Land filling is another major practice of dumping huge volumes of lignocellulosic biomass. This leads to the release of noxious gases such as CO, CO_2, SO_2, CH_4, and NO_x into the environment which contributes to global warming/climate change.

This text covers the applications of various extremophiles such as thermophiles, psychrophiles, barophiles, acidophiles, and alkaliphiles for the enhanced biodegradation of lignocellulosic biomass for bioenergy applications. Production of lignin-degrading extremozymes, namely, lignin peroxidase and polyphenol oxidase, and cellulose-degrading thermozymes, namely, endoglucanase, cellobiohydrolase, and β-glucosidase, has been documented in the literature. There is a growing demand for the extremophilic processes in the industrial sector because of the robust nature of the extremophiles. Extremophilic bioprocessing is widely used for the hydrolysis of lignocellulosic biomass. The higher growth rate and the catalytic rates will help in accelerating the rates of hydrolysis of lignocellulosic biomass in an economical, eco-friendly, and efficient manner. This book begins with the basic concepts of microbial processes for motivating the beginners and increases the pace toward the forthcoming chapters. The book introduces basic concepts about the recalcitrant nature of the lignocellulosic biomass and their pretreatment processes. This book includes different industrial processes such as production of bioethanol, biobutanol, biodiesel, hydrogen, biogas, exopolysaccharides, polyhydroxybutyrates, and value-added products from lignocellulosic biomass. The book also addresses the bioconversion of greenhouse gases (e.g., methane) to value-added products. A chapter on photobiocatalysis for the production of limonene from CO_2 using extremophilic cyanobacteria is also included. Chapters have been included on the Techno-economical Assessments of the process for the production of biofuels from lignocellulosic biomass.

The book covers various pretreatment processes for lessening the recalcitrant nature of the lignocellulosic biomass. In addition, the book provides different bioprocess characteristics such as reactor design, unit operations, and downstream processing of the biofuels and value-added products. The book provides the knowledge on engineer extremophiles using genetic and metabolic engineering strategies for enhanced conversion of lignocellulosic feedstocks to biofuels and value-added products. This book is unique in that it is suited to a wide range of readers in academia, research, and industry. The book gives a clear understanding on this upcoming field of science and engineering of extremophilic bioprocesses in such a way besides understanding the concept that they will be in a position to design the bioprocesses for production of the biofuels and value-added products. The basic

concepts in extremophilic bioprocessing are provided in detail for the understanding of the beginners. The take-home message for each chapter is also included at the end of every chapter to improve the reasoning ability of the reader in a specific topic.

Chapter 1 introduces the basic concepts in extremophile biology. It discusses in detail the different classification of extremophiles and their applications in bioenergy, bioremediation, and production of value-added products. It provides the details on its advantages over chemical and enzymatic process. A brief outline about the extremophiles and extremozymes and their advantages over others are also described in this chapter. It covers the various sources of extremozymes such as thermophiles, psychrophiles, barophiles, acidophiles, alkaliphiles, desiccation-resistant microorganisms, etc. It emphasizes the need for knowledge, understanding, and skill on working with extremophilic enzymes.

Chapter 2 deals with the conversion of lignocellulosic feedstocks into bioethanol using extremophiles. Lignocellulosic biomass is the most abundant available feedstock, and its use for microbial processes can greatly help in lowering down the costs of operation. The chapter provides the advantages of thermophiles for bioethanol production and the physiology of thermophilic microorganisms. The chapter covers the various processes for bioethanol production including pretreatment methods of lignocellulosic biomass, enzymatic hydrolysis and saccharification, fermentation, and product recovery. A section on integrated processes for ethanol production from lignocellulose addresses the separate hydrolysis and fermentation, simultaneous saccharification and fermentation and simultaneous saccharification and co-fermentation, and consolidated bioprocessing. The chapter also covers strain improvement strategies such as evolutionary adaptation and genetic engineering of thermophiles.

Chapter 3 covers extremophilic production of biobutanol using lignocellulosic biomass. Biobutanol has several advantages over ethanol such as higher energy content, lower water absorption, and better blending ability. The chapter discusses the fermentative production of butanol including biomass selection for butanol production, fermentation techniques, pathways, and enzymes. The chapter also discusses the microorganisms in the production of biobutanol and the role of recombinant microorganisms in improving biobutanol production.

Chapter 4 describes in detail the production of biodiesel from extremophilic microalgae. Algal biomass is one of the promising lipid sources for biodiesel production because of its very high photoautotrophic growth rates, CO_2 fixation, and accumulation of carbon storage metabolites such as triglycerols. The chapter describes strategies for harvesting algae including flocculation, sedimentation, filtration, centrifugation, and drying. The chapter also discusses in detail bioprospecting algae from extremophilic sources for biodiesel production.

Chapter 5 discusses biohydrogen ($BioH_2$) production from lignocellulosic feedstocks using extremophilic microorganisms. This chapter describes in detail the different routes of $BioH_2$ production from lignocellulosic biomass using microorganisms including direct process as well as two-stage process. The direct process involves the production of $BioH_2$ using single microorganism that is capable of hydrolyzing the cellulose/hemicellulose and producing $BioH_2$. The two-stage

process, on the other hand, involves two sets of microorganisms, one for hydrolyzing the cellulose and the other for producing $BioH_2$. The chapter also discusses the production of $BioH_2$ from lignocellulosic biomass without pretreatment using extremophiles and their molecular mechanisms in $BioH_2$ production.

Chapter 6 describes in detail the production of biogas from extremophiles. This chapter discusses different phases of biogas production, namely hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Further, the chapter discusses in detail the effects of different process parameters such as substrates, organic loading rate, hydraulic retention time, temperature, pH, volatile fatty acids, ammonia, and nutrients. The chapter also discusses the different biogas reactors such as continuous stirred tank reactor (CSTR), UASB (up flow anaerobic sludge blanket reactor), plug flow, fixed dome, and floating drum reactors. The chapter discusses psychrophilic digestion and thermophilic digestion in detail including their advantages and disadvantages. The different applications of biogas and their economic outlook are also addressed at the end of this chapter.

Chapter 7 describes in detail the production of biogas from lignocellulosic biomass. The chapter begins with the recalcitrant nature of the lignocellulosic biomass and discusses in detail the different pretreatment strategies including physical methods such as mechanical treatment, extrusion, stream explosion, hydrothermolysis, and irradiation and chemical methods such as acid hydrolysis, alkaline hydrolysis, oxidative pretreatment, ionic liquid pretreatment, and biological pretreatment. The chapter covers the key parameters affecting the biogas production such as temperature, reaction medium, carbon/nitrogen (C/N) ratio, retention time, agitation, organic loading rate/total solid content, feedstock and nutrient concentrations, inhibitory product, moisture content, and co-digestion. The chapter also discusses the different analytical methods for quantifying biogas and strategies for purification.

Chapter 8 discusses the bioconversion of methane to value-added products. The chapter describes the different basic concepts about methanotrophic bacteria, their classification, metabolism, cultivation, and their applications. This chapter describes the biosas to liquid biofuels (Bio-GTL) and the safety measures. The chapter discusses the process development for biological conversion of methane into desired products in regard to the enhancement of mass transfer efficiency and the development of bioreactor design, including continuous stirred-tank bioreactor, bubble column bioreactor, loop and airlift bioreactor, trickle-bed bioreactor, monolithic biofilm bioreactor, and membrane biofilm bioreactor.

Chapter 9 describes developing a recombinant cyanobacteria for synthesizing limonene from CO_2. Limonene is a third-generation biofuel and used in jet fuel and diesel applications. The chapter discusses the physical properties, chemical properties, sources, and production of limonene for use as fuel. The chapter also discusses the different metabolic routes for isoprenoid synthesis such as MVA pathway, MEP pathway, and convergence of MEP and MVA pathways through IPP/DMAPP Formation. The chapter also discusses the reaction mechanism and conserved sequences of limonene synthases. The chapter describes in detail engineering recombinant cyanobacteria as cellular factories for limonene production.

Chapter 10 deals with the production of a value-added product—exopolysaccharide (EPS) from extremophiles. The chapter discusses EPS-producing microorganisms and their biosynthetic pathways. The chapter also discusses different applications of exopolysaccharides such as anti-inflammatory, antiviral, and drug delivery applications. The chapter also discusses chemical and structural characterization, purification, compositional analysis, and molecular weight distribution.

Chapter 11 addresses the production of polyhydroxyalkanoates (PHA) from renewable and waste materials using extremophiles. The chapter discusses the different extremophilic sources for PHA, its biochemistry, chemical composition, mechanical properties, biodegradability, and its applications. The chapter discusses in detail the effects of different substrates such as glycerol from biofuel productions, crude and waste plant oils, oil mill effluents, surplus whey from the dairy industry, wastes from the sugar industry, lignocellulosic wastes, and municipal wastes on the yield of PHA in a metabolic perspective.

Chapter 12 covers the concepts about the electroactive microorganisms and their potential to mediate bioelectrocatalysis. The chapter clearly discuses the principle, construction, operation, and application of microbial fuel cells, microbial electrolytic cells, microbial desalination cells, and microbial electrosynthesis.

Chapter 13 is on the integrated bioprocesses for conversion of lignocellulosic feedstock to biofuels and value-added bioproducts. The application of CBP of lignocellulosic biomass using extremophiles can aid in cost-effective production of biofuels. This chapter addresses developing an integrated CBP by the combination of CBP with several other bioprocesses for high value-added products, such as biopolymers. The chapter discusses on how recombinant DNA technologies such as metabolic engineering can help in improving the performance of CBP.

Chapter 14 deals with the production of value-added products from wastes using extremophiles in biorefineries. It provides detailed information about the biorefineries and clearly discusses process modeling, simulation, and optimization tools.

Overall, these chapters cover the different extremophilic processes for a wide range of applications. Adequate basic concepts have been included for the beginners.

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Contents

Chapter 1
Bioprospecting of Extremophiles for Biotechnology Applications

Navanietha Krishnaraj Rathinam and Rajesh K. Sani

What Will You Learn from This Chapter?
This chapter aims to acquaint the readers with basic concepts about extremophiles, extremophilic bioprocesses, and their advantages over other chemical and biological systems. The chapter introduces the concept of extremophiles and extremophilic bioprocesses for wide range of biotechnological applications. Finally, the chapter addresses the applications of extremophiles in different sectors such as bioenergy, bioelectrochemical systems, bioremediation, and production of value-added products.

1.1 Introduction

It is well-known that all components of the universe are driven by catalytic reactions. Catalytic reactions are indispensable to fundamental aspects of life including food, water, and energy (Sani and Rathinam 2017). Catalysis helps in accelerating the thermodynamically favorable reactions, wherein, catalysts provide alternative routes

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for the reactions that forms intermediate products with lower activation energy. However, the catalysts do not lower the activation energy of the reaction by itself (Alberts et al. 2002).

Catalytic reactions play a key role in any chemical/biochemical processing industry. Owing to environmental concerns, it has been realized that the biocatalysts are promising for industrial applications. The microbial/enzymatic processes are environmentally benign and eco-friendly. They help to avoid the use of chemicals/solvents as in the case of chemical processes or avoids the need the sophisticated facilities/extreme conditions as in the case of physical methods. In the case of microbial catalysis, microorganisms act as whole cell catalysts to accelerate the chemical reactions. Microorganisms make use of the complex enzymatic machinery for mediating the biochemical reactions. In addition, microorganisms can make use of organic waste materials as feedstocks to produce desired products. This helps in the safe disposal of wastes as well as production of desired products in an inexpensive manner.

Microbial processes have been exploited for a variety of industries including food processing, beverages, biofertilizers, pharmaceuticals, polymers, and bioremediation (Adrio and Demain 2014; Yamada and Shimizu 1988). According to the report of an Economic Impact Analysis of the US Biobased Products Industry sponsored by the US Department of Agriculture (USDA), in 2013 the overall bio-based products industry supported 4 million jobs with a total contribution of $369 billion to the US economy (Golden et al. 2015). The report also showed that bio-based products currently displace about 300 million gallons of petroleum per year. The report "US Biobased Products: Market Potential and Projections Through 2025" projected that the market share of bio-based chemicals in the global chemical industry will have a drastic increase from 2% in 2008 to 22% in 2025. Poor enantiomeric specificity to synthesize the specific products (which have lots of significance in pharmaceuticals) is a major limitation in the chemical processes, and this can be overcome by the use of the enzymatic process. The use of bioprocesses is more ideal for industrial applications as they can work under mild operating conditions of temperature, pH, and atmospheric conditions unlike most chemical processes. Realizing the several advantages of biocatalysis over conventional chemical processes, industries have made a drastic move to harness the potential of the microorganisms for replacing the conventional chemical processes in the industries (Cooper 2000).

Bioprocesses have limitations that they can mediate catalysis only under mild conditions. The use of extremophiles/extremozymes helps to broaden the range of operating conditions and helps to overcome the limitations of traditional bioprocesses. Extremophiles are interesting class of microorganisms which can resist adverse environmental conditions such as high/low temperature, acidic/alkaline environments, toxic environments, metal ions, solvents, and so on. It has great scope for industrial applications. The extremophiles have shown to be promising for the production of biofuels, biopolymers, and value-added products and for bioremediation of recalcitrant compounds (Bhalla et al. 2013; Coker 2016; Niehaus et al. 1999). Extremophiles are generally categorized in two broad groups as extremophilic organisms and extremotolerant organisms. Extremophilic organisms demand one or more extreme

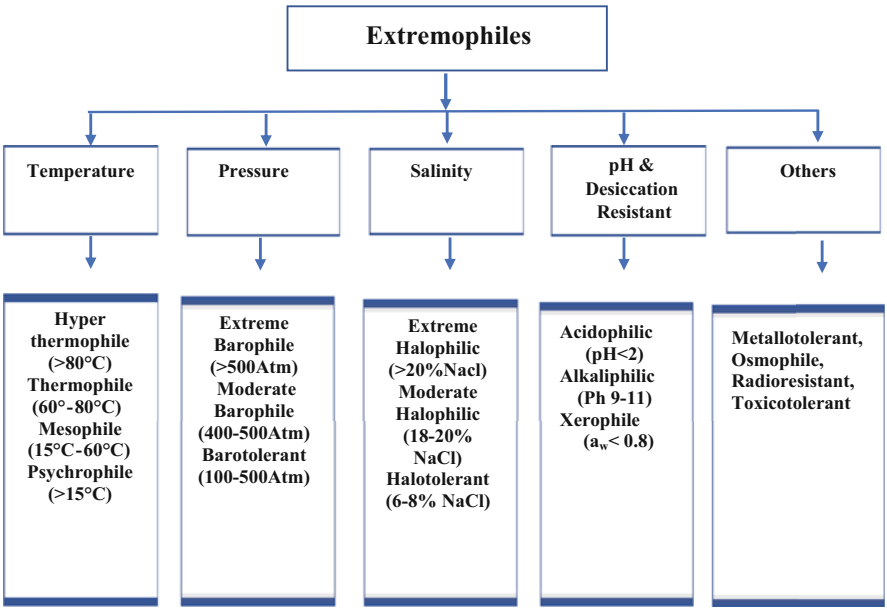

Fig. 1.1 Classification of extremophiles

conditions to grow, whereas the extremotolerant organisms have the potential to tolerate extreme values of one or more physicochemical conditions (Podar and Reysenbach 2006; Pikuta et al. 2007).

Depending on the type of environment the extremophiles grow, they are classified as thermophiles and hyperthermophiles (organisms that grows at high temperatures), psychrophiles (organisms that grows best at low temperatures), acidophiles and alkaliphiles (organisms that can grow in acidic or basic pH values, respectively), barophiles (organisms that are adapted to grow under pressure), and halophiles (organisms that demands high salt concentration for growth) (Fig. 1.1). Polyextremophiles are another interesting group of extremophiles which can grow in environments where various physicochemical parameters reach extreme values. For example, some extremophiles can grow in environments having different extreme conditions such as high temperature, acidic, and high concentrations of toxic metals at the same time (Rampelotto et al. 2013). Some of the examples for polyextremophiles include aerobic and anaerobic alkalithermophiles (Wiegel 1998, Wiegel and Kevbrin 2004) and anaerobic halophilic alkalithermophiles (Mesbah and Wiegel 2008).

This chapter briefly discusses the fundamentals of microbial processes and introduces the basic concepts about the different types of microbial processes based on biochemical and thermodynamic aspects and their applications with focus on extremophiles.

1.2 Three Domains of Extremophiles

As in the case of general microbial classification, the extremophiles can be categorized as bacteria, archaea, and eukarya. It has been reported often that archaea constitute the major proportion of extremophiles. The category of extremophile also includes lower protists such as bacteria; higher protists such as algae, fungi, and protozoa; and multicellular organisms.

1.2.1 Extremophilic Archaea

The archaea are comparatively a new domain which is devoid of nucleus and other organelles. Archaea are known to often inhabit extreme environments such as hot springs, extremely alkaline or acid waters, anoxic muds of marshes, and bottom of the ocean and even thrive in petroleum deposits deep underground. They can easily adapt to extreme environments. The cell membranesof archaea confer a high permeability barrier and the potential to maintain the liquid crystalline phase with isoprenoid chains which helps to adapt lipids to changes in the environmental temperature (Koga 2012).

Depending on the protein adaptations to extreme environments, they are classified as thermophilic, psychrophilic, and halophilic. *Archaeoglobus fulgidus* are hyperthermophilic sulfur-reducing strain of type Archaeoglobales that are found in hydrothermal vents and deep subsurface of the oil fields (Klenk et al. 1997). They have an optimum growth temperature of 83 °C and a minimum division time of 4 h. An aerobic and thermoacidophilic crenarchaeon, *Sulfolobus tokodaii*, has been isolated from Beppu hot springs in Kyushu, Japan, in 1983. It is an obligate aerobe that has the optimal growth temperature of 80 °C and optimal pH range of 2.5–3 under aerobic and chemoheterotrophic growth conditions (Suzuki et al. 2002). Another novel thermoacidophilic archaeon, *Sulfolobus tengchongensis* sp. nov., isolated from a hot spring in Tengchong, China, was reported in the literature. The isolated strain grows aerobically in lithotrophic or heterotrophic conditions with the optimal growth temperature of 80 °C and optimal pH of 3.5. The genome of the isolated strain was found to have G + C content of 34.4 mol%. This strain was shown to mediate oxidation of sulfur, tryptone, D-xylose, D-arabinose, D-galactose, maltose, sucrose, D-fructose, or L-glutamic acid. However, it did not utilize D-glucose and D-mannose. Different halophilic archaea that has high salt resistance are also reported in the literature (Xiang et al. 2003). The haloarchaeon, *Haloterrigena turkmenica*, has been used for the production of extracellular halophilic α-amylase (Santorelli et al. 2016).

McDuff et al. (2016) isolated the different CO-oxidizing strains of the euryarchaeotes, namely, *Haloarcula*, *Halorubrum*, *Haloterrigena*, and *Natronorubrum*, from the Bonneville Salt Flats (UT) and Atacama Desert salterns (Chile). *Halorubrum lacusprofundi* is an extreme halophilic archaeal strain within the phylum Euryarchaeota that is isolated from Deep Lake, Antarctica. Most species of *Halorubrum* are shown to

be haloalkaliphiles. Archaeal strains such as *Halorubrum lacusprofundi* ACAM 34 isolated from a water sediment source from Deep Lake, Antarctica, are psychrotolerant and halophilic. They can grow at a wide range of temperature between −1 °C and 40 °C with an optimal growth temperature of 36 °C. They can grow with the salt concentration of 1.5–4.5 M with an optimum salt concentration and optimum magnesium concentration of 3.5 M and 0.1 M, respectively. These cells were prone to lysis in distilled water (Anderson et al. 2016). *Methanogenium frigidum*, a stenopsychrophilic archaeon isolated from methane-saturated waters in Ace Lake, Antarctica, has the lowest known upper growth temperature of 18 °C and is shown to utilize H_2–CO_2 (Franzmann et al. 1997). Sinha and Datta (2016) cloned and overexpressed a putative β-glucosidase gene (O08324) in the hyperthermophilic archaeon *Thermococcus* sp. in *Escherichia coli* to overcome the glucose inhibition of β-glucosidase. The enzyme produced from the clones had maximum activity at 78 °C and pH 5–6.8. It was thermostable and had a half-life of 860 min at 78 °C in the presence of 1.5 M glucose. In addition, the enzyme did not have glucose inhibition up to a concentration of 4 M and also displayed no decrease in activity in the presence of up to 4 M of sodium chloride or potassium chloride. Archaeal stains belonging to genera, *Pyrodictium* and *Pyrolobus*, are known to survive for even 1 h of autoclaving (Stetter 2006). Their modes of nutrition are either organoheterotrophic with carbon or can be lithoautotrophic with hydrogen, thiosulphate, and carbon dioxide.

1.2.2 Extremophilic Bacteria Including Cyanobacteria

Several extremophilic strains of bacteria, and cyanobacteria, have also been isolated from different extreme environments. Thermophilic and hyperthermophilic bacteria have been isolated from hot springs, volcanic sites, geothermal sites, deep biospheres, and mining areas. The thermophilic bacteria are generally classified as spore forming and non-spore forming. *Aquifex aeolicus* is one of the versatile examples for a hyperthermophile. It can grow at around 95 °C. It is a chemolithoautotroph that it can use inorganic carbon sources such as hydrogen, oxygen, carbon dioxide, and mineral salts for biosynthesis. The G + C content of the stable RNAs can provide a clear indication about the high growth temperature of the organism (Deckert et al. 1998). *Methanococcus jannaschii* is another hyperthermophilic methanogen that was isolated from a deep-sea hydrothermal vent. It can grow at a temperature of 80 °C. It had a maximum specific growth rate of 0.56 h^{-1} and a maximum specific methane productivity of 0.32mol g^{-1} h^{-1}(Tsao et al. 1994). *Heliobacterium modesticaldum*, grown at 95 °C, has been isolated from the Yellowstone hot spring microbial mats and volcanic soils from Iceland (Kimble et al. 1995). Scanning electron micrographs of the thermophiles isolated from Sanford Underground Research Facility (Lead, S.D.) is shown in the Fig. 1.2 (Dr. Sani's lab unpublished data).

Depending on the optimal pressure at which the barophilic microorganisms survive/grow, they are classified as barotolerant, barophilic, or obligately barophiles. Sometimes, they are also referred to as piezophiles and hyperpiezophiles. These

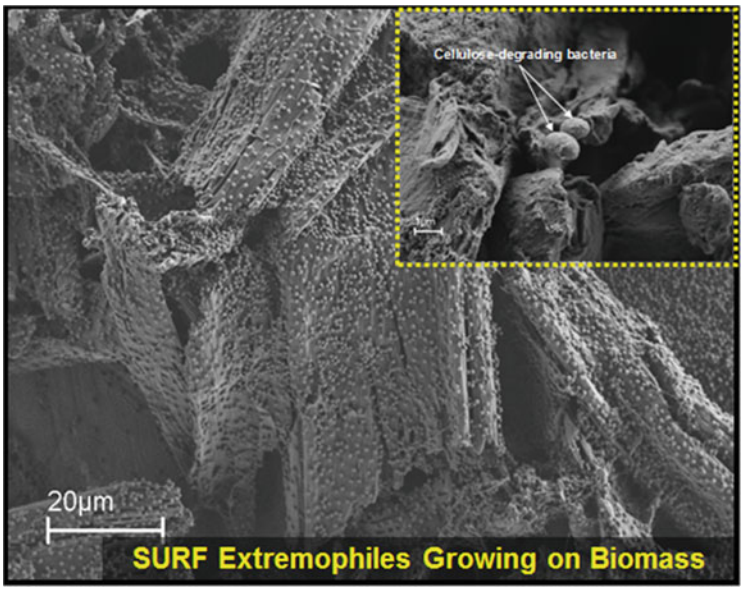

Fig. 1.2 Thermophiles isolated from Sanford Underground Research Facility. Inset shows the SEM images of the cellulose degrading thermophiles

organisms are known to grow up to a maximum growth pressure of 1400×10^5 Pa (Yayanos et al. 1979, 1982). Yang et al. (2007) isolated a novel psychrophilic bacterium, LT17T from deep-sea sediments (3300 m depth) of the East Sea (Sea of Japan) that is closely related to *Shewanella japonica*, *Shewanella pacifica*, *Shewanella olleyana*, *Shewanella frigidimarina*, and *Shewanella gelidimarina*. The isolated strain was shown to grow between the hydrostatic pressure of 0.1 and <30 MPa and had an optimal growth at a hydrostatic pressure of 10 MPa. This strain displayed catalase, oxidase, lipase, β-glucosidase, and gelatinase activities and was able to reduce nitrate to nitrite. This strain also had interesting feature to produce predominant cellular fatty acids which are iso-C13: 0, iso-C15: 0, C16: 0, C16: 1omega7, and C20: 5omega3. Attempts were also made to isolate barophiles from intestinal contents of deep-sea fishes (*Coryphaenoides yaquinae* and *Ilyophis* sp.) retrieved from depths of 4700 to 6100 m in the northwest Pacific Ocean. The isolated barophiles grow well between 20.7 and 82.7 MPa and had optimal growth at 41.4 MPa. However, they did not grow at atmospheric pressure (Nakayama et al. 1994). Phylogenetic analysis between the genome sequences of the barophiles showed that the distinct subgroup of the genus *Shewanella* have been found to be barophiles (Kato and Bartlett 1997). *Shewanella psychrophila* sp. nov. and *Shewanella piezotolerans* (type strain WP3T) have been isolated from west Pacific deep-sea sediment, and they grew in the hydrostatic pressure range 0.1–50 MPa, with optimal growth at 20 MPa (Xiao et al. 2007).

Acidophilic bacteria which can tolerate very low pH have been isolated from different sources such as acid mines, fruit orchards, and geothermal sites. For example, Johnson et al. (2009) isolated thermophilic acidophilic bacteria (*Ferrimicrobium acidiphilum* gen. Nov., sp. nov. and *Ferrithrix thermotolerans* gen. Nov., sp. nov.) from geothermal (30–83 °C) and acidic (pH 2.7–3.7) sites in Yellowstone National Park. The acidophilic isolates include Firmicutes, *Sulfobacillus*-like isolates, *Actinobacterium*, *Methylobacterium*-like isolate, and *Acidisphaera* sp. These isolates grew in the temperature range of 50–65 °C and pH range of 1.0–1.9. These isolates were able to mediate dissimilatory oxidation of ferrous iron, and some of them could mediate the reduction of ferric iron to ferrous iron in anaerobic cultures, and few isolates also oxidized tetrathionate. *Acidithiobacillus* is one of the common acidophilic sulfur- and iron-oxidizing microbes that survive in acidic environments that are rich in iron ores and other heavy metals. These acidophilic organisms play key role in metal solubilization and leaching from such ores (Golyshina and Timmis 2005).

Haloalkaliphilic *Candidatus Desulfonatronobulbus propionicus* was isolated from hypersaline soda lakes in Kulunda Steppe (Altai, Russia). This isolate was salt-tolerant (up to 1.2 M salt) and has the optimal pH of 10. The strain was shown to oxidize propionate incompletely to acetate (Sorokin and Chernyh 2016). The haloalkaliphilic strains *Desulfonatronobacter acidivorans* gen. Nov., sp. nov., and *Desulfobulbus alkaliphilus* sp. nov., isolated from anoxic sediments of hypersaline soda lakes in Kulunda Steppe (Altai, Russia), were shown to reduce sulfate. These strains are shown to use propionate and pyruvate as the electron donor and sulfate or sulfite as the electron acceptor (Sorokin et al. 2012; Sorokin and Chernyh 2016). Cha et al. (2014) isolated a γ-radiation-resistant bacterium *Deinococcus radiotolerans* sp. nov. from γ ray-irradiated soil. These strains are shown to be resistant to γ-radiation with a D10 value in excess of 8 kGy. These strains were found to have predominant respiratory quinone such as menaquinone MK-8 and phosphoglycolipid as major polar lipid.

Certain members of cyanobacterial genera such as *Leptolyngbya*, *Gloeocapsa*, *Gloeocapsopsis*, *Stigonema*, *Fischerella*, *Synechocystis*, *Microcoleus*, *Cyanobacterium*, *Chroococcus*, and *Geitlerinema* have been isolated from geothermal springs in Algeria with water temperatures ranging from 39 °C to 93 °C. It has been reported that the members of *Leptolyngbya*, *Synechococcus*, and *Gloeomargarita* were dominant in hottest springs (70 °C), whereas *Oscillatoriales* other than *Leptolyngbya*, *Chroococcales*, and *Stigonematales* were dominant in lower temperatures springs (40–45 °C) (Amarouche-Yala et al. 2014). Some of these extremophilic cyanobacterial strains are also shown to be toxic. Mohamed et al. (2008) reported the presence of toxic cyanobacteria and cyanotoxins in public hot springs in Saudi Arabia. Cyanobacterial strains of *Oscillatoria limosa* and *Synechococcus lividus* have been identified from the hot springs, and the spring waters had toxins such as microcystins and endotoxins at the concentration levels of 5.7 μg l^{-1} and 640 EU ml^{-1}, respectively.

Strains of *Fischerella* have been isolated from nuclear reactor cooling water reservoirs at the Savannah River Site in Aiken, South Carolina. These strains are shown to be viable even after exposure to high temperature of 60 °C for 2 h or low temperature of 15 °C for 2 days. Psychroplilic cyanobacterial strains of *Hydrocoryne* cf. *spongiosa*, *Nostoc* sp., *Nodularia* cf. *harveyana*, *Oscillatoria* cf. *subproboscidea*,

Phormidium cf. *autumnale*, *Schizothrix* sp. have been isolated and identified from Lake Fryxell (McMurdo Dry Valleys, Antarctica). The isolated psychrophilic strains that are cold adapted grow at 5 °C in benthic gradient chamber and the oxygenic photosynthesis was optimal at 10 °C (Taton et al. 2003). Halophilic cyanobacterial strains belonging to *Phormidium* sp., *Oscillatoria* sp., and *Lyngbya* sp. have been identified from the hypersaline meltwater ponds in south of Bratina Island, McMurdo Ice Shelf, Antarctica (Jungblut et al. 2005). *Oscillatorian* cyanobacteria are shown to be predominant in benthic microbial mat communities in many polar freshwater ecosystems. Nadeau et al. (2000) isolated two different isolates of psychrophilic and psychrotolerant oscillatorians from meltwater ponds on Antarctica's McMurdo Ice Shelf. They displayed a maximum growth at 8 °C, with doubling rates of 0.12 and 0.08 per day, respectively. The psychrotolerant isolate has very slow growth at 4 °C and a rate of 0.9 doublings per day at its optimal temperature of 23 °C. The psychrophilic oscillatorians adapted at 8 °C had highest photosynthetic rates, and those acclimated at 3 °C still carried out active photosynthesis.

On the other hand, the psychrotolerant isolate had maximum photosynthetic rates at 24 °C. The cyanobacterial communities belonging to the genus *Chroococcidiopsis* are more predominant in the halite deposits of the hyper-aridity core of the Atacama Desert. Investigations have shown that the dessert strains of *Chroococcidiopsis* had great potential to resist adverse conditions such as prolonged desiccation and can survive even after several years of storage. Dried strains of *Chroococcidiopsis* were shown to survive even after exposure of 10 min (30 kJ/m^2) under a simulated martian UV flux, which depicts the higher tolerance of this strain than *Bacillus subtilis* spores. These strains were also resistant to Mars-like UV radiation (200–400 nm) and space vacuum for up to 1.5 years permanence in low Earth orbit experiments indicating that this strain is promising for space biology experiments (Wierzchos et al. 2006; Stivaletta et al. 2012; Bishop et al. 2011).

1.2.3 Eukarya: Microalgae, Yeast, Fungi, and Mold

Algae include the autotrophic eukaryotes that can mediate photosynthesis. Investigations have shown that, among the different groups, Kingdom Protista is shown to be more tolerant to extremophilic conditions than other taxa (Varshney et al. 2015). Extremophilic algae are very rarely investigated and most of them remain untapped. Mezhoud et al. (2014) made the first investigation of thermophilic Chlorophyta isolated from the geothermal springs in the north of Tunisia at water temperature of 60 °C. The isolated thermophilic strain was identified as a genus *Graesiella*, and it had maximum similarity of 99.8% with two *Graesiella* species, namely, *Graesiella emersonii* and *Graesiella vacuolata*, using 18S rDNA molecular identification. This strain was shown to be facultative with an optimal growth temperature of 30 °C. Further attempts were made to produce exopolysaccharides (EPS) from this strain,

and its EPS yield was found to be 11.7 mg L^{-1} per day at the temperature of 40 °C and light intensity of 20 μmol photons m^{-2} s^{-1}.

Psychrophilic *Mesotaenium berggrenii* has been identified on bare glacier surfaces in alpine and polar regions. This extremophilic strain is shown to produce brownish vacuolar pigments in very large amounts, whose chemical constitution and ecological function is largely unknown until now. This strain also produces purpurogallin carboxylic acid-6-*O*-β-D-glucopyranoside which seems to have lot of ecophysiological relevance like analogous protective pigments (Remias et al. 2012). A acidophilic and psychrophilic green alga, *Chlamydomonas species* has been identified in an acid mine drainage lake having strong acidity and high metal concentrations in Anhui Province, China. The acidophile mediated the oxidative dissolution of metal sulfides (Hao et al. 2017).

Chlamydomonas acidophila, a extremophilic green microalga, grows well in acidic waters having a pH range of 2.3–3.4 and where CO_2 is the sole source of carbon. This species can accumulate inorganic carbon and has high affinity for CO_2 utilization under low-CO_2 (air-equilibrium) conditions. However, the accumulation of inorganic carbon and CO_2 utilization is downregulated under high-CO_2 (4.5% CO_2) conditions (Spijkerman et al. 2014). The genes coding for novel phytochelatin synthase (PCS) have been identified in two extremophilic green algae, namely, *Chlamydomonas acidophila* and *Dunaliella acidophila*. PCS gene was upregulated by cadmium, and it confers cadmium resistance (Olsson et al. 2017). *Chlorella vulgaris* BA050, alkaliphilic alga that can grow at a high pH of around 9.8, was isolated from Soap Lake, Washington. This strain is promising for high lipid production (Bell et al. 2015).

Certain extremophilic strains of green algae such as *Chlorella ohadii* was isolated from desert soil crust. These strains were shown to display enhanced photosynthetic O_2 evolution upon increasing the radiation. *C. ohadii* lessens irradiation-mediated photodamage resistance by minimizing singlet oxygen formation and photoinhibition by activating a nonradiative electron recombination route. Excessive light also did not affect the light-harvesting antenna and carotene composition of *C. ohadii*. *C. ohadii* exhibited structural, compositional, and physiological changes upon irradiation with excess light, and it also showed increase in lipid content, carbohydrate content, photosynthetic rate, and inorganic carbon cycling. *C. ohadii* also mitigates photodamage by a modified function of photosystem II (Treves et al. 2016).

Thermophilic mold *Sporotrichum thermophile* has been used for the enzyme phytase production using lignocellulosic substrates (sugarcane bagasse and wheat bran). The enzyme was found to be thermostable with a melting temperature (Tm) of 73 °C (Maurya et al. 2017). Psychrophilic fungal strains belonging to ascomycetous and basidiomycetous yeasts and melanized fungi including those from genera *Cladosporium*, *Aureobasidium*, and *Penicillium* have been identified from an Arctic environment. These strains grow at seawater, in melted sea ice, and in melted glacier ice in very low water activity (Gunde-Cimerman et al. 2003). The psychrophilic fungal strains are known to resist and thrive over a wide range of conditions including low temperature, desiccation resistance, low water availability due to freezing, high concentrations of ions, low levels of nutrients in the liquid, and high

UV irradiation. Halophilic fungi that grow at very high salt concentrations are classified into two categories as kosmotropic and chaotropic. Kosmotropic fungi can grow at high concentrations of kosmotropes (stabilizing salts) such as NaCl, KCl, and $MgSO_4$, whereas chaotropic fungi can grow at high concentrations of chaotropes (destabilizing salts) such as NaBr, $MgCl_2$, and $CaCl_2$ (Zajc et al. 2014).

Fungus belonging to basidiomycetous genera is dominant in the Arctic circle. *Wallemia ichthyophage* is the most halophilic fungus that can grow with at least 10% NaCl and can grow even in solutions saturated with NaCl (Zalar et al. 2005; Zajc et al. 2013). The halophiles can also be categorized as thalassohaline and athalassohaline depending on the type of hypersaline brines and their origin of formation. Thalassohaline organisms are those that thrive in environments that are rich in sodium and chloride ions such as marine ponds, salt marshes, and solar salterns. Athalassohaline organisms grow in environments that are dominated by $MgCl_2$ and $CaCl_2$. Certain fungal strains belonging to genus *Cladosporium* are found in environment foods, preservatives, salt marshes, rhizosphere of halophytic plants, and the phylloplane of Mediterranean plants. This group of organisms is classified as xerotolerant, and *Cladosporium sphaerospermum* is an example as it grows with the minimal water activity of 0.82 (Maurya et al. 2017).

1.3 Roles of Extremophiles in Environment

Extremophiles play several key roles in the environment. Their robust nature to resist, survive, and mediate catalysis at extreme environmental conditions not only makes them unique but also promising for conserving the environment. Extremophiles including the marine microorganisms and microorganisms in deep biosphere significantly contributes to the geochemical cycles in the atmosphere. They maintain the chemical balance in the environment, help in lessening the greenhouse gases (GHGs) in the atmosphere, and detoxify the hazardous chemicals from the environment. Tortell et al. (1999) reported that the subarctic North Pacific marine microorganisms belonging to cyanobacteria, eukaryotic phytoplankton, *Prochlorococcus*, heterotrophic bacteria, and heterotrophic protozoa contribute to more than 50% assimilation of the dissolved Fe. Haloalkaliphilic bacteria and archaea that inhabit in the soda lakes helps in regulating carbon cycle (carbon fixation and heterotrophic carbon utilization), methane cycle, nitrogen cycle, sulfur cycle, and elemental sulfur disproportionation (Sorokin et al. 2014).

Deep subsurface microbes carry out processes that alter the chemical makeup of minerals, degrade pollutants, and alter the mineral content of groundwater. Many of them can also break down petroleum substances, which have been utilized in cleaning up oil spills and other accidents of that nature. These microbes thriving in deep-sea sediments are a group of extremophiles that can tolerate high pressures and low temperatures. Certain extremophiles that can thrive under the alkaline conditions expected in cement-based radioactive waste have also been discovered. Some of these radiation-resistant extremophiles can use radionuclides such as uranium and

neptunium as terminal electron acceptor, thus contributing to safe disposal of nuclear wastes. One of the first radiotolerant microbes *Deinococcus radiodurans* has multiple antioxidant systems, namely, enhanced ROS-scavenging and ROS-detoxifying activities, modified metabolic activities that result in a decreased ROS production, and downregulating the expression of the proteins containing Fe–S clusters and respiratory chain enzymes that confers resistance to radiation and mitigating its effects (Krisko and Radman 2013). For instance, radiation levels of 5 Gy are lethal to the average human; radiation of 1000 Gy is lethal to *Escherichia coli*. However, this radiation-resistant extremophile, *D. radiodurans*, is resistant up to a chronic radiation (60 Gy/h). *D. radiodurans* are resistant to other DNA-damaging conditions such as exposure to desiccation, UV, and hydrogen peroxide (Lange et al. 1998). Thermophilic sulfate-reducing bacteria, namely, *Desulfotomaculum reducens*-HA1 and *Desulfotomaculum hydrothermale*-HA2, have resistance to high concentration of metal ions and are known to remove heavy metals such as copper, chromium, and nickel (Hussain and Qazi 2016).

1.4 Applications of Extremophiles

Extremophiles and extremozymes have the potential to mediate catalysis in a wide range of operating conditions including extreme environments and enhanced catalytic rates. These interesting features of the extremophiles and extremozymes make them promise for a wide range of real-time application. They contribute to bioremediation, agriculture, polymers, bioenergy, and other commercial applications in industry. The thermoacidiphilic microorganisms from the sediments from the different sites of geothermal springs in Yellowstone National Park having a high-temperature range of 48–89 °C and low pH range of 1.9–5.02 play a crucial role in N_2 fixation in the natural environment (Hamilton et al. 2011). These thermophiles are shown to mediate biological reduction of nitrogen to ammonia contributing to nitrogen fixation or diazotrophy in terrestrial systems. Chen et al. (2007) investigated the use of thermotolerant microorganisms, *Streptomyces thermonitrificans* NTU-88, *Streptococcus* sp. NTU-130, and *Aspergillus fumigatus* NTU-132, isolated from compost for the production of biofertilizer with enhanced quality.

Chang and Yang (2009) reported the production of multifunctional biofertilizer using certain thermotolerant phosphate-solubilizing microorganisms including bacteria, actinomycetes, and fungi that are isolated from different compost plants and biofertilizers. The isolated thermophilic isolates produce a wide range of enzymes including amylase, carboxy methylcellulase, chitinase, pectinase, protease, lipase, and nitrogenase. These thermophilic consortia displayed potential to solubilize calcium phosphate, Israel rock phosphate, aluminum phosphate, iron phosphate, and hydroxyapatite, thereby contributing toward improving the quality of multifunctional biofertilizer.

Extremophilic microorganisms have been shown to be promising for the production of wide range of biologically active compounds for biomedical applications with huge industrial demands. Sahli et al. (2017) reported the antiradical,

antimicrobial, antiviral, and cytotoxic activities of the extracts of extremophilic plants, *Juncus maritimus* and *Limonium virgatum*. An acid mine waste fungal extremophile is shown to produce berkelic acid, a novel spiroketal, and γ-pyrone and spiciferone which have high anticancer activity (Stierle et al. 2006, Andrea et al. 2015). *Pleurostomophora* sp., acid mine extremophile strain isolated from the Berkeley Pit, an acid mine waste lake, has been shown to produce azaphilones such as berkchaetoazaphilones A–C, the red pigment berkchaetorubramine, and the known compound 4-(hydroxymethyl) quinoline (Stierle et al. 2015).

A halophile *Halomonas smyrnensis* AAD6T is used for the microbial levan production (Sarilmiser et al. 2015). A psychrotrophilic marine bacterium, *Pseudoalteromonas haloplanktis*, is shown to produce sterols/steroids and their derivatives via cholesterol catabolism which have applications in biopharmaceutical production (Gelzo et al. 2014). Attempts have been made to produce probiotics from marine psychrophile, *Psychrobacter namhaensis* SO89, which indicates that this psychrophile is promising to use as feed diet additive (Makled et al. 2017). Iron- and sulfur-oxidizing chemolithotrophic acidophiles such as *Leptospirillum ferriphilum*, *Acidithiobacillus (At.) ferrooxidans*, and *At. caldus* are shown to produce glycolic acid (Nancucheo and Johnson 2010). Alkaliphiles have been used for the production of different organic acids such as propionic acid, succinic acid, lactic acid, pyruvic acid, acetic acid, formic acids, isobutyric acids, isovaleric acids, α-oxoisovaleric acids, α-oxo-β-methylvaleric acids, α-oxoisocaproic acids, and phenylacetic acids (Kulshreshtha et al. 2012; Paavilainen et al. 1994). Extremophiles have been used for the production of pigments such as violacein and deoxyviolacein that have huge commercial applications (Nakamuraa et al. 2002).

1.4.1 Bioenergy

Bioenergy and bioremediation are the two major areas where applications of extremophiles have been well explored. In the bioenergy sector, extremophiles have been used for production of bioethanol, biobutanol, biodiesel, and gaseous fuels such as methane, hydrogen, and syngas (Kumar et al. 2015; Wang et al. 2018; Rathinam et al. 2017). Ji et al. (2016) reported a single step process for the conversion of different components of brown algae to bioethanol using a thermophilic bacterium *Defluviitalea phaphyphila*. Fermentations were carried out at 60 °C and obligate anaerobic conditions with the different components of brown algae such as glucose, mannitol, and alginate (Ji et al. 2016). Recently, Dhiman et al. (2017) developed a one-pot CRUDE (Conversion of Raw and Untreated Disposal into Ethanol) process for simultaneous hydrolysis and fermentation of untreated food waste into ethanol using thermophilic anaerobic conditions (65 °C). In CRUDE process, hydrolysis and fermentation steps were also totally free from supply of any external enzymes.

Several extremophilic microorganisms have been shown to produce significant amounts of fatty acids for the production of biodiesel. Amaretti et al. (2010) reported

the use of *Rhodotorula glacialis* DBVPG 4785, an oleaginous psychrophilic yeast isolated from a glacial environment that can accumulate lipids between −3 and 20 °C. Different types of fatty acids with chain length ranging from 14 to 18 carbons, including their saturated, monounsaturated, and polyunsaturated forms, have been produced, of which 18-carbon FA (69.0–83.1%) and 16-carbon fatty acids (14.0–27.6%) are dominant among the saturated fatty acids and palmitoleic (C16:1Δ9), oleic (C18:1Δ9), linoleic (C18:2Δ9,12), and α-linolenic (C18:3Δ9,12,15) acids are dominant among the unsaturated fatty acids.

Lipase, which plays crucial roles in mediating the transesterification reaction for biodiesel production, has been reported to be produced from different extremophilic sources. It is evident from the literature that the lipases that are resistant to extreme conditions have been produced from thermophilic, psychrophilic, acidophilic, alkaliphilic, and halophilic bacteria (Lee et al. 1999; Ramle and Rahim 2016; Verma and Kanwar 2010; Saranya et al. 2014; Pérez et al. 2011). Attempts were also made to clone the two novel genes coding for lipases from strictly anaerobic extreme thermophiles *Thermoanaerobacter thermohydrosulfuricus* (*LipTth*) and *Caldanaerobacter subterraneus* subsp. *tengcongensis* (*LipCst*) and expressed in *E. coli*. The recombinant enzymes displayed a broad temperature of 40-90 °C, pH range of pH 6.5 and 10, and the half-life of 48 h at 75 °C and pH 8.0 (Royter et al. 2009). Several reports are also available on the production of lipases from the thermophilic and psychrophilic fungus. Razak et al. (1997) documented the production of extracellular lipases from thermophilic fungi, *Rhizopus oryzae* and *Rhizopus rhizopodiformis*, isolated from palm oil mill effluent. The isolated lipases from these two sources had an optimum pH of 6.0 and temperature of 45 °C. Ryu et al. (2006) reported the production of psychrophilic lipase from *Photobacterium lipolyticum* sp. nov. isolated from an intertidal flat of the Yellow Sea in Korea. This cold-adapted lipase exhibited better catalysis at low-temperature range of 5–25 °C and had an activation energy of 2.07 kcal/mol. The extremophiles have been documented as promising candidates for the degradation of recalcitrant lignocellulosic biomass (Bhalla et al. 2013; Blumer-Schuette et al. 2014).

Extremophiles have been shown to produce lignin-degrading enzymes such as laccase, peroxidase, polyphenol oxidase, as well as cellulose hydrolyzing enzymes, namely, endoglucanase, cellobiohydrolase, and β-glucosidase (Zambare et al. 2011). The extremophiles not only produce this group of enzymes that are required for the hydrolysis of cellulose/oxidation of lignin but also produce enzymes at higher yield and better catalytic activity (Acharya and Chaudhary 2012; Ou et al. 2009). The lignocellulolytic activity of the extremophiles has been harnessed for the production of different biofuels such as biohydrogen, biomethane, and bioethanol from lignocellulosic feedstocks. Extremophilic microorganisms are known to produce biohydrogen via dark fermentation and photofermentation. Willquist et al. (2010) reported the production of biohydrogen (i.e., 4 mol H_2/mol hexose) from carbohydrate-rich substrates via dark fermentation process using the hyperthermophilic bacterium *Caldicellulosiruptor saccharolyticus*. *Clostridium* sp. strain PROH2, isolated from a shallow submarine hydrothermal chimney (Prony Bay, New Caledonia), was used for the fermentative hydrogen production in alkaline and mesothermic

environments with different feedstocks such as cellobiose, galactose, glucose, maltose, sucrose, and trehalose (Mei et al. 2014, Nan et al. 2014). Paulo et al. (2004) reported the thermophilic anaerobic conversion of methanol to methane and acetate at 55 °C via methanogenesis and homoacetogenesis using a sludge consortium. *Bacillus methanolicus*, a facultative methylotrophic and thermophilic bacterium, is used for the conversion of methanol to value-added products such as commercially important amino acids L-glutamate and L-lysine at 50 °C via methylotrophy (Müller et al. 2015).

1.4.2 Bioelectrochemical Systems

The electrocatalytic activity of extremophiles to mediate electrooxidation/electroreduction of electron donor/electron acceptor and their electron transfer characteristics have been investigated in great detail (Hawkins et al. 2011, Sokolovskaya et al. 2015). The electrocatalytic activity of the extremophiles has been harnessed for the different bioelectrochemical applications (Dopson et al. 2016). Microbial fuel cells have been developed making use of electrocatalytic activity of extremophiles for bioelectricity generation from different substrates such as glucose, xylose, cellobiose, acetate, lignocellulosic materials, wastewater, heavy metals, and dyes in anode compartment or cathode compartment depending on whether it is an electron donor or electron acceptor, respectively (Wrighton et al. 2008; Jong et al. 2006; Lusk et al. 2015). Mathis et al. (2008) reported the bioelectricity generation of 209–254 mA/m^2 at 60 °C using a thermophile closely related to *Thermincola carboxydophila* with cellulose or acetate as a electron donor. Attempts have been used to develop microbial fuel cells with barophilic microorganisms using the deep-sea sediments as electron donors (Richter et al. 2015). The use of electrochemical activity of the extremophiles for biohydrogen production in a microbial electrolysis cells (MECs) is also reported in the literature (Lu et al. 2011). Lusk et al. (2007) developed a microbial electrolysis cells with thermophilic bioanode consisting of phylum *Firmicutes* with the *Thermoanaerobacter* and *Thermincola* genera. The developed MECs operated at 60 °C with cellulose as a substrate in anodic compartment produced a current densities of 6.5 A m^{-2} and coulombic efficiency (CE) of 84% without production of CH_4 (Lusk et al. 2007). A report has been documented in the literature on the simultaneous bioelectrosynthesis of formate and acetate and reduction of CO_2 using thermophilic *Moorella thermoautotrophica* at 60°C (Yu et al. 2017).

1.4.3 Bioremediation

Extremophiles have been realized as promising candidates for the bioremediation of wastes because of their innate potential to resist toxic environments as well as to degrade a wide range of pollutants (Sar et al. 2013; Margesin and Schinner 2001). Fardeau et al. (2000) isolated a new thermophilic strain *Thermoanaerobacter*

subterraneus sp. nov. from an oil field water which was shown to mediate the fermentation of a range of carbohydrates to acetate, L-alanine, lactate, H_2, and CO_2 and reduced thiosulfate and elemental sulfur. The thermophilic consortium that has dominant Caldisericales, "Shengli Cluster," and Synergistetes mediated the simultaneous degradation of crude oil *n*-alkanes and methane production at 55 °C with the yield of 2.8 ± 0.3 mmol of methane per gram oil (Cheng et al. 2014). Xia et al. (2015) isolated thermophilic *Anoxybacillus* sp. with four alkane monooxygenase genes that can degrade a wide range of hydrocarbons (C8–C22) at 67 °C and produce oligosaccharide–lipid–peptide bioemulsifier. It displayed an excellent emulsification activity with various oil phases (EI24 > 60%), and it had the potential to increase cell surface lipophilicity during degradation. Psychrophilic strains, *Pseudomonas simiae* G1-10O, *P. taiwanensis* Y1–4, and *P. koreensis* Gwa2 isolated from oil contaminated soil, were shown to degrade kerosene,gasoline, and diesel as carbon sources, at 10 °C (Pham et al. 2014). Extremophiles such as *Geobacillus caldoxylosilyticus* T20 are shown to utilize toxic pesticides such as organophosphates as the sole phosphorus source at 60 °C (Obojska et al. 2002). Certain extremophiles were also shown to degrade a wide range of pharmaceutical compounds such as sulfadiazine, sulfamethoxazole, and sulfamerazine which indicates that extremophiles are promising for degradation of pharmaceutical compounds (Pan et al. 2017). Several investigations are also underway on the extremely high UV-C radiation-resistant extremophiles for space biology applications (Paulino-Lima et al. 2016).

1.4.4 Value-Added Products

Biopolymers such as polyhydroxyalkanoates, extracellular polysaccharides, and alginate have been produced using different extremophilic microorganisms with improved physical properties. The thermophile *Cupriavidus* sp. strain was shown to synthesize polyhydroxybutyrate (PHB) from glucose at 50 °C. Further, genetic engineering approaches have been carried out to insert the *pha* genes of *Cupriavidus* sp. in *E. coli* for PHA synthesis in improved thermostability (Sheu et al. 2012). The genome sequence of *Yangia* sp. CCB-MM3, a halophile, isolated from soil sediment in the estuarine Matang Mangrove, Malaysia, had been confirmed to have the presence of pathway for production of propionyl-CoA and gene cluster coding for PHA production (Lau et al. 2017). Sardari et al. (2017) reported the production of exopolysaccharides using the two strains of the thermophilic bacterium *Rhodothermus marinus* with lactose as feedstocks.

1.4.5 Extremozymes

Last, but not the least, the extremophiles are the sources of extremozymes that can mediate catalysis specifically at accelerated rates. The extremozymes have been

promising in a wide range of sectors including diagnostics, therapeutics, detergents, bioenergy, food processing, tanning, and others in other industrial biotechnology applications. The enzyme FMN-dependent NADH azoreductase isolated from the thermophile *Geobacillus stearothermophilus* is thermostable and is shown to mediate the degradation of methyl red at its optimal temperature of 85 °C (Matsumoto et al. 2010). Different enzymes, amylases, proteases, lipases, cellulases, esterases, and oxidoreductases, have been produced from different extremophiles (Karan et al. 2012, Fuciños et al. 2012, López-López et al. 2014). Thermostable DNA polymerase has been used for several PCR-based diagnostics and research (Abu Al-Soud and Rådström 1998; Morgan et al. 1998). Extremophiles also serve as the sources of extremely thermostable restriction enzymes. Bacterial β-lactamase, an antibiotic, has been produced by the psychrophile, *Psychrobacter immobilis* A8, isolated from the Antarctic station Dumont d'Urville on frozen organic debris. The isolated β-lactamase was found have a molecular weight of 41,000 Da and pI of 5.3. The enzyme was stable at very low temperatures (less than 10 °C) and pH over a range of 4–10 (Feller et al. 1995).

Take-Home Message

Microbes have served and continue to serve as one of the largest and useful sources of many enzymes.

Compared to microbial catalysis, the enzymes have several advantages such as higher productivity and simpler product purification and do not demand sophisticated bioreactors as in the case of microbial bioprocesses.

Extremophile includes the microorganisms growing at extreme environments including thermophiles, psychrophiles, halophiles, acidophiles, alkaliphiles, desert-resistant microorganisms, drug-resistant microorganisms, and radiation-resistant microorganisms. Extremophiles has the advantage that they can mediate catalysis over a wide range of conditions as well as high catalytic rates.

Extremophiles have wide applications in bioenergy, biopolymer production, production of value-added products, biomining, bioremediation, and production of enzymes.

The use of extremozymes will help to overcome the limitations of the enzymatic processes such as high costs of pure enzyme, fragile nature of the enzyme, very narrow reaction conditions, low half-life of the enzyme, and need for exogenous cofactors.

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Chapter 2
Conversion of Lignocellulosic Feedstocks into Bioethanol Using Extremophiles

Sean Michael Scully and Johann Orlygsson

What You Will Learn

1. Advantages of using thermophiles for bioethanol production
2. Considerations for using thermoanaerobes for processing lignocellulosic biomass
3. Utilization of combined bioprocesses such as SSF and CBP for bioethanol production
4. Recent advances in genetically modified thermoanaerobes for bioethanol production

2.1 Introduction

While the original intention of Henry Ford was for Americans to grow their own feedstock and power their cars using ethanol, this never achieved market proliferation. It was not until the energy crises of the 1970s and early 1980s that bioethanol began to receive attention as a viable alternative to petrol-based energy sources. Until recently, Brazil has dominated the first-generation bioethanol market (from sugar cane). Presently, the USA is the largest producer in the world, using corn as their main substrate (Renewable Fuel Association 2013). The production of ethanol from sugar- and starch-based material (first-generation production) has been heavily criticized for competing with food-based agriculture and its environmental impact. More recently, a growing demand for bioethanol derived from renewable and environmentally friendly feedstocks has been driven by governmental targets to curb CO_2 emissions, because of volatility in crude oil prices and increasing demand

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for energy independence. Thus, ethanol produced from lignocellulose has emerged as a future alternative and is termed as second-generation ethanol production (Tan et al. 2016).

Ethanol production from carbohydrates has been widely investigated among both mesophilic and thermophilic microorganisms. The most powerful known ethanol producers are both mesophiles: the yeast *Saccharomyces cerevisiae* and the bacterium *Zymomonas mobilis* both with reported yields above 1.90 moles of ethanol per mole of hexose (Pereira et al. 2012; Rogers et al. 1979). The utility of these microorganisms is however limited by their narrow substrate spectra as the wild types are only capable of utilizing simple sugars. The earliest studies on bioethanol production by thermophiles originated after the oil crisis in the mid-1970s. High growth rates and high ethanol titers make ethanol-producing thermophilic anaerobes attractive candidates for large-scale industrial bioethanol production. Furthermore, their broad substrate spectra make them of particular interest for the fermentation of lignocellulosic biomass hydrolysates.

Lignocellulose is composed of three different components: cellulose, hemicellulose, and lignin (Sánchez and Cardona 2008). Hemicellulose is comprised of several heteropolymers composed of a variety of hexoses and pentoses including glucose, galactose, mannose, xylose, arabinose, glucuronic acid, galacturonic acid, and L-rhamnose. The more complex mixture of sugars thus presents a challenge to traditional ethanologenic microorganisms. Furthermore, hemicellulose and lignin are tightly embedded in the plant material necessitating that the lignin needs to be removed and the sugar polymers released in order to make them accessible for the subsequent hydrolytic/enzymatic steps. This additional pretreatment step is the main difference between the utilization of simple biomass (starch and sugars) and complex biomass. Additionally, the sugar pool released in the latter is more heterogeneous as compared with first-generation substrates (Sánchez and Cardona 2008; Taylor et al. 2009). This recalcitrant structure of lignocellulose and extensive pretreatment needed pose a significant challenge in terms of large-scale feasibility. The food versus fuel problem is addressed by second-generation bioethanol production since it is derived from nonfood (lignocellulosic) biomass demonstrating more sustainability. Lignocellulosic biomass is often an agricultural by-product such as straw produced from wheat or corn (Sánchez and Cardona 2008).

This review focuses on thermophilic anaerobes, their physiology, ethanol production capacity from complex biomass, process advances, and recent advances in the genetic modification of thermophilic ethanologens with a particular emphasis on second-generation bioethanol production and the amenability of thermophiles for consolidated processes.

2.2 Advantages of Thermophiles for Bioethanol Production

For the production of ethanol from biomass to be commercially successful, several key processes and characteristics of organisms of interest need to be considered (Sánchez and Cardona 2008; Taylor et al. 2009; Chang and Yao 2011). Table 2.1 highlights the requirements needed from three points of view: the physiological properties of the ethanologen used, the feedstock, and the process. Of particular interest are strains that do not exhibit the so-called glucose effect in which glucose is preferentially ustalized over other carbon sources present; the simultaneous co-utilization of hexoses and pentoses present in lignocellulosic biomass hydrolysates is critical and eliminates the need for a co-fermentation. At present, there is no single organism that possesses all features highlighted in Table 2.1. While genetic manipulation is a valuable tool, it has yielded only modest improvements for ethanologens, and these techniques rarely yield stable transformants (Ostergaard et al. 2000; Jeffries 2006).

Thermophilic bacteria have many properties making them suitable for second-generation ethanol production as they typically have broad substrate ranges; do not require extensive heating, cooling, or mixing (Taylor et al. 2009; Chang and Yao 2011) and have broad tolerances to environmental conditions such as salt, pH, and low nutritional requirements (Taylor et al. 2009). From a process perspective, mixing operations are easier at elevated temperatures due to reduced viscosity and increased substrate loadings. Furthermore, mass transfer rates are higher at increased temperatures, and the risk of mesophilic contamination is lower (at higher process temperatures).

Table 2.1 Requirements of bioethanol organisms, feedstocks and processes

Requirements		
Ethanologen	Feedstock	Process
• Minimal by-product formation • High productivity (>1 g/L/h) • GRAS status • Broad tolerance to environmental conditions • High ethanol tolerance (pH, temperature, osmotic strength) • Broad substrate spectra • No “glucose effect” (catabolite repression) • High cellulolytic activity • Tolerance to inhibitory compounds (or bioconversion of) • Tolerance of high solid and substrate loadings • Simple nutritional needs • Low biomass production • Ease of genetic manipulation	• Renewable • High productivity • Low-cost pretreatment • High carbohydrate content • Minimal lignin content • Does not compete with food • Minimal environmental impact	• >90% of theoretical yield • High ethanol titers [>5% (v/v)] • Minimum number of process steps • Minimal process cooling • Recyclable cells • Co-fermentation of substrates • Limited or no pretreatment • Minimal waste stream • Inexpensive media formulation

2.3 Thermophilic Ethanologens

The interest in thermophilic ethanologens is mostly due to their broad substrate spectra including biopolymers, hexoses, and pentoses, as well as their ethanol yields and tolerance toward many environmental variants (Taylor et al. 2009; Chang and Yao 2011). Species of highly ethanologenic thermophilic anaerobes are typically members of the genera *Clostridium*, *Caldanaerobacter*, *Thermoanaerobacter*, or *Thermoanaerobacterium* (Taylor et al. 2009; Scully and Orlygsson 2015) although thermophilic ethanol producers can be found within other genera, e.g., *Bacillus*, *Geobacillus*, *Paenibacillus*, and *Caloramator*. The physiology and ethanol production potential of species within most genera have not been widely investigated; typically studies have focused upon one or two members within each genus. The maximum ethanol yields reported are by *Thermoanaerobacter ethanolicus* or 1.90 mol ethanol/mol glucose and 1.64 mol ethanol/mol xylose (Wiegel and Ljungdahl 1981; Lacis and Lawford 1988). Other thermoanaerobes with high yields are, e.g., *Thermoanaerobacter pseudoethanolicus* (1.88 mol ethanol/mol glucose) (Lovitt et al. 1988), *Thermoanaerobacter* strain AK5 and *Thermoanaerobacter* strain J1 (both 1.70 mol ethanol/mol glucose) (Brynjarsdottir et al. 2012; Jessen and Orlygsson 2012), *Thermoanaerobacter pentosaceus* (1.68 mol ethanol/mol glucose) (Tomas et al. 2013), *Caloramator boliviensis* (1.53 mol ethanol/mol glucose) (Crespo et al. 2012), and *Thermoanaerobacterium* strain AK17 (1.50 mol ethanol/mol glucose) (Almarsdottir et al. 2012). For further information regarding the substrate spectra, ethanol tolerance, inhibitor tolerance, and fermentation end products of these strains, the reader is directed to the recent overview by Scully and Orlygsson (2015) and the references therein.

2.4 Physiology of Thermophilic Anaerobic Bacteria

Most thermophiles use the Embden-Meyerhof-Parnas pathway (EMP) for sugar degradation to pyruvate (Taylor et al. 2009). They differ however in context with pyruvate degradation compared with yeast and facultative bacteria by reducing pyruvate to acetyl-CoA via pyruvate ferredoxin oxidoreductase (PFOR) instead of pyruvate decarboxylase. A second reduction step is then performed via alcohol dehydrogenase leading to the production of ethanol. These two reduction steps lead to a redox imbalance, which is not observed during yeast fermentation. This forces the microorganism to make an oxidation, most often by producing acetate via an acetyl phosphate intermediate using phosphotransacetylase (PTA) and acetate kinase (AK). Finally, mixed acid fermentation can also include the formation of lactate (Fig. 2.1), formate, and alanine (not shown in Fig. 2.1) from pyruvate via lactate dehydrogenase (LDH), pyruvate formate lyase, and alanine transaminase, respectively. One of the major obstacles to achieve high ethanol titers in thermophiles is to minimize the variety of end products formed. Pentoses, including xylose and arabinose, are

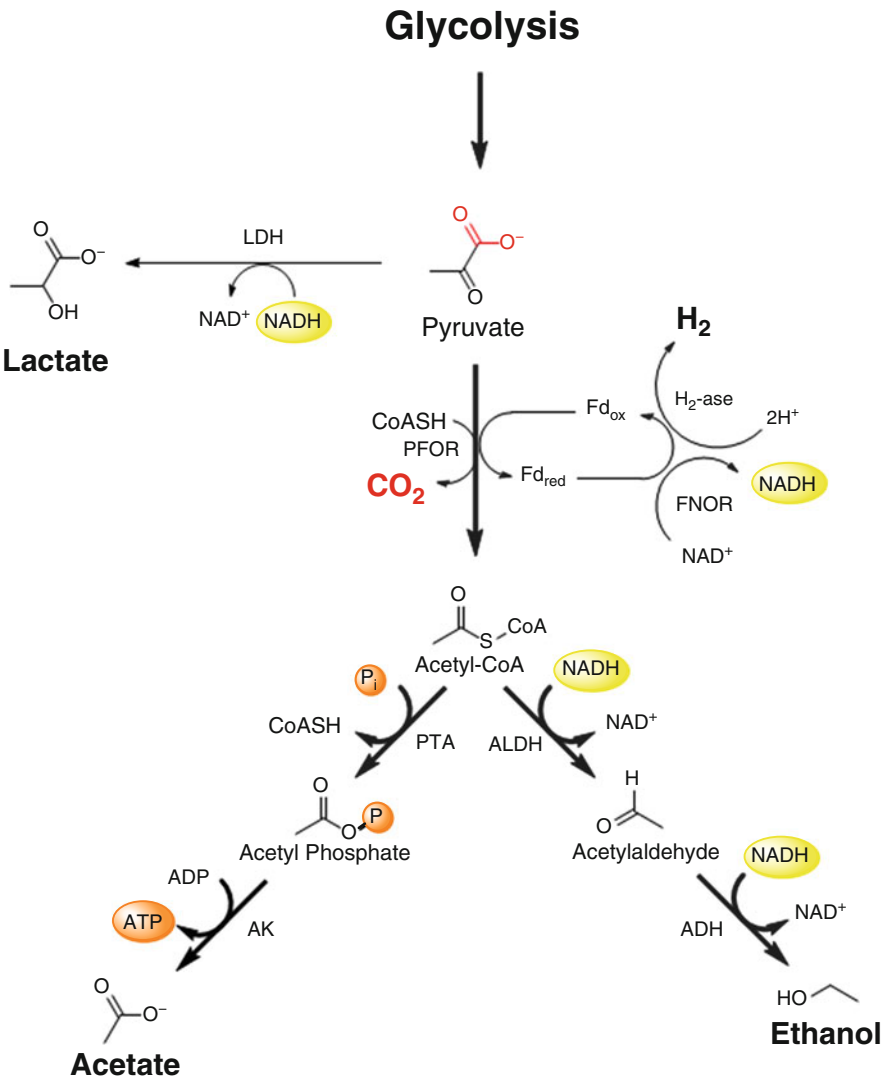

Fig. 2.1 Simplified scheme of glucose degradation to various end products by strictly anaerobic bacteria. Abbreviations: *ADP* adenosine triphosphate, *ATP* adenosine triphosphate, *ALDH* acetaldehyde dehydrogenase, *ADH* alcohol dehydrogenase, *AK* acetate kinase, *CoASH* acetyl coenzyme A, *fd* ferredoxin, *FNOR* ferredoxin oxidoreductase, *H_2-ase* hydrogenase, *LDH* lactate dehydrogenase, *NAD^+* nicotinamide adenine dinucleotide (oxidized), *NADH* nicotinamide adenine dinucleotide (reduced), *P_i* inorganic phosphate, *PFOR* pyruvate ferredoxin oxidoreductase, *PTA* phosphotransacetylase. Modified from Scully and Orlygsson (2015)

degraded via the nonoxidative pentose phosphate pathway which leads to the formation of intermediates that are directed into the glycolysis pathway (Taylor et al. 2009; Chang and Yao 2011). Theoretical yields of ethanol are 2 moles/mole glucose and

1.67 moles/mole xylose (Eqs. 2.1 and 2.2, respectively). However, these yields are never obtained since a part of the substrate is converted to biomass or directed toward other end products (Fig. 2.1).

$$1 \text{ glucose} \rightarrow 2 \text{ ethanol} + 2\ CO_2 \tag{2.1}$$

$$1 \text{ xylose} \rightarrow 1.66 \text{ ethanol} + 1.66\ CO_2 \tag{2.2}$$

Various factors are known to influence the production of ethanol by thermophilic bacteria: the substrate types, partial pressure of hydrogen, initial substrate concentration, pH, and temperature. Increased partial pressure of hydrogen (pH_2) is known to increase the production of reduced end products (ethanol, lactate) and minimize the production of oxidized end products (acetate, butyrate). The main reason for this is (because of) the thermodynamics involved and inhibitory effects on the key enzymes that are responsible for hydrogen production, namely, pyruvate ferredoxin oxidoreductase and NAD(P) oxidoreductase. Thus, especially at lower temperatures, microorganisms are forced to transfer electrons produced in previous oxidation steps on pyruvate leading to the production of ethanol and lactate but not hydrogen. The effect of pH_2 has been investigated for several ethanologenic thermophiles, such as *Thermoanaerobacter* strain AK5 and *Thermoanaerobacter* strain J1 (Brynjarsdottir et al. 2012; Jessen and Orlygsson 2012). The main findings of these studies show that scavenging hydrogen by either adding thiosulfate or by cocultivating the sugar-degrading strain together with a hydrogenotrophic methanogen results in a shift in end-product formation, namely, away from ethanol toward acetate production.

It has been well established that thermophilic bacteria are inhibited at relatively low substrate concentrations as compared with yeasts and *Z. mobilis* (Taylor et al. 2009). By increasing the sugar concentrations in batch cultures of thermophilic bacteria, inhibition of substrate degradation often occurs between 10 and 30 mM (Almarsdottir et al. 2012; Brynjarsdottir et al. 2012; Tomas et al. 2011). Although this inhibition may also be caused by accumulated hydrogen or low pH, it could also be an intriguing factor for thermophiles. Recent investigation showed that high partial pressure of hydrogen resulting from high initial substrate concentration is more likely to be the main inhibitory factor for further substrate degradation rather than the substrate or end low pH (Viponik et al. 2016).

One of the main reasons for the success of *Z. mobilis* and especially *S. cerevisiae* is the adaptation of these industrial strains to high ethanol tolerance. Good ethanol producers should tolerate at least 4% (v/v) ethanol for an economical ethanol recovery to occur (Taylor et al. 2009). Most thermophiles tolerate less than 3% (v/v), which is considerably less as compared with yeasts and *Z. mobilis*. This is mainly due to their fatty acid membrane structure, i.e., proportionally containing more unsaturated fatty acids. The presence of ethanol in the fermentation broth causes membranes to leak, resulting in loss of energy during cellular metabolism and finally cell lysis. Additionally, ethanol has a strongly denaturing effect on proteins. Substantial efforts have been made to increase the ethanol tolerance of thermophilic species. Sometimes these experiments have resulted in strains with higher ethanol tolerance but often showing lower ethanol yields (Lovitt et al. 1984). There is still

much debate concerning ethanol tolerance of thermophilic anaerobes with many strains being highly sensitive to relatively low concentrations (Baskaran et al. 1995; Lin et al. 2013). Natural ethanol tolerance is typically below 3–4% (v/v) although several exceptions such as *Thermoanaerobacter* strain J1 have been noted. Strains can be naturally manipulated by indirect mutagenesis; their ethanol yields often drop substantially. Clearly, a better understanding of the fundamental ethanol tolerance mechanism of thermoanaerobes, such as cell membrane alterations and enzymatic regulation, is needed (Lovitt et al. 1988; Timmons et al. 2009).

Other environmental factors of importance for thermophilic bacteria are their pH and temperature growth optimum, their tolerance toward inhibitory compounds like furfuraldehyde and 5-hydroxymethyl-furfuraldehyde (5-HMF), and their need for trace elements and vitamins often originating from complex medium supplements like yeast extract.

2.5 Production of Ethanol from Lignocellulose: Processes for Ethanol Production

2.5.1 Production of Ethanol from Lignocellulosic Substrates

The production of bioethanol from lignocellulosic biomass by thermoanaerobes has been widely reported in the literature mainly from bacteria within *Clostridia* including the genera of *Clostridium*, *Thermoanaerobacterium*, and *Thermoanaerobacter*. There is a large variation in the type and concentration of biomass used, fermentation processes (batch, semi-batch, continuous), pretreatment methods, as well as whether pure or mixed cultures are used.

The maximum ethanol yield obtained from glucose fermentation is 2 mol ethanol/mol glucose (0.51 g/L or 11.1 mM/g glucose). Near theoretical yields are however never obtained which may be partially explained by the complex structure of lignocellulosic biomass contributing compounds that may be inhibitory such as lignin derivatives, aldehydes formed from sugars during the pretreatment process, and Table 2.2 shows selected data of ethanol yields obtained by thermophilic bacteria using various complex biomass. Early experiments on ethanol yields from complex biomass were conducted with *Thermoanaerobacter ethanolicus* and *Clostridium thermocellum* using hemicellulose from birch- and beechwood (Wiegel et al. 1983). These early reports showed promising results and a mutant strain of *T. ethanolicus* produced 4.5 mM/g xylose equivalent used. *Clostridium thermocellum* produced 7.2–8.0 mM/g from Whatman paper (Table 2.2). Similar yields were obtained from paddy straw and sorghum stover, pretreated with alkali (Rani et al. 1997). *Thermoanaerobacterium saccharolyticum* HG8 produced 6.3 mM of ethanol per gram of xylan from wheat straw hydrolysates (Ahring et al. 1999). The highest ethanol yields reported from complex biomass have been by *Thermoanaerobacter* BG1L1 grown on corn stover and wheat straw (Georgieva and

Table 2.2 Examples of ethanol production from lignocellulosic biomass by thermophilic bacteria

Organisms	Substrate type	Fermentation mode	Substrate conc. (g/L)	Pretreatment	Ethanol yields (mM/g)	Temp (°C)	References
Clostridium thermocellum	Whatman paper	Batch	8.0	None	7.20–8.00	60	Rani et al. (1997)
Clostridium thermocellum	Paddy straw	Batch	8.0	None	6.10–8.00	60	Rani et al. (1997)
Clostridium thermocellum	Sorghum stover	Batch	8.0	None	4.80–8.10	60	Rani et al. (1997)
C. Thermocellum and C. Thermolacticum	Microcrystal cellulose	Batch	10.0	None	9.1	57	Xu and Tschirner (2014)
Thermoanaerobacter mathranii	Wheat straw	Batch	60.0	WO/E	5.30	70	Ahring et al. (1999)
Thermoanaerobacter ethanolicus	Beet molasses	Batch	30.0	None	4.81	65	Avci and Donmez (2006)
Thermoanaerobacter BG1L1	Corn stover	Batch	25.0–150.0	WO/E	8.50–9.20	70	Georgieva and Ahring (2007)
Thermoanaerobacter BG1L1	Corn stover	Con	25.0–150.0	WO/E	8.50–9.20	70	Georgieva and Ahring (2007)
Thermoanaerobacter BG1L1	Wheat straw	Batch	30.0–120.0	WO/E	8.50–9.20	70	Georgieva et al. (2008)
T. Ethanolicus	Wood HL	Batch	8.0	E	3.30–4.50	70	Wiegel et al. (1983)
Thermoanaerobacter AK5	Whatman paper	Batch	2.25	E	7.7	65	Brynjarsdottir et al. (2012)
Thermoanaerobacter AK5	Grass	Batch	4.5	A/E	4.31	65	Brynjarsdottir et al. (2012)
Thermoanaerobacter J1	Whatman paper	Batch	4.5	E	7.5	65	Jessen and Orlygsson (2012)
Thermoanaerobacter J1	Hemp	Batch	4.5	A/E	4.3	65	Orlygsson (2012)
T. Saccharolyticum	Xylan	Batch	10.0	WO	6.30	60	Ahring et al. (1996)
Thermoanaerobacterium AK17	Cellulose	Batch	2.5	E	8.6	60	Almarsdottir et al. (2012)
Thermoanaerobacterium AK17	Grass	Batch	2.5	A/Alk/E	5.5	60	Almarsdottir et al. (2012)

A acid, *Alk* alkaline, *E* enzymatic, *WO* wet oxidation, *con* continuous. Modified from Scully and Orlygsson (2015)

Ahring 2007; Georgieva et al. 2008). The biomass was pretreated with acid or wet oxidation, and ethanol yields were up to 9.2 mM/g for biomass hydrolysates.

More recent studies on ethanol production by *Thermoanaerobacterium* strain AK17, isolated from an Icelandic hot spring, grown on various types of lignocellulosic biomass were reported by Sveinsdottir and co-workers (Sveinsdottir et al. 2009). Batch culture studies using 7.5 g/L of cellulose, grass, and newspaper, pretreated with heat and enzymes, showed ethanol yields of 2.0 (paper), 2.9 (grass), and 5.8 (cellulose) mM/g biomass, respectively. Optimization experiments were recently done on this strain where ethanol yields on grass and cellulose were increased to 4.0 and 8.6 mM/g, respectively. The main environmental factors that showed to increase ethanol yields were the use of acid/alkali for pretreatment and lowering of the substrate concentration from 7.5 to 2.5 g/L (Almarsdottir et al. 2012). Recent investigations on two *Thermoanaerobacter* strains, AK5 and J1, showed promising results from various types of hydrolysates prepared from chemically and enzymatically pretreated lignocellulosic biomass as shown in Table 2.2.

2.5.2 Processes for Ethanol Production

The cost of pretreatment and enzymatic hydrolysis is the main barrier of using lignocellulosic biomass as a feedstock for bioethanol production. Most emphasis for improving the economic feasibility of second-generation ethanol production has been on reducing the number of process steps, e.g., by using integrated process technologies that lead to decreased processing time, reduced pretreatment costs, and increased energy efficiency of bioethanol production.

Types of bioethanol processes vary due to feedstock, pretreatment, and organism selection. In general the process of converting complex lignocellulosic substrates into ethanol involves four steps: (1) physical and chemical pretreatment of biomass, (2) hydrolase production (cellulases, hemicellulases) and saccharification (enzymatic hydrolysis of polymers to hexoses and pentoses), (3) fermentation (of both pentoses and hexoses), and (4) product recovery.

There are several review articles on ethanol production from lignocellulosic biomass (Taylor et al. 2009; Chang and Yao 2011; Lynd et al. 2005). Below is a short description of each step.

2.5.2.1 Pretreatment of Biomass

Before fermentation, lignocellulosic biomass requires pretreatment to separate its components, increasing the enzymatic accessibility during hydrolysis and thus facilitating the conversion of the polymeric carbohydrates into sugars. The main aim of pretreatment is to minimize energy consumption while maximizing the amount of sugars liberated. Still, this remains a major challenge with no single pretreatment method being universally applicable (Sánchez and Cardona 2008; Mosier et al. 2005). There are various chemical, physical, physiochemical, and

biological pretreatment methods available. Usually, the biomass is treated at a high temperature and pressure in a mildly acidic solution for liberation of lignocellulose from the biomass matrix. In this case, thermophiles have a clear advantage over mesophilic organisms because the pretreated biomass does not require as much cooling prior to subsequent process steps (saccharification and fermentation). One of the major drawbacks of hot acidic pretreatments is the formation of aldehydes, 5-HMF, and furfuraldehyde from glucose and xylose, respectively. Additionally, phenolic compounds may be formed from lignin. For more comprehensive description of pretreatment methods, recent review articles should be examined (Menon and Rao 2012; Talebnia et al. 2010). The main outcome of pretreatment is the removal of lignin and separation of cellulose and hemicellulose.

2.5.2.2 Enzymatic Hydrolysis and Saccharification

The main hindrance of producing bioethanol from complex biomass is the cost of commercial enzymes for conversion of the hemicelluloses and celluloses to sugars (Elleuche et al. 2014). An ideal enzyme system for biorefinery applications should be generated in situ and be useable in continuous culture and possess high activity and a long half-life. Additionally, the enzymes need to be stable under process conditions including elevated temperatures and be resistant to inhibitory compounds such as aldehydes. Still today, there are no commercially available enzyme suitable for high-temperature applications (>55 °C); most have been isolated from mesophilic organisms with operating temperatures below 50 °C and have poor yields and relatively slow rates of hydrolysis (Bhalla et al. 2013).

Using in situ methods to degrade cellulose and hemicellulose, i.e., biological pretreatment, is an interesting emerging method. There are several well-known cellulase-producing fungi and bacteria that can attack lignocellulosic biomass, making it more accessible to enzymes. Examples include *T. reesei*, *Aspergillus niger*, and the bacterium *C. thermocellum* (Talebnia et al. 2010). This chemical-free method is inherently more environmentally friendly and thus makes it a particularly desirable pretreatment option. Other advantages include a low required energy input. The drawbacks, however, are that it is a very time-consuming process requiring large volumes and active control of growth conditions (Elleuche et al. 2014).

2.5.2.3 Fermentation

Many thermophilic bacteria have broad substrate spectra which makes them an excellent choice for using lignocellulose as raw biomass material. Furthermore, cultivation under elevated temperatures increases metabolic conversion rates and limits the risk of mesophilic contamination (Turner et al. 2007). Many thermophiles degrade hexoses and pentoses simultaneously, while mesophiles like *Z. mobilis* and *S. cerevisiae* typically use glucose first, before degrading other substrates (Tomás 2013).

Given that the biochemical conversion of sugars to ethanol is an exothermic process, mesophilic organisms require active cooling. This problem is somewhat mitigated with the use of thermophilic organisms and can be coupled to in situ ethanol removal thus decreasing the amount of external energy needed for process cooling and product recovery while driving end production formation and limiting ethanol inhibition. This is particularly relevant in the context of consolidated bioprocesses.

2.5.2.4 Product Recovery

After fermentation, ethanol is separated from other components by distillation. Ethanol production at high temperatures could theoretically be removed in situ, by the so-called self-distillation. At atmospheric pressure, ethanol boils at 74.8 °C which is slightly higher than the temperature optimum for many ethanologenic thermophiles. Thus, ethanol, being volatile, could be continuously removed at sub-boiling temperatures to further drive the process to completion.

2.5.3 Integrated Processes for Ethanol Production from Lignocellulose

After pretreatment, complex biomass can be converted to ethanol via one of the following ways: (1) separate hydrolysis and fermentation (SHF), (2) simultaneous saccharification and fermentation (SSF), (3) simultaneous saccharification and co-fermentation (SSCF), and (4) consolidated bioprocessing (CBP). The most relevant processes for thermophilic ethanol production are SHF, SSCF, and CBP since there is no need for a separate hexose and pentose fermentation as used in the SSF process. The main differences of these processes are outlined in Fig. 2.2. During the

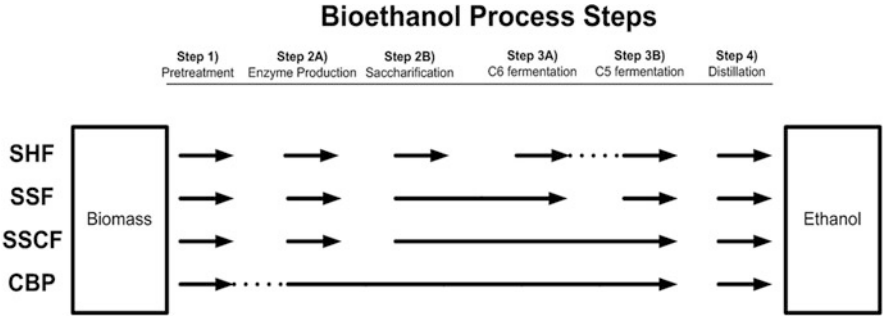

Fig. 2.2 Process steps of bioethanol production processes. *SHF* separate hydrolysis and fermentation, *SSF* simultaneous saccharification and fermentation, *SSCF* simultaneous saccharification and co-fermentation, *CBP* consolidated bioprocessing, *C6* hexose, *C5* pentose. From Scully and Orlygsson (2015)

SHF process, polymer hydrolysis and fermentation are performed in two separate steps, whereas SSF and SSCF are both single-step processes. The difference between the two is that the latter includes both pentose and hexose fermentation but the former only hexose fermentation. Finally, the CBP applies when enzymatic production, cellulose degradation, and fermentation occur in a single step.

2.5.3.1 Separate Hydrolysis and Fermentation

The two-stage SHF process is the most traditional method for ethanol production with the main advantage being that both polymer hydrolysis and fermentation occur under optimum conditions. The main disadvantage, however, is the fact that glucose production during the first step can strongly inhibit further cellulase activity.

2.5.3.2 Simultaneous Saccharification and Fermentation and Simultaneous Saccharification and Co-fermentation

Both SSF and SSCF are performed in a single fermentation vessel and have been developed to avoid the inhibitory effect of sugars on cellulases as well as reducing the number of process steps. One of the major advantage of SSF is increased hydrolysis rates and thus lower enzyme loadings needed, as well as less need for sterile conditions, decreased reaction times, and lower reactor volume (Balat et al. 2008). Simultaneous consumption of released sugars decreases the risk for product inhibition, while rapid consumption of monosugars minimizes the risk of contamination by other saccharolytic organisms and keeps substrate levels low thus avoiding inhibition of both the fermentative organisms and the hydrolytic enzymes (Abdel-Banat et al. 2010). The challenge of these processes is the compatibility of the optimal conditions for the hydrolysis and the microorganisms used for fermentation. Enzymatic hydrolysis is usually performed at 50 °C, while most common mesophilic fermentative microorganisms (used) exhibit temperature optimum of 28 °C–37 °C. This results in lower efficiency and lower ethanol yields (Menon and Rao 2012; Abdel-Banat et al. 2010).

A number of challenges, however, need to be solved to address the production of bioethanol from lignocellulosic biomass in an adequate way. As the enzymatic conversion of polymers to monosugars is typically performed at 50 °C and pH 4.5 (Vohra et al. 2014), the use of fermentative mesophiles either means operating at a "trade-off" temperature compromising either the efficiency of the enzyme or growth at a non-optimum temperature. While the thermotolerant yeast *Kluyveromyces marxianus* has broad substrate spectra, the wild type cannot utilize cellobiose limiting its usefulness as the ethanologenic organism in SSF applications. Moderate thermophiles may be a very good option under these conditions as they fall within the operating temperature range of currently available enzymes. A recent SSF study using *Thermoanaerobacterium saccharolyticum* ALK2 grown on avicel at a

concentration of 20 g/L found that the enzyme systems used were dramatically inhibited by anaerobic conditions and high ethanol titers (Bhalla et al. 2013).

2.5.3.3 Consolidated Bioprocessing

Consolidated bioprocessing has recently been developed where all steps of bioethanol production occur in a single vessel by either a single organism or a co-culture as first proposed in 1996 (Lynd 1996). Here, the enzymes used in saccharification of biomass are produced in situ in the fermentation vessel leading to lower cost and high efficiencies (Lynd et al. 2002). Being a one-step process makes the choice of microorganism of great importance. The microorganism must have enzymatic machinery to produce both a variety of hemicellulases and cellulases as well as being a good ethanol producer (Lynd et al. 2005). Presently, there is no wild-type microorganism that possesses all these desired properties. Several attempts have been made to genetically modify microorganisms for use in CBP processes, using mainly two strategies, CBP I and CBP II (Xu et al. 2009). In CBP I the microorganism uses its native ability to degrade lignocellulose and is then further engineered to become a powerful ethanol producer. In the CBP category II, however, the aim is to genetically engineer a microorganism that is a good ethanol producer but does not have the ability to produce the enzymes needed for the biomass breakdown (Xu et al. 2009).

2.6 Evolutionary Adaptation and Genetic Engineering of Thermophiles

To date, no single organism meets all the requirements needed for highly efficient ethanol production from lignocellulosic biomass. There are two general strategies of enhancing ethanol production by wild-type microorganisms: evolutionary adaptation of natural strains and genetic modification. Historically, classical methods such as the clone selection and nonspecific mutagenesis were used to improve ethanol production in thermophiles. These methods can be time-consuming (Lynd et al. 2002). Genetic modification has been used more in recent times but is however not without drawbacks, as modified strains can exhibit poor growth and unexpected shifts in end-product formation.

2.6.1 Evolutionary Adaptation

The use of classical evolutionary adaptation methods, such as nonspecific mutagenesis and artificial selection, to enhance specific traits of microorganisms for industrial

bioethanol production has been applied to thermophilic anaerobes on a limited basis. As previously mentioned, one of the major drawbacks of working with thermophilic anaerobes is their low substrate and ethanol tolerance compared to their mesophilic counterparts.

Three new strains of *Thermoanaerobacter ethanolicus* were obtained by selection of pyruvate and iron deprivation (He et al. 2009) leading to enhanced ethanol tolerance (10% v/v) at substrate concentrations above 10 g/L. Recent investigation on *Clostridium thermocellum* showed increased ethanol tolerance (up to 5% v/v) by stepwise increasing and transferring cultures to increased ethanol concentrations (Shao et al. 2011). While adapting mesophilic strains to high substrate concentrations as well as to inhibitory substances are quite common, there are few examples of these techniques being used for thermoanaerobes. *Thermoanaerobacter pentosaceus* has though been gradually adapted to higher substrate concentrations and demonstrated higher ethanol tolerance and substrate utilization (Sittijunda et al. 2013). Thus, evolutionary adaptation may still be used for the evolving of wild-type strains with enhanced tolerance to high ethanol titers, high substrate concentrations, and increased concentrations of 5-HMF and furfuraldehyde.

2.6.2 Genetic Engineering

The main drawback of thermophiles is their production of mixed end products resulting in lower ethanol yields and the fact that highly ethanologenic organisms are not natively cellulolytic and vice versa. Efforts to metabolically engineer thermophilic organisms for consolidated bioprocessing (CBP) have focused on two strategies: increasing the ethanol yields of cellulase-producing organisms and expressing cellulases in highly ethanologenic organisms (Shaw et al. 2009). The first approach involves increasing ethanol yields by eliminating other fermentation products and improving ethanol tolerance, whereas the second approach involves addition of cellulolytic genes to the genome of a good ethanol-producing bacterium.

The first thermophile to be genetically modified in order to increase its ethanol production was *Thermoanaerobacterium saccharolyticum* (Desai et al. 2004). In the past 10 years, several other thermophiles have been genetically modified in an effort to increase ethanol titers and minimize the formation of other end products such as acetate and lactate (Table 2.3).

Deletion of genes responsible for production of other end products may increase ethanol production capacity. Lactate dehydrogenase has been knocked out in several thermophiles, e.g., *Thermoanaerobacterium saccharolyticum* (Shaw et al. 2008, 2010), *Thermoanaerobacter mathranii* (Yao and Mikkelsen 2010a, b), *Clostridium thermocellum* (Argyros et al. 2011), and *Geobacillus thermoglucosidasius* (Cripps et al. 2009).

Clostridium thermocellum is both cellulolytic and a good ethanol producer and has thus been investigated intensively (in this area). The first successful transformation of the species was performed in 2006 (Tyurin et al. 2006), leading to the

Table 2.3 Ethanol yields of genetically engineered thermophilic bacteria from different substrates and fermentation conditions

Strain	Genotype	Substrate type	Substrate conc. (g/L)	Mode	Ethanol yields (mol/mol hexose)	References
C. Thermocellum	ΔpyrF, Δpta::gapDHp-cat	Ce	5.0	Batch	0.59	Tripathi et al. (2010)
C. Thermocellum	ΔpyrF, Δpta::gapDHp-cat	Av	5.0	Batch	0.71	Tripathi et al. (2010)
C. thermocellum adhE* (EA) Δldh	Δhpt, Δldh	Ce	5.0	Batch	0.37	Biswas et al. (2014)
C. Thermocellum	Δhpt, Δldh, Δpta (evolved)	Av	19.5	Batch	1.08	Biswas et al. (2014)
C. thermocellum/ *T. saccharolyticum*	Δhpt, Δldh, Δpta (evolved) and Δpta, ΔAK, Δldh	Av	19.5	Batch	1.26	Biswas et al. (2014)
T. saccharolyticum TD1	Δldh	X	5.0	Batch	0.98	Biswas et al. (2014)
T. saccharolyticum ALK2	Δpta, ΔAK, Δldh	Ce	70.0	Con	ND	Shaw et al. (2008)
T. saccharolyticum HK07	Δldh, Δhfs	Ce	1.8	Batch	0.86	Shaw et al. (2009)
T. saccharolyticum M0355	Δldh, ΔAK Δpta	Ce	50.0	Batch	1.73	Argyros et al. (2011)
T. saccharolyticum M1051	Δldh, ΔAK Δpta, ureABCDEFG	Ce	27.5	Batch	1.73	Shaw et al. (2009)
G. thermoglucosidasius TM242	Δldh-, pdh up, pflB-	G	34.0	Batch	1.73	Cripps et al. (2009)
G. thermoglucosidasius TM242	Δldh-, pdh up, ΔpflB-	G	34.0	Batch	1.84	Cripps et al. (2009)
G. thermoglucosidasius TM242	Δldh-, Δpdh up, ΔpflB-	X	29.0	Batch	1.37	Cripps et al. (2009)
T. mathranii BG1L1	Δldh	WS	30–120	Con	1.53–1.67	Georgieva et al. (2008)
T. mathranii BG1G1	Δldh, GldA	G + gly	5.0	Batch	1.68	Yao and Mikkelsen (2010a)

(continued)

Table 2.3 (continued)

Strain	Genotype	Substrate type	Substrate conc. (g/L)	Mode	Ethanol yields (mol/mol hexose)	References
T. mathranii BG1G1	Δldh, GldA	X + gly	5.0	Batch	1.57	Yao and Mikkelsen (2010a)
T. mathranii BG1G1	Δldh, GldA	X + gly	12.8 and 7.2	Con	1.53	Yao and Mikkelsen (2010a)
Thermoanaerobacter Pentocrobe 411	Δldh, ΔAK Δpta	WS	65.0	Con	1.84	Andersen et al. (2015)
Thermoanaerobacter Pentocrobe 411	Δldh, ΔAK Δpta	OP	130.0	Con	1.92	Andersen et al. (2015)

AK acetate kinase, *GldA* glycerol dehydrogenase A, *hfs* hydrogenase, *hpt* hypoxanthine phosphoribosyl transferase, *pdh* pyruvate decarboxylase, *pyrF* orotidine-5-phoshate decarboxylase, *pfl* pyruvate formate lyase, *ure* urease, *Ce* cellobiose, *Av* avicel, *X* xylose, *G* glucose, *Gly* glycerol, *WS* wheat straw, *OP* oil palm frond. Data from Scully and Orlygsson (2015), Asterisk denote mutant

development of genetic systems to knock out the PTA gene and thus acetate formation (Argyros et al. 2011). This modified strain however grew abnormally but retained its cellulase activity. More recent work with *C. thermocellum* showed improved ethanol yields in a Δhpt, Δldh, and Δpta evolved strain as well as successful use of co-culture of this strain with *Thermoanaerobacterium saccharolyticum* (Argyros et al. 2011).

Early work on *Thermoanaerobacterium saccharolyticum* involved electroporation and the use of shuttle vectors (Mai et al. 1997). Later work involved insertion of a cellobiohydrolase gene from *Clostridium thermocellum* into its genome (Mai and Wiegel 2000), a LDH gene knockout (Desai et al. 2004), and a double knockout of both LDH and AK (Shaw et al. 2008). By knocking out acetate formation, ethanol formation was increased, but at the same time, the strain gained less energy and thus build up less cell biomass, from both glucose and xylose. Another double knockout of *Thermoanaerobacterium saccharolyticum* focuses on the electron transfer system of the bacterium. When the hfs and ldh gene clusters, which encode for hydrogenase and LDH, respectively, a considerable increase in ethanol (44%) production was obtained as compared with the wild type (Shaw et al. 2009).

Within the genus *Thermoanaerobacter*, *T. mathranii* has undergone most genetic modification. The first mutant was generated, by knocking out the ldh gene (strain BG1L1). This strain showed more than a twofold increase in ethanol production as compared with the wild type, up to 1.52 mol ethanol/mol xylose (Sommer et al. 2004). Further modifications of this strain have involved the overexpression of a NAD(P)H-dependent bi-functional aldehyde/ADH protein, resulting in the strain BG1E1. Clearly, this enzyme is of great importance for ethanol production, and its overexpression resulted in higher ethanol yields (Yao and Mikkelsen 2010a). The electron balance for sugar degradation was additionally focused upon with this strain when mannitol, which is more reduced than glucose and xylose, was used as a substrate leading to more ethanol production (Yao and Mikkelsen 2010b). One more mutant of *Thermoanaerobacter mathranii* was finally developed, BG1G1 where the gene encoding for NAD^+-dependent glycerol dehydrogenase was inserted. This increased ethanol production by 40% as compared with the wild type. Additionally, the strain utilized the highly reduced glycerol and expressed co-metabolism of glycerol and sugars.

A recent paper using a lactate dehydrogenase, phosphotransacetylase, and acetate kinase knockout strain of *Thermoanaerobacter* BG1, dubbed Pentocrobe 411 (DSM 23015), was recently described by Andersen et al. (2015). Pentocrobe 411 achieved impressive ethanol titers (1.84–1.92 mol ethanol/mol sugar equivalent) nearing the maximum theoretical yield from hexoses and pentoses on various pretreated biomass in continuous culture.

Thermophilic bacteria within the genus of *Geobacillus* have also attracted increased interest due to their ethanol production capacity. These bacteria are facultative anaerobes and can ferment various sugars to pyruvate by pyruvate dehydrogenase to acetyl coenzyme A (Cripps et al. 2009). Under aerobic conditions, however, pyruvate formate lyase is used and a variety of end products are formed. A research group led by Cripps (Cripps et al. 2009) manipulated *Geobacillus thermoglucosidasius*, obtaining an

upregulated expression of pyruvate dehydrogenase under anaerobic conditions in a lactate dehydrogenase-inactivated strain. Several mutants were developed (TM89; ldh knockout; TM180; ldh knockout and upregulated pdh; TM242; ldh, pdh up, and pfl). The TM180 strain produced 1.45 mol ethanol/mol hexose (the wild type produced 0.39 mol ethanol/mol hexose and TM89 0.94 mol ethanol/mol hexose). The triple mutant TM242 produced 1.65 mol ethanol/mol hexose. This mutant also showed good yields on xylose (1.33 mol/mol) and good productivity rates. *Geobacillus thermoglucosidasius* has recently been genetically modified by expressing pyruvate decarboxylase from *Gluconobacter oxydans* with ethanol yields as high as 1.37 mol ethanol/mol glucose (van Zyl et al. 2014).

Overall, efforts to engineer thermophilic anaerobes to increase ethanol titers have resulted in modest gains in yields while minimizing or eliminating the formation of unwanted end products. Future targets for genetic manipulation might include the inclusion of the cellulolytic machinery of *C. thermocellum* into highly ethanologenic *Thermoanaerobacter* and *Thermoanaerobacterium* strains.

2.7 Conclusions

Ethanol production from heterogenic lignocellulosic biomass requires robust microorganisms with complementary pretreatment strategies. Thermophiles have broad substrate spectra and can degrade both hexoses and pentoses simultaneously. Some thermophiles natively degrade complex carbohydrates and operate at temperatures that minimize contamination risk of mesophiles. Additionally, recent advances in genetic engineering have improved ethanol yields, often by knocking out metabolic pathways affecting end-product formation. However, no large-scale bioethanol plants with genetically modified microbes are currently operating. Ethanol yield and tolerance toward inhibitory compounds are lower as compared to mesophiles; thermophiles are more sensitive toward various environmental variations, especially partial pressure of hydrogen, high substrate concentrations, and ethanol tolerance.

From the process point of view, the lack of compatible enzyme systems for consolidated processes such as SSF and CBP remains an issue. Most commercially available enzymes have temperature optima between room temperature and 50 °C, and commercially available cellulases are inhibited by anaerobic conditions and by ethanol. This emphasizes that there is an urgent need for enzyme systems that are compatible with an oxygen-free environment so that thermophilic consolidated bioprocesses become a feasible alternative.

Take-Home Messages

1. Ethanol production with thermophiles may be more feasible on lignocellulosic biomass as compared with yeast and *Zymomonas mobilis.*
2. Thermophiles have much broader substrate spectrum compared with yeast and *Zymomonas mobilis*.
3. Variety of end products is however common feature with thermophilic bacteria.

4. Genetic engineering has been widely used for thermophilic bacteria, mainly making them homoethanolgenic.
5. Consolidated bioprocesses have been used for thermophilic bacteria with some success.

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Chapter 3
Biobutanol Production Using Recombinant Microorganisms

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What Will You Learn from This Chapter?
This chapter reviews microbial biobutanol production and discusses the possibilities, remaining challenges, and prospects of biobutanol. The chapter also discusses the use of lignocellulosic biomass and development of mutant strains having higher butanol yield, selectivity, and tolerance to inhibition.

3.1 Introduction

Petroleum is a finite resource and eventually will be depleted (Bentley 2002). At the same time, global energy consumption is projected to increase by up to 50% by 2025 (Ragauskas et al. 2006). However, the current oil reserves are estimated to run out within the next 50 years (Arifin et al. 2014). Therefore, the production of fuels from renewable resources such as lignocellulosic biomass is vital to secure our energy needs. For example, the United States annually generates more than 1 billion tons of lignocellulose that could be used to produce 80–100 billion gallons of renewable fuels, or 70–80% of current gasoline

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demand. In the past decade, biofuels have become a promising solution to solve the energy security, environmental, and economic challenges connected with petroleum need.

One of the most widely used biofuels is ethanol which is biochemically produced from carbohydrates (Fortman et al. 2008). Global ethanol production is reaching 1.5 million barrel per day (http://www.eia.gov/). However, there are several drawbacks of ethanol, such as its energy content being only 70% the energy of gasoline. Also, reconstruction of new pipelines and storage infrastructure is needed as ethanol is hygroscopic and corrosive (Arifin et al. 2014). Butanol (C_4H_{10}) is a biofuel that has several advantages over ethanol. As a biofuel, biobutanol shows even better potential than bioethanol in the transportation industry as it contains 30% more energy (per volume) than bioethanol (Lee et al. 2008). Additionally, its use as a blend with gasoline and diesel does not require any modification to existing vehicles (Demain 2009). Furthermore, butanol heat of vaporization is only half of ethanol's heat of vaporization. Consequently, the engine running on butanol is easier to start during cold weather than the one running on ethanol (Arifin et al. 2014). Butanol also offers the potential to be upgraded to aviation jet fuel, a product generally not associated with biofuels (www.greenbiologics.com). Butanol fermentation can use the same feedstocks as bioethanol and can be transported through existing infrastructure (Bibra et al. 2014). A difficulty in butanol fermentation is the inhibition caused by the product as butanol concentrations around 20 g/L inhibit microbial growth (Knoshaug and Zhang 2009). In addition, the *Clostridium* species used for fermentation are strictly anaerobes (Wang et al. 2010), and the anaerobic conditions need to be established before the beginning of the fermentation and the reactor must be remain closed during the process (Samsuri et al. 2009). This leads to increased downstream separation costs and larger production equipment. Using extremophiles or their genes in the production of butanol could mitigate the abovementioned problems.

3.2 History of Biobutanol Production

The formation of butanol in the microbial fermentation was reported first by Louis Pasteur in 1861 (Gabriel and Crawford 1930). The microorganism *Clostridia acetobutylicum* used for fermentation was first isolated by Weizman (Patent US1315585), a student of Louis Pasteur. Industrial production of butanol began in 1916. The so-called ABE fermentation (acetone/butanol/ethanol, 3:6:1) became the second largest biotechnological process ever performed. It is beaten in volume only by ethanol fermentation, usually performed in much smaller facilities. ABE fermentation process was used up to 1920 exclusively for producing acetone intended for manufacturing smokeless powder cordite during the time of First World War. However, at the fermentation, the yield of each pound of acetone was accompanied by formation of two pounds of butanol. In the 1920s nitrocellulose was mixed with butanol which yielded a drying lacquer. Three years later, automobile industry changed the whole market completely, and till 1927 butanol became the main product of ABE process. The economics of the biobutanol production are largely dependent on the cost of the fermentation substrate. For biobutanol to have a meaningful impact as an

alternative fuel, the biomass feedstock must be widely available at low cost (Lynd et al. 1999). Effective bioconversion of the feedstock and having the lowest possible environmental impact in its use and production are also key issues. In many countries, there is also ongoing debate about the use of food crops for biofuel production. As a result, lignocellulose biomass has been identified as the most suitable feedstock for biofuel production since it consists of approximately 75% polysaccharide sugars (Bayer et al. 2007; Lynd et al. 1991). Sources of lignocellulose include agricultural waste such as corn stover, bagasse, wood, grass, municipal waste, and dedicated energy crops such as miscanthus and switch grass (Bibra et al. 2014; Gomez et al. 2008).

3.3 Fermentative Production of Butanol

Traditionally, an ABE process is used for biobutanol production. This is one-stage batch process which uses *clostridium* strain (generally *C. beijerinckii or C. acetobutylicum*) to produce solvents. First, fermentation produces a mixture of butyric, lactic, and acetic acid. Later, the culture pH drops and butanol, acetone, and ethanol are produced in a 6:3:1 by mass ratio, respectively. Typically, the total concentration of solvents (acetone, butanol, and ethanol) in ABE fermentation broth is 20 g/L (ratio of butanol, acetone, and ethanol is 6:3:1) with butanol around 13 g/L (Ezeji et al. 2004a, b; Westhuizen et al. 1982).

3.3.1 Biomass Selection for Butanol Production

On the basis of utilization of feedstocks, biofuels including biobutanol were classified into first-generation and second-generation biofuels. In the first-generation biofuels, raw materials were sugarcane and cereal grains, while in the second-generation biofuel, lignocellulosic materials (e.g., agriculture and forest wastes) were used as feedstocks. Traditional attempts, utilizing cereal grains and sugar as feedstocks in ABE fermentation for industrial-scale production, were encouraged by the availability of these raw materials and vast requirement of fermentation products (Durre 1998). However, utilization of these substrates was criticized as "price hikers" as well as contributing to food shortages except in sugarcane- and cereal grain-rich countries like Brazil (Dürre 2007; Zhang et al. 2010; Yan et al. 2009). This concern forced to explore inexpensive and non-food competitive raw materials for ABE fermentation. Inexpensive and renewable feedstocks, such as lignocellulosic materials, can decrease the production cost of butanol. The major sources of this kind of raw material are agricultural residues and wastes such as rice straw, wheat straw, wood (hardwood), by-products left over from the corn milling process (corn fiber), annual and perennial crops, waste paper (Qureshi et al. 2007), and sweet sorghum (Whitfield et al. 2012).

These raw materials consist of three types of polymers: cellulose, hemicellulose, and lignin. *Clostridium beijerinckii* is being explored as a promising strain to produce biobutanol from cellulosic materials (Kumar and Gayen 2011).

Lignocellulosic materials require additional pretreatment before hydrolysis and fermentation. This is because of the complex structure of lignocelluloses including cellulose crystallinity, cellulose protection by lignin, and low accessibility of enzymes to the lignocellulosic compounds. The sheathing by hemicelluloses and the degree of hemicellulose acetylation additionally hinder the biomass processing (Hsu 1996). Pretreatment alters the structure of cellulosic biomass so as to improve its ability to form sugars by hydrolysis. This is achieved by breaking the lignin seal, removing lignin and hemicellulose, or increasing the porosity of the biomass (Mosier et al. 2005; Sun and Cheng 2002). It is crucial that the selected pretreatment method is cost-efficient as it is one of the most costly steps of the conversion process from biomass to fermentable sugars. Pretreatment should improve the yield of the formation of fermentable sugars while avoiding the degradation or loss of carbohydrates and the formation of inhibitors for subsequent hydrolysis and fermentation processes (Garcia et al. 2011).

3.3.2 Fermentation Techniques

Traditionally, batch fermentations were commonly used for butanol production. During the 1940s and 1950s, biobutanol production on an industrial scale (Terre Haute, IN, and Peoria, IL) was carried out using large batch fomenters ranging in capacity from 200,000 to 800,000 L. The industrial process used 8–10% corn mash, which was cooked for 90 min at 130–133 °C. Sugarcane molasses was also used to produce biobutanol in a commercial plant in South Africa until the early 1980s. The main difficulty in butanol fermentation is the inhibition caused by the product as butanol concentrations around 20 g/L inhibit microbial growth (Knoshaug and Zhang 2009). Product toxicity results in low butanol concentration in the reactor. In addition, productivity in batch reactors is often low due to downtime and long lag phase. While downtime and lag phase can be eliminated using a continuous culture, the problem of product inhibition remains. This problem can be eliminated by the application of novel product removal techniques. In addition to continuous culture, fed-batch techniques can also be applied to the fermentation process. Fed-batch fermentation is an industrial technique, which is applied to processes where a high substrate concentration is toxic to the culture. In such cases, the reactor is initiated in a batch mode with a low substrate concentration (non-inhibitory to the culture) and a low-medium volume, usually less than half the volume of the fermenter. As the substrate is used by the culture, it is replaced by adding a concentrated substrate solution at a slow rate, thereby keeping the substrate concentration in the fermenter below the toxic level for the culture. In this type of system, the culture volume increases in the reactor over time. The culture is harvested when the liquid volume is approximately 75% of the volume of the reactor. Since butanol is toxic to bacterial cells, the fed-batch fermentation technique cannot be applied unless one of the novel product recovery techniques is applied for simultaneous separation of the product. As a result of substrate reduction and reduced product inhibition, greater cell growth occurs and reactor productivity is improved. This process was employed for butanol

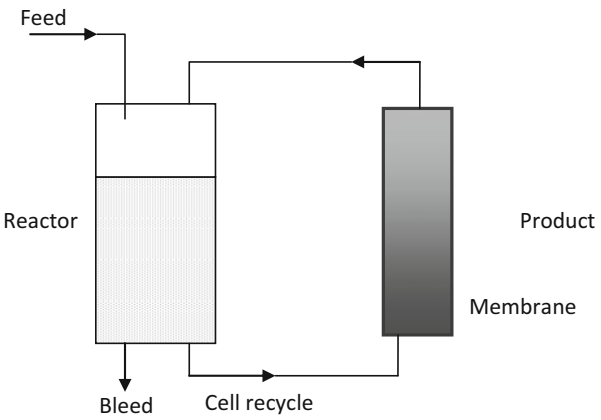

Fig. 3.1 Membrane cell recycle reactor (redrawn from Ezeji et al. 2004a, b)

fermentation using *C. beijerinckii* where a glucose solution containing 500 g/L glucose was fed and product was recovered by gas stripping or pervaporation.

The cell concentration inside the bioreactor can be increased by one of two techniques, namely, "immobilization" or "cell recycle." In a study to explore different cell supports (e.g., clay brick) for *C. beijerinckii* cells, Qureshi et al. (2005) were able to improve reactor productivity to 15.8 g/L/h. In another approach, Huang et al. (2004) immobilized cells of *C. acetobutylicum* in a fibrous support and used these in a continuous reactor to produce ABE; a productivity of 4.6 g/L/h was obtained. Cell recycle is where cells are returned to the bioreactor using a filter and clear liquid is removed and, thus, can also be used to increase cell concentration in the reactor and to improve reactor productivity. Membrane cell recycle reactors are another option for improving reactor productivity (Mehaia and Cheryan 1986). In such systems, the reactor is initiated in a batch mode and cell growth is allowed. Before reaching the stationary phase, the fermentation broth is circulated through the membrane. The membrane allows the aqueous product solution to pass while retaining the cells. The reactor feed and product (permeate) removal are continuous and a constant volume is maintained in the reactor (Fig. 3.1). In such cell recycle systems, cell concentrations of over 100 g/L can be achieved. However, to keep the cells productive, a small bleed should be withdrawn (<10% of dilution rate) from the reactor. Some investigators have employed this technique, and reactor productivity on the order of 6.5 g/L/h has been achieved in these systems (Afschar et al. 1985; Pierrot et al. 1986). Although superior membranes have been developed, fouling of the membrane with the fermentation broth remains a major obstacle (Ezeji et al. 2004a, b).

A flash fermentation technology was proposed by Mariano et al. (2009) to overcome the low productivity hurdle in synthesizing butanol. This innovative technology consisted of three interconnected units, viz., fermenter, cell retention system, and vacuum flash vessel (for continuous recovery of butanol from broth). The positive aspects about the flash fermentation process are the solvent productivity, the use of concentrated sugar solution, and the final butanol concentration. The last two features can be responsible for a meaningful reduction in the distillation

costs and result in environmental benefits due to lower quantities of wastewater generated by the process.

3.3.3 *Fermentation Pathways and Enzymes Involved*

Fermentation depends on the level of metabolic activities of the organism. Therefore, understanding of metabolic network of an organism is essential to engineer the strain leading better productivity. Insights of the metabolic pathway and the metabolic network analysis are key steps for metabolic engineering (Kumar and Gayen 2011). It was observed that *C. acetobutylicum* typically performs standard butyric acid fermentation, with acetate, butyrate, CO_2, and H_2 as major products. About twice as much butyrate is produced compared to acetate. At the end of the exponential growth, a major metabolic switch takes place in *C. acetobutylicum*. The organism slows down acid production and takes up excreted acetate and butyrate and converts them into the solvents acetone and butanol (approximately two times more butanol than acetone).

The first enzyme, required for solventogenesis, is an acetoacetyl-CoA:acetate/butyrate-coenzyme A transferase (CoA transferase, CtfA/B) that converts butyrate and to a lesser extent also acetate into butyryl-CoA and acetyl-CoA. The latter is recycled to acetoacetyl-CoA, the starting point of the reaction (Fig. 3.2).

Enzyme thiolase (ThlA) is also needed for butyrate formation. This has implications for the regulation of its gene. Acetoacetate, one of the products of the CoA transferase action, is converted into acetone and CO_2 by acetoacetate decarboxylase (Adc), a reaction required to "pull" the thermodynamically unfavorable butyryl-CoA formation. This can also be achieved using different substrates, resulting in

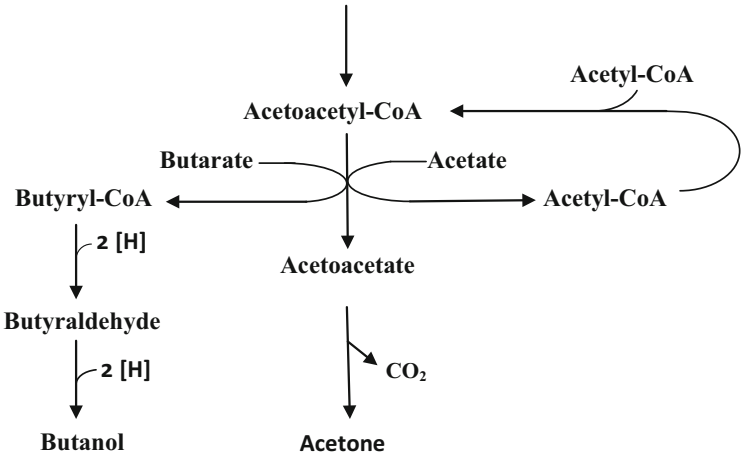

Fig. 3.2 Schematic diagram of solventogenesis (redrawn from Dürre 2007)

massively increased butanol production over acetone (Bahl et al. 1982). Finally, butyryl-CoA is reduced to butyraldehyde and butanol by numerous enzymes. AdhE is a bifunctional butyraldehyde/butanol dehydrogenase and catalyzes initiation of solventogenesis, whereas BdhB, a butanol dehydrogenase, takes over later and is responsible for the enormous production. BdhA is an alcohol dehydrogenase that uses acetaldehyde and butyraldehyde almost equally well (Welch et al. 1989), assuming its physiological role to be just a sink for surplus reducing equivalents and resulting in minor ethanol and butanol formation. AdhE2 is another bifunctional butyraldehyde/butanol dehydrogenase that is only formed when *C. acetobutylicum* is growing on reduced substrates (e.g., a mixture of glucose and glycerol), which leads to a so-called alcohologenic fermentation (only butanol and ethanol are formed, but no acetone) (Girbal and Soucaille 1998; Fontaine et al. 2002).

3.4 Microorganisms in the Production of Biobutanol

A large variety of clostridial cultures capable of producing acetone, butanol, isopropanol, and ethanol, with varying yield and proportions, have been known for more than half century (Beesch 1952). These strains were able to utilize 7.5% fermentable sugar with solvent concentrations in the range 18–23 g/L and a yield of 30–33% (Table 3.1). Hongo (1960) patented cultures that produced up to 70% butanol. George et al. (1983) published a comparative study of as many as 34 strains representing 15 species of *Clostridia* for production of acetone, butanol, isopropanol, and ethanol. The principal species that produced solvent in reasonable amounts (>1 mmol/L) in a peptone, yeast extract, and glucose medium were *C. beijerinckii*, *C. butylicum*, *C. acetobutylicum*, *C. aurantibutyricum*, *C. pasteurianum*, *C. sporogenes*, and *C. cadaveris*. The strain *C. aurantibutyricum* produces acetone, butanol, and isopropanol, whereas *C. tetanomorphum* produces equal amount of ethanol and butanol. The industrial strains of clostridia were categorized among four species, namely, *C. acetobutylicum*, *C. beijerinckii* (formerly *C. butylicum*), *C. saccharoperbutylacetonicum*, and *C. saccharobutylicum* (Keis et al. 1995; Jones and Keis 1995; Johnson and Chen 1995; Johnson et al. 1997). Another related species of industrial solvent-producing clostridia is *C. pasteurianum*. This species was initially known as an acid producer that fermented carbohydrates to butyrate, acetate, CO_2, and H_2 (Gottschalk 1986). However, Harris et al. (1986) reported that this species also could produce significant quantities of acetone, butanol, and ethanol, when grown in media of high glucose content.

Clostridium ABE fermentation is considered to be the main process of biobutanol production; however, the low butanol yield as well as the low product tolerance of these organisms is forcing researchers to look at alternative routes, including solvent-tolerant organisms (Bibra et al. 2014). Most microorganisms are unable to grow at butanol concentrations above 2% (Knoshaug and Zhang 2009; Barnard et al. 2010). However, there are certain organisms, such as certain species of *Bacillus*, that

Table 3.1 Butanol production by clostridial cultures on different substrates and fermentation processes

Microorganism	Fermentation process	Substrate	Maximum titer of ABE (g/l)	Reference
C. beijerinckii P260	Batch	Barley straw	26.64	Qureshi et al. (2010)
C. beijerinckii P260	Batch	Wheat straw	21.42	Qureshi et al. (2008a, b)
C. beijerinckii P260	Batch	Corn stover and switch grass	21.06	Qureshi et al. (2010)
C. beijerinckii P260	Batch	Switch grass	14.61	Qureshi et al. (2010)
C. beijerinckii P260	Fed-batch	Wheat straw	16.59	Qureshi et al. (2008a, b)
C. beijerinckii BA101	Fed-batch	Glucose	25.3	Qureshi and Blaschek (2000)
C. beijerinckii BA101	Continuous	Synthetic medium	8.8	Qureshi et al. (2004)
C. beijerinckii BA101	Batch	Corn fibers	9.3	Qureshi et al. (2008a, b)
C. beijerinckii BA101	Batch	Glucose	17.7	Ezeji et al. (2003)
C. beijerinckii BA101	Fed-batch	Glucose	17.7	Ezeji et al. (2004a, b)
C. beijerinckii BA101	Continuous	Starch and glucose	9.9	Ezeji et al. (2005)
C. beijerinckii BA101	Batch	Liquefied corn starch	18.4	Ezeji et al. (2007)
C. beijerinckii BA101	Continuous	Degermed corn	14.28	Ezeji et al. (2007)
C. acetobutylicum P262	Continuous	Whey permeate	8.6	Qureshi and Maddox (1995)
C. acetobutylicum P262	Batch	Whey permeate	7.72	Qureshi et al. (2005)
C. acetobutylicum P262	Fed-batch	Whey permeate	7.72	Qureshi et al. (2005)
C. acetobutylicum P262	batch	Whey permeate	8.7	Maddox et al. (1995)
C. acetobutylicum P262	Continuous	Defidered-sweet-potato slurry	7.73	Badr et al. (2001)
C. acetobutylicum 824A	Continuous	Lactose and yeast extract	1.43	Napoli et al. (2010)
C. acetobutylicum ATCC 55025	Continuous	Corn	12.50	Huang et al. (2004)

(continued)

Table 3.1 (continued)

Microorganism	Fermentation process	Substrate	Maximum titer of ABE (g/l)	Reference
C. acetobutylicum ATCC 824	Fed-batch	Glucose	–	Qureshi et al. (2001)
C. acetobutylicum DSM 1731	Fed-batch	Potato wastes	19	Grobben et al. (1993)
C. saccharobutylicum DSM 13864	Continuous	Sago starch	9.1	Liew et al. (2005)
C. saccharobutylicum N1	Fed-batch	Synthetic medium with butyric acid	16.0	Tashiro et al. (2004)

are able to tolerate butanol concentrations as high as 2.5–7% (Sardessai and Bhosle 2002). Gram-negative bacteria, namely, certain strains of *Pseudomonas* and some *E. coli* mutants, have devised various novel adaptive mechanisms which enable them to thrive in the presence of supersaturating amounts of toxic organic solvents. These adaptive mechanisms mainly consist of modifications in cell envelope so as to increase cell membrane rigidity and decrease permeability, by increasing rate of membrane repair enzymes, secreting special solvent-inactivating enzymes, having active efflux of solvents by means of solvent efflux pumps (*tol C/mar/rob/sox S/acr AB* genes), and releasing of membrane vesicles with solvent molecules adhering to it. However, there is a large void in the available data on such mechanisms in Gram-positive bacteria (Sardessai and Bhosle 2002). It is believed that organic solvent emulsifying/deactivating/solubilizing enzymes/substances could play a very important role in diminishing solvent toxicity in Gram-positive bacteria (Abe et al. 1995). There are reports where *Pseudomonas* achieves high solvent tolerance by removal of solvent using efflux pumps and physicochemical changes of their membrane lipids (Segura et al. 2004; Ramos-González et al. 2002). The *P. putida* S12 has inherent moderate tolerance to butanol (De Carvalho et al. 2004), while other *P. putida* strains have evolved to tolerate 6% w/v butanol (Ruhl et al. 2009). This fact and the recent engineering of *P. putida* to produce butanol (Nielsen et al. 2009) open up a new field of research for the production of butanol from solvent-tolerant organisms.

3.5 Recombinant Microorganisms for Improvement of Biobutanol Production

Low tolerance to higher concentration of butanol is one of the main drawbacks of the butanol-producing microorganisms. It was found that majority of microbial strains, including *Clostridia*, were not able to tolerate 2% of butanol (Gottschal and Morris 1981). Hermann et al. (1988) and Jain et al. (1993) used mutagenesis approach to

resolve butanol tolerance issue by getting mutant with higher butanol resistance. Tomas et al. (2003) reported that in *C. acetobutylicum*, overexpression of groESL, a class I heat shock protein gene, can increase butanol tolerance and found the final solvent titer 40% higher than that of the wild type. A butanol production process by the fermentation of *C. beijerinckii* BA101, a hyperamylolytic and higher butanol-tolerant mutant strain, isolated by treating the parent strain chemically with nitrosamines such as MNNG (N-methyl-N′-nitro-N-nitrosoguanidine) produced 18–21 g/L of butanol which was almost double the amount produced by the parent strain (Blaschek et al. 2002; Annous and Blaschek 1990). Inactivation of sporulating protein (SpoIIE) and keeping the cells into vegetative phase for improved solvent production was also tried in *C. acetobutylicum* and was quite successful (Desai and Papoutsakis 1999; Bennett and Scotcher 2007).

For large-scale production of butanol, it is very difficult to operate fermentation under anaerobic conditions, thus limiting the commercial applications of *Clostridium* strains. Anaerobic fermentations generally result in low biomass formation due to low ATP gain. Continuous culture conditions, butanol toxicity, and serial subculturing also cause *Clostridium* species to lose their ability to produce high amount of butanol. Due to these inherent drawbacks, researches have been directed toward the use of other microbial strains which are not a natural producer of biobutanol but can have a potential after some genetic modifications (Table 3.2). *Escherichia coli*, *Saccharomyces cerevisiae*, and *B. subtilis* are the most well-studied microbial strains that can potentially induce butanol production (Kharkwal et al. 2009). It was reported that highly concentrated *E. coli* cells, modified by the expression of genes, *thiL*, *hbd*, *crt*, *bcd-etfB-etfA*, and *adhe1* (or *adhe*), coding for acetyl-CoA acetyltransferase (THL), β-hydroxybutyryl-CoA dehydrogenase (HBD), 3-hydroxybutyryl-CoA dehydratase (CRT), butyryl-CoA dehydrogenase (BCD),

Table 3.2 Potential microbial strains other than *clostridium* producing biobutanol (table modified from Kharkwal et al. 2009)

Microbe	Comments	References
E. coli	Butanol produced by transforming the genes for the enzymes catalyzing butanol metabolic pathway of *Clostridium*	Feist et al. (2007), Inui et al. (2008), Shen and Liao (2008), Atsumi and Liao (2008), Atsumi et al. (2008), Atsumi et al. (2008), Papoutsakis et al. (2008), Lee et al. (2008), Nielsen et al. (2009)
Lactobacillus sp.	Reported as a capable butanol-producing strain	Donaldson et al. (2008)
P. putida	Reported as capable butanol-producing strain	Donaldson et al. (2008), Puchalka et al. (2008)
S. cerevisiae	Butanol production by transforming the genes for the enzymes catalyzing butanol metabolic	Sikorski and Hieter (1989), Donaldson et al. (2008), Madeliene et al. (2008), Uvini et al. (2008), Steen et al. (2008), Nookeaw et al. (2008)
K. lactis	Butanol production reported	Madeliene et al. (2008)
Pichia sp.	Good for ethanol production. Reported as a capable candidate for butanol production	Donaldson et al. (2008), Madeliene et al. (2008)

butyraldehyde dehydrogenase (BYDH), and butanol dehydrogenase (BDH), six different enzymes of butanol synthetic pathway, showed production of butanol with a concentration of 16 mM (Inui et al. 2008). Papoutsakis et al. (2008) developed a novel method for producing butanol from a recombinant *E. coli* strain by generating butyryl-CoA initially as an intermediate and then converting butyryl-CoA to butanol. The citramalate pathway directly converts pyruvate to 2-ketobutyrate bypassing threonine synthesis. This technology whereby *E. coli* is used for butanol production has been adopted by the biotechnology company Gevo™ (www.gevo.com; Barnard et al. 2010). Several genetically modified microbes of the genera *Zymomonas*, *Enterococcus*, *Rhodococcus*, *Pichia*, *Candida*, *Saccharomyces*, *Pseudomonas*, and *Klebsiella* are also reported as potential butanol producers though not explored extensively (Donaldson et al. 2008).

Beyond lab-scale experiments, the challenge for commercial production of biobutanol is removing butanol from the fermentation broth in a way that is economical and environmentally-friendly. A possible way to improve the economic efficacy of acetone/butanol/ethanol fermentation is to increase the butanol ratio by eliminating the production of other by-products, such as acetone. Hence, consideration to screen those microbes which can be used as an alternative source to *Clostridium* species and produce biobutanol without any side products is important. Some research is in progress for butanol production from *E. coli*, *S. cerevisiae*, *B. subtilis*, and other microbes as an alternative to *Clostridium* spp., but the main challenge is still to increase the yield. Though the functional butanol pathways were successfully constructed in *E. coli*, *P. putida*, and *S. cerevisiae*, in comparison to *C. acetobutylicum*, butanol yield is very low. We can also target some ethanol-producing microorganisms like *Z. mobilis*, *Pichia* spp., *Candida* spp., *Kluyveromyces lactis*, and *Klebsiella* spp. as a potential butanol producers (Kharkwal et al. 2009). In order to enhance the butanol production, it is highly required to select appropriate microbial strain(s) as well as fermentation process for the butanol production and unveil the role of genetic manipulation for strain and process improvements.

Take-Home Message

- Increasing oil prices and awareness of global warming have brought significant attention to the production of biofuels from biomass.
- Most of the commercially viable technologies for biofuel production depended on food grade or expensive raw materials.
- Economically, biofuels will not be able to restore the demand for fossil fuels unless lignocellulosic biomass and wastewater are used in the fermentation processes.
- One of the biggest challenges in the future will be the scaling-up process in a cost-effective manner.
- At this point extremophile and their genes will help in increasing butanol tolerance and high production of butanol.
- In the future, microbial biofuel will play an important role in commercial biobutanol production.

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Chapter 4
Biodiesel (Microalgae)

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What You Will Learn

- About the two predominant groups of microalgae, chlorophytes (green algae) and diatoms, and their respective primary carbon fixation methods
- A diverse range of extreme environments where microalgae have been found, some of the characteristics of the microalgae found in these environments, and how to target different environments for algal product production
- How microalgae are used in a conceptual biofuel production strategy including the various methods for growing, harvesting, and converting algal lipids and other products to biofuels and bioproducts

4.1 Introduction

Algal biomass represents a promising renewable energy system due to fast photoautotrophic growth rates, CO_2 fixation, and accumulation of carbon storage metabolites which can be used as precursors for fuels and specialty chemicals; moreover, it offers a solution to offset the global dependence on conventional fuels. Currently,

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fuels make up approximately 66% of the global energy demand (Bhateria and Dhaka 2015). Correspondingly, extensive use of nonrenewable energy sources has increased the global carbon dioxide (CO_2) concentration approximately 43% since the use of these fuels was significantly intensified since the Industrial Revolution, around 1750 (USEPA 2016). Fossil fuels are subject to volatile price swings based on geopolitical issues and availability of crude oil. As sources of crude oil become depleted, prices associated with fuels and other petroleum-derived products will experience rapid increases in response. Biofuels and other bio-products derived from microalgae have potential to contribute significantly to this market, yet how large their impact on the market will be remains to be seen (Hill et al. 2006).

Microalgae are oxygenic, phototrophic eukaryotes, which are abundantly found across diverse environments ranging from acidic hot springs to arctic ice and snow. Like other phototrophs, microalgae require light energy, water, and a few inorganic nutrients (carbon dioxide, nitrogen, phosphorus, iron, etc.) which they convert to biomass with diverse biochemical composition (Markou et al. 2014). There are several advantages to utilizing microalgae for biofuel production. First, microalgae have increased theoretical photosynthetic efficiency (10–12%) over terrestrial plants (4–6%) and high cell division rates (1–3 per day) which leads to overall improved biomass yield per unit area, which when paired with the ability to be cultivated continuously year-round further improves their productivity over terrestrial plants (Brennan and Owende 2010). Additionally, microalgae can be grown using brackish, salt, and wastewater sources reducing their demand for freshwater and can utilize nitrogen and phosphorus from agricultural, industrial, and municipal wastewaters as low-cost nutrient sources and as a method for remediation of the wastewater (Hu et al. 2008). Furthermore, algae have the potential to reduce carbon emissions if co-located with a power plant to sequester portions of the emitted CO_2 before it enters the atmosphere (Schenk et al. 2008). Lastly, and perhaps most importantly, microalgae frequently have higher lipid content than terrestrial plants (Amin 2009), and with the combination of the other added benefits, often result in higher biofuel productivities on a per biomass basis. The inherent advantages to using microalgae for sustainable biofuel and bio-product formation are well known, though several bottlenecks still exist on the path from lab bench to full-scale production of microalgae.

One of the primary challenges associated with scale-up of biofuel production is algal species selection. For rapid growth in large-scale open systems, a robust species that tolerates moderate temperature, pH, and salinity changes must be selected to keep productivity high. Extremophilic algae are a compelling choice for biofuel production because of their innate ability to survive and even thrive on the boundary of extreme conditions (Seckbach 2007). Extremophilic microalgal strains have an added benefit of growing in conditions that inhibit growth of many competing microorganisms, which may allow higher biofuel productivity of targeted strains. Some strains isolated from alkaline or halophilic environments have been shown to contain very high concentrations of lipids, primarily in the form of triacylglycerol (TAG). Further, alkaline environments have greater flux of atmospheric CO_2 into the algal growth medium, thus increasing inorganic carbon

available for fixation. Therefore, extremophilic, microalgal strains have the potential to improve algal biofuel viability by providing a more cost-effective production with a greater potential for algal biodiesel productivity and decreased probability for significant contamination.

4.2 Microalgae

Algal biofuels are derived from two predominant groups, green algae and diatoms, both of which are unicellular, photosynthetic eukaryotes (Fig. 4.1). Some strains are known to store high concentrations of triacylglycerol (TAG) that can be converted into biodiesel. Diatom strain, RGd-1, was found to produce 30–40% (w/w) TAG and 70–80% (w/w) biofuel potential (BP) for ash-free dry weight (Moll et al. 2014). An isolate from the Heart Lake area of Yellowstone National Park, RGd-1, is able to grow in exceptionally high silica concentrations that are often inhibiting for marine diatoms (Hildebrand et al. 2012). Moll et al. (2014) found that RGd-1 maintained the best growth and TAG accumulation when grown in 2 mM Si, which is roughly an order of magnitude greater than the silica concentration in seawater. They went on to further stimulate TAG accumulation by adding 25 mM $NaHCO_3$ just prior to nutrient depletion. The greatest lipid accumulation occurred during the stress of a combined Si and NO_3^- limitation with $NaHCO_3$ addition which yielded a nearly a two-fold increase in TAG accumulation compared to Si limitation alone (Fig. 4.2). Further, $NaHCO_3$ addition increased TAG accumulation compared to only nutrient limitation (Moll et al. 2014).

Chlorophytes (green algae) are thought to utilize the C_3 photosynthesis pathway for carbon fixation, whereas diatoms, including *Thalassiosira pseudonana,* are thought to use the C_3 and C_4 pathways (Armbrust et al. 2004; Reinfelder et al. 2004; Roberts et al. 2007). However, both mechanisms utilize ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCo) to catalyze the first step in the

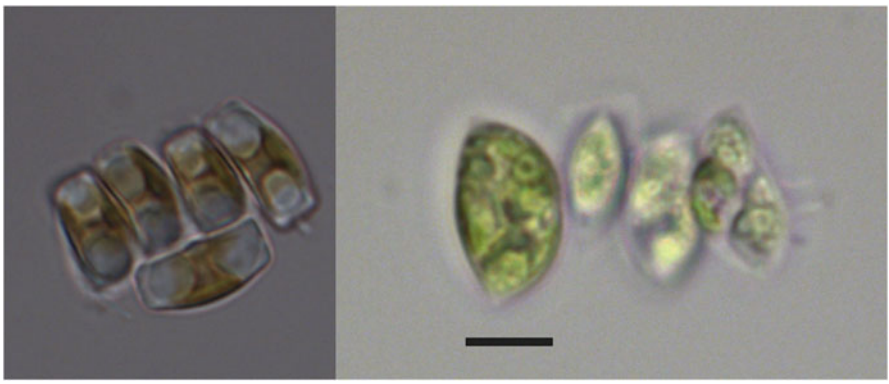

Fig. 4.1 YNP diatom strain RGd-1 (left) and YNP green algal WC-1 (right, scale bar 10 μm)

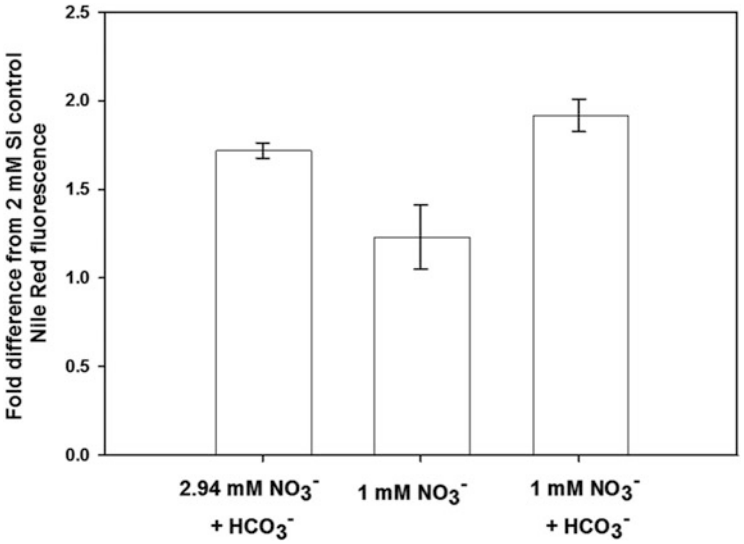

Fig. 4.2 Each bar represents the fold difference in Nile Red fluorescence intensities at 15 days for each treatment compared to the 2 mM Si control

Calvin cycle. RuBisCo has a relatively low affinity for CO_2 and is less than half saturated under normal atmospheric conditions (Giordano et al. 2005). By consequence, microalgae have evolved carbon concentrating mechanisms (CCMs) to increase the carbon flux to RuBisCo (Kaplan and Reinhold 1999; Moroney and Somanchi 1999; Moroney and Ynalvez 2007). Algal CCMs are essentially a two-phase process: in the first phase, inorganic carbon is acquired from the environment and shuttled to the chloroplast, while during the second phase generates elevated HCO_3 in the chloroplast stroma (Moroney and Ynalvez 2007). Microalgae have a number of carbonic anhydrases and bicarbonate transport channels to move inorganic carbon across the periplasmic membrane, through the cytosol, into the chloroplast, and convert the carbon to CO_2 in the direct vicinity of the Rubisco in the pyrenoid. Interestingly, C_4 pathways have the extra ability to convert HCO_3 directly to a C_4 organic acid molecule which is shuttled to the pyrenoid and reconverted to CO_2 to be used by RuBisCo (Roberts et al. 2007; Radakovits et al. 2012).

Alkaline environments (e.g., Soap Lake, Washington) often have high bicarbonate ion concentrations. Given the current understanding of CCMs, it is not surprising that soda lakes are highly photosynthetically productive. Organisms that thrive in these extreme environments have physiological adaptations that allow them to be successful under conditions that would be lethal to other microorganisms. Diatoms are uniquely suited to living in alkaline environments due to their C_4 metabolism. Evidence from the *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* genomes indicates a propensity for C_4 metabolism. Valenzuela et al. (2012) found evidence for *P. tricornutum* using C_3 and C_4 metabolism when dissolved inorganic carbon concentrations were low. Further, they found an increase in expression for

P. tricornutum pyruvate carboxylase, malic enzyme, and malate dehydrogenase which indicates the presence of the C_4 pathway (Valenzuela et al. 2012). This is advantageous by providing another pathway for CO_2 fixation, especially given that C_4 carboxylases are high affinity molecules allowing carbon to be concentrated in the chloroplast. As more extremophilic microalgae are isolated, identified, and characterized, further advances in biotechnology for biofuels and renewable biochemicals will become available.

Under replete growth conditions, microalgae capable of TAG accumulation will synthesis TAG during light hours and utilize the stored carbon for cellular maintenance during dark hours (Bigelow et al. 2011; Gardner et al. 2012, 2013b). However, when the cellular cycling is stressed or arrested due to nutrient limitation (Eustance et al. 2013; Stephenson et al. 2010; Valenzuela et al. 2013), environmental stress (e.g., pH, light, temperature stress) (Sharma et al. 2012; Gardner et al. 2010; Guckert and Cooksey 1990), or by chemical addition (Gardner et al. 2013a; Hunt et al. 2010), many algal strains will accumulate and maintain TAG vacuoles within the cell. Thus, industrial algal biofuel systems producing TAG as a biofuel substrate have to balance rapid growth with a means of impeding the cell cycle when the culture has reached a desired density (Borowitzka 1992; Griffiths and Harrison 2009). Typically, this is accomplished by timing cellular density with the depletion of nitrogen in the growth medium; however, this can often make the culture susceptible to contamination or predation from other microorganisms. However, use of extremophilic strains as an industrial algal biofuel platform is an under studied tactic and merits additional investigation focused on cellular cycling and TAG accumulation.

4.3 Extreme Environments

Microalgae have been isolated from extreme environments such as Arctic/Antarctic regions and acidic hot springs, as well as from alkaline and/or hypersaline environments. Examples of such environments include Yellowstone National Park; Soap Lake, Washington; Mono Lake, California; Great Salt Lake, Utah; and the East African Soda Lakes. In addition to providing a selective advantage for algal growth, with increased pH, there is increased CO_2 solubility, leading to enhanced algal growth (Stumm and Morgan 2012). Soda lakes accumulate very high concentrations of sodium carbonate salts due to the limited Mg^{2+} and Ca^{2+} concentrations with pH 8–12 (Jones et al. 1998). The East African Soda Lakes are among the most productive lakes in the world with gross photosynthetic rates up to 36 g O_2 m^2 per day for Lake Nakuru, Kenya (Melack and Kilham 1974). Another example of a soda lake from which high lipid containing strains have been isolated is Soap Lake, Washington, which is pH 9.9 and contains very high concentrations of sodium carbonate and sodium bicarbonate at 6870 mg L^{-1} (0.7%) and 5209 mg L^{-1} (0.5%), respectively (Richards 2007). Halophiles are uniquely adapted to their

Fig. 4.3 Inputs from thermal hot springs into Witch Creek (research in Yellowstone was conducted under an approved Yellowstone Research Permit [Permit # 5480])

environments by keeping high concentrations of intracellular K^+ to compensate for the high extracellular Na^+ concentrations. Pick et al. (1986) found that when *Dunaliella salina* was grown in 1–4 M NaCl, intracellular Na^+ concentrations were 20–100 mM and K^+ concentrations were 150–250 mM.

Witch Creek is an alkaline, freshwater creek located in the Heart Lake area of Yellowstone National Park (USA). Witch Creek is approximately two miles long and is fed by a combination of fresh groundwater and effluent channels (Fig. 4.3) from alkaline hot springs with high concentrations of metals such as arsenic (~300 ppb) and silicon (~72 ppm). Regular inputs from these thermal features into Witch Creek make the creek alkaline, leading to the growth of microorganisms including microalgae that are adapted to the alkaline conditions found in Witch Creek. Such microalgae have been isolated and characterized for biofuel applications.

4.3.1 Targeting Extremophiles

For microalgae, extremophilic organisms are those considered to have improved growth outside of "normal" environments. For microalgae, these defined "normal" environmental parameters are outlined as in Seckbach (2007) as those having an optimum temperature range between 4 and 40 °C, a pH range of 5–8.5, and a salinity range between that of fresh and salt water (0%–3.5%). The bulk of extremophilic microalgae species fall into the alkaliphile, acidophile, or halophile classifications although there are thermophilic microalgae as well (Seckbach 2007). Although the majority of microalgae species find their optimum growth somewhere in these

Table 4.1 Examples of extremophilic microalgae and their desirable temperature, pH, and salinity conditions with each having at least one environmental condition outside of normal range

Extremophilic organism	Extremophilic condition			Reference
	Temp (°C)	pH	Salinity (%)	
Dunaliella salina	0–38	6–9	3–31	Borowitzka (1990)
Cyanidium caldarium	35–55	2–3	<3	Doemel and Brock (1971)
Yellowstone Diatom Isolate Rgd-1	28	9.3	<3	Moll et al. (2014)
Yellowstone Green Isolate WC-1 *Scendesmus* sp.	24	9.3	<3	Gardner et al. (2010)
Chlamydomonas nivalis	1.5–20	6–8	<3	Remias et al. (2005)
Aphanothece halophytica	30	7.5	15–30	Madigan et al. (2008)
Cyanodioschyzon sp.	45–55	2.5	<3	Skorupa et al. (2014)

defined ranges, extremophilic species have been targeted for use in biofuel production because of the generalized acceptance that product formation of lipids and other products is increased when the cell cycle is ceased and environmental stresses are implemented (Cooksey 2015; Markou and Nerantzis 2013). Table 4.1 highlights several extremophilic algae including acidophiles, alkaliphiles, psychrophiles, thermophiles, and halophiles and the conditions from which they were isolated.

4.3.2 Bioprospecting

Bioprospecting for potential strains that can be used for biodiesel production begins by matching desired growth conditions (e.g., high pH and salinity values) with natural environments that contain those conditions. In addition, locations for microbiological sampling are either on public or private property, and written permission to collect samples should be obtained for any samples collected. For finding extremophilic microalgae, find environments with a pH value below 5 or above pH 8.5, and a salinity range above that of salt water (3.5% w/w) up to sodium chloride saturation (35% w/w). For temperature limits, the upper limit for microalgal (eukaryotic) growth is approximately 57 °C (Seckbach 2007). However, around 45 °C phototrophic growth may be dominated by cyanobacterial species. Therefore, with regard to temperature, microalgal growth will be primarily in the mesophilic range (20–45 °C) (Madigan et al. 2008). As shown in Fig. 4.4, microbial and microalgal communities can change significantly over very short distances due to gradients in temperature, pH, salinity, or nutrient availability; therefore, care should be taken to characterize specific sampling locations for optimum selection of targeted microorganisms. Once areas have been targeted for sampling and written

Fig. 4.4 Typical heterogeneity of a sampling site containing green algae, diatoms and cyanobacteria (research in Yellowstone was conducted under an approved Yellowstone Research Permit [Permit # 5480])

permission is obtained, samples can be taken from the area of interest and returned to the lab for isolation.

In the approach recommended here, samples should be disaggregated and inoculated into (5 mL) of various microalgal media types (e.g., Bold's basal medium) to determine which would provide the best conditions for growth. Typically, standard media are not ideal for isolation of extremophilic microalgae, and so must be adjusted to higher or lower pH, higher salinity, or temperature that must be controlled at a higher or lower value. Samples should also be streaked for isolation on the appropriate solid growth media. Once the colonies have grown to sufficient size, individual, isolated colonies should be aseptically "picked" from the agar plate and inoculated into liquid medium (1 mL) and grown until they change the color of the medium (often green or brown, for green algae or diatoms, respectively) and subsequently transferred to liquid medium (5 mL). After approximately two weeks or once they have reached substantial growth, cultures should be streaked for isolation again and re-picked from agar plates for a total of three rounds of streaking for isolation and transferring to new medium to ensure isolation from other algal species. Following each round of isolation, strains should be observed under transmitted light microscopy to determine the cellular morphology.

Each isolated strain should be screened for TAG content by staining with Nile Red dye or Bodipy 505-515 and observed using epifluorescent microscopy (Cooksey et al. 1987). Fluorescence of lipid vacuoles appear bright yellow for Nile Red dye under epifluorescent light (Fig. 4.5) or green when stained with Bodipy. Strains that have the highest TAG content should be selected for further characterization, since these will likely have the highest biodiesel potential. Strains found to have high concentrations of TAG during the screening process should also have their growth rates measured, since a combination of fast growth rate and high lipid production is desirable. Strains that show faster growth rates and TAG production should be inoculated into approximately 150 mL of liquid medium in 250 mL baffled flasks and grown in triplicate at various temperatures to determine the optimal growth and TAG accumulation conditions for each strain.

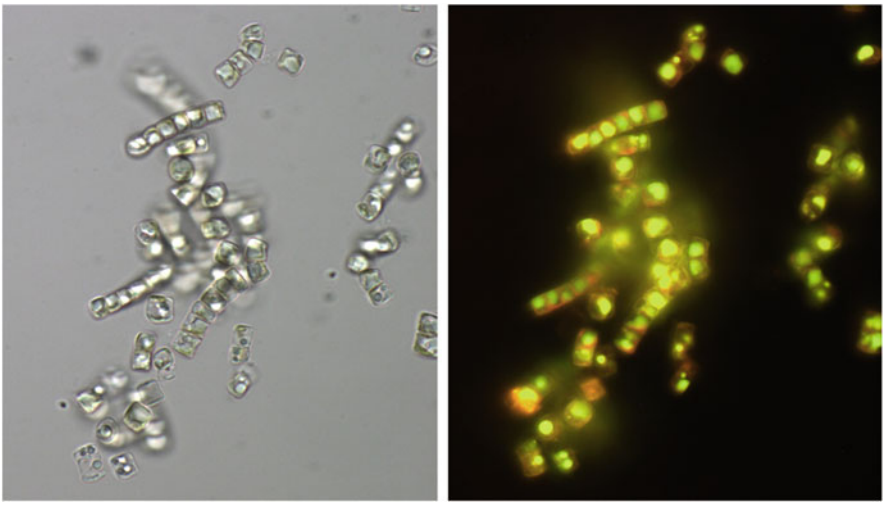

Fig. 4.5 RGd-1 transmitted light (left) and Nile Red fluorescence under epifluorescent light (right)

4.4 Algae as Biofuels

Manufacturing of biofuels and bioproducts from microalgae at industrial scale has many proposed methodologies for start-to-finish generation of targeted end products. Similar to a conventional petroleum refinery which makes multiple products and fuels from crude petroleum, a biorefinery would produce biofuels and other products from algal biomass. An operation such as this would have a variety of different steps, but the major sequences are as follows: cultivation of algae for biomass generation, harvesting of algal biomass, and extraction/conversion of algal biomass. Significant life cycle analyses and techno-economic analyses will be needed with regard to each step of this process to determine the most advantageous strategy for each phase of the operation (Davis et al. 2011).

Primary cultivation systems for the production of algal biomass are through the use of raceway ponds (Fig. 4.6) or photobioreactors (Fig. 4.7). Raceway ponds are typically large closed-loop ponds, in which microalgal culture is continuously circulated through a designed path, generally with the use of revolving paddle wheels (Chisti 2007). Algae are most often grown in large outdoor raceway ponds because it is one of the most cost-efficient ways to grow large quantities of algae (Schenk et al. 2008); however, raceway ponds can suffer from large evaporative losses and poor mass transfer properties for the application of CO_2 (Terry and Raymond 1985). Still, the major caveat of this type of system is that it is non-sterile and has a potential for undesirable contamination from faster growing microorganisms that are not biofuel productive. One advantage of using extremophilic algae in outdoor raceway ponds is to create pond conditions that are favorable for extremophilic algal growth while being inhibitory for faster growing

Fig. 4.6 Outdoor raceway pond (2000 L) at Utah State University, Logan, UT

microorganisms that do not produce biodiesel precursors, but are relatively low cost to build and operate. Conversely, photobioreactors are collections of small-to-medium diameter (<10 cm) transparent tubes which are all oriented parallel to each other and usually stacked vertically to increase the reactor volume in a given footprint (Chisti 2007). Primary concerns regarding the use of photobioreactors are the design limitations on tube length, which is dependent on the degree of O_2 production, CO_2 depletion, and pH variation (Brennan and Owende 2010). While photobioreactors can cost more to build, they typically offer a more controlled environment and higher productivity than open raceway ponds (Sheehan et al. 1998).

Even dense cultures of microalgae require removal of excess water for downstream processing to some extent, and cost-effective and energy-efficient processes for the removal of water and subsequent harvesting of algal biomass are required for economical production of algal biofuels (Schenk et al. 2008). Harvesting of the algae is an energy-intensive step because many conversion pathways require the algal biomass to be at substantially higher concentrations than cultures grow in nature. Typically, even for extremely productive strains, biomass concentrations will not exceed 5% (*w/w*) suspensions while most conversion strategies require a minimum 20% biomass slurry and can require even more dewatering and drying. There are many different approaches to harvesting of algae, but the major developed strategies for harvesting algae are flocculation and sedimentation, filtration, and centrifugation. Each of these methods has advantages and disadvantages, and these methods are often used in combination to reach the desired final algae to water ratio. Flocculation

Fig. 4.7 Photobioreactor illuminated (Green Wave Energy, Inc.) by artificial light in pilot-scale laboratory setting at Montana State University, Bozeman MT

and sedimentation is a routine method for harvesting algae which do not settle out in well-maintained reactors because of their small cell size (Bhateria and Dhaka 2015). Flocculation can be obtained through chemical additives such as alum, lime, polyacrylamide polymers, or surfactants. Following flocculation of the cells, the cells are allowed to settle, and excess water can be removed from the top of the cell sediment. Flocculation is also commonly used with dissolved air flotation (DAF) where the flocculated biomass is driven upward by the attachment of microbubbles where it can be collected at high concentration at the tank surface. Another common form of harvesting microalgae is centrifugation. It is likely that centrifugation will play a minor role in harvesting of culture where other harvesting methods fail to reach the desired algae content for slurry, as centrifugation can reach higher concentration biomass slurries than flocculation with sedimentation or other harvesting

alternatives. However, centrifugation is an energy-intensive process which makes it seemingly unappealing for scale-up of algae cultivation for biofuels (Williams and Laurens 2010). Filtration is another possible alternative for harvesting algae, but has lost some appeal in scale-up from laboratory testing because of the potential for membrane or screen fouling as well as the labor-intensive process of operating such a system. Combinations of these practices for harvesting algae can reach biomass concentrations in the 20%–30% (*w/w*) range required for conversion methods utilizing wet biomass, but are not ideal for methods that require whole or dry biomass. If further dewatering is needed after the culture harvesting, a drying step will be necessary for removing excess water from algae paste or slurry. Thermal drying using methane drum dryers is most commonly practiced, but other oven-type dryers have been used, as well as solar drying and freeze-drying of algae slurry (Bhateria and Dhaka 2015).

Conversion of algal biomass can be accomplished through different methods which are generally categorized into two categories: thermochemical conversion or biochemical conversion (Amin 2009). From cultivated microalgal biomass, there are two major conversion strategies for making usable biofuels. The first, and more well known, is biochemical conversion. Most common for microalgal biodiesel production is the process of transesterification. Through the use of heat and an acid or base catalyst, algal lipids are converted to fatty acid methyl esters (FAMEs) and glycerol. These FAMEs are crude biodiesel and are similar in composition to those produced from transesterification of vegetable oils. The high lipid content in microalgae, primary TAG, makes transesterification an efficient process with production yields between 70% and 90%. While transesterification is primarily a straightforward chemical reaction, it falls into the biochemical conversion category because it does not require the significant energy requirements for high temperature and pressure systems typical with thermochemical conversion pathways. Another biochemical conversion pathway is fermentation of an algal slurry to produce ethanol. Fermentation of algae is a less common method for energy production, mostly because of the difficulties associated with the process of separating produced ethanol after the fermentation process as well as the relatively low starch content of microalgae compared with alternative lignocellulosic biomass. Still, fermentation of lipid extracted algae for conversion of the residual carbohydrates may offset costs associated with biofuel production from microalgae.

As an alternative to biochemical conversion, thermochemical conversion is currently being heavily studied for its application to microalgal biomass. Of the many different techniques for thermochemical conversion, hydrothermal liquefaction and pyrolysis are emerging as the two benchmark technologies, while gasification and hydrogenation will likely play smaller roles in utilizing all products from the conversion process (Amin 2009). Hydrothermal liquefaction is a process which uses subcritical water at moderate temperature and pressure (~300 °C and 10 MPa) to convert wet biomass into a liquid fuel called primary oil. This oil can be separated and purified using a solvent such as dichloromethane. Other products from hydrothermal liquefaction such as the aqueous and gas phases can be recycled to supply nutrients for more algae cultivation or used in a gasification or hydrogenation

process for other products. Pyrolysis is another thermochemical conversion process to produce energy rich compounds such as biofuel, charcoal, and gaseous products from algal biomass. Short residence times and high temperatures (500 °C) are used to crack biomass into short chain molecules which can then be rapidly cooled into a liquid phase to produce biofuel. Pyrolysis has a high-energy input required because it requires algal biomass to be completely dried, adding the necessity for 20%–30% algal slurries to have all remaining residual water removed through one of the processes mentioned previously. There is still no general consensus on whether biochemical or thermochemical conversion processes will be the ultimate solution to producing biofuel from microalgae; however, life cycle analysis and techno-economic analysis considering the entire production of algae to biofuels is being completed with considerations for both types of conversion methods (Hise et al. 2016).

4.4.1 Other Secondary Products

Microalgae not only offer a source of sustainable biodiesel, but can be used to make an assortment of products such as food supplements, fertilizers, bioplastics, nutraceuticals, and cosmetics (Markou and Nerantzis 2013). For algal biofuels to be economically viable, additional coproducts will need to be formed in concert with biofuel. In particular, those products which have a combination of the highest yields and highest specific selling price (e.g., $/lb.) will be the optimum targets for coproducing with biofuels. Two examples of these higher value compounds are carotenoids and unsaturated fatty acids, which are both produced naturally by microalgae. Carotenoids are colorful pigments which can be used as food and feed additives, as well as nutraceutical supplements to promote health. The two most common carotenoids produced naturally by microalgae are β-carotene and astaxanthin (Markou and Nerantzis 2013). Two specific organisms produce these compounds in much greater quantity than any others studied, *Dunaliella salina* and *Haematococcus pluvialis* for β-carotene and astaxanthin production, respectively. Coincidentally, *Dunaliella salina* is a halophile with optimum sodium chloride concentrations between 10% and 27% depending on targeted growth regime, but also produces culture rich in β-carotene. Alternatively, *Haematococcus pluvialis* produces high concentrations of astaxanthin when environmentally stressed with sodium chloride concentrations in the range of 4%–6%. Furthermore, both these organisms can withstand high light environments and even can be induced to make more of their targeted carotenoids with light induced stress (Markou and Nerantzis 2013).

Mono- and poly-unsaturated fatty acids being another alternative secondary product, they must be valued separately from being simply a biofuel precursor. Depending on the intended application, omega-3 fatty acids such as α-linolenic acid or eicosapentaenoic acid can be sold as health supplements for much higher value as opposed to being converted to biofuel. Similarly, the monounsaturated fatty acid

oleic acid is a valuable precursor to 9-decenoic acid, which can be used to create valuable products such as surfactants, lubricants, and polyester, amongst other things (Burns 2010). A feasible production strategy would make not only biofuels, but some other valuable secondary products as well.

Take Home Message

- Microalgae are promising candidates for biodiesel production due to their fast growth rates, can be cultivated on nonarable land, can use brackish or wastewater, and avoid competing with food supplies.
- Some microalgae strains accumulate high concentrations of TAG that can be converted into biodiesel.
- Extremophilic algae are uniquely suited for growth because their growth conditions inhibit growth for most contaminating (biofuel nonproductive) microorganisms.

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Chapter 5
Biohydrogen Production from Lignocellulosic Feedstocks Using Extremophiles

Raman Rao, Rajesh K. Sani, and Sachin Kumar

What Will You Learn from This Chapter?

- Different pretreatment methods for hydrogen production from lignocellulosic biomass
- Microbial process for hydrogen production
- Thermophilic hydrogen production from untreated lignocellulosic biomass
- Mechanism of extremophilic microbial hydrogen production

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5.1 Introduction

Fossil fuels are the major energy provider for current economy and day-to-day life (Demirbas 2007). Maximum percentage of fossil fuel like petrol, diesel, compressed natural gas (CNG), and liquefied natural gas (LPG) are used in the transportation sector. Fossil fuels also emit the greenhouse gases (GHG: CO, CH_4, CO_2), which affect the environment adversely. Therefore, researchers around the world are focusing on finding the alternate energy resources, which are environmental friendly and renewable in nature (Singh et al. 2013a, b). Hydrogen (H_2) is one such attractive energy source, which has the highest energy yield (calorific value of 143 MJ/kg) among any known fuel. It can be easily transported through conventional means and has been accepted globally as environmentally safe energy resource (Das 2009). Due to high current global demand for H_2 (>45 million tons per annum), a vast array of physical, chemical, physiochemical, and biological processes is currently being employed for H_2 production (Rittmann and Herwig 2012). These include water electrolysis, steam reformation, catalytic steam gasification of biomass, biomass pyrolysis, supercritical water gasification, photolysis of water, and microbial fermentation.

To date, however, 96% of the current H_2 supply comes from fossil fuels (49%, natural gas; 29%, crude oil; and 18%, coal) through steam reforming, and 4% H_2 comes through electrolysis as shown in Fig. 5.1 (Evers 2008; Suresh et al. 2010). Fossil fuel reforming generates greenhouse gases and is not renewable. On the other hand, the biohydrogen ($BioH_2$) production process is eco-friendly (nonpolluting in nature), generates no GHG, and renewable as it can be produced from biomass. Therefore, generating H_2 from microbial origins can meet the requirements of a viable biofuel prospect, providing a cost-effective, pollution-free, and energy-saving alternative to current production practices. Several options for the biological production of H_2 are being investigated such as biophotolysis of water through microalgae and

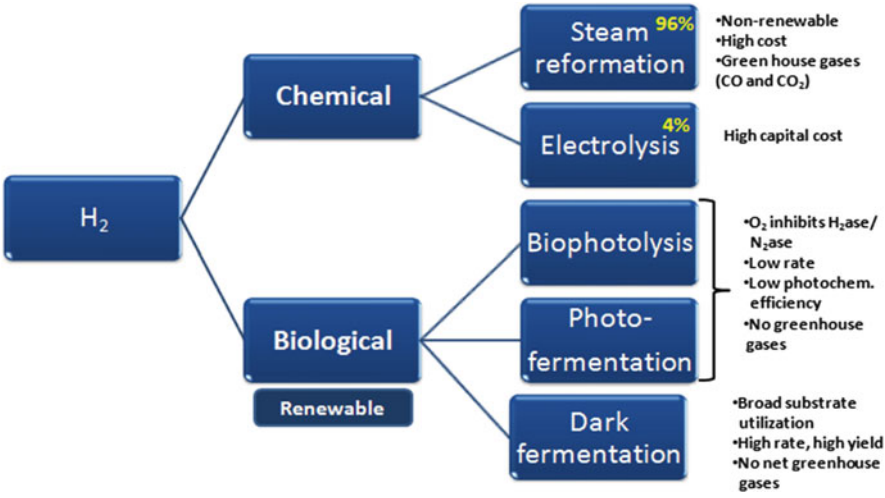

Fig. 5.1 Hydrogen production methods

cyanobacteria, the use of photosynthetic bacteria for the photo-fermentation of organic substances, and dark fermentation of organic substances by anaerobic organisms. The last approach, i.e., dark fermentation, is generally preferred because it does not rely on the availability of light sources. The major advantages of dark fermentation are (1) its simplicity of reactor design, (2) process operation, (3) its wide variety of feedstocks utilization, and (4) higher H_2 production rates compared to other biological methods of H_2 production (Kumar et al. 2015; Saripan and Reungsang 2013).

The utilization of biomass for energy, food, and chemical could solve waste disposal problems and help in finding the alternate route to meet the future energy demands by providing a convenient and renewable source of energy (Ragauskas et al. 2006). Therefore, it has been proposed that the use of inexpensive renewable resources such as lignocellulosic biomass (LCB) for $BioH_2$ production, especially by using extremophiles, can fulfill the huge demand of future energy supply (Lynd et al. 2008). These LCB must be first pretreated in order to remove lignin and hemicelluloses and to increase the surface area of material to enhance the release of sugars (Mosier et al. 2005).

Pretreatment methods improve the fermentability of LCB by overcoming recalcitrance of the lignocellulosic complex by altering its structure, which makes the cellulose and hemicelluloses accessible to the enzymes (Mosier et al. 2005). One of the methods of interest is pretreatment with an alkaline agent at relatively low temperatures (<100 °C). There are few reports on thermophilic bioprocessing (≥ 55 °C) of LCB, which results in higher $BioH_2$ yield as compared to mesophilic conditions (30–40 °C), due to favorable thermodynamical conditions at high temperatures and reduced variety in by-product formation (de Vrije and Claassen 2003; Hallenbeck 2005; Jones 2008). However, the rate of production of $BioH_2$ from complex substrates using thermophiles is too low to be commercially viable. Further, many thermophilic bacteria, including *Thermotoga neapolitana* and *Caldicellulosiruptor saccharolyticus*, can utilize different substrate ranging from simple sugars to complex carbohydrates (Blumer-Schuette et al. 2008). Several investigators have reported a $bioH_2$ yield, closer to theoretical yield 4.0 mol-H_2/mol-glucose by using extremophiles (Munro et al. 2009; d'Ippolito et al. 2010). The use of elevated temperature to produce $BioH_2$ offers several benefits including higher mass transfer rates leading to substrate solubility, enhanced hydrolysis of LCB, and lower risk of microbial contamination.

5.2 Lignocellulosic Biomass and its Preprocessing

Plant cell walls are the major source of renewable biomass with annual production of $150–170 \times 10^9$ tones (Pauly and Keegstra 2008). Few studies have been reported to produce $BioH_2$from the feedstocks such as agricultural wastes and other waste materials. Plant cell wall is the major source of LCB including solid materials such as wheat straw waste (Fan et al. 2006), delignified wood fibers (Levin et al. 2006), and other cellulose waste materials (Magnusson et al. 2008). Other biomass like corn stalks and corn stover have also been used for $BioH_2$ production followed by pretreatment and hydrolysis to obtain soluble biomass with mixture of sugars and oligosaccharides (Datar et al. 2007; Ren et al. 2008a, b).

Conversion of LCB includes hydrolysis of feedstock to produce reducing sugars and production of H_2 and higher valuable products via fermentation. Porosity of waste materials, crystallinity of cellulose fiber, and lignin and hemicelluloses content affect the hydrolysis of LCB (McMillan 1994). Two classes of processing strategies have been explored in a way so that the hemicellulose and cellulose fractions can be processed together or separated and processed individually. Due to the close association of cellulose and hemicellulose with lignin in the plant cell wall, pretreatment is necessary to expose the cellulose present in the plant biomass (Radeva et al. 2012; Behera et al. 2014). The goal of the pretreatment process is to break down the lignin structure and disrupt the crystalline structure of cellulose, so that the acids or enzymes can easily access to hydrolyze the cellulose (Mosier et al. 2005). The process can enhance the bio-digestibility of the wastes for $BioH_2$ and ethanol production and increase accessibility of the enzymes to the materials. It results in enrichment of the difficult biodegradable materials and improves the yield of $BioH_2$ from the wastes (Taherzadeh and Karimi 2008).

5.2.1 Physical Treatment

The mechanical disruption (such as grinding, milling, or chipping) of LCB is an environmentally friendly pretreatment process. The milling pretreatment has several advantages for lignocelluloses, including the higher accessible surface area, decreased crystallinity, and no loss of low molar mass components (Lin et al. 2010). In mechanical pretreatment, reduction of particle size and crystallinity of lignocellulosic feedstock should be achieved in order to increase the specific surface and reduce the degree of polymerization. It includes chipping, grinding, or milling depending on the final particle size of the material (10–30 mm after chipping and 0.2–2 mm after milling or grinding). Different types of milling processes such as ball milling, two-roll milling, hammer milling, colloid milling, and vibro-energy milling can be used to improve the biodegradability of LCB. Irradiation processes such as gamma and electron rays can also be used to break the lignocellulose structure by changing physical and chemical structure of cellulose (Shin and Sung 2008).

5.2.2 Physicochemical Treatment

Physicochemical methods include steam explosion, hot water treatment, ammonia fiber explosion, wet oxidation, and CO_2 explosion. Steam explosion method breaks the LCB by sudden release of applied pressure. However, it is necessary to maintain the reaction condition (temperature, moisture content and chip size, etc.) in order to reduce the inhibitor production. In the AFEX method, biomass is treated with liquid ammonia at high temperature and pressure, whereas CO_2 explosion method includes the use of supercritical fluid under high pressure, where lignin solubilizes effectively. In liquid hot water (LHW) treatment, hot water at a temperature of 160–240 °C is used to remove the

lignin and to hydrolyze the hemicelluloses. However, due to recondensation of soluble components, complete delignification is not possible (Cara et al. 2007). Okuda et al. (2008) used the liquid hot water method at 100–300 °C for 30 min to treat the green algae (*Monostroma nitidum*) and red algae (*Soleria pacifica*). Maximum glucose yield from cellulose using enzymatic hydrolysis were 79.9% and 87.8% for *M. nitidum* and *S. pacifica*, respectively. Mosier et al. (2005) reported the increase of enzymatic hydrolysis of corn stover with controlled pH by using liquid hot water pretreatment. Maximum glucose yield of 90% was obtained using 16% slurry of corn stover at 190 °C for 15 min. In addition, by using liquid hot water pretreatment (230–240 °C for 2–15 min) prior to enzymatic saccharification process, the glucose yield of the poplar (*Populus nigra*) biomass can be improved by 60% (Negro et al. 2003).

5.2.3 Chemical Treatment

This method involves the chemical reactions for disruption of the LCB structure, which includes acid treatment, alkali treatment, liquid hot water, ionic liquids, organosolv, and ozonolysis. In acid treatment, the LCB is hydrolyzed to fermentable sugar by using both dilute and concentrated acid. Sulfuric acid is the most used acid for the acid pretreatment process followed by nitric acid, hydrochloric acid, and phosphoric acid. In alkali treatment, lignin is removed from biomass along with acetyl and other uronic acid substitutions, which decreases the accessibility of enzyme to the cellulose surface (Chang and Holtzapple 2000). In ionic liquid (IL) methods, there is a formation of electron donor-electron acceptor complexes (EDA) which interacts with ionic liquids. ILs such as imidazolium salts like 1-n-butyl-3-methylimidazolium chloride (BMIMCl), N-methylmorpholine-N-oxide monohydrate (NMMO), 3-methyl-N-bytylpyridinium chloride (MBPCl), 1-allyl-3- methylimidazolium chloride (AMIMCl), and benzyldimethyl (tetradecyl) ammonium chloride (BDTACl) are used for fractionation of biomass (Lee et al. 2009; Li et al. 2009). In ozonolysis method, the lignin and hemicelluloses present in the feedstock like wheat straw, rice straw, etc. are degraded by using ozone. This method does not produce any toxic inhibitor and is carried out at room temperature and pressure. However, this method is expensive because large quantity of ozone is required for efficient pretreatment.

5.3 Microbial Routes for BioH_2 Production from Lignocellulosic Biomass

BioH_2 can be produced by biophotolysis of water by blue-green algae, dark fermentation in anaerobic conditions, and photo-fermentation by photo heterotrophic bacteria. BioH_2 production by microbial routes has been studied for the last few years using polysaccharide derived from plants. During the dark fermentation, the anaerobic culture utilizes the carbohydrates to produce bioH_2 in mesophilic (25–55 °C),

thermophilic (60–75 °C), or hyperthermophilic (75–90 °C) environment. Higher yields of H_2 have been obtained by using thermophilic and mixed hyperthermophilic microorganisms with different simple sugar as well as complex substrates (Table 5.1). Thermophiles or hyperthermophiles contain membrane-bound NADPH-dependent hydrogenase enzyme which is responsible for the thermodynamic feasibility of the process and higher yields of H_2. There are two methods available for the conversion of LCB into bioH_2 production including direct process and two stage process. In direct process, single microorganism is capable to hydrolyze the cellulose/hemicellulose and to produce bioH_2 in a single step; whereas in two stage process, the cellulose is hydrolyzed by pure or mixed cultures, and H_2 is produced using the different culture separately.

It has been reported that extremophiles can effectively act on pretreated biomass to produce H_2. In a study, thermophiles *C. saccharolyticus* and *T. neapolitana* were employed for H_2 production from lignocellulosic energy crop *Miscanthus*. The *Miscanthus* was pretreated using alkali pretreatment, and H_2 yield of 2.9–3.4 mole of H_2/mole of hexose was obtained which is about 74–85% of the theoretical yield (de Vrije et al. 2009).

One of the extreme thermophiles, *C. saccharolyticus* DSM 8903, has been reported for its efficiency to produce BioH_2 from untreated and dried biomass like sweet sorghum, sugarcane bagasse, maize leaves, wheat straw, silphium, and pine wood. Wheat straw produced maximum H_2 yield of 44.7 L/Kg of dry biomass which is equivalent to 3.8 mole H_2/mol of glucose (Ivanova et al. 2008, 2009). Further study was conducted using 0.5% (w/v) untreated pine wood biomass for up to 91 days to produce BioH_2 by the same strain DSM 8903. It was found that significant amount of H_2 was produced for up to 55 days (Ivanova et al. 2008). However, the total yield of H_2 was low, but this study provided possible use of DSM 8903 strain in future to enhance the H_2 production from untreated plant biomass. Further, the strain DSM 8903 produced H_2 from untreated switch grass (SWG) without any chemical or physiochemical treatments yielding 11.2 mmol H_2/g of SWG in a single step (Talluri et al. 2013).

5.4 BioH_2 Production from Untreated Lignocellulosic Biomass Using Thermophiles

Literature suggests that the use of thermophilic microorganisms such as *Caldicellulosiruptor saccharolyticus* or *Thermotoga maritima* has shown promising results for H_2 production (Talluri et al. 2013; Willquist et al. 2010; Ivanova et al. 2009). Thermophilic H_2 fermentations have higher H_2 yields than mesophilic ones due to the suppression of H_2-consuming bacteria such as methanogens and sulfate-reducing bacteria (Hallenbeck et al. 2012; Ren et al. 2010). For example, higher H_2 yield of H_2 (19.01 mmol H_2/g of sugar) was obtained at a temperature of 80 °C in CSTR by a mixed culture as compared to yield of 15.2 mmol H_2/g of sugar at 55 °C

Table 5.1 BioH_2 production by thermophilic and hyperthermophilic culture from simple and complex substrates

Organism	Substrate	Temperature (°C) and culturing type	End products	Yield (mol/mol)[a]	Reference
Thermotoga neapolitana	Xylose	75 °C, batch	Acetic acid, lactic acid	2.22	Ngo et al. (2012)
Thermotoga neapolitana DSM 4359	Glucose	80 °C, batch	Acetic acid, lactic acid	3.8	Eriksen et al. (2011)
Thermoanaerobacter mathranii A3N	Glucose	70 °C, batch	Acetic acid, lactic acid, butyric acid, ethanol	2.64	Jayasinghearachchi et al. (2012)
Pyrococcus furiosus DSM 3638	Cellobiose	70 °C, batch	Acetic acid, alanine, ethanol	3.8	Chou et al. (2008)
Thermobrachiumcelere, *Thermoanaerobacterium aotearoense*, *Clostridium thermopalmarium*	Xylose	70 °C, batch	Acetic acid, ethanol	1.84	Zhao et al. (2010)
Thermococcus kodakaraensis TSF100	Starch	85 °C, batch	Acetic acid, alanine	3.3	Kanai et al. (2005)
Thermoanaerobacterium, *Thermoanaerobacter*	Glucose	70 °C, batch	Acetate, butyrate, ethanol, propionate	2.38	Lu et al. (2012)
Mixed enriched culture	Glucose	70 °C, UASB	Acetate, butyrate	2.47	Kotsopoulos et al. (2006)
Thermococcus onnurineus	CO/Sodium formate/ Starch	72 °C, batch	Acetic acid, butyric acid. ethanol	3.13	Bae et al. (2012)
Thermotoga neapolitana	Rice straw	75 °C, batch	N/A	2.7 mmol of H_2/g rice straw	Nguyen et al. (2010)
Caldicellulosiruptor saccharolyticus	Miscanthus hydrolysate	72 °C, batch	Acetic acid, lactic acid	3.4	de Vrije et al. (2009)
Caldanaerobacter subterraneus, *Thermoanaerobacter subterraneus*, *Thermoanaerobacterium thermosaccharolyticum*	Wheat straw hydrolysate	70 °C, CSTR	Acetic acid, butyric acid, propionic acid, lactic acid, formic acid, ethanol	9.46 mmol H_2/g sugars	Kongjan and Angelidaki (2010)

(continued)

Table 5.1 (continued)

Organism	Substrate	Temperature (°C) and culturing type	End products	Yield (mol/mol)[a]	Reference
Thermotoga neapolitana	Miscanthus hydrolysate	80 °C, batch	Acetic acid, lactic acid	2.9	de Vrije et al. (2009)
Thermoanaerobacterium saccharolyticum, T. thermosulfurigenes, Bacillus sp., Geobacillus sp.,	Sago starch	60 °C, batch	Acetate, ethanol	19.72 mmol H_2/g Starch	Hasyim et al. (2011)
Anaerobic mixed microflora	Cellulose	60 °C, CSTR	Acetic acid, butyric acid	19.01 mmol H_2/g Cellulose	Gadow et al. (2012)
Thermotoga neapolitana	Potato stem Peels	75 °C, CSTR	Acetic acid	2.6–3.8	Mars et al. (2010)
Thermoanaerobacterium sp.	Sweet sorghum bagasse	70 °C, batch	Acetate, lactate	2.6	Panagiotopoulos et al. (2010)

[a]Mole of H_2 produced/mole of substrate consumed

and 0.6 mmol H_2/g of sugar at 37 °C (Gadow et al. 2012). In addition, several thermophiles can produce H_2 from both C_5 and C_6 sugars. The thermophile *C. saccharolyticus* produces H_2 from pentose sugars, while *Thermoanaerobacterium thermosaccharolyticum* W16 was shown to ferment a biomass hydrolysate containing a mixture of glucose and xylose to H_2 (Ren et al. 2008a, b). Microbial production of H_2 has been studied for the past few years, but it has not yet been developed to an economically viable status. As discussed above, most of the microorganisms including thermophiles produce higher yields of BioH_2 only when pretreated LCBs are used. This suggests that conversion of lignocellulose to H_2 will require pretreatment, removal of inhibitors released during pretreatment, hydrolysis, and fermentation. Pretreatment of LCBs, detoxification of hydrolysates, and the high current costs of lignocellulose-deconstructing commercial enzymes make dark fermentation more challenging. These factors warrant the development of a cost-effective H_2 production route. Using acid-treated sugarcane bagasse hydrolysate, *T. thermosaccharolyticum* KKU-ED1 produced only 1.12 mole-H_2/mol sugar. *C. saccharolyticus* has been shown to achieve the theoretical Thauer limit of 4 moles of H_2/mole of glucose used (Willquist et al. 2010); however, it generated only 11.2 mmol H_2/g switchgrass (Talluri et al. 2013).

The commercial H_2 production is hindered by high cost of pretreatment steps and formation of some inhibitory compounds like organic acids, aromatics, and hydroxyl methyl furfural (HMF) in acid hydrolysates. A viable option to lower the costs of feedstock and lignocellulolytic commercial enzymes costs is to screen for and identify H_2-producing microorganisms capable of utilizing cellulose and hemicellulose directly from the biomass without pretreatment at higher temperatures ($\geq$60 °C). This would eliminate the need for the separate steps of pretreatment, lignocellulolytic enzyme production, and enzymatic hydrolysis of biomass and would improve the process sustainability and economics. However, information on such microorganisms which produce H_2 from untreated LCB is relatively scarce. Table 5.2 summarizes the recent studies of H_2 production from untreated LCB using thermophiles. All these reports mentioned in Table 5.2 have used different parameters for H_2 yield calculations. Therefore, it was not feasible to compare H_2 yields. Some of these reports did utilize pretreatments, e.g., physical pulverization, treatment with α-amylase (Chen et al. 2012; Mars et al. 2010).

5.5 Mechanism of H_2 Production by Extremophiles

Most of the extreme thermophilic bacteria from the phylum *Clostridia* follow the Embden-Meyerhof-Parnas pathway (EMP) to metabolize hexose sugars to pyruvate followed by formation of BioH_2via decarboxylation of pyruvate to acetyl CoA, and proton are reduced to H_2 by reduced ferredoxin (Fd_{red}) (Verhaart et al. 2010) as shown in Fig. 5.2. Pyruvate is formed at the end of glycolysis, and it can be reduced to lactate by lactate dehydrogenase but most of the anaerobic bacteria oxidize pyruvate into acetyl CoA by enzyme POR (pyruvate oxidoreductase), and the end

Table 5.2 BioH_2 gas production from untreated lignocellulosic feedstocks

Feedstock	Bacterial strain	Pretreatment	Temp (°C)	Reference
Switch grass and microcrystalline cellulose	*Caldicellulosiruptor saccharolyticus* DSM 8903	None	65	Talluri et al. (2013)
Rice straw	Heat treated sludge	Physical pulverization	55	Chen et al. (2012)
Potato stem peels	*Thermotoga neapolitana*	Treatment with α-amylase (Novozymes)	75	Mars et al. (2010)
Potato stem peels	*Caldicellulosiruptor saccharolyticus*	Treatment with α-amylase (Novozymes)	72	Mars et al. (2010)
Maize leaves	*Caldicellulosiruptor saccharolyticus*	None	70	Ivanova et al. (2009)
Poplar, switch grass, napier and bermuda grasses	*Anaerocellum thermophilum* DSM 6725	None	70	Yang et al. (2010)
Dried distillers grain, barley hulls, and Fusarium head blight contaminated barley hulls	*Clostridium thermocellum* ATCC 27405	None	60	Magnusson et al. (2008)

product formed is acetic acid/butyric acid depending upon the microbes involved and environmental conditions. Two pathways are available for bioH_2 production by strict anaerobes: first, from a NAD(P)H by GAPDH (glyceraldehyde-3-P dehydrogenase) and from pyruvate ferredoxin oxidoreductase (PFOR) (Jones 2008). BioH_2 production from either ferredoxin or NAD(P)H is thermodynamically unfavorable; therefore, the H_2 yield observed by mesophilic and thermophilic bacteria is low (Jones 2008; Hallenbeck 2009). The redox potential of Fd_{red}/Fe_{ox} couple depends on the microorganism and temperature involved. In nature, the activity of methanogens and sulfate-reducing bacteria reduces the partial pressures of H_2. This results in a low partial pressure of BioH_2 which is favorable for a complete oxidation of glucose to acetate and CO_2. Higher temperatures and partial pressure of BioH_2 do not affect the activity of key enzymes responsible for bioH_2 production. Hence, extremophilic bacteria are able to produce up to 4 moles of bioH_2 along with 2 moles of acetate in pure cultures and also for the fact that microorganisms growing at lower temperatures direct their end product formation to other reduced products.

It has been estimated that conversion of 1 mole of glucose can yield 12 mol of BioH_2. The H_2 production with different end products using glucose as substrate is shown below:

If the end product is acetic acid, 1 mole of glucose gives 4 moles of H_2.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (5.1)$$

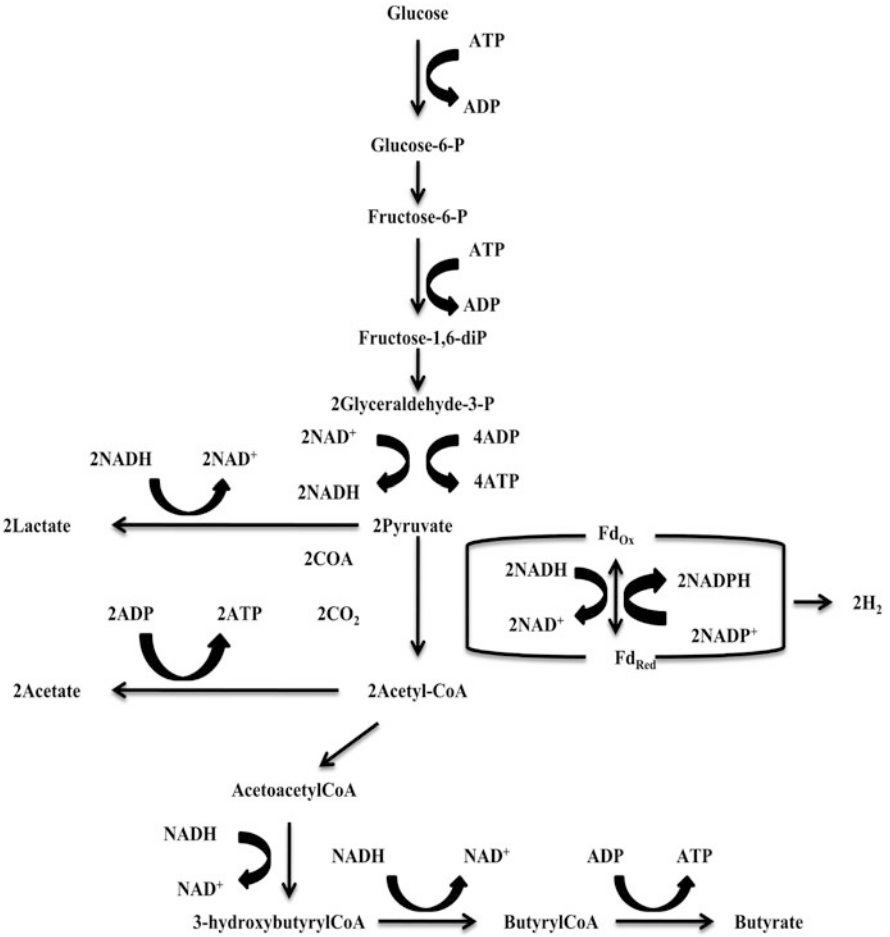

Fig. 5.2 Embden-Meyerhof-Parnas pathway for $BioH_2$ production by extremophiles (adapted from Abreu et al. 2012)

If the end product is butyric acid and formic acid, 1 mole of glucose produces 2 and 1 moles of H_2, respectively.

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (5.2)$$

$$HCOOH \rightarrow CO_2 + H_2 \quad (5.3)$$

All the fermentative methods to produce H_2 depend on the activity of enzymes (nitrogenase and hydrogenase) whose properties are shown in Table 5.3. Hydrogenase is the main enzyme responsible for $BioH_2$ production and is of two types (Fe hydrogenase and Ni-Fe hydrogenase) depending upon their metal content. During the fermentative EMP pathway, the H_2 is produced during the conversion of

Table 5.3 Properties of nitrogenase and hydrogenase (Ni et al. 2006)

Property	Nitrogenase	Hydrogenase
Substrates	ATP, H^+ or nitrogen	H^+, hydrogen
Products	H_2, NH_4^+	ATP, H^+ or nitrogen
Number of proteins	2 (Mo-Fe and Fe)	1
Metal components	Mo, Fe	Ni, Fe, S
Optimal temperature	30 °C (*A. vinelandii*)	55 °C (*R. rubrum*) 70 °C (*R. capsulatus*)
Optimal pH	7.1–7.3 (*A. vinelandii*)	6.5–7.5 (*R. sulfidophilus*)
Inhibitors	N_2, NH_4^+, O_2, high N:C	CO, EDTA, O_2 and some organic compounds
Stimulators	Light	Absence of organic compounds

pyruvate to acetyl CoA. In this step, NADH is oxidized, and ferredoxin is reduced to produce molecular H_2. *C. saccharolyticus* anaerobe has been reported to produce $BioH_2$ by producing some cellulolytic enzymes, which degrade the LCB like switchgrass, sweet sorghum, paddy straw, etc. *C. saccharolyticus* and *T. tengcongensis* show a different and modified form of EMP (Kumar et al. 2015). *C. saccharolyticus* produces high yield of molecular H_2 as the level of lactic acid and ethanol produced is low. Further research on the genetic level of this microbe should explore its potential to produce $BioH_2$. In the pathway, glucose molecule is metabolized in to glyceraldehyde-3-phosphate (GAP). Electron carrier NADH is formed by further conversion of GAP into pyruvate. Pyruvate is further oxidized into acetyl CoA by enzyme POR (pyruvate oxidoreductase), and ferredoxin is reduced in this step. The acetyl CoA is then converted into acetic acid. However, *T. tengcongensis* does not grow on the LCB. The enzyme present in *T. tengcongensis* is NADH-dependent hydrogenase, which uses direct NADH to produce H_2 at low concentration of H_2 (Soboh et al. 2004). Studies have shown that both species of extremophiles can use NADH directly for H_2 production. On increasing the pressure, enzyme lactate dehydrogenase will utilize NADH to produce lactate instead of acetate and $BioH_2$ thereby decreasing the $BioH_2$ yield.

5.6 Future Scope

The laboratory scale production of $BioH_2$ from LCB includes several steps which ultimately increase the production cost. The combination of several steps will reduce the overall cost efficiency of the process. Therefore, an efficient and cost-effective single step process can degrade and ferment the untreated lignocelluloses. As a result, there will be no need of any additional step like pretreatment, lignocellulolytic enzyme production, enzymatic hydrolysis of biomass, and fermentation. One of such practice is presented with consolidated bioprocessing (CBP) process in which enzyme production, enzyme hydrolysis of the biomass, and fermentation step are combined in a single step. This single step CBP process can reduce the production

cost up to 50% by eliminating the cost associated with enzyme production as in other methods like simultaneous saccharification and fermentation (SSF) or co-fermentation (Xu et al. 2009). The use of extremophiles offers several advantages like increase mass transfer rates, favorable thermodynamic conditions, less chances of microbial contamination, etc., thereby, increasing the overall economics of the process.

Combining dark fermentation with photo-fermentation or combining dark fermentation with methanogenesis is also an alternative or holistic approach for $BioH_2$ production from LCB. The volatile acids such as acetic acid, butyric acid, etc. are present in the dark fermentative effluent, which can be used by methanogens to produce methane gas. As a result, the combined hybrid system will increase the overall energy recovery from biomass. In a different novel approach, the lignocellulose-based $BioH_2$ system can be designed by combining sequencial dark and photo-fermentation with microalgae photoautotrophic process, where microalagae will utilize all CO_2 produced in sequencial dark and photo-fermentation. Microbial biomass produced can be used further in biorefinery to produce value-added biofuels as shown in Fig. 5.3.

If the composition of substrate is complex, the use of microbial consortia to degrade the biomass might increase the $BioH_2$ yield as compared to pure culture. In vitro system can also be designed to build a modified $BioH_2$ production pathway. For example, glucose substrate can be converted into H_2 and glucuronic acid by combining glucose dehydrogenase from *Thermoplasma acidophilum* and NADPH-

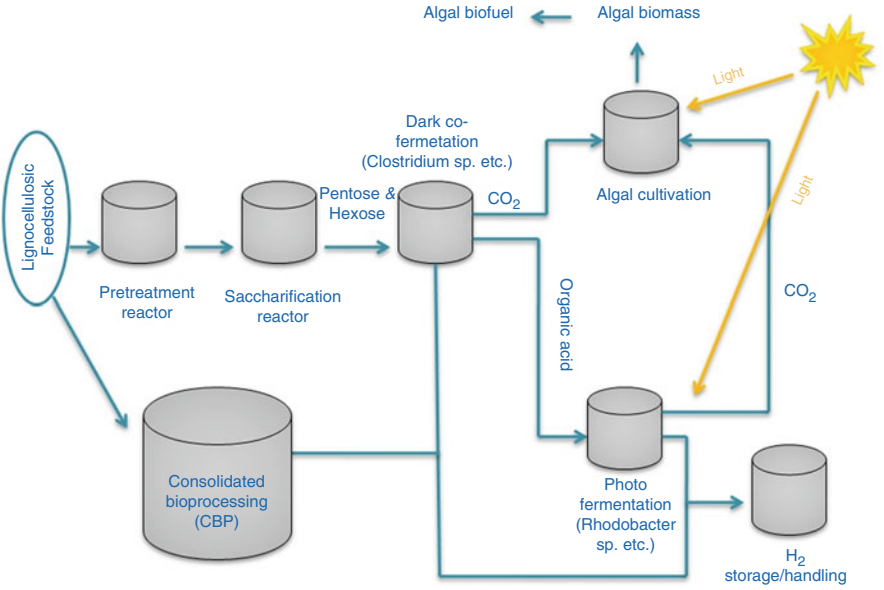

Fig. 5.3 Conceptual approach of sequential dark fermentation and photo-fermentation augmented with microalgal photoautotrophic process

dependent hydrogenase from *Pyrococcus furiosus.* Metabolic engineering has also been reported to increase the $BioH_2$ production rate. The modification in the native pathways depends on redirecting the metabolic flux which blocks those pathways which compete with H_2 production. However, the yield cannot be increased above the network potential. Therefore, nonnative pathways are employed which involves the expression of nonnative hydrogenase. For example, the nonnative hydrogenase from *E. cloacae* can be over expressed in non-H_2-producing *E. coli* to get the enhanced production rate of $BioH_2$ as compared to yield obtained from wild strain *E. cloacae*. Also, the significant progress has been achieved by using genetic tools, which improved our understanding of extremophilic microorganisms by manipulating the genetic characteristics for H_2 and carbon metabolism. Further, research and development in bioprocessing of extremophiles can lead to the commercialization of extremophile-based application in the near future.

5.7 Summary and Outlook

Conversion of LCB to $BioH_2$ shows an attractive pathway to meet the future demand of energy. These feedstocks are abundant and can be efficiently degraded by microorganisms to produce $BioH_2$. The methods involved in the production of $BioH_2$ from LCB are pretreatment, hydrolysis, removal of inhibitors, and fermentation. Use of extremophiles in producing renewable H_2 from LCB is gaining attention due to its advantages over other biological methods.

Existing processes of $BioH_2$ production from complex wastes, such as LCB, utilize several steps. The inclusion of several steps reduces the overall cost-efficiency of the process. An alternative to the use of various steps in H_2 production is the development of an efficient and cost-effective single-step process utilizing untreated lignocellulose-degrading and fermentative thermophiles (second-generation consolidated bioprocessing, CBP). Growth at high temperature favors the thermodynamics of stoichiometric H_2 yield and decreases the possibility of contamination by unwanted microorganisms that compete for the same substrates. The use of elevated temperatures also offer several potential advantages such as (1) improved hydrolysis of cellulosic substrates, (2) higher mass transfer rates leading to better substrate solubility, and (3) lowered risk of potential contamination, thus improving the overall economics of the process (Bhalla et al. 2013). The innovative CBP may impact current multiple-step conversion processes of complex wastes to biofuels by providing a safe, more efficient, sustainable, and economical process. However, this method needs further research and development to make this process feasible for commercial application. In a long term, progress in lignocelluloses breakdown and genetic tools to manipulate the H_2 production capabilities in thermophiles is expected to offer unique advantages to the design, construction, and application of an economically viable $BioH_2$ production system.

Take-Home Message

The use of second-generation feedstocks (lignocelluloses) to produce $BioH_2$ can be a promising and efficient method, which fulfill the future demand of energy. Recently $BioH_2$ production using extremophiles has gained high attention due to fast production rate without any preprocessing or mild processing of plant biomass. Extremophiles are reported to produce $BioH_2$ and other value-added products even from untreated lignocellulosic biomass. This chapter presented a review and in-depth analyses of extremophilic $BioH_2$ production from lignocellulosic biomass. The chapter also provided the knowledge on how to develop a more efficient and economical integrated processes for enhanced conversion of lignocellulosic feedstocks to $BioH_2$.

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Chapter 6
Biogas from Extremophiles

Karthik Rajendran and Gergely Forgacs

What Will You Learn from This Chapter?
This chapter provides an elementary understanding of biogas production processes and applications. The chapter covers the important parameters affecting biogas production, different types of digesters, and technology used in anaerobic digestion.

6.1 Introduction

Biogas is a mixture of methane and carbon dioxide produced as a result of anaerobic digestion (AD) of organic compounds. The methane fraction in the biogas is an energy-rich compound (~39.4 MJ/m^3), which can be used for different applications including cooking, heating, and electricity production and also can be used as fuel for vehicles. The history of biogas dates back to 3000 BC, where the Sumerians used the anaerobic digestion concepts to treat waste. Later in 1776, Alessandro Volta collected the gas from a lake and reported that the gas formation was due to a fermentation process and it can form an explosive mixture in the presence of air. However, only in 1821, the structure of methane was first elucidated. In 1897, Pasteur reported the first application of biogas by electrifying street lamps using biogas from wastewater treatment. Since then the application and market for biogas are widely increasing (Deublein and Steinhauser 2008).

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Biogas production can be divided into four different phases including hydrolysis, acidogenesis, acetogenesis, and methanogenesis. In the first phase, the complex substrates are broken down into their monomeric forms and long-chain fatty acids, for instance, cellulose to glucose, proteins to amino acids, and fats into fatty acids. In acidogenesis, the monomers and long-chain fatty acids are converted into short-chain volatile acids including valeric acid, propionic acid, and butyric acid. The short-chain acids are further converted into acetic acid, hydrogen, and carbon dioxide in acetogenesis phase. Finally, the products of acetogenesis are converted to methane and carbon dioxide in the methanogenesis phase. Approximately 70% of the biogas production is obtained from the acetic acid conversion and 30% from the hydrogen and carbon dioxide pathway in the acetogenesis phase (Rajendran et al. 2013).

The biogas is usually produced at three different temperature ranges: psychrophilic <25 °C, mesophilic 25–35 °C, and thermophilic >45 °C. The scope of this book is limited to extremophiles, and hence the main focus of this chapter will be on psychrophilic and thermophilic anaerobic digestion. This chapter focusses on anaerobic digestion from extremophiles including different process parameters and digester designs. Nextly, a comparative assessment of two extremophilic conditions, i.e., psychrophilic and thermophilic, was discussed. Later, the technology-wise comparison was made based on solids loading. The last section of this chapter covers on the overview on applications and economic outlook on biogas productions (Deublein and Steinhauser 2008).

6.2 Substrates

In theory, any organic substrate or biomass can be used for biogas production when they contain carbohydrates, proteins, fats, cellulose, and hemicelluloses. Some of the conventional substrates for biogas production include cattle manure, MSW, sludge, and food waste. Recently, certain new substrates are being explored including seaweeds, algae, and water hyacinth, and in addition sometimes a mixture of the substrate is used in the same digester called as co-digestion. In co-digestion systems, optimal C/N ratio and nutrient balance can be achieved, which leads to a higher biogas productivity (Al Seadi 2008).

There are certain factors which need to be considered when choosing a substrate for biogas production:

1. Content of organics in the substrate
2. Nutritional value
3. Pathogen-free substrates
4. Less heavy metals and other hazardous content
5. Expected methane content for further application
6. Treatment of digestate as a fertilizer

Table 6.1 Different substrates and their biogas yield

Substrate	Substrate classification	Dry matter (%)	Ash (%)	Biogas yield
Manure	Cow	38	14	0.6–0.8 m^3/kg TS
	Pig	20–25	NA	0.27–0.45 m^3/kg TS
	Poultry	89	33	0.3–0.8 m^3/kg TS
	Horse	28	NA	0.4–0.6 m^3/kg TS
Agricultural residues	Rice straw	91	13	0.55–0.62 m^3/kg TS
	Wheat straw	91	8	0.18 m^3/kg VS
	Maize straw	86	NA	0.4–1.0 m^3/kg TS
	Grass	88	6	0.28–0.55 m^3 /kg VS
	Corn stalk	80	7	0.35–0.48 m^3/kg VS
	Cassava peels	NA	NA	0.66 m^3/kg VS
Food wastes	Vegetable waste	5–20	NA	0.4 m^3/kg TS
	Kitchen/restaurant wastes	27/13	8	0.50/0.65 m^3 CH_4/kg VS
	Leftovers	14–18	NA	0.20–0.50 m^3/kg TS
	Food	25	NA	0.97–0.98 m^3/kg TS
Aquatic plants or seaweeds	Algae	NA	NA	0.38–0.55 m^3/kg VS
	Water hyacinth	7	NA	0.2–0.3 m^3/kg VS
	Caboma	NA	NA	0.22 m^3/kg VS
	Salvinia	NA	NA	0.15 m^3/kg VS

The list of some of the substrates and their expected biogas yield are provided in Table 6.1.

6.3 Process Parameters

6.3.1 Organic Loading Rate (OLR)

The organic loading rate (OLR) defines the load or the material intake that a reactor can handle. Usually, this is a design parameter, and it is in the correlation between HRT, biogas yield, and economics of the biogas plant. The OLR for the psychrophilic digestion varies between 0.2 and 1.2 kgVS/ m^3/day, while the thermophilic biogas production varies between 3 and 4 kgVS/m^3/day (Al Seadi 2008). The OLR can be calculated by the following equation:

$$\mathrm{OLR} = m \times \ c/V_R$$

where m is a mass of the substrate fed per time (kg/d), c is concentration of organics (%), and V_R is the volume of the digester (m^3).

6.3.2 Hydraulic Retention Time (HRT)

Hydraulic retention time determines the time that the organic load that stays inside the digester. The retention time determines the volume of the digester, for instance, a shorter retention time decreases the volume of the digester, while higher retention time which is often used in psychrophilic digestion increases the reactor volume. This is the reason why most psychrophilic digestion has a larger volume of the digester to treat the same amount of waste when compared to the thermophilic digestion. The HRT for the psychrophilic process varies between 60 and 80 days, while for the thermophilic process it is between 15 and 25 days (Al Seadi 2008). The HRT is given by the following equation:

$$\mathrm{HRT} = V_R/f$$

where HRT is retention time (days), V_R is volume of the digester (m^3), and f is influent flow rate (m^3/day).

6.3.3 Temperature

Temperature is another important parameter in anaerobic digestion processes. The biogas production can usually occur in three different temperature ranges: psychrophilic, mesophilic, and thermophilic digestion. The psychrophilic digestion is usually carried at temperatures less than 25 °C, and it is common in household biogas digester such as fixed dome/floating drum or balloon digesters. The mesophilic (25–35 °C) and thermophilic (>45 °C) digestion systems are quite common in industrial setups; however, the hydrolysis is more favorable in the thermophilic temperature range. In addition, the pathogens need to be treated, and if the digestion happens at a mesophilic temperature, the treatment of pathogens needs additional energy pushing the world trend toward biogas production from thermophilic digesters (Kabir et al. 2015).

6.3.4 pH

pH measures the alkalinity/acidity in the digester, and it influences the growth of microorganisms present in the digester. It also affects the dissociation of some compounds including ammonia, organic acids, sulfide, etc. Different microorganisms favor different pH, while most of the methanogens are active at pH 7.0–8.0, and the hydrolytic bacteria are active at pH 5.5–6.5. The dissolution of carbon dioxide in

water decreases with the increase in temperature, and as a result of dissolution, carbonic acid is formed, which decreases the pH in the system (Deublein and Steinhauser 2008; Al Seadi 2008).

6.3.5 Volatile Fatty Acids (VFA)

The stability of the anaerobic digestion process is affected by the volatile fatty acids (VFA) including acetate, butyrate, propionate, and valerate which are formed during the different stages of anaerobic digestion. The accumulation of the VFA causes a disturbance in the system, and it is a relative unit, where the same VFA level in two different systems can act differently due to the combination of microorganisms present in the system (Deublein and Steinhauser 2008).

6.3.6 Ammonia

The amounts of ammonia present in the digester play an important role in the inhibition of the anaerobic digestion process. The free ammonia is more toxic compared to the ammonia in ionic forms. Methanogens are mostly affected by the ammonia inhibition causing turbulence in the system, and the ammonia concentration should be maintained less than 1500 mg/l for an optimum process. The ammonia inhibition is about to happen more in thermophilic temperatures than mesophilic anaerobic digestion (Al Seadi 2008). The ammonia inhibition is calculated by the following equation:

$$\mathrm{NH_3} = \frac{T - \mathrm{NH_3}}{1 + \frac{H^+}{k_a}}$$

where k_a is dissociation constant.

6.3.7 Nutrients

The nutrients can be divided into macro- and micronutrients. The macronutrients include carbon, nitrogen, phosphor, and sulfur, and an optimum ratio is 600:15:5:1. The micronutrients include iron, nickel, cobalt, selenium, molybdenum, and tungsten, which are relatively important for the growth of the microorganisms.

6.4 Types of Biogas Digesters

6.4.1 CSTR

CSTR is the acronym for the continuous stirred tank reactor, which is the most common anaerobic digestion system on the industrial scale. In CSTR, the digester contains a mixer in the middle, which rotates continuously. The produced biogas is stored and collected in a balloon or a reservoir. The mixer is usually centered vertically and rotates in a low axial speed to avoid fouling and provide enough mixing for the substrate to get in contact with the microorganisms (Al Seadi 2008).

6.4.2 UASB

UASB, the acronym for upflow anaerobic sludge blanket reactor, which is mainly used to treat wastewater and the biogas is a by-product. In this type of digesters, the feed is provided from the bottom, and it flows through the agglomerated biomasses which are mostly methanogens. The rapid contact between agglomerated biomass and wastewater results in higher rate of biogas production. Usually, the retention time varies between 6 h and 8 days, which is much shorter compared to the retention time for solid substrates and conventional biogas systems. In addition, the methane content is high from the UASB due to two reasons: (1) the dissolution of carbon dioxide at lower temperatures and (2) the produced biogas goes through the long column, where the CO_2 gets absorbed (Al Seadi 2008).

6.4.3 Plug Flow

The plug flow designs are more common in the tropical countries for the domestic biogas production. The balloon and PVC-based digesters are quite popular which are operated in the ambient conditions. In the plug flow digesters, the movement of the substrate will happen from one end to another end without mixing. Since there are no moving parts in this digester, the lifetime of this digester is considerably long. In addition, the plug flow digesters are helpful in handling fouling problems associated with biogas production (Rajendran et al. 2012).

6.4.4 Fixed Dome

There are about 30 million fixed dome digesters available in China and India, while it is the most common digesters for household and agricultural purposes. The fixed

dome digesters contain three chambers including the feed tank, digester, and digestate collection pit. These digesters are usually placed beneath the ground. These digesters are not temperature controlled and can affect the biogas production during winter seasons (Rajendran et al. 2012).

6.4.5 *Floating Drum*

In the floating drum design, an iron or fiber drum is placed on top of the digester on the vertical axis. This allows the drum to move up and down based on the accumulation of the biogas produced. The stored biogas has enough pressure to use it for cooking application. The constraint with this digester system is that the size cannot be increased more than 50 m^3 due to the weight of the drum and some fibrous materials could block the movement of the drum. Figure 6.1 shows the schematic sketch of the different biogas digester designs (Rajendran et al. 2012).

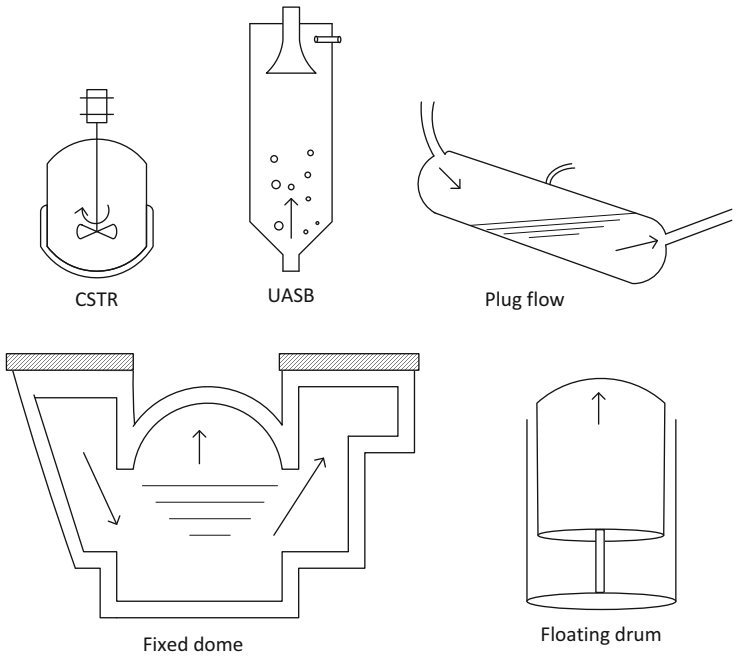

Fig. 6.1 Different biogas digester designs

6.5 Psychrophilic Digestion

6.5.1 Overview

Psychrophilic digestion takes place at temperatures less than 25 °C, and it usually happens on the domestic scale, especially during the winter season. The consequence of the psychrophilic digestion is that it increases the retention time which subsequently increases the size of the digester. The common retention time for the psychrophilic digestion is about 60–80 days. Another disadvantage with the psychrophilic digestion is that effective hydrolysis may not happen as it requires higher temperatures, and as a result, the overall efficiency of the process is reduced.

Another aspect in the psychrophilic digestion is the dissolution of carbon dioxide. In lower temperatures, the carbon dioxide is dissolved in the system leaving a high concentration of methane, which is beneficial for the application purposes. However, the dissolution of carbon dioxide results in the formation of carbonic acid which reduces the pH and thus affects the overall stability of the systems. This is the reason why most domestic biogas systems fail and the OLR cannot be increased higher than 1.5 gVS/L/day.

6.5.2 Advantages and Disadvantages

The advantage for the psychrophilic digestion is the less energy requirement, and it is much suitable for the tropical conditions or the household digesters, where the temperature effect is negligible. The digestate after treatment in psychrophilic processes contains higher TS than the thermophilic processes, which is why it is easy to handle as a fertilizer. On the other hand, the disadvantages include the treatment of harmful pathogen after treatment, before releasing to the environment. The longer retention times in psychrophilic digestion increases the total digester volume is another hassle.

6.6 Thermophilic Digestion

6.6.1 Overview

Thermophilic anaerobic digestion (50–60 °C) is an alternative to conventional mesophilic anaerobic digestion (35 °C). Thermophilic AD has been studied since the 1930s. Since the late 1980s, it was known that thermophilic AD can achieve higher efficiency and can operate under lower retention time than comparable mesophilic digestion (Willis and Schafer 2006). During this period pathogen reduction was also recognized. However, the application was hindered by the major concern of process stability and the odor formation. These concerns were dispersed

in the 1990s, where the stable operation of thermophilic digestion was proved. Currently, there are still more mesophilic AD plants, but the gap between mesophilic and thermophilic plant tends to decrease.

6.6.2 *Advantages and Disadvantages*

Traditionally, AD plants have operated in the mesophilic temperature range, since it was difficult and costly to maintain a high temperature in the digester. However, it was recognized that thermophilic AD holds several advantages over the mesophilic. Thermophilic digesters operate at a faster rate (shorter hydraulic retention time) and with higher loading. They archive higher methane production and enhanced pathogen removal. Due to certain legislation, many feedstocks have to undergo a sanitation process at elevated temperature (i.e., 70 °C for 60 min). In these cases, since the feedstock is already heated, thermophilic digestion can be more favorable from an economic point of view. The main disadvantages are higher energy input and a higher degree of operation and monitoring. Typically, thermophilic methanogens are more sensitive to changes in operation conditions; therefore without proper control, ammonia and/or volatile fatty acid (VFA) inhibition is more likely to happen (Forgács 2012). Table 6.2 presents a detailed comparison between the conventional mesophilic and thermophilic AD.

6.7 Wet and Dry Digestion Technology

Anaerobic digesters are categorized based on their total solid (TS) content as wet ($\leq$10% TS) and dry ($\geq$15% TS). Both wet and dry anaerobic digestion processes have their advantages and disadvantages (as summarized in Table 6.3). Wet digestion systems are designed to utilize dilute organic slurries containing typically less than 15% TS which is in liquid form. Solid waste also can be used in wet AD systems; however, water addition is required to reduce the solids content. Moreover, due to a

Table 6.2 Comparison of mesophilic and thermophilic anaerobic digestion

	Mesophilic AD	Thermophilic AD
Temperature	25–45 °C	50–60 °C
Digestion period	Longer (18–60 days)	Shorter (8–18 days)
Gas production rate	Slower	Faster
Reactor volume	Bigger	Smaller
Operating cost	Cheaper	Dearer
Capital cost	Cheaper	Dearer
Pathogen kill	Lower	Higher
Toxicity problem	Less	More
Loading rate	Lower	Higher

Table 6.3 Comparison of wet and dry digestion

	Wet anaerobic digestion	Dry anaerobic digestion
Dry matter	5–10%	15–50%
Reactor design	Complete mixing	Plug flow, complete mixing
Reactor volume	Larger	Smaller
Capital cost (setup)	Higher	Cheaper
Operating cost	Dearer	Cheaper
Gas production/unit feedstock	Lower	Higher
Mass removal rate	Lower	Higher
Gas quality	Stable	Not stable
Toxicity problem	More	Less
Digestate dewatering	Expensive	Cheaper
Suitability	Ideal for slurry, wastewater, and manure	Ideal for silage, straw-based feedstock

large amount of water in the AD reactor, the system requires a large amount of energy for heating. The digestate of wet AD process has high water content and relatively low solids, and it can generate difficulties in the digestate management, for example, storage and transportation, and limits its potential application (Li et al. 2011).

Dry AD digesters are designed to operate under high TS content, typically, a thick slurry containing more than 20% TS is utilized in dry systems. Typically, the fresh feedstock is mixed with partly digested material from the digesters to accelerate the digestion process and adjust the moisture level. In other systems, leachate is recycled into the digester to speed up the anaerobic process. It is worth to mention that increasing the solid content has a limitation; several researchers reported that solid content over 30% strongly inhibits the methanogens. For handling the solid waste, most designs involve conveyor belts, screws, and powerful pumps, which are more expensive and in some cases more energy-intensive; however, heating has minimal energy requirements. Dry systems are considered to be more robust and flexible than wet systems. They are not sensitive to contaminants including glass, plastic, and grit. Dry digesters can operate higher organic loading and require smaller digesters. Moreover, management of the digestate is much easier due to its lower moister content. The digestate can be further treated by composting. It is stackable, and it can be stored in the open and in stockpiles on site; therefore there is no need for large storage tanks or lagoons for the digestate (Li et al. 2011).

6.8 Application of Biogas

As it stated in previous section, biogas is typically composed of 50–70% methane (CH_4) and 30–50% carbon dioxide (CO_2). Moreover, it usually contains impurities such as nitrogen (0–5%), oxygen (1%), hydrocarbons (1%), hydrogen sulfide (0.5%), ammonia (0.05%), water vapor (1–5%), and siloxanes (0–50 mg/m^3).

Biogas can be utilized in many ways including cooking, heat, and/or electricity production or as vehicle fuel; however, in many cases most or all part of the impurities should be removed before application (Holm-Nielsen et al. 2009).

6.8.1 Cooking

Cooking is one of the simplest applications for biogas. It is widespread in developing countries, including China and India, where biogas is typically produced in small-scale facilities. For this purpose, the biogas produced is distributed directly through a pipe from the household digester (1–3 m^3) to the kitchen, where the gas is burned for cooking on a gas stove. Biogas can be used in its raw form (cleaning or upgrading the biogas is not required). Currently, more than 30 million household operates in China. By 2020, the number of digesters is expected to exceed 80 million supplying over 300 million people with biogas for cooking propose (Rajendran et al. 2012).

6.8.2 Electricity Production

Producing electricity and heat in combined heat and power plants (CHP) is the most common utilization form of biogas in industrial countries. Electricity from biogas is considered as green electricity, and most of the European countries developed a financial support system to promote it. In a CHP unit, the biogas is converted to heat and electricity. The efficiency of the CHPs varies depending on the size; typically electrical efficiency varies around 30–40%, while thermal efficiency is around 40–50%. The requirements for the quality of the gas in a CHP unit are quite strict; however, the H_2S level should be lower than 250–500 ppm, and the siloxanes should be removed to guarantee a long operation of the CHP plant (Ryckebosch et al. 2011).

6.8.3 Vehicle Fuel

Upgraded biogas, usually referred to as biomethane, can be used as a vehicle fuel or injected directly to the natural gas grid. Several upgrading techniques exist, such as water scrubbing, pressure swing adsorption, chemical absorption, as well as cryogenic and membrane separation. During the upgrading carbon dioxide, hydrogen sulfide, ammonia, particles, and water (and sometimes other trace components) are removed to obtain a product gas with methane content above 97%. Worth to mention that, the regulation and specification of biomethane varies from country to country (Nijaguna 2006).

6.9 Economic Outlook

The economics of an AD plant varies widely depending on the location, the size of the plant, the technology used, and other factors such as the funding sources, supply chain, and market demand situation. This section presents the main economic aspects that should be considered, without any cost figures were given.

Anaerobic digestion can only be successful and economical if there is a continuous demand for its products. Therefore, the first step for identifying the optimal location is a comprehensive market demand analysis. This analysis should include the following aspects:

- Identification of market size and ideal form of the products (i.e., electricity, heat, biomethane, etc.).
- Identification of all possible customers for biogas and/or digestate.
- What is the customers' willingness and ability to pay?
- Which other factors influence the sales of products from AD?
- Current regulation and legislation which can affect the business.

The market demand analysis is typically followed by a cost-benefit analysis, which evaluates that implementation of the AD system is sustainable or not. This analysis includes all cost related to an AD installation as well all the predicted revenues (Rajendran et al. 2014).

6.9.1 Investment Costs [Also Called Capital Expenditures (CAPEX)]

The investment cost depends on several factors such as:

- Size and technology of the AD system
- Land space required and costs of land acquisition
- Planning studies and required surveys
- Civil works at the facility including support structures and buildings
- Construction of the digester
- Biogas pipes
- Large and small mechanical equipment (e.g., shredder)
- Transport of materials (including insurance)
- Customs duties, taxes

6.9.2 Operational Costs [Also Called Operational Expenditures (OPEX)]

Operation costs include all costs associated with the operation of the service and maintenance of the system. At a household level, operational costs are negligible,

because waste material is used as feedstock and the operation of the digester mostly carried out by family members. For industrial biogas plants, operation cost includes insurance, electricity, water, transport of feedstock, spare parts, staff salaries, etc.

6.9.3 Revenue

The major sources of income from anaerobic digestion come from the sale of biogas and digester-related products; however, in many cases, the AD plants have other income sources as well. The most common revenues are:

- Sales of biogas (or a product from its processing such as heat and electricity)
- Sales of digestate as fertilizer
- Income from financial support system (e.g., renewables obligation certificates, feed tariffs)
- Gate fees

6.10 Conclusion

This chapter summarizes the different technologies used in anaerobic digestion specific to the purpose of extremophiles, i.e., psychrophilic and thermophilic AD technologies. Furthermore, the important process parameters in AD processes were discussed on how it will affect both psychrophilic and thermophilic digestion. Later, the technologies on wet and dry digestion were compared on the different solid loading levels and its importance in the handling of digestate and how it can reduce the overall volume of the digester. The briefings on economic outlook suggest the important factors, which should be considered when designing a biogas plant.

Take-Home Message

- The choice of the digester type used depends on the substrates, cost, and location of the plant.
- Thermophilic digestion technology produces more biogas compared with psychrophilic digestion.
- Profitability of the biogas plant is influenced by the purity of raw material/waste obtained and the type of product produced.
- Dry digestion technology is preferred over wet digestion due to the easiness of handling digestate after digestion.
- Financial support/Incentivization system plays a crucial role in the profitability of anaerobic digestion.

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Chapter 7
Conversion of Lignocellulosic Feedstocks into Biogas

Ritika Verma, Abhilash Kumar Tripathi, and Sudhir Kumar

List of Abbreviations

BMIMCI	1-*N*-butyl-3 methylimidazolium chloride
C/N	Carbon/nitrogen
CNG	Compressed natural gas
CSTR	Continuous stirred-tank reactor
DMEA	Dimethyl ethanol amine
DSEAR	The Dangerous Substances and Explosive Atmospheres Regulations
EBA	European Biogas Association
EU	European Union
FAO	Food and Agricultural Organization
FID	Flame ionization detector
FYM	Farmyard manure
GC	Gas chromatography
HDPE	High-density polyethylene
HRT	Hydraulic retention time
ILs	Ionic liquids
MBPCI	1-Allyl-3-methylimidazolium chloride, 3-methyl-*N*butylpyridinium chloride
MEA	Ethanol amine
NBMMP	National Biogas and Manure Management Programme
NDIR	Nondispersive infrared
NMMO	*N*-methylmorpholine-*N*-oxide monohydrate
OFMSW	Organic fraction of municipal solid waste

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OLR	Organic loading rate
PEG	Polyethylene glycol
PEM	Proton exchange membrane
PFR	Plug flow reactors
PSA	Pressure swing adsorption
SRT	Solid retention time
TCD	Thermal conductive detector
TS	Total solid content
UASB	Upflow anaerobic sludge blanket
UTs	Union territories
VFAs	Volatile fatty acids
VOCs	Volatile organic compounds
VS	Volatile solids
WRAP	Waste and Resources Action Programme

Words "biomass" and "lignocellulosic feedstock" are used interchangeably in the chapter.

What Will You Learn from This Chapter?

This chapter will discuss in detail about the different processes involved in conversion of lignocellulosic feedstocks into biogas and the shortcomings of the conventional processes. The different methods of physical, chemical, and biological pretreatment for the lignocellulose will be discussed. The chapter also addresses methods of biogas production, analysis, and the different biogas reactors.

Highlights

- Biogas production using lignocellulosic feedstock as a cost-effective raw substrate.
- Pretreatment methods as a panacea to combat recalcitrant structure of lignocellulosic feedstock.
- A comprehensive review of biogas production process along with diverse pathways opted by the microbial communities indulged, contribution of extremophiles, biogas reactors used, and different parameters affecting the production.
- Biogas analysis, upgradation methods, and utilization as vehicle fuel, PEM fuel cell, for heating applications.
- Co-digestion and lignin extraction process which aid in enhancing the energy and economic efficiency.
- Correlation of biogas in developing sustainable environment is presented, along with safety issues.

7.1 Introduction

7.1.1 History of Biogas

Plinius and Van Helmont were the early observers of biogas emanating from swamps and decaying organic matter in the seventeenth century. By 1776, a man named Volta reported the explosive nature of this gas when mixed with air (Barker 2001; Sasso et al. 2012). Nature and chemical composition of biogas was established by Dalton, Henry, and Davy (1804–1810). Microbiological nature of the process was determined by Bechamp and Tappeiner in the late 1800s. A French student, Gyon, produced first man-made gas by fermenting methane in 1884. Bechamp and Gyon were students of Louis Pasteur who himself devoted some time to understand the process of biogas production. Biogas from sewage was used to illuminate streetlamps in Exeter, England, in 1896. In 1900 biogas generating plant from human feces was established in an asylum for lepers in Matunga, India (Sathiananthan 1975). Extensive development in biogas generation happened in mid-1900 in many countries like Germany, France, the USA, India, Japan, Italy, Kenya, Russia, Israel, and the Philippines. This was the time when scientist studied the stoichiometry of the bioreactions, factors affecting biogas production, energy production, and isolated methane-producing bacteria, i.e., *Methanosarcina barkeri* and *Methanobacterium formicicum*. Today successful biogas technology depends upon efforts of the early researchers.

7.1.2 Present Global Scenario and Scope

Germany dominates biogas sector being a market leader and biggest producer (Fig. 7.1). Germany produced 18,244 gigawatts hours (Gwh) biogas power in 2012. The existence of the Renewable Energy Act and support of German government in development and operation of methanization plants strengthened their countrywide program. Various substrates like agricultural residues (mainly corn), cow manure, and industrial and food wastes are used to run these biogas plants (Wieland 2003). Germany and Sweden are also advanced in cleaning and upgradation of plants for biogas which are 120 and 53 in number, respectively (Thrän et al. 2014a, 2014b). Italy, Austria, and Belgium also developed biogas as an alternative source of renewable energy and positive in setting up of upgrading technologies to produce biomethane. European Biogas Association (EBA) promotes the development of biogas in Europe; on the other hand, Italy, Brazil, and the USA have maximum gas-fueled cars, but their biogas upgradation is in a nascent state. The USA relies mainly on cow manure, and capturing of landfill gas is a popular means of energy generation with a potential of reducing 4% greenhouse gases of the country (Cuellar and Michael 2008). In the UK, sewage and food waste biogas plants are common, and their grid used biogas first time in 2010. Switzerland is also

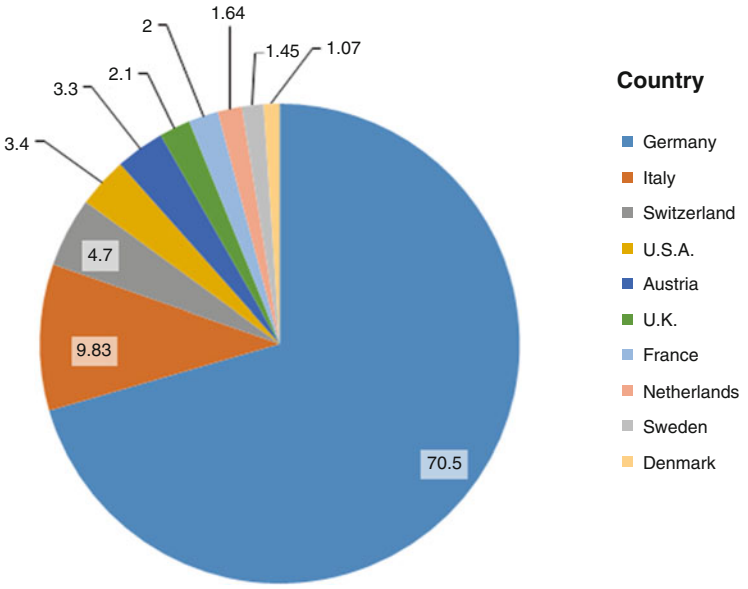

Fig. 7.1 Percentage of biogas digesters in IEA bioenergy member countries (2012) [adapted from Thrän et al. (2014)]

focusing on biogas program with more than 600 biogas plants. India and China have established domestic biogas technology where rural households use cow manure or sewage. Biogas plants are mainly low-cost fixed dome-type concrete structures. Kitchen waste-based biogas plants are also becoming popular. Biogas is called as "gobar gas" (gobar means cow manure) in Indian subcontinent (India, Pakistan, Nepal, Bangladesh). Biogas is used as an important renewable energy source across the world, though biomethanation technology is not yet widespread.

7.2 Biomass for Biogas Production

7.2.1 Conventional Sources

When the biogas technology laid its foundation stone, the only source of feedstock was animal manure. That may be cow manure, pig manure, or chicken manure (either in liquid or solid form). With time, dependence on other sources for biogas feedstock grabbed the pace and got satisfactory outcomes as well. Other sources include domestic waste (fruit and vegetable waste), organic fraction of municipal solid waste (OFMSW), waste from food industry (whey, brewery waste, fruit waste, etc.), and energy crops (corn, millet). However, in 2008, Food and Agricultural Organization (FAO) gave a report which concluded that many of the proposed

energy crops to be used in biofuel synthesis double as food crop. So in order to avoid circumstances of food insecurity, other alternatives should be opted (Kigozi et al. 2014). Though the use of OFMSW as a feedstock for biogas production has been in the headlines, as an environmentally sustainable energy source, we cannot let the lignocellulosic feedstock go waste, which is in bulk as MSW. Eventually, realizing the abundance of lignocellulosic material distributed in the environment, a very sustainable option of feedstock source for biofuels like biogas and bioethanol came into existence.

7.2.2 Lignocellulosic Biomass: Waste to Fuel Approach

At present we need to produce fuels (biogas) from the biomass that is renewable as well as available in abundance. Lignocellulosic biomass thus appears to be the most promising sustainable option. As of now, bulk lignocellulosic residues (forestry, agriculture, and agro-industrial residues) go waste, either disposed off in the landfills or burned in the fields. Instead, a better and intelligent act would be to earn money and energy out of this waste by using it as feedstock for energy (biogas) production. Lignocellulosic biomass has high sugar content because it chemically comprises of cellulose, hemicellulose, and lignin, each of which being polymer of different sugars could be digested, fermented, and converted to various value-added products including biogas.

7.2.3 Hurdles Offered by Recalcitrant Structure of Feedstock

Plant cell wall is made up of a complicated lignocellulosic material, meant for prevention and protection of plants from microbial degradation. The cross-linked major constituents of lignocellulose, namely, cellulose (35–50%), hemicellulose (20–35%), and lignin (10–25%), are responsible for recalcitrance (resistance to degradation by enzymes and microbes) (Saha 2003). Lignin acts as a shielding barrier for the degrading enzymes and hence prevents access to the utilizable cellulose and hemicellulose content. The hurdle of this compact structure needs to be removed so that cellulose and hemicellulose could be accessed and degraded by microbial enzymes to produce fermentable sugars. To achieve this, the lignocellulosic biomass needs to be pretreated first. Pretreatment solubilizes the substrate, removes lignin, and reduces cellulose crystallinity, thereby enhancing the biodegradability of the lignocellulosic substrate (Cara et al. 2008).

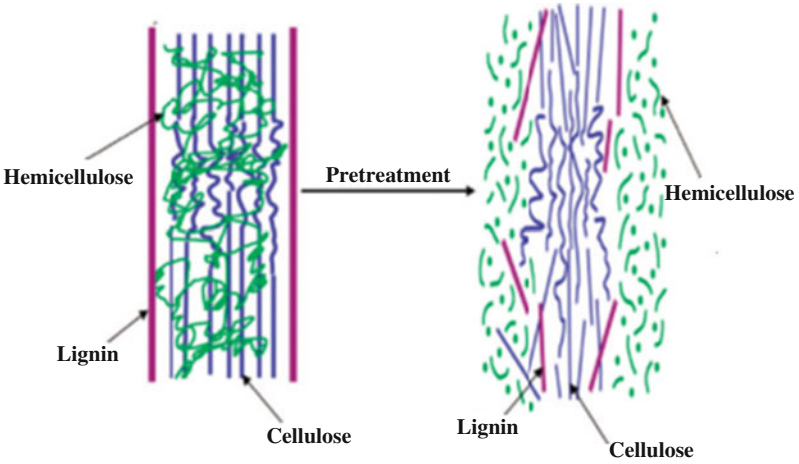

Fig. 7.2 Schematic of the role of pretreatment in the conversion of biomass to fuel [adapted from Hsu et al. (1980)]

7.3 Various Pretreatment Methods

The recalcitrance of the lignocellulosic biomass demands for certain treatment to be done, before the biomass is fed as a substrate into the biogas digesters. The objective of biomass pretreatment is to create certain structural alterations in the biomass, such that the maximum sugar yields are availed from the cellulose or hemicellulose fraction. Pretreatment opens up the complex structure, exposing different lignocellulose constituents to be then easily acted upon by the degrading microbial enzymes (Fig. 7.2) (Hsu et al. 1980). The method of pretreatment is formulated as per the type of substrate, whose bioavailability is to be increased (Mosier et al. 2005). Pretreatment can thus be considered as a rate-limiting step because efficiency of this step decides the final yield of desired products.

7.3.1 Physical Pretreatment

7.3.1.1 Mechanical Pretreatment

It is carried by mechanically comminuting the lignocellulosic biomass by means of chipping, grinding, or milling, so as to increase the accessibility of the biomass by reducing the crystallinity of cellulose and increasing the surface area. Grinding or milling usually results in particle size ranging from 0.2 to 2 mm, whereas chipping gives particle size of 10–30 mm (Sun and Cheng 2002). However, due to high power requirement, the method turns out to be very expensive. In 2010, Menind and

Normark used knife milled hay as a feedstock in digester and observed approximately 10% increase in biogas yield (Menind and Normak 2010).

7.3.1.2 Extrusion

The biomass is subjected to forces like high shear, temperature, and pressure which causes the breakdown of cellular structure of biomass into an accessible and digestible form. Faster rate of biogas production has been observed with the extruded biomass (straw, fresh grass, solid manure, and cattle litter) as compared to the untreated one (Hjorth et al. 2011). High electricity cost and wearing of screws are a drawback.

7.3.1.3 Steam Explosion (Autohydrolysis) and Hydrothermolysis

In autohydrolysis, lignocellulose substrate is collected in a closed system and heated at a high temperature (160–260 °C) and high pressure (0.69–4.83 MPa) for duration of several seconds to few minutes. Sudden release of pressure makes rapid loss of moisture content from the biomass which causes explosion (Sun and Cheng 2002) and thus leads to certain degree of lignin transformation and hemicellulose hydrolysis. In 2010, Bruni and his coworkers achieved 29% increase in methane yield by using steam digested biofibers (separated from digested manure) (Bruni et al. 2010). Another method is hydrothermolysis, in which at elevated temperature, water is maintained in its liquid state by applying pressure (Rogalinski et al. 2008). Chandra et al. practiced hydrothermolysis of wheat straw and found 29% increased methane production (Chandra et al. 2012).

7.3.1.4 Irradiation

It includes pretreatment with microwave, ultrasound, gamma ray, and electron beam, causing degradation by heating. However, microwave technology is the most preferred one as it causes very rapid heating of a large volume of substrate, saving both time and energy (Zheng et al. 2014). There are reports where microwave treated wheat straw (used as substrate) was found to increase methane yield by 28% (Jackowiak et al. 2011). However, at very high temperatures of 200 or 300 °C, decrease in gas yield occurs (Sapci 2013).

7.3.2 Chemical Pretreatment

7.3.2.1 Acid Hydrolysis

Acid pretreatment is carried either using dilute acid (e.g., 0.1%) at high temperature (e.g., 230 °C) or concentrated acid (e.g., 30–70%) at low temperature (e.g., 40 °C). Dilute acid treatment results in efficient hemicellulose hydrolysis (up to 100%), in addition to lignin disruption. While concentrated acid causes effective cellulose hydrolysis, but due to its toxicity and corrosive nature, it is comparatively less preferred. Both organic and inorganic acids are used for dilute acid pretreatment, but sulfuric acid is the most commonly used (Zheng et al. 2014).

7.3.2.2 Alkaline Hydrolysis

It causes saponification and cleavage of cross-linkages between lignin and other polymers, which increases biodegradability of lignocellulosic biomass. Different alkalies like NaOH, $Ca(OH)_2$, KOH, and $NH_3.H_2O$ are used, but sodium hydroxide is the most popular one (Tarkow and Feist 1969). However, lime [$Ca(OH)_2$] is another alkali which is extensively preferred, because of its low cost, high recovery, and minor environmental effects. It also controls pH changes during anaerobic digestion and increases the efficiency of digestion process. In recent studies, Song et al. 2013, 74% enhancement in methane yield has been observed on using $Ca(OH)_2$ for digesting rice straw (Song et al. 2013).

7.3.2.3 Oxidative Pretreatment

This method makes use of oxidizing agents like hydrogen peroxide, ozone, oxygen, or air to carry removal of lignin for the biomass. It causes hemicellulose hydrolysis, but the main purpose is delignification. Song et al. achieved 50–120% increased methane yield from rice straw treated with hydrogen peroxide (1–4%) (Song et al. 2012). In 2013, Cesaro and Belgiorno studied the effect of ozone treatment with OFMSW and observed 37% increment in gas production (Cesaro and Belgiorno 2013). Wet oxidation is another type of oxidative pretreatment in which water is used along with an oxidizing agent.

7.3.2.4 Ionic Liquid (IL) Pretreatment

Pretreatment with ionic liquids decreases the crystallinity of cellulose and results in its dissolution. The process is cost-effective due to its occurrence under mild conditions (90–130 °C and ambient pressure), recovery of ILs at the end of the process, and easy downstream processing (Heinze et al. 2005). Various ILs,

including *N*-methylmorpholine-*N*-oxide monohydrate (NMMO), 1-*n*-butyl-3 methylimidazolium chloride (BMIMCl), 1-allyl-3-methylimidazolium chloride, 3-methyl-*N*butylpyridinium chloride (MBPCl), and benzyldimethyl (tetradecyl) ammonium chloride, have been studied for pretreatment of lignocellulosic biomass (Zheng et al. 2014).

7.3.3 Biological Pretreatment

Since different conventional pretreatment methods demand for high energy and economic input, biological pretreatment has been opted as an efficient, eco-friendly, and cheap alternative (Taherzadeh and Karimi 2008). Several fungi classes, including brown-, white-, and soft-rot fungi, have been used for pretreatment of lignocellulosic biomass for biogas production, but white-rot fungi are the most effective (Sun and Cheng 2002). In 2013, Zhao reported 154% increase in methane yield by pretreating yard trimmings with a white-rot fungi *Ceriporiopsis subvermispora* (Zhao 2013). As compared to fungal pretreatment, a microbial consortium usually results in high cellulose and hemicellulose degradation. In 2011, a thermophilic consortium was used by Zhang and his coworkers to treat cassava residues and achieved 96% higher methane yield than untreated residues. Sometimes, enzyme pretreatment is also practiced (Zhang et al. 2011; Lin et al. 2010).

7.3.4 Combined Pretreatment

This method includes a combined effect of various pretreatment methods. In 2013 Michalska and Ledakowicz tried a combination of NaOH with enzymatic pretreatment on *Sorghum* Moench and achieved 50% and 300% higher methane yield in comparison to the biomass treated with enzyme and NaOH alone, respectively (Michalska and Ledakowicz 2013).

7.4 Biogas Production

7.4.1 Bioreactions

Anaerobic digestion (AD) is the sequential multistep process, involving degradation of organic material into methane in the absence of oxygen. The digestion is completed in four stages which involve metabolic reactions carried by various participating microbial communities. The four stages are hydrolysis/liquefaction, acidogenesis, acetogenesis, and methanogenesis (Fig. 7.3).

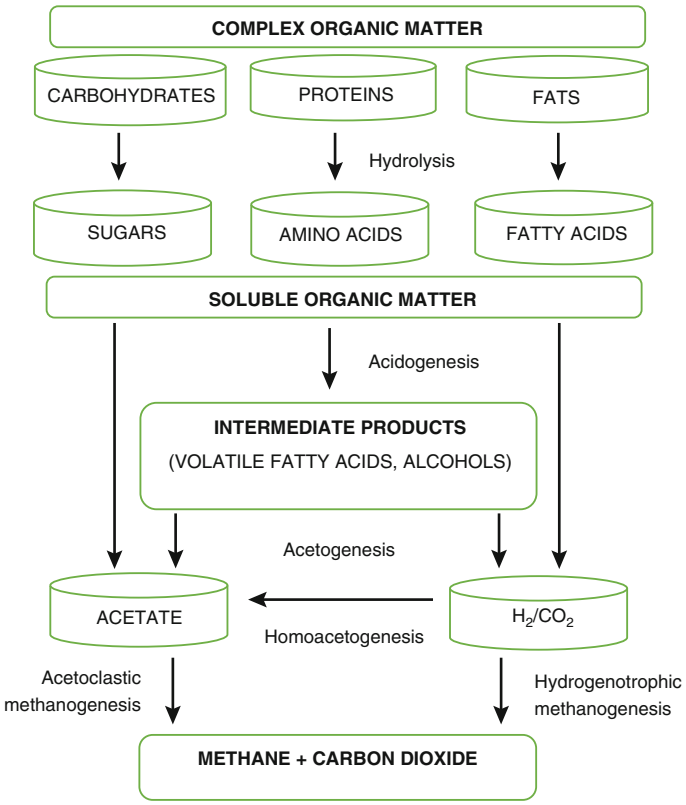

Fig. 7.3 Schematic diagram of anaerobic digestion

7.4.1.1 Hydrolysis

In this step the complex polymers of organic biomass are converted into simple monomers by the action of various hydrolytic enzymes (cellulase, amylase, lipase, or protease), produced by facultative hydrolytic anaerobes. If substrate particles are large in size with low surface to volume ratio, then hydrolysis is considered as a rate-limiting step. While if the substrate particles are easily degradable, then acetogenesis and methanogenesis become the rate-limiting steps (Bjornsson et al. 2001; Vavilin et al. 1996). Bacteria of genus *Bacteriocides*, *Clostridia*, *Streptococci*, *Enterobacteriaceae*, and *Bifidobacteria* participate in this phase. At the end of this stage, the substrate molecules become soluble enough to be transportable through the microbial cell membrane and available for fermentation in the following steps of AD.

Hydrolysis/Liquefaction Reactions

1. Fats → fatty acids

2. Carbohydrates → sugars
3. Protein → amino acids
4. Nucleic acids → purines and pyrimidines

7.4.1.2 Acidogenesis (Fermentation)

The soluble organic molecules, produced after hydrolysis, are acted upon by fermentative bacteria, which are facultative and obligate anaerobes. They convert sugars, amino acids, and fatty acids to hydrogen, acetate, and carbon dioxide. The intermediates such as volatile fatty acids (VFAs) like propionate, butyrate, and acetate and alcohols and lactates are also produced. The bioreactions involved in conversion of glucose to acetate, ethanol, and propionate are shown below (Kangle et al. 2012):

1. $C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$
2. $C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2$
3. $C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O$

Acidogenic bacteria consume all the oxygen present inside the digester to produce acids and hence create anaerobic conditions for the methanogens. Also, these bacteria remove all degradable organic molecules and convert them into those substrate molecules which are readily available to methanogenic bacteria, along with some short-chain fatty acids and alcohols (Kangle et al. 2012).

7.4.1.3 Acetogenesis

Bacteria participating in this stage are also known as acid formers. Acetogenic bacteria convert VFAs and alcohols into acetate, H_2, and CO_2, and homoacetogenic bacteria further convert H_2 and CO_2 into acetate (Sterling et al. 2001). Bacteria involved in the process are obligated proton reducing in nature and belong to genus *Clostridium*, *Syntrophobacter*, *Syntrophomonas*, *Peptococcus*, *Lactobacillus*, and *Actinomyces*. During acetogenic reactions, low H_2 partial pressure is the condition for syntrophic bacteria to be thermodynamically favorable (Schink 1997). The principal products formed at the end of this step include acetic acid, propionic acid, butyric acid, and ethanol.

7.4.1.4 Methanogenesis

During this stage, methane formers convert the products of acetogenesis into methane and carbon dioxide and also maintain low H_2 partial pressure. Methanogenic bacteria are facultative anaerobes and are highly sensitive to the environmental fluctuations inside the digester. These bacteria may be acetate consumers, e.g., *Methanosarcina barkeri*, *Methanococcus mazei*, and *Methanothrix soehngenii*, or

H_2/CO_2 consumers. Thus, based on the type of substrate consumed by the methanogenic bacteria, methanogenesis can be categorized as below (Kangle et al. 2012):

- **Hydrogenotrophic methanogenesis:** Only methane is produced by consuming CO_2 and H_2 as shown below:
  $CO_2 + 4\ H_2 \rightarrow CH_4 + 2H_2O$
- **Acetotrophic or acetoclastic methanogenesis:** Methane and CO_2 are produced by consumption of acetate as shown below:
  $CH_3COOH \rightarrow CH_4 + CO_2$

Out of the total methane produced, 70% fraction is produced by the conversion of acetate, while H_2/CO_2 acts as precursor for the rest of the 30% fraction (Smith and Mah 1966). This step is the slowest among all and depends on factors like type of feedstock, temperature, loading rate, and pH.

7.4.1.5 Role of Extremophiles in Anaerobic Digestion

As the name indicates, extremophiles are robust organisms having incredible capability of thriving extreme environmental conditions like low/high pH, low/high temperature, acidity, salinity, etc. By far, this potential has uplifted the contribution of extremophiles in many industrial processes, and the current hot spot is a contribution in biofuel synthesis (bioethanol, biogas, biohydrogen, biodiesel). Methanogens have been found in the extreme environments (15 °C to 100 °C) in which all electron acceptors other than CO_2 are depleted. In methane production, many of the thermophilic methanogens rule the process of AD (*Methanosarcina*, *Methanococcus*, *and Methanothermococcus*). Besides this, extremophiles tolerating extreme acidic conditions also hold an important contribution in biogas production.

7.4.2 Key Parameters Affecting Biogas Production

7.4.2.1 Temperature

A wide range of temperature ranging from very low temperature (<20 °C) to extreme high temperature (>60 °C) is reliable for carrying AD. The choice of the temperature thus depends on the type of participating microorganisms, for example, psychrophiles (12–24 °C), mesophiles (22–40 °C), and thermophiles (50–60 °C) are having their own respective temperature regimes for digestion (Vintila et al. 2010). But mainly thermophilic and mesophilic conditions rule the environment inside the biogas plant. Recent reports depict that biogas production is more rapid in thermophilic range than in mesophilic range. However, it has been found that thermophilic methanogenic bacteria show much more sensitivity toward temperature fluctuations, while mesophilic bacteria sustain temperature fluctuations without showing any

significant influence on biogas production. Digestion carried in thermophilic conditions shares certain advantages, as it reduces the retention time, enhances the digestion process, and effectively destroys weeds and pathogens. Higher energy consumption, high degree of instability, and higher rate of ammonia inhibition put on some limitations on the thermophilic digestion as well. But still the higher energy consumption is offset by the higher biogas productivity under the thermophilic conditions (Dobre et al. 2014). The type of substrate also decides the required temperature conditions, for example, fat-containing substrates are very well degraded at thermophilic range.

7.4.2.2 Reaction Medium pH

AD comprises of different stages which require specific pH range to conduct respective bioreactions, because the microflora involved in different stages have different pH optima. During AD, hydrolysis and acidogenesis reactions occur at a pH range of 5.5–6.5, while methanogenesis occurs at pH 6.5–8. Any deviation from the respective optimum pH range would result in either lesser yield or complete inhibition of the process, for example, methane formers are very sensitive to acidity, and so slight drift toward acidic range could be detrimental for methanogenesis process. During digestion, decrease in pH is the result of excess accumulation of volatile fatty acids and CO_2 inside the reactor (Sibiya and Muzenda 2014). On the other hand, increase in pH occurs because of accumulation of ammonia produced by protein degradation. Temperature also influences the pH, as at high temperature, solubility of CO_2 decreases leading to high pH, whereas at low-temperature CO_2 being easily solubilized in the reaction medium forms carbonic acid, resulting in low pH. So, the major task is to maintain the acidity and alkalinity ratio during digestion. If conditions become more acidic, lime could be added for neutralization, while hydrochloric acid could be added to compensate extreme alkaline conditions. However, generally the bicarbonate buffer system controls the pH variations inside the anaerobic digester (Dobre et al. 2014).

7.4.2.3 Carbon/Nitrogen (C/N) Ratio

The type of the feedstock fed into the digester decides the fate of C/N ratio. If the feedstock has high proportion of carbon content, C/N ratio is also high, thus causing deficiency of ammonia and low methane yield. Whereas if feedstock has high nitrogen content, then the C/N ratio will be low which results in access to ammonia accumulation and causes toxicity to methanogenic bacteria. The optimum C/N ratio for AD is 30:1, as it has been observed that in an anaerobic process, the carbon utilization by bacteria is 30 times more than nitrogen (Sorathia et al. 2012). In order to maintain the desired optimum ratio, additional substrates could be fed into the digester to make up for the missing element.

7.4.2.4 Retention Time

This time is referred to as the duration for which the fermentable material remains inside the digester. HRT (hydraulic retention time) is the time for which soluble component of the reaction medium remains inside the reactor and SRT (solid retention time) represents residence time of solid component (bacteria). On an average, the retention time lies within the range of 30–50 days, but it varies according to many other factors like climatic conditions, location of the digester, loading rate, type of feedstock, and prevailing temperature conditions inside the digester. Digestion carried in thermophilic range has lower retention time relative to one in mesophilic range. Since methanogenic bacteria have longer generation time, SRT should be of sufficient duration to avoid the wash out of active microbial community causing low gas yield (Gerardi 2003).

7.4.2.5 Agitation

Agitation is practiced to ensure maximum and efficient interaction of bacterial population with the feedstock. It also avoids scum formation and thermal stratification within the medium, maintains physical and chemical uniformity throughout the digester, and causes rapid dispersion of metabolic waste and toxic products (minimizing their inhibitory effects on methane production). Rate of agitation and equipment used for agitation vary according to the digester design and the components to be agitated (Lemmer et al. 2013).

7.4.2.6 Organic Loading Rate (OLR)/Total Solid Content (TS)

OLR refers to the amount of organic matter fed into the digester with respect to the volume of the digester per day. Usually it is expressed as kilograms of volatile solids (VS) fed to the digester per day per cubic meter of the volume of the digester. There is an optimum value of loading rate which depends on the capacity of digester, retention time, activity of microflora, and digestion temperature. Deviation from this optimum value (whether under loading or overloading) results in decreased gas production. Recommended value of organic loading rate is 0.2 kg VS/m^3/day. The solid content of the reaction medium also needs to be in an optimum ratio with the water content; usually 10% of solid content with 90% of water is preferred (Sorathia et al. 2012). Highly concentrated and highly diluted media both have negative impact on the yield of gas. The formula for OLR calculation is as follows (Mattocks 1984):

$$\mathrm{OLR} = \frac{Q^* S}{V}$$

Where:

OLR: Organic loading rate (kg substrate/m^3/day),

Q: Flow rate of input (m^3/day)
S: Concentration of VS in the input (kg/m^3)
V: Reactor volume (m^3)

7.4.2.7 Feedstock and Nutrient Concentration

All biodegradable substrates that constitute carbohydrates, fats, proteins, cellulose, and hemicellulose as major components can be used as feedstock in biogas plant. Composition of the feedstock directly affects the yield of gas, for example, highly lignified substrate (wood) is not preferred, and fat-rich substrate gives maximum gas yield, whereas carbohydrate- and protein-containing substrates give comparatively lesser gas yield. But for the efficient AD, along with feedstock, some macro- and micronutrients are also required by the anaerobic microflora, for growth and survival. Trace elements like iron, nickel, cobalt, selenium, molybdenum, and tungsten are important for the growth of microorganisms. Iron is required in higher concentration of 1–10 mg/l, whereas other micronutrients are used in very low concentration of 0.05 and 0.06 mg/l (Bischoff 2009). Nickel is an indispensable nutrient for methanogenic bacteria because it helps in the synthesis of cofactor F_{430} which participates in methane formation.

7.4.2.8 Inhibitory Products

Inside the digester, various harmful materials are present that could inhibit the growth of microorganisms and thus affect methane formation. Some inhibitory products may enter the digester along with feedstock, while some are produced from various metabolic reactions occurring during AD. Ammonia, detergents, sulfides, and heavy metals are toxic if present in high concentrations and thereby inhibit the fermentation (Sorathia et al. 2012).

7.4.2.9 Moisture Content

Optimum moisture content has to be maintained inside the digester for the efficient processing of metabolic reactions by the microorganisms. Generally 90% moisture content to the total volume of feedstock is maintained. If the conditions are too moist, it will lead to decrease in the gas production, whereas if moisture content is too low, reaction medium will become too acidic because of accumulating acids and will inhibit the methane formation (Sorathia et al. 2012).

7.4.3 Co-digestion: A Concept to Boost Biogas Production

With a desire for the betterment of the AD process, the term co-digestion has come into existence and is gaining popularity because of its appreciable outcomes. Co-digestion implies the simultaneous digestion of homogeneous mixture of multiple biodegradable organic feedstock in a digester. Co-substrates used in the digestion process establish a positive synergism, leading to better nutritional balance (C/N ratio) and required moisture content inside the digester, and, hence, boost up the yield of biogas. Traditionally, only one substrate was used in AD, but later on it was realized that the biogas production could be enhanced and stabilized, if some additional substrates could be added, compensating for the missing nutrients. For example, animal manure is considered to be a rich source of ammonia and can be inhibitory to AD (Dobre et al. 2014). Likewise, lignocellulosic materials due to recalcitrance create hindrance in digestion. However, co-digestion of the two feedstocks results in better yield due to their complementary effect, as all the nutrients required are supplied by the manure and C/N balance is maintained by the carbon-rich lignocellulosic substrate (Lehtomaki et al. 2007).

7.4.4 Analytical Methods

7.4.4.1 Collection of Biogas Sample

Sampling is a crucial step in biogas analysis as it directly affects the final results. The point of collection of sample should assure that the biogas is properly mixed and stable in terms of temperature and pressure.

7.4.4.2 Basic Composition Analysis of Biogas

Gas chromatography (GC) is mostly an opted technique, for getting specific and accurate results. Often, it is used in combination with mass spectrophotometry to enhance the accuracy in detection. In GC, thermal conductive detector (TCD) is used for analyzing gases like CO_2, methane, propane, butane, and pentane isomers. Flame ionization detector (FID) is used to analyze C_6 or C_8 hydrocarbons. Portable gas analyzers working on the principle of infrared absorbance nondispersive infrared (NDIR) absorbance are also used, for example, galvanic cells and electrochemical sensors. Volatile organic compounds (VOCs) can be detected by using FID equipped gas analyzers (Wojdyla et al. 2012).

7.4.4.3 Analysis of Condensate, Dust, and Oils

Karl Fischer method is used for the detection of water content, and gravimetric methods are employed for the detection of dust and oil (Wojdyla et al. 2012).

7.4.4.4 Detection of H_2S, Ammonia, Organic Sulfur Compounds, Chlorine, and Fluorine

Specified spectrophotometric and colorimetric methods as well as analysis using portable gas analyzer give satisfactory results. For H_2S detection, electrochemical sensors could be used, but due to the interferences created in a gas mixture, NDIR sensors are preferred. While for detection of organic sulfur compounds, chlorine and fluorine, GC can be performed; using alkali-flame ionizer detector, flame-photometric detector (FPD) and electrochemical detector (Wojdyla et al. 2012).

7.4.4.5 Siloxane Detection

Due to chemical instability of siloxanes, their sampling and analysis are quite problematic. Individual siloxanes are identified and quantified using gas chromatography equipped with FID and FPD detectors. Mass spectrometry can also be used with gas chromatography (Matsui and Imlamura 2010).

But among all the analytical methods for biogas, GC has so far been considered as the most reliable method in terms of giving very fast and precise results of biogas analysis (Wojdyla et al. 2012).

7.5 Biogas Reactors

Biogas reactors have gained importance due to its wide acceptance of biogas technology. Correctly designed biogas reactors satisfy the users' need and are convenient to operate and maintain. Appropriate biogas plants, which are technically advanced and cost-effective, are needed for economical and sustainable development.

All biogas reactors operate on the same basic principle. They hold the manure and other organic wastes under oxygen-scarce conditions and maintain an ecological niche for methane-forming microbes (methanogens) to flourish. Some of the commonly used digesters are:

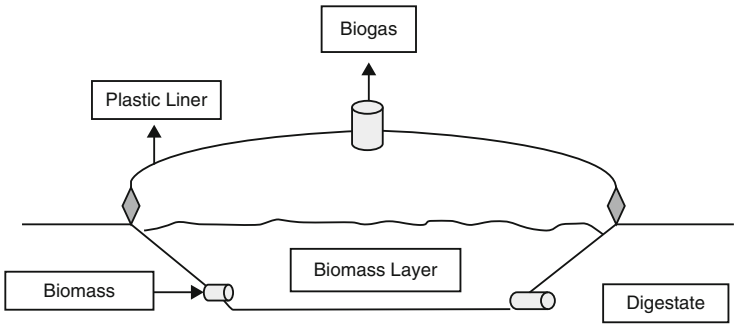

Fig. 7.4 One cell covered anaerobic lagoon

7.5.1 Covered Anaerobic Lagoon

This system has been mostly used with dairy and swine manure wastes. It consists of at least one storage cell, covered with flexible gas tight cover, usually made from high-density polyethylene (HDPE) (Fig. 7.4). Single-cell system must be large enough to sustain 3–6 months of manure storage and excess rainfall. On the other hand, in a multiple cell system wastewater, excess rainwater can be stored in one dedicated cell. Overtime, excess solids and organic material can form sludge which remains in digester for years. This sludge reduces the excessive loading of phosphorus, nitrogen, and potassium in the effluent wastewater. Therefore, a lagoon provides treatment as well as storage. Depending on the management of the facility, HRT of this system can vary between 60 and 360 days (Beddoes et al. 2007). This reactor is dependent on temperature for efficient biogas production; hence these are sometimes referred to as "ambient temperature digesters." They are most suited for tropics than temperate regions.

7.5.2 Plug Flow Reactors (PFR)

PFR (Fig. 7.5) digests organic waste and manure as they move through the reactor in an axial direction having minimal mixing compared to other reactors. The manure entering the reactor displaces an equally likely volume of manure inside the digester. Since mechanical stirring or agitation may not be part of system design, PFR requires a high solid content so as to keep the particles suspended as waste flows through the reactor. Low solid content in the waste "short-circuits" the digester (Beddoes et al. 2007). PFR is made of a concrete tank which is rectangular and horizontal buried in the ground in an inclined position. The manure tank has straight or U-shaped flow pattern and has retention time of 18–20 days. PFR is a two-phase system separating acidogenesis and methanogenesis. The covering of the digester may either be of concrete (fixed) or polypropylene (flexible) for biogas recovery.

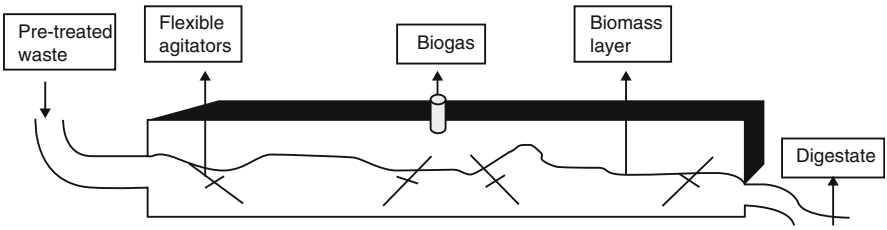

Fig. 7.5 Plug flow reactor

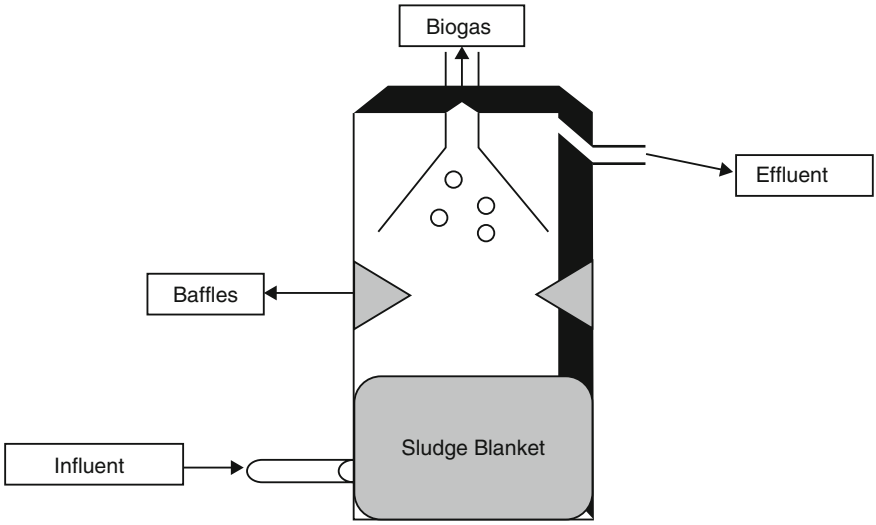

Fig. 7.6 Upflow anaerobic sludge blanket reactor

High total solid (TS) requirement in waste makes PFR unsuitable for waste with low solid content (<10%). As PFR requires some type of bedding, incompatibility of bedding material such as sand creates problem (Beddoes et al. 2007).

7.5.3 Upflow Anaerobic Sludge Blanket (UASB) Reactor

UASB comprises of a single tank (Fig. 7.6) in which the waste to be treated is introduced at the bottom and effluent is collected from the top. The microorganisms form a bed of activated sludge and break down organic matter by AD producing biogas. Gas recirculation ensures good contact between microbes and substrates even at low gas production rates and high hydraulic loading rates. This system is easier to construct and can tolerate high organic and hydraulic loading rates (Karthikeyan and Kandasamy 2006). It is highly suitable for tropical regions because

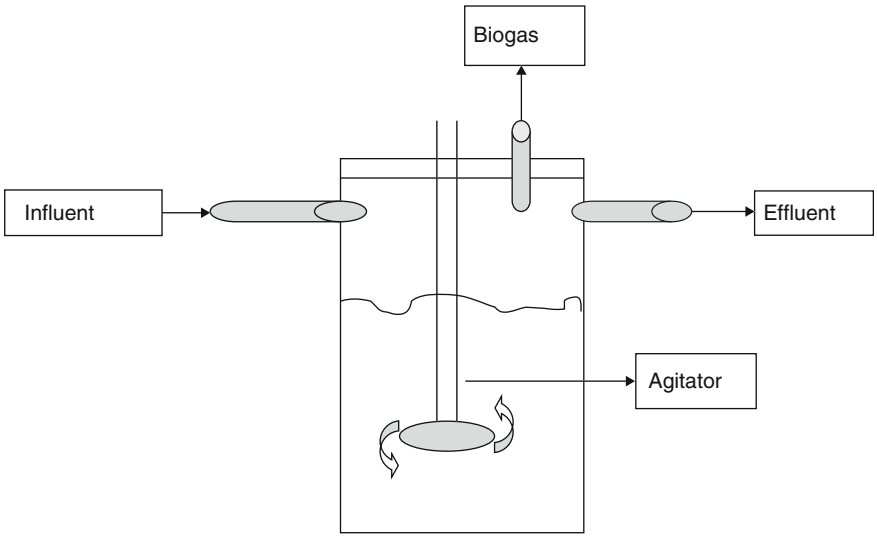

Fig. 7.7 Continuous stirred-tank reactor

they work best at mesophilic temperatures. The effluent can be stabilized using appropriate posttreatment technologies and used for agricultural practices.

7.5.4 Continuous Stirred-Tank Reactor (CSTR)

These digester types are also known as complete mix systems. They can tolerate toxicity issues and handle shock loading better than PFR. Most of CSTR reactors are vertical digesters which are made of reinforced concrete or steel tanks that often have a conic bottom for easy stirring. They are outfitted with stirrer or pumps and are insulated (Fig. 7.7). The gas produced is usually stored in an external storage facility or is collected by building a roof of concrete or steel on top of the digester. The HRT of this system varies between 5 and 20 days (Beddoes et al. 2007). However, one major disadvantage of this reactor is the improper immobilization of anaerobic biomass. Due to this some of the influent biomass is discharged without being digested, which can harm the system's efficiency.

7.6 Biogas Digestate: A Valuable Resource

Biogas production from organic substrates, especially agricultural residues, is a holistic approach which takes into consideration the socioeconomic and environmental benefits. AD as a sustainable technology not only results in energy and heat

production but also gives digestate as a by-product. Digestate is a rich source of macronutrients like nitrogen, phosphorus (P), and potassium (K). Thus, it can be used as biofertilizer as value addition of the entire process. Apart from biofertilizers, production of other value-added products has been a hot topic of research. Digestate liquor (obtained after dewatering) can be used as substrate for growth of microalgae due to its high organic content (Uggetti et al. 2014). One other possibility is the cultivation of mushroom using biogas digestate mixed with agricultural waste (Savoie et al. 2011).

7.7 Biogas Upgrading and Utilization

Upgradation means cleaning of unwanted gases like carbon dioxide, hydrogen sulfide, and nitrogen along with water vapors and particulates. Removal of carbon dioxide which is the second most abundant (30–40%) component of biogas makes it upgraded to natural gas, though natural gas also has higher level of hydrocarbons. To avoid corrosion of the pipelines, water removal from the biogas is necessary. H_2S removal is necessary as it has toxic effects on methanogens and corrodes the surface and pipelines. For application of biogas as vehicle fuel or for grid connection, biogas needs to be upgraded to have high energy content (Fig. 7.8). Further, scarcity of electricity and rising oil and natural gas prices warrant the upgradation of biogas. Upgradation technologies should be sustainable, nonpolluting with lower investment and operational cost. Methane loss should be minimized as this has 20 times more potent greenhouse gas compared to CO_2.

7.7.1 Need for Upgradation and Standards

To enable the biogas to be injectable into natural gas grid or to use as vehicle fuel, upgradation is required to separate out CO_2, H_2S, and water vapors. However, prior to injection certain standards have to be maintained. In this regard, propane is usually added to increase the calorific value of biogas (Hussey 2013). There are some safety standards (Table 7.1) that need to be maintained before injecting upgraded biogas into transmission or distribution networks (Potts et al. 2008).

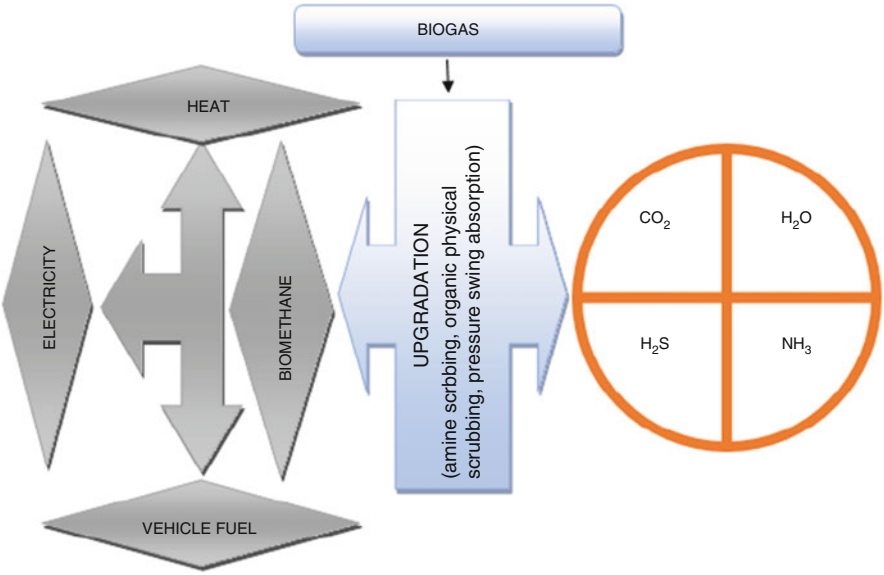

Fig. 7.8 Biogas upgradation and utilization

Table 7.1 Natural gas network requirements [Adapted from Potts et al. (2008)]

Parameter	Quality requirement
Carbon dioxide	2.5 mol%
Gross Wobbe Index	47.2–51.4 MJ/Nm^3
Gross calorific value	36.9–42.3 MJ/Nm^3
Hydrogen	0.1 mol%
Hydrogen sulfide	5 mg/Nm^3
Oxygen	0.001 mol%
Total sulfur	50 mg/Nm^3

7.7.2 Upgradation Technologies

7.7.2.1 Removal of CO_2

Chemical Scrubbing

The principle of this technology is the use of chemical reagents that selectively bind to CO_2, removing it from the biogas, e.g., amines are the commonly used reagents such as ethanol amine (MEA) and dimethyl ethanol amine (DMEA) (Appl et al. 1982). This is an efficient method with CH_4 losses $< 0.1\%$ and regeneration of liquid after removing CO_2 by heating. This is a mature technology of removing CO_2 to upgrade biogas (Bauer et al. 2013). The principle of this technology is solubility differences of CO_2 and CH_4. CO_2 has more solubility than CH_4 in amine solutions.

Water Scrubbing

It is a commonly available upgrading technique. The principle of this technology also relies on better solubility of CO_2 than CH_4 in water. Solubility of CO_2 at 20 °C is 1.7 (approximately) g gas per kg water, whereas CH_4 has 0.025 g gas per kg water. Water scrubbing is a physical process, and biogas is pressurized (6–10 bars) preferably at low temperature dissolving CO_2 in biogas slurry and in turn increasing the concentration of CH_4 in the outgoing gas. CO_2 is then released from the water using desorption column by addition of counterflow of air at atmospheric pressure. Water is regenerated after scrubbing the pressurized gas. This technology is successfully demonstrated with a most suitable flow of biogas at 30–100 N m^3/h (Petersson and Wellinger 2009). Counterflow of gas and water needs to be maintained to avoid CH_4 loss and energy minimization and to enhance CO_2 solubility.

Organic Physical Scrubbing

Organic solvents such as polyethylene glycol (PEG) are used for absorption of CO_2 rather than water. CO_2 solubility is about five times higher in PEG than water (Tock et al. 2010). Digester size can be smaller since lower flow and volume of PEG are required due to higher solubility of CO_2 into it. PEG can be regenerated by depressurizing and heating. Genosab and Selexol are the commercial solvents used in organic physical scrubbing (Burr and Lyddon 2008).

Adsorption Technologies

Pressure Swing Adsorption (PSA)

PSA is a technique used to separate CO_2 from biogas by adsorption at elevated pressure. Adsorption columns usually have zeolites and activated carbon as the adsorbing material. When the adsorbing material is saturated with CO_2, pressure is reduced in continuous steps, i.e., pressure swing adsorption (PSA) using several columns, desorbing CO_2 releasing to atmosphere with a 2–10 min cycle (Grande 2011; Spoorthi et al. 2010). PSA is a commercialized low-cost dry technology with an output of up to 98% CH_4 without consuming any water or producing effluent (Santos et al. 2011). Removal of H_2S and CO_2 is required before feeding the biogas into PSA columns, to prevent the damage to adsorbing material.

7.7.2.2 Removal of H_2S from Biogas

H_2S removal from biogas is necessary due to its toxic effects on methanogens and to avoid corrosion of pipelines. Depending upon application and H_2S amount,

physicochemical or biological methods of desulphurization can be used, e.g., precipitation of FeS by addition of Fe salt solutions ($FeCl_3/FeSO_4/FeCl_4$) and adsorption on iron oxide-coated (like Fe_2O_3) material. Application of extremophiles (*Thiobacillus* sp. and *Sulfolobus* sp.) in biological desulphurization is also common using oxygen (max. limited to 12% v/v to avoid the risk of explosion). The use of trickling filters having immobilization of microorganisms (*Thiobacillus* sp. and *Sulfolobus* sp.) is also preferred to increase the efficiency of removal of H_2S. Lowering of pH indicates the microbial desulphurization, and then sulfur-containing solution is removed (Abatzoglou and Boivin 2009; Ryckebosch et al. 2011).

7.7.2.3 Removal of Particulates and Water

Removal of particulates and water vapors is also required to enable the biogas to inject to natural gas grid or to use as vehicle fuel. Mechanical filters are used to separate particulates of biogas. Water-saturated biogas is either passed through adsorbents such as SiO_2 and activated charcoal to absorb water or alternatively water is condensed by increasing pressure and decreasing temperature. Glycol is also in use as absorption medium for water in certain cases.

7.7.2.4 Membrane Separation

The principle of separation is selective permeability of membranes mainly for CO_2 retaining CH_4 (>98%). These membrane systems made of glassy polymers are in use since 1990 (Petersson and Wellinger 2009), and now improved versions are fabricated in countries like France, Germany, and Austria. Particulate matters and water vapors need to be removed before feeding the biogas to membrane systems. H_2S reduction is also advisable before membrane separation (activated charcoal may be used); this leads to purified upgraded biomethane from landfill gas.

7.7.3 Applications

Biogas use for cooking and heating applications is ongoing since long. The heat provided by biogas plant is available throughout the year, irrespective of the seasonal changes (as in case of solar energy), and makes the technology economically viable. The heat produced is used for drying processes, e.g., hay drying, for conversion of liquid manure into fertilizer by heating, cleaning of milk equipments, heating greenhouses, and heating industrial plants or swimming pools (Deublein and Steinhauser 2008). Now, injection of biogas into grid also supports end users that are not near to the site of biogas production. The need and demand of using upgraded biogas as vehicle fuel have seen a steady increase in the last decade. This is primarily

due to high price of gasoline fuels and their global warming potential. In contrast to this, the vehicles running on CNG reduce CO_2 emission by more than 95% (Potts et al. 2008). Also there is significant reduction in emission of particulates as compared to vehicle operating on diesel and petrol engines. The use of proton exchange membrane fuel cell (PEM fuel cell) system on biogas is another step toward efficient and environment-friendly power generation from biogas. The fuel cell system works by carrying an electrochemical process in which the source fuel (e.g., hydrogen from biogas) being fed into the system gets directly converted into an electric current, on reacting with an oxidant (e.g., oxygen from air) in the presence of a catalyst (Scholz and Ellner 2011). The ongoing process of fabrication of biotoilets based on biogas technology is a recent application in Indian Railways (Kumar 2013).

7.8 Biogas and Environment

7.8.1 Regulations and Safety Guidelines

In different countries, the respective authorities and organizations put forth set of certain regulations and guidelines for the promotion of safe and efficient handling of biogas plants. The objective of these regulations is to prevent or minimize the probability of hazards due to biogas plants. German biogas association presents some of the hazards arising from biogas plants, as listed below (Deublein and Steinhauser 2008):

- Leakage of gases (NH_3, H_2S, CH_4) from the plant, causing suffocation, irritation, odor problem, and risk of explosion
- Mechanical, electrical, or fire hazards resulting in loss of life and property
- Severe falls for workers causing serious injuries or even death

With respect to such hazards arising from the biogas plants, some general regulations and safety guidelines are designed, which vary among different countries. Few are discussed ahead.

7.8.1.1 Regulations for Construction of Biogas Plants

- There should be a legal framework of plant construction and operation.
- All designing and technical installations of the plant need to be approved by respective authorities and also should undergo inspection periodically.
- Location of biogas plant should be such that it does not conflict with public interest.
- It should be approachable because of its demand on agricultural products and its requirement to remove the residue and also to serve the public supply of electricity, gas, and heat.

- Proper safety valves and security system must be installed. Danger-prone parts must be labeled.

In order to encourage the construction of biogas plants, governments of the countries even provide subsidies for constructing the plant. Like in India, the Ministry of New and Renewable Energy is implementing the National Biogas and Manure Management Programme (NBMMP) in all the states and UTs of the country, under which the Ministry provides subsidy for constructing family-type biogas plants (National Biogas and Manure Management Programme, Ministry of New and Renewable Energy, Govt. of India. http://mnre.gov.in/schemes/decentralized-systems/schems-2/).

7.8.1.2 Feedstock and Residue (Digestate) Used

- German biogas association states that animal manure and slurries from diseased livestock should not be fed to the digester.
- Biomass used in AD should be pathogen-free. According to the European Regulation EC 1774/2002, separate pre-sanitation of specific feedstock categories is mandatory.
- Residues produced after AD are used as fertilizer, according to the laws of respective country.

There is a quality protocol developed by the Environment Agency and WRAP (Waste & Resources Action Programme), applicable in England, Wales, and Northern Ireland, which sets up a criteria to produce quality digestate from AD of source-segregated biodegradable waste (Anaerobic Digestate: End of Waste Criteria for the Production and Use of Quality Outputs from Anaerobic Digestate of Source Segregated Biodegradable Waste, Quality Protocol Report (2012) http://www.biofertiliser.org.uk/).

7.8.1.3 Feeding Biogas to the Gas Network

According to the EU guideline, there should not be any restriction in feeding biogas to the gas network, until it creates any technical problem or impairment of security. The law says that the biogas producer will have to arrange the client on his own, for selling his biogas (Deublein and Steinhauser 2008).

7.8.1.4 Regulations Related to Safety, Health, and Environment Issues

- These include laws and guidelines to avoid fire and explosion hazards, leakage of toxic gases, and prevention from injuries.
- Different electrical components and appliances should be installed in accordance to the locations of the explosion-endangered spaces around the plant.

- Positive pressure needs to be maintained all the time to avoid explosion due to forced entry of air under negative pressure. Emergency alarming system should be there.
- Well-insulated gas pipes should be there to give protection against continuous fire, and certified flame traps must be there.
- The operation of engines, machines, and plant must correspond to the state of the art of noise protection.
- Biogas asphyxiation is reported in an enclosed space causing deaths in the past (Osbern and Crapo 1981). Similar concerns are raised for the use of biotoilets in Indian Railways, because toilets are small in railways and methane displaces oxygen in an enclosed space causing asphyxiation (Kumar 2013).

In England, anaerobic digester operators have to comply with certain health and safety regulations. Lists of regulations like the Confined Spaces Regulations 1997, Work Equipment Regulations 1998 (PUWER), and the Workplace (Health, Safety and Welfare) Regulations 1992 are followed to avoid any risk to safety and health, especially for the workers working in confined spaces near the plant (Confined spaces: a brief guide to working safely (2016) http://www.hse.gov.uk/). For the protection from fire, explosions, and substances corrosive to metals, the Dangerous Substances and Explosive Atmospheres Regulations 2002 (DSEAR) are implemented.

7.8.2 Positive Impact on Environment

The biogas technology has effectually complied with being an environment-friendly source of energy. Remarkable environmental benefits that have been availed from this technology include:

7.8.2.1 Contribution to Climate Change

Reduction of Greenhouse Gas Emission

Methane is a potent greenhouse gas. US Environmental Protection Agency states that methane is over 20 times more effective at trapping heat in the atmosphere than carbon dioxide over 100 years (Inventory of US Gas House Emissions and Sinks 2015, EPA430-R-15-004. http://www.epa.gov/climatechange/Downloads/Biogas-Roadmap.pdf). This shows that entrapping methane gas will largely contribute to prevent climate changes. Landfills release methane on the decomposition of the lignocellulosic residues and promote global warming. Biogas plant comes with a solution to capture this methane for creating energy rather than escaping into the atmosphere (Hughes et al. 2013).

Maintains Closed Carbon Cycle in the Environment

Though biogas combustion also releases CO_2, still it adds no extra carbon to the atmosphere. The reason being that this CO_2 fraction originally comes from the organic plant material formed during the photosynthesis. So by the combustion of biogas, stored fraction of CO_2 is again returned to the atmosphere and further again comes back to plant. Hence a complete closed or neutral carbon cycle is maintained.

7.8.2.2 Positive Impetus to the Agricultural Sector

Provides Quality Organic Fertilizer

Farmyard manure (FYM) and composted manure have been so far the only options to be looked upon as a source of organic fertilizer. But onset of biogas technology has given a revolutionary blow to the agricultural sector by producing good quality of organic fertilizer for sustainable agriculture (Kumar et al. 2015). AD occurring inside biogas plant produces a by-product called biogas slurry which comprises of substantial amount of macro- and micronutrients imperative for plant growth (Alam 2006). Biogas slurry is nutrient efficient like the conventional organic fertilizers (Ishikawa et al. 2006) making it a good source of quality organic fertilizer. Since biogas slurry fulfills nutritional demand for plant growth, there is reduced relying on synthetic fertilizers (Khan et al. 2014).

Positively Adds to Crop Yield and Quality

Digested slurry from biogas plant is rich in good amount of organic matter as well as supplies all the indispensable macro- and micronutrients for good growth and development of crops. Application of biogas slurry has efficiently increased the yield of various crops including field crops, tobacco, caster, onion, peas, mustard, banana, pearl millets, and sugarcane (Kumar et al. 2015). Not only crop yield, but it also enhances the crop quality by increasing the nutritive value and biomass. Its application results in better-quality vegetable crops as compared to that with synthetic fertilizers (Krishna 2001), and it also inhibits diseases (Liu et al. 2008). In some cases, even the slurry could be used in combination with chemical fertilizers to get outstanding improvements. For example, protein content of baby corn cob was increased by 100% and total sugar content by 4%, when biogas slurry and nitrogen fertilizer were used in combination (Khan et al. 2014).

7.8.2.3 Mitigation of Environmental Pollution

The biogas technology checks on different forms of pollution. Being a substitutive energy source (cooking, heating, electricity generation), it reduces air pollution from

burning of fossil fuels. The use of biogas as a fuel minimizes deforestation for fuel wood and thus also checks desertification and soil erosion (Khoiyangbam 2011). During AD, organic nitrogen gets transformed into ammonia, which is readily uptaken by plants, before it gets converted to nitrate. So this prevents nitrate leaching and thus checks pollution of groundwater (Khan et al. 2014).

7.9 Concluding Remark

- Biogas as a renewable fuel is continuously gaining importance, and upgraded biogas can be an alternative solution to the production of heat and electrical energy.
- Processed biogas can be used as a substitute for compressed natural gas (CNG) for transportation purposes. Many biogas upgrading technologies have been developed, and they have been continually improved.
- Pretreated lignocellulosic biomass has emerged as an important substrate for biogas production vis-a-vis a conventional substrate like MSW and cow manure.
- Type of digesters and biogas production depend upon the landscape, environmental factors, and requirement of the end users. Inclusion of biogas as a fuel under the renewable energy acts will strengthen the biogas program worldwide.

Take Home Message

- Biogas is a versatile fuel easily available from renewable sources of energy.
- Production of biogas from lignocellulosic feedstocks is an environment friendly approach of creating energy from waste.
- Appropriately upgraded biogas is an efficient candidate to cater various domestic and commercial requirements.
- In nutshell, lignocellulosic waste should not be burned and dumped in open fields to check the environmental pollution; rather used for generating biogas for overcoming future energy crisis.

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Chapter 8
Bioconversion of Methane for Value-Added Products

Qiang Fei and Philip T. Pienkos

What Will You Learn from This Chapter?
This chapter will briefly summarize the background of methane production from natural gas and biogas together with an introduction to the biomolecular basis of methanotrophy and the actual and potential commercial applications. This chapter also discusses the safety considerations for using methane in laboratory and safe development of methane-based bioprocesses. In the end, this chapter will focus on the process development for biological conversion of methane into desired products with attention to the enhancement of mass transfer efficiency and the development of bioreactor designs.

8.1 Introduction

Methane (CH_4) is a colorless, odorless, nontoxic, and flammable gas and is the simplest and most energy dense alkane with a specific energy of 55 MJ/kg. It is one of the most common gases in the universe where it was produced as part of the same processes that formed the stars, planets, and other celestial bodies. On earth, CH_4 formed underground from organic material as a fossil fuel along with coal and petroleum. It is usually found in both wetlands and oceans, where it often finds its way to the surface and into the atmosphere. Approximately 36% of the CH_4 released into the atmosphere is due to natural geological activities (Bousquet et al. 2006).

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However, most of the rest comes from human activities, such as burning fuel, leakage from natural gas systems, and raising livestock. In the USA, energy extraction (natural gas and petroleum), agriculture (enteric fermentation), and waste management (landfills) account for the highest CH_4 production (EPA 2015). CH_4 is a greenhouse gas, and its production has kept pace with the world's population growth. Due to its high global warming potential that is 80 times that of CO_2 over a 20-year time frame, more than 5.4 quads (1.47×10^5 million cubic meter) of natural gas has been flared annually at oil production sites around the globe (World Bank 2013). CH_4 can also be harnessed as an energy source, which has played a vital role of the world's supply of energy for years. Natural gas containing about 80–95% (v/v) CH_4 mixed with other heavier alkanes is one of the major sources for CH_4 production. With the heating value of 1020 BTU per standard cubic foot (BTU/scf), it is one of the major fuels used throughout the country. As a fossil fuel, natural gas is commonly used as an energy source for transportation, heating, cooking, and electricity generation. More than 24.6 quads (2.4×10^4 billion cubic feet) of natural gas has been extracted from the ground every year in the USA since 2010 (EIA 2016). The International Energy Agency estimates that the extraction of natural gas will keep increasing with projections that 25% of global energy will be derived from natural gas by 2035. This is due in large part to a tremendous increase in natural gas extraction in the USA since 2007 because of the shale gas development. Shale gas is one form of unconventional natural gas that is trapped within relatively nonporous shale formations, compared to the conventional sources found in multiple, relatively small, porous zones in various naturally occurring rock formations such as carbonates, sandstones, and siltstones. The unconventional gas reservoirs have large volumes that previously had been difficult to develop. However, advanced shale gas technologies, primarily hydraulic fracturing or "fracking," have not only improved the extraction capacity of natural gas but have also reduced natural gas costs from \$13/MM BTU to \$3/MM BTU with prices expected to remain stable for a long time (www.eia.gov), which allows CH_4 to be available as an economic substrate for bioprocesses. As the primary carbon source, glucose (corn syrup) was projected at a cost of approximately \$645/ton from a techno-economic analysis in 2011 cost (Davis et al. 2015), which is equal to \$0.194/carbon mole.[1] The price of wellhead natural gas was \$2.62/MM BTU (spot price of Henry Hub) in 2015 that gives \$146/ton or \$0.0023/carbon mole.[2] Therefore, the cost of CH_4 on per mole of carbon is eight times cheaper than the cost of glucose per mole of carbon. Taking account of CH_4 as the most reduced carbon source available, it is clearly a more suitable carbon source for the production of reduced products (e.g., fatty acids) compared with glucose.

[1] 1 ton glucose gives 33,333 (1,000,000 g/180 × 6) carbon mole. Glucose price = \$645/ton = \$645/33,333 carbon mole = \$0.0194/carbon mole.

[2] Natural gas (NG) price = \$2.62/MM BTU = \$2.62/1000 ft^3. Considering NG density of 0.7 kg/m^3 and 1 m^3 = 35 ft^3, NG price = \$2.62/20 kg = \$131/ton. Therefore CH_4 price is \$146/ton when NG contains 90% CH_4. 1 ton CH_4 gives 62,500 (1,000,000g/16) carbon mole. CH_4 price = \$146/ton = \$146/62,500carbon mole = \$0.0023/carbon mole.

Besides the fossil-based CH_4 derived from natural gas, biogas, a form of renewable energy produced from organic matter through the biological process of methanogenesis, is another major source of CH_4. Biogas has a lower energy content (400 BTU/scf) than natural gas and is mainly composed of 55–70% CH_4 and 30–45% carbon dioxide (CO_2). It may also contain small amounts of moisture, siloxane, hydrogen sulfide, ammonia, nitrogen, hydrogen, and aromatic hydrocarbons (Tsavkelova and Netrusov 2012). Biogas is commonly produced via a process called anaerobic digestion, which is a complex process that involves two stages. In the first stage, decomposition is performed by acidogenic bacteria that metabolize the waste components (primarily protein, carbohydrate, cellulose, and hemicellulose) into mainly volatile fatty acids (acetic, propionic, and butyric acid) and ammonia along with CO_2 and hydrogen (H_2) gases. In the second stage, most of the organic acids and all of the H_2 are metabolized by methanogens, with the end result being production of generating biogas.

An important source of biogas is agricultural activities typically generated from enteric fermentation taking place within ruminant animals (e.g., cows, sheep, goats, cattle, buffalo, and camels), which is responsible for 25% of anthropogenic CH_4 production (EPA 2015). Methanogenic microbial communities responsible for the enteric fermentation reside in the stomachs of these animals and convert organic matter into CH_4, which is exhaled, eructated, or released via flatus. Between 1990 and 2013, CH_4 production from agricultural activities increased by 11.3%, which is mainly due to the increased animal husbandry to provide increasing amounts of meat in the diet of the world's population. In 2014, more than 0.26 quads (257 billion cubic feet) of CH_4 was estimated to be produced from agriculture in the USA (USDA et al. 2014). Global livestock production has increased substantially since the 1960s and is expected to continue rising. Rice farming is another agricultural source of biogas. Rice paddies, which are essentially artificial wetlands, are characterized by high moisture content, oxygen depletion, and ample organic material. This creates a great environment for methanogenic communities that decompose the organic matter for CH_4 generation.

Landfill gas is another type of anthropogenic biogas typically derived from anaerobic methanogenic communities present in water and soil samples which can convert a diverse range of agricultural, industrial, domestic, and municipal wastes and biodegradable solid waste. Landfills are the third largest source of CH_4 production in the USA presenting 0.29 quads (284 billion cubic feet) of CH_4 produced in 2014 (USDA, EPA 2014). The peak production of biogas is from 5 to 7 years after the feedstock is dumped at landfills (EPA 2000). However, the bacteria will continue to decompose the buried waste and emit methane slowly for years after a landfill is closed.

8.2 Methanotrophic Bacteria

Methanotrophic bacteria are a group of bacteria that are capable of utilizing CH_4 as their sole carbon and energy source. Methanotrophs, first discovered in 1906, were isolated and characterized beginning in the 1970s by Whittenbury and his coworkers (Whittenbury et al. 1970), establishing the basis of the current classification of methanotrophic bacteria. Methanotrophic bacteria are gram-negative bacteria, and most were isolated from sewage, bogs, wetlands, lake basins, or ruminants (in other words, in environments where methane is plentiful), where they can grow well. Although methanotrophs are now known to be able to grow in both aerobic and anaerobic environments, most methanotrophs are usually specified as aerobic microorganisms that can oxidize methane to methanol and beyond for catabolism and anabolism. In this chapter, we will focus on aerobic methanotrophic bacteria and their cultivation. Readers who are interested in anaerobic methanotrophs can find more details from recent reviews (Caldwell et al. 2008; Knittel and Boetius 2009).

Methanotrophs are now classified into three groups replacing earlier categories such as Type I, Type II, and Type X (Fei et al. 2014b). Group I contains *Gammaproteobacteria* methanotrophs (formerly Type I and X) that are able to utilize the ribulose monophosphate (RuMP) cycle for single carbon assimilation, including the genera *Methylobacter Methylococcus*, *Methylohalobius*, *Methylomonas*, *Methanosphaera*, *Methylosoma*, *Methylomicrobium*, *Methanothermus*, *and Methanosarcina.* Group II contains *Alphaproteobacteria* methanotrophs (formerly Type II) capable of using the serine cycle to assimilate single carbon sources, including the genera *Methylosinus*, *Methylocapsa*, *Methylocella*, and *Methylocystis*. Recently, a new division of methanotrophs known as *Verrucomicrobia* methanotrophs have been assigned to Group III (Dunfield et al. 2007; Pol et al. 2007). This group can oxidize methane to generate metabolic energy to assimilate carbon at the level of CO_2 using the Calvin-Benson-Bassham cycle. All of the methanotrophs mentioned above are obligate methylotrophs that can only use the C1 molecules of methane, methanol, methylamine, or trimethylamine as the carbon and/or energy source. However, two recently discovered species, *Methylocella* and *Methylocystis*, are facultative methylotrophs, able to utilize not only methane/methanol as their sole energy source but also acetate and ethanol (Dedysh et al. 2005; Im et al. 2011).

Methanotrophs can metabolize CH_4, because of a unique enzyme, methane monooxygenase (MMO), which catalyzes the conversion of methane to methanol, the first intermediate in methane metabolism. Two forms of MMO are known as soluble methane monooxygenase (sMMO) present in the cytoplasm and particulate methane monooxygenase (pMMO) bound to intracellular membranes. Most methanotrophs employ pMMO when grown in the presence of copper and iron (needed for pMMO activity) (Dalton 1992), but the synthesis of sMMO is observed in copper-limited environments. In addition to copper and iron availability, a number of environmental factors can affect the type and activity level of MMO. These include nutrient availability including O_2, N, and P, gas transfer rate, pH, and temperature

(Fei et al. 2014b). There are two separate cycles (ribulose monophosphate (RuMP) cycle and serine cycle) by which formaldehyde, produced as an intermediate in methane oxidation, is assimilated into microbial biomass and metabolites (Fei et al. 2014b). The RuMP cycle is usually found in Group I methanotrophs for their C1 assimilation. These organisms have incomplete TCA cycles. Group II methanotrophs using the serine cycle to assimilate C1 substrates have functioning TCA cycles. However, some Group I methanotrophs not only use RuMP cycle but also possess genes for serine cycle enzymes (Lieberman and Rosenzweig 2004).

Metabolic engineering tools are beginning to play a critical role in the development of industrial methanotrophic biocatalysts. The development of mutagenesis techniques, gene transfer methods (knock-in and knockout of genes between chromosomal and plasmid DNA), and gene expression systems (development of promoters and other regulatory elements) offers a means to enhance nutrient uptake and alter metabolic flux toward desirable products, such as single-cell protein, chemicals, and fuels (biological conversion of gas to liquid fuels and chemicals or Bio-GTL&C), and other high-value co-products.

8.3 Applications

8.3.1 Single-Cell Protein (Amino Acids)

Because of the challenges to supply sufficient protein for the world's growing population in the 1960, the concept of using methane and methanol for the production of microbial biomass termed single-cell protein (SCP) was pursued by several companies including ICI, Hoechst, and Phillips Petroleum. SCP derived from biomass of methanotrophs is composed of nutritionally acceptable amino acids and has been mainly used as a promising replacement for animal nutrition supplement and human protein consumption. Enormous R&D efforts were devoted to SCP production by methanotrophs using CH_4 as the carbon source. *Methylococcus capsulatus* (Bath) has been researched considerably because of its high efficiency in production of SCP with CH_4 and some criteria such as amino acid composition, digestibility, and animal performance and health. Bewersdorff and Dostálek (Bewersdorff and Dostálek 1971) determined that 71% crude protein on dry cell weight base was produced in a mixed bacterial culture grown using CH_4 with a yield of 0.64 protein/g CH_4. In a continous cultivation with a dry cell weight of 2.2 g/L, the amino acid composition of the protein (g/100 g protein) was shown to be 10.2 of glutamic acid, 8.8 of aspartic acid, 7.7 of alanine, 7.0 of leucine, 5.9 of valine, 5.6 of glycine, 5.4 of lysine, 4.9 of isoleucine, and 4.3 of threonine along with smaller amounts of the other amino acids (Bewersdorff and Dostálek 1971). Protein from methanotrophic biomass has been used as animal supplement for pigs, chickens, mink, fox, dog, and fish (Øverland et al. 2010). Although several novel techniques for the production of SCP from methane were developed by Shell, viable commercial processes never materialized, largely due to economical considerations (Strong

et al. 2015). Although the increases in agricultural productivity brought on by advances made during the "Green Revolution" greatly mitigated the challenges of providing food for an increasing world population, this problem is beginning to surface again, leading to a reconsideration of the potential production of microbial protein. Due to the abundant supply and cheapness of natural gas, the production of methanotrophic protein from natural gas could be realistic on an industrial scale. UniBio A/S, a pioneer company of methanotrophic protein production, based in Odense, Denmark, opened its first commercial plant of SCP using natural gas as a nutritional food protein feed for animals in Nov 2016 (Vallenet et al. 2013). This plant improves UniBio's annual capacity of SCP production up to 80 ton. Calysta, Inc., a company located at Menlo Park, CA, USA, is also a major player focusing on the production of SCP using CH_4 as the sole carbon source. Calysta's FeedKind™ protein, a new fish and animal feed ingredient, has been targeting industrial markets of aquaculture and livestock feeds (Cantera et al. 2017).

8.3.2 Bio-Based Chemicals (Bio-GTC)

The same reason for renewed attention to the production of methanotrophic SCP makes it attractive as a potential source of biochemicals. Soluble metabolites produced by methanotrophs (e.g., biopolymer, organic acids, keto acids, carboxylic acids, ectoine, and vitamins) are all potential products from CH_4 with multiple industrial uses and high global demand. Recent interests in industrial applications have been focused on the bioconversion of natural gas/CH_4 into bulk chemicals (Bio-GTC). Methanol, formaldehyde, and formate are the initial products of CH_4 metabolism by methanotrophs. However, higher rates, yields, and titers would be necessary for economic processes. Poly-3-hydroxybutyrate (PHB) is a bio-derived and biodegradable plastic, a polyester made up of repeating units of 3-hydroxybutyric acid, with similar physical properties to other industrial plastics, such as polypropylene. The production of PHB has been observed in both Group I and Group II methanotrophs (Anthony 1982). However, the serine pathway used by Group II methanotrophs is the most efficient metabolic machinery for PHB biosynthesis (Wendlandt et al. 2001). When isocitrate dehydrogenase is used for NADPH regeneration, the theoretical yield of PHB is 0.56–0.67 g/g (Asenjo and Suk 1986; Yamane 1993), which is higher than that from glucose of 0.48 g/g and sucrose of 0.5 g/g (Yamane 1993), attesting to the higher energy content of methane compared to sugars. Wendlandt et al. reported that PHB content was as high as 51% in a continuous culture of *Methylocystis* sp. using methane as the sole carbon source (Wendlandt et al. 2001). Mango Materials, a new biology start-up founded in 2011, recently announced its new process of converting waste biogas into bioplastics instead of conventional petroleum-based plastics (Whitworth 2014). According to an economic model developed by researchers at Stanford University, the cost for PHB produced from Mango Materials' process could be as low as $1.2/kg (Roland-Holst et al. 2013).

Due to the development of genetic tools, manipulation of pathways to overproduce metabolic intermediates or compounds not naturally biosynthesized by methanotrophs is a topic of current interest. Recently, Calysta Energy announced that it has successfully demonstrated a lab-scale production of lactic acid from methane, under a research collaboration with NatureWorks (Calysta 2014). Intrexon's patent also indicates the feasibility of the production of 1-butanol, fatty alcohols, fatty acid esters, and 2,3-butanediol by metabolically engineered methanotrophs (Coleman et al. 2014). Besides the production of chemicals mentioned above, succinic acids, acetic acids, ectoine, vitamins, and astaxanthin are alternative value added products with large markets that could be produced by methanotrophs (Strong et al. 2015).

8.3.3 Biofuel (Bio-GTL)

Biofuel is another valuable product from methanotrophs with an enormous demand. A comprehensive review about the bioconversion of natural gas into liquid fuel (Bio-GTL) has been published discussing its opportunity and challenge (Fei et al. 2014b). However, until now there are still few studies regarding the feasibility of utilizing the Bio-GTL concept for the production of liquid fuels, due in large part to the lack of a robust, suitable production strain. A program developed by the Advanced Research Projects Agency-Energy (ARPA-E) of the US Department of Energy (DOE) known as Reducing Emissions using Methanotrophic Organisms for Transportation Energy (REMOTE) was initiated in 2013 to accelerate the development of economic Bio-GTL processes. A continuous gas delivery system with safety control and precautions has been developed at the National Renewable Energy Laboratory for converting CH_4 into lipids in high cell density culture of oleaginous methanotrophs—*Methylomicrobium buryatense* (Scanlon 2014). The microbial lipids can be catalytically upgraded to diesel blend stocks for biofuel. Downstream processing of microbial lipids for renewable diesel production follows a sequential catalytic upgrading process familiar to petroleum refining consisting of hydrotreating (hydroprocessing) followed by catalytic cracking and isomerization of the fatty acyl chains to fit the properties of renewable diesel. A techno-economic analysis (TEA) was performed for an integrated biorefinery process using biological conversion of methane considering such parameters as carbon yield, process efficiency, productivity (both lipid and acid), natural gas, and other raw material prices, etc. (Fei et al. 2014d). This preliminary cost analysis of Bio-GTL based solely on raw material costs and yields projected a CH_4-derived diesel cost range from \$0.7 to \$10.8/gal (Fei et al. 2014b).

Intrexon Corporation, a synthetic biology company, has formed a joint venture named Intrexon Energy Partners to scale up its Bio-GTL platform in 2014 targeting isobutanol for gasoline blending. Recently, Intrexon Corporation announced the achievement of farnesene production from CH_4, a potential feedstock for diesel fuel. Considering the recent volatility of crude oil prices and the potential for future

shortages, the utilization of CH_4 as a substrate for liquid fuel production has tremendous potential.

8.4 Safety Control

8.4.1 Explosive Limits

CH_4 is a combustible gas, which can cause fires or explosions in the presence of O_2 and an ignition source. As shown in the stoichiometric equation below, the combustion of CH_4 is highly exothermic.

$$CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O \quad \Delta H = -890\,\mathrm{KJ/molA} \tag{8.1}$$

Therefore, the presence of CH_4 in the atmosphere is a potential hazard in industrial, commercial, and domestic environments. Due to the colorless and odorless nature of gaseous hydrocarbons, a trace amount of the organic sulfur compound methanethiol (CH_4S) is added to give commercial natural gas its distinctive smell. This safeguard makes gas leaks readily detectable and reduces the potential for serious explosion or asphyxiation. To support CH_4 combustion in air, however, the ratio of CH_4 and air/O_2 must be within well-defined explosive range, determined experimentally and referred to as explosive limits. The lower explosive limit (LEL) of CH_4 is the lowest CH_4 percentage by volume in air that can cause an explosion if ignited, and the upper explosive limit (UEL) of methane is the highest CH_4 percentage in air capable of producing an explosion (Gas 2013). These values are 5% for the LEL and 15% for the UEL. Below the LEL, CH_4 is too lean to burn, and above the UEL, CH_4 is too rich to burn. Therefore, CH_4 concentrations outside those limits are nonflammable. The potential for CH_4 explosions is also associated with the limiting oxygen concentration (LOC). The LOC is the minimum concentration of O_2 in a homogeneous mixture of CH_4, air, and an inert gas (e.g., N_2, CO_2) that will propagate a fire or explosion, independent of the concentration of CH_4 (Zlochower and Green 2009). The effect of adding an inert gas into a fuel gas mixture is to reach a level, below which this mixture can no longer be made flammable. Therefore, using air instead of pure O_2 can reduce the possibility of explosion and avoid the flammable mixture zone. The LOC for methane is 12.1 vol% and 14.6 vol% with N_2 and CO_2 as an inert gas, respectively (Hamer et al. 1967). Thus, by maintaining the O_2 concentration in a gas mixture below the LOC, certain safe operation can be predicted and guaranteed. A triangular diagram exhibits the regimes of flammability in a homogeneous mixture of CH_4, air, and N_2. This flammability diagram also depicts explosive limits (LEL and UEL), LOC, combustion stoichiometric line, and N_2 purge concentrations to avoid the flammable zone (orange-colored envelope determined experimentally). More explanations of this diagram are available (Hamer et al. 1967).

As discussed above, the flammability of CH_4 is calculated based upon its combustion stoichiometry (Eq. 8.1). However, during the culture of aerobic methanotrophic bacteria, the concentrations of CH_4 and O_2 vary with the gas specific utilization rate. For example, the utilization efficiency of CH_4 and O_2 will increase as the cell density increases and will either plateau or decrease in the stationary phase, which means the concentration of CH_4 and O_2 in the off-gas from a bioreactor will change with the growth phase of methanotrophs. Therefore the metabolic stoichiometry of methanotrophs should also be considered for the safe usage of gas mixtures during cultivation.

8.5 Control and Precaution

To grow aerobic methanotrophic bacteria, CH_4 is required as carbon source and energy source, and O_2 is required as source of the oxygen atom in methanol and as electron acceptor. Continuous supply of these two gases is mandatory during cultivation in order to maintain a healthy growth environment. Therefore, safety considerations for controls and precautions of continuous gas supply are needed before designing a bioprocess and carrying out experiments. Risk assessment is the first step to estimate the likelihood and magnitude of the occurrence of an unwanted event scenario and is used in such potentially dangerous fields, such as fire management, petroleum and natural gas extraction, processing and delivery, coal mine activity, and nuclear science and engineering. A risk assessment process for CH_4 usage in a laboratory should not only review restrictions, regulations, and standards for the institution but also consider the specifics of hazardous materials used for experiments, system pressure, size and ventilation of lab room, fire and explosion, volume/quantity, gas mixture composition, and flow rate of the gas mixture. A matrix shown in Table 8.1 is a simple tool to estimate the risk level of different scenarios.

Based on the assessment, risk management and control are also required for using CH_4 in laboratory (Schaufele 2013). In general, risk control can be categorized as administrative controls, engineering controls, and work practice controls (safe operating procedure). Administrative controls can be in the form of general policies or laboratory-specific standard operating procedures established at an administrative level and implemented by the principal investigator, laboratory supervisor, department chair, department safety committee, or office of environmental health and safety at an institute or a university. Engineering controls consist of various measures for eliminating hazards or reducing exposure to hazards at their sources before they are created. In the laboratory, examples of engineering controls for CH_4 usage include pressure relief valves, flow check valves, continuous leakage detection systems, use of nonflammable gas mixtures, limitation of maximum flow rates of CH_4 (e.g., installation of a restrictive flow orifice), and utilization of local exhaust systems such as a fume hood or ventilation system. A continuous gas delivery system for a Bio-GTL&C process can include a transient leakage detection system

Table 8.1 Risk assessment tool

	Risk Level				
	Insignificant (minor problem easily handed by normal day to day processes)	Minor (some disruption possible, e.g. damage equal to $500k)	Moderate (significant time/resources required, e.g. damage equal to $1 million)	Major (operations severely damaged, e.g. damage equal to $10 million)	Catastrophic (business survival is at risk damage, e.g. damage equal to $25 million)
Likelihood	Consequences				
Very likely (>90 chance)	High	High	Extreme	Extreme	Extreme
Likely (50-90% chance)	Moderate	High	High	Extreme	Extreme
Possible (10-50% chance)	Low	Moderate	High	Extreme	Extreme
Unlikely (3-10% chance)	Low	Low	Moderate	High	Extreme
Rare (<3% chance)	Low	Low	Moderate	High	High

that is able to turn off a solenoid valve at the CH_4 source if CH_4 is detected outside the cultivation system and an off-gas monitor system that is able to adjust the flow rate and ratio of CH_4 and air/O_2 through the mass flow controller to avoid flammable gas mixture (Hamer et al. 1967).

8.6 Cultivation of Methanotrophs

8.6.1 Operating Strategy of Methanotrophic Cultivation

Besides the safety management and control, one of the first decisions is the choice of the cultivation mode, which is essential to fermentation performances in terms of growth rate and productivity. The production of PHB and SCP by methanotrophs was a topic of interest for the last two decades. In those works, the researchers investigated batch, fed-batch, and continuous cultivation scheme for the methanotrophic culture. It is important to note that gaseous substrates (CH_4 and O_2) need to be supplied constantly during the cultivation of methanotroph to achieve high cell densities and productivities.

Batch cultivation in a closed culture system is the simplest mode for the production of cell mass and desired products (Fei et al. 2011b, 2013a, b, 2014a; Kennedy and Krouse 1999). Because nutrients (including CH_4 and O_2) are not added, nor waste products (other than CO_2) removed in the course of the entire fermentation, batch cultures can only allow a limited generation of microorganisms before growth stops. In the early stage of process development and research study, batch culture is a

good choice due to the low capital investment and simple operation. Serum vials and screw-cap bottles have been widely used in the methanotrophic cultivation for strain screening and genetic manipulation works (Whittenbury et al. 1970). In order to overcome the limitations of low cell density and productivity in batch cultivation, fed-batch cultures with controlled nutrient feeding are more suitable at large scale.

For the fed-batch culture of methanotrophs, one or more nutrients (especially CH_4, O_2, and nitrogen sources) are supplemented to the sealed container or bioreactor during cultivation, and the primary product remains in the bioreactor until the end of the fermentation (Fei et al. 2011a, 2015, 2016; Kennedy and Krouse 1999; Park et al. 2014). A higher cell density culture of methanotrophs can be achieved easily by only adding CH_4 and O_2 continuously (Fei et al. 2014c). In contrast to the closed/semi-closed system of batch/fed-batch culture, an open system with continuous cultivation provides steady-state cell growth rate by continuously supplying fresh medium as well as gaseous substrates such as CH_4 and O_2 for the methanotrophic cultivation.

This third option has several advantages over batch and fed-batch operations, including minimizing equipment downtime and production time lost due to the lag phase of the microbial culture. Shah et al. reported that both cell-specific growth rate and pMMO productivity were improved in a continuous culture of *M. trichosporium* OB3b (Park et al. 1992). Although the limitation of CH_4 and O_2 can be solved in fed-batch and continuous cultures, the mass transfer of these gases in the aqueous phase is the greatest technical challenge for the methanotrophic cultivation at large scale.

8.7 Mass Transfer Efficiency

Gas-liquid mass transfer efficiency is one of the primary technology obstacles in aerobic microorganism cultivation. The mass transfer efficiency, which can be expressed as the volumetric mass transfer coefficient $K_L a$, has been described by Klasson (Klasson et al. 1993), as shown in the following equation:

$$\boldsymbol{\gamma}_S^G = \frac{K_L a}{H}\left(P_S^G - P_S^L\right)$$

where $\boldsymbol{\gamma}_G$ (mol/L/h) is the volumetric mass transfer rate of the gaseous substrate; P_S^G and P_S^L (atm) are the partial pressures of the gaseous substrate in gas and liquid phase, respectively; and H (L×atm/mol) is Henry's law constant. According to the above equation, it is obvious that the partial pressure differential (between gas and liquid phase) is the main driving force for mass transfer and thus controls the availability of the substrate. Since H is a temperature-dependent constant, the mass transfer efficiency is affected by the culture temperature as well.

Because of the importance of gaseous substrates, CH_4 and O_2, gas mass transfer efficiency is one of the most important parameters in methanotrophic cultivation. The low solubility of CH_4 (22.7 mg/L) and O_2 (14.6 mg/L) in the water/fermentation

broth significantly limits their consumption and conversion efficiency for the cell growth of methanotrophs and calls for continuous transfer from the gas phase to the liquid phase. In order to improve the gas transfer rate, gas molecules need to travel into gas-liquid interface and subsequently diffuse efficiently through the culture media and then through the microbial cell surface, where they can participate in metabolic reactions. In an ideal situation, the bubble size of gaseous substrate should be small and uniform throughout the bioreactor. Therefore, significant effort has gone into the design of bioreactors that can provide a higher mass transfer rate by generating more gas-liquid-cell interfacial area from smaller and uniform gas bubbles.

8.8 Bioreactor Design

Because both CH_4 and O_2 acting as the electron donor and acceptor are gaseous and the solubility of these two gases is extremely low in the culture medium, the cultivation of methanotrophs will be severely limited by the mass transfer efficiency. Therefore, the choice of the bioreactor and the operating strategy for methanotrophic cultivation is one of the most important decisions in regard to the bioprocess development, which determines the final product titer, carbon conversion efficiency, and productivity and whether sustainable, reliable performance can be achieved. In addition to the bioreactor design and culture strategy, other aspects (e.g., the composition of gas mixture) should also be considered for high mass transfer. The gas mixture topics to be considered are air/pure O_2, pure methane, natural gas, biogas, and source of natural gas and biogas to provide workable mixtures of CH_4, higher alkanes, CO_2, and minor contaminants such as H_2S. Alvarez-Cohen reported that the highest cell growth of methanotrophs in a suspended cultivation is estimated at 130–200 mg/L/h with feeding CH_4/O_2 mixture and 40–70 mg/L/h with feeding CH_4/air mixture (Alvarez-Cohen 1993). The use of the water-immiscible organic solvent for improving methane transfer rate has been tested to achieve a high cell density culture (Han et al. 2009). Recently, a method of adding nanoparticles with functional groups into gas fermentation system has been developed to exploit the extensive adsorption capability of the functionalized nanoparticles to increase the mass transfer coefficiency (Zhu et al. 2010).

8.8.1 Continuous Stirred-Tank Bioreactors

Continuous stirred-tank bioreactors (CSTRs) are the most widely used bioreactors for growing methanotrophic bacteria. By increasing the agitation speed or modifying the bioreactor's impellor, smaller-size bubbles can be generated for increasing the gas-liquid interfacial area. However, high shear rates from excessive agitation could damage cells and inhibit growth rate, and power input for these strategies greatly

reduce the economic viability of industrial-scale fermentations. Consequently, microbubble sparging reactors, bubble column reactors, loop and airlift reactors, trickle-bed reactors, and membrane-based reactors are some of the other configurations developed for enhancing the mass transfer efficiency.

8.8.2 Microbubble Sparging CSTRs

In order to generate smaller gas bubbles, a microbubble distributor has been developed and equipped for CSTRs. A microbubble sparger breaks up the gaseous substrate into extremely fine and, in some cases, surfactant-stabilized bubbles in a high shear zone, which have lower rise velocities for longer liquid retention time (Bredwell et al. 1999). Compared with traditional CSTRs, the microbubble distributor provides a more energy efficient method to increase mass transfer efficiency.

8.8.3 Bubble Column Bioreactors

Bubble column bioreactors that are basically a cylindrical vessel with a gas sparging system at the bottom are widely used for industrial applications with large working volumes due to advantages in design and operation as compared to other reactors. Excellent heat and mass transfer, low maintenance requirements, and low operational costs are primary merits for methanotrophic cultivation. It was found that a bubble column reactor provided a higher conversion rate of methane than CSTR did, which resulted in a threefold greater propylene oxide titer (Hill et al. 1990).

8.8.4 Loop and Airlift Bioreactors

Similar to the mechanical agitation of bubble column bioreactors, loop and airlift bioreactors are characterized by recirculating the liquid in a defined cyclic pattern through channels that connect the gas-liquid separator on top of the main bubbling section or riser to its lower part. The patterns of fluid circulation are designed specifically for enhancing the mass transfer. It was found that a forced-liquid vertical tubular loop bioreactor can provide a twofold cell density improvement compared with an external airlift loop reactor and a horizontal tubular loop bioreactor (Yazdian et al. 2012). Rahnama et al. developed and compared a bubble column and a vertical loop reactor for the PHB production from natural gas, and a PHB content of 51% w/w was achieved in their vertical tubular loop bioreactor (Rahnama et al. 2012). UNIBIO, a company from Denmark, has also demonstrated commercial production of SCP as animal feed from methane using a patented U-loop bioreactor (UNIBIO 2011).

8.8.5 Trickle-Bed Bioreactor

Unlike bubble column bioreactors, gas and liquid in a trickle-bed bioreactor move cocurrently downward over a packed bed, or gas is fed countercurrently upward, while the liquid moves downward. Trickle-bed bioreactors have been mainly used for waste gas removal by methanotrophs (Reij et al. 1998). In the trickle-bed reactor, methanotrophic bacteria as catalysts are packed on the bed, which allows them to degrade the pollutants after the waste gas diffuses through the water phase. The mass transfer rate in the trickle-bed reactor is relatively dependent on the gas flow rate and operating temperature (Barton et al. 1999).

8.8.6 Monolithic Biofilm Bioreactors

Monolithic biofilm bioreactors resemble trickle-bed reactors, in which the gaseous substrate is allowed to pass through a bed of carrier material with low-pressure drop. Microorganisms are present as a biofilm attached on the bed, which have been used in CH_4 bioconversion platforms. In the culture of methanotrophs, attached methanotrophic bacteria in the biofilm utilize CH_4 efficiently for the production of extracellular products and cell mass (Arcangeli and Arvin 1999).

8.8.7 Membrane Biofilm Bioreactors

In a membrane biofilm bioreactor (MBfR), a biofilm is directly attached to a membrane instead of carrier media in monolithic biofilm bioreactors. Gases pass through the interior of a membrane to a biofilm growing on the membrane surface. Composite hollow fiber MBfRs have been proposed as technologically and economically feasible for nitrate and trichloroethylene (TCE) removal from contaminated waters with methane (Rishell et al. 2004). In the MBfRs, CH_4 and O_2 are diffused through the walls of membranes without forming bubbles. The methanotrophic biofilms on the outer wall of the membranes continuously consume gaseous substrates and pollutants for the development of a thick biofilm, which gave a TCE removal efficiency up to 90% (Clapp et al. 1999). This innovative MBfR offers a significant advantage in providing a high transfer rate of sparingly soluble gaseous substrates directly to the microorganisms, preventing losses to the atmosphere or effluent liquid and potentially reducing the gas volume (Henstra et al. 2007).

8.9 Conclusions and Future Perspectives

In this chapter, we have reviewed current knowledge of bioconversion of methane derived from either natural gas or biogas into high-value chemical products and biofuels instead of conventional methane application such as electricity generation or heating. Methane-derived products could replace a significant amount of petroleum-based commodities in the USA while at the same time capturing value from a wasted resource and mitigating climate change issues exacerbated by vented and flared natural gas. With the advent of economic and efficient tools of systems biology especially genomics, transcriptomics, and metabolomics, the potential to construct recombinant methanotrophic bacteria for methane-derived products is becoming much more accessible. Nevertheless, the challenges in moving from proof of concept to scale-up and commercialization still remain to be solved. The biggest technical challenges for methane bioconversion in commercial-scale application remain to be the mass transfer efficiency of poorly soluble gaseous substrates in the culture medium. Consequently, the development of bioreactors for methane fermentation has been the most popular research topic within recent years. CSTR, microbubble sparging CSTR, bubble columns reactors, loop reactors, airlift reactors, trickle-bed reactors, and membrane-based reactors are some of reactor types that have been investigated to improve mass transfer efficiency. The organic solvent and nanoparticle addition can also enhance the mass transfer but with a high cost. Meanwhile, efforts to optimize culture medium and fermentation conditions as well as exploring bioprocess technology are being pursued to enhance productivity and reduce production cost. Besides the consideration of the optimization of the bioprocess control, the safety assessment, risk management, and engineering and administrative control for CH_4 usage are also essential to the entire bioprocess.

Take-Home Message

- As a promising class of biocatalysts, methanotrophs are able to convert the CH_4 derived from natural gas or biogas into single-cell protein (SCP), biochemicals, and biofuels.
- Safety controls for CH_4 use are available and are a key factor for the development of bioconversion of CH_4 at both laboratory and industrial scales.
- To improve the mass transfer efficiency of CH_4 in batch, fed-batch, and continuous culture modes for the methanotrophic cultivation, bubble column bioreactor, loop and airlift bioreactor, trickle-bed bioreactor, monolithic biofilm bioreactor, and membrane biofilm bioreactor have been explored and have shown promise in enhancing both methanotroph growth and product kinetics.

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Chapter 9
Synthetic Biology Enables Photosynthetic Production of Limonene from CO_2 and H_2O

Charles Halfmann, Liping Gu, William Gibbons, and Ruanbao Zhou

What You Will You Learn from This Chapter:

- The physical and chemical properties of limonene, a C_{10} isoprenoid with applications in green solvents, pharmaceuticals, perfumes, and food flavorings
- An overview of efforts to genetically engineer cyanobacteria to synthesize limonene
- Perspectives on developing integrated systems to produce limonene at the industrial-scale

9.1 Introduction

Photosynthesis is the ultimate life-sustaining biological process on the planet and is the main driver behind the worldwide production of food, fiber, and renewable fuels. With the human population projected to increase by ~2 billion by 2050 (Cohen 2003), innovations in agriculture that will meet the demand for these resources are critically important. More solar energy reaches the Earth's surface every hour (4.3×10^{14} MJ) than is consumed annually (4.1×10^{14} MJ), making solar radiation a plentiful energy resource (Zhu et al. 2008). However, the energy losses of incident solar radiation from cell interception to the formation of chemical energy create a limit on biomass yields and productivity. This is especially true for most crop plants, which exhibit relatively low photosynthetic efficiencies (2–4%) (Zhu et al. 2008). Furthermore, the extensive energy needed for planting, pesticide and herbicide application, harvesting, and year-round land management means that modern

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agriculture is a major energy user and contributes to deforestation, soil erosion, and pollution (Foley et al. 2005; Montgomery 2007). To ensure sustainable agriculture for the future, innovations that can more efficiently fix and sequester carbon without negative effects to the environment are key areas for scientific investigation and economic investment.

Microalgae and cyanobacteria are photosynthetic microorganisms that have been researched extensively as models for carbon fixation (Parmar et al. 2011; Chisti 2007). They exhibit higher photosynthetic efficiency and biomass productivity than traditional oil-seed crops, can be grown on nonarable land, and can utilize a wide variety of water sources (e.g., wastewater). These characteristics, along with their abundant composition of lipids and sugars, give them an advantage over terrestrial crops as a carbon feedstock (Quintana et al. 2011). Over the last few decades, advancements in genomics, recombinant DNA technology, and synthetic biology have provided researchers with a sophisticated toolset for genetically engineering cyanobacteria to overproduce high-value compounds originally harvested from crops. These include naturally synthesized carbon feedstocks, such as fatty acids and triacylglycerides (TAGs), and fermentable sugars (sucrose, glycogen) that can be used for biodiesel and bioethanol, respectively (Quintana et al. 2011). Furthermore, genetic engineering efforts have created cyanobacteria able to directly synthesize and secrete tailor-made compounds from their cells, eliminating the need for harvesting, processing, or fermentation (Fig. 9.1). These new products include fuels, as well as a diverse portfolio of medicines, polymers, food flavorings, fragrances, and industrial solvents (Ducat et al. 2011).

One group of compounds that have gained recent attention as a commercial product from engineered cyanobacteria is the isoprenoids (also referred to as terpenoids), a large class of organic molecules that are naturally synthesized by plants, animals, bacteria, and archaea (Vranova et al. 2013). Their energetic composition make them ideal precursors for drop-in diesel and jet fuels (Rude and Schirmer 2009). Given their structural diversity, they also have industrial applications as solvents, nutraceuticals, natural pesticides, and drugs (Harrewijn 2001). During the last 15 years, a number of genetic engineering projects have made great strides in reprogramming different microorganisms to overproduce and secrete isoprenoids from their cells (Wang et al. 2015). In time, these achievements may become the foundation of commercial systems that can utilize CO_2, H_2O, and solar radiation to produce isoprenoids and similar molecules for human use.

Although great achievements have been made in this field, the cost of making isoprenoids from cyanobacteria and microalgae still outweigh the risk for most commercial investors. This is primarily due to low productivities from currently engineered strains, technicalities of isoprenoid collection, and high start-up costs (Parmar et al. 2011). To make large-scale isoprenoid production from single-celled photosynthetic microorganisms a reality, future research and ingenuity will be needed to make the photons-to-bioproduct strategy economically viable. Increasing our knowledge of cyanobacterial genetics, metabolism, and energy distribution and defining the regulatory networks involved in isoprenoid biosynthesis will expand our ability to predict the outcomes of genetic manipulation. This information,

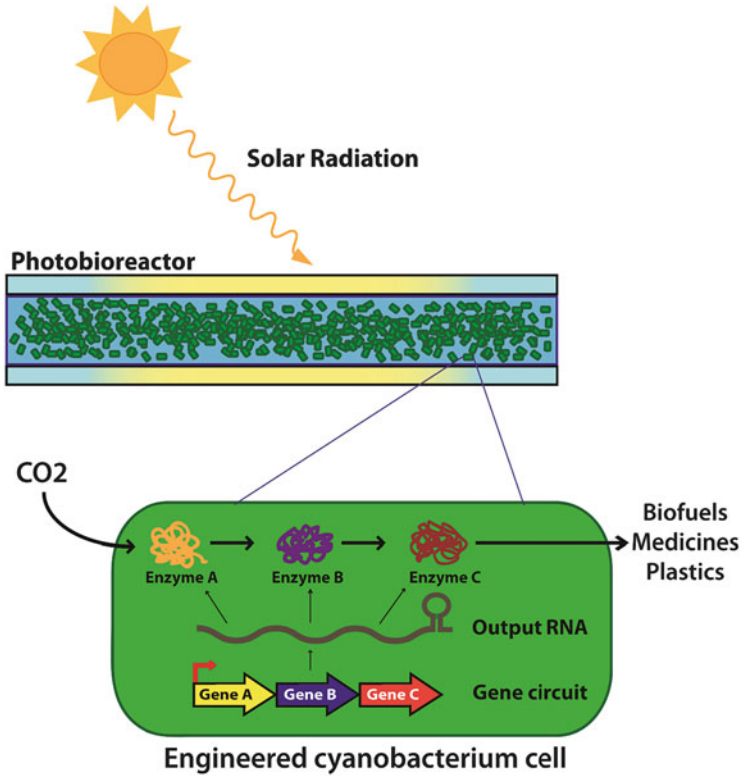

Fig. 9.1 Engineered cyanobacteria with designed gene circuits in a photobioreactor (PBR) have demonstrated advanced biofuel production technology that can utilize solar radiation to transform CO_2 directly to renewable fuels, medicines, and biomaterials

coupled with advancements in gene editing and programming, will enable us to design better engineering strategies that increase productivity in large-scale photosynthetic systems.

In this chapter, we discuss using engineered cyanobacteria for the photosynthetic production of limonene, a C_{10} cyclic isoprenoid that has applications not only as an alternative fuel but also in green solvents, pharmaceuticals, perfumes, and food flavorings.

Table 9.1 Chemical and physical properties of limonene

Parameter	Value	References
Melting point	−74.3 °C	Lide (1991)
Boiling point	175.5–176 °C	Budavari (1989)
Density	0.8411 g ml^{-1} at 20 °C	Larrañaga et al. (2016)
Solubility	Insoluble in water. Soluble in benzene, carbon tetrachloride, diethyl ether, ethanol, and petroleum ether. Slightly soluble in glycerine	Lide (1991), STN International (1992)
Stability	Oxidizes to film in air. Store in dark at −18 °C	Larrañaga et al. (2016) Ranganna et al. (1983)
Refractive index	1.4730	Lide (1991)
Optical rotation	125.6°	Lide (1991)
Flash point	50 °C	Lide (1991)
Energy density		
Volatility (vapor pressure)	133 Pa at 14 °C; 665 Pa at 40.4 °C	National Toxicology Program (1991)

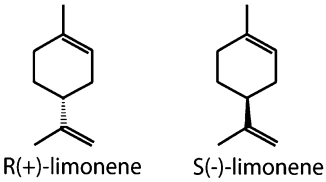

Fig. 9.2 Limonene structure, showing both *R*- and *S*- enantiomers

9.2 Limonene: A Cyclic Chemical with Important Societal Benefits

9.2.1 Physical and Chemical Properties

Limonene ($C_{10}H_{16}$, molecular weight 136.24 g/mol) is classified as a cyclic terpene, with the properties listed in Table 9.1. It is also named 1-methyl-4-(1-methylethenyl) cyclohexene under the National Institute of Standards and Technology. Limonene is a chiral molecule, occurring in two enantiomers (Fig. 9.2). The most common is *R* (+)-limonene (also called *d*-limonene), which is mainly found in citrus fruits, and gives the characteristic scent of orange or lemon. The other enantiomer is *S*(-)-limonene (also called *l*-limonene), which has an earthy, mint aroma or the scent of pine. The racemic mixture of both isoforms is also known as dipentene (Simonsen 1947). Limonene is found mainly in the oils of citrus fruit rinds but is also found in other fruits and vegetables. It has been reported that limonene is present in the essential oils of more than 300 species (Flavor and Extract Manufacturers'

Association 1991). The *d*-form comprises the majority (98–100%) of limonene in citrus oils, while the *l*-form is most common in citronella and lemongrass oils (Burdock and Fenaroli 2010; Clayton et al. 1991; Larrañaga et al. 2016).

9.2.2 Safety Issues

d-Limonene is generally recognized as safe for human consumption by the US Food and Drug Association as a flavoring substance (US Food and Drug Administration 1991). However, limonene and its oxidized products can be skin and respiratory irritants during long-term industrial exposure (IARC 1993). High doses of limonene have resulted in renal cancer in male rats (National Toxicology Program 1990), but the IARC classifies *d*-limonene as a Group 3 carcinogen: *not classifiable as to its carcinogenicity to humans* (IARC 1993). In fact, limonene is being evaluated for chemopreventative or anticancer effects (Crowell 1999; Tsuda et al. 2004).

9.2.3 Sources and Production

The first reported extraction and purification of limonene was in Florida in the early 1940s from evaporator condensate of citrus molasses (Schulz 1972). Thereafter production expanded, with the primary feedstocks being orange, grapefruit, and lemons (Verghese 1968). Several methods can be used to recover limonene. Citrus peels are mechanically processed to rupture the oil sacs and release the oil into an aqueous emulsion from which the peel oil is recovered by centrifugation (Ranganna et al. 1983). The resulting peel oil can contain up to 95% *d*-limonene. Alternatively citrus peels and pulp can be cold pressed, and the resulting press liquor is evaporated to produce citrus molasses. The condensate from this process is referred to as stripper oil, and it may contain >95% *d*-limonene (Ranganna et al. 1983). *d*-Limonene may also be recovered during other aspects of processing, including deterpenation of citrus oils (Burdock and Fenaroli 2010). Distillation may be used to further concentrate *d*-limonene, due to its relatively high thermal stability.

9.2.4 Production Levels and Use

On a global basis, ~50,000 tons of limonene are extracted annually, primarily from the residue of harvested citrus fruits (Brennan et al. 2012). Production has only grown slightly over the past 30–40 years and largely comes from the southern USA, Mexico, the Caribbean basin and Southern Hemisphere countries. Since its discovery, limonene has been used primarily as a flavor or fragrance additive in cosmetics, beverages, and foods (Duetz et al. 2003). Another major use has been as a chemical

intermediate in resin and adhesive production, as a solvent and cleaner, and in some paints and air freshener products (Schulz 1972; Burdock and Fenaroli 2010; Larrañaga et al. 2016). Due to its low toxicity to mammals and acute effects on many insects, limonene has also been used as alternative to synthetic insecticides (Karr and Coats 1988; Hooser et al. 1986; Hooser 1990). Another relatively minor volume use has been as an alternative to treat gastroesophageal reflux disease and heartburn (Sun 2007).

Future demand for green solvents is expected to rise due to consumer preference for environmentally safe products with minimal health hazards, and younger generations are willing to pay more for sustainable, eco-friendly products up from 55% in 2014 to 72% in 2015 (The Nielsen Company 2015). Green solvents are included in cleaners, paints, coatings, printing inks, and pharmaceutical and personal care products such as cosmetics and toiletries. Major companies in the green solvents space include Bio Amber Inc. (USA), Cargill Inc. (USA), Florida Chemicals Inc. (USA), Dow Chemicals (USA), DuPont (USA), and Vertec Biosolvents (USA), BASF (Germany), and CSM (the Netherlands).

9.2.5 Use as a Fuel

A new use for limonene is as a third-generation biofuel (Brennan et al. 2012), especially in jet fuel and diesel applications due to its immiscibility in water, combustibility, high energy density, and low freezing point (Hellier et al. 2013). Limonene also may be further functionalized through cyclopropanation to increase its energetic content (Langlois and Lebel 2010). The key factor limiting limonene's use in biofuels is that global limonene production is far from sufficient to meet potential demands. As a result, many groups have sought to engineer microbes to produce limonene. This has included heterotrophic bacteria to synthesize limonene from sugars (Alonso-Gutierrez et al. 2013), as well as cyanobacteria to produce limonene from CO_2 and H_2O (Halfmann et al. 2014b; Davies et al. 2014; Kiyota et al. 2014), which will be in later sections.

Limonene is viewed as an attractive target compound for large-scale production due to its potential applications in many different industrial sectors. Furthermore, its chemical similarities to diesel and jet fuels make it a prime candidate as a third-generation biofuel. Photosynthetic limonene production from engineered cyanobacteria represents a viable alternative biofuel platform and could possibly be a future innovation in agriculture. Understanding the biosynthetic pathways that produce limonene and similar compounds will be critically important in designing engineering strategies that can create biofuels and bioproducts via photosynthesis with high productivity and yield. As discussed below, limonene belongs to a large family of functional compounds that are naturally synthesized in many organisms.

9.3 Isoprenoids: Functions in Biological Systems

Limonene is a member of the isoprenoid family, a structurally diverse group of compounds that function as primary and secondary metabolites (Chappell 1995; Rohmer 1999). The roles of isoprenoids in living organisms have been researched extensively, mostly in plants, but also in animals, yeasts, bacteria, and green algae. There are many excellent reviews which highlight the diverse roles of isoprenoids in cellular function (Vranova et al. 2013; Lichtenthaler 1999). As secondary metabolites, they play a central role in membrane fluidity, respiration, photosynthesis, protein prenylation, and the regulation of growth and development through hormones synthesis. A major proportion of isoprenoids synthesized in plants are used to construct photosynthetic pigments that participate in light harvesting, photoquenching, and transferring resonance energy in photosynthetic reaction centers (PRCs). Chlorophylls, which consist of a magnesium-bound tetrapyrrole ring attached to an isoprenoid-derived phytol chain, are essential molecules in the PRC for photon absorption and energy transfer. Accessory pigments, such as carotene and xanthophylls, are also derived from isoprenoids and are important for quenching excess excitation energy and protecting the light-harvesting complexes from photodamage. Plant sterols are steroid-like molecules derived from isoprenoid precursors and, like cholesterols in vertebrates, function to regulate the rigidity and integrity of cell membranes. Monoterpenes emitted from plants have a pleasant aroma and are utilized to attract insect pollinators and seed-dispersing animals. Isoprenoids also participate in plant-pathogen interactions and can aid in protection against herbivory.

9.3.1 Metabolic Routes for Isoprenoid Synthesis

All isoprenoids are formed through the universal precursor isopentenyl pyrophosphate (IPP) and its isomer, dimethylallyl pyrophosphate (DMAPP) (Vranova et al. 2013). The 1:1 condensation of IPP (C_5) and DMAPP (C_5) catalyzed by geranyl pyrophosphate synthase (GPPS; EC 2.5.5.1) creates geranyl pyrophosphate (GPP; C_{10}), the precursor for all C_{10} isoprenoids (monoterpenes), including limonene. The condensation of IPP to GPP creates farnesyl pyrophosphate (FPP), the precursor for all sesquiterpenes (C_{15}), the major constituents of hops in beer. The addition of IPP to FPP creates geranylgeranyl pyrophosphate (GGPP; C_{20}), the precursor for photosynthetic pigments (chlorophylls and carotenoids) and quinones involved in the light-dependent reactions (plastoquinones and ubiquinones). Subsequent additions of IPP subunits to longer prenylated pyrophosphates create the precursors needed to create triterpenes (C_{30}), tetraterpenes, (C_{40}), etc. IPP and DMAPP are synthesized from two separate pathways: the mevalonate (MVA) pathway and the more recently discovered 2-*C*-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate (MEP/DOXP) pathway (Vranova et al. 2013; Lichtenthaler 1999). Animals, archaea,

some gram-positive cocci bacteria, and yeast contain the MVA pathway, while most gram-negative bacteria, cyanobacteria, and green algae use the MEP pathway exclusively.

While most organisms have either one or the other terpenoid biosynthetic pathway, higher plants and some marine alga (e.g., the red alga *Cyanidium caldarium*) contain both the MVA and MEP pathway. Genomic analyses have revealed higher plants compartmentalize these pathways, with the MVA pathway operating in the endoplasmic reticulum and cytosol and the MEP pathway functioning in the chloroplast. Why plants have retained both pathways is currently unknown but brings an interesting discussion on evolutionary aspects regarding isoprenoid synthesis. By compartmentalizing the pathways in different organelles, plants may overcome the disadvantages of immobility by having a more stringent control over isoprenoid synthesis (e.g., localizing chlorophyll synthesis in the plastid; phytosterol synthesis in the cytoplasm). This would allow accurate and faster responses to environmental cues that affect growth, development, and metabolism (Vranova et al. 2013).

9.3.2 Isoprenoid Synthesis Through the MVA Pathway

Isoprenoids synthesized in the cytosol and mitochondria are synthesized through the MVA pathway (Fig. 9.3b) (Vranova et al. 2013; Lichtenthaler 1999). The pathway starts with the Claisen condensation of two molecules of acetyl-coenzyme A (Ac-CoA) to create acetoacetyl-CoA (AcAc-CoA) in a reversible reaction, catalyzed by the enzyme AcAc-CoA thiolase (AACT; EC 2.3.1.9). Thiolases are divided into two classes: class I, which are characterized as degradative thiolases and are involved in fatty acid oxidation, and class II, which are biosynthetic thiolases and can catalyze the first reaction of the MVA pathway. Following models of pathway regulation, AACT activity strictly adheres to substrate/product ratios and was found to be extremely sensitive to free CoA. In an effort to increase *n*-butanol production in *Clostridium acetobutylicum*, the AACT enzyme ThlA was engineered to be less sensitive to feedback inhibition through free CoA by substituting three amino acids in the protein. This lead to an increase of ethanol and butanol titers by 46% and 18%, respectively (Mann and Lutke-Eversloh 2013).

AcAc-CoA is further condensed to 3-hydroxy-3-metylglutaryl-CoA (HMG-CoA) by HMG synthase (HMGS; EC 2.3.3.10). In yeast and most plants, these enzymes are found in paralogs (a set of genes created from a gene duplication event) within the chromosome. The exception is found in *Arabidopsis thaliana*, where HMGS is encoded by a single gene found to complement *erg11* and *erg13* yeast mutants lacking HMGS (Vranova et al. 2013).

Next, HMG-CoA is converted to mevalonic acid (MVA) in a two-step reduction process, requiring NADPH as a reducing equivalent. This reaction is catalyzed by 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR; EC 1.1.1.34), a major rate-limiting enzyme in the MVA pathway. *Saccharomyces cerevisiae* harbors two paralogs of HMGR (*hmg1p* and *hmg2p*), each consisting of two major domains:

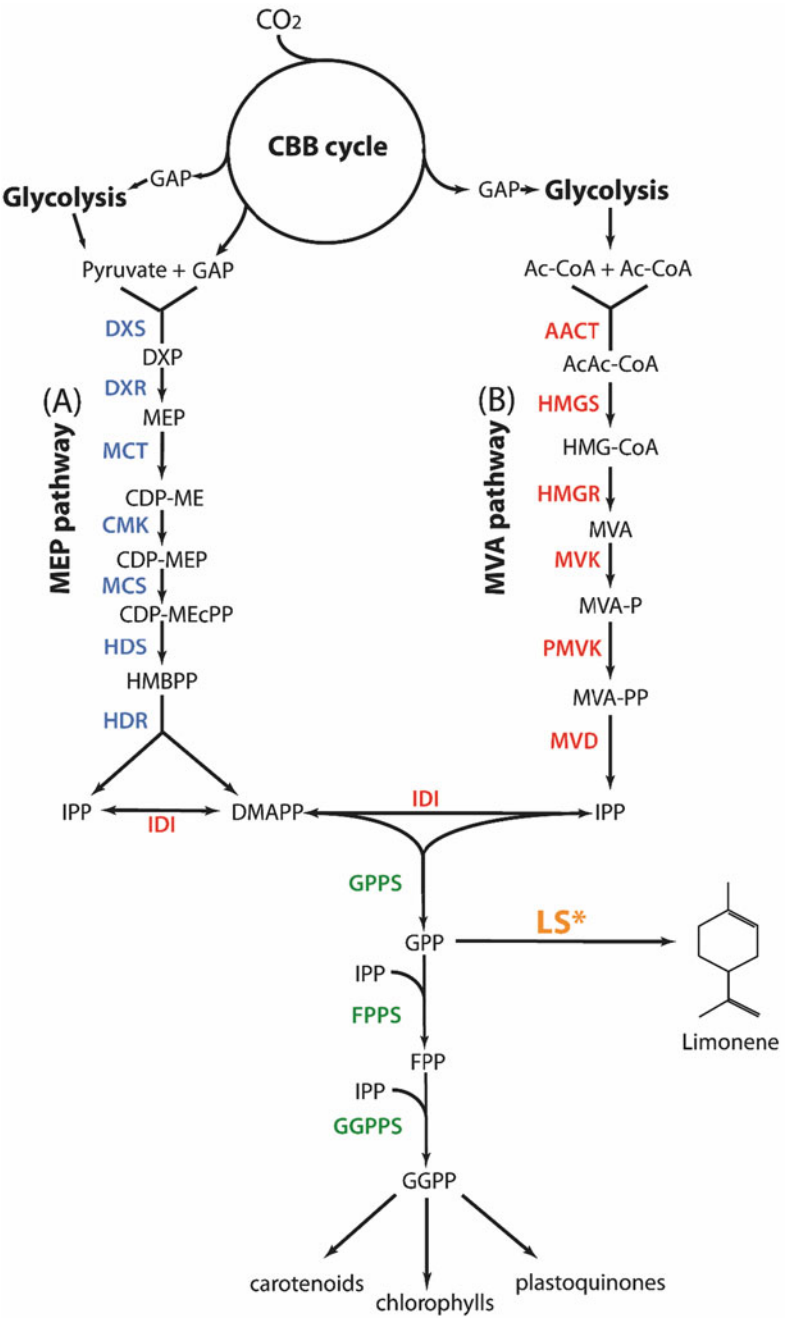

Fig. 9.3 Diagram of the two biosynthetic isoprenoid pathways. (**a**) The MEP pathway is found in most gram-negative bacteria, cyanobacteria, green algae, and plant plastids. (**b**) The MVA pathway is found in animals, archaea, fungi, plant cytosol, and some gram-positive cocci. In photosynthetic

an anchoring transmembrane domain associated with the endoplasmic reticulum (ER) and a catalytic domain facing the cytosol. Research has shown removal of the N-terminal anchoring domain and overexpression of the catalytic domain increased HMGR activity and the accumulation of squalene in yeast (Donald et al. 1997).

In two successive phosphorylations, mevalonate kinase (MVK; EC 2.7.1.36) phosphorylates MVA to create mevalonate-5-phosphate (MVA-P), which is then phosphorylated by phospho-MVA kinase (PMVK; EC2.7.4) to produce MVA-5-pyrophosphate (MVA-PP). These two enzymatic steps were identified as major bottleneck points in the MVA pathway, and overexpression of both genes in *E. coli* leads to improved productivity of desired bioproducts (Redding-Johanson et al. 2011).

The last steps of the pathway involve the decarboxylation of MVA-5-pyrophosphate to IPP, followed by the reversible isomerization of IPP to DMAPP. MVA-5-pyrophosphate to IPP is catalyzed by the enzyme MVA-pyrophosphate decarboxylase (MVD; EC 4.1.1.33). This step relies on the input of ATP and results in the decarboxylation of MVA-5-pyrophosphate to IPP, along with the release of CO_2. IPP isomerase (IDI; EC 5.3.3.2) then acts to convert IPP to its isomer DMAPP by transposing the position of a C-C double bond on the IPP molecule. This isomerization reaction is a noted rate-limiting step in the pathway, with equilibrium favored toward DMAPP production (Sun et al. 1998).

9.3.3 Isoprenoid Synthesis Through the MEP Pathway

The MEP pathway, also referred to as the 1-deoxy-D-xylulose 5-phosphate (DOXP) pathway, is an alternative route to the formation of IPP and DMAPP and is found in bacteria, various apicomplexan parasites, and plant chloroplasts (Lichtenthaler 1999) (Fig. 9.3a). The pathway starts with the condensation of pyruvate and glyceraldehyde-3-phosphate (GAP) to produce 1-deoxy-D-xylulose 5-phosphate (DXP). This reaction is catalyzed by the enzyme 1-deoxy-D-xylulose-5-phosphate synthase (DXS; EC 2.2.1.7), which requires a divalent cation (Mg^{2+} or Mn^{2+}) and thiamine pyrophosphate (TPP) as a cofactor. DXS plays an important regulatory role in the MEP pathway, since excess IPP and DMAPP can inhibit DXS activity by competing with TPP for binding with the enzyme (Banerjee and Sharkey 2014). In this way, excess IPP and DMAPP (created downstream in the pathway) can effectively slow or shut down MEP flux through an inhibitory feedback loop.

Fig. 9.3 (continued) organisms, substrates for both pathways are created during photosynthesis through the Calvin-Benson-Bassham (CBB) cycle. The isomerization of GPP through LS creates limonene, a cyclic isoprenoid with industrial applications. Enzymes in the MEP pathway are labeled in blue; enzymes in the MVA pathway are labeled in red. Enzymes downstream of both pathways are labeled in green. LS is labeled in orange and asterisked. Details on enzymes and pathway intermediates are described in the text

Next, DXP is converted to methylerythritol 4-phosphate (MEP) via DXP reductoisomerase (DXR, also known as IspC; EC 1.1.1.267). DXR requires reducing power in the form of NADPH, gained from the photosynthetic electron transport chain in photosynthetic organisms. Enzyme activity is controlled by phosphorylation of a particular serine residue in bacteria (Ser177 in *Francisella tularensis*; Ser186 in *Escherichia coli*) and acts as an important residue for binding of substrate (Banerjee and Sharkey 2014). The serine residue is conserved within plant DXRs; however, there is no information at present whether this same mechanism plays any role in regulating the MEP pathway.

The enzyme methylerythritol 4-phosphate cytidylyltransferase (MCT, also known as IspD; EC 2.7.7.60) converts MEP to diphosphocytidyl methylerythritol (CDP-ME) in a CTP-dependent reaction. Like DXR, this enzyme is thought to be dependent on a phosphorylation site on a threonine residue (Thr141 in *F. tularensis*; *Thr140 in E. coli*), which could also play a role in substrate binding (Banerjee and Sharkey 2014). CDP-ME is then phosphorylated from ATP to produce diphosphocytidyl methylerythritol 2-phosphate (CDP-MEP), catalyzed by CDP-ME kinase (CMK; EC 2.7.1.148). These two steps in the MEP pathway both require CTP and ATP, respectively.

The cyclic conversion of CDP-MEP to CDP-ME-cyclo-pyrophosphate (CDP-MEcPP) is performed by MEcPP synthase (MCS, also known as IspF; EC 4.6.1.12) and represents a major regulatory checkpoint in the MEP pathway (Banerjee and Sharkey 2014). Crystal structural analysis of the MCS from *E. coli* has shown a hydrophobic cavity present in the enzyme that may bind to different isoprenoids containing pyrophosphate moieties, such as IPP/DMAPP, GPP, and FPP. Furthermore, sequence alignments show strong conservation of this motif among MCSs from various organisms, suggesting a selective pressure for this specific structure (Banerjee and Sharkey 2014). These results suggest the motif in MCS might play a specific role in feedback regulation in the MEP pathway.

The next two steps in the MEP pathway consist of successive redox reactions. The first enzyme, hydroxy-methylbutenyl diphosphate synthase (HDS, also known as IspG or GcpE; EC 1.17.7.1), converts CDP-MEcPP to hydroxyl-methylbutenyl pyrophosphate (HMBPP), which is then reduced to IPP and DMAPP by HMBPP reductase (HDR, also known as IspH or LytB; EC 1.17.1.2). Both enzymes utilize [4Fe-4S]-clusters and involve double one-electron transfers in their reaction mechanism. It was demonstrated in *A. thaliana* and the thermophilic cyanobacterium *Thermosynechococcus elongatus BP-1* that HDS reduces MEcPP through reduced ferredoxin, generated by the photosynthetic electron transport chain (Okada and Hase 2005; Seemann et al. 2006). In contrast, *E. coli* HDS requires the flavodoxin/flavodoxin reductase/NADPH system to reduce MEcDP (Xiao et al. 2009). The highest reported HDS activity in *E. coli* is remarkably low at 74–99 nmol min^{-1} mg^{-1}, which is roughly 300-fold lower than HDR (30.4 μmol min^{-1} mg^{-1}) and 100- to 5000-fold lower than all of the other MEP enzymes (10–500 μmol min^{-1} mg^{-1}). HDS and HDR both require a very negative reducing power to catalyze their reactions, which are naturally supplied by ferredoxin (plants and cyanobacteria) or the NADPH/flavodoxin system (bacteria). Reducing power is

often a limiting factor for HDS and HDR activity. Exchanging NADPH/flavodoxin for an artificial redox partner with a lower reducing potential (methyl viologen; −446 mV) increased the activity of the *E. coli* HDS to 550 nmol min^{-1} (Xiao et al. 2009).

9.3.4 Convergence of MEP and MVA Pathway Through IPP/DMAPP Formation

In contrast to the MVA pathway, which creates IPP and isomerizes it to DMAPP through IDI, the enzyme HDR catalyzes the simultaneous formation of both IPP and DMAPP in an approximate 5:1 proportion (Adam et al. 2002). This formation of IPP and DMAPP is the one step linking both the MVA and MEP pathway, although the pathways are separated by different organisms, or in the case of plants, different organelles. Two classes of IDI enzymes have been discovered which show no sequence similarities, display different reaction mechanisms, and require different cofactors for proper catalysis (Perez-Gil and Rodriguez-Concepcion 2013). The type I enzyme (IDI-I) is found in many bacteria (including *E. coli*) and is similar to IDIs found in fungi, plants, and animals. The type II enzyme (IDI-II) is found in archaea and some bacteria (*Streptomyces*) but is absent from plants and animals. Interestingly, genome sequencing has revealed some bacteria contain either IDI-I or II enzymes, while others contain both or neither. Since IDI activity is essential to produce DMAPP in organisms only containing the MVA pathway, it may not be surprising to find its absence in organisms harboring the MEP pathway, since they can form IPP and DMAPP through HDR. Some species of cyanobacteria, including *Synechocystis*, *Synechococcus*, and *Cyanothece*, contain orthologs of IDI-II, yet these enzymes are deemed nonessential for isoprenoid synthesis (Perez-Gil and Rodriguez-Concepcion 2013).

As stated earlier, the head-to-tail linking of IPP and DMAPP creates the prenyl pyrophosphate GPP, the substrate for all monoterpene synthases. Next, we focus on the monoterpene synthase that catalyzes the conversion of GPP to limonene.

9.3.5 Limonene Synthases in Nature

Limonene synthase (LS) is a monoterpene cyclase that catalyzes the cyclization of GPP to limonene. To date, there are two major classes of LS found in nature, each producing the separate *R*- and *S*-enantiomers of limonene. The most common is (4*R*)-LS (EC 4.2.3.20), which produces *R*(+)-limonene (*d*-limonene). The other major LS is (4*S*)-LS (EC 4.2.3.16), which produces S(-)-limonene (*l*-limonene). The first LSs to be characterized were the (4S)-LSs from the oil glands of mint plants (Alonso et al. 1992; Colby et al. 1993; Rajaonarivony et al. 1992). This opened the

door to the isolation and functional expression of LS cDNA from many members of the mint, herb, and citrus families. Across plant species, most LSs have similar features: they are localized to the chloroplast, have a molecular weight of ~65 kD, require divalent metal ions (Mg^{+2} or Mn^{+2}) for substrate binding and catalysis, and have a pI of ~5 and optimal pH of ~7 and a $k_{cat} < 1\ s^{-1}$ (Hyatt et al. 2007).

9.3.6 Reaction Mechanism and Conserved Sequences

Monoterpene synthases, such as LS, utilize GPP as a natural substrate. Since GPP cannot be directly cyclized due to the location of double bond between the 2 and 3 carbons (C2 and C3), the reaction mechanism involves isomerization and cyclization steps (Bohlmann et al. 1998). First, GPP is ionized with the help of a divalent metal ion (Mg^{+2} or Mn^{+2}) bound to the LS active site. The interaction between the resulting C2 carbocation and the migration of the pyrophosphate anion to C3 yields the enzyme-bound intermediate (3R)- or (3S)-linalyl pyrophosphate (LPP). Whether limonene takes either 4R- or 4S-form depends on the initial folding of the GPP substrate. After rotation from a trans-to-cis formation, LPP ionizes again and cyclizes to form the corresponding 4R- or 4S-α-terpinyl cation. From here, the cation intermediate is deprotonated, creating the final limonene product. Minor products, including α-pinene, β-pinene, and myrcene, have been reported from preparative LS assays, although their concentrations are significantly lower (Colby et al. 1993; Martin et al. 2004). The ability to create multiple products is an unusual enzymatic attribute of LS, yet common among characterized monoterpene synthases.

Comparative genomics have revealed sequence similarities between LSs and other isoprenoid synthases, giving us insight into important structural and catalytic elements of these enzymes (Bohlmann et al. 1998). In general, LS is composed of two distinct structural domains: an N-terminal transit domain and a C-terminal active site domain (Fig. 9.4). Transit peptides located in the N-terminus target the newly synthesized LS enzyme to the chloroplast. Posttranslational modifications then remove the N-terminal residues upstream of a conserved, tandem arginine repeat (RRx_8W), creating a mature protein. The active site of the LS is composed of an aspartate-rich DDxxD motif, which coordinates two Mg^{2+} ions for aligning the GPP substrate to the catalytic pocket of the enzyme. Mutagenesis of any of the three aspartates of the DDxxD motif reduces catalytic activity by 1000-fold, proving the absolute necessity of this element for LS activity (Bohlmann et al. 1998).

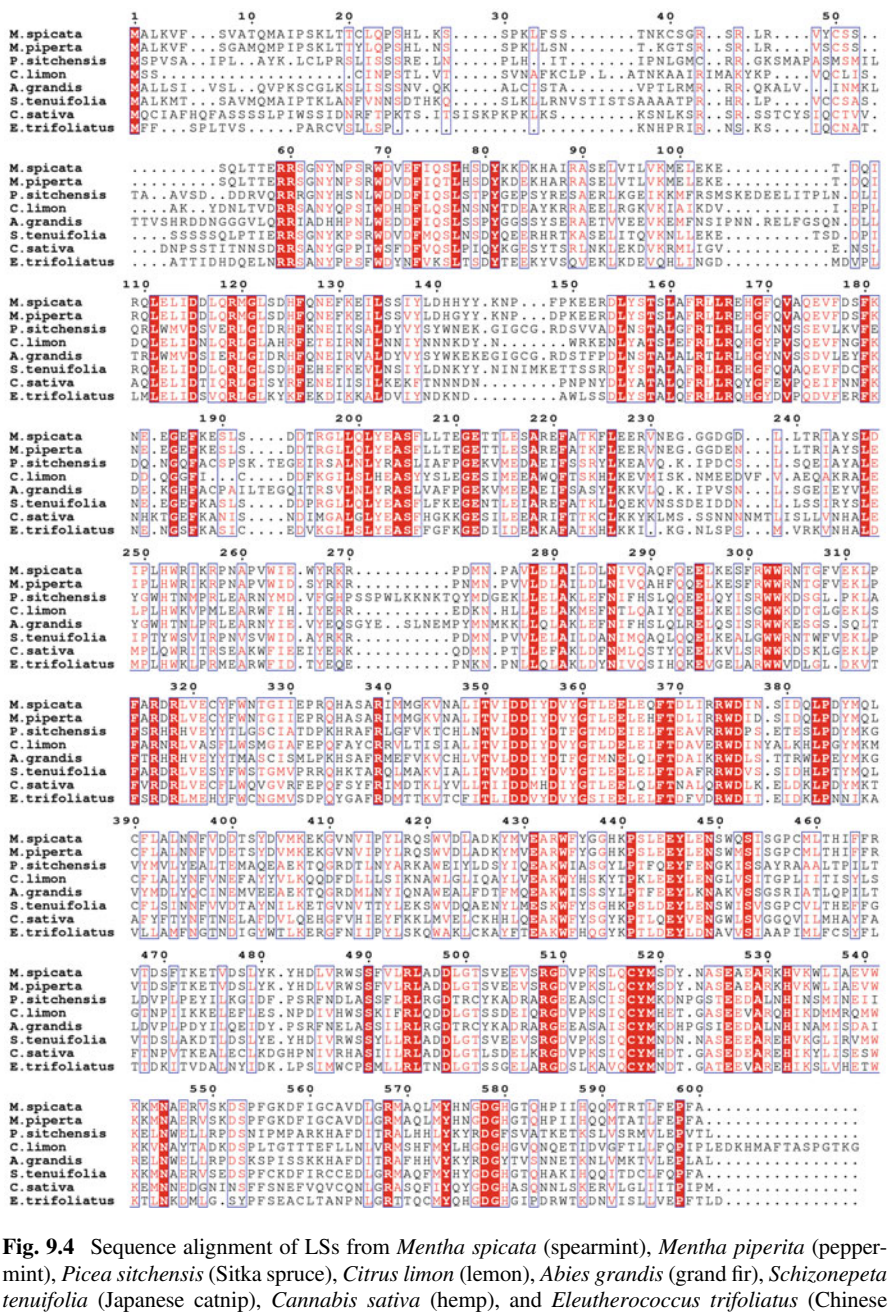

Fig. 9.4 Sequence alignment of LSs from *Mentha spicata* (spearmint), *Mentha piperita* (peppermint), *Picea sitchensis* (Sitka spruce), *Citrus limon* (lemon), *Abies grandis* (grand fir), *Schizonepeta tenuifolia* (Japanese catnip), *Cannabis sativa* (hemp), and *Eleutherococcus trifoliatus* (Chinese herb). Conserved amino acids are labeled in red, including the RRx_8W and DDxxD motif

9.4 Designing Cyanobacteria as Cellular Factories for Limonene Production

The last 20 years have seen a revolution in genome sequencing, unveiling the complete chromosome and plasmid sequences of many different cyanobacteria species (http://genome.microbedb.jp/cyanobase/). These efforts have allowed researchers to characterize the genes that create metabolic networks and to understand the extraordinary processes that convert CO_2 into sugars, amino acids, nucleotides, lipids, pigments, and other cellular components. Advancements in DNA-editing technology provide new opportunities to not only improve production of metabolites naturally produced by an organism but to add new genetic components to allow an organism to generate new metabolites. In the case of limonene, this would require insertion of new genes into the cyanobacterium to redirect intermediate metabolites into limonene. The expression and regulation of genes involved in biosynthetic pathways can boost carbon flux toward limonene synthesis, resulting in the higher limonene productivities needed for commercialization.

Inserting new genes into a cell is not a simple process, and many researchers have dedicated their lives to understand how genes can be packaged into organized structures and shipped across the host's cell membrane. Once inside the cell, the new genes must either be integrated into the chromosome or maintained as plasmids in the cytosol, where they express proteins that influence native metabolic networks. Most importantly, recombinant proteins must be regulated in a manner that eliminates the build-up toxic intermediates, prevents pathway bottlenecks, and allows high flow of substrate to product (Peralta-Yahya et al. 2012). This takes careful consideration in the design and construction of genetic components and their subsequent integration and regulation in the cyanobacterium.

9.4.1 Transfer and Recombination of Genes into the Cyanobacterium Genome

The introduction of genes that divert fixed carbon into a desired molecule has been the main approach in engineering cyanobacteria to produce valuable chemicals (Ducat et al. 2011; Ruffing 2011). Ever since the first report of exogenous DNA transfers into a cyanobacterium (Shestakov and Khyen 1970) and the development of recombinant DNA technologies in the 1970s, our knowledge of genetic engineering in cyanobacteria has expanded greatly. Descriptions of recombinant DNA engineering in cyanobacteria are widespread throughout the literature (Golden et al. 1986; Frigaard et al. 2004; Koksharova and Wolk 2002; Eaton-Rye 2004). Most genetic manipulations of cyanobacteria were originally performed to gain knowledge into metabolic, genetic, and photosynthetic processes of these organisms. These studies are now the backbone of research focused on the genetic manipulation of cyanobacteria for applied purposes.

The primary challenge regarding the insertion of DNA into a cyanobacterium is the efficiency of DNA transfer into the cell. Fortunately, several species of unicellular cyanobacteria, such as *Synechocystis* sp. PCC 6803, *Synechococcus elongatus* PCC 7942, and *Synechococcus* sp. PCC 7002, are naturally competent and can transport DNA across the cell membrane with moderate to high efficiency (Grigorieva and Shestakov 1982). Consequently, this defining feature makes these cyanobacteria species very popular for genetic research. Other cyanobacterial species, such as *Nostoc punctiforme* ATCC 29133, *Anabaena variabilis* ATCC 29413, and *Anabaena* sp. PCC 7120, have attributes that make them advantageous hosts, due to their ability to produce hydrogen or fix nitrogen. However, DNA insertion into these strains is problematic, due to highly active restriction enzymes that degrade foreign DNA that enters the cell. Mechanisms that protect foreign DNA, such as methylation factors, have been developed for these strains (Elhai et al. 1997). Other transformation techniques, including conjugation and electroporation, have also been developed (Elhai and Wolk 1988; Thiel and Poo 1989; Koksharova and Wolk 2002). Often, selectable markers are incorporated alongside the inserted gene, which helps screen for successful transformants after transformation. These are often antibiotic-resistant markers, which are dependent upon the antibiotic sensitivity of the host, as well as the ability of the host to produce the functional protein product of the antibiotic gene.

The insertion of a gene(s) in a cyanobacterium host can be accomplished using two methods: (1) by integration of the gene(s) into the host chromosome using homologous flanking regions or (2) by expressing the gene(s) outside of the chromosome on a self-replicating plasmid, sometimes referred to as a "shuttle vector" (Wolk et al. 1984). Each of these approaches has its advantages and disadvantages. Although genes that are integrated into the chromosome are more stable in the progeny of transformed lines, the known chromosomal loci that can be disrupted without corresponding negative effects are limited. Chromosomal neutral sites that allow for gene integration and disruption without harmful side-effects to the cell have been identified in cyanobacteria (Clerico et al. 2007). Shuttle vectors harbor the genes-of-interest outside of the chromosome, which preserves chromosomal integrity, and contain replication origins (the site where DNA replication takes place) that are easily recognized by the host cell. However, these plasmids replicate independently of cell division, creating daughter cells with inconsistent plasmid copy numbers. Most shuttle vectors are derived from native cyanobacterial plasmids with uncharacterized plasmid copy numbers, making control of gene expression unpredictable. Antibiotic selection pressure is also required to maintain these plasmids, and this is a concern for scale-up and commercialization.

Gene expression plays a crucial role in developing engineered microorganisms, since it is the regulation of genes that will ultimately influence production of the target compound. Engineered pathway genes can be controlled in several ways (Peralta-Yahya et al. 2012). The number of copies of a pathway gene (gene copy number) can be changed by increasing the number of genes integrated into the genome or by modifying the strength of the origin of replication (site of DNA replication). The rate at which a gene(s) is transcribed to mRNA can be controlled

by changing the strength of the promoter. Promoters can even be designed to be constitutive (always "on") or inducible (turned "on" and "off" during a desired time), giving researchers the power to regulate parts of a pathway, or multiple pathways, independently. Genes can be engineered with synthetic transcriptional terminator sites (TTS) that can control transcriptional efficiency. The strength of the ribosomal-binding site (RBS) upstream of the gene can be predicted using computer modeling, allowing optimized protein translation. Protein turnover (the balance between protein synthesis and degradation) can be regulated by adding a specialized "degradation tag" to a recombinant protein, which marks a protein for destruction. The combinatorial use of these genetic elements allows control over gene expression on the transcriptional, translational, and posttranslational level.

In recent years, the field of synthetic biology has revolutionized genetic engineering and is a crucial tool in designing complex gene components. Synthetic biology is the design and construction of new biological parts, devices, and systems, as well as the redesign of existing biological systems for useful purposes (http://syntheticbiology.org). Genetic components, such as promoters, RBSs, terminators, and replication origins, are organized in the "Registry of Standard Biological Parts," a repository that consists of thousands of genetic parts (available at http://partsregistry.org). These components, also known as "BioBricks," can be assembled using established cloning techniques and used for the rapid construction of engineered microorganisms. Although most of the work in synthetic biology has been performed in *E. coli*, some researchers have dedicated to creating a BioBrick format for cyanobacteria (Huang et al. 2010).

9.4.2 *Demonstrating Limonene Production from Cyanobacteria*

To this date, over 50 cyanobacteria species have completely sequenced genomes, with new cyanobacteria genome projects increasing at a rapid pace. These efforts have revealed orthologs for all known MEP pathway genes in most of the well-studied species (http://genome.microbedb.jp/CyanoBase). Although genome surveys have identified the necessary components to a functional MEP pathway in cyanobacteria, no gene similarities to annotated plant LSs have been found, which explains limonene's absence from volatile profiles from cyanobacteria. This has prompted researchers to investigate the possibility of adding a new carbon sink by inserting a plant LS gene into its genome, thereby channeling natively produced GPP into limonene.

In 2014, a number of research groups showed proof-of-concept demonstrations of limonene production from several different cyanobacteria species using genetic engineering. A (4*S*)-LS from *Picea sitchensis* (Sitka spruce) was linked to a dual *nir-psbA1* promoter and expressed in the filamentous, nitrogen-fixing cyanobacterium *Anabaena* sp. PCC 7120, using a replicating shuttle vector (Halfmann et al.

2014b). Volatized limonene emitted from the transgenic *Anabaena* were captured using a hydrophobic resin column and analyzed using gas chromatography-mass spectrometry (GC-MS). With expression of the (4*S*)-LS from *P. sitchensis*, the transgenic *Anabaena* yielded 114.3 ± μg limonene L^{-1} during 14 days of continuous growth. Limonene yield was increased 2.3-fold during the same time frame by co-expression of three rate-limiting enzymes (DXS, IDI, GPPS) in the MEP pathway. Optical density and chlorophyll measurements were similar between the wild-type (WT) and transgenic cultures, concluding that limonene's effects on culture growth were minimal to nonexistent. However, an increase in photosynthetic activity was also observed, suggesting limonene acting as an additional sink for carbon fixation.

In both transgenic strains (LS and LS-DXS-IDI-GPPS *Anabaena*), limonene productivity decreased during the duration of the growth trials, presumably from the inability of light to evenly disperse as the cultures became more dense (the "self-shading effect") (Halfmann et al. 2014b). Increasing the light intensity from 50 to 150 μE m^{-2} s^{-1} greatly improved limonene productivity upon initial culture growth (3.6 ± 0.5 μg limonene L^{-1} $O.D.^{-1}$ h^{-1}) but dropped after cell density reached a critical point ($O.D._{700nm} > 1.0$). It is assumed that the drop in productivity is the result of light limitation, which could reduce activity of the MEP pathway by limiting reductants created through photosynthesis, or reduce activity of the psbA1 promoter, which is known to be light-activated.

Kiyota et al. used a similar approach to engineer a limonene-producing cyanobacteria. They expressed a codon-optimized LS from the Chinese medicinal herb *Schizonepeta tenuifolia* in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803. Limonene was collected using a gas stripping method, in which outgas from a bubbling culture was released in the octane phase of a cold trap. The system produced limonene at a rate of 41 μg L^{-1} day^{-1} when expressing the LS alone and increased to 56 μg L^{-1} day^{-1} when co-expressing three MEP pathway enzymes (DXS, IPI, CrtE). Sustained limonene production was observed for up to 300 h and decreased to nearly one-third of the initial production rate after 700 h of continuous growth (Kiyota et al. 2014).

A (4*S*)-LS from *Mentha spicata* (spearmint) was expressed in the unicellular cyanobacterium *Synechococcus* sp. PCC 7002, and limonene was collected by applying a dodecane ($C_{12}H_{26}$) overlay on top of the culture (Davies et al. 2014). The hydrophobic layer maintained separation from the culture during growth trials and was able to trap limonene secreted from the cells. Using this methodology, total limonene titers reached 1.7 mg limonene $gDCW^{-1}$ (4 mg L^{-1}) after 96 h. Several factors may attribute to the superior performance of limonene production in this engineered strain. The superior kinetics and high specificity of the (4*S*)-LS from *Mentha spicata* may play a significant role in the fast turnover of GPP to limonene. Also, the dodecane overlay may actively sequester limonene away from the cell, thereby relieving negative feedback pressures exerted by the products and promote the forward reaction of GPP to limonene. Two-phase bioreactors that trap secreted products were also shown to prevent evaporative loss of limonene and other isoprenoids (Bentley and Melis 2012).

Glycogen is a highly branched polymer of glucose and serves as the main carbon and energy reserve in cyanobacteria. The inactivation of glycogen synthesis in several cyanobacteria strains was shown to cause a number of physiological effects during nutrient stress (Gründel et al. 2012; Suzuki et al. 2010). Studies on glycogen-deficient *Synechocystis* and *Synechococcus* mutants showed that under nitrogen depletion, partially oxidized metabolites (α-ketoglutarate, pyruvate, succinate, acetate, and α-ketoisocaproate) were found to accumulate in the culture media (Gründel et al. 2012; Davies et al. 2014). Rerouting these precursors through the MEP pathway would be one strategy to distribute flux from a major carbon sink into limonene synthesis. However, expressing an LS into glycogen-deficient *Synechococcus* sp. PC 7002 resulted in no significant difference in limonene production (Davies et al. 2014). It is therefore presumed that other bottlenecks or regulatory checkpoints in the MEP pathway can prevent carbon flux to limonene, even with the accumulation of precursors in the beginning of the pathway. Interestingly, it was found that these organic acids increased in accumulation with the expression of LS. This mysterious dynamic between limonene synthesis and metabolic overflow in glycogen-deficient cyanobacteria mutants is an interesting area for future research.

9.5 Discussion

Compared to other carbon pathways, cyanobacteria invest a tiny percentage of daily fixed carbon toward isoprenoids, ranging from 2 to 5% of total fixed carbon (Lindberg et al. 2010). Limonene flux of engineered cyanobacteria is even lower, with estimates as low as 0.1% of the total carbon pool (Kiyota et al. 2014). Similar levels of chlorophyll and carotenoids between engineered and WT cyanobacteria suggest that LS activity is a major limiting factor to limonene productivity. To obtain a significant increase in limonene productivity, it may be necessary to express a more optimal LS to synthesize limonene from the GPP pool. Data mining plant genomes can reveal highly active LSs, as well as more suitable enzyme candidates for upstream pathway reactions. Redesigning proteins for higher catalytic activity and specificity is another approach that has seen some success as an engineering strategy (Wen et al. 2009).

There are many potential factors behind the limited allocation of carbon into MEP pathway products in cyanobacteria. These include rapid degradation of unstable intermediates, competition with other carbon pathways that create cell architecture and the photosynthetic apparatus, and the inactivation of enzymatic steps through various host control mechanisms (Kudoh et al. 2014). Increasing the pool of IPP and DMAPP is necessary to provide the needed precursors to isoprenoid products. Expressing the alternative MVA pathway in cyanobacteria that operates in parallel to the MEP pathway can increase flux toward isoprenoid synthesis, since the host organism would not possess the required MVA pathway regulatory elements to control it. This was demonstrated by the expression of a whole MVA pathway in a

Synechocystis sp. PCC 6803, which the cyanobacterium used to produce the C_5 isoprene alongside its native MEP pathway (Bentley et al. 2014). This increased isoprene productivity from ~60 to ~150 μg isoprene L^{-1} over 196 hours of culture growth, an approximate 2.5-fold increase.

Operating whole-engineered pathwaysin microorganisms for chemical production is a formidable task, since most biosynthetic pathways are extremely complex and are regulated at multiple biological levels. Careful consideration of transcriptional and translational efficiencies, enzymatic activities, and pathway cross-talk is needed by the designer to allow for the rapid transformation of the initial substrate to the final product (Keasling 2012). A common misconception is that this can be accomplished by highly expressing all genes in the pathway. However, this is a flawed concept, since all pathway reactions involve enzymes with different kinetics, require different cofactors, are influenced by diverse inhibitory and activating agents, and so on. Expressing a gene at too high a level can erroneously shift metabolites away from the desired product and reduce productivity or in some cases cause the buildup of toxic intermediates that are detrimental to cell maintenance (Keasling 2012). Instead, recombinant enzymes need only to be produced in sufficient amounts that allow for the transformation of intermediates into the desired products at a sufficient rate. This involves coordinating gene expression in a way that takes into account each reaction step from the initial substrate to final product (Kirby and Keasling 2009). Other strategies, such as introducing efflux pumps to prevent feedback inhibition, have also been implemented (Dunlop et al. 2011).

Although additional engineering work has improved isoprenoid production in cyanobacteria, current productivities reported from photosynthetic microorganisms are much lower than engineered heterotrophic bacteria, most notably *E. coli*. For instance, the expression of a heterologous MVA pathway in *E. coli* while co-expressing the *M. spicata* LS resulted in limonene titers reaching 450 mg limonene L^{-1} over 72 h (Alonso-Gutierrez et al. 2013), much higher than any limonene-producing cyanobacteria in the literature (4 mg L^{-1} in 96 h, Davies et al. 2014). This "productivity gap" between engineered cyanobacteria and *E. coli* reflects the infancy of cyanobacteria engineering, since *E. coli* is a popular model organism with a more established bioengineering repertoire. The engineering strategies used in *E. coli* can be adopted for cyanobacteria to increase isoprenoid productivity and titers.

As stated above, *E. coli* has been a highly used model for microbial isoprenoid production, most notably for the production of the sesquiterpene amorpha-4,11-diene, the precursor for the antimalarial drug artemisinin (Martin et al. 2003). Improvements in pathway engineering through optimizing gene codons and copy numbers, alleviation of pathway bottlenecks, and an efficient fed-batch fermentation process boosted amporpha-4,11-diene titers to more than 27 g L^{-1} (Tsuruta et al. 2009). Scientists at the University of California, Berkeley, brought this technology to the commercial level through the biotech company Amyris, which now manufactures artemisinin-based drugs and more recently biofuels from engineered *Saccharomyces cerevisiae* (https://amyris.com/products/fuels/). In the future, we may all benefit from a diverse variety of specialized medicines and fuels that are made exclusively by microorganisms.

9.6 Conclusions

Proof-of-concept demonstrations of isoprenoid production from metabolically engineered cyanobacteria are widespread in the literature, including isoprene (Lindberg et al. 2010), farnesene (Halfmann et al. 2014a), phellandrene (Bentley et al. 2013), squalene (Englund et al. 2014), bisabolene (Davies et al. 2014), and limonene (Davies et al. 2014; Halfmann et al. 2014b; Kiyota et al. 2014). These engineering efforts were successful, thanks to decades of advancements in genomics, molecular biology tools, and recombinant DNA technology. For these biofuel/chemical platform technologies to be successfully commercialized, proof-of-concept studies will need to mature to pilot-scale demonstrations that show the technology can be a cost-effective alternative to petroleum.

The transition from lab-bench to pilot scale can be aided by techno-economic modeling, which analyzes the economic feasibility of industrial-scale cyanobacteria cultivation, harvesting, and chemical extraction from biomass. The Farm-level Algae Risk Model (FARM) was previously used to simulate operation of a commercial limonene-production facility, revealing that photosynthetic limonene productivity would need to reach ~1 mg limonene L^{-1} day^{-1} to be economically viable (Johnson et al. 2016). Advancements in engineering (molecular and industrial) are the likely routes to achieving these rates while also utilizing resources such as natural sunlight, flue gas, and wastewater to be converted into valuable fuels and chemicals. Photobioreactors designed to optimize mass transfer, temperature, nutrients, and solar radiation will also be critical. Furthermore, biomass-biofuel separation techniques must be engineered to collect the biofuel efficiently and alleviate cell toxicity/product feedback. In all, this will require multidisciplinary efforts from molecular biology, biochemistry, biophysics, computational biology, and industrial engineering.

The idea of direct CO_2 conversion into fuels and chemicals has already garnered significant interest from the investment world. Joule Unlimited has brought photosynthetically derived fuels into recognition through the production of *Sunflow*® products, ethanol and diesel fuels produced from engineered algal cells (Berry 2010). Recently, Joule Unlimited announced the development of a 4-acre test site in Hobbs, New Mexico, for planned production of up to 25,000 gallons of ethanol and 15,000 gallons of diesel per acre annually, at an estimated price of $50/barrel (Hepler 2015). Algenol, headquartered in Fort Myers, Florida, confirmed they had exceeded production rates of 9000 gallons of ethanol/acre/year from their 4-acre, outdoor Process Development Unit in Lee County, Florida. This facility previously achieved continuous ethanol production at 7000 gallons ethanol/acre/year in September 2013, up from a projected 6000 gallons ethanol/acre/year (Lane 2013). Company CEO Paul Woods announced that the Direct-to-Ethanol technology allowed the production of ethanol for around $1.00/gallon, using only sunlight, carbon dioxide, and seawater. Whether these companies or similar startups can achieve the same successes with isoprenoids is yet to be seen.

The milestones reached by industry to engineer algae and cyanobacteria to convert inorganic carbon, waste water, and light into diesel and ethanol bring hope for future production of advanced biofuels. However, as noted above, significant improvements in key areas are needed to achieve this goal of using cyanobacteria-based technologies to convert CO_2 into valuable fuels and chemicals.

Take-Home Message

- Limonene, a cyclic C_{10} isoprenoid, is a valuable commodity chemical with applications in solvents, perfumes, flavorings, pharmaceuticals, and biofuels.
- As consumer interest in "all-natural" chemicals continues to grow, attention has turned to utilizing fourth-generation biofuel technologies for limonene production.
- Cyanobacteria can serve as a photosynthetic chassis for synthesizing economically relevant fuels and chemicals. Proof-of-concept studies demonstrate the potential use of genetically modified cyanobacteria to produce a wide range of green solvents, including limonene.
- Future advancements in genetic and industrial engineering may potentially lead to the direct conversion of CO_2 into limonene in industrial facilities.

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Chapter 10
Exopolysaccharide Productions from Extremophiles: The Chemical Structures and Their Bioactivities

Paola Di Donato, Annarita Poli, Giuseppina Tommonaro, Gennaro Roberto Abbamondi, and Barbara Nicolaus

What Will You Learn from This Chapter

This chapter will discuss some remarkable examples of extremophilic bacteria, isolated from different ecosystems, which produce exopolysaccharides (EPSs). The chapter will also cover the properties of EPSs produced by extremophiles and their possible commercial applications ranging from pharmaceutical to food processing, detoxification and bioremediation. This chapter will also cover the techniques that are used to purify, analyse and structurally characterise the bacterial EPSs; the state of the art in the field of bacterial EPSs research, with mention to the main examples of well-studied and commercially exploited EPSs; an overview of the main EPSs' producing extremophiles that have been isolated from both aquatic and terrestrial environments; the description of the main biosynthetic routes leading to the EPSs production in archaeal and bacterial extremophiles, with reference to the enzymes involved and to the genetic manipulations for biosynthesis's tailoring; the analysis of the most interesting biological properties of extremophiles' EPSs (that can act as anti-inflammatory, immunomodulating and antiviral agents) and of their biotechnological applications in drug delivery systems; and the compendium of the most used techniques to perform the purification, the analysis of chemical composition, the

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identification of glycoside linkage position and substitution pattern, the determination of the molecular-weight distribution and the structural analysis of EPSs.

10.1 Introduction

The exopolysaccharides (EPSs) are high-molecular-weight heterogeneous polymers composed by some most recurring monomers like hexoses, pentoses, uronic acids and amino sugars that are often substituted either by organic non-carbohydrate or inorganic groups. Some remarkable examples of well-studied and commercially exploited EPSs include xanthan gum (produced by *Xanthomonas* species), gellan (produced by bacteria belonging to *Sphingomonas* genus), alginate (found in bacteria from the genera *Azotobater* and *Pseudomonas*), hyaluronan (produced by different bacterial strains, e.g. *Pseudomonas aeruginosa*) and levan (produced by species of the genera *Bacillus*, *Rahnella*, *Aerobacter*, *Erwinia*, *Streptococcus*, *Pseudomonas* and *Zymomonas* and recently found also in a species of *Halomonas* genus) (Freitas et al. 2011). Several studies have described the production of large quantities of these polymers also by bacterial species living in extreme environments, for example, Antarctic marine environments or hydrothermal vents, where they are thought to complex with various metal ions and contribute to their mobility and entry into the food web (Mancuso Nichols et al. 2004 and references therein). Bacterial EPSs usually are found as biofilm, a complex mixture constituted by proteins, nucleic acids and lipids besides the polysaccharides. The bacterial species that are able to produce EPSs include several extremophiles, a group of microorganisms that have developed various adaptation strategies, enabling them to compensate for the deleterious effects of harsh environmental conditions such as high temperatures and salt concentrations, low pH or temperature and high radiation. Among these strategies, the EPS biosynthesis is one of the most common protective mechanisms. Exopolysaccharides can be found as in capsular material or as dispersed slime in the surrounding environment. Considerable progress has been made in discovering and developing new microbial EPSs that possess novel industrial significance. In recent years the increased demand for natural polymers for pharmaceutical, food and other industrial applications has led to a remarkable interest in polysaccharides produced by microorganisms (Poli et al. 2011; Finore et al. 2014; Nicolaus et al. 2010).

This chapter will give a short review of extremophilic microorganisms as a source of EPSs with particular attention to their production, structural characterisation methods and biological activities.

10.2 Examples of Archaeal and Bacterial EPS Producers and Their Biosynthetic Pathway

EPS-producing microorganisms have been isolated from different natural sources of both aquatic and terrestrial environments, as well as in extreme niches such as hot springs, cold, hypersaline and halophilic environments, such as salt lakes and salterns (Poli et al. 2009, 2010; Yasar Yildiz et al. 2014).

EPSs produced by extremophiles embody promising biotechnological applications: their rheological properties, biological activities, metal-binding capabilities and new sugar composition make these biopolymers suitable for many applications. Indeed their potential biotechnological spectrum of application is very wide, ranging from antiviral and thickening agents to bioflocculanting and viscosifying agents and from biosurfactant and bioemulsifier to vaccine adjuvants. Moreover, for their metal-binding and pollutant bioadsorption activities, EPSs could be employed in the bioremediation processes (Finore et al. 2014).

Microorganisms belonging to *Bacteria* and *Archaea* domains have been described as EPS producers: some representative examples are listed in Table 10.1. In the *Archaea* domain, *Sulfolobus solfataricus* strain MT4, *Haloferax mediterranei*, *Haloferax gibbonsii* and *Haloarcula japonica* strain T5 represent some example of species able to produce EPS for which the participation in biofilm formation and solid surface adhesion has been suggested (Poli et al. 2011). In the range of thermophilic EPS producers (Table 10.1), species like *Thermus aquaticus* strain YT-1, *Thermotoga maritima* and several species belonging to the *Geobacillus* genus (*G. thermantarcticus* strain M1, *G. tepidimans* strain V264, *G. thermodenitrificans* strain B3-72, *Geobacillus* sp. strain 4004) showed the EPS production. Moreover, species of *Bacillus* genus (such as *B. licheniformis* strain T4 isolated from a shallow hydrothermal vent of Panarea Island, Italy) and *Aeribacillus* genus (*Aeribacillus pallidus strain* 418 isolated from the Bulgarian Rupi hydrothermal springs) have been reported as responsible for the synthesis of novel EPSs, as shown in Table 10.1 (Kambourova et al. 2009; Nicolaus et al. 2010; Spanò et al. 2013 and references therein).

Pseudoalteromonas strain 721 isolated from deep-sea hydrothermal vents produced an EPS that exhibited gel formation and viscoelastic behaviour at increasing temperature. In the Antarctic and Arctic ecosystems (see also Table 10.1), *Pseudoalteromonas* sp. SM9913, *Pseudoalteromonas* strain CAM025 and CAM036 and *Colwellia psychrerythraea* strain 34H have been described as source of EPS from cold marine environments: in the case of *Pseudoalteromonas* CAM 025 and CAM036, sulphated heteropolysaccharides with high levels of uronic acids and acetyl groups have been described, and cryoprotection activities have been suggested (Mancuso Nichols et al. 2004 and references therein).

Table 10.1 Most remarkable examples of microbial species producing bioactive or biotechnologically useful EPSs

Group	Microbial species	Activities/biotechnological applications	References
Archaeabacteria	*Sulfolobus solfataricus* strain MT4 *Haloferax mediterranei* *Haloferax gibbonsii* *Haloarcula japonica* strain T5	EPS-producer strains Participation in biofilm formation and solid surface adhesion suggested	Poli et al. (2011) and references therein
Thermophiles	*Thermotoga maritima* *Geobacillus* genus*:* *G. thermantarcticus* strain M1 *G. tepidimans* strain V264 *Geobacillus* sp. strain 4004	Responsible for the synthesis of novel EPSs	Kambourova et al. (2009), Nicolaus et al. (2010), Spanò et al. (2013) and references therein, Mastascusa et al. (2014)
	Thermus aquaticus strain YT-1	TA-1 Immunomodulatory activity TA-1 stimulated macrophages cells to produce the cytokines TNF-α and IL-6	Lin et al. (2011)
	Geobacillus thermodenitrificans strain B3-72	EPS-2 stimulate the cytokine production (IL-12, IFN-γ, TNF-α and IL-18) in PBMC cells in a concentration-dependent manner EPS-2 treatment in PBMC affected on HSV-2 replication, increasing the inflammatory response	Arena et al. (2009)
Hydrothermal springs/vents	*Bacillus* genus (*Bacillus licheniformis* strain T4)	EPS-1, containing tetrasaccharide-repeating units formed by sugars with a mannano-pyranosidic configuration Immunomodulatory effect: production of IFN-α, IL-12, IFN-γ, TNF-α, IL-18 in vitro Inhibition of HSV-2 replication in PBMC (human peripheral blood mononuclear cells) by upregulating	Spanò et al. (2013), Arena et al. (2006)

(continued)

Table 10.1 (continued)

Group	Microbial species	Activities/biotechnological applications	References
		the expression of pro-inflammatory cytokines	
	Aeribacillus pallidus strain 418	EPSs with high molecular weight and high thermostabiliy	Kambourova et al. (2009), Nicolaus et al. (2010) and references therein
	Pseudoalteromonas strain 721	EPS with gelling properties and viscoelastic behaviour at increasing temperature	Poli et al. (2010) and references therein
Antarctic and Arctic ecosystems	*Pseudoalteromonas* sp. SM9913 *Colwellia psychrerythraea* strain 34H	Source of EPS from cold marine environments	Mancuso Nichols et al. (2004) and references therein
	Pseudoalteromonas strain CAM025 *Pseudoalteromonas* strain CAM036	Sulphated heteropolysaccharides with high levels of uronic acids and acetyl groups Cryoprotective agent	
Halophiles	*Halomonas maura* strain S-30 *Halomonas anticariensis* strain FP35 *Halomonas ventosae* strain A112 *Halomonas eurihalina* strain F2-7	Highly sulphated EPSs Formation of stable emulsions	Poli et al. (2009) and references therein
	Halomonas smyrnensis	First examples of levan producer *Halomonas* species Cytoprotective activity against toxic agents: Brine Shrimp Test Drugs delivery system: nano-carrier system for peptide and protein	Poli et al. (2009) and references therein Sezer et al. (2011)
	Halomonas alkaliantarctica	Fructo-glucan polymer with high viscosity at low pH values/high NaCl concentration Proposed as viscosity control agent	Poli et al. (2010) and references therein
	Salipiger mucosus A3T	EPS rich of fucose. Proposed as source of fucose	Llamas et al. (2010)
	Zunongwangia profunda SM-A87	First marine bacterium that produces EPS with the high yield of 8.90 g/L	Qin et al. (2010)

Saline soils collected from salterns and sediments from salt lakes represent the extreme niches in which halophiles EPSs producers have been isolated: some main examples are reported in Table 10.1. High sulphate content EPSs have been described for the EPSs produced by *Halomonas maura* strain S-30, *Halomonas anticariensis* strain FP35, *Halomonas ventosae* strain A112 and *Halomonas eurihalina* strain F2-7: for these two latters, the formation of stable emulsions has been also recorded (Poli et al. 2010 and references therein). *Halomonas smyrnensis*, isolated from a saltern area in Turkey, represents the first examples of levan producer *Halomonas* species (Poli et al. 2009). This microorganism could be considered an alternative cheap source of levan polymer for which bioflocculant properties in the treatment of industrial wastewaters have been described (Table 10.1). Levan from *H. smyrnensis* has been tried as a nano-carrier system for peptide and protein drugs delivery. *Halomonas alkaliantarctica* isolated from a salt lake in Antarctica produced a fructo-glucan polymer that presented a high viscosity at low pH values and at high NaCl concentration, resulting as a viscosity control agent in proposed biotechnological applications (Poli et al. 2010). *Salipiger mucosus* A3T (Table 10.1), a halophilic species belonging to the *Alphaproteobacteria* genus and isolated from the Spanish Mediterranean seaboard, produced an EPS rich of fucose that was synthesised essentially during the exponential phase growth (Llamas et al. 2010): the authors suggested its use as source of fucose in spite of fucose-rich polysaccharides usually extracted from brown algae with more laborious and expensive procedures. *Zunongwangia profunda* SM-A87 isolated from deep-sea sediment in southern Okinawa Trough is the first marine bacterium that produces EPS with the high yield of 8.90 g/L (Qin et al. 2010).

Bacterial polysaccharides are comprised of repeating units of sugar moieties, which are synthesised by a group of enzymes named 'glycosyltransferases'. In some cases the EPS biosynthetic pathway is known, and the genetic manipulation has been employed in order to not only optimise the yield of EPS production but also to tailor the EPS chemical composition and structure. In fact, in addition to elucidation of biosynthesis mechanisms, it is possible to improve the microbial productivity via strain improvement strategies or to modify physicochemical and/or rheological properties of the biopolymer by changing its composition, length or degree of branching (Nicolaus et al. 2010). There are three main biosynthetic routes for microbial EPSs. The synthesis of some glucan- or fructan-type homopolysaccharides is carried out in the extracellular environment through the action of specifically secreted sucrase enzymes (Nicolaus et al. 2010). These glucansucrases and fructansucrases are glycoside hydrolases that act on sucrose and catalyse the transglycosylation reactions forming the polymer chain. Biosynthetic pathways of microbial heteropolysaccharides and of some homopolysaccharides are more complex and involve five distinct steps: (1) uptake of sugar subunits and their activation with a high-energy bond through their conversion into sugar nucleotides, (2) assembly of the repeating monosaccharide unit on an isoprenoid lipid carrier by sequential transfer of monosaccharides from sugar nucleotides by glycosyltransferases, (3) addition of any acyl groups, (4) polymerisation of the repeating unit and (5) secretion of the polysaccharide from the cell membrane into the extracellular environment. Despite the structural diversity of EPSs, there are only three

mechanisms for the polymerisation, namely, ABC-transporter-dependent, synthase-dependent and the most commonly used wzy-dependent pathways (Nicolaus et al. 2010).

Analysis of the flanking regions of a mini-Tn5 insertion site in an EPS-deficient mutant of *Halomonas maura* strain TK71 led to the identification of five ORFs (epsABCDJ), which form part of a gene cluster (*eps*) with the same structural organisation as others involved in the biosynthesis of group 1 capsules and some EPSs. The possibility that mauran, the polysaccharide produced by *H. maura*, might be synthesised via a Wzy-like biosynthesis system is reported by Arco et al. (2005).

The genomes of three model extreme thermophiles, an archaeon, *Pyrococcus furiosus* (*T*opt of 98 °C), and two bacteria, *Thermotoga maritima* (*T*opt of 80 °C) and *Caldicellulosiruptor saccharolyticus* (*T*opt of 70 °C), encode numerous carbohydrate-active enzymes, many of which have been biochemically characterised in their native or recombinant forms. In *Streptococcus thermophilus* Sfi6 the biosynthesis of exopolysaccharides is regulated by eps gene cluster of 14.5-kb region comprised of 13 genes, namely, epsA to epsM (Stingele et al. 1996). Gene epsA located at the beginning of the cluster is involved in the regulation of EPS expression, and the central region (epsE, epsF, epsG, epsH and epsI) of the gene cluster is involved in the biosynthesis of the tetramer repeating unit. Gene epsE encodes the galactosyltransferase, catalysing the first step of biosynthesis of the repeating unit. Genes upstream (epsC and epsD) and downstream (epsJ and epsK) of the central region regulate polymerisation and export of the EPS (Stingele et al. 1996). In the case of *Zunongwangia profunda* SM-A87, its genome was the first sequenced in the phylum of *Bacteroidetes* (Qin et al. 2010). It possesses two polysaccharide biosynthesis gene clusters, and the genome analysis reveals its adaptation to the deep-sea environment and ecological role in sedimentary organic nitrogen degradation.

For a systems-based approach to the EPS biosynthetic pathways, microbial genome sequence is considered as a starting point, and from this point of view, next-generation sequencing (NGS) technologies play a significant role by enabling high-throughput genomic data at very high speed with a relatively low cost. When the whole-genome sequence of the EPS-producer microorganism is not available, sequence data of a taxonomically close species could also be used for systems-based studies. This approach has been used for the improvement of levan production by the halophilic strain *Halomonas smyrnensis* AAD6^T, where first, the available whole-genome sequence of a taxonomically close microorganism, *Chromohalobacter salexigens* DSM 3043, was used to construct a comprehensive genome-scale metabolic model, and then this model was recruited and adopted to the producer strain via integration of the available biochemical, physiological and phenotypic features of *H. smyrnensis* AAD6^T. With metabolic system analysis of this generic metabolic model, significant improvement in levan yields was obtained (Ateş et al. 2011, 2013).

Considering that the microbial biodiversity of marine and terrestrial ecosystems is relatively unexplored, it is reasonable to hypothesise that the isolation and identification of new microorganisms will provide wide opportunities for new industrial fields.

10.3 Most Remarkable Examples of Bioactive EPS

Natural bioactive polysaccharides isolated from several sources have attracted much attention in the field of biochemistry and pharmacology: indeed several polysaccharides or their glycoconjugates have been shown to exhibit multiple biological activities including anti-inflammatory, immunostimulating, antiviral, antioxidant, etc.

Moreover, polysaccharides are biodegradable materials expressing biocompatibility; thus, they could represent as versatile tools for application in biomedical fields such as tissue engineering, drug delivery, prostheses and medical devices. İn the following sections, some examples of bioactive EPS, also listed in Table 10.1, are discussed in detail.

10.3.1 Anti-inflammatory/Immunomodulating

Inflammation is a complex and well-coordinated response of the innate and adaptive immune system following infection or injury. This process is characterised by a vascular response and recruitment of circulating leukocytes, defined initially by polymorphonuclear granulocytes followed by monocytes, which differentiate locally into macrophages. Host-defence mechanisms are divided into two distinct, but inextricably linked, pathways: the innate and the adaptive immune responses. The first one mounts a rapid response to injury by means of phagocytosis, production of reactive oxygen species (ROS) and release of cytokines, autacoids and lipid mediators that coordinate and amplify the local inflammatory response. On the contrary, the adaptive immune response mounts more slowly and furnishes a more focused response mechanism that requires the identification of specific molecular structures and depends on the generation of large numbers of antigen receptors (i.e. T-cell receptors and immunoglobulins). The resolution of inflammation occurs mainly via clearance of apoptotic cells by phagocytosis and by the production of anti-inflammatory mediators, such as IL-10 and TGF-β, in the lesion by tissue macrophages that phagocyted apoptotic cells.

An exopolysaccharide named EPS-1, containing tetrasaccharide-repeating units formed by sugars with a mannano-pyranosidic configuration, was isolated from a halophilic and thermotolerant *Bacillus licheniformis* strain (Table 10.1). EPS-1 was tested for its immunomodulatory effect by means of the production of different cytokines (IFN-α, IL-12; IFN-γ, TNF-α, IL-18) involved in the immune response in human peripheral blood mononuclear cells (PBMC) during the HSV-2 virus infection. Results showed that EPS-1 affected on cytokines production in a dose-dependent manner stimulating IFN-α production. Moreover, EPS-1 induced the production of IL-12 at considerable levels; IFN-γ and TNF-α were also detected (Arena et al. 2006). Later, Arena et al. (2009) (Table 10.1) reported the immunomodulatory and antiviral effects of an exopolysaccharide EPS-2, isolated from a strain of *Geobacillus thermodenitrificans*. EPS-2 also stimulated the cytokine

production in PBMC cells in a concentration-dependent manner, like EPS-1. A high level of IL-12, IFN-γ, TNF-α and IL-18 was revealed after EPS-2 treatment (Arena et al. 2009).

Bacteria produce a high quantity of exopolysaccharides when they form biofilm, which is a consortium of microorganisms immobilised and penned within EPS that is able to limit the diffusion of substance and antimicrobial compounds. From the biofilm of the thermophilic bacteria *Thermus aquaticus* YT-1, a novel EPS named TA-1 with interesting immunomodulatory effects was isolated (Table 10.1). TA-1 stimulated macrophages cells to produce the cytokines TNF-α and IL-6 (Lin et al. 2011).

10.3.2 Antiviral

Pathogenesis is the process by which virus infection leads to disease. Pathogenic mechanisms include implantation of the virus at a body site, replication at that site and then spread to and multiplication within sites (target organs) where disease or shedding of virus into the environment occurs. Viruses cannot synthesise their genetic and structural components, and so they rely almost exclusively on the host cell for these functions. Pathogenesis at the cellular level can be viewed as a process that occurs in progressive stages leading to cellular disease. An essential aspect of viral pathogenesis at the cellular level is the competition between the synthetic needs of the virus and those of the host cell.

The antiviral activity linked to the immunoregulatory effect of bacterial polysaccharides was reported by Arena et al. (2006, 2009). In the first paper, the antiviral effect of a novel exopolysaccharide EPS-1 produced by a strain of thermotolerant *Bacillus licheniformis* (Table 10.1), isolated from a hot spring of Vulcano Island (Italy), has been reported. EPS-1 inhibited HSV-2 replication in PBMC (human peripheral blood mononuclear cells) by upregulating the expression of pro-inflammatory cytokines. In a later paper, an antiviral exopolysaccharide EPS-2, produced by a strain of *Geobacillus thermodenitrificans*, is reported. EPS-2 treatment in PBMC, at a concentration of 200 and 300 μg/mL, is affected on HSV-2 replication, in a concentration-dependent way, increasing the inflammatory response (Arena et al. 2009).

10.3.3 Application in Drug Delivery Systems

EPSs have the capacity to establish polymeric matrices. This ability enables their in vitro manipulation to create novel structures in which bioactive compounds are encapsulated, developing new applications in drug delivery systems.

Halomonas smyrnensis strain AAD6 (JCM 15723) (Table 10.1) was reported as a producer of high levels of levan exopolysaccharide. Studies on biocompatibility

were performed, and the results exhibited that this levan did not affect cellular viability and proliferation of osteoblasts and murine macrophages. Moreover, the toxicity test carried out by Brine Shrimp Test showed a protective effect of levan against a toxic agent (Poli et al. 2009). Because of the amphiphilic nature of levan, it is able to form nanoparticles by self-assembly in water. The levan EPS isolated from strain AAD6 was studied for its potential use as a biopolymer in nanoparticle drug delivery system. Levan nanoparticles encapsulated with bovine serum albumin (BSA) were prepared and used as a model to investigate their different properties. The size of nanoparticles varied between 200 nm and 537 nm, and their encapsulation capacity also varied (ranged between 49.3% and 71.3%) depending on the levan concentration used. Moreover, the increasing in vitro release of BSA from the nanoparticles was displayed to be controlled release of proteins (Sezer et al. 2011).

10.4 Chemical and Structural Characterisation

The complete characterisation of an unknown EPS requires the definition of the polymer's chemical composition and its structural analysis. The chemical composition is defined on the basis of the total carbohydrate content and of other molecules, such as proteins, and on the identification of the monosaccharide unit type of their relative molar ratio and of the presence of substituting groups. The structural analysis is defined by determining the sequence of glycosidic bonds and the possible tridimensional distribution of monomer components, i.e. the polysaccharide conformation. These tasks are accomplished by combining different analytical and instrumental techniques, i.e. chromatography, spectrometry and spectroscopy, which are used along all the steps of exopolysaccharide characterisation. The different methods and techniques required for the complete EPSs characterisation are listed in Table 10.2 and will be discussed in detail in the following sections.

10.4.1 Purification

The purification of EPSs is the first step of the study: usually intact cells are removed by heating the bacterial culture broth at 90–95 °C (in order to denature enzymes that hydrolyse the polysaccharide) followed by centrifugation. The EPS can be separated from the broth by means of precipitation (by adding alcohol or complexing metal ions) or ultrafiltration. The crude EPS obtained is then purified by combining different chromatography techniques, for example, gel filtration chromatography and anion-exchange chromatography. Usually the purified EPS is freeze-dried to be stored for long times in order to perform the chemical and structural characterisation.

Table 10.2 Summary of main methods required for EPSs characterisation and relative information obtained

Technique/method	Type of information
Dubois method	Total carbohydrate content
Bradford method	Total protein content
• TFA hydrolysis at 110–120 °C/ per acetylation or silylation/ GC analysis • TFA hydrolysis at 110–120 °C/HPAE-PAD analysis	Monosaccharide composition
Methylation analysis (MA)/ GC-MS analysis of alditol acetates	Determination of linkage positions
• Size-exclusion chromatography (SEC) • HPSEC with refractive index (RI) detection • HPSEC with multi-angle laser light scatter (MALLS) detection	Molecular-weight distribution
Sedimentation analysis	Intrinsic viscosity η
Nuclear magnetic resonance: • 1D NMR (^{1}H NMR/^{13}C NMR) • 2D homo- and heteronuclear correlation (COSY, HSQC, HMQC or HMBC, TOCSY, NOESY)	• Presence and nature of substituting groups • Number/type of monomer sugar residues • Anomer configurations • Monosaccharide sequence in the polymer backbone
Fourier transform-infrared spectroscopy (FT-IR)	Presence of functional and substituting groups

10.4.2 Compositional Analysis

The gross chemical composition of an EPS is determined by measuring the total carbohydrate content, the protein and nucleic acid contents: the total carbohydrate content can be assessed by means of the Dubois method based on the use of phenol-sulphuric acid reagent; the total protein content is estimated using the Bradford method; finally the nucleic acids' content is determined spectrophotometrically by measuring absorbance at λ 260 nm. Uronic acid content is another important chemical feature of EPS that can be determined according to the method described by Blumenkrantz and Asboe-Hansen (Spanò et al. 2013).

10.4.3 Determination of Monosaccharide Composition, Glycoside Linkage Position and Substituting Groups

The most common sugars that can be found as constituents of bacterial EPS include hexoses (D-glucose, D-galactose, D-mannose, D-allose, L-fucose, L-rhamnose), pentoses (D-ribose, D-arabinose, D-xylose), uronic acids (D-glucuronic, D-mannuronic and D-galacturonic acids) and amino sugars (D-glucosamine, D-galactosamine). The nature and the molar ratio of the monosaccharides that constitute an EPS can be

determined by chemolytic methods, i.e. total hydrolysis of EPS that is carried out at high temperature (up to 110–120 °C) in strong acidic conditions by using concentrated trifluoroacetic (TFA) or formic or sulphuric acids. The resulting hydrolysis mixture can be analysed after reduction followed by per-acetylation or silylation that converts the released monomer sugars in the respective volatile derivatives (alditol acetates or methylsilanes) that can be analysed by means of gas chromatography coupled with mass spectrometry (GC-MS). Alternatively, the hydrolysis mixture can be analysed without any derivatisation of monosaccharides, by means of liquid chromatography methods like the high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD), that is, a well-established method that allows either qualitative and quantitative analysis of monomer sugars released by acid. Chemolytic methods, for example, the methylation analysis (MA), are also employed for the determination of linkage positions. Commonly MA requires the following steps: methylation, by addition of methyl iodide; cleavage of glycosidic bonds, by classic acidic hydrolysis or by methanolysis (with methanolic HCl); reduction with $NaBH_4$ followed by acetylation or silylation; and GC-MS analysis of the resulting mixture of volatile alditol acetates or methylsilanes. This treatment causes methylation of the free OH groups and acetylation or silylation only for the OH groups involved in the glycoside bonds: in such a way the position of glycosidic linkages will be identified as those corresponding to the non-methylated OH groups present in the alditol derivatives. Different organic and inorganic substituent groups can be present in the EPS that also significantly affect their chemical and biological properties. The most common organic substituents include ester-linked residues of acetate, succinate, propionate, glycerate and hydroxybutanoate, the ketal-linked pyruvate unit or amino acids like L-glutamate or serine. The most common inorganic substituents are sulphate and phosphate groups. The presence of the substituting groups can be assessed by means of Fourier transform-infrared spectroscopy (FT-IR) or nuclear magnetic resonance (NMR) (Mishra and Jha 2013).

10.4.4 Molecular-Weight Distribution

The molecular-weight (M_w) distribution of an EPS is an important structural feature that often also affects biological activity of this kind of polymer. Different techniques can be employed for this purpose, for example, light scattering, sedimentation analysis in analytical ultracentrifugation, intrinsic viscosity determination, size-exclusion chromatography (SEC) or the more modern high-performance size-exclusion chromatography (HPSEC) techniques with refractive index (RI) detection or multi-angle laser light scatter (MALLS) detection.

Light scattering allows to measure the absolute M_w using the relationship, stated by the Rayleigh theory, between the intensity of light scattered by a molecule and its molecular weight and size. Sedimentation analysis is based on the determination of sedimentation velocity of polysaccharides during ultracentrifugation: the value of

M_w is deduced from the changes in the refractive index of the polysaccharide solution when it is subjected to high force fields. The measure of the intrinsic viscosity η is used for M_w determination at infinite dilution. In such conditions, η is a function of the average molecular weight (M_w) and can be calculated by means of the Mark-Houwink-Sakurada equation $\eta = K M_w{}^{\alpha}$ where K and α are constants for a given polymer/solvent/temperature system.

Size-exclusion chromatography (SEC) is widely employed for the M_w characterisation of different kinds of polymers. This technique is based on the use of soft gels, for example, Sepharose CL-4B or porous polystyrene-divinylbenzene resins, and the M_w is calculated by comparison with external standard calibration curves derived by using pullulans and dextrans as standard polymers. Significant improvements in the use of this technique have been achieved by coupling with online detectors like light-scattering detectors and viscometers. More recent developments of SEC are represented by high-performance size-exclusion chromatography (HPSEC) technique using online detectors like refractive index (RI) or multi-angle laser light scatter (MALLS) detectors. Although widely used, such methods present some major drawbacks like longer time of analysis and need of higher amount of samples. For such reasons the use of HPSEC methods is emerging since they afford faster analyses and requires smaller quantities of samples. HPSEC columns are made of rigid matrixes of small porous silica microspheres as such or bonded with glycerylpropyl groups, of agarose or methacrylate polymers. The online detection is often performed with RI, a universal detector that is useful for the determination of M_w distribution since there is a linear correlation between the EPS concentration and its M_w. Nevertheless, higher accuracy can be achieved by using a MALLS detector that allows the absolute determination of M_w that is based on the calculation of the amount of scattered light at each angle detected, thus not requiring external calibration curves (Gómez-Ordonez et al. 2012).

10.4.5 Structural Analysis by NMR and FT-IR Techniques

NMR spectroscopy is a useful tool for the determination of both polysaccharides' composition and conformation. The structure elucidation of EPSs relies on the use of both 1D and 2D ^{1}H- and ^{13}C-NMR techniques and of relevant databases such as Carb-Bank, SUGABASE or CASPER. Indeed ^{1}H and ^{13}C chemical shift and coupling constant values are available in literature for many monosaccharides and can be used for assignment of polysaccharide resonances. 1D ^{1}H and ^{13}C techniques can afford a good estimation of the number of sugar residues present in an EPS: identification and integration of signals in the anomeric region of ^{1}H, corresponding to δ 4.4 – 5.5 ppm, can give such an information that is confirmed by the analysis of the anomeric region of 1D-^{13}C spectrum. Alternatively, this information can be gained by applying 2D heteronuclear techniques like HSQC, HMQC or HMBC that are more sensitive than single mono-dimensional analysis since they can resolve complex signal patterns that usually characterise the anomeric region of ^{1}H

spectrum. The following step is represented by the determination of the type of monosaccharides that constitute the EPS that can be identified by means of two-dimensional homonuclear and heteronuclear techniques since the typical chemical shift values and J-coupling patterns of most monosaccharides are listed in several databases. The homonuclear TOCSY or DQF-COSY spectra afford information on the spin system of single monosaccharides: such techniques allow resolution of the so-called bulk region of ^{1}H spectrum in which usually the resonance relatives to non-anomeric protons strongly overlap. The ^{13}C shift values relative to such protons are then easily deduced by comparison with HSQC or HMBC spectra. NMR can also be used for assignment of anomeric configuration since usually the β-anomers of pyranose sugars resonate at higher field than the corresponding α-isomers; in addition the calculation of direct coupling constant $^{1}H–^{13}C$ for the anomer proton ($J_{C1,H1}$) can also be useful, since usually its value is about 170 Hz for the α-anomeric sugars, while for the β forms it is ~ 160 Hz.

The sequence of the monosaccharide residues within the EPS can also be achieved by means of 2D techniques like NOESY and HMBC. In particular, NOESY spectrum allows to identify NOEs (nuclear Overhauser effects) between signals belonging to directly linked monosaccharide residues, thus affording information on glycosidic linkages sequence along the polysaccharide backbone.

Finally, NMR can be used for identification of substituents since the signals of most organic groups, for example, acyl or methyl groups, do not overlap to carbohydrate resonances, being found in high field regions at about 0.2–0.5 ppm. On the other hand, the presence of inorganic groups like phosphates can be assessed by means of heteronuclear $^{31}P–^{1}H$ long-range coupling measurements. The interpretation of such complex data can be supported by the use of software programmes, for example, PRONTO freely available at this link: http://www.crc.dk/chem/pronto/welcome.html (Duus et al. 2000).

Fourier transform-infrared spectroscopy (FT-IR) is another spectral technique that can be applied to EPS structural investigation. Indeed some typical signals support polysaccharide recognition like the signals in the fingerprint region, i.e. below 1500 cm^{-1}, where broad stretching bands relative to C–O–C and C–O (at 1700 cm^{-1}) ring bonds and C–H bending (at 1410 cm^{-1}) are typically found; like the stretching peak of hydroxyl groups (usually at around 3300 cm^{-1}); or, finally, like the signals around 2900 cm^{-1} attributable to the C–H stretching of either methyl or methylene groups.

10.5 Conclusions and Future Perspectives

EPSs are extracellular biopolymers produced by many extremophilic microorganisms that use them as a valuable adaptation strategy to extreme environmental conditions. EPSs protect extremophiles from the deleterious effects of extreme pH, temperature, salt concentration or radiation by forming capsular materials or dispersed biofilm in the surrounding environment. Indeed, several extremophiles,

isolated either from aquatic or terrestrial environments, have been shown to be EPSs producers. Notably, these biopolymers represent a promising class of compounds for applications in several fields, i.e. pharmaceutical, food or biotechnology sectors since they possess interesting properties, for example, biological and biotechnological properties. Indeed several examples of bioactive EPSs acting as anti-inflammatory, immunomodulating or antiviral agents are described in literature. Other promising applications in the pharmaceutical fields are as dug delivers or as vaccine adjuvants. Moreover thanks to their features they can act as metal binders, thus affording a valuable tool for bioremediation processes, or as biosurfactant or bioemulsifier, thanks to their rheological properties like viscosity or thermostability. Many other possible applications can be found for extremophiles EPSs that in future could play a pivotal role in biotechnology.

Take-Home Message

- EPSs are produced by several extremophilic microorganisms, belonging to both *Archaea* and *Bacteria* domains that have been isolated from different ecosystems.
- The biosynthesis of EPSs can take place either into the cell or in the extracellular milieu; it follows three main routes, all regulated by a group of enzymes named 'glycosyltransferases'.
- The genetic manipulation of the EPSs' producing bacteria has been employed to optimise the yield of and for tailoring polymers' chemical composition and structure.
- The structural characterisation of EPSs requires a combination of analytical and instrumental techniques that include chromatography, IR spectroscopy, 1D and 2D NMR (^{1}H NMR/^{13}C NMR).
- The extremophiles' EPSs possess unique properties that can be exploited for a variety of biological and biotechnological applications.

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Chapter 11
Polyhydroxyalkanoates Production from Renewable and Waste Materials Using Extremophiles/Recombinant Microbes

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Abbreviations

3HV	3-Hydroxyvalerate
3HPA	3-Hydroxypropionaldehyde
CDW	Cell dry weight
mcl	Medium-chain length
MMC	Mixed microbial culture
PHAs	Polyhydroxyalkanoates
PHB	Poly(3-hydroxybutyrate)
PHBV	Poly(3-hydroxyvalerate)
PHP	3-Hydroxypropionate
scl	Short-chain length
T_d	Thermodegradation temperature
T_g	Glass transition temperature
T_m	Melting temperature
VFA	Volatile fatty acids

What Will You Learn from This Chapter?

- Polyhydroxyalkanoates (PHAs) are biodegradable polyesters produced by various species of *Bacteria* and *Archaea* as reserves of energy and carbon in nutrient poor environments.

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- Being biodegradable and biocompatible, PHAs have found many industrial and medical applications as attractive bio-based alternatives to petroleum-based polymers.
- To compete with petroleum-based polymers and garner a bigger market share, cost-effective PHA production processes are needed.
- Therefore, renewable, cheap, sustainable, and readily available carbon sources from industrial wastes and agricultural by-products should be considered in PHA production.

11.1 Introduction

Since their discovery, petroleum-based plastics have turned out to be the most widely utilized materials in almost every area of daily life such as packaging, home appliances, electronic devices, and medicine. In accordance with the environmental concerns caused by discarded petrochemical plastics, increasing carbon dioxide emissions, global warming, and fluctuations of petroleum prices, development of eco-friendly biodegradable bio-based plastics from cheap, renewable resources is becoming increasingly important. In order to produce bioplastics with chemical and mechanical properties matching those of conventional plastics, microorganisms belonging to *Bacteria* and *Archaea* domains are employed.

Various monomeric building blocks such as lactic acid, bioethylene, *cis*-3,5-cyclohexadiene-1,2-diol, and 1,3-propanediol can be produced by microorganisms and used to synthesize polylactic acid, polyethylene, poly(*p*-phenylene), and poly (trimethylene terephthalate), respectively. Polyhydroxyalkanoates (PHAs) are linear aliphatic polyesters composed of hydroxycarboxylate monomers. They differ from these microbial originated biopolymers because their polymerization is conducted in vivo as cytoplasmic inclusions in *Bacteria* and *Archaea* under certain nutrient-deprived growth conditions (Chen 2010).

More than 300 bacterial and archaeal species have been found to synthesize PHAs consisting of approximately 150 different (*R*)-hydroxyalkanoic acid monomers (Arcos-Hernández et al. 2013). According to their chemical and physical properties, PHAs may be classified into two major groups: often stiff and brittle short-chain-length (scl) PHAs consisting of C3–C5 monomers and medium-chain-length (mcl) PHAs consisting of C6–C16 monomers which are elastomeric in nature (Fig. 11.1). Due to the stereospecificity of the enzymes involved in the biosynthesis, all of these monomers are in the *R*-configuration (Steinbüchel and Valentin 1995).

Their structural diversity and unique properties such as biodegradability and biocompatibility allow PHAs to be used in various applications, including packaging materials, biomedical implants, biofuels, carriers in drug delivery, and bioactive compounds (Tan et al. 2014).

Despite their great advantages, PHAs held only a relatively small fraction of the biopolymer market share because of their relatively high production cost and concurrent availability of low-cost petrochemical plastics. Since most of the cost

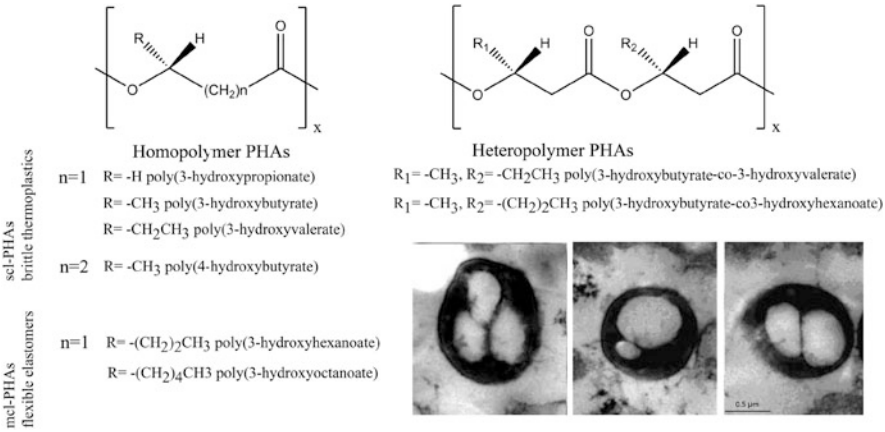

Fig. 11.1 General structure of common polyhydroxyalkanoates and transmission electron microscope pictures of PHBV granules in extremely halophilic archaea *Natrinema* 1KYS1 isolate

arises from the need for expensive carbon sources, industrial, agricultural, or municipal wastes may be used as feedstock for bacterial production of PHAs in order to overcome this obstacle (Anterrieu et al. 2014). Hence, in this chapter we focused on the biosynthesis and applications of PHAs from renewable resources and waste materials.

11.2 Biosynthesis and Applications of PHA

PHA polymers can be synthesized biologically or chemically; while obtaining materials with desired structures is easier with the latter approach, biosynthesis of PHAs via microorganisms has certain advantages. Biosynthetic PHAs generally have high molecular weights. Since they can be produced from sustainable resources, by-products of large industrial processes, and waste materials, they are accepted as eco-friendly (Chen 2010).

PHAs are synthesized by numerous organisms in the domains of *Bacteria*, *Archaea*, and *Eukarya* (fungi and animals including higher vertebrates). In *Bacteria* and *Archaea*, when essential nutrients such as nitrogen, oxygen, and phosphorus are limited and there is an excess carbon source present, PHA biosynthesis and accumulation as carbon and energy sources are favored (Chee et al. 2010). Molecular structure and properties of the synthesized PHAs are dependent on the particular bacterial and archaeal species in use, type of the feedstock, and substrate specificities of the enzymes in the biosynthetic pathways (Lu et al. 2009). Accumulation of PHAs by *Pseudomonas aeruginosa*, *Aeromonas caviae*, *Chelatococcus daeguensis* TAD1, *Lactobacillus reuteri*, *Azotobacter vinelandii* UWD, *Bacillus subtilis*, *Escherichia coli*, *Ralstonia eutropha*, and *Burkholderia cepacia* was investigated by researchers

(Koller et al. 2010; Insomphun et al. 2014; Cui et al. 2015; Linares-Pastén et al. 2015; Oh et al. 2015; Rodríguez-Contreras et al. 2015).

The most extensive studies were done for PHA synthesis in *Cupriavidus necator*, *Pseudomonas aeruginosa*, and *Pseudomonas putida*. More than eight different pathways for the synthesis of PHAs have been discovered to date in various organisms (Tan et al. 2014).

In addition to bacterial strains, extremely halophilic archaea have a remarkable capacity for accumulation of PHAs, which are used for production of biodegradable and biocompatible plastics (Quillaguamán et al. 2010). Poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) are the most familiar and analyzed PHAs (Tan et al. 2014). Researchers demonstrated that species belonging to genera *Haloferax*, *Halorubrum*, *Haloarcula*, *Halococcus*, *Halobacterium*, *Haloterrigena*, *Natronobacterium*, *Natrinema*, *Natronorubrum*, *Halostagnicola*, *Halobiforma*, *Halogeometricum*, and *Halalkalicoccus* produced PHAs (Table 11.1).

In addition to species listed in Table 11.1, *Halorubrum coriense* DSM 10284T (Legat et al. 2010), *Natronococcus occultus* DSM 3396T (Legat et al. 2010), *Halobiforma haloterrestris* (Hezayen et al. 2002), *Halopiger aswanensis* (Hezayen et al. 2010), *Halococcus hamelinensis* JCM 12892T (Legat et al. 2010), *Halococcus saccharolyticus* DSM 5350T (Legat et al. 2010), *Halococcus qingdaonensis* JCM 13587T (Legat et al. 2010), *Halorubrum chaoviator* DSM 19316T (Legat et al. 2010), *Halorhabdus utahensis* (Wainø et al. 2000), *Haloquadratum walsbyi* (Burns et al. 2007), *Halorhabdus tiamatea* (Antunes et al. 2008), and *Natrinema altunense* (Xu et al. 2005) have been recognized as PHA producers.

The first study on PHB accumulation by extremely halophilic archaebacterium (*Haloarcula marismortui* previously known as *Halobacterium* sp. from the Dead Sea) was carried out by Kirk and Ginzburg (1972). PHB accumulation of *Haloferax mediterranei* in the test medium containing glucose, yeast extract, and marine salts was subsequently observed by Fernandez-Castillo et al. (1986). Researchers found that PHB accumulation of this strain increased to 45% cell dry weight (CDW) in a medium with glucose, yeast extract, and low salt concentration. Lillo and Rodriguez-Valera (1990) discovered that high PHB accumulation of *Haloferax mediterranei* (60% CDW) could be obtained in the test media containing 2% (w/v) starch.

Although different species of halophilic archaea produce biopolyesters, most studies on PHAs were done using *Haloferax mediterranei*, which accumulates high quantities of PHBV. *Haloferax mediterranei* is a highly preferable source for inexpensive and easy production of high-quality PHA because of its stable genetic structure, rapid growth rate, minimum sterility requirement, growth in high saline waste materials, aseptic condition, broader substrate spectrum, and capacity for high-yield accumulation of PHA (Lillo and Rodriguez-Valera 1990; Koller et al. 2007; Koller 2015). It was emphasized that the other advantage of this strain was the production of PHA without antibiotic addition (Koller 2015).

Don et al. (2006) reported that PHA accumulated by *H. mediterranei* was PHBV. Koller et al. (2007) explained that *H. mediterranei* DSM 1411 accumulated 50% (CDW) poly-3-(hydroxybutyrate-co-8%-hydroxyvalerate) from hydrolyzed whey

Table 11.1 PHAs produced by extremely halophilic archaea

Extremely halophilic archaea	Carbon sources	Biopolymer	References
Haloferax mediterranei	Yeast extract/glucose	PHB	Fernandez-Castillo et al. (1986)
Haloferax volcanii	Yeast extract/glucose	PHB	Fernandez-Castillo et al. (1986)
Haloferax gibbonsii	Yeast extract/glucose	PHB	Fernandez-Castillo et al. (1986)
Haloarcula hispanica	Yeast extract/glucose	PHB	Fernandez-Castillo et al. (1986)
Haloferax mediterranei ATCC 33500	Starch	PHB	Lillo and Rodriguez-Valera (1990)
Haloferax mediterranei DSM 1411	Hydrolyzed whey	PHA	Koller et al. (2007)
Haloferax mediterranei DSM 1411T	Glucose	PHB/ PHBV	Legat et al. (2010)
Haloarcula hispanica DSM 4426T	Glucose	PHB/ PHBV	Legat et al. (2010)
Halococcus dombrowskii DSM 14522T	Yeast extract/hy-case	PHB/ PHBV	Legat et al. (2010)
Halococcus salifodinae DSM 8989T	Yeast extract/hy-case	PHB/ PHBV	Legat et al. (2010)
Halobacterium noricense DSM 9758T	Yeast extract/tryptone	PHB/ PHBV	Legat et al. (2010)
Natronobacterium gregoryi 2189T	Yeast extract/ casamino acids	PHB/ PHBV	Legat et al. (2010)
Haloarcula marismortui	Raw vinasse	PHB	Pramanik et al. (2012)
Haloarcula japonica T5	Molasses	PHB	Nicolaus et al. (1999)
Haloarcula marismortui ATCC 43049	Glucose	PHA	Han et al. (2007)
Halalkalicoccus tibetensis CGMCC 1.3240	Glucose	PHA	Han et al. (2010)
Haloferax mediterranei CGMCC 1.2087	Glucose	PHA	Han et al. (2010)
Haloarcula amylolytica 26–3	Glucose	PHA	Han et al. (2010)
Haloarcula argentinensis CGMCC 1.7094	Glucose	PHA	Han et al. (2010)
Halobacterium cutirubrum CGMCC 1.1962	Glycerol	PHA	Han et al. (2010)
Halobiforma nitratireducens CGMCC 1.1980	Fructose	PHA	Han et al. (2010)
Halobacterium halobium PM CGMCC 1.1952	Glycerol	PHA	Han et al. (2010)
Halococcus morrhuae CGMCC 1.2153	Glucose	PHA	Han et al. (2010)
Haloferax gibbonsii CGMCC 1.2148	Glucose	PHA	Han et al. (2010)
Halorubrum litoreum 12–2	Glucose	PHA	Han et al. (2010)

(continued)

Table 11.1 (continued)

Extremely halophilic archaea	Carbon sources	Biopolymer	References
Halorubrum trapanicum CGMCC 1.2201	Glucose	PHA	Han et al. (2010)
Halostagnicola larsenii 24–25	Glucose	PHA	Han et al. (2010)
Haloterrigena turkmenica CGMCC 1.2364	Glucose	PHA	Han et al. (2010)
Haloterrigena hispanica	Carrot waste	PHB	Di Donato et al. (2011)
Natrinema altunense CGMCC 1.3731	Glucose	PHA	Han et al. (2010)
Natrinema pallidum JCM 8980	Glucose	PHA	Han et al. (2010)
Natrinema pellirubrum JCM 10476	Glucose	PHA	Han et al. (2010)
Natronobacterium gregoryi CGMCC 1.1967	Glucose	PHA	Han et al. (2010)
Natronorubrum tibetense CGMCC 1.2123	Glucose	PHA	Han et al. (2010)
Natrinema sp. XA3–1	Glucose	PHA	Han et al. (2010)
Halogeometricum borinquense strain TN9	Glucose	PHA	Salgaonkar et al. (2013)
Natrinema pallidum (KYS1)	Starch	PHA	Danis et al. (2015)
Haloferax mediterranei ATCC 33500	Extruded rice bran and corn starch	PHA	Huang et al. (2006)

PHA polyhydroxyalkanoates, *PHB* poly (3-hydroxybutyrate), *PHBV* poly (3-hydroxybutyrate-co-3-hydroxyvalerate)

without addition of 3-hydroxyvalerate (3HV) precursors (Koller et al. 2007). Han et al. (2007) found that *Haloarcula marismortui* accumulated 21% (CDW) PHB in the test medium containing 2% glucose. Moreover, investigators examined the PHA accumulation of 28 haloarchaeal strains belonging to genera of *Halalkalicoccus*, *Halobacterium*, *Haloarcula*, *Halobiforma*, *Halostagnicola*, *Haloterrigena*, *Halococcus*, *Halorubrum*, *Haloferax*, *Natrinema*, *Natronococcus*, *Natronobacterium*, *Natronomonas*, *Natronorubrum*, and *Natrialba* in the test media containing, separately, different carbon sources such as glucose, glycerol, fructose, and acetate (Table 11.1). Eighteen of the twenty-eight tested strains accumulated PHA at levels in the range of 0.8% to 22.9% (w/w) of CDW (Han et al. 2010), and the highest PHA production (22.9% (w/w) of CDW) was detected in *Natrinema pallidum* JCM 8980 grown in glucose medium (Han et al. 2010).

Previous experiments clearly show that extremely halophilic archaeal species may be potentially important resources for industrial production of PHAs. Production of PHA by halophilic archaea may have several advantages. As it is known, extremely halophilic archaea grow in high-salt concentrations (2.0–5.2 M) wherein other non-halophiles cannot survive (Lillo and Rodriguez-Valera 1990). PHA produced from nonpathogenic haloarchaeal species reduces the risk of microbial contamination during cultivation (Koller et al. 2007; Quillaguamán et al. 2010). Researchers suggested that a production system such as open pond, which is used

for sewage treatment, may be used for PHA production of haloarchaeal strains (Lillo and Rodriguez-Valera 1990). These microorganisms can be continuously grown in unsterile conditions without risk of contamination (Yin et al. 2015). Due to low microbial contamination during cultivation, energy can be very efficiently conserved as sterility is irrelevant (Koller et al. 2007). Industrial and household waste products or cheap materials can be used as carbon sources by extremely halophilic archaea to produce PHA (Koller et al. 2007; Pramanik et al. 2012; Danis et al. 2015). Using industrial waste for PHA production is also a promising solution to the waste disposal problem and its huge expense (Koller et al. 2007). Hence, production of PHA from extremely halophilic archaea may be implemented for cost-effectiveness. Furthermore, high-quality polyesters are derived from some strains of halophilic archaea (Koller et al. 2007). Isolation of PHA from extremely halophilic *Archaea* is easier than that of *Bacteria* (Koller et al. 2007) because of disintegration of haloarchaeal cells in water (Ventosa and Nieto 1995). Additionally, investigators mentioned that genome sequence of *H. mediterranei* was determined, and biotechnological applications could be applied for PHA accumulation (Bhattacharyya et al. 2015).

11.2.1 Biochemistry of PHA Synthesis

The key components for the biosynthesis of PHA are acetyl-CoA produced from carbohydrates and acyl-CoA generated by the β-oxidation of fatty acids. For the synthesis of scl-PHA, many bacterial and some archaeal species use a three-step reaction: production of acetoacetyl-CoA from two molecules of acetyl-CoA by the activity of β-ketothiolase, reduction of the product to 3-hydroxybutyrl-CoA by NADPH-dependent acetoacetyl-CoA reductase, and finally polymerization of PHB catalyzed by the action of PHA synthase. 3-Hydroxyvaleryl-CoA is synthesized by the reaction of acetyl-CoA and propionyl-CoA and used for polymerization of PHBV (Shirastav et al. 2013). Mcl-PHAs or copolymers of scl and mcl PHAs may be synthesized from the acyl-CoA produced by the β-oxidation of fatty acids in certain bacteria. Bacteria such as *Pseudomonas aeruginosa* and *Aeromonas caviae* have PHA synthase enzymes with broad substrate specificity; therefore, they can accumulate PHAs with different monomer lengths (C6–C12) (Insomphun et al. 2014). In another pathway, the production of mcl PHAs is achieved by the polymerization of (*R*)-3-hydroxyacyl intermediates of de novo fatty acid biosynthesis from structurally unrelated substrates such as glycerol, glucose, and sucrose (Philip et al. 2007). This pathway is especially important for the utilization of industrial and agricultural by-products and wastes for the synthesis of PHAs.

PHA biosynthesis is regulated by both high level of NAD(P)H and the ratio of NAD(P)H to $NAD(P)^+$. β-ketothiolase, the first enzyme of the PHA synthesis, is inhibited by the high intracellular CoA levels. However, when a nutrient is limited, $NADH/NAD^+$ ratio increases, and subsequently, as a result of the inhibition of the

enzymes of TCA cycle, CoA levels fall. This phenomenon in turn cancels the inhibition of β-ketothiolase (Shirastav et al. 2013).

11.2.2 Chemical and Mechanical Properties of PHA

Since PHAs are semicrystalline in structure, their thermal and mechanical properties are generally defined with their melting temperatures (T_m), glass transition temperatures (T_g), and thermodegradation temperatures (T_d) (Anderson and Dawes 1990). Due to their diverse structural varieties, PHAs have variable T_m, T_g, and T_d within these respective ranges: non-observable to 177 °C, −52 to 4 °C, and 227 to 256 °C (Tan et al. 2014). They also possess a varying degree of Young's modulus, elongation at break, and tensile strength values within these respective ranges: 0.008 to 3.5×10^3 MPa, 2% to 1000%, and 8.8 to 104 MPa (Chen 2010; Tan et al. 2014).

Due to the stereoregular structure of PHB, it has a high degree of crystallinity, and therefore its use as a polymeric material is limited. In order to overcome this mechanical weakness, researchers usually employed chemical modifications such as copolymerization with alternative PHA monomers or blending with other polymers. Incorporation of PHB with 3HV resulted in the production of PHBV, which has more suitable physical properties—lower T_m, less stiffness, and increased toughness—for commercial applications (Anderson and Dawes 1990).

Mcl-PHAs such as 3-hydroxyoctanoate and 3-hydroxydecanoate have significantly lower T_m and crystallinity. These qualities make them more elastomeric in structure when compared to scl-PHAs and, therefore, broaden the range of applications for PHA (Davis et al. 2013).

11.2.3 Biodegradability and Biocompatibility of PHA

Most of the plastics produced in each year are used in a short period of time and usually discarded, either to be recycled, incarcerated, or sent to landfill. One of the unique advantages of biological PHA materials over traditional petroleum-based polymers is their biodegradability in various natural environments such as soil and water by a number of bacteria and fungi (Shirastav et al. 2013). The rate of biodegradability depends on the various properties of the environment such as temperature, pH, and microbial population and composition of PHA.

Since PHB and its degradation product 3-hydroxybutyrate naturally exist in the human body as a normal blood constituent, PHB and other PHAs in general considered as biocompatible, which means they are not toxic and their existence does not elicit an immunological tissue response (Yang et al. 2014).

11.2.4 Applications of PHA

Just like conventional petroleum-based plastics, PHAs can be used in a broad range of applications. Furthermore, because of their biodegradability, biocompatibility, and other superior properties, they are attractive candidates to replace petrochemicals (Chee et al. 2010).

In accordance with the growing environmental awareness and demands from the consumers for the use of sustainable and eco-friendly materials in packaging, bioplastics, especially PHAs, are attracting renewed attention. PHAs are utilized as shopping bags, personal hygiene products, and disposable tableware (Chen 2010).

PHAs, due to their biocompatibility and biodegradability, have more expedient attributes when compared to conventional petroleum-based polymers with biomedical applications. PHAs have been used to develop devices including cardiovascular and nerve repair devices, sutures, bone plates, stents, bone marrow scaffolds, skin substitutes, wound dressings, and other medical purposes (Koller et al. 2010).

One important application area of PHAs is as controlled drug carriers. By inserting biodegradable PHAs impregnated with a drug into tissue, it is possible to achieve that drug's controlled release over a period of time. The use of PHA-based vehicles for the delivery of drugs such as anticancer agents, anesthetics, and antibiotics has been previously reported (Bonartsev et al. 2007). Our group reported the use of PHBV films as a carrier for the antituberculosis drug rifampicin (Danis et al. 2015).

PHA monomers also find application area as drugs themselves. 3-Hydroxybutyrate and its derivatives were found to be effective in decreasing cell apoptosis of mouse glial cells (Xiao et al. 2007). O'Connor et al. (2013) describe the purification of 3-hydroxydodecanoic acid, and they demonstrated that upon conjugation, it improved the anti-proliferation activity of peptide DP18L. Moreover, PHAs are also used in agriculture as controlled release vehicles for substances such as insecticides (Philip et al. 2007).

11.3 The Importance of Reutilization of Renewable and Waste Materials

Bio-based and biodegradable polymers are viable replacements for petroleum-derived plastics (Steinbüchel and Valentin 1995; Braunegg et al. 1998). As mentioned before, PHAs are produced by a variety of prokaryotic strains from such sources as starch, glucose, sucrose, lipids, vegetable oils, alcohols (e.g., glycerol), and organic acids when a nutrient is insufficient in the growth medium. Usage of edible raw materials is not economically profitable; starch, which is the most used raw material for industrial PHA production, is relatively expensive.

Today, despite the numerous advantages of using biodegradable plastics and interest in biotechnological production of PHAs from renewable resources, PHA production has made notable progress as a profitable enterprise (Ciesielski et al. 2015).

The high production cost of PHAs is primarily due to the significant reliance on carbon substrate. Efficient bacterial strains, fermentation, and recovery processes have been developed in a number of studies, but low-cost, waste-based substrates have only recently been employed for PHAs production (Koller et al. 2007; Han et al. 2010). Thus, the selection of proper carbon substrates is very demanding and influences the overall performance of the bacterial fermentation and the cost of PHA. Hence, for large-scale production of PHAs and microbial growth, the selection of renewable, cheap, and most readily available carbon sources should be considered as potential renewable feedstock (Chee et al. 2010). The cost of complex nitrogen nutrients is another financial consideration in phosphate-limited biosynthesis of PHAs. Both cheap carbon and nitrogen sources can be extracted from many sorts of industrial and agricultural wastes. Utilizing these waste materials for PHA production as a carbon source does not only reduces the substrate cost but also reduces the expense of waste disposal and cuts the pollution load (Braunegg et al. 1998).

Waste streams can be grouped as waste streams from biofuel production, surplus whey from the dairy industry, wastes from sugar industry (molasses and bagasses), lignocellulosic materials and their waste by-products, and municipal solid and wastewater treatment plants.

11.3.1 Glycerol from Biofuel Production

Crude glycerol is generated in large quantities from the transesterification of vegetable oils as a coproduct mainly from biodiesel plants. With the increase in production of global biodiesel, large quantities of glycerol are discharged. It is very costly to refine the crude glycerol to a pharmaceutical grade for use in foods, pharmaceuticals, and cosmetics. Many methods for disposal and utilization of this crude glycerol have been attempted (Koller et al. 2005). One of the innovative utilization of crude glycerol is PHA production. Different species of microorganisms metabolize glycerol as a sole carbon and energy source due to the plentiful occurrence of glycerol in nature (Posada et al. 2011). Cui et al. (2015) showed that using glycerol at low concentrations as the only carbon source, with the addition of mixed nitrate (NH_4Cl, yeast extract, and tryptone), stimulated PHB accumulation in thermophilic *C. daeguensis* TAD1, while excess glycerol inhibited PHB accumulation.

Working with cells of native *Lactobacillus reuteri* in an early stage, Linares-Pastén et al. (2015) designed a production system for the conversion of glycerol to 3-hydroxypropionaldehyde (3HPA) by transformation of the 3HPA to poly (3-hydroxypropionate) (PHP) using recombinant *Escherichia coli* strain co-expressing highly active coenzyme A-acylating propionaldehyde dehydrogenase

from *L. reuteri* and polyhydroxyalkanoate synthase (PhaCs) from *Chromobacterium* sp. PHP content was found up to 40% CDW using crude glycerol as a waste substrate together with organic matter in the form of nonvolatile fatty acids. Moita et al. (2014) investigated the feasibility of manufacturing PHA production by a mixed microbial culture (MMC) capable of consuming both glycerol and methanol present in the crude. The aerobic mixed culture generated a maximum PHB content of 47% (CDW) and a productivity of 0.27 g PHB/Ld. A moderate halophile *Yangia* sp. ND199, isolated from mangrove soil sample in Vietnam, was found to accumulate PHBV when cultivated in a medium containing 4.5% (w/v) NaCl, with glycerol as carbon and yeast extract as nitrogen source. The content of value-added polymer PHBV and productivity were found as 40.6% (CDW) and 0.25 g/Lh, respectively (Van-Thuoc et al. 2015). *Cupriavidus necator* and *Burkholderia sacchari* were used by Rodríguez-Contreras et al. (2015) to produce value-added PHB biopolymers with low molecular masses. When glycerol was used together with glucose in fermentation with *C. necator*, high cell dry mass and growth rate were obtained. However *B. sacchari* used only glycerol as a sole carbon source and accumulated low-molecular-weight PHB.

Hermann-Krauss et al. (2013) compared the accumulation of PHA co- and terpolyesters of *Haloferax mediterranei* from inexpensive crude glycerol phase (1%, w/v) obtained from biodiesel production and from pure glycerol (1%, w/v). When the pure glycerol and crude glycerol phase were used, 13.4% and 16.2% (g/L) PHA accumulated, respectively, in the test strain.

11.3.2 Crude and Waste Plant Oils and Oil Mill Effluents

Bacterial polyesters from crude and waste plant oils, which can be difficult to dispose of, can be recovered and used as disposable crude, and waste plant oils can be used to grow bacterial polyesters (Ciesielski et al. 2015).

Acidic oil cake of *Calophyllum inophyllum* which is nonedible was examined under dark and photo fermentation conditions by using a coculture composed of a dark fermentative (*E. aerogenes*) and a photo fermentative (*R. sphaeroides*) bacteria for biohydrogen and PHA production. With the use of a minimal salt media and alternate dark-photo fermentative periods, cost of production has reduced (Arumugam et al. 2014). Mozejko and Ciesielski (2013) investigated the synthesis of mcl-PHAs from *Pseudomonas* sp. Gl01 cultivated in a biofermentor containing saponified waste palm oil as the only carbon source. Martino et al. (2014) used cooking oil as the only carbon source for production of PHB by *C. necator* DSM 428. The resultant biomass was used for extraction of the PHB granules with a solvent-free approach using sodium dodecyl sulfate, ethylenediaminetetraacetic acid, and the enzyme alcalase in an aqueous medium. In another study, *C. necator* DSM 7237 strain was grown in crude glycerol, sunflower meal hydrolysates, and levulinic acid as the only fermentation feedstock in a bioreactor. Levulinic acid

could be combined with biodiesel industry by-products for the generation of high content, industrially useful PHB and PHBV (Kachrimanidou et al. 2014).

Taking into account of their higher volatile fatty acids (VFA) concentration, fermented olive oil effluents might be a potential source for PHA production (Dionisi et al. 2005). The feasibility of producing PHAs by feeding a pure culture of *C. necator* with dephenolized and fermented olive mill wastewater as the carbon source for PHAs generation was demonstrated at shaken-flask scale (Martinez et al. 2015). Alpecin, which is an effluent of olive oil wastewater, is a harmful environmental contaminant due to its considerably high phenolic content concentration. *Pseudomonas putida* KT2442, containing plasmid harboring *C. necator*, grew in high concentrations of alpecin and accumulated considerable amounts of PHA (Ribera et al. 2001). In another study, researchers demonstrated that *A. chroococcum* H23 was capable of accumulating substantial amounts of PHA in the media with high alpecin content (Koller et al. 2010).

11.3.3 Surplus Whey from the Dairy Industry

Whey is a by-product of cheese or casein manufacture which is regarded as a waste and surplus material. It constitutes about 80–90% of the volume of processed milk. The amount of whey that is produced globally is about 120 million tons per year. Roughly half of all whey is turned into whey beverages, additives for food processing (e.g., meat products and ice cream), and animal feed, and the remainder is disposed of as waste material (Koller et al. 2010).

Lactose in whey when discharged into the environment becomes a hazard because of its high biochemical oxygen demand. Hence, many studies have aimed to recover and find suitable uses for lactose (Braunegg et al. 2007; Koller et al. 2007; Nikodinovic-Runic et al. 2013; Koller 2015). While the higher-grade lactose obtained from whey is used in infant formulae and as an excipient for pharmaceutical products, the amounts of purified lactose required for these uses make up only 5–10% of what can be potentially derived (Nikodinovic-Runic et al. 2013). While the direct application of recovered lactose may not be possible for all bacteria strains of interest, one technique is to hydrolyze lactose into glucose and galactose, each of which is readily and efficiently fermented. The glycosidic bond can be enzymatically hydrolyzed by β-galactosidase or by acid hydrolysis. Fermentations using concentrated, high lactose content whey solution and recombinant *E. coli* CGSC 4401 having *Alcaligenes* PHA synthase genes were performed in laboratory-scale bioreactors, reaching a final PHB concentration of 96.2 g/L (Nikodinovic-Runic et al. 2013). Wong and Lee (1998) also reported 50 g/L PHA with *E. coli* CGSC 6576 containing plasmids of *C. necator* PHA synthase genes.

The potential of various microorganisms to convert whey lactose to PHAs has been studied by Koller et al. (2007) with *H. mediterranei*, *Hydrogenophaga pseudoflava*, and *Pseudomonas hydrogenovora*, as industrial scale PHAs producers from the hydrolyzed whey feedstock. Among the strains only *H. mediterranei* has

significantly accumulated (50%, CDW) of PHBV from hydrolyzed whey without necessity of expensive propionic or valeric acids and 3-hydroxyvalerate (3HV) precursors.

Koller (2015) examined the reutilization of waste fermentation broth, obtained from previous PHA production of *H. mediterranei* in whey, for a second PHA production using the same microorganism. It was observed that the waste fermentation broth may be used instead of fresh saline fermentation medium to produce PHA. However, he mentioned that 29% of yeast extract can be substituted by cell debris from the previous fermentation broth.

11.3.4 Wastes from the Sugar Industry

Molasses is a by-product of sugarcane or sugar beet processing containing high amount of sugar and has been commonly used as a carbon source in industrial-scale fermentations due to its low price and abundance (Du et al. 2012). PHA production was reported in 1992 by *Azotobacter vinelandii* UWD using sugar beet molasses. *Bacillus* sp. JMa5 which was isolated from molasses contaminated soil was osmotolerant and able to grow at high temperatures. This strain produced 70 g/L PHB (25–35%, CDW) using sugar beet molasses (Wu et al. 2001). In another study, *Bacillus subtilis* and *E. coli* isolated from industrial contaminated soil samples were grown on cane molasses yielding a maximum PHA production of 54.1% and 47.16%, respectively (Gomaa 2014). Albuquerque et al. (2007) developed a three-stage PHA production process from sugarcane molasses using MMC. This culture reached a maximum PHA content of 74.6%. In a Brazilian company manufacturing sucrose and ethanol from sugarcane, economically competitive PHA was produced by *C. necator* DSM 545. This process employed the factory's two main waste streams: bagasse and fusel alcohols. Bagasse was used for energy generation, while fusel alcohols (mainly isopentyl alcohol), which are less harmful than chloroform, are used for the PHA extraction (Koller et al. 2010).

11.3.5 Lignocellulosic Wastes

Lignocellulosic material, containing lignin, cellulosic, and hemicellulosic fibers, constitutes the most abundant renewable resource on the earth. With the development of optimization methods for the bioconversion of cellulose and hemicellulose into microbially usable carbon sources such as monosaccharides, it will be possible to use lignocellulose and cellulose wastes for the production of high-value biopolymers and other materials (Braunegg et al. 1998).

Davis et al. (2013) investigated the potential of delignified and hydrolyzed grass biomass as a feedstock for *Pseudomonas* strains such as *P. putida* W619, *P. putida* KT2440, and *P. fluorescens* 555. Tested strains accumulated 20–34% of CDW.

Cesário et al. (2014) utilized wheat straw hydrolysates and *B. sacchari* cells accumulated PHB (70%, CDW). The PHB volumetric productivities attained were the highest ever achieved on agricultural waste hydrolysates. Extruded rice bran and extruded cornstarch (1:8 w/w) were utilized as carbon sources to produce PHA by *H. mediterranei*, and PHA amount was found as 77.8 g/L (Huang et al. 2006). Researchers examined the usage of vegetable wastes as growth media for extremophilic microorganisms that produce biopolymers. They found that biomass fermentation provides a cheaper way to produce PHA (Di Donato et al. 2011). The *Haloterrigena hispanica* strain FP1 cells grown on carrot wastes as sole carbon source were able to produce a comparable amount of PHB (0.13%, CDW) with respect to that produced when cells were cultivated on complex standard media (0.14%, CDW). This result suggests an alternative and low environmental impacting method for vegetable waste management (Poli et al. 2011). In our previous study, whey, melon, apple, and tomato wastes and sucrose as carbon source were evaluated on PHA production from Natrinema 1KYS1 (Danis et al. 2015), and the strain was able to grow and produced 19.9%, 10.5%, 3.1% and 12.1% of CDW PHBV, respectively.

Wine lees, pretreated with crude enzymes of *Aspergillus oryzae*, were converted into a fermentation nutrient for the strain *C. necator* DSM 7237. This process maintained 30.1 g/L of PHB concentration (71.3%, CDW) and a productivity of 0.56 g/Lh during fed-batch fermentation (Dimou et al. 2015). The synthesis of PHAs by activated sludge with aerobic dynamic feeding process was conducted in a sequencing batch reactor by using food wastes and excess sludge fermentation liquid as the carbon source (Zhang et al. 2014).

Hardwood spent sulfite liquor, a complex feedstock originating from the pulp industry, was tested as a substrate for a MMC identified as α-(72.7%), β-(11.1%), and γ-proteobacteria (10.3%), and maximum PHA content of 67.6% CDW was achieved under aerobic dynamic feeding conditions (Queirós et al. 2014).

H. mediterranei, with its supreme advantages mentioned before, can utilize waste stillage from the rice-based ethanol industry for PHA production. This strain accumulated PHBV (63%, CDW) in the simple plug-flow reactor configuration of the activated sludge process (Bhattacharyya et al. 2015).

Rice bran treatment process for the production of hydrolysate solution containing 24.41 g/L of glucose and small amount of fructose was developed and used to produce PHAs. Recombinant *E. coli* expressing *R. eutropha* phaCAB genes and *R. eutropha* were found to produce PHB with the polymer contents of 90.1% and 97.2% of CDW, respectively (Oh et al. 2015). Utilization of hydrolyzed hemicellulose and cellulose fractions of bagasse as carbon source for PHA production was examined using *B. cepacia* and *Burkholderia sacchari* IPT 101 on a laboratory scale (Silva et al. 2004). Promising results were reported for the latter; accumulation of 62% PHA CDW was accomplished. Production of PHA by *Haloarcula marismortui* from vinasse, which is a waste of the ethanol industry, was evaluated. This microorganism accumulated PHB (23%, CDW) in the test medium containing raw vinasse (10%, v/v) (Pramanik et al. 2012).

11.3.6 Municipal Wastes

Municipal waste consists of waste originated from households, offices, and various sources and collected by municipal authorities. Especially in the developing countries, municipal wastes are not well managed and pose a serious threat to the environment. The use of municipal waste as a resource could be useful to cut greenhouse gas emissions (Nikodinovic-Runic et al. 2013). Waste-based PHA production by bacterial enrichments generally follows a strategy in which the wastewater is converted into a volatile fatty acid rich stream. A non-fermented substrate was studied by Moralejo-Gárate et al. (2014), supporting the development of pure culture-based PHA production as a replacement for bacterial enrichment. Pittmann and Steinmetz (2014) investigated the production of PHAs as a side stream process on a municipal wastewater treatment plant. He also studied the effect of substrate concentration, pH, temperature, and cycle of installed feast/famine regime on PHA production. High PHA production up to 28.4% of CDW was maintained with optimal of lower substrate concentration, neutral pH value, 20 °C, and a 24 h cycle time.

The biosynthesis of PHB directly from carbon dioxide (CO_2) is a sustainable alternative for nonrenewable, petroleum-based polymer production. The conversion of CO_2 implies a reduction of greenhouse gas emissions. Through an autotrophic conversion, hydrogen-oxidizing bacteria such as *C. necator* have the ability to store PHB using CO_2 as a carbon source. A mathematical model based on mass balances was simulated taking into account the stoichiometry and kinetics of biomass growth and PHB formation, as well as physical transfer from the gas phase to the liquid fermentation broth. The developed model was calibrated and validated for *C. necator* based on independent experimental datasets from literature. The obtained simulation results accurately described the dynamics of autotrophic biomass growth and PHB production. The effect of oxygen (O_2) and/or nitrogen stress conditions, as well as the effect of the gas mixture composition in terms of O_2 and hydrogen, was investigated through scenario analysis (Mozumder et al. 2015).

11.4 Concluding Remarks and Future Perspectives

With the increasing demand for healthy and natural products and awareness of environmental issues, biopolymers have emerged as attractive candidates. Being both biocompatible and biodegradable, PHAs are the most promising and most extensively studied biopolymers. Besides these excellent properties, it is possible to produce various PHA formulations with distinct physical and chemical properties for a variety of applications. However, the expensive carbon sources for the bio-based PHA production and the lack of efficient large-scale processes make PHAs uncompetitive with petroleum-based plastics. PHAs therefore have a limited

market share. In order to reduce the need for expensive carbon sources which constitute the major part of the production cost, it is possible to use waste-based substrates. This decision also has a positive impact in so far as it reduces environmental pollution and carbon dioxide emissions.

The production cost is remarkably reduced in processes in which halophilic archaea are used. Expenses for sterile conditions are not needed, and expensive organic solvents are not utilized for PHA extraction. Also, for the production of monomers like 3-hydroxyvalerate from bacterial sources, addition of substrates such as propionate is required, but archaeal strains can produce this monomer solely from waste materials.

Future work should focus on discovering new PHA-producing microorganisms as well as improving the production processes depending on minimum energy and solvent requirements.

Take-Home Messages

- Polyhydroxyalkanoates are produced by more than 300 microorganisms including various species of *Bacteria* and *Archaea* for carbon and energy storage in an environment that is carbon-rich but poor in certain nutrients such as phosphorus and nitrogen.
- More than eight different pathways for the synthesis of PHAs have been discovered to date in various organisms; however, many species use a three-step reaction: production of acetoacetyl-CoA, its reduction to 3-hydroxybutyrl-CoA, and finally polymerization of PHB.
- Due to their diverse structural varieties, PHAs have variable chemical and mechanical properties such as glass transition temperatures, thermodegradation temperatures, Young's modulus, and tensile strength.
- Being biocompatible and biodegradable, PHAs have superior attributes when compared to conventional petroleum-based polymers in biomedical applications such as cardiovascular repair devices, sutures, and skin substitutes.
- PHA production cost is not compatible to petroleum-based polymers, primarily due to the significant reliance on expensive carbon substrates.
- It is critically important to develop cost-effective and technically performing PHAs by utilizing renewable and waste materials to meet the market demand for green polymers.

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Chapter 12
Rewiring Extremophilic Electrocatalytic Processes for Production of Biofuels and Value-Added Compounds from Lignocellulosic Biomass

Navanietha Krishnaraj Rathinam, Rajesh K. Sani, and David Salem

What Will You Learn from This Chapter?

This chapter will introduce the basic concepts of bioelectrocatalysis and the advantages of extremophiles for bioelectrochemical systems. The chapter will discuss electrogenic activity and electron transfer characteristics of extremophiles and their applications in microbial fuel cells, microbial electrolytic cells, microbial desalination cells, and

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microbial electrosynthesis. The use of extremophilic bioprocesses for production of bioenergy and value-added products from lignocellulosic biomass will also be discussed.

12.1 Introduction

Tremendous advancements in science, technology, and medicine all over the world have led to remarkable growth in industrialization, supporting an ever-growing population, which in turn threatens the supply of commodities, including electricity from non-renewable resources. The primary energy consumption in the United States was nearly 96 quadrillion BTU in 2016 (U.S. Energy Information Administration, Monthly Energy Review, April 2017). For electricity generation, most countries, including the United States, mainly depend on non-renewable sources of energy, such as coal, and on nuclear sources. Nuclear power plants can have drastic negative impacts on mankind and the environment (Kyne et al. 2016). Coal power plants emit radionucleotides which cause respiratory disorders and other deleterious effects (Pandit et al. 2011). Although non-renewable sources provide maximum power output, they are depleting rapidly, have grave environmental consequences, and cause major health hazards to biota, including humans (Navanietha Krishnaraj and Yu 2015).

On the other hand, several countries are moving towards predominant use of renewable energy sources, and this trend is likely to grow. While most renewable sources of energy—such as solar, wind, and tidal energy—are ecofriendly and help to mitigate the issues of global warming/climate change (Panwar et al. 2011), they demand huge costs for installation, operation, and maintenance.

Bioelectrochemical systems (BES) are a promising approach to producing bioelectricity (Pant et al. 2012). BES are electrochemical devices that make use of the complex enzymatic machinery of electroactive microorganisms and its electron transfer characteristics for mediating bioelectrocatalysis. The microorganisms or enzymes act as electrocatalysts in these bioelectrocatalytic systems and are referred to as microbial electrocatalysts and enzymatic electrocatalysts, respectively (Schröder and Harnisch 2013). In addition to bioelectricity generation, the BES make use of the electrocatalytic activity of the electroactive microorganisms, which have a wide range of applications such as production of biofuels (bioelectricity, biohydrogen, methanol, biodiesel), water treatment (including desalination), biosensing of analytes, and production of value added compounds (Sleutels et al. 2012).

BES help to convert chemical energy (wastes) into electrical energy and vice versa. They have several advantages over conventional energy systems because of their low cost, eco-friendly nature, high conversion efficiency, and mild operating conditions. Different configurations of bioelectrochemical systems such as a Biological Fuel Cell, Microbial Electrolytic Cells, Microbial Desalination Cells, Microbial Electrosynthesis, and Electrochemical Biosensors have been reported in the literature (Logan et al. 2006, 2015; Navanietha Krishnaraj et al. 2015).

The use of microorganisms in electrochemical systems, or any other bioprocessing operation, has limitations in that they can thrive and mediate electrocatalytic reactions only in a narrow range of operating conditions (temperature, pH, pressure, etc.). Enzymes have

high catalytic rates and could confer better sensitivity and selectivity when compared with microbial catalysis. On the other hand, they are fragile and become denatured at elevated conditions. These limitations of the microorganisms/enzymes can be circumvented by the use of extremophilic systems. The use of extremophilic bioelectrocatalysts in electrochemical systems has the advantage that they can catalyze a wide range of substrates, including the recalcitrant lignocellulosic biomass (Turner et al. 2007; Bhalla et al. 2014a, b). Lignocellulosic biomass is generated at very large volumes from agricultural and municipal wastes, and the use of lignocellulosic biomass in extremophilic electrocatalytic process will greatly help in cutting down BES operating costs. This chapter will provide the basic concepts of BES and will discuss the scope of using extremophiles as electrocatalysts in lignocellulosic biomass fed Bioelectrochemical Systems.

12.2 Electroactive Extremophiles

Electroactive microorganisms are those organisms that can exhibit electrocatalytic activity. They can produce/consume electrons upon oxidation/reduction of electron donor and electron acceptor, respectively, and transfer the electrons across the electrode–electrolyte interfaces. They are the key players in any bioelectrochemical system and can serve as electrocatalysts in electrochemical reactions. The electron transfer in microbial electrocatalysis becomes difficult if the electrocatalytic reactions occur deep within the cell. However, wiring the redox sites of the enzymes/microorganisms to the electrode surface is difficult, as the respiratory proteins in the Gram-positive bacteria are covered by a thick peptidoglycan layer and a periplasmic space. This problem can be circumvented by careful selection of electroactive microorganisms. A good electroactive microorganism should contain the conductive proteins on the surface of the cell wall and, besides having good oxidation ability, must have good electron transfer characteristics from the microorganism to the electrode at the electrode–electrolyte interface. Beside these features, other properties are also advantageous, such as electrochemical activity over a wide range of pH and temperature, resistance to substrate/product inhibition, and resistance to toxins. Microorganisms can transfer electrons either by the direct electron transfer mechanism or using electron shuttling compounds. Direct electron transfer is carried out by the microorganisms using c-type cytochromes, pili (commonly referred as microbial nanowires), or extracellular minerals. Reguera et al. (2005) reported the pili-mediated electron transfer in *Geobacter sulfurreducens*.

Recently, reports also revealed that these microbial nanowires have metallic-like conductivity (contrary to the previous assumption that electron transfer in biological system is via electron tunneling), which helps in direct interspecies electron transfer between syntrophic organisms, in addition to having the ability to transfer electrons between the electron donors/electron acceptors (Malvankar and Lovley 2012). Organisms such as *Shewanella oneidensis* MR-1 perform direct electron transfer with the c-type cytochromes located on the periplasmic membrane (Schuetz et al. 2009). Extracellular polymeric substances are also shown to contain redox proteins such as c-type cytochromes and biofilm promoting proteins, thereby mediating direct electron

transfer reactions at biofilm–electrode (electron acceptor) interfaces. Certain microorganisms produce mediators or electron shuttling compounds such as flavin, quinone, and phenazine for mediating the electron transfer reactions (Schuetz et al. 2009; Rabaey et al. 2004). Genome analysis and microarray have been used to study the regulation of electron transfer genes in the electroactive biofilm grown onto the electrode surface (Holmes et al. 2006). Reports are also available on the morphological characteristics as well as the basis for conductivity in pili nanowires of certain electroactive microorganisms (Malvankar et al. 2015). Vargas et al. (2013) showed that aromatic amino acids are essential to confer conductivity to pili in *Geobacter sulfurreducens*.

The use of extremophiles as electrocatalysts in bioelectrochemical systems will aid in improving the electrocatalytic activity and the overall performance of the bioelectrochemical system. Extremophilic organisms are promising candidates for developing electrochemical systems that can operate at extreme environments such as high/low temperatures, high/low pH, high/low pressures, saline environments, etc. In addition, the extremophiles can mediate the oxidation/reduction of a wide range of electron donors/acceptors at very high rates. They can also oxidize recalcitrant materials such as lignocellulosic biomass. The use of extremophiles will have the added advantage of developing a robust system for cost-effective commercialization, which has been a major limitation of any microbial/enzymatic systems. For instance, Rastogi et al. (2010) isolated different thermophiles from compost samples using a cellulose-degrading enrichment culture technique. The results of the 16S rRNA analysis of the isolated cultures showed that the sequences were related to *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Deinococcus-Thermus*, *Firmicutes*, and *Proteobacteria*. Different isolates belonging to the *Geobacillus*, *Thermobacillus*, *Cohnella*, and *Thermus* that displayed potential to degrade cellulose, carboxymethyl cellulose (CMC), or ponderosa pine sawdust were identified. Among the different isolates, *Geobacillus* sp. WSUCF1 was selected based on its higher growth rate and cellulase activity. The optimal pH and temperature for carboxymethyl cellulase (CMCase) activity of WSUCFI was reported as 5.0 and 70 °C. The WSUCF1 CMCases had a km value of 1.08 mg/mL and retained 89% of the initial CMCase activities after incubation at 70 °C for 1 day. The whole genome sequence of this *Geobacillus* sp. strain WSUCF1 revealed several genes encoding lignocellulose degradation (Bhalla et al. 2013). The results of the genome annotation showed that among the 865 ORFs that are responsible for carbohydrate metabolism, 70 open reading frames (ORFs) were related to polysaccharide degradation, 3 ORFs for cellulose degradation, and 13 ORFs were annotated as xylan-degrading enzymes. This strain was also shown to produce endoglucanase, xylanase, and β-xylosidase (Bhalla et al. 2014a, b, 2015). Recent investigations have shown that the thermophilic strain WSUCF1 can respire onto the carbon felt electrode and perform direct electron transfer reactions (our unpublished data) indicating the value of this strain for applications in bioelectrochemical systems with lignocellulosic substrates as feedstocks. The use of an inexpensive and abundant lignocellulosic biomass in any bioelectrochemical system will greatly help to decrease the cost of operation as well as to provide safe disposal of these wastes, generated in huge volumes from different sources of environment.

12.3 Biological Fuel Cells

Biological fuel cells are electrochemical systems that aid in converting chemical energy into electrical energy with the aid of either electroactive microorganisms or isolated enzymes (Rathinam et al. 2018; Shrestha et al. 2018). These devices operate on the principles of microbial or enzymatic electrocatalysis and are referred to as Microbial Fuel Cells (MFC) and Enzymatic Fuel Cells, respectively. In a biological fuel cell, the microorganism/enzymes are used to oxidize the electron donor and transfer the electrons onto the anode. The electrons received by the anode travel across the external circuit and are transferred to the cathode where microorganism/enzymes are used to reduce the electron acceptor. The anode and cathode compartments are separated by proton exchange membrane. A scheme showing the construction and operation of MFC is shown in Fig. 12.1. In a biological fuel cell, microorganisms/enzymes can be used as electrocatalyst for oxidation, reduction or both. If the electroactive microorganisms used in the microbial fuel cell are capable of performing direct electron transfer onto the electrode, then it is referred to as mediator-less MFC. The use of robust catalysts such as extremophiles in MFC help in the oxidization/reduction of a wide range of electron donor/electron acceptor at accelerated rates. In the case of lignocellulose based bioelectrochemical systems, the electroactive microorganism must be able to oxidize lignin using polyphenol oxidase, laccase, lignin and peroxidase, and hydrolyze cellulose with the help of endoglucanase and cellobiohydrolase. In addition, they must be able to oxidize the

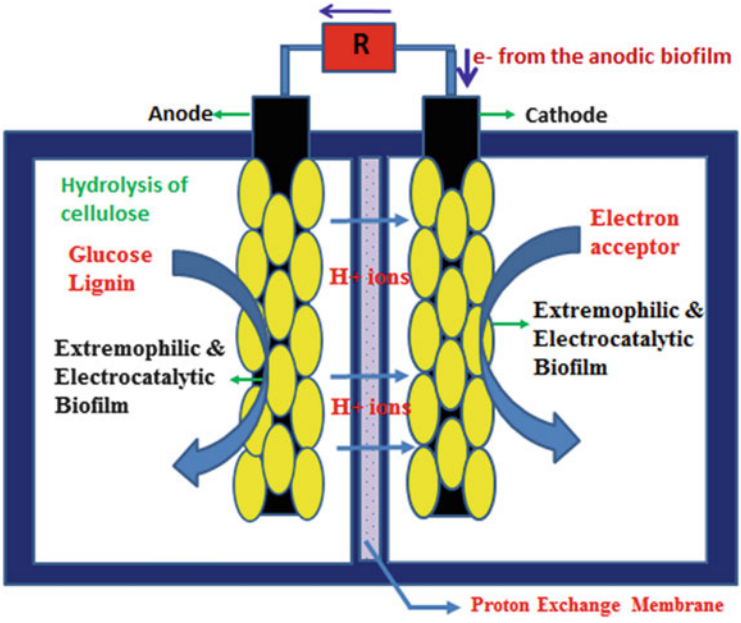

Fig. 12.1 Construction and operation of MFC

hydrolyzed cellulose to produce electrons and efficiently transfer the electrons onto the anode with the help of direct electron transfer or mediated electron transfer mechanisms. MFC have been shown to be promising for treatment of different wastewaters containing glucose, ethanol, cellulose, acetate, and soak-liquor (Navanietha Krishnaraj et al. 2013; Bhuvaneswari et al. 2013; Selvaraj et al. 2016). Different configurations of MFC, electrode materials, electrode functionalization strategies, and membranes have been reported in the literature (Navanietha Krishnaraj et al. 2014; Karthikeyan et al. 2016; Bella et al. 2016).

Different electroactive microorganisms have been harnessed as electrocatalysts in lignocellulose fed bioelectrochemical systems. For example, Ren et al. (2007) reported a two-chamber MFC with the co-culture of *Clostridium cellulolyticum* and *Geobacter sulfurreducens* as electrocatalyst and cellulose as substrate for the generation of bioelectricity. It was reported that *C. cellulolyticum* were found attached to cellulose particles in suspension, whereas *G. sulfurreducens* adhered to the electrode. This showed that the *C. cellulolyticum* was involved in the hydrolysis of lignocellulosic biomass whereas *G. sulfurreducens* performed electrocatalysis of glucose. The bacterial distribution and biofilm architecture of *C. cellulolyticum* and *G. sulfurreducens* fed in a two-chamber MFC containing cellulose have been investigated in detail (Ren et al. 2007, 2008). Fluorescence in situ hybridization (FISH) and quantitative reverse transcription-polymerase chain reaction (QRT-PCR) analyses were performed to analyze the ecology of the electroactive microorganisms in MFC. This investigation suggested that there is a distinct function-related distribution of these two bacteria in the MFC.

Rezaei et al. (2009) designed a U-tube MFC with *Enterobacter cloacae* as a catalyst for the simultaneous degradation of cellulose with and without the use of an exogenous mediator. Two different strains of *E. cloacae* were used for the electrocatalysts of cellulose in a MFC. *E. cloacae*, isolated from a waste water treatment plant, produced a current density of 127 ± 14 mA/m^2 at 1.8 ± 0.02 mW/m^2. The electrocatalytic activity of *E. cloacae* with different carbon sources such as glycerol, glucose, and *N*-acetyl-D-glucosamine showed that these substrates generated a higher power output than cellulose. These results suggest that the hydrolysis of cellulose is the limiting factor in the bioelectricity generation with *E. cloacae*.

A new bacterial strain from the cellulose fed bioelectrochemical system was identified (Kodama and Watanabe 2011). The isolated strain was found to be Gram-negative, non-spore-forming, straight or slightly curved rods. The cells had one or two polar prosthecae, and reproduced by binary fission or by budding. They oxidized a wide range of sugars and produced lactate, acetate, and fumarate. They could reduce nitrate, ferric iron, oxygen, and fumarate, but not sulfate and malate. The DNA G+C content of the newly isolated strain was found to be 64.7 mol%. Phylogenetic analysis based on the 16S rRNA gene sequence revealed that the new strain belonged to the genus *Rhizomicrobium* and named as a *Rhizomicrobium electricum* sp. nov.

There are a few reports in the literature on the bioelectricity generation from complex polysaccharides using pathogenic microorganisms. MFC with *Clostridium butyricum* as electrocatalyst for the bioelectricity generation from molasses and starch was developed (Niessen et al. 2004). The mean current density of 1.1 mA/cm^2 was

generated with polytetrafluoroaniline modified platinum electrodes. However, the use of pathogenic microorganisms as the entire cell bioelectrocatalyst in MFC is very risky and might pose several ethical issues. Hassan et al. (2012) demonstrated cellulose fed MFC with potassium ferricyanide as a catholyte using the mixed and pure cultures of *Nocardiopsis* sp. KNU (S strain) or *Streptomyces enissocaesilis* KNU (K strain). MFCs with pure cultures of *Nocardiopsis* sp. KNU and *Streptomyces enissocaesilis* KNU were supplemented with cellobioase enzyme for the hydrolysis of cellulose and they produced a power output of 162 mW/m^2 and 145 mW/m^2 respectively. MFC with the mixed culture produced a power output of 188 mW/m^2 at a current of 0.5 mA with 1 g/L cellulose as substrate without the use of cellobiase enzyme. The use of enzymes in MFC is not economically feasible and will not be suitable for practical applications.

Wang et al. (2009) produced bioelectricity with a single-chamber, air-cathode MFC using corn stover as a substrate. The MFC with the mixed culture and corn stover produced a maximum power of 331 mW/m^2. The MFC was operated for over 60 days, and the denaturing gradient gel electrophoresis showed that the presence of *Rhodopseudomonas palustris* was involved in the electrochemical reaction. The major limitation with the mesophiles in any bioelectrochemical system is their limited potential to perform both hydrolysis of lignocellulosic biomass as well as electrocatalysis of the hydrolyzed sugars. Most of the reports either rely on more than one species of microorganisms, or consortium, to mediate these two reactions simultaneously. Alternatively, there have also been reports on engineering new configurations of bioelectrochemical systems for bioelectricity generation from lignocellulosic biomass.

The use of *Canna indica* (canna), a lignocellulosic aquatic plant, was reported as a substrate for MFC without pretreatment (Zang et al. 2010). Rumen microorganisms were used as MFC bioelectrocatalysts. A novel three chambered MFC design for the simultaneous degradation of lignocellulosic materials for bioelectricity generation and pigment production, coupling the catalytic activities of a lignocellulolytic cyanobacterium and an electrogenic acetic acid bacterium has been reported (Navanietha Krishnaraj et al. 2015). The three-chamber MFC comprises a first compartment for pretreatment; a second compartment as the anode, and a third compartment as the cathode. *Oscillatoria annae* was used for the hydrolysis of cellulose in the pretreatment compartment, acetic acid bacteria were used for the electrooxidation of sugars in the anode compartment and ferricyanide was used as electron acceptor in the cathode compartment. Gregoire and Becker (2012) designed an integrated reactor coupling the tubular air cathode MFCs and leach-bed bioreactors to develop a new solid-substrate MFC with a single chamber wherein monomerization of cellulose, fermentation, and anode respiration occurs. The solid-substrate MFC with corncob pellets yielded continuous power output for more than 60 days. Exposure to oxygen at regular intervals limited methanogenesis leading to the enhanced generation of bioelectricity. Furthermore, the use of *Geobacter metallireducens* for bioaugmentation improved the power output of the MFC.

The extremophilic electrochemical systems have several advantages such as operation at robust conditions, stability of the bioelectrocatalysts for a longer time, and better electrocatalytic activity. However, these systems suffer from several limitations. The use of thermophilic or psychrophilic systems demands suitable

electrolytes which can mediate electron transfer at high or low temperatures. Evaporation will be a major issue in the case of thermophilic systems, and it demands external energy to maintain the high temperature. The choice of membrane is also a major factor in these electrochemical systems. A suitable membrane should have good proton conductivity even at extreme conditions such as high or low temperatures, acidic/alkaline environments, and high pressure. The membrane must resist the high pressures, and should be able to resist the entry of oxygen and hydrogen gases. The configuration of the biofuel cell for operation in an extreme environment is a major challenge and materials that resist high/low temperatures/pressures/pH are required for the fabrication of an electrochemical system.

The use of extremozymes will have several advantages over normal enzymes in developing enzymatic fuel cells. The use of extremozymes will help to oxidize the electrons at much faster rates compared to microbial systems. They will be more promising in developing implantable fuel cells to power low energy devices such as pacemakers. Different types of extremophiles have been reported in the literature for biological fuel cell applications. The use of thermozyme based biological fuel cells have several advantages such as better mixing, high substrate solubility, good mass transfer rate, and decreased risk of contamination. Several reports are also available on the use of different sugars as substrates in extremophilic microbial fuel cells. However, the cellulose fed microbial fuel cell systems are limited.

Thermophilic microbial fuel cells with Firmicutes as electrocatalyst and 10-mm acetate as the sole electron donor have been developed (Wrighton et al. 2008). The thermophilic MFC were operated at 55 °C over 100 days of operation and produced a power density of 37 mW/m^2 and a coulombic efficiency of 89%. Choi et al. (2004) developed a microbial fuel cell with thermophilic microorganisms such as *Bacillus licheniformis* and *Bacillus thermoglucosidasius* operated at 50–70 °C. It was shown that the developed microbial fuel cell could oxidize a wide range of electron donors such as fructose, galactose, glucose, lactose, maltose, mannitol, mannose, sorbitol, starch, sucrose, and trehalose. Abramov et al. (2013) reported a thermophilic process using *Thermoanaerobacterium* (including *T. thermosaccharolyticum* and *T. aotearoense*) and *Clostridium* genus including *C. cellulosi* and *C. thermocellum* for hydrogen production from cellulosic wastes coupled with bioelectricity production using membrane-bound [NiFe]-hydrogenase from *Thiocapsa roseopersicina* strain BBS. Different wastes such as paper (filter paper, newsprint or magazine paper, 15.0 g/L), wheat bran (10.0 g/L), wood sawdust (15.0 g/L), kitchen waste (15.0 g/L), straw (15.0 g/L), and different wastes from the brewing industry (yeast, 15.0 g/L and spent grains, 15.0 g/L) were treated by this process. The use of psychrophilic microorganisms as electrocatalysts have also been reported in the literature. Catal et al. (2011) reported single-chamber air-cathode mediator-less microbial fuel cells with the samples obtained during anaerobic digestion of grass silage as substrate. The psychrophilic system (15 °C) produced a power output of 31 ± 1 Wm^3, and removed the chemical oxygen demand (COD) and total phenolics over 90% and 30–75%, respectively.

In summary, the electroactive microorganisms are promising candidates for a lignocellulose fed microbial fuel cell. They should be able to hydrolyze cellulose as

well as oxidize glucose to generate electrons. The extremophilic microorganisms can also be used as electrocatalysts for the reduction of electron acceptors in the cathode compartment of the MFCs. Suraniti et al. (2013) reported the immobilization of thermostable bilirubin oxidase (BOD) from *Bacillus pumilus* onto the electrode for developing a bioelectrode for bioelectrochemical applications. The thermostable BOD was immobilized in a cross-linked redox-active hydrogel film having pendant osmium moieties grafted on a polyvinylimidazole backbone of the electrode. They displayed high electrocatalytic activity in the electron shuttling compounds at a broad pH range of 7–10 and temperature of 70 °C. Similar reports for the reduction of CO_2, a thermophilic biocathode, were also made based on electromethanogenesis. The biocathodes containing thermophiles (55 °C) such as *Methanothermobacter*-related methanogen and *synergistetes*- and *thermotogae*-related bacteria mediated the electrocatalysis to produce CH_4 at high rates of 1103 mmol m^{-2} day^{-1} at an applied voltage of 0.8 V (Fu et al. 2015). Kobayashi et al. (2017) analyzed the draft genome of a novel *Coriobacteriaceae* sp. strain EMTCatB1, isolated from the metagenome of a thermophilic electromethanogenic biocathode that actively catalyzes electromethanogenesis.

12.4 Microbial Electrolytic Cells

Microbial electrolytic cells (MECs) are electrochemical devices that operate on the principles of bioelectrocatalysis, as in the case of microbial fuel cells. However, the MECs differ from MFCs in the way that the MECs convert electrical energy to chemical energy whereas the MFCs convert chemical energy to electrical energy. The operational principle of an MEC is the reverse of an MFC. The MEC produces hydrogen with the aid of the external voltage, as in the case of electrolytic cells, but the MEC makes use of the voltage produced by the microorganism in addition to the external voltage. In the anode compartment, the substrate (electron donor) is oxidized by the electroactive microorganism and it produces electrons and protons (H^+ ions). The electrons are collected by the anode and reach the cathode through an external circuit. The protons generated by the anodic reaction reach the cathode through the electrolyte. The cathodic reaction mediates the generation of hydrogen by combining the H^+ ions. Figure 12.2 depicts the operational principle of MEC. The electrochemical potential produced by the bioelectrocatalytic oxidation reaction in the anode compartment is insufficient to provide the reducing power required for the hydrogen evolution reaction (HER) at the cathodic site. It requires an additional voltage (normally 0.2 V–1.0 V) for the hydrogen evolution reaction (Logan et al. 2008). MEC requires a very small supplementary external voltage when compared to the much higher voltage (>1.2 V) needed in the case of conventional water electrolysis processes. MEC for biohydrogen production is therefore an energy-efficient option.

Extremophilic microorganisms can be used for the oxidation of substrate in the anode compartment or hydrogen evolution reaction in the cathode compartment. Lignocellulosic biomass would be the feedstock of choice in the anode

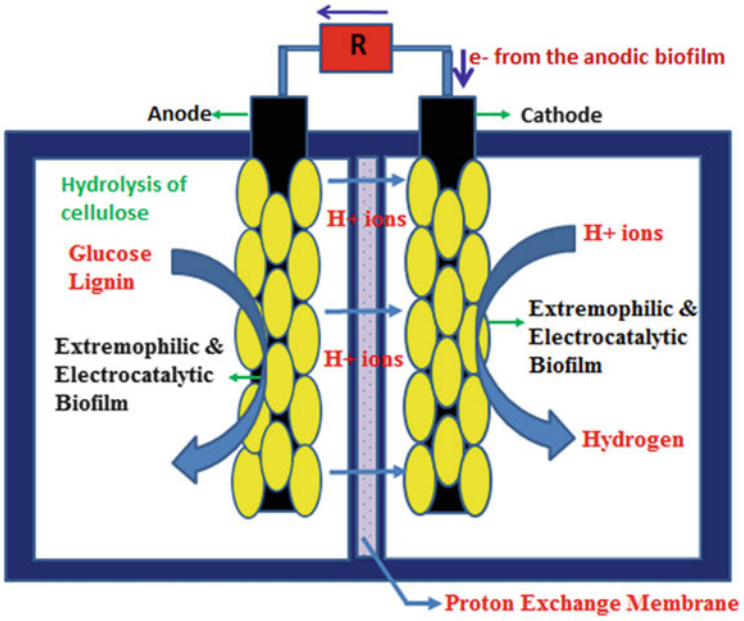

Fig. 12.2 Construction and operation of MEC

compartment. As in the case of MFC, individual extremophilic microorganism co-cultures/consortia having both hydrolytic activity and electrogenic activity will be useful to produce electrons from the lignocellulosic biomass. Different organisms have been reported in the literature for use as electrocatalysts for the electrocatalysis of lignocellulose in the anode compartment as well as reduction of protons to hydrogen in the cathode compartment of MECs. Recently Shehab et al. (2017) reported the use of brine pools from three different locations of the Red Sea, namely, Valdivia, Atlantis II, and Kebrit for the enrichment of the anodic compartment of MECs. The developed MEC operated under thermophilic (70 °C) and hypersaline (25% salinity) conditions and produced a high current of 6.8 ± 2.1 A/m^2 in MECs operated at a set anode potential of +0.2 V vs. Ag/AgCl (+0.405 V vs. standard hydrogen electrode). Fu et al. (2013) reported a thermophilic biocathode containing six different phyla (predominantly Firmicutes) for hydrogen production in a two compartment MEC. The developed biocathode produced a current density of 1.28 ± 0.15 A/m^2 and hydrogen production rate of 376.5 ± 73.42 mmol day^{-1} m^{-2}. Wang et al. (2014) developed a psychrophilic biocathode for hydrogen production in molasses wastewater fed MEC. The developed MFC with biocathode (operated at a low temperature of 9 °C) produced an overall hydrogen recovery of 45.4% with an applied voltage of 0.6 V. Lu et al. (2011) reported that the psychrophilic single-chamber MECs operated at low temperatures of 4 °C or 9 °C with anodic biofilm containing *Geobacter psychrophilus*. The rates of hydrogen production in the acetate fed MEC ranged from 0.23 ± 0.03 to 0.53 ± 0.04 m^3 H_2 m^{-3} d^{-1}, and it produced the

maximum hydrogen yield of 2.94 ± 0.02 mol H_2 mol^{-1} acetate. Lu et al. (2012) reported the synergistic effect of methanogenesis and homoacetogenesis for hydrogen production in a MEC at 25 °C. The hydrogen yield of the single-chamber MEC operated at 4 °C amounted to 6 mol H_2 mol^{-1} glucose and reached a maximum rate of around 0.37 ± 0.04 m^3 H_2 m^{-3} d^{-1}.

12.5 Microbial Desalination Cells

Microbial desalination cells (MDCs) are those electrochemical systems that make use of the electrocatalytic activity of the microorganisms for simultaneous bioelectricity generation and desalination of water. Basically, the operation principle of MDCs is similar to electrosmosis and electrodialysis (Cao et al. 2009; Qu et al. 2012). In MDC, the anodic and cathodic compartments are separated by a desalination chamber and the electrochemical potential of the microorganisms is utilized to drive the transport of ions. The anode and desalination chambers are partitioned by an anion exchange membrane whereas the cathode and the desalination chamber are partitioned by a cation exchange membrane. The schematic diagram showing the principle of MDC is shown in Fig. 12.3. Microorganisms are utilized for the oxidation of electron donors in the anode compartment and reduction of electron

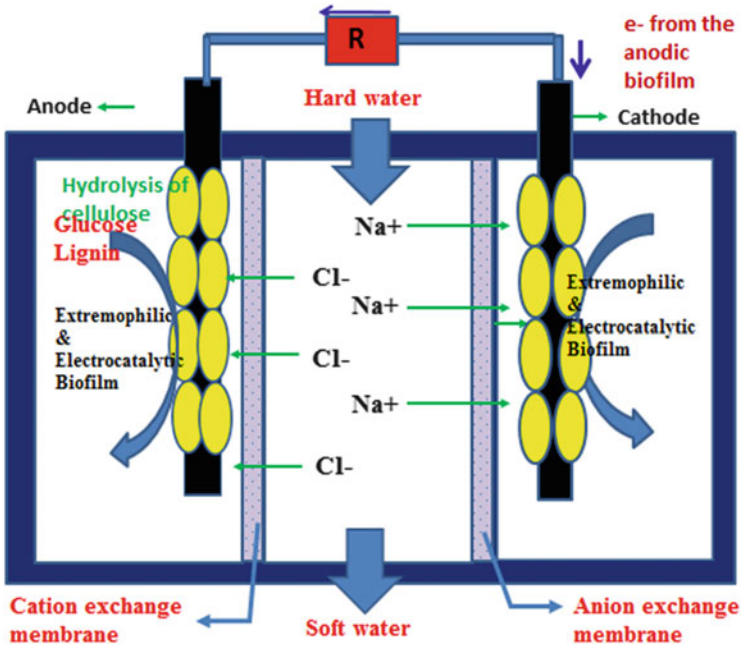

Fig. 12.3 Construction and operation of MDC

acceptors in the cathode compartment, as in the case of MFCs. The potential gradient developed in this process is used for driving the transport of dissolved Na^+ and Cl^- ions through the selective ion exchange membranes toward the cathode and anode, respectively. The larger the potential difference between the anode and the cathode, the higher is the rate of desalination. Hence, for any bioelectrochemical systems, the ideal choice of bioanode should have more negative anodic potential, and the biocathode should have more positive cathodic potential.

MDCs are hybrid strategies that makes use of electrochemical and membrane separation techniques. When compared with the conventional desalination systems, MDC has the advantage that it has minimal energy consumption, accelerated rates of desalination, and minimal damage/fouling to the membrane. In addition, unlike the constant pressure or constant volume filtration systems, MDS is independent of pressure and does not demand special configurations or reactor systems to resist high pressures. However, in terms of electrocatalysis, MDCs are similar to MFCs. MDCs differ from MFC in configuration/construction.

12.6 Bioelectrosynthesis

Bioelectrosynthesis is a bioelectrochemical process by which electroactive microorganisms/enzymes make use of the electrochemical potential for the synthesis of value added products (Rabaey and Rozendal 2010). This is similar to the microbial or enzymatic processes, but differs in that the oxidation or reduction potential is applied to the bioelectrocatalyst (electroactive enzyme/microorganism). The scheme depicting the concept of bioelectrosynthesis is shown in Fig. 12.4. The use of oxidation and reduction potential helps in accelerating the electrooxidation/electroreduction of electron donor/electron acceptor. In a microbial electrosynthesis process, electrical energy is transformed into chemical energy.

Microorganisms have been well explored for the synthesis of several industrially important compounds such as organic acids, amino acids, vitamins, antibiotics, therapeutic compounds, etc. Unlike the enzymatic processes, the microorganisms make use of a series of reactions to synthesize the product. The electrocatalysts aid in mediating the oxidative/reductive synthesis of the desired product from the reactants. Reports are available on the electro organic synthesis approaches for the treatment of waste waters by oxidizing/reducing the toxic electron donors/electron acceptors into nontoxic forms. The bioelectrosynthesis strategy is a hybrid approach making use of catalytic activity of microbial/enzymatic catalysts as well as electrochemical potential. When compared with bioprocesses, the bioelectrosynthetic processes have much higher specificity. The use of specific applied potential on the bioelectrodes also has the additional benefits of decreasing the side reactions/by products which are a major limitation in the conventional microbial systems that greatly demands serious downstream processing strategies.

Photobioelectrocatalysts such as photosynthetic bacteria, algae, or cyanobacteria can also be used as electrocatalysts for the bioelectrosnthesis of value added

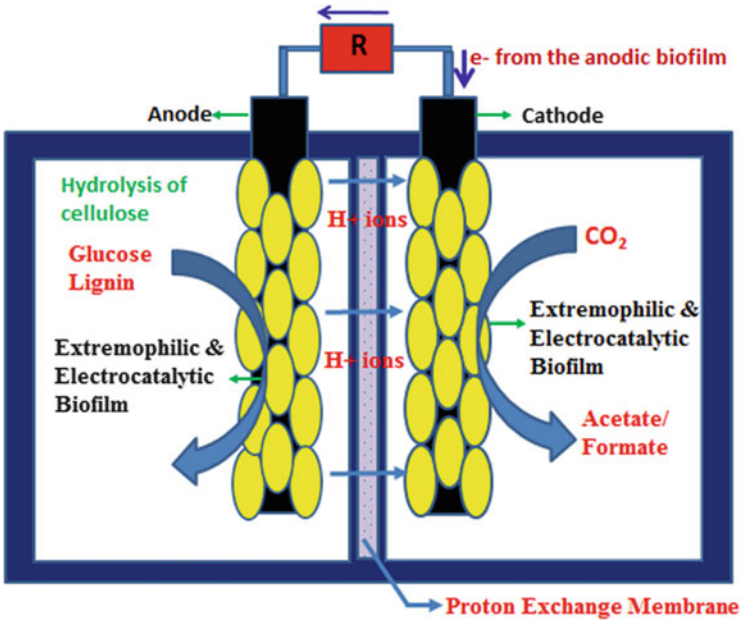

Fig. 12.4 Concept of bioelectrosynthesis

products. In principle, on irradiation with photons, the photosynthetic organisms produce electrons which are used to drive the metabolic machinery. Photomicrobial electrosynthetic processes will likely improve the production of biofuels and value-added products. Several reports are available on the use of acetogenic microorganisms' bioelectrosynthesis of acetate (also known as electroacetogenesis) (May et al. 2016). Reports have also been documented in the literature on the use of thermophiles for enhanced electrosynthesis of acetate. Electrochemical investigations on electron uptake rate of *Moorella thermoautotrophica* at a cathode potential of −0.4 V (vs. standard hydrogen electrode) showed the temperature dependence and demonstrated a maximum current density of 63.47 mA/m^2 at 55 °C. Further, it has been shown that an increase in temperature from 25 to 50 °C increased the electrosynthesis rates of formate and acetate by 23.2- and 2.8-fold, respectively (Yu et al. 2017). In addition, the effect of immobilizing the thermophilic *Moorella thermoautotrophica* along with carbon nanoparticle showed that rates of electrosynthesis of acetate and formate significantly increased by 14- and 7.9-fold reaching to 58.2 and 63.2 mmol m^{-2} day^{-1} with 65% coulombic efficiency.

Take Home Message

- Bioelectrochemical Systems (BES) are a promising strategy for the synthesis of biofuels and value-added products due to their ecofriendly nature and ability to catalyze at normal operating conditions.

- Use of extremophiles in BES can aid in circumventing the limitations of the conventional biological processes.
- The extremophiles/extremozymes can help facilitate immobilization and increase stability/activity leading to improved electron transfer and enhanced electrocatalysis.
- Use of lignocellulosic biomass will help to cut down the cost of the electrochemical process. Limitations from the recalcitrant nature of lignocellulosic biomass can be overcome with the aid of highly efficient extremophilic microorganisms. This seems to be promising for commercial applications in the future.

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Chapter 13
Integrated Consolidated Bioprocessing for Conversion of Lignocellulosic Feedstock to Biofuels and Value-Added Bioproducts

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What Will You Learn from This Chapter?
This chapter will provide basic information about consolidated bioprocessing (CBP), including native and recombinant strategies and their application in biofuel production. It will address the integrated CBP process to produce biopolymers (e.g., polyhydroxyalkanoates, extracellular polysaccharides), organic compounds (e.g., 1,3-propanediol), and biogas (methane). The chapter will also discuss the production of biofuels by integrating the CBP process with fuel cells and other bio-electrochemical systems. A detailed discussion will be provided on the thermophilic anaerobic digestion (TAD) process to produce methane from agricultural biomass using thermophilic microorganisms as well as biological oxidation of methane to methanol using methanotrophic bacteria. The chapter will conclude with presenting different approaches in modeling CBP processes for existing applications.

13.1 Introduction

Recent decades witnessed an exponentially increasing trend in industrialization and motorization throughout the world. This has led to rapid depletion of fossil fuels and increase in emission of greenhouse gases (GHGs) and other toxic pollutants in the environment. The use of fuel alternatives to replace the depleting fossil fuels will help to meet the growing energy demand as well as mitigating environmental issues. Biofuels are one of the most promising sources of energy for replacing fossil fuels, and they can supplement solar, wind, and other clean energy resources in the renewable energy industry in the foreseeable future (Krishnaraj and Yu 2014).

Biofuels are the renewable fuel products that are produced from biomass using biological processes. They include biohydrogen, bioethanol, biobutanol, biodiesel, biogas (biomethane), and biomethanol (Barnard et al. 2010). Biofuels are economical and eco-friendly sources of energy for sustainable development, since they provide renewability, biodegradability, and generate cleaner exhaust gases, greatly contributing to lessening the emission of GHGs and improving air quality (Sani and Krishnaraj 2017). The use of lignocellulosic biomass as a feedstock for biofuel production would be an added advantage.

Lignocellulosic biomasses are derived from trees, grass, aquatic plants, agricultural wastes, municipal wastes, and various industrial by-products, which are locally available (Chong et al. 2009; van Maris et al. 2006). They are the structural components of plant cell wall and mainly composed of cellulose, hemicelluloses, and lignin, in the range of 20–50%, 12–50%, and 10–40% (w/w), respectively. The composition of the different lignocellulosic biomass varies with different species. For instance, the composition of cellulose and hemicellulose in hardwood ranges from 40–55% to 24–40%, respectively. In wheat straw, the composition of cellulose and hemicellulose constitutes around 30% and 50% of the biomass, respectively (Bajpai 2016). These lignocellulosic substrates can be utilized as promising feedstocks for liquid and gaseous biofuel production through different bioprocess operations.

Lignocellulosic woody biomasses possess strong physical structure and have high lignin content which make these considerably recalcitrant to degradation by microorganisms (Zhu et al. 2010). The cellulose and hemicellulose in lignocellulosic materials are tightly bound to lignin; therefore, pretreatment processes for delignification and depolymerization are often necessary to obtain fermentable sugar substrates such as pentose and hexose. The lignin constitutes around 27–32% in woody plants and ranges around 14–25% in herbaceous plants (Chen et al. 1996). The lignin content of alfalfa fibers, pine straw, wheat straw, and flax fibers constitutes about 34%, 23%, 20%, and 15%, respectively (Watkins et al. 2015).

Different strategies for the pretreatment of lignocellulosic biomass such as mechanical comminution, pyrolysis, steam explosion, ammonia fiber explosion, carbon dioxide explosion, ozonolysis, acid hydrolysis, and the organosolv process have been reported in the literature (Kumar et al. 2009). However, these pretreatment processes for degradation of lignocellulosic biomass raise serious concerns in upstream processing, including the increased energy requirement leading to decreased overall efficiency of the process and high cost of the product.

The microbial digestibility of the resulting hydrolysates, downstream processing, industrial scalability, and biorefinery protocols can also be affected by the pretreatment processes of lignocellulosic feedstock. Different pretreatment methods also demand sophisticated techniques for chemical recovery and wastewater treatment to comply with certain environmental regulations (Lin and Tanaka 2006; Chong et al. 2009; Zhu et al. 2010). Meanwhile, it is challenging to develop an economical and efficient pretreatment process to degrade lignocellulosic materials. In many cases, the pretreatment processes also generate toxic compounds inhibiting the microbial growth and lessening the rates of bioprocessing and product yield (Talluri et al. 2013). Minimizing the total energy consumption during biofuel production has been considered quite critical to achieve the maximum yield and efficiency.

Some of the above-mentioned limitations can be overcome by the use of extremophiles, which can improve the rates of utilization of lignocellulose as well as improving the efficiency of the bioprocess. The potential of these organisms to thrive over a wide range of operating conditions and to metabolize a broad range of substrate including recalcitrant materials will be an added advantage. Extremophiles are microorganisms that can thrive and mediate catalysis in extreme conditions of temperature, pressure, acidity, alkalinity, or salinity, in which most of the other organisms cannot survive (Rampelotto 2010). They can synthesize stable enzymes and other macromolecules even at harsh environmental conditions. The extremophiles and their enzymes have a broad range of applications. It is now widely accepted that extremophilic microorganisms will provide valuable resources for exploitation in novel biotechnological processes including integrated bioprocesses for biofuel production (Bhalla et al. 2013). There has been a lot of interest worldwide to develop bioprocesses using different kinds of extremophiles to produce different biofuels which include first-, second-, third-, and fourth-generation biofuels. The second-generation biofuels which includes biofuels from nonedible lignocellulosic materials have several advantages over the first-generation biofuels that are produced from food crops. The use of second-generation biofuels helps to

overcome the ethical issues raised by the first-generation biofuels related to conflicts with food supply and agricultural land usage (Barnard et al. 2010). The extremophilic bioprocesses for production of second-generation biofuels from energy crops or lignocellulosic biomass will help to overcome the limitations of the mesophilic bioprocesses, such as poor rates or efficiency.

The extremophilic organisms are promising candidates for integrated bioprocessing because they can mediate a wide range of catalytic reactions. As in the case of any other bioprocess, choosing ideal microorganisms is one of the key requirements for CBP. In an integrated extremophilic bioprocess, different extremophilic bioprocesses are developed and integrated with each other to complete full transformation of feedstocks to the desired products (biofuels), and unutilized fractions can be further bioprocessed to generate other value-added products. Thus, an integrated continuous and sequential process can be developed to produce biofuels and other value-added bioproducts from lignocellulosic wastes in a sustainable and economical manner.

Integrated extremophilic bioprocessing is therefore a higher value approach to microbial biofuel production from lignocellulosic materials and wastes, compared to conventional bioprocesses using lignocellulosic biomass in which the unutilized fractions and by-products are discarded, reducing economic viability and ecological advantage. Different microorganisms are known to produce biogas through a thermophilic aerobic digestion (TAD) process or to produce value-added products such as exopolysaccharides (EPSs), polyhydroxyalkanoates (PHAs), and 1,3-propanediol (1,3-PDO) (Gebreeyessus and Jenicek 2016; Nicolaus et al. 2010; Chen 2009; Saxena et al. 2009). These value-added products can be refined through downstream processing, and they have different applications in different sectors including plastics, biomedical products, food packaging, and pharmaceutical delivery systems.

This chapter will focus on integrated extremophilic bioprocessing to produce biofuels which involves several steps starting from lignocellulosic materials as a primary feedstock. In this processing scheme, CBP will be used as the first step to produce biofuels including biohydrogen, bioethanol, and biobutanol from lignocellulosic feedstocks. These liquid and gaseous biofuels will be transformed to electrical energy using fuel cells/bioelectrochemical systems. The organic by-products and unhydrolyzed fractions from CBP will be further utilized as substrates for production of methane (through TAD process), biopolymers, and 1,3-PDO. The methane obtained can be utilized to generate methanol and lipid with methanotrophs. The latter can be transesterified to produce biodiesel. The glycerol generated from the biodiesel production process as by-product can be fed back as substrate for biopolymer and 1,3-PDO production. The unutilized liquid fraction from TAD and aerobic fermentation of value-added products can also be recycled as nitrogen and water sources. Finally, the heat required by CBP can be generated by combustion of methane and bioH_2 generated through TAD and CBP. This integrated bioprocessing will be an environmentally friendly biofuel producing process with high production efficiency and minimal waste generation, thus increasing the scope of commercialization. A scheme showing an Integrated CBP for production of biofuels and other value-added products is shown in Fig. 13.1.

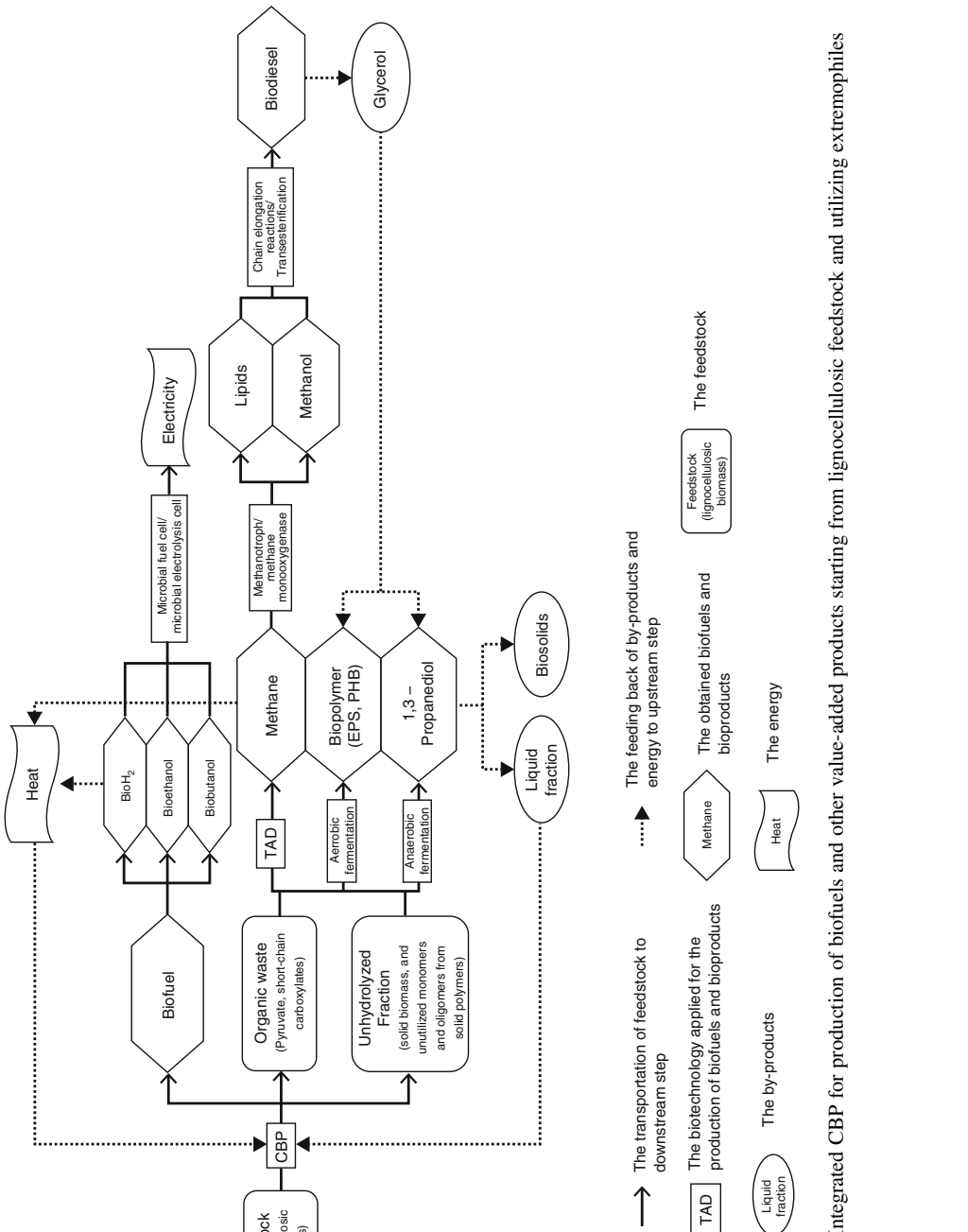

Fig. 13.1 Integrated CBP for production of biofuels and other value-added products starting from lignocellulosic feedstock and utilizing extremophiles

13.2 Consolidated Bioprocessing (CBP) by Extremophilic Microorganisms

CBP is a one pot bioprocessing strategy for the conversion of lignocellulosic substrates into desired products such as biofuels using a single bioreactor. CBP combines enzyme production, cellulose hydrolysis, and fermentation for final products in only one step without adding hydrolytic enzymes. CBP is widely considered as a promising procedure for hydrolysis and fermentation of lignocellulosic woody biomass and wastes with appealingly low cost (Olson et al. 2011; Lynd et al. 2005). Since there is no need to externally supplement enzymes to the system for the hydrolysis of lignocellulosic woody biomass, the processing cost of CBP can be considerably lower than that of separated hydrolyzation and fermentation steps, especially when the enzyme loading for hydrolysis is high. CBP can also attain extensive saving due to high yield of the substrate and feedstock utilization efficiency. CBP using extremophiles, especially thermophiles, is promising because of the high efficacy of lignocellulosic hydrolases, higher operating temperature, and enzyme–microorganism synergy, or combination of these. The cost of CBP can be much lower than that of traditional process for biofuel production from woody biomass. In a traditional process, the hydrolysates of lignocellulose inhibit the lignocellulolytic enzymes, thereby lowering the rates of saccharification. On the other hand, in CBP, the hydrolysates will be fermented immediately and thus the product inhibition for saccharification will be no longer effective. Although CBP has several advantages over traditional processes, especially the use of a single reactor, it has certain challenges. Some operating conditions may not be optimal for both saccharification and fermentation. The organic acids which are mostly generated as by-products in CBP lead to a lower biofuel yield compared with the theoretical maximum value. The heat energy that is required to maintain thermophilic CBP is also relatively intensive. Finally, the leftover lignocellulosic substrates are considered hardly utilizable for further bioprocessing to produce other bioproducts (Mazzoli 2012).

The CBP process has been developed using different thermophilic strains such as *Caldicellulosiruptor saccharolyticus*, *Caldicellulosiruptor bescii*, and *Thermoanaerobacterium thermosaccharolyticum* for biohydrogen production (Talluri et al. 2013; Cao et al. 2014; Cha et al. 2013); *Clostridium thermocellum* and *Thermoanaerobacterium saccharolyticum* for bioethanol production (Schuster and Chinn 2013); and mesophilic strains such as *Caldicellulosiruptor cellulolyticum* and *Clostridium* spp. for biobutanol production (Higashide et al. 2011; Mazzoli 2012). The ideal microorganisms for CBP can express and synthesize different kinds of glycoside hydrolases such as cellulase and xylanase. They help in the enzymatic hydrolysis of lignocellulosic feedstocks to soluble monosaccharides and disaccharides which can be used for biofuel production. All the enzymes involved in CBP should demonstrate proper synergy and can be well coordinated, which means the products from one enzymatic step will not inhibit the activities of any other enzymes for the next several steps. The biosynthesis of hydrogen, ethanol, and butanol takes place through several series of redox biochemical reactions involving the Embden–Meyerhof route and NAD/NADP, ATP, and ferredoxin, which also generates several kinds of organic acids as by-products including lactate, acetate, and butyrate. Higher biofuel yields can

be attained by the repression of competing metabolic pathways coupled with certain biofuel producing pathways. Metabolic engineering strategies are aimed at developing microorganisms with resistance over adverse operating conditions and which can produce biofuels such as hydrogen, ethanol, or butanol at high rates.

13.2.1 The Native Strategy of CBP

The native strategy of CBP makes use of the microorganisms with inherent capability to decompose insoluble lignocellulosic biomass to fermentable monosaccharides (Olson et al. 2011). Several thermophilic strains, such as *Thermoanaerobacter* sp., *Caldicellulosiruptor* sp., *Thermoanaerobacterium saccharolyticum*, and *Geobacillus thermoglucosidasius* have been shown to utilize a broad range of lignocellulosic substrates to produce biofuel with high yield through an anaerobic process which is favorable for CBP (Cao et al. 2014; Talluri et al. 2013; Cha et al. 2013; Bhalla et al. 2013; Argyros et al. 2011; Shaw et al. 2008).

Thermophiles are capable of producing a range of extracellular enzymes and can hydrolyze a broad range of different organic substrates. They can yield high rates of production in a relatively short period when compared with traditional mesophilic fermentation process. Thermophilic microbial strains also possess versatile metabolic pathways which are advantageous for utilizing a wide range of lignocellulosic biomass and waste to produce biohydrogen and other value-added products by anaerobic CBP (Bhalla et al. 2013). Table 13.1 summarizes the different native consolidated extremophilic bioprocesses that are documented in the literature.

Thermophilic anaerobic bacterium *Clostridium thermocellum* can naturally hydrolyze lignocellulosic substrates and utilize the hydrolysates primarily to produce bioethanol and biohydrogen in one single-step conversion. *C. thermocellum* is suitable for CBP since it performs fermentation using lignocellulosic substrate to produce bioethanol and acetic acid as major final products. Although *C. thermocellum* can hardly utilize pentose sugar including xylose from degradation of hemicellulose, CBP can still be carried out with co-cultures of *C. thermocellum*, *C. thermosaccharolyticum*, and *Thermoanaerobacter ethanolicus* (Harish et al. 2010), which makes the whole process promising for continuous and efficient lignocellulose hydrolysis, monosaccharides utilization, and bioethanol production . Ethanol production through co-cultures coupling *C. thermocellum* with *C. themosacharolyticum* using banana waste could attain up to 0.41 g ethanol/g substrate, which is relatively higher compared with that of the single wild type of *C. thermocellum*, which reached 0.08–0.37 g ethanol/g substrate (Harish et al. 2010). The use of thermophilic co-cultures for simultaneous lignocellulosic material hydrolyzation and simple sugar utilization can be a preferred strategy, especially when the whole CBP for biofuel production cannot be accomplished by a single strain.

Caldicellulosiruptor saccharolyticus, an anaerobic and thermophilic strain, can be considered as an ideal candidate for biohydrogen production using CBP as they can efficiently hydrolyze cellulose. This strain could degrade untreated switchgrass

Table 13.1 Consolidated bioprocessing by native extremophiles

Organism	Substrates	Biofuel products	Yield	Operating conditions	Reference
Clostridium thermocellum	Banana agro-waste	Ethanol	0.41 g/g substrate	60 °C	Harish et al. (2010)
Caldicellulosiruptor saccharolyticus	Switchgrass	Hydrogen	11.2 mmol/g substrate	65 °C	Talluri et al. (2013)
Thermoanaerobacterium thermosaccharolyticum	Microcrystalline cellulose, corn cob, corn stalk, and wheat straws	Hydrogen	10.86, 3.27, 3.47, and 3.53 mmol/g substrate	60 °C	Cao et al. (2014)
Clostridium thermocellum	Dried distillers grain, barley hulls, and fusarium head blight contaminated barley hulls	Hydrogen	1.27, 1.24, and 1.18 mmol/g glucose equivalent utilized	60 °C	Magnusson et al. (2008)
Caldicellulosiruptor saccharolyticus	Wheat straw	Hydrogen	3.8 mol/mol glucose equivalent consumed	70 °C	Ivanova et al. (2009)

and micro-crystalline cellulose and produce biohydrogen (23.2 mmol/L) using hydrolysates in a single step (Talluri et al. 2013). Combined saccharification and fermentation for biohydrogen production utilizing lignocellulosic substrate may be developed to provide a breakthrough that would provide a more effective process at lower cost.

Some other thermophilic microorganisms have also been reported as promising strains for CBP of biohydrogen production, due to their ability to degrade untreated woody biomass. *Caldicellulosiruptor saccharolyticus* is a gram-positive thermophilic anaerobic strain, which can utilize various substrates including lignocellulose, starch, pectin, pentose, and hexose for growth. The ability of this organism for simultaneous fermentation utilizing untreated lignocellulosic woody biomass represents an excellent feature of this bacterium for biohydrogen production through CBP (Ivanova et al. 2009).

Clostridium thermocellum, a gram-positive, acetogenic, cellulolytic, thermophilic bacterium, was shown to synthesize a series of cellulolytic enzymes and form cellulosome, which is critical for the bacteria to attach to lignocellulosic feedstock and hydrolyze the polysaccharides to simple sugars such as glucose, which can be utilized for biohydrogen production (Magnusson et al. 2008). Therefore *C. thermocellum*, which represents a group of thermophilic strains producing cellulosome, provides the potential for directly producing biohydrogen through CBP from lignocellulosic materials.

Clostridium sp., a gram-positive anaerobic bacterium, is widely used for biobutanol production, and this process is usually considered as acetone–butanol–ethanol fermentation (ABE) since the strains can produce acetone, butanol, and ethanol simultaneously in the ratio of 3:6:1 using a series of carbohydrate feedstock including polysaccharides and lignocellulosic substrates. Several reports are available on the production of biobutanol from hydrolysates of lignocellulosic materials as carbon source; however, the concentration of hydrolysates is limited and can inhibit cell growth and butanol yield. Meanwhile, inhibition from the final product (butanol) is another issue in the ABE process. CBP can overcome this drawback by removing the accumulated butanol immediately in the continuous process. As the by-products during butanol fermentation—acetone and ethanol—account for 40% of the final products, attempts to decrease the by-products during butanol production through metabolic engineering techniques can benefit from the CBP process.

13.2.2 The Recombinant Strategy of CBP

In a recombinant CBP strategy, a recombinant strain is developed with capacities including efficient depolymerization of lignocellulosic materials, a wide range of utilization of different kinds of monosaccharides, and tolerance for toxic compounds from hydrolysates and metabolites during CBP. This strategy attempts to develop a recombinant microorganism for degrading lignocellulosic substrates using a heterogeneous genome to overexpress lignocellulolytic enzymes towards production of biofuels and value-added products (Olson et al. 2011). Therefore, one of the most

challenging steps for recombinant strains is the expression of heterogeneous enzymes with sufficient amount and activity. The cellulases for hydrolysis of lignocellulosic substrates can be either extracellular enzymes or surface binding enzymes as cellulosomes. However, developing the strains for the synthesis of extracellular enzymes will be an advantage. The extracellular release of enzymes will help to overcome the mass transfer limitations and will help in increasing the rates of reaction/product.

Until now more than 400 different genes related to the hydrolysis of lignocellulosic woody biomass have already been reported in the literature (Parisutham et al. 2014). Multiple genes may be required in order to express and synthesize an enzyme system for efficient degradation of lignocellulosic biomass. The gene of β-glucosidase can also be transferred into recombinant strains to lessen the effect of feedback inhibition on cellulase by cellobiose during hydrolysis of lignocellulosic substrate. The activities of different enzymes need to be controlled to avoid feedback inhibition. For example, the activity of β-glucosidase for cellobiose degradation in the recombinant strain should be higher than those of the enzymes for glucose metabolism in order to decrease the cellobiose accumulated during CBP (Martinez et al. 2008). The composition of cellulose, hemicellulose, and lignin in lignocellulosic materials varies with different sources. The ideal recombinant strains for CBP should be able to hydrolyze them irrespective of their varying ratios, and it requires the strains to express different kinds of enzymes for degrading variable lignocellulosic substrates.

Besides the transfer and expression of heterogeneous genes for lignocellulolytic enzymes, the recombinant strains should possess an efficient protein secreting system in order to produce sufficient amounts of enzymes with higher activities for degradation of lignocellulosic substrates. On the other hand, attempts can also be made to engineer the enzymes with higher catalytic rates in order to improve the overall efficiency of the process. One native thermophilic and cellulolytic strain *Clostridium thermocellum* can be considered as a promising candidate for CBP of biobutanol production, due to the high-level conversion of substrate, low risk of contamination, and improved product recovery (Higashide et al. 2011).

Metabolic engineering techniques can be used in certain microorganisms which have already been shown to be promising as native biofuel producing strains in CBP for improving efficient process and decreased by-products. A pyrF-based genetic system in *Clostridium thermocellum* has been developed by deleting pyrF gene through metabolic engineering for making targeted gene knockouts which could not produce acetate, and can rapidly solubilize cellulose during biofuel fermentation (Tripathi et al. 2010). Some other genes of crucial enzymes that are responsible for the production of undesired by-products during CBP have been deleted, such as the genes encoded lactate dehydrogenase (ldh) and phosphotransacetylase (pta). The genetic system modification provides a method to build engineered CBP strains with high yield of bioethanol compared with the wild-type strain. The developed Δldh and Δpta mutant of *Clostridium thermocellum* exhibited a high ethanol selectivity of 40:1 ethanol and has 4.2 times higher ethanol yield when compared with the wild-type strain. (Argyros et al. 2011).

Metabolic engineering can be carried out for minimizing certain by-products. Gene knock out is an ideal way for developing higher yield of cellulosic biofuels by deleting the genes responsible for unrelated products. *Clostridium cellulolyticum*, a mesophilic strain, was modified as metabolically engineered strain for CBP to produce isobutanol from cellulosic substrates through directing the conversion pathway of pyruvate to isobutanol (Higashide et al. 2011). The thermophilic strain *Anaerocellum thermophilum* is a strictly anaerobic bacterium which grows optimally at 75 °C, and this strain is able to utilize different substrates such as crystalline cellulose and untreated plant biomass to produce hydrogen and organic acids as final products (Kataeva et al. 2009). This strain is a good source for different thermostable lignocellulolytic enzymes. The genes for these enzymes can be isolated, cloned, and expressed in other genetically engineered strains for CBP.

Thermostable endo-xylanase from a thermophilic strain was cloned and expressed heterologously in *E. coli*, and the recombinant enzymes exhibited high specific activity of 461 U/mg of protein at 70 °C on xylan. (Bhalla et al. 2014a). The thermostable hemicellulases can be promising for CBP processes in the biofuel industry. In addition, the use of high temperature in thermophilic CBP helps in increasing the mass transfer and decreasing the viscosity, leading to enhanced solubility of substrates and products, thereby contributing to better performance of the bioprocess and biofuel production. Depending on the improved thermostability at relatively high temperature, the hydrolysis performance of recombinant endo-xylanase and β-xylosidase can be improved for thermophilic CBP. The recombinant lignocellulolytic enzymes have a longer active life under higher temperature, broader active pH range, broad substrate specificity, high specific activity, and thermostability, which significantly favor commercial applications in CBP.

Xylanolytic enzymes are required for complete degradation of hemicellulose in lignocellulosic biomass. Among the different xylanolytic enzymes, β-xylosidase is responsible for hydrolyzation of xylo-oligosaccharides to xylose as monosaccharide. An active β-xylosidase can also lessen the end-product inhibition issues for xylanases and cellulases raised by xylose oligomers. Endo-xylanases and β-xylosidases are both hemicellulases which aid in the conversion of the xylan fraction in lignocellulosic biomass and enhance the performance of cellulases. The enzyme cocktail of endo-xylanase, β-xylosidase, and cellulase has been shown to have a synergistic effect on lignocellulose hydrolyzation, generating fermentable monosaccharides (Bhalla et al. 2014b) (Table 13.2).

13.2.3 Integrated Biofuel Production and Fuel Cell/Microbial Fuel Cell

Fuel cells are electrochemical energy devices that convert chemical energy into electrical energy. Unlike batteries, fuel cells require continuous sources of fuels (e.g., electron donor and electron acceptor) to carry out the reaction in order to generate electricity (Edwards et al. 2008; Winter and Brodd 2004). Fuel cells are

Table 13.2 Consolidated bioprocessing by recombinant extremophiles

Organism	Substrates	Biofuel products	Yield	Operating conditions	Recombinant strategy	Reference
Clostridium thermocellum and *Thermoanaerobacterium saccharolyticum*	Avicel	Ethanol	38.1 g/L	55 °C	Deletion of lactate dehydrogenase and phosphotransacetylase	Argyros et al. (2011)
Thermoanaerobacterium saccharolyticum	Xylan	Ethanol	37 g/L	50 °C	Knockout of genes involved in organic acid formation (acetate kinase, phosphate acetyltransferase, and L-lactate dehydrogenase)	Shaw et al. (2011)
Caldicellulosiruptor bescii	Switchgrass	Hydrogen	Around 23 mmol/L	75 °C	Deletion of the L-lactate dehydrogenase gene	Cha et al. (2013)
Clostridium cellulolyticum	Crystalline cellulose	Isobutanol	660 mg/L	34 °C	Expressing enzymes that convert pyruvate to isobutanol by using an engineered valine biosynthesis pathway	Higashide et al. (2011)

promising strategies for direct transformation of the biofuels produced from CBP to applicable energy.

Fuel cells consist of anode, cathode, and electrolyte in which, for instance, hydrogen, methane, ethanol, sugars, and acetate produced from CBP can be used as electron donors, and carbon dioxide produced in CBP can be used as electron acceptors. The oxidation of the hydrogen produces electrons which travel across the external circuit to reach the cathode, where they reduce the electron acceptor, thereby producing electricity. The electrons flow through the external circuit and produce DC current. The CBP process can be integrated to improve the overall performance of the system, which includes higher yield of products, minimal wastes, and lower cost of the products. The use of crude wastes produced from CBP in an electro-chemical system will decrease the power output and may have greater chance of fouling/damaging the membrane electrode assemblies. Under those conditions, the wastes that are obtained through CBP should be processed before feeding them to fuel cells as sources of electron donors/acceptors.

Microbial fuel cells (MFCs) are similar to chemical fuel cells, but differ in the way they make use of the metabolic machinery of microorganisms for bioelectricity generation. The fuels and the electrode surfaces in a MFC must be nontoxic to microorganisms. The microorganisms act as a whole cell bioelectrocatalyst which can be used to oxidize the electron donor or reduce the electron acceptor at the anode or cathode, respectively. Methane and hydrogen can be oxidized in the anode whereas carbon dioxide can be reduced at the cathodic side of the MFC using suitable microorganisms (Pham et al. 2006; Rabaey and Verstraete 2005; Du et al. 2007). In most cases, the microorganisms that are used in MFCs as electrocatalysts are consortia from waste water (Logan et al. 2006). However, the use of extremophiles will be advantageous for developing MFCs which utilize the waste from CBP. MFCs have already been used for hydrogen production using a microbial electrolysis cell (Wang et al. 2011). For integrated bioprocessing, MFCs can be combined with a CBP reactor wherein the wastes from CBP can be used as fuels for MFC and the electrical energy generated from the MFC can be utilized for operating/supplementing the CBP process. Reports are also available on the computational molecular approaches for screening the electroactive microorganisms for electrocatalytic activity and cellulolytic activity (Krishnaraj et al. 2014a, 2017; Dodda et al. 2016; Krishnaraj and Pal 2017). These techniques will be useful for identifying suitable microorganisms for MFCs in the developed CBP process.

Different configurations of MFC have been reported in the literature for the production of bioelectricity with different substrates such as lignocellulosic biomass, glucose, ethanol, acetate, winery effluent, food industry effluent, and tannery effluent (Bhuvaneswari et al. 2013; Krishnaraj et al. 2014b, 2015; Rajeswari et al. 2016). Reports have been made on the different electrode functionalization strategies that allow the growth and proliferation of the microorganisms on the electrode surface and contribute to better substrate utilization and higher power output (Krishnaraj et al. 2013; Karthikeyan et al. 2016). Attempts have also been made to develop economical proton exchange membranes using polyvinyl alcohol, polyvinyldine

chloride, and polystyrene sulfonic acid for fuel cell applications, which would help to cut down the costs of installation by replacing the Nafion (Bella et al. 2016).

13.3 Integrated CBP Utilizing Organic Waste and Unhydrolyzed Fraction

The organic wastes from CBP mainly contain organic acids acetate, propionate, lactate, and butyrate (Lin and Tanaka 2006), which are valuable products and can be further processed through secondary bioprocesses. Due to the unutilized lignocellulosic fractions and the organic acids generated from upstream CBP, opportunities exist to transform these waste streams to biogas and other value-added products in order to maximize the value of these streams. Meanwhile, the remaining nutrients and water can also be recycled. The organic wastes obtained from CBP can be used as organic feedstocks to produce biopolymers, short-chain carbohydrates, and methane. The microbial strains that are used for integrated CBP should be able to breakdown the complex and variable substrates and must overcome substrate/product inhibitions as well. Several biological processes can be integrated with the production of organic acids from lignocellulosic substrates through CBP, such as reduction of carboxylates to certain alcohols, and biological elongation of short-chain carboxylates to longer chain biopolymers (Agler et al. 2011). In this section, the other bioprocesses in the integrated CBP system will be reviewed, including methane generation through the TAD process, biopolymers and 1,3-PDO production through aerobic or anaerobic fermentation, and biodiesel production.

13.3.1 Biopolymers

13.3.1.1 Exopolysaccharides

Exopolysaccharides (EPSs) are high molecular weight carbohydrate biopolymers that are composed of sugar residues and are secreted by microorganisms into the surrounding environment (Nicolaus et al. 2010; Poli et al. 2010). Microorganisms can synthesize a wide variety of EPSs that serve a broad and diverse range of functions, such as intercellular signal transmission, molecular recognition, protection against predation, construction of a comfortable extracellular environment, pathogenic process, and so on (Nicolaus et al. 1999; Moriello et al. 2003). A great number of microorganisms are able to produce EPSs and excrete them out of the cell either as soluble or insoluble polymers. The EPSs can attach to the cellular surface or exist in extracellular medium as glue with indefinite form. The industrial microbial EPSs such as xanthan, dextran, curdlan, gellan, and pullulan are usually generated by pathogenic mesophilic strains, and their production processes are costly. Lignocellulosic biomass resources are considered inexpensive feedstocks for EPS production. Extremophilic microorganisms can be advantageously harnessed for the production of EPSs as they can thrive in a wide range of conditions, possess robust

Table 13.3 EPS produced by extremophiles

Organism	Substrates	Yield	Operating conditions	Monosaccharide composition	Reference
Bacillus Licheniformis	Sucrose	366 mg/L	50 °C	Fructose, fucose, glucose, galactosamine, mannose	Spanò et al. (2013)
Halomonas sp.	Beet molasses	12.4 g/L	137.2 g/L NaCl	Fructose	Küçükaşik et al. (2011)
Pseudoalteromonas sp.	Lactose	5.25 g/L	15 °C	Glucose, arabinose, galactose, xylose,	Qin et al. (2007)
Cronobacter sakazakii	Sucrose	3.15 g/L	pH 10	Glucose, mannose, galactose, xylose, arabinose	Jain et al. (2012)

hydrolytic machinery for lignocellulose degradation, and can produce EPSs with unique characteristics, such as high thermostability (Nicolaus et al. 2010).

In order to survive in extreme conditions, extremophiles have to adapt to the hostile environments through unique mechanisms, and the biosynthesis of EPSs is one of the vital mechanisms in extremophiles which allow them to survive under harsh environments. As a kind of response and adaptation under extreme conditions, extremophiles produce EPSs to protect themselves to endure the extremes of temperature, pressure, acidity, alkalinity, or salinity. The aerobic extremophilic strains can synthesize lignocellulolytic enzymes which enhance the lignocellulose utilization and thereby enhance EPS production using the organic wastes and unhydrolyzed fractions from upstream CBP in biofuel production. Thus, the lignocellulolytic extremophiles can degrade the unutilized fractions from CBP and secrete EPSs. The cellulase activities of extremophiles need to be optimized during the EPS production, since the EPS production can influence bacterial cellulase activity (Öner 2013). To provide a value-added polymer bioproduct, EPS biosynthesis combined with integrated bioprocessing can be feasible through extremophilic process with significant lignocellulolytic activity (Table 13.3).

13.3.1.2 Polyhydroxyalkanoates (PHAs)

Polyhydroxyalkanoates (PHAs) are polyesters composed of hydroxyl fatty acids. They are synthesized by microorganisms and can be stored as lipid inclusions. Some extremophiles are able to produce and accumulate lipid inclusions containing PHAs which are utilized as intercellular carbon and energy sources (Poli et al. 2011). Being derived from renewable feedstock and having biodegradable and biocompatible properties, PHAs are attractive alternatives to petroleum-based plastics which have many deleterious effects on the environment. Halophilic strains including *Haloferax*, *Haloarcula*, *Natrialba*, *Haloterrigena*, *Halococcus*, *Haloquadratum, Halorubrum*, *Natronobacterium*, *Natronococcus, Halobacterium*, and *Halomonas* have been

shown as promising extremophilic sources for production of PHAs (Du et al. 2012; Poli et al. 2011).

The traditional PHA fermentation is costly due to the use of relatively expensive carbon source as feedstocks, which represents approximately 50% of the overall cost (Dietrich et al. 2013). Therefore, it is necessary to use inexpensive lignocellulosic substrates as carbon source for PHA production process. The unhydrolyzed fractions from upstream CBP of biofuels can be used for PHA synthesis. This will help to cut down the operation costs of the processes. To improve the physicochemical properties and meet a broad range of industrial needs, PHAs can be engineered with different monomer composition and molecular structures. However, the organic acids such as acetic acid are prone to inhibit the PHA synthesis, and the tolerance of PHA-producing strains for these kinds of inhibitors need to be considered before developing a process (Dietrich et al. 2013). The study of inhibitor tolerance and PHA production may identify ways to screen and improve PHA production using unhydrolyzed lignocellulosic material from CBP.

13.3.2 1,3-Propanediol (1,3-PDO)

1,3-Propanediol (1,3-PDO) is a valuable bifunctional molecule, which can be produced from renewable resources using microorganisms. Due to the presence of two hydroxyl groups, 1,3-PDO finds applications in the synthesis of polymers, such as polyesters and polyurethanes. The natural producers of 1,3-PDO are mainly bacteria such as *Klebsiella*, *Clostridia*, *Citrobacter*, *Enterobacter*, and *Lactobacilli* (Saxena et al. 2009; Nakamura and Whited 2003).

The by-product stream generated during CBP biofuel production can be utilized as feedstock for 1,3-PDO production, and the crude glycerol generated from biodiesel production can be utilized with the stream (Saxena et al. 2009). As a major by-product from the biodiesel industry, glycerol is an economical substrate for 1,3-PDO production. The surplus glycerol waste from biodiesel production can be used to produce 1,3-PDO (Rastogi et al. 2013).

To maximize the production of 1,3-propanediol, genetic engineering strategies such as fermentation through immobilized cells and two-stage fermentation can be used since these are applicable in achieving high conversion rate. Lama et al. (2017) reported a metabolic engineering approach for producing 1,3-propanediol (1,3-PDO) from glucose using *Klebsiella pneumoniae* J2B. Homologous overexpression of glycerol dehydratase and 1,3-PDO oxidoreductase from *Saccharomyces cerevisiae* were overexpressed, and disruption of glycerol oxidation pathways in *Klebsiella pneumoniae* J2B increased the production of 1,3-PDO with 0.27 and 0.52 mol/mol with glucose and glycerol, respectively. These strategies will aid in 1,3-PDO production from unutilized CBP hydrolysates. The coculture fermentation is also able to utilize mixtures of glycerol and other monosaccharides such as glucose as substrate for 1,3-PDO production. Co-fermentation improves the growth rate and yield of 1,3-PDO by suppressing the formation of other by-products.

13.3.3 Methane

13.3.3.1 Thermophilic Anaerobic Digestion (TAD)

Thermophilic anaerobic digestion (TAD) is a process engineered to decompose organic substrates using different thermophilic, anaerobic microorganisms to produce biogas having methane as a major component. The digestate from the TAD process is rich in nitrogen (Li et al. 2011; Weiland 2010), which can be fed back to CBP of biofuel as a nitrogen source. Agricultural wastes, food wastes, and wastewater sludge can be used as the feedstock for the TAD process. The chemical oxygen demand (COD) and biological oxygen demand (BOD) of these kinds of wastes can be reduced through TAD (Li et al. 2011). TAD has been considered as a developed technology for waste-to-energy conversion, and during the TAD process the methane is released directly without any downstream separation steps.

TAD can be separated into two types: solid-state TAD and liquid-state TAD. In solid-state TAD, the solid content of the feedstocks is usually no less than 15% (Li et al. 2011; Weiland 2010). One of the advantages of solid-state TAD is that the residues of the process can be further fed back as feedstocks for CBP of biofuels. During TAD, the unhydrolyzed fractions from CBP can be continuously degraded into simple soluble molecules with the aid of microorganisms or extracellular enzymes. Consortia of microorganisms are broadly used for the TAD process which makes it a synergistic process, and the members of the consortia for TAD usually include lignocellulolytic bacteria, acetogenic bacteria, and methanogenic bacteria. For the TAD process, a wide range of organic solid wastes with varying compositions and characteristics can be used as feedstocks, and the thermophilic conditions accelerate the anaerobic digestion process and increase the yield of biogas.

The methanogenic consortia for TAD utilize the acetate produced by acetogenic bacteria to generate methane as the final product. Therefore, the organic wastes from CBP can be further utilized as feedstock for the TAD process for methane production, such that TAD will not be limited by the hydrolysis of lignocellulosic substrates. The growth rate of thermophilic consortia is significantly higher than those in mesophilic processes, which makes TAD more efficient and economical. Thermophilic processes for methane production also require a smaller reactor volume and shorter hydraulic retention time. Meanwhile, an optimum carbon/nitrogen ratio in the feedstock can avoid the accumulation of acetate and improve the methane yield and stability of the TAD process. The optimum pH value for TAD usually ranges from 7.0 to 8.0, and it needs to be prudently controlled in order to avoid inhibition for methanogens. The proper amount of trace elements is also necessary for the growth of methanogenic consortia (Weiland 2010). The energy required for maintaining thermophilic temperature during TAD is not extremely energy intensive, since the heat can be provided by methane itself and the biofuels generated through upstream CBP. The water can be recycled after TAD and fed back for CBP. For a highly efficient TAD process, the mixing and process control also need to be optimized. Additionally, a well-developed

and stable microbial consortia is crucial for methane production through the TAD process since they can influence the final yield of methane.

13.3.3.2 Biological Oxidation of Methane to Methanol Using Methanotrophs

Methane is a gas at normal atmospheric temperature and pressure which makes it expensive for storage and transportation. Hence, production of liquid fuels from renewable substrates is advantageous over gaseous fuels. In addition, methane is a strong GHG, having an effect that is 23 times stronger than carbon dioxide (Weiland 2010). Given that current industrial production of methanol is energy intensive and is not environmentally benign, the development of CBP to convert methane produced from the TAD process offers important advantages.

Methane can be oxidized to methanol through a biological process using methanotrophic bacteria or methane monooxygenase with high selectivity under mild conditions (Park and Lee 2013). However, based on the current literature, the conversion rate of methane to methanol is not high enough for industrial application. Methanotrophs are capable of utilizing methane as carbon and energy source through methane monooxygenase under aerobic conditions (Khoshtinat et al. 2010). Some of the methanotrophic strains thrive in extreme environmental conditions (Dedysh et al. 2000; Vorobev et al. 2011). Methanol is not the final product of the metabolism pathway of methanotrophic bacteria, and it can be further used as a precursor for further biosynthetic process of other metabolites. Methanol can be further oxidized to formaldehyde using methanol dehydrogenase, and formaldehyde can be subsequently oxidized to formate (Khoshtinat et al. 2010). Development of genetically or metabolically engineered methanotrophic strains is a way forward to attain high selectivity for the production of methanol.

Methane monooxygenase can be used for the conversion of methane to methanol directly. The engineered methane monooxygenase is required for enhanced and stable catalytic activity for a technically and economically applicable oxidation process. The methanol produced in this process can be further utilized as raw material for the production of biodiesel through transesterification in the CBP (Khoshtinat et al. 2010). It would be of significant value to combine the conversion of methane to methanol in the integrated CBP, producing methanol continuously using the methane obtained from the upstream TAD process.

The methanotrophic strains can also generate lipids through oxidation of methane (Blumenberg et al. 2004). There are a few reports on harnessing the methanotrophic communities for the production of lipids, but so far the lipids from methanotrophic strains have not been explored for industrial application. The specific lipid composition associated with certain consortia can be analyzed and recognized, so that these consortia-specific lipids can provide important information on the structure of microbial communities and the identification of methanotrophic bacteria (Jahnke et al. 1995). Both nonpolar lipids and polar lipids can be produced using methanotrophic strains. The methanotrophs also produce unique monounsaturated fatty acid that are suitable for biodiesel production. The lipid production through methanotrophic strains

can be combined with integrated CBP for biodiesel production along with the generation of methanol using methanotrophs.

13.3.4 Applications of the Bioproducts Obtained Through Integrated CBP

In order to commercialize bioproducts, e.g., EPSs, the cost of their production and downstream processing need to be optimized. Until now, microbial extremophilic EPSs have not been well explored for use as industrial biopolymers, although several reports have been made. Due to some superior properties, certain microbial EPSs are promising alternatives to traditional polysaccharides and have several advantages over a number of synthetic polymers. In the light of recent reports on bacterial EPSs with unique physiochemical properties, new commercial applications of the EPSs will be explored. Current high-value markets for EPSs with great potential include cosmetics, pharmaceuticals, and biomedical industries (Kumar et al. 2007; Freitas et al. 2011; Nicolaus et al. 2010).

PHAs can be degraded to carbon dioxide and water by several microorganisms. The materials made with PHAs are promising alternatives to replace traditional petroleum-based plastics (Du et al. 2012). PHAs can also be blended with other polymers or copolymerized, increasing their property range and providing a range of potential applications in packaging, drug delivery, and medical bio-implants. PHA-based copolymers also find application in the production of bulk chemicals such as heat-sensitive adhesives, latex, and smart gels and can be used as processing aids in plastics and textile manufacturing. PHAs are processed into fibers for various biomedical substrates and are used for developing controlled drug release matrices (Chen 2009).

1,3-PDO is also a value-added product which has great industrial demand. For example, it finds several applications in the polymer and cosmetic industries. It can be used as a monomer for synthesis of polyester and biodegradable plastics, and its properties can be modified in order to satisfy different requirements. Additionally, 1,3-PDO has been studied as an industrial biocide and preservative.

Biodiesel is biodegradable, nontoxic, sulfur-free, renewable, and can be produced from agricultural and woody wastes. Biodiesel can be produced from edible, nonedible, and waste vegetable oils. Transesterification is an important method to produce biodiesel from lipids. During the transesterification process, the biodiesel and triglycerides are used to produce glycerol and other alkyl esters (Agarwal 2007). The methanol, ethanol, and butanol obtained from integrated CBP can be used for transesterification to biodiesel. Besides the application as biofuels, the alcohols and methane produced during integrated CBP can be used to generate heat directly to maintain the temperature for thermophilic processes in CBP. The liquid fraction obtained from integrated CBP can be fed as a nitrogen source for various processes in integrated CBP in order to provide a no-waste bioprocess.

13.4 Modeling of CBP

To develop integrated CBP with higher reliability and efficiency, simulation software such as ASPEN Plus can be used for evaluating the economic performance of CBP through integrated flowsheet configuration. A combined framework can be generated through ASPEN Plus to simulate the integrated bioprocesses for biofuel production. The simulation strategy estimates several different parameters for biofuel production processes, and the parameters can be optimized through the model. In this way, ASPEN Plus can be used to design advanced reactors for the whole CBP process.

Traditional ethanol production through bioprocess needs an enzymatic pretreatment step of the lignocellulosic materials, which leads to increased cost for the commercialization of biofuel production process. The total cost can be estimated through ASPEN Plus software which includes the cost for commercial enzymes. In the case of bioethanol, companies such as Poet and DuPont have already reported that the cost of enzymes is approximately $0.50 per gallon of ethanol (Geddes et al. 2011). Therefore, utilization of CBP without the pretreatment step using supplementary lignocellulolytic enzymes provides a way to decrease the total cost of biofuel production significantly. The ASPEN modeling framework, developed by the National Renewable Energy Laboratory (NREL), has been used to simulate the cost of ethanol production through CBP and compare it with simultaneous saccharification and co-fermentation (SSCF) processes using dedicated lignocellulolytic enzymes (Lynd et al. 2005; Cardona and Sánchez 2007).

The cost of dedicated cellulase production and SSCF is ¢9.85/gallon ethanol and ¢8.98/gallon ethanol, respectively, for a total of ¢18.9/gallon ethanol. However, the total cost of CBP for bioethanol production is just ¢4.23/gallon ethanol, which means the cost of supplementary enzymes can be more than half the total cost of ethanol production through SSCF in order to maintain appropriate hydrolysis rate. Meanwhile, the reacting time required for SSCF is 7 days which is much longer than that of CBP (1.5–3 days). Without supplementary enzymes, the total cost of ethanol production through CBP is around one-fourth of the total cost of SSCF (Lynd et al. 2005). The ASPEN simulation can also be used to evaluate the processing cost of the biofuel production using switchgrass by CBP and electricity generation from the biofuel obtained (Laser et al. 2009). CBP has great potential to cut down costs as well as improve ethanol yield; additionally, the power required for CBP is considerably reduced compared with traditional bioconversion technology. For integrated CBP, the thermochemical process of biodiesel production and related heat and power plant required can be simulated by ASPEN Plus software (Wu et al. 2006). The choice of process technology, configuration, feedstock, and size of the reactors can be postulated using simulation software to provide detailed information for industrial application.

As a carbon-free energy carrier, biohydrogen is a fuel resource with zero emission. The ASPEN Plus simulation program can also be used to solve mass and energy balances for biohydrogen produced through CBP. The pH value of process

streams can be calculated using the data generated for ionic species through the ASPEN model, in order to manage the effects of recirculation on the osmolality of the fermentation broth. The parameters obtained through the model can be compared with the experimental results generated from large-scale fermentation of biohydrogen via CBP. The thermophilic fermentation of biohydrogen should be deployed in anaerobic conditions and at high temperature. In the simulation model, the integrated pathway of hydrogen biosynthesis can be simplified and described in the following reactions:

$$\text{For hexose } C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2CH_3COOH + 2CO_2$$
$$\text{For pentose } 3C_5H_{10}O_5 + 5H_2O \rightarrow 10H_2 + 5CH_3COOH + 5CO_2$$

The inhibition of hydrogen production from increasing hydrogen partial pressure also needs to be considered during the design of the configuration for simulation modeling. The effluent streams can be recycled to reduce the water and heat consumption of CBP. Cost analysis has already been carried out which demonstrates that, currently, the cost of biohydrogen production is still not competitive with traditional fossil fuels. Therefore, significant system improvements are necessary for biohydrogen production through CBP (Foglia et al. 2009, 2011a, b; Choi and Anh 2015; Yasin et al. 2013; Wukovits et al. 2007; Hay et al. 2013).

13.5 Conclusions and Future Perspectives

In order to remove commercial barriers and attain competitive yield and cost compared with traditional fossil fuels, a number of efforts have been made to improve CBP over the past several years. The establishment of economically feasible fermentation processes for biofuels requires the microorganisms to be in an integrated CBP, to attain high yield and a relatively low product inhibition effect. The strains should be able to utilize lignocellulosic substrates with considerable differences in compositions. Engineered cellulolytic-recombinant strains help to improve the performance of CBP. For other kinds of value-added bioproducts, it is important to identify suitable strains, especially extremophiles, to utilize the organic wastes and residues from the upstream steps with high biosynthetic rate and final yield of the products. Since most of the residues and unutilized fractions will be fed to the next steps or back into the CBP, the whole integrated CBP leads to minimal waste generation. For further improvement of the integrated CBP, novel extremophilic strains with desired properties need to be developed. In addition, various biotechnology strategies such as genetic engineering, metabolic engineering, mutagenesis, and protein engineering can be used for developing an integrated CBP in order to produce value-added bioproducts with improved yields and properties. In summary, due to the application of extremophiles and lignocellulosic substrates, integrated CBP can be economical and eco-friendly. In the near future, biofuels and other value-added bioproducts obtained from integrated CBP promise to be competitive alternatives to traditional fossil fuels and petroleum-based synthetic materials.

Take Home Message

- Biofuels are renewable, eco-friendly, and economical energy resources and are promising alternatives to traditional fossil fuels.
- The application of CBP with extremophiles using lignocellulosic substrates as feedstock can provide cost-effective production of biofuels, and pretreatment processing of lignocellulosic materials can be eliminated by using CBP with lignocellulolytic extremophiles.
- Integrated CBP is a sequential or continuous process to produce several kinds of gaseous and liquid biofuels and other value-added bioproducts using lignocellulosic substrates as primary feedstock. An integrated CBP can be developed by the combination of CBP for biofuels with several other bioprocesses for the production of biopolymers, 1,3-PDO, methane, and biodiesel.
- In integrated CBP, the liquid residues can be fed back to the upstream bioprocesses as nutrient, while the heat required for CBP can be generated directly from the biofuels obtained. The development of integrated CBP will be a feasible method for sustainable and efficient production of different kinds of biofuels and value-added bioproducts with minimal waste generated.
- Future research on integrated CBP will focus on removing commercial barriers by attaining competitive yields and costs compared with similar products from fossil fuels. The extremophilic microorganisms involved in integrated CBP should be able to degrade lignocellulosic substrates and utilize the organic wastes and residues from upstream steps with low inhibition effects and with high biosynthetic rate and final yield. The development of engineered lignocellulolytic recombinant strains is a worthwhile strategy to improve the performance of integrated CBP and apply the technology to current commercial needs.

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Chapter 14
Value-Added Products from Wastes Using Extremophiles in Biorefineries: Process Modeling, Simulation, and Optimization Tools

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List of Abbreviations

ATP	Adenosine triphosphate
CHP	Combined heat and power
CSTR	Continuously stirred tank reactor
GHG	Greenhouse gas
GWP	Global warming potential
LCA	Life cycle assessment
MFA	Metabolic flux analysis
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenosine dinucleotide phosphate
PHB	Poly-3-hydroxybutyrate
PLA	Polylactic acid
SDS	Sodium dodecyl sulfonate

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What Will You Learn from This Chapter?

- An overview of value-added production using extremophiles as well as the advantages and challenges for process development
- Process development using extremophiles for value-added products from wastes in a biorefinery concept
- A modeling framework that includes microorganism's metabolism, growth kinetics, and bioreactor models as well as process simulation
- Economic analysis and life cycle assessment and application to continuous production of poly-3-hydroxybutyrate (PHB) using the halophilic bacteria *Halomonas* sp.

14.1 Introduction

A biorefinery can be defined as a complex system for the sustainable processing of biomass by a systematic integration of physical, chemical, biochemical, and thermochemical processes to obtain a range of products such as chemicals, nutraceuticals, pharmaceuticals, polymers, and energy such as biofuels, heat, and power (Sadhukhan et al. 2014). By producing a mix of products through flexible and efficient processes, biorefineries have the potential to make biomass processing profitable. Biorefineries also represent a sustainable and low-pollution system alternative to petroleum processing. The projected market created throughout the entire value chain of biomass to value-added products in biorefineries is $295 billion by 2020 (King 2010). However, biorefineries have been developed based on simple biofuel plants producing bioethanol or biodiesel and low-value coproducts such as dried distillers' grains with solubles (DDGS) or glycerol. Current first-generation biofuel plants use mainly food crops as feedstock that create socioeconomic conflicts such as the food vs. fuel debate, and they still use fossil resources for their energy and auxiliary raw material requirements. Indeed, the Mexican government limits the use of food crops such as maize and cereals for bioethanol fuel production to avoid this food vs. fuel concern (Diario Oficial 2008). With increasing concerns on climate change, resource scarcity, and food poverty, a shift to lignocellulosic biomass processing is required (Aburto et al. 2008).

In order to improve economics and sustainability, a truly integrated biorefining approach that allows utilization of wastes for resource circularity is much needed (Satchatippavarn et al. 2016). The use of lignocellulosic residues has been widely explored in the recent years as second-generation feedstock using a biochemical platform. However, pretreatment, conversion, and downstream processes still need extensive efficiency improvements to make it more attractive, thus making biorefinery products economically, environmentally, and socially sustainable. Several approaches have been applied to improve the efficiency and the environmental sustainability of biofuel production. On one hand, genetic and metabolic engineering to improve microorganism's performance in converting lignocellulose into products

has been extensively explored in the recent years. On the other hand, process engineering tools have been developed for on-site energy and raw material supply and value-added production (Martinez-Hernandez et al. 2013b; Sadhukhan et al. 2014) as well as for economic and environmental assessment (Martinez-Hernandez et al. 2013a). Although the choice of microorganism and growing conditions will affect the ideal pretreatment and downstream processing, the two approaches are often carried out separately. Furthermore, current microorganisms are designed to grow in carefully controlled mild conditions that require more processing steps, which increase both capital and operating costs.

With the aim to address the challenges of processing wastes with minimum requirements and process steps and in a wide range of conditions, the use of extremophiles has been recently proposed (González-García et al. 2013; Bhalla et al. 2013; Bosma et al. 2013; Zambare et al. 2011; Ramírez et al. 2006). All these organisms grow at unusual and extreme conditions that prevent microbial contamination and may have some advantages for processing feedstocks and downstream recovery and purification of products. For example, extremophiles growing at high temperatures could be used for ethanol production at a temperature at which ethanol would evaporate, thus avoiding product inhibition and easing purification (Zambare et al. 2011). Another example of value-added product from extremophiles is the production of poly-3-hydroxybutyrate (PHB) using halophilic bacteria (Lorantfy et al. 2014; Garcia-Lillo and Rodriguez-Valera 1990). Halophilic bacteria grow at high-salinity conditions and lyse when salinity decreases, thus releasing PHB, which is an internal constituent of cell biomass. The exploration of the potential of extremophiles for chemical production has just emerged, and more studies are needed to achieve high conversion efficiency, productivities, and economic profitability. For several extremophiles, genetic and process engineering tools are already available, but these are mostly used for the optimization of ethanol production. Engineering organisms and processes for the conversion of wastes into chemicals would be an important next step. Interesting questions for designing processes using extremophiles are the implication for suitable product recovery and purification steps, equipment materials, energy consumption, and environmental impact.

This chapter presents a framework for modeling, simulation, and optimization tools for process development using extremophiles for sustainable production of value-added products from wastes in a biorefinery concept. This is crucial for developing extremophiles such as industrial platform organisms and optimizing biorefinery processes. This chapter starts with an overview of value-added production using extremophiles as well as the advantages and challenges for process development. Then, the various elements of the modeling framework include microorganism's metabolic model, growth kinetics and bioreactor model, process simulation, and economic and environmental impact analysis. These are illustrated with examples for PHB production using the halophilic bacteria *Halomonas* sp. (strain KM-1 recently studied by Jin et al. 2013). A biorefinery process scheme for extremophilic conversion of sugarcane bagasse into PHB is developed under the Mexican context. Furthermore, economic and life cycle analysis and implications for

whole process optimization are devised in order to guide future research on biorefinery process development based on extremophiles.

14.2 Extremophile Processing for Value-Added Production from Waste

Examples of some extremophile types and their implications for processing in terms of advantages and challenges are summarized in Table 14.1. Main value-added products being explored by using extremophiles include hydrogen, ethanol, lactic acid, ectoine, and poly-3-hydroxybutyrate (PHB) (Bosma et al. 2013).

Table 14.2 shows examples of extremophile processing of waste for value-added production. Lactic acid is a value-added chemical with a market demand of 500 kton and current production of 300–400 kton per year (NNFCC 2016). Lactic acid is the building block for polylactic acid (PLA) and other products. The conventional production process uses calcium hydroxide to neutralize the fermentation broth and precipitate calcium lactate. The acid is then recovered by acidification with sulfuric acid which produces gypsum ($CaSO_4$) which has low commercial value and needs to be disposed of. Development of microorganisms which can tolerate acidic conditions (lower pH) can reduce the unit cost of recovery and purification using an extraction process (Kumar et al. 2006). In this sense, acid-tolerant thermophilic

Table 14.1 Extremophile types and implications for the biorefinery process

Extremophile type	Example	Advantages	Challenge
Thermophilic	*Thermoanaerobacter* ($T > 70$ °C) for ethanol production (Georgieva et al. 2007)	Easier recovery of volatile products such as ethanol Lower product inhibition Sterilization may not be required and microbial contamination is avoided	Higher fermentation temperatures may imply higher utility consumption for heating and cooling
Acidophilic	Thermophilic *Acidophilic Bacillus* sp., pH = 5.0 and $T = 55$ °C for lactic acid production (Patel et al. 2005)	Lower neutralization agent required, thus less salt waste	Anticorrosive materials needed for equipment
Halophilic	*Halomonas* sp. and *Haloferax mediterranei* (salinity 3–15% weight/volume basis) for PHB production (Jin et al. 2013; Rathi et al. 2013)	Easier recovery of intracellular PHB product by osmotic lysis leads to less processing steps	Anticorrosive materials needed for equipment Salts may need to be discarded

Table 14.2 Examples of extremophile processing of waste for value-added production

Feedstock	Process and product	Microorganism and conditions	Reference
Glycerol	Fermentation for acetate and carotenoids	*Haloferax mediterranei*, 37 °C and hypersaline (min 10% weight and salt concentration)	Lorantfy et al. (2014)
Sugarcane bagasse	Simultaneous saccharification and fermentation (SSF) to produce L(+)-lactic acid	Thermophilic *Acidophilic Bacillus* sp. (36D1), pH = 5.0 and 55 °C	Patel et al. (2005)
Corn stover and prairie cord grass	Hydrolysis and consolidated bioprocessing to ethanol	*Geobacillus* sp. R7, 70 °C and 15–20% solid content	Zambare et al. (2011)
Oil palm empty fruit bunch	Fermentation to lactic acid	*Bacillus coagulans* JI12 50 °C	Ye et al. (2014)
Sugarcane bagasse	Fermentation to lactic acid	*Bacillus* sp., 50 °C and pH = 5	Patel et al. (2004)
Sugarcane molasses	Batch fermentation to lactic acid	*Lactobacillus delbrueckii*	Dumbrepatil et al. (2008)
Whey	Fermentation to PHB	*Haloferax mediterranei*	Koller et al. (2008)

bacteria such as *Bacillus coagulans* could help to produce L-lactic acid from lignocellulose sugars in a more environmentally friendly manner.

Halophilic bacteria *Haloferax mediterranei* was studied by Garcia-Lillo and Rodriguez-Valera (1990) for the production of poly-3-hydroxybutyrate (PHB). The minimum of 90 g L^{-1} of NaCl is required for growth and tolerance is up to 300 g L^{-1}. This strain accumulates PHB during exponential growth; thus, using one bioreactor stage was feasible for continuous production. Despite the advantages of halophilic bacteria for PHB production (e.g., solvent-free recovery and purification, can use inexpensive carbon source), several drawbacks exist. High salinity requires expensive bioreactor materials. Recycling of the salts and nutrients may be needed to avoid environmental problems due to waste disposal. In this chapter, the use of a recently studied strain of *Halomonas* sp. for a biorefinery concept producing PHB polymer is modeled and analyzed as follows.

14.3 Modeling a Biorefinery for PHB Production Based on Extremophile Processing

From a process systems engineering perspective, a biorefinery can be viewed as a multilayer as shown in the onion model in Fig. 14.1. This figure illustrates the various modeling and optimization tools and objectives depending on the layer. A holistic view is encouraged in order to develop any new biorefinery concept based on extremophile processing, and therefore in applying these tools, there should be

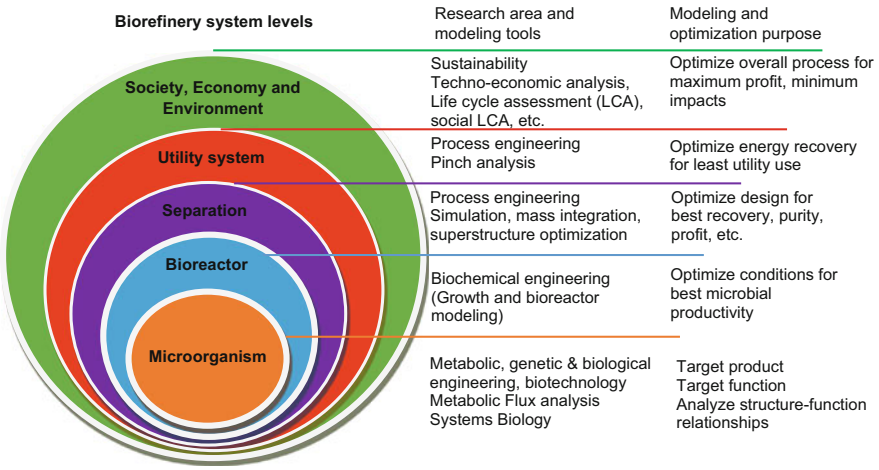


Fig. 14.1 Onion model of biorefinery system and the modeling tools and optimization purposes at each level

feedback between and across the various levels. The basic steps of the modeling framework illustrated in this book chapter are shown in Fig. 14.2. The framework offers a tool for accelerating biorefinery process development and is illustrated for PHB production. Modeling of PHB production has been widely studied in the literature (Koller et al. 2006; Koller and Muhr 2014) with special focus on bioreactor modeling but not the whole process and rarely using halophilic bacteria (Dotsch et al. 2008).

14.3.1 Metabolic Modeling: Microorganism's Cells as the Core of Biochemical Processes

A few decades ago, the reactions occurring at the cellular level in a bioreactor were traditionally taken somewhat as a black box due to the simplicity for parametrization and practicality for design. As such, the core of the process was simply the bioreactor, and only the macroscopic nature of this process unit was considered for modeling and optimization. With the advancement of metabolic and genetic engineering as well as computational capabilities, the approach is shifting toward viewing not the reactor vessel per se as the core of a biochemical process but the microorganism cells. It is in the cells where all the reactions that transform raw material into products actually occur, and, as such, the microorganism cells are the micro-reactors that need to be understood first to optimize a biochemical process. To this end, metabolic engineering has largely contributed with mathematical modeling of biochemical reaction pathways or networks within microorganism cells, thus

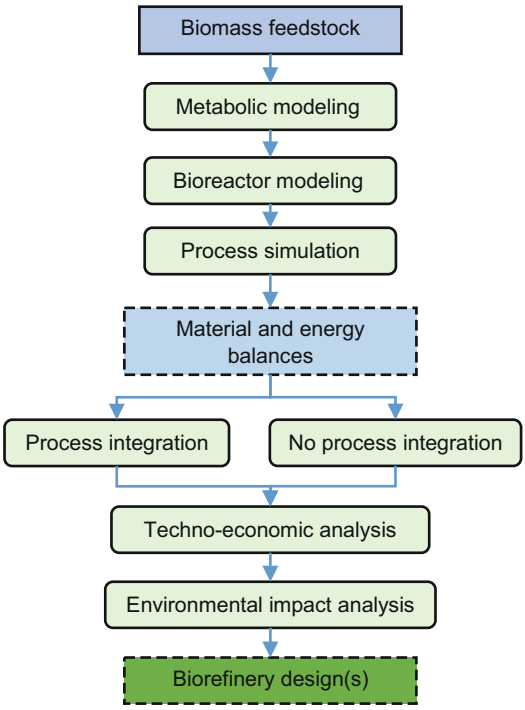


Fig. 14.2 Modeling framework for biorefinery process development

helping to understand the relation between metabolism, culture conditions, and the productivity.

The analysis of the reaction pathways leading to each of the metabolism products and biomass constituents is called metabolic flux analysis (MFA). MFA is a mathematical modeling tool used to target reactions, enzymes, and genes that enhance a desired product or inhibit an undesirable by-product (Xu et al. 2008). This information then is passed on to genetics which can then target enzymes, chromosomes, and genes to engineer a microorganism to deploy certain functionality. Another tool is the metabolic balance, used to determine macroscopic parameters used in the modeling of bioreactors, thus leading to a multiscale model. Metabolic modeling requires more details about what is going inside the cells that requires specialized experimental techniques. Furthermore, the modeling does not consider spatial and temporal variations that may occur in a bioreactor. However, MFA is becoming more common in developing a bioprocess and will definitely play a role in the biorefineries based on the biochemical platform using extremophiles.

The construction of a metabolic model was formalized by Stephanopoulos et al. (1998). The model illustrated here involves the representation of the stoichiometry and kinetics of metabolic reactions in matrices and solving the resulting equation system in order to find the macroscopic specific yield coefficients. The basic steps can be summarized and illustrated as follows for halophilic bacteria producing PHB from glucose.

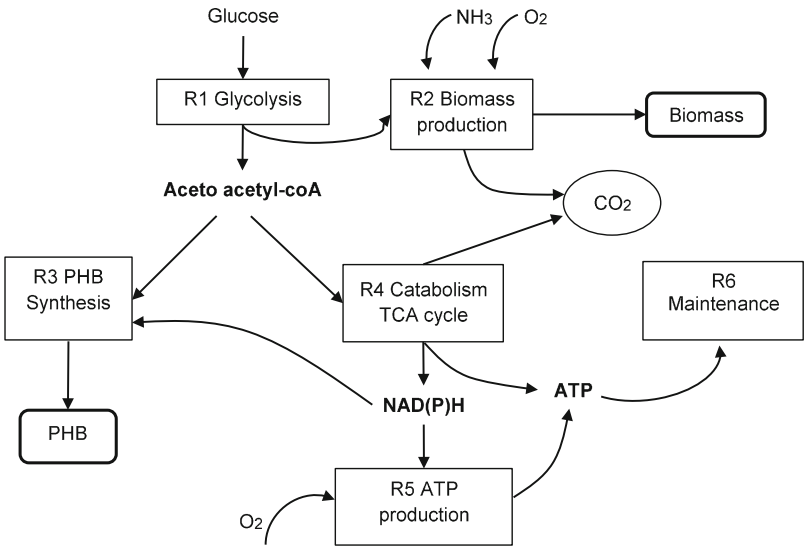


Fig. 14.3 Simplified metabolic model for PHB production from *Halomonas* sp.

1. Identify the reactions and chemical species participating (consumed or produced) in the metabolic pathways of interest.

The simplified metabolic pathway for PHB production in the halophilic bacteria *Halomonas* sp. KM-1 (Jin et al. 2013) is shown in Fig. 14.3. Four types of species can be distinguished: substrates, metabolic products, biomass constituents, and intracellular metabolites. The *substrate* is glucose which is the raw material to be metabolized by the glycolysis reaction pathway. During these and other internal reactions, intermediates and building blocks are produced as *intracellular metabolites*. Glycolysis produces the intermediate acetyl-CoA. This is further metabolized by two pathways: the tricarboxylic cycle (TCA) and the PHB synthesis pathway. The TCA cycle is a pathway to generate energy via NADH (nicotinamide adenine dinucleotide) and NADPH (nicotinamide adenosine dinucleotide phosphate) by aerobic organisms. From this cycle, *metabolic products* (e.g., succinate, acetate, and ethanol) are excreted to the culture medium by some microorganisms and can be recovered as products from the fermentation broth. PHB is rather a cell *biomass constituent* together with other macromolecules such as RNA, DNA, lipids, proteins, and carbohydrates. This means that the cells need to be broken down to recover PHB, and thus cells cannot be recycled as in other fermentation processes.

Jin et al. (2013) identified 53 metabolites for *Halomonas* sp. The authors found that accelerating the TCA cycle produces the NADPH required for the PHB synthesis pathway as a coenzyme for the conversion of acetoacetyl-CoA into (R)-3-hydroxybutyryl-CoA (3-HB-CoA). This 3-HB-CoA is then converted into PHB. There could be hundreds of reactions occurring during microbial growth, but it would not be possible to include all for a metabolic model, as solving the system would be computationally expensive. The discrimination of which reactions to

Table 14.3 Metabolic model for PHB production

Reaction	Reaction equation
R1 Glucose conversion to acetyl-CoA	$C_6H_{12}O_6 \rightarrow$ AcetylCoA + 2NAD(P)H + 0.5ATP + CO_2
R2 Biomass production (net reaction from glucose via acetyl-CoA)	$(1+a)C_6H_{12}O_6 + \frac{1}{5}NH_3 + \left(\frac{2a-1}{4}\right)O_2 \rightarrow CH_{1.74}O_{0.46}N_{0.19} + aCO_2$
R3 PHB synthesis from acetyl-CoA	2AcetylCoA + NAD(P)H + 0.5ATP $\rightarrow$ PHB
R4 Acetyl-CoA catabolism in the TCA cycle	AcetylCoA $\rightarrow$ 4NAD(P)H + ATP + $2CO_2$
R5 ATP production	NAD(P)H + $0.5O_2 \rightarrow \delta$ATP
R6 ATP use for maintenance (nongrowth associated)	$-$ATP $= 0$

include is assisted by considering what is called the *relaxation time* and comparing with the time of the macroscopic process. This is the time that a reaction would take to complete when approximated as a first-order reaction (Stephanopoulos et al. 1998). For example, any process happening at slower rate than cell growth in a fermentation process can be neglected (e.g., mutations). Another basic concept that helps to simplify the metabolic model construction is the pseudo-steady-state assumption for very fast reactions (e.g., enzymatic catalysis). Thus, by assuming steady state, the intermediates are considered not to accumulate as they are consumed and generated fast. For illustration purposes, only the reactions shown in Fig. 14.3 will be used.

2. Construct matrix of reaction stoichiometry. This involves first balancing the basic metabolic reactions and then representing the resulting algebraic equation systems in a matrix to find the missing stoichiometric coefficients.

Table 14.3 shows the six reactions in the simplified metabolic model. Note that the notation NAD(P)H means either NADH or NADPH can participate, and to further simplify the exercise, they are lumped in a single pool of reducing species (Stephanopoulos et al. 1998).

The chemical stoichiometry can be represented by:

$$AS + BP + CX + DM = 0 \tag{14.1}$$

where A contains the coefficients for the substrates, B those for products, C for biomass constituents, and D for intracellular metabolites. These matrices represent the J reactions in the rows and the species I in the columns so that the coefficient υ_{ij} in each element shows the coefficient of a species i in reaction j. The vectors S, P, X, and M represent the concentrations of the species. For the PHB production model, Eq. (14.1) can be expanded to:

$$\begin{bmatrix} -1 & 0 & 0 \\ -(1+a) & -\left(\frac{2a-1}{4}\right) & -0.2 \\ 0 & 0 & 0 \\ 0 & 0 & 0 \\ 0 & -0.5 & 0 \\ 0 & 0 & 0 \end{bmatrix} \begin{bmatrix} S_{\mathrm{G}} \\ S_{\mathrm{O2}} \\ S_{\mathrm{NH3}} \end{bmatrix} + \begin{bmatrix} 1 \\ a \\ 0 \\ 2 \\ 0 \\ 0 \end{bmatrix} [P_{\mathrm{CO2}}]$$
$$+ \begin{bmatrix} 0 & 0 \\ 1 & 0 \\ 0 & 1 \\ 0 & 0 \\ 0 & 0 \\ 0 & 0 \end{bmatrix} \begin{bmatrix} X_{\mathrm{B}} \\ X_{\mathrm{PHB}} \end{bmatrix} + \begin{bmatrix} 1 & 2 & 0.5 \\ 0 & 0 & 0 \\ -2 & -1 & -0.5 \\ -1 & 4 & 1 \\ 0 & -1 & \delta \\ 0 & 0 & -1 \end{bmatrix} \begin{bmatrix} M_{\mathrm{AcCoA}} \\ M_{\mathrm{NAD(P)H}} \\ M_{\mathrm{ATP}} \end{bmatrix}$$
$$= \begin{bmatrix} 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \end{bmatrix} \tag{14.2}$$

where concentrations of the three substrates are denoted by S_{G}, S_{O2}, and S_{NH3} for glucose, oxygen, and ammonia, respectively, those for CO_2 product by P_{CO2}, and the biomass constituents by X_{B} and X_{PHB} and the intracellular metabolites as M_{AcCoA}, $M_{\mathrm{NAD(P)H}}$, and M_{ATP}. By convention, υ_{ij} is negative for reactants and positive for products. Assuming that a and δ are known, there are a total of nine variables and six equations, resulting in a degree of freedom equal to three.

3. Formulate reaction rate expressions and apply the pseudo-steady-state assumption to intracellular metabolite production rates in order to have a fully determined algebraic equation system.

In this step, the production rates for each species are formulated from the reaction rates. This is to express production or consumption rates using reactions that can be easily measured experimentally. In general, the production rates of biomass, PHB, and CO_2 or consumption of glucose and other nutrients is measurable. The matrix R containing the reaction rates is related to each specie's production or consumption rate r_i by their respective coefficient matrix as:

$$R_i = K^T \times R \tag{14.3}$$

where R_i is a one column matrix containing the production rates of substrates r_{G}, r_{O2}, and r_{NH3}, metabolite products r_{CO2}, biomass constituents r_{B} and r_{PHB}, or intracellular metabolites r_{AcCoA}, $r_{\mathrm{NAD(P)H}}$, and r_{ATP}. The matrix R contains the rates r_1 to r_6 for the reactions 1–6 in the metabolic model (Table 14.3 in this example). K^T is the transpose matrix of the corresponding coefficient matrix (A, B, C, or D from Eq. 14.2). The pseudo-steady state is then applied for the intracellular metabolites,

so that their net consumption or production can be assumed equal to 0. For the matrix M Eq. (14.2) containing r_{AcCoA}, $r_{\mathrm{NAD(P)H}}$, and r_{ATP}, Eq. (14.3) gives:

$$\begin{bmatrix} r_{\mathrm{AcCoA}} \\ r_{\mathrm{NAD(P)H}} \\ r_{\mathrm{ATP}} \end{bmatrix} = \begin{bmatrix} 1 & 2 & 0.5 \\ 0 & 0 & 0 \\ -2 & -1 & -0.5 \\ -1 & 4 & 1 \\ 0 & -1 & \delta \\ 0 & 0 & -1 \end{bmatrix}^{\mathrm{T}} \times \begin{bmatrix} r_1 \\ r_2 \\ r_3 \\ r_4 \\ r_5 \\ r_6 \end{bmatrix} = \begin{bmatrix} 0 \\ 0 \\ 0 \end{bmatrix} \tag{14.4}$$

This results in the following expressions:

$$\begin{pmatrix} r_{\mathrm{AcCoA}} \\ r_{\mathrm{NADPH}} \\ r_{\mathrm{ATP}} \end{pmatrix} = \begin{pmatrix} r_1 - 2 \cdot r_3 - r_4 \\ 2 \cdot r_1 - r_3 + 4 \cdot r_4 - r_5 \\ 0.5 \cdot r_1 - 0.5 \cdot r_3 + r_4 + \delta \cdot r_5 - r_6 \end{pmatrix} = \begin{pmatrix} 0 \\ 0 \\ 0 \end{pmatrix} \tag{14.5}$$

Applying Eq. (14.3) to other species (this time without the steady-state assumption), the following expressions are obtained:

$$\begin{pmatrix} r_{\mathrm{G}} \\ r_{\mathrm{O2}} \\ r_{\mathrm{NH3}} \\ r_{\mathrm{CO2}} \\ r_{\mathrm{B}} \\ r_{\mathrm{PHB}} \end{pmatrix} = \begin{bmatrix} -r_1 - r_2 \cdot (1 + a) \\ -0.5 \cdot r_5 - r_2 \cdot \left(\dfrac{2a - 1}{4} \right) \\ -0.2 \cdot r_2 \\ r_1 + 2 \cdot r_4 + a \cdot r_2 \\ r_2 \\ r_3 \end{bmatrix} \tag{14.6}$$

ATP consumed for nongrowth associated maintenance can be correlated to the cell biomass by the specific maintenance factor m_{ATP} as:

$$r_6 = m_{\mathrm{ATP}} X_{\mathrm{B}} \tag{14.7}$$

Thus, the equation system is now completely determined, and Eqs. (14.6 and 14.7) can be combined with Eq. (14.5) to derive the expressions for the measurable reaction rates:

$$\begin{pmatrix} -r_{\mathrm{G}} \\ -r_{\mathrm{O2}} \\ r_{\mathrm{CO2}} \end{pmatrix} = \begin{bmatrix} \dfrac{0.667}{1 + 4\delta} \cdot m_{\mathrm{ATP}} \cdot X_{\mathrm{B}} + \dfrac{6.\delta + 1.667}{1 + 4\delta} \cdot r_{\mathrm{PHB}} + \dfrac{a + 4\delta + 4a\delta + 1}{1 + 4\delta} \cdot r_{\mathrm{B}} \\ \dfrac{2 m_{\mathrm{ATP}} \cdot X_{\mathrm{B}}}{1 + 4\delta} + \dfrac{0.5 \cdot r_{\mathrm{PHB}}}{1 + 4\delta} + \dfrac{(2a - 1) \cdot r_{\mathrm{B}}}{4} \\ \dfrac{2.667}{1 + 4\delta} \cdot m_{\mathrm{ATP}} \cdot X_{\mathrm{B}} + \dfrac{8.\delta + 2.667}{1 + 4\delta} \cdot r_{\mathrm{PHB}} + a \cdot r_{\mathrm{B}} \end{bmatrix} \tag{14.8}$$

4. Find yield coefficients. The yield coefficients are the ratio of mass of species i formed per mass of species i' consumed. For example, the yield coefficients are related to the specific substrate consumption rate (q_S) using the Herbert-Pirt equation

for substrate distribution toward specific growth (μ), specific product formation (q_P), and maintenance (m_s):

$$-q_S = m_S + \frac{q_P}{Y_{\mathrm{PS}}^{\max}} + \frac{\mu}{Y_{\mathrm{XS}}^{\max}} \tag{14.9}$$

From Eq. (14.8), the specific rates for glucose, oxygen, and CO_2 can be obtained by dividing the biomass concentration X_{B}, resulting in the following Herbert-Pirt relationships:

$$\begin{pmatrix} -q_{\mathrm{G}} \\ -q_{\mathrm{O2}} \\ q_{\mathrm{CO2}} \end{pmatrix} = \begin{bmatrix} \frac{0.667}{1+4\delta} \cdot m_{\mathrm{ATP}} + \frac{6.\delta + 1.667}{1+4\delta} \cdot q_{\mathrm{PHB}} + \frac{a + 4\delta + 4a\delta + 1}{1+4\delta} \cdot \mu_{\mathrm{B}} \\ \frac{2m_{\mathrm{ATP}}}{1+4\delta} + \frac{0.5 \cdot q_{\mathrm{PHB}}}{1+4\delta} + \frac{(2a-1) \cdot \mu_{\mathrm{B}}}{4} \\ \frac{2.667}{1+4\delta} \cdot m_{\mathrm{ATP}} + \frac{8.\delta + 2.667}{1+4\delta} \cdot q_{\mathrm{PHB}} + a \cdot r_{\mathrm{B}} \end{bmatrix} \tag{14.10}$$

By inspection of the general form in Eq. (14.9) and the first expression in Eq. (14.10), the maximum theoretical yields of biomass and PHB from glucose are:

$$Y_{\mathrm{XS}}^{\max} = (1 + 4\delta)/(a + 4\delta + 4a\delta + 1) \tag{14.11}$$

$$Y_{\mathrm{PS}}^{\max} = (1 + 4\delta)/(6\delta + 1.667) \tag{14.12}$$

Assuming $\delta = 3$, the values are $Y_{\mathrm{XS}}^{\max} = 0.5$ g g^{-1} and $Y_{\mathrm{PS}}^{\max} = 0.66$ g g^{-1}. These values can then be compared with those reported in the literature but may need to be fine-tuned using experimentation and macroscopic bioreactor models. These yield coefficients will appear when the development of mass balances in bioreactors as shown later. The next sections of the modeling framework are based on the materials presented by Sadhukhan et al. (2014) for biorefinery process design, integration, and sustainability.

14.3.2 Growth Kinetics and Bioreactor Modeling

Kinetic models allow the prediction of microorganism's performance and serve as the basis for bioreactor modeling, design, and scale-up. Kinetic modeling involves experimentation from small-scale reactor experimentations in order to obtain model parameters. The dynamics of microorganism growth comprises a lag phase, an exponential phase, a stationary phase, and a death phase. Different products may be predominant at different stages. For example, PHB accumulation occurs mainly in the stationary phase, while the growth of microorganism cells occurs mainly in the exponential phase. Thus, a strategy used in PHB production is a two-stage reaction where growth happens in the first reactor and PHB accumulation in the second

reactor. This system potentially improves substrate conversion, yield, and also PHB content in the cell biomass, thus improving productivity.

14.3.2.1 Specific Growth Constant and Monod Equation

The kinetic parameters are usually obtained from the exponential phase of growth during batch experiments. In a batch reactor, there is no continuous input or output flows. If growth inhibition conditions are avoided during experimentation and mortality and maintenance are neglected, as they are much slower than growth in the exponential phase, then the rate of cell biomass growth during the exponential phase can be written as:

$$\frac{dX}{dt} = \mu X \tag{14.13}$$

where X is the total cell biomass concentration (including PHB accumulated inside the cells), μ is the specific growth rate as introduced in the previous section, and t is time. Integration of Eq. (14.13) from concentration X_1 at $t = t_1$ and X_2 at time t_2 results in the following form:

$$\ln(X_2) = \mu t + \ln(X_1) \tag{14.14}$$

Thus, it is possible to determine the value of μ from the slope of the line obtained by plotting the values of the logarithm vs. time.

Growth data extracted for *Halomonas* sp. KM-1 is shown in Table 14.4. The values of μ can be graphically determined from the linear fit shown in Fig. 14.4 as $\mu_1 = 0.2245\ h^{-1}$ and $\mu_2 = 0.2316\ h^{-1}$ for the initial glucose concentrations of 50 and 100 g L^{-1}, respectively.

Biomass growth is affected by several factors such as temperature, product, and substrate inhibition. The most common model for microorganism's growth used to capture such effects is the Monod kinetic equation. When describing the growth of a single culture limited by substrate concentration S, the Monod equation is written as:

$$\mu = \frac{\mu_{\max,S}\, S}{K_S + S} \tag{14.15}$$

Table 14.4 Growth data for *Halomonas* sp. KM-1 (Jin et al. 2013) at two different initial substrate concentrations

S1 = 50 g L^{-1}			S2 = 100 g L^{-1}		
Time (h)	X	S	Time (h)	X	S
10	3.5	35.89	12	3.559	83.47
12	4.26	32.78	14	5.69	70.29
14	7.7	25.1	15	8.03	64.44
15.2	10.9	21.99	18	14.58	44.07

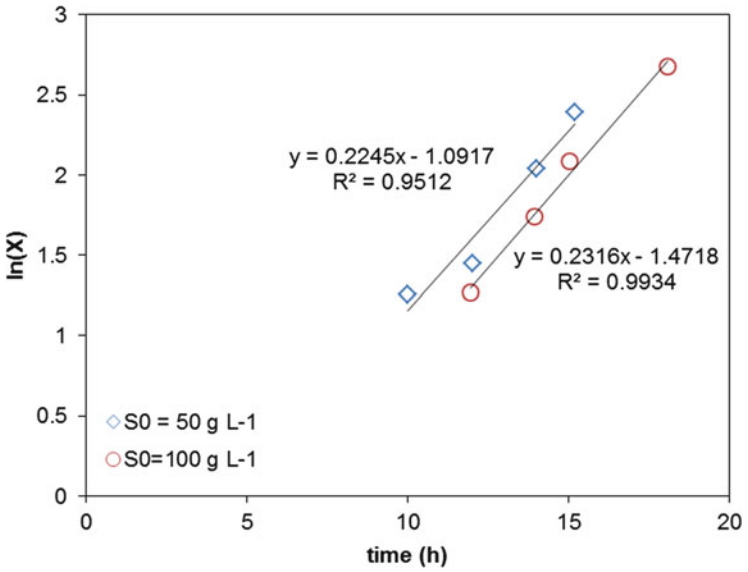


Fig. 14.4 Plot of ln(X) vs. time for two different initial concentrations (blue diamond: 50 g L^{-1}, red circle: 100 g L^{-1}) to find the specific growth rate of *Halomonas* sp. KM-1

where $\mu_{\max,S}$ is the maximum achievable growth rate and h^{-1} under the influence of substrate concentration. K_S is the saturation constant. This assumes that all the other factors are in optimum conditions. The parameters $\mu_{\max}$ and K_S can be estimated from the specific growth rate as a function of substrate concentrations. From Table 14.4, two data points are available for different initial substrate concentrations (S1 and S2). Therefore, two Monod equations can be formulated using Eq. (14.15). From the known values previously found for μ_1 and μ_2, such equations can be solved to obtain $\mu_{\max,S} = 0.24\ h^{-1}$ and $K_S = 3.27\ g\ L^{-1}$. These values are comparable with values reported in the literature for other halophilic bacteria (e.g., $\mu_{\max,S} = 0.39$ and $K_S = 2.98$ for *H. mediterranei* (Koller et al. 2006).

When more data points are available, a better estimate could be obtained by linearizing Eq. (14.15). The resulting equation is the well-known Lineweaver-Burk equation, which allows obtaining $\mu_{\max}$ and K_S from a plot of $(1/\mu)$ vs $(1/S)$:

$$\frac{1}{\mu} = \frac{K_S}{\mu_{\max,S}}\frac{1}{S} + \frac{1}{\mu_{\max,S}} \tag{14.16}$$

This simple form of the Monod equation can be extended to include other fitting parameters. There could also be one Monod equation for each substrate. For example, the following model has been proposed for the effect of saline concentration Z on specific growth for halophilic bacteria (Dotsch et al. 2008):

$$\mu = \frac{\mu_{\max,\,Z}\, Z}{K_Z + Z + Z^2/k_1 + Z^3/k_2} \tag{14.17}$$

where $\mu_{\max,\,Z}$ is defined similarly to $\mu_{\max,\,S}$ and k_1 and k_2 are additional parameters.

14.3.2.2 Bioreactor Modeling

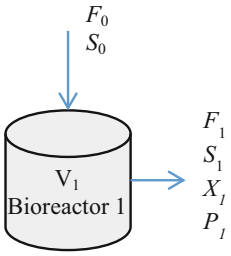
Batch reactors are one of the major reactors used both experimentally and industrially for biochemical production. The main advantage of batch reactors is that high product concentrations can be obtained. A variety of the batch reactor is the fed-batch reactor where a substrate or nutrient is fed periodically when there is inhibition to high concentrations or when a deficiency of nutrients favors the metabolism toward a desirable product. However, in order to improve process economics, a continuous reactor system can be used. High productivity, but lower concentrations, is achieved in continuous reactors. Dilution makes separation less efficient and may require more downstream processing steps. Thus, the trade-offs between productivity and purity of the product need to be carefully evaluated.

PHB production in a continuous process is desirable in order to improve process economics and also has consistency in the quality and properties of the polymer. Thus, this section will develop a model for an ideal continuously stirred tank reactor or CSTR as shown in Fig. 14.5. This reactor model allows assuming a homogeneous system and steady-state operation.

A CSTR reactor is modeled as follows. First, the mass balance for each component of interest is formulated, biomass and PHB in the case shown here. Remember that PHB is part of the internal cell biomass, but here the balance is done separately for PHB and the residual biomass (i.e., total biomass – PHB). The component mass balance has this general form: accumulation = input – output + generation – consumption (or death in case of microorganisms). Using the symbols in Fig. 14.5, cell biomass balance around the first reactor volume V_1 can be expressed as:

$$V_1 \frac{dX_1}{dt} = F_0X_0 - F_1X_1 + \mu_1X_1V_1 - k_dX_1V_1 \tag{14.18}$$

Several assumptions can be made to simplify this expression such as constant volume, no biomass in the feed flow ($F_0X_0 = 0$), and that the death rate can be



F input flow rate
S substrate concentration
X biomass concentration
P product concentration
V volume

Fig. 14.5 Scheme showing the variables for modeling a continuous bioreactor for PHB production

neglected assuming it is much smaller than growth ($k_d \ll \mu_1$). Dividing by V_1, Eq. (14.18) is reduced to:

$$\frac{dX_1}{dt} = (\mu_1 - D_1)X_1 \tag{14.19}$$

where D_1 is known as the dilution rate equal to F_1/V_1. Now, CSTR reactors reach a point in time where concentrations are constant; therefore it can be found that at steady state:

$$\mu_1 = D_1 \tag{14.20}$$

This suggests that to achieve the maximum growth, the dilution rate at which a CSTR bioreactor operates should be tuned to the specific growth rate. Note that parameters for the Monod equation can be also found using a CSTR reactor by varying the dilution rate at constant fermentation volume.

The reactor operates under limiting substrate; therefore, a mass balance for glucose can be written as:

$$V_1 \frac{dS_1}{dt} = F_0 S_0 - F_1 S_1 - \frac{1}{Y_{XS}^{\max}} \mu_1 X_1 V_1 - m_s X_1 V_1 - \frac{1}{Y_{PS}^{\max}} q_{PHB} X_1 V_1 \tag{14.21}$$

At steady state and neglecting PHB generation, as it is much slower than the biomass growth under nitrogen rich conditions $\left(\frac{q_{PHB} X_1}{Y_{PS}^{\max}} \ll D(S_0 - S_1)\right)$, and dividing by V_1, Eq. (14.21) simplifies to:

$$D_1(S_0 - S_1) = \frac{1}{Y_{XS}^{\max}} \mu_1 X_1 + m_s X_1 \tag{14.22}$$

Dividing by μ_1 and biomass concentration X_1, and since at steady state $\mu_1 = D_1$, Eq. (14.22) becomes:

$$\frac{1}{Y_{XS}} = \frac{1}{Y_{XS}^{\max}} + \frac{m_s}{\mu_1} \tag{14.23}$$

where Y_{XS} is the overall yield of biomass from glucose and $Y_{XS}^{\max}$ is the theoretical yield coefficient of biomass from glucose (as in Eq. 14.9). With known experimental yield data, the values of $Y_{XS}^{\max}$ and m_s can be obtained and used to calibrate the metabolic model studied in Sect. 14.3.1.

Finally, the product mass balance can be written as follows:

$$V_1 \frac{dP_1}{dt} = F_0 P_0 - F_1 P_1 + q_{PHB} X_1 V_1 \tag{14.24}$$

At steady state and with $P_0 = 0$:

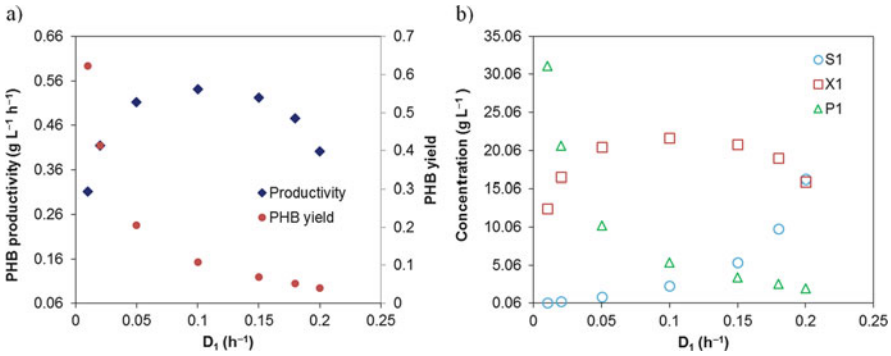


Fig. 14.6 Optimization of PHB production at various dilution rates (D_1) in one CSTR showing (**a**) blue diamond: productivity and red circle: yields and (**b**) blue circle: concentrations of substrate (S1), red square: residual biomass (X1), and green triangle: PHB (P1)

$$q_{\text{PHB}} = \frac{1}{X_1} D_1 P_1 \tag{14.25}$$

Thus, solving the equation system (Eqs. 14.20, 14.22, and 14.25), the values of outlet concentration of substrate, biomass, and PHB can be calculated for various dilution rates. Subsequently, the optimum D_1 for the maximum productivity P_{PHB} (g L^{-1} h^{-1}) can be obtained. Productivity can be calculated as:

$$P_{\text{PHB}} = D_1 P_1 \tag{14.26}$$

Figure 14.6 shows the concentrations and values of productivity and PHB yield (kg PHB per kg glucose input) at different dilution rates after solving the mass balance equations using the parameters found previously for *Halomonas* sp.: $\mu_{\max} = 0.24$ h^{-1}, $K_S = 3.27$ g L^{-1}, $m_S = 0.02$ h^{-1}, $Y_{XS}^{\max} = 0.5$, and $q_{\text{PHB}} = 0.025$ h^{-1}. The initial glucose in a sugarcane bagasse hydrolysate was assumed to be concentrated up to 50 g L^{-1}. Note how the highest productivity is obtained at around $D_1 = 0.1$ h^{-1} with a PHB yield of 10.8% and a concentration of 5.41 g L^{-1}. The PHB content in biomass can be calculated as 20%. Thus, the residual biomass was 21.67 g L^{-1} and the total biomass (residual + PHB) is 27.1 g L^{-1}.

As mentioned previously, to improve yield and PHB concentration and content in biomass, the strategy used is a two-stage reactor system. Equations for the second bioreactor in the system can be derived in a similar way to the one shown here for the first reactor. However, as the second reactor operates with nitrogen limitation, the effect of nitrogen can be captured in the model by a Monod equation.

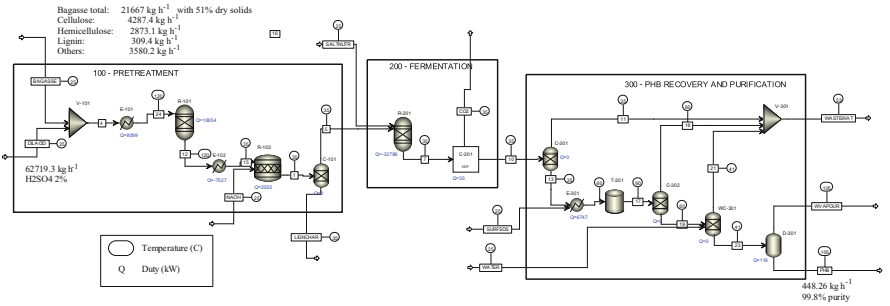


Fig. 14.7 Simulation flow sheet of PHB production process in a biorefinery using halophilic bacteria

14.3.3 Process Simulation of Extremophile-Based Process for PHB Production

PHB production using *Halomonas* sp. is analyzed in terms of its economics and greenhouse gas (GHG) emissions. The flow sheet simulated in the process simulator Aspen Plus® is shown in Fig. 14.7. The basis for the simulation is the production of 2000 t per year of PHB from sugarcane bagasse containing 51% of dry matter. The dry matter composition is 38.8% glucan, 23.5% xylan, 2.5% arabinan, 2.8% ash, and 32.4% lignin and others. The whole process consists of the following areas:

A-100: Pretreatment. Bagasse is pretreated by diluting H_2SO_4 (2% mass basis) at 120 °C and followed by enzymatic saccharification. The whole pretreatment has been simulated as one unit R-101. Then, the hydrolysate is neutralized using NaOH in R-102. The lignin, ash, and char formed are then separated as solids by centrifugation in C-101, and sugars are recovered in the liquid stream which is then sent to the next area.

A-200: Fermentation. The hydrolysate is then sent to fermenter R-201 for conversion of sugars into PHB, cell biomass, and CO_2. The CO_2 from cell metabolism and respiration is vented out but could ideally be recovered for further conversion, thus avoiding process of GHG emissions. The fermentation broth is then sent to area A-300.

A-300: PHB recovery and purification. Here, the broth passes through centrifuge C-301 to separate the total biomass containing the PHB from the liquid stream. Since PHB is a microorganisms' intracellular constituent, the cells need to be lysed, in order to recover this product. The main advantage of using halobacteria is that cells are easily lysed by a sudden change in salt concentration between the fermentation broth and pure water. The osmotic differential breaks down the cells and releases the PHB granules. The recovery and purification are thus simplified as no further treatment and no solvent are required, unlike the current typical process for PHB production with non-halophilic bacteria. Therefore, biomass treatment is carried out in T-301 as described. To improve recovery yield and

purity, sodium dodecyl sulfonate (SDS) solution at 0.1% (weight/volume basis) is used as the surfactant to assist the separation of the residual biomass from PHB granules (Rathi et al. 2013). The PHB granules are recovered from C-202 and is then washed and centrifuged to remove any remaining soluble components in WC-301. Afterward, the PHB granules are spray-dried in D-201 to obtain a mass purity of >98%. The use of the halophilic bacteria shows reduction of process steps and avoids using hazardous chemicals and solvents. Furthermore, the separation of the biomass from the liquid phase allows recycling of salts and nutrients required for the growth of halophilic bacteria. However, the use of high salinity would need corrosion-resistant equipment.

The overall process yield of PHB relative to bagasse was 2.1% despite using the optimum dilution rate (D_1) determined from the bioreactor modeling section. Thus, it is necessary to look at the whole picture in order to select the optimum D_1 at the process level and not only at the bioreactor or microorganism level. Thus, the process can still be optimized by selecting the appropriate dilution rate in the bioreactor. Another strategy would be the two-stage bioreactor system. But in order to decide the best option, some economic and environmental impact analysis might be required, as shown in the following sections.

14.3.4 Economic Analysis of PHB Production in a Extremophile-Based Biorefinery

Economic analysis was performed according to Sadhukhan et al. (2014), and the currency used was US dollars ($). The plant operates only 4380 h per year while there is sugarcane available to obtain the bagasse. The bagasse price was 16 $ t^{-1}, and the prices and costs were obtained for Mexico when information was available (Barrera et al. 2016). The biorefinery plant included an effluent treatment plant. Two cases were analyzed:

Case A: dilution rate $D = 0.1$ for maximum bioreactor productivity (Sect. 14.3.2.2). The PHB yield in the bioreactor was 10.8%, the glucose conversion was 95.3%, and the PHB content in biomass was 20% with a productivity of 0.54 g L^{-1} h^{-1}. The overall PHB yield in respect to bagasse input was 2.1%.

Case B: dilution rate $D = 0.01$ for high PHB yield in the bioreactor = 62%. The glucose conversion was 99.7%, and the PHB content in biomass was 71.4% with a productivity of 0.31 g L^{-1} h^{-1}. The overall PHB in respect to bagasse input was 11.9%. This case includes a combined heat and power (CHP) plant from solid residues.

The objective of the economic analysis was to analyze the minimum selling price for profitability. The discount rate was set at 10% and the plant lifetime was 15 years. Figure 14.8 shows the results of economic analysis. Figure 14.8a shows that capital costs contribute to total annual costs by up to 51%. The direct operation costs are

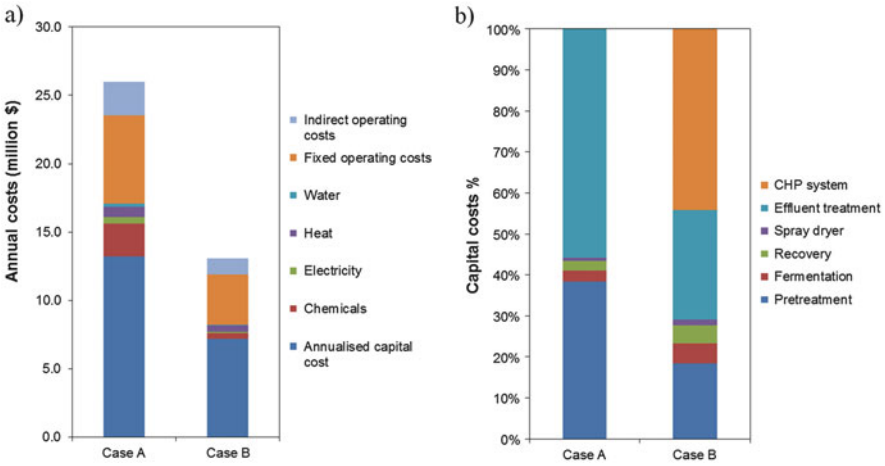


Fig. 14.8 (**a**) Annual operating cost and (**b**) capital cost contribution by various sections of the PHB production process for case A (low yield and no CHP plant) and case B (high yield and with CHP plant)

contributed mainly by chemicals (9% of total) and then energy (5% of total). Figure 14.9 shows that the minimum selling price for a positive netback from bagasse would need to be 13.76 \$ kg^{-1} in case A. This would make PHB from halophiles not competitive with other production technologies and other biopolymers such as polylactide- and starch-based polymers with values reported in the range of 5–12 \$ kg^{-1} (Mudliar et al. 2008; Choi and Lee 1997).

Figure 14.8 shows how the operating costs decrease by 50% in case B due to increased overall yield. Lower bagasse needs to be processed (for the same PHB production of 2000 t per year) and thus lower chemical requirements and lower effluents. When looking at hot spots in the capital costs in Fig. 14.8b, the effluent treatment had a high contribution in case A which is reduced significantly in case B. Furthermore, case B allowed the integration of a combined heat and power (CHP) plant using the solid residues to supply 80% of electricity and 34.5% of heat requirements. This on-site energy supply will have a positive effect on the environmental performance as shown in the next section. Sensitivity analysis in Fig. 14.9 showed that minimum selling price in case B is reduced to 7.05 \$ kg^{-1}. This price is within the range of values reported in the literature which means that PHB production from halophiles could be competitive for specialty applications. However, it would be difficult to compete as a commodity with petrochemical-based polymers, which prices are just around 1.2 \$ kg^{-1} (e.g., low density polyethylene).

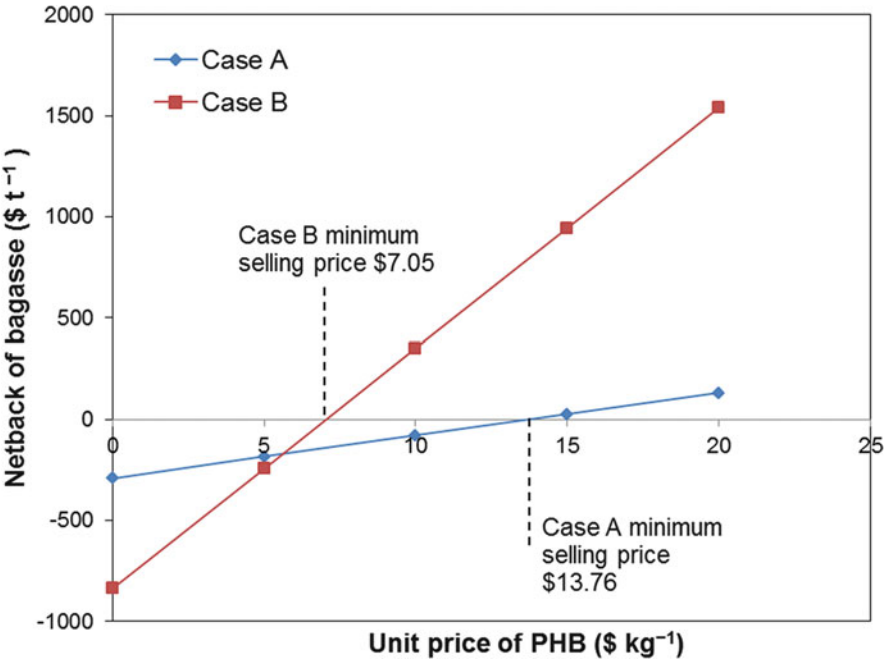


Fig. 14.9 Sensitivity analysis to determine minimum PHB selling price for positive netback in blue diamond: case A and red square: case B

14.3.5 Life Cycle Assessment (LCA) of PHB Production System

The LCA is a holistic and systematic environmental impact assessment tool in a standardized way and format for cradle to grave systems. According to the International Organization for Standards (ISO) 14040, 14041, and 14044, the LCA is carried out in four phases: goal and scope definition, inventory analysis, impact assessment, and interpretation (ISO 1997). All these phases are interdependent, as the result of one phase determines the execution of the next phase. Although LCA is a standard technique, it needs a practitioner's expertise to ensure that the system is correctly defined, inventories are robust, and impacts assessed and interpretations are comprehensive (Sadhukhan et al. 2014).

The LCA study follows these ISO guidelines, practical implementation of which has been discussed in Sadhukhan et al. (2014). The system boundary includes the direct, indirect, and embedded inputs and outputs. The inlet and outlet mass and energy flowrates of the system were extracted from the process modeling and simulation discussed in earlier sections. For each inlet or outlet flow, inventory data were extracted from ecoinvent 3.0 and characterized and aggregated for life cycle impacts in various categories using GaBi 6.0 (Thinkstep 2016). The most relevant and important impact characterizations for the system are global warming,

Table 14.5 Inlet and outlet raw material and energy flowrates of the system, for which inventory data were extracted from Ecoinvent 3.0, for cases A and B

Per kg PHB production	Case A (low yield, no CHP)	Case B (high yield, CHP)
Direct emission from the plant (i.e., CO_2 emission from the fermenter), kg CO_2	0.2125	0.2125
Quantity of raw materials causing indirect impacts of the plant		
Heat from combustion of natural gas, MJ	58.7791	
Sodium hydroxide, kg	2.2844	0.3977
Grid electricity, MJ	5.8950	
SDS, kg	0.5197	0.0521
Sodium chloride, kg	1.1434	0.1285
Sulfuric acid, kg	0.2798	0.0487
Make-up water, kg	131.7748	15.5924
Embedded or captured CO_2 in PHB, kg CO_2	−2.0465	−2.0465

acidification, eutrophication, freshwater aquatic ecotoxicity, human toxicity, and photochemical ozone creation potentials.

The direct greenhouse gas (GHG) emission from the PHB production process, indirect GHG emission due to sourcing of raw materials needed by the PHB production process, and embedded or sequestrated carbon in PHB have been taken into account in the estimation of the life cycle global warming potential over 100 years (GWP). The inlet and outlet flowrates of the system, for which inventory data were extracted from Ecoinvent 3.0, are shown for cases A and B, in Table 14.5.

Case A gives a total of 7.0114 kg CO_2 equivalent GWP (per kg PHB) from the PHB production system with utilities sourced externally. However, if separated solids (primarily containing lignin) are used for combined heat and power (CHP) generation using biomass boiler, heat recovery steam generator, and steam turbines (Wan et al. 2016), the needs for natural gas heating and grid electricity can be completely eliminated, such as in case B. Case B with high-yield and on-site CHP generation thus has a reduction in GWP impact by 90%, i.e., 0.7083 kg CO_2 equivalent per kg PHB production, that is, without the consideration of embedded or captured CO_2 in PHB. Figure 14.10 shows the GWP impact proportions of various direct and indirect attributes in case B, without consideration of embedded or captured CO_2 in PHB. The highest to the lowest impact hot spots are sourcing of sodium hydroxide, direct CO_2 emission, and sourcing of SDS, sodium chloride, sulfuric acid, and makeup water, respectively. The GWP was lower than the best value of 1.96 kg CO_2 equivalent per kg PHB production, reported in the literature (Harding et al. 2007). This has been achieved by process integration and optimization strategy developed here.

For the primary impact categories shown in Table 14.6, considerable differences between case B with high-yield and on-site CHP generation and equivalent fossil-based polymer production system exist. The GWP of impact of case B with high-yield and on-site CHP generation here takes account of the embedded or captured

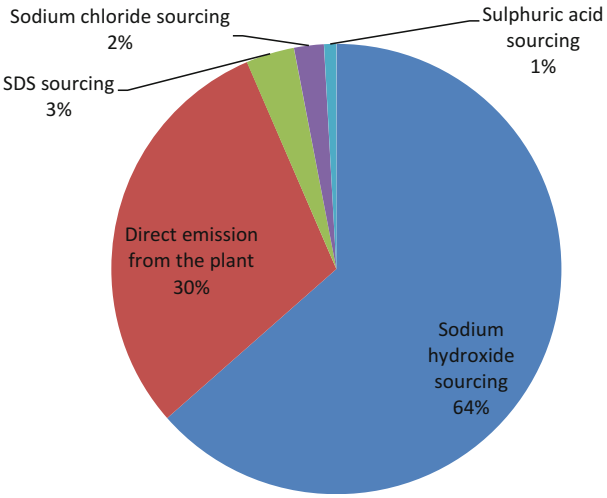


Fig. 14.10 GWP impact proportions of various direct and indirect attributes in case B with high-yield and on-site CHP generation (without the consideration of embedded or captured CO_2 in PHB)

CO_2 in PHB, i.e., = 0.7083 – 2.0465 = –1.3382 kg CO_2 equivalent per kg PHB production. This gives a percentage reduction in GWP of greater than 100%, while for all other categories, the percentage reduction in environmental impacts is 76–96%. This once again consolidates the importance of process integration tools for integrated biorefinery design. The greater the sourcing of raw materials and energy on-site by in-process material and energy integration, the higher is the sustainability of the integrated biorefinery system.

Performance can still be optimized by reducing the amount of effluents, energy, and chemicals. Biomass pretreatment also plays an important contribution to economic costs, and thus the PHB production may be best when combined with pretreatment technologies other than dilute acid. Furthermore, the separation and conversion of xylose could be beneficial for the overall biorefinery performance.

Take-Home Message

- A set of modeling and analysis tools can be systematically applied for biorefinery development based on extremophile processing, as illustrated for PHB production using halophilic bacteria.
- It is important to relate models at the various scales and to look at the whole process picture to optimize the economic and environmental performances of biorefineries.
- The potential for using halophile bacteria against other technologies is demonstrated to be competitive in terms of economics and environmental impacts; however, high yields and content are required.
- Extremophile processing will play a key role in making biorefineries more profitable and sustainable.

Table 14.6 Primary impacts, for which considerable differences between case B with high-yield and on-site CHP generation and equivalent fossil-based polymer production system exist

Per kg polymer production	Case B: PHB production with CHP generation	Fossil-based equivalent polymer production	Savings by biopolymer production system	% savings by biopolymer
Acidification potential, kg SO_2 equivalent	0.0032	0.0191	0.0159	83.4846
Eutrophication potential, kg phosphate equivalent	0.0016	0.0137	0.0121	88.2027
Freshwater ecotoxicity potential, kg DCB equivalent	0.2814	1.1807	0.8993	76.1698
GWP, kg CO_2 equivalent	−1.3382	7.5146	8.8528	117.8074
Human toxicity potential, kg DCB equivalent	0.3856	7.3761	6.9906	94.7726
Marine ecotoxicity potential, kg DCB equivalent	833.6787	3489.2043	2655.5256	76.1069
Urban smog, kg ethylene equivalent	0.0002	0.0042	0.0040	95.5579
Terrestrial ecotoxicity potential, kg DCB equivalent	0.0121	0.0534	0.0413	77.3540
DCB: 1,4-dichlorobenzene				

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