Vipin Chandra Kalia *Editor*

Microbial Factories

Biofuels, Waste treatment: Volume 1

Springer

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Vipin Chandra Kalia
Microbial Biotechnology and Genomics
CSIR-Institute of Genomics
and Integrative Biology
Delhi University Campus
Delhi, India

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Dedicated to my family and friends

Preface

Human beings are an integral part of the environment. Biological activities have a strong influence on physical and chemical components of the ecosystem. Plants are the major contributors as producers of bioproducts, to be used by animals and microbes. In the animal kingdom, human beings are the most aggressive consumers, and their needs are increasing geometrically with time. Unlike animals, human needs extend beyond food and shelter. The innovative nature of man has led to discoveries and inventions, which apparently are for the benefit of human beings. However, these developments are a big drain on the available natural resources with a cascade effect. At the base of this chain reaction, the most adversely affected is the energy sector. The demand for energy is increasing rapidly because of the needs and attitude of humans, who are thus transforming to a society of high-end consumers. Since fossil fuels are the major source of energy, their consumption is the root cause of irreparable damage to the environment. Another factor which adds to the ever-increasing environmental pollution is the unmanageable quantities of wastes. The conventional means of disposal of wastes and waste waters, adopted in most parts of the world, pollutes the land, atmosphere, and the water bodies. Here, we may need to approach the most efficient organisms on the planet Earth. These efficient organisms are the microbes, which can metabolize organic matter content of the biowastes, especially those produced due to human activities. These bioproducts are eco-friendly, biodegradable, and highly energy efficient. Microbes can be exploited as factories for producing energy (biofuels), biopolymers (bioplastics), and bioactive molecules (antimicrobial, anticancer, antidiabetic, antioxidants, etc.). There has been a vigorous scientific pursuit to exploit microbes for the welfare of human beings. The most exciting are the possibilities of generating clean fuels (biohydrogen, biodiesel, etc.) and biodegradable plastics as an alternative to nondegradable plastics. Apart from these, the most curiosity-driven activities have been to learn about those microbes which are yet to be cultured. During the last 2–3 decades, many scientific activities have been demonstrated and published in scientific journals of repute; however, it is yet to reach the curious young minds – the graduate and postgraduate students – of our future scientists. This compilation, contributed by the experts in these research domains, speaks a lot about the present status of microbial factories and their future potential for the welfare of human beings. In principle, experts exist in all domains; however, most of the times, they are too busy in their pursuits to spare time for such activities. The young, curious, and tender minds are eager to learn, but those who know what and how to say do not get the right platform and access. I am extremely thankful to all those who readily agreed to share their expertise for the *Ignited Minds*, to whom the book is dedicated. Although it is impossible to acknowledge the reality and true worth of the efforts of the contributing authors, however, I am still indebted to their prompt responses and dedicated efforts. My inspiration to learn well and transmit the knowledge to the next generation burgeons from the tireless efforts and constant support of my close ones – Mrs. Kanta Kalia and Mr. R.B. Kalia (parents); Amita (wife); Sunita and Sangeeta (sisters); Ravi, Vinod, and Satyendra (brothers); Daksh and Bhrigu (sons); and my teachers and friends Rup, Hemant, Yogendra, Rakesh, Atya, Jyoti, Malabika, Neeru, and Ritushree – to write this book. I must also acknowledge the selfless and dedicated support of my next-generation colleagues – Prasun, Sanjay, Subhasree, Shikha, Anjali, and Jyotsana.

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About the Editor

Vipin Chandra Kalia is presently working as chief scientist at Microbial Biotechnology and Genomics, CSIR-Institute of Genomics and Integrative Biology, Delhi. He is a professor at the Academy of Scientific and Innovative Research (AcSIR), Delhi. He obtained his M.Sc. and Ph.D. degrees in genetics from the Indian Agricultural Research Institute, Delhi. He has been elected as (1) Fellow of the Association of Microbiologists of India (FAMI) and (2) Fellow of the National Academy of Sciences (FNASc). His main areas of research are microbial biodiversity, genomics, and evolution, bioenergy, biopolymers, antimicrobials, quorum sensing, and quorum quenching. He has published 85 papers in scientific journals such as (1) *Nature Biotechnology*, (2) *Biotechnology Advances*, (3) *Trends in Biotechnology*, (4) *Critical Reviews in Microbiology*, (5) *Bioresource Technology*, (6) *International Journal of Hydrogen Energy*, (7) *PLoS ONE*, (8) *BMC Genomics*, and (9) *Gene*. His works have been cited 2080 times with an *h* index of 25 and an i10 index of 39. He has edited a book: *Quorum Sensing Versus Quorum Quenching: A Battle with No End in Sight* (2015, Springer India). He is presently the editor in chief of the Indian *Journal of Microbiology* and editor of (1) *PLoS ONE*, (2) *Journal of Microbiology and Biotechnology* (Korea), (3) *Applied Biochemistry and Biotechnology* (USA), (4) *International Scholarly Research Notices* (energy), (5) *Dataset Papers in Science* (microbiology), and (6) *Journal of Molecular and Genetic Medicine*. He is a life member of the following scientific societies: (1) Society of Biological Chemists of India; (2) Society for Plant Biochemistry and Biotechnology, India; (3) Association of Microbiologists of India; (4) Indian Science Congress Association; (5) BioEnergy Society of India; and (6) the Biotech Research Society of India (BRSI). He is also a member of the American Society for Microbiology. He can be contacted at vckalia@igib.res.in;vc_kalia@yahoo.co.in

Microbes: Factories for Bioproducts

1

Vipin Chandra Kalia

Abstract

A quest for rapid progress has forced man to indiscriminately tap diverse natural resources. As a result, a scenario of rapid depletion of fossil fuels and unmanageable wastes have become major challenges. Fermentation of biological wastes and burning of fossil fuels release obnoxious gases, which are the major cause of worry for Environmentalists and Health departments. Efforts to meet these challenges have brought to the foreground certain innovative biological solutions especially the exploitation of microbial metabolisms. The advent of molecular biological techniques along with Bioinformatic tools have lead to the emergence of synthetic biology. These together have expanded the limits to which biological processes can be exploited for human welfare.

1.1 Introduction

In the quest for rapid progress, man has indiscriminately tapped diverse natural resources. Consequently, we are facing a scenario of rapid depletion of fossil fuels on one hand and unmanageable quantities of wastes on the other (Raizada et al. 2002; Kalia 2007). Environmental pollution due to fermentation of biological wastes and release of obnoxious gases on burning of fossil fuels is a major cause of concern among environmentalists and health departments. It is no surprise that these have become a major cause of deteriorating human health, especially the respiratory problems caused by inhalation of these obnoxious gases. Another factor which contributes to the worries of health departments is the slow and uncontrolled fermentation, a perfect ground for release of greenhouse gases, breeding, and spread of pathogenic microbes. Efforts to meet these challenges, (1) the limited reservoirs of ever-polluting fossil fuels and (2) an ever-increasing quantum of wastes being generated, have brought to the foreground certain innovative biological solutions. Among these potential solutions, microbial metabolisms are the most attrac-

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V.C. Kalia (✉)
Microbial Biotechnology and Genomics,
CSIR-Institute of Genomics and Integrative Biology,
Delhi University Campus, Mall Road,
Delhi 110007, India
e-mail: vckalia@igib.res.in; vc_kalia@yahoo.co.in;
http://scholar.google.co.in/
citations?hl=en&user=XaUw-VIAAAAJ

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tive. Microbes are the most versatile organisms, which have been studied and exploited. The advent of molecular biological techniques has provided greater insights into microbial genomes. Bioinformatic tools and metagenomic techniques have expanded the limits to which biological processes can be extended and exploited for human welfare (Kalia et al. 2003a, b; Rani et al. 2008; Porwal et al. 2009). A new dimension to these R&D works has been added by the emergence of synthetic biology.

Among the various abilities of the microbes, the most lucrative are those leading to generation of bioenergy, biopolymers, bioactive molecules, enzymes, etc. The economy of all these bioprocesses depends upon the cost of the feed and the recovery process. Compared to pure substrates, the use of biowastes as feed has been shown to be economical. The best part of microbial metabolisms is their versatility to operate under aerobic and anaerobic conditions. For utilizing biowastes with the dual objectives of producing biomolecules and stabilizing them, anaerobic digestion (AD) process is more advantageous in comparison to aerobic processes (Kalia 2007). Anaerobic process results in degradation of more than 95 % of the organic matter into methane and carbon dioxide. AD, being a multistep process, is carried out by a diverse set of microbes. At each stage, it is possible to exploit the intermediates into various value-added products – hydrogen, biopolymers such as polyhydroxyalkanoates (PHAs), and bioactive molecules such as enzymes, volatile fatty acids, sugars, amino acids, etc. – as precursors for other biotechnological and medical applications.

1.2 Bioenergy

Bioenergy from renewable sources such as biomass is likely to substitute fossil fuels, which are available in relatively limited amounts. At present, bioenergy production is not economical, primarily on account of high cost of feed, feedstock productivity, and other factors. Currently, the emphasis is on developing environment-friendly technologies to increase energy supplies and efficient usage (Kalia and Purohit 2008). Worldwide, different countries like China, Germany, France, Sweden, and the USA are seriously implementing major national programs for the production of biofuel. The top-ranking technical challenges are the search for an industrially robust microbe and identification of cheap raw material. Of the various possibilities of generating bioenergy, microbial production of biohydrogen has gained significant recognition worldwide recently due to its high efficiency and eco-friendly nature.

1.2.1 Biohydrogen

H_2 as a fuel for the future was recognized as it meets the characteristics which are a must in an energy provider (Kalia and Purohit 2008; Patel and Kalia 2013). H_2 is an attractive energy source, as its burning does not generate any significant pollutants, such as traces of NOx. The major product on burning is water, which can be recycled. At this stage, H_2 contributes to around only less than 3 % of the total energy consumption, but it is expected to grow significantly in the near future. Biohydrogen is produced most commonly by algae and bacteria (Kalia et al. 2003b; Kalia and Purohit 2008). It is playing a major role in the key metabolism of these organisms. Pure cultures have been widely used to study BHP under dark- and photo-fermentative conditions. Dark- and photo-fermentative microorganisms have a very diverse physiology and metabolism and, therefore, operate different mechanisms to generate H_2. Theoretically, a maximum of four moles of H_2 can be generated by either dark- or photo-fermentation of one mole of glucose. However, H_2 yields primarily depend on the type of metabolite produced as an intermediate during fermentation. For achieving the maximum theoretical H_2 yields, it is important to integrate the two routes and exploit the various combinations of physiological conditions and substrate utilization (Kalia et al. 1994; Kumar et al. 1995, 2015a, b; Patel et al. 2010, 2012a, b). To further enhance the process efficiency, one can use cocultures of defined bacteria (Patel et al. 2015), or the effluents from these processes may be employed for production of biopolymers and finally methane (CH_4) (Kalia and Joshi 1995; Kumar et al. 2014a).

1.2.2 Biomethane

Biogas (rich in CH_4) production occurs during the microbial decomposition of organic matter in terminal stage of AD (methanogenesis) (Kalia 2007). It is a well-adapted mechanism of biogas production worldwide and offers several environmental, agricultural, and socioeconomic benefits. Biologically, CH_4 is produced by methanogens from a wide range of biological materials (Kalia et al. 1992a, b, 2000b; Sonakya et al. 2001).

1.2.3 Bioethanol

Ethanol is considered as a renewable source of energy due to its production by plants that utilize light from the Sun. It is the most commonly used biofuel, which accounts for more than 90 % of total biofuel usage. It can also be used by blending with either gasoline or diesel. Pure sugars and lignocellulosic biomass are major sources used for the production of bioethanol. By-product of sugar industries (molasses) has been used extensively as a raw material for bioethanol production in India. Among others, feedstocks, cereal crops, corn (maize), potatoes, sorghum, and cassava have been used for its production. New and innovative production technologies are being developed to establish competitive domestic biofuel industries for processing and improving the overall efficiency of bioethanol production.

1.2.4 Biodiesel

Biodiesel is fatty acid ester, which is produced by reactions between lipids (such as vegetable oils and animal fats) and alcohols. It is catalyzed by acids, alkalis, or lipase enzymes. The production of biodiesel consists of two steps: (1) the oil extraction and (2) further conversion of oil to biodiesel. Global biodiesel production has increased six- to tenfold during the last decade.

1.3 Biopolymers

Biopolymers especially polyhydroxyalkanoates (PHAs) are macromolecules having properties quite similar to synthetic plastics but are biodegradable in nature (Kalia et al. 2000a; Reddy et al. 2003; Kumar et al. 2015c; Singh et al. 2015). Many microbes have abilities to accumulate biopolymers such as reserve food material. The production of PHA occurs as a result of nutritional imbalance (e.g., high carbon/nitrogen ratio). PHA may be accumulated up to 90 % of the dry cell weight of organisms (Singh et al. 2009). Previously, only few photosynthetic organisms – *Rhodopseudomonas* and *Rhodospirillum* spp. – having abilities to produce H_2 and PHA had been reported (Kalia et al. 2003a). However, extensive efforts during the last few years have helped in identifying dark-fermentative organisms such as *Bacillus* spp. with abilities to produce H_2 and PHA from pure substrates and biowaste (Porwal et al. 2008; Kumar et al. 2009; Patel et al. 2015). In addition, integrative approaches to combine aerobic and fermentative conditions have also been attempted (Patel et al. 2011; Singh et al. 2013; Kumar et al. 2015a, b). The feasibility of these processes may be further improved by selective feed and organism or even ecobiotechnological approaches (Kumar et al. 2014b, 2015c; Patel et al. 2015).

1.4 Biodiversity

Microbial diversity has been a very important resource for almost all biotechnological applications. The culturable techniques have been in practice for the last few centuries (Selvakumaran et al. 2008, 2011; Verma et al. 2010, 2011). However, metagenomics has open up this field in such a manner that the enthusiasm of microbiologists has shoot to new heights. This method allows the identification of bacteria present in the community without actually culturing them (Rani et al. 2008). A few new dimensions have been added by the development of genomics tools for identifying novel microbial lineages and may lead to identify novel *Candidatus* taxa as well (Porwal et al. 2009).

1.5 Waste Treatment

Microbial processes for treatment of biological and chemical wastes, domestic and industrial wastewaters, and agricultural wastes have been developed by novel genomic techniques. Molecular methods of genetic engineering have now been combined with metagenomic techniques for genetically engineered microbial strains with abilities to hyper-produce hydrolytic enzymes, which are used in treating effluents from highly polluting industries (Kalia 2007).

1.6 Perspective

There is a need to consolidate the solutions to energy crisis and take a holistic approach to (1) identify and select (a) the microbe(s) with high H_2-producing abilities from a range of substrates (pure sugars and complex organic matter), (b) substrate as feed(s) which are biodegradable and available in large quantities (biological wastes or specially grown plants), (c) hydrolytic bacteria and their associates (enhancers and augmenters); (2) select physiological conditions promoting growth of H_2 producers and suppressing H_2 quenchers; (3) maintain the population of H_2 producers optimal for H_2 production and suppressing alternative metabolic routes (ethanol, lactic acid, etc.); and (4) look for those microbes which can produce value-added products without affecting H_2 yields, such as polyhydroxybutyrate (PHB) production, antibiotics, industrially important enzymes, etc. (Kumar et al. 2013). In light of these available information, it may be desirable to develop consortia of microbes and feed for optimal and economically feasible H_2 production. Since food is constantly required and waste is constantly produced, biowaste may be a good feed material.

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Vipin Chandra Kalia M.Sc. and Ph.D. Genetics from IARI, New Delhi. He is presently the Chief Scientist at CSIR-IGIB and Professor of AcSIR. He is a Fellow of Association of Microbiologists of India and Fellow of National Academy of Sciences (FNASc). He is the Editor-in-Chief of the Indian Journal of Microbiology (India). His current interests are bioenergy, biopolymers and quorum sensing. Google Scholar: http://scholar.google.co.in/citations?hl=en&user=XaUw-VIAAAAJ. Website: http://www.igib.res.in/?q=V.C.Kalia

Exploration of Microbial Cells: The Storehouse of Bio-wealth Through Metagenomics and Metatranscriptomics

2

Ravi Ranjan, Asha Rani, and Rajesh Kumar

Abstract

Microbes are omnipresent, most abundant, versatile, have been studied exhaustively, and used historically for the human welfare. This enormous diversity of microbes serves as tiny cellular factories and has been used to generate "bio" energy, gas, fuel, and polymers and in waste management. However, to date majority of these microbes remain unexplored and thus remain unexploited for bioprospecting. Recent advancements in molecular biology techniques, next-generation high-throughput sequencing, and bioinformatics have aided to circumvent this caveat by providing insights in the genomes and biological process of these microbes. These recent developments in scientific research have advantages but also present the researchers with economic and computational challenges, but none the less it has led to tremendous discoveries compared to decades ago. In this review, we focus on the technological advancements and the recent studies using metagenomics and metatranscriptomics for exploration of "microbial cell factories" – the storehouse of biological wealth.

2.1 Introduction

Microbes are ubiquitous and found in diverse habitats and in a colossal diversity. Soil, sediments, and aquatic (marine) habitats are probably the most complex natural niches with respect to the microbial community size and species diversity. The number of prokaryotes is estimated to be over $4–6 \times 10^{30}$, exceeding, by various orders of magnitude, all plant and animal diversity (Woese 1987; Amann et al. 1995; Whitman et al. 1998; Curtis et al. 2002; Ward 2002; Schloss and Handelsman 2004). These tiny microorganisms represent the richest repertoire of molecular and

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RR and AR have contributed equally, and considered as co-first and co-contributing authors

R. Ranjan, Ph.D. (✉) • A. Rani, Ph.D. (✉)
Department of Medicine, University of Illinois,
Chicago, IL 60612, USA
e-mail: ranjan.du@gmail.com; aasha511@gmail.com

R. Kumar
Department of Botany, Hindu College,
University of Delhi, Delhi 110007, India
e-mail: rajesh_bot@yahoo.com

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chemical diversity and possess huge potential for bioprospecting (Madigan et al. 2010). These microbes have been used to generate bioenergy, biogas, biofuel (ethanol and diesel), biopolymers, biocatalysts, and antibiotics and in waste management (Kalia et al. 2003a, b; Ni et al. 2007; Kalia and Purohit 2008; Gulder and Moore 2009; Kumar et al. 2009, 2013, 2014a, b; Singh et al. 2009; Williams 2009; Weiland 2010; Kuhad et al. 2011; Patel et al. 2011, 2015; Soares et al. 2012; Vermelho et al. 2012; Patel and Kalia 2013; Singh et al. 2013; Kalia 2014; Koch et al. 2014; Latif et al. 2014; Ling et al. 2015). However, only a miniscule fraction (~1–5 %) can be readily cultured using the routine laboratory culture techniques – which is appropriately termed as the "great plate count anomaly" (Staley and Konopka 1985; Kellenberger 2001; Tanaka et al. 2014). This indicates that the majority (~95–99 %) of the microbes remain "unculturable" and thus unexploited for commercial applications and bioprospecting. With the advancement in culture-independent techniques (metagenomics and metatranscriptomics) and advent of high-throughput next-generation DNA sequencing, the limitation of culturing the microbes has been circumvented, and peeking in their genome is feasible than before (Handelsman 2004; Streit and Schmitz 2004; Sharma et al. 2005; Warnecke and Hess 2009; Metzker 2010).

Over the past decade, these advances have yielded a massive amount of genomic, transcriptomic, metagenomic, and metatranscriptomic data of microbes and microbial communities. Analyzing the functional potential has become a common but a challenging task, and this has led to the generation of bioinformatics and computational, statistical, and analytical software packages to make sense of the huge genomic data (Meyer et al. 2008; Gilbert and Hughes 2011; Mitra et al. 2011; Kuczynski et al. 2012a; Luo et al. 2013; Gifford et al. 2014; Ladoukakis et al. 2014; Nilakanta et al. 2014). These developments have widely transformed our capacity to investigate and exploit the "microbial cell factories" for biotechnological applications.

2.2 Sequencing the New Generation Way

Since the inception of chain termination-based DNA sequencing (Sanger et al. 1977), which was the major source of automated sequencing offered by Applied Biosystems (www.appliedbiosystems.com/), there has been tremendous advancements in the sequencing techniques – "next generation" and development of "third generation" by various companies using different technologies (Schadt et al. 2010). Improvements in next-generation sequencing (NGS) technologies in the last decade have significantly doubled the data output, reduced the cost per genome, and surpassed the analogous improvement in computer technologies as predicted by Moore's law (www.genome.gov/sequencingcosts/). In recent years, the Illumina Inc. (http://www.illumina.com/index.html) has emerged as one of the leading manufactures of the NGS instruments. It acquired the Solexa's Genome AnalyzerIIx, and its technology is based on "sequencing by synthesis" (SBS), which uses fluorescently labeled reversible-terminator dyes. It offers a wide variety of sequencers, ranging from its benchtop versions [MiSeq and NextSeq 500] for small research lab to commercial versions [HiSeq 2500, 3000, and 5000, xFive, and xTen]. The company launched a multimillion dollar product, HiSeqX Ten, which would provide large-scale whole-genome sequencing for $1,000 per genome. Roche Diagnostics Corporation (www.roche.com/index.htm) is also one of the vendors for NGS instruments. It acquired the 454 Life Sciences, which uses the pyrosequencing technology, in which pyrophosphates are generated, while the DNA polymerase adds nucleotides to the template DNA (King and Scott-Horton 2008). Roche offers the GS Junior Plus (benchtop version) and GS-FLX+ System. The Junior boasts up to 700-base read length, and the FLX+ boasts 1,000-base read length. Life Technologies Inc. (www.lifetechnologies.com/), which was acquired by Thermo Fisher Scientific, has two NGS platforms, SOLiD and Ion Torrent. The SOLiD was earlier owned by Applied Biosystems and is based on the Sequencing by Oligonucleotide Ligation and Detection. Currently, the 5500xl SOLiD and 5500 SOLiD (benchtop version) are available. This advanced sequencers have enabled researchers to sequence more samples (genomic, metagenomic, and metatranscriptomic) in less time with high output. Many bacterial genomes and metagenomes have been sequenced using the NGS technologies, which are tabulated in Table 2.1. One has to take into consideration factors like amount of data output, read length and number of reads, number of samples multiplexed in one sequencing run, sequencing time, and cost. These factors are important as it determines the sequencing depth and coverage for the samples. The more number of samples multiplexed in one sequencing run, the more savings on cost per sample, but it results in low number of reads or data per sample (Sims et al. 2014).

Currently, there are few emerging companies with novel sequencing technologies offering the Third-Generation Sequencers (TGS). Oxford Nanopore Technologies Limited (https://nanoporetech.com/) is developing "strand sequencing" technology that passes intact DNA polymers through a protein nanopore, sequencing in real time as the DNA translocates through the pore (Branton et al. 2008; Metzker 2010; Guy et al. 2012; Benowitz 2014; Laszlo et al. 2014). It is expected to achieve long reads, low cost, and high speed with minimal sample preparation and instrumentation. One of the advantages of the nanopore technology is that it is designed to perform the direct analysis of RNA strands and direct, electronic analysis of proteins. Pacific Biosciences of California, Inc. (PacBio) (http://www.pacificbiosciences.com) is an emerging TGS venture, which projects a new method to sequence DNA cheaply and rapidly by watching an array of single DNA molecules being replicated in real time, called as "SMRT" – for Single-Molecule sequencing in Real Time (Eid et al. 2009). Currently it offers the PacBio RS II system, which is capable of extraordinarily long reads (depending upon starting library, half of the data are in reads more than 14,000 base pairs long with the longest reads over 40,000 base pairs), extremely high accuracy, and exquisite sensitivity. Helicos BioSciences Corporation platform was the first DNA-sequencing instrument to operate by imaging individual DNA molecules. Helicos technology is based on a "Virtual Terminator" technology. The reversible terminators are tethered inhibitors and are efficiently incorporated with high fidelity while preventing incorporation of additional nucleotides (Bowers et al. 2009). Complete Genomics (http://www.completegenomics.com) is offering a proprietary novel technology termed as "DNA nanoball or DNB" (Drmanac et al. 2010; Carnevali et al. 2011). This technology causes each long single molecule to consolidate, or ball up, into a small DNB particle. These DNBs are approximately 200 nm in diameter, and a library contains millions of DNBs that together represent the complete genome. Further, the Combinatorial Probe-Anchor Ligation (cPAL) chemistry attaches one of four possible fluorescent-labeled probes to the DNB anchor, depending on the sequence. This allows the fluorescence signals to be detected and read very efficiently by the sequencer. While none of the emerging TGS technologies have been thoroughly applied and tested with metagenomics and metatranscriptomics, nonetheless it offers promising alternatives, even further cost reduction, higher data output, and reduced time for sequencing.

2.3 Culture Dependent *vs* Culture Independent (Metagenomics)

Pure culture of microbes and the single-cell genomics are powerful tools for exploring for microbes for their biotechnological potential. In culture-dependent methods, bacteria are isolated from environmental samples by growing them in growth medium and under optimal physiological conditions. While many efforts and advances have been made in microbiological culture techniques, it is still challenging to culture a majority of bacterial species using the available laboratory culturing techniques (Staley and Konopka 1985; Stewart 2012; Tanaka et al. 2014). This limitation has severely impacted the limits for commercial

Table 2.1 List of bacterial genomes sequenced by NGS

		Bioprospecting						
Organism	Isolation source	PHA	Hydrogen	Bioethanol	Other products	NGS platform	^{a}Genome size (Mbp)	References
Azotobacter vinelandii strain CA6	nr	nr	Yes	nr	Yes	454 GS-FLX Titanium	nr	Noar and Bruno-Barcena (2013)
						Ion Torrent PGM		
Bacillus cereus strain A1	Activated sludge	nr	Yes	Yes	Yes	Illumina HiSeq 2000	5.6	Zhang et al. (2014)
Bacillus cereus strain tsu1	Agar-cellulose plate	Yes	nr	nr	Yes	Illumina HiSeq 2000	5.8	Li et al. (2014)
Brevundimonas naejangsanensis strain B1	Activated sludge	nr	Yes	nr	Yes	Illumina HiSeq 2000	2.9	Su et al. (2014)
Caloramator celer strain JW/YL-NZ35	Hot spring sediments	nr	Yes	Yes	Yes	Illumina HiSeq 2000	2.6	Ciranna et al. (2013)
Clostridium bifermentans strain WYM	Landfill leachate sludge	nr	Yes	Yes	Yes	Illumina MiSeq	3.4	Wong et al. (2014a)
Clostridium intestinale strain URNW	nr	nr	Yes	Yes	Yes	Roche/454 GS-FLX	4.6	Lal et al. (2013b)
						Illumina HiSeq 2000		
Clostridium pasteurianum NRRL B-598	nr	nr	Yes	Yes	Yes	Roche GS Junior	6	Kolek et al. (2014)
Clostridium pasteurianum strain ATCC 6013	nr	nr	Yes	Yes	Yes	454 GS-FLX	4.4	Pyne et al. (2014)
						Illumina MiSeq		
Clostridium perfringens strain JJC	Landfill leachate sludge	nr	Yes	nr	Yes	Illumina MiSeq	3.2	Wong et al. (2014b)
Clostridium sp. strain Ade.TY	Landfill leachate sludge	nr	Yes	nr	Yes	Illumina MiSeq	3.1	Wong et al. (2014c)
Clostridium termitidis strain CT1112	Termite gut (*Nasutitermes lujae*)	nr	Yes	Yes	Yes	Roche/454 GS-FLX	6.4	Lal et al. (2013a)
Erythrobacter litoralis strain DSM 8509	Cyanobacterial mat	Yes	nr	nr	Yes	Illumina mate pair	3.1	Wang et al. (2014)

Erythrobacter longus strain DSM 6997	Surface of green seaweed	Yes	nr	nr	Yes	Illumina mate pair	3.5	Wang et al. (2014)
Halanaerobium saccharolyticum strain DSM 6643T	nr	nr	Yes	Yes	Yes	Illumina Roche 454	2.8	Kivisto et al. (2013)
Haloarcula hispanica strain N601	Solar saltern	Yes	nr	nr	Yes	Roche GS-FLX Illumina GAIIx	3.9	Ding et al. (2014)
Halomonas hydrothermalis MTCC 5445	Seacoast	Yes	nr	nr	Yes	Roche 454 GS-FLX	nr	Bharadwaj Sv et al. (2015)
Pseudomonas mediterranea strain CFBP 5447^T	Tomato plants	Yes	nr	nr	Yes	Illumina GAIIx	6.3	Licciardello et al. (2014)
Rhizobium lupini HPC(L)	Saline desert soil	Yes	nr	nr	Yes	Ion torrent	5.2	Agarwal and Purohit (2013)
Streptomyces exfoliatus DSMZ 41693	Shotgun library	Yes	nr	nr	Yes	454 GS-FLX	8.8	Martinez et al. (2014)
Sulfurospirillum sp. strain MES	Microbial electrosynthesis system metagenome	nr	Yes	nr	Yes	Illumina MiSeq Pacific Biosciences	2.6	Ross et al. (2015)
Thermoanaerobacterium aotearoense SCUT27	Hot spring	nr	Yes	Yes	Yes	Illumina HiSeq 2000	2.8	Ai et al. (2014)

nr not reported, *PHA* polyhydroxyalkanoate

[a] Genome size Mbp: estimated genome size (megabase pairs)

utilization of these microbes. The 16S ribosomal RNA (rRNA) gene is routinely used to explore the microbial diversity and determine evolutionary and phylogenetic relationships between microbes (Woese and Fox 1977; Pace et al. 1986; Woese 1987). The present census of the microbial diversity, based on 16S rRNA genes of pure cultures and "unculturable" populations in environmental samples, includes at least 52 phylum-level bacterial and 20 phylum-level archaeal phylogenetic lineages; however, majority of the environmental microbes are not available as pure culture (Rappe and Giovannoni 2003; Schloss and Handelsman 2004). Interestingly, the human gastrointestinal tract and other body parts also harbor various microbes (Koren et al. 2011; Human Microbiome Project 2012; Schommer and Gallo 2013; Kliman 2014; Rajilic-Stojanovic and de Vos 2014). This recent surge of research in molecular microbial ecology based on 16S rRNA provides a convincing evidence for the existence of many diverse lineages of bacterial phyla encompassing novel unculturable bacteria (Rani 2008). However, the 16S rRNA gene information helps to access the microbial diversity, and it is limited to offer any genomic insights and functional potential of the microbe.

Metagenomics (also referred to as environmental and community genomics) is the culture-independent genomic analysis of assemblage of microorganisms of any environmental sample (Handelsman et al. 1998; Handelsman 2004). Metagenome analyses are initiated by the isolation of pure quality, high molecular weight environmental DNA, or metagenomic DNA from the environmental sample without culturing the microbes. The DNA isolation protocol should be such that maximum cells are lysed to maximize the yield with minimal shearing of DNA. Precaution should be taken to avoid the degradation of released nucleic acids because of the ubiquitous presence of environmental nucleases and also concomitant release of cellular nucleases during cell lysis. Simultaneously high molecular weight contaminants (humic acid and fulvic acid) also coprecipitate along with DNA, which hinder the downstream applications (Ranjan 2008). Many commercial DNA isolation kits are available from various manufacturers and have been compared to isolate DNA from different environmental samples (Dineen et al. 2010; Mahmoudi et al. 2011; Claassen et al. 2013; Vishnivetskaya et al. 2014). To date there is no accepted universal method for DNA isolation of different environmental samples; however, the major emphasis of the DNA isolation should be an unbiased genomic representation of all the microbial species. The metagenomic DNA can be used to amplify the 16S rRNA and sequenced to access the microbial diversity – called as amplicon sequencing metagenomics. The metagenomic DNA can be cloned in various cloning vectors [such as plasmids, cosmids, fosmids, bacterial artificial chromosome (BAC) vectors] and screened for functional genes, called as functional metagenomics, or can be sequenced using the next-generation sequencing, called as shotgun metagenomics (Fig. 2.1) (Ranjan et al. 2005; Ranjan 2008; Vester et al. 2015). Since the availability of the first two human pathogenic bacterial genome sequences (Fleischmann et al. 1995; Fraser et al. 1995) and the first two monumental shotgun metagenome sequencing of the Sargasso Sea and acid mine drainage, the paradigm in microbial genomics has been revolutionized (Tyson et al. 2004; Venter et al. 2004). In an effort to characterize the Earth microbial communities, The Earth Microbiome Project was formed. It includes collaborations among scientists worldwide and proposes to analyze 200,000 samples from various environmental communities using metagenomics, metatranscriptomics, and amplicon sequencing. This will help to generate a global Gene Atlas, environmental metabolic models for each biome, and approximately 500,000 reconstructed microbial genomes (www.earthmicrobiome.org/). To date there are more than 5000 bacterial genome sequences available and metagenomes sequenced from various environmental samples (www.ncbi.nlm.nih.gov/genome/browse/, http://metagenomics.anl.gov/).

The next-generation sequencing (NGS) technology has enabled us to perform massive parallel ultra-deep sequencing and transformed the landscape of microbial genomics through their ability to generate hundreds of megabases in a

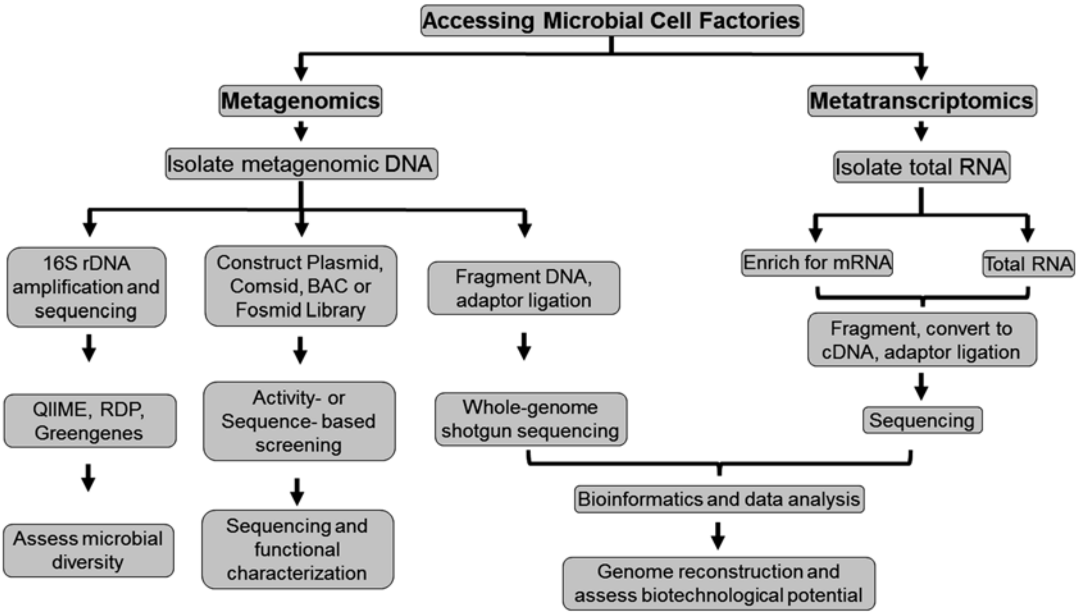

Fig. 2.1 Schematic overview of the metagenomic and metatranscriptomic approach to explore the "microbial cell factories"

single run. However, the use of these instruments requires the metagenomic DNA to be processed (called as library preparation) uniquely for different sequencers, as these are based on different sequencing technologies. The library preparation involves random fragmentation of the metagenomic DNA, using Covaris instrument [which is based on Adaptive Focused Acoustics (AFA) technology] or the transposome-mediated fragmentation technique or nebulized or enzymatically fragmented. The fragments are modified with the ligation of an adapter or barcode (sequence of nucleotides) and amplified. These double-stranded DNA are then denatured, and the nucleotide base composition on the single-stranded DNA molecules is detected by the sequencers. These procedures are dependent on the sequencing platform/instrument (Head et al. 2014). Additionally, the commercial DNA library prep kits (Illumina, Nextera XT DNA Sample Preparation Kit; Bioo Scientific Corporation, NEXTflex Rapid DNA-Seq Kit; and other similar kits) have eliminated the requirement of high amount of input DNA required for library preparation. Recently, Parkinson et al. described the process of preparation of DNA libraries from 20 pg of DNA from *Escherichia coli* (*E. coli*) using a modified transposome-mediated fragmentation technique (Parkinson et al. 2012).

2.4 Metatranscriptomics: Getting to the Functionality

Next-generation high-throughput sequencing technologies have enabled researchers to directly explore the microbial processes at transcriptional or the messenger RNA (mRNA) level, termed as "metatranscriptomics." The process basically involves three steps: (1) isolating the high-quality total RNA directly from the environmental sample and enriching for mRNA, (2) converting to cDNA, and (3) sequencing using NGS (Fig. 2.1). However, there are few options that a researcher can tailor based on their desired applications. The total RNA in a cell contains majority (~95–98 %) of ribosomal RNA (rRNA) and transfer RNA (tRNA), and the remaining (1–4 %) is mRNA. Since the mRNA has very short half-life and is prone to degradation, precaution should be taken at the time of collection and storage, as this can alter the microbial transcriptional profile (Deutscher 2006). Commercial RNA preservation and storage reagents [RNAlater (Life Technologies, Inc.), LifeGuard Soil Preservation Solution (MO Bio Laboratories Inc), etc.] should be added to alleviate the degradation. Most of the RNA isolation from different environmental samples involves the bead beating or homogenization using glass or zirconium beads for efficient lysis of microbial cells. Other methods involving microwave-based rupture, liquid nitrogen grinding, and enzymatic lysis have been shown to be less efficient than those involving bead beating. However, till now, there has been no universal method for RNA extraction for different environmental samples (Wang et al. 2012; Carvalhais and Schenk 2013). Currently, there are few commercial RNA extraction kits like PowerSoil Total RNA Isolation Kit (MO Bio Laboratories, Inc.), FastRNA Pro Soil-Direct kit (MP Biomedicals), E.Z.N.A. Soil RNA kit (Omega Bio-Tek), etc. These kits boast of the high purity and yield. As there is only a tiny fraction of mRNA in the total RNA pool, it may be enriched by rRNA subtractive hybridization. The contaminating rRNA can be subtracted by using the MICROBExpress Bacterial mRNA Enrichment kit (Life Technologies Inc) and Ribo-Zero rRNA Removal Kit – Bacteria (Illumina, Inc.). Another alternative is an Exonuclease (5′-phosphate-dependent exonuclease) treatment of total RNA, which is based on the principle to degrade the bacterial rRNA as it possesses a 5′-monophosphate. In few instances, the total RNA can be subjected to subtractive hybridization followed by exonuclease treatment, which results in highly enriched mRNA and maximal removal of the rRNA (Zoetendal et al. 2006; Mettel et al. 2010; Carvalhais and Schenk 2013). Once the enriched mRNA is obtained or the total RNA can be converted to cDNA, it must be noted that in case if the mRNA or the RNA is low in concentration, it can be converted to cDNA and amplified to increase the yield using multiple displacement amplification [MDA] or multiple annealing and looping-based amplification cycles [MALBAC] (Gilbert et al. 2008; Zong et al. 2012; de Bourcy et al. 2014; Motley et al. 2014). Recently, researchers have reported the method to prepare whole-transcriptome cDNA libraries from a minute (500 pg) amount of total RNA (Tariq et al. 2011). Similar steps as DNA libraries can then be followed to prepare cDNA libraries based on the NGS platform.

2.5 Challenge: Generating to Analysis

The NGS data contains random microbial community DNA (metagenomics) and mRNA/RNA sequences (metatranscriptomics), and the question is to know which microbes are present, understand what they are doing, and how can we use them for biotechnological applications. Regardless of the NGS platform used for sequencing, there are three basic steps involved for analysis: (1) trimming of poor quality of sequences based on Phred scores or the ASCII characters, (2) assembly of reads and binning, and (3) gene prediction, annotation, and data interpretation. Several labs have established bioinformatic resources and computational and programming (Linux based, Perl, R) capabilities can configure their own modules and tools for data analysis, canonically termed as “pipeline.” Many of the research labs have limited resources and are unable to develop their own data analysis tools. There are few online high-throughput pipelines (MG-RAST server, EBI Metagenomics server) that a researcher can use to upload raw sequence data and obtain the phylogenetic, taxonomic, and functional information (Meyer et al. 2008; Hunter et al. 2014). Commercial software such as CLC Genomics Workbench (Qiagen – CLC Genomics) is available as a complete package and has all the major software modules for performing the data analysis. The comparison of different pipelines and tools for metagenomic data analysis is reviewed by Wooley and Ye (2009), Carvalhais et al. (2012), Thomas et al. (2012), Luo et al. (2013), Ladoukakis et al. (2014), and Nilakanta et al. (2014) and tabulated in Table 2.2.

Table 2.2 List of software, online pipeline, bioinformatics, and statistical tools for NGS data analysis

Software/pipeline/online tool	Application	References/web-page link
AliView (ALignment Viewer)	Fast alignment viewer and editor for large datasets	Larsson (2014) www.github.com/AliView
BaCoCa (BAse COmposition Calculator)	Software tool to identify sequence biases in large datasets of gene and taxon	Kuck and Struck (2014) http://software.zfmk.de
BRENDA (BRaunschweig ENzyme Database)	Enzymes information for a metagenome data with the use of functional biochemical and molecular data	Schomburg et al. (2013) http://www.brenda-enzymes.org
CAMERA (The Community Cyberinfrastructure for Advanced Marine Microbial Ecology Research and Analysis)	Online computing service and high-performance computing infrastructure for metagenomic data analysis	Seshadri et al. (2007) http://camera.calit2.net
CARMA/WebCARMA	Pipeline for short, unassembled reads for species composition and genetic potential of microbial datasets	Krause et al. (2008) and Gerlach et al. (2009) http://webcarma.cebitec.uni-bielefeld.de
CLC Genomics workbench	Commercial pipeline, for almost all the NGS platforms (SOLiD, Ion Torrent, Illumina, and Roche 454). NGS data analysis for genomics, transcriptomics, and epigenomics	http://www.clcbio.com/products/clc-genomics-workbench/
CoMET (Comparative METagenomics)	Platform for comparative metagenomics of big datasets for metagenomic short reads	Lingner et al. (2011) http://comet.gobics.de
DAMBE5 (Data Analysis for Molecular Biology and Evolution)	Software package for descriptive and comparative sequence manipulation and analysis	Xia (2013) http://dambe.bio.uottawa.ca
DOTUR (Distance-Based OTU and Richness)	Operational taxonomic units and species richness estimation	Schloss and Handelsman (2005) www.plantpath.wisc.edu/fac/joh/dotur.html
EnGenIUS (Environmental Genome Informational Utility System)	Metagenome research toolset for large (>250,000) sequence reads	Kaplarevic et al. (2008)
EBI metagenomics	Raw sequence data upload for taxonomic and functional analysis	Hunter et al. (2014) http://www.ebi.ac.uk/metagenomics/

(continued)

Table 2.2 (continued)

Software/pipeline/online tool	Application	References/web-page link
EnvDB	Database for most complete and updated catalog of environmental distribution of prokaryotes based on 16S rDNA sequences currently stored in GenBank	Pignatelli et al. (2009) http://metagenomics.uv.es/envDB
GAAS (Genome relative Abundance and Average Size)	Software package for metagenomic sequences to estimate relative genome abundance and average genome length	Angly et al. (2009) http://sourceforge.net/projects/gaas/
Greengenes	Chimera detection, sequence alignment, and taxonomy identification using multiple published taxonomies	McDonald et al. (2012) http://greengenes.lbl.gov
IMG/M (the Integrated Microbial Genomes)	Comparative analysis of publicly available genomes, a community resource	Markowitz et al. (2012) http://img.jgi.doe.gov
KEGG (Kyoto Encyclopedia of Genes and Genomes)	Functional database for genomic, chemical, and systemic functional analysis	Kanehisa et al. (2014) http://www.genome.jp/kegg/kegg1.html
MEGAN (MEtaGenome Analyzer)	Comparative metagenomic and metatranscriptomic data analysis using NCBI taxonomy, and SEED, COG, and KEGG for functional classifications	Huson and Weber (2013) http://www-ab.informatik.uni-tuebingen.de
MetaBioME	Identification of novel homologues for known enzymes (of high commercial use) in completed bacterial genomes and metagenomic datasets	Sharma et al. (2010) http://metasystems.riken.jp/metabiome/
METAGENassist	Comparative metagenomic web server for amplified 16S rRNA and shotgun metagenomic data	Arndt et al. (2012) http://www.metagenassist.ca
MG-RAST (Metagenomics Rapid Annotations using Subsystems Technology)	Freely available and commonly used, automated pipeline for phylogenetic and functional analysis of metagenomes	Meyer et al. (2008) http://metagenomics.anl.gov/
MoCAT	Metagenomic sequence assembly and gene prediction pipeline for small and large metagenomic data produced by the Illumina sequencing	Kultima et al. (2012) http://www.bork.embl.de/mocat/
mothur	Comprehensive software to analyze microbial community sequence data	Schloss et al. (2009) http://www.mothur.org/

NCBI (the National Center for Biotechnology Information)	Community server for gene and function annotation of the metagenome and metatranscriptome data	http://www.ncbi.nlm.nih.gov/
PAST (PAleontological Statistics)	Statistical software for univariate and multivariate statistics	Hammer et al. (2001) http://folk.uio.no/ohammer/past/
PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States using 16S)	Functional composition prediction of metagenome data using gene data and reference genome database	Langille et al. (2013) http://picrust.github.com
QIIME (Quantitative Insights Into Microbial Ecology)	Comparative data analysis of microbial communities using 16S and metagenomic data	Kuczynski et al. (2012b) http://qiime.org
RDP (the Ribosomal Database Project)	Website for rRNA gene sequence data analysis	Cole et al. (2009) http://rdp.cme.msu.edu/
RITA (Rapid Identification of High-Confidence Taxonomic Assignments)	Accurate classification of very short sequences using different classification groups and varying degrees of confidence	MacDonald et al. (2012) http://kiwi.cs.dal.ca/Software/RITA
SEED	Genome annotations for multiple genomes for developing de novo annotations	Overbeek et al. (2005) http://pubseed.theseed.org/
STAMP (STatistical Analysis of Metagenomic Profiles)	Statistical software for statistical tests and analyzing taxonomic and functional profiles	Parks et al. (2014) http://kiwi.cs.dal.ca/Software/STAMP
WebMGA	Fast metagenomic data analysis web server for taxonomic and functional data analysis	Wu et al. (2011) http://weizhongli-lab.org/metagenomic-analysis/

2.6 Microbial Genomes, Metagenomics, and Metatranscriptomics

Many draft genomes, metagenomes, and metatranscriptome are now available because of these advancements and have enabled researchers to explore microbes at both the genomic and functional levels. *Pseudomonas mediterranea* strain CFBP 5447T produces cyclic lipopeptides and medium-chain-length polyhydroxyalkanoates (PHA) from various carbon sources and is known to convert biodiesel-derived glycerol to PHA. Cyclic lipopeptides such as cormycin A and corpeptins are produced by this strain, with antimicrobial and biosurfactant activities. The genome sequence of this species will enable applications at industrial level with cost-effective strategies for bioprospecting (Licciardello et al. 2014; Solaiman et al. 2005). *Halomonas hydrothermalis* MTCC 5445, a halophilic, Gammaproteobacteria, can grow in high salt (5 % NaCl) concentrations and produces polyhydroxybutyrate (PHB). *H. hydrothermalis* accumulates PHB intracellularly (75 % of dry weight) and can utilize waste glycerol from biodiesel to produce PHB. It can ferment glucose, sucrose, maltose, fructose, and ribose sugars. The whole-genome sequence of this species will aid in the efforts of high industrial production of PHAs (Bharadwaj Sv et al. 2015). Due to high commercial value of *Streptomyces*, multiple genomes of this group have been completely sequenced, and numerous are ongoing. Streptomycetes produce antibiotics and bioactive and biological compounds for industrial and biotechnological applications. *Streptomyces exfoliatus*, DSMZ 41693, contains genes for poly-3-hydroxyoctanoate depolymerase, used for the synthesis of (*R*)-3-hydroxyalkanoic acids. The genome sequence of this strain has revealed many genes for biosynthesis of different metabolites including polyketides and terpenes. *S. exfoliatus* can degrade poly-3-hydroxyalkanoates, poly-3-hydroxybutyrate, and poly-3-hydroxyoctanoate. This strain is a potential novel producer of (*R*)-3-hydroxyalkanoic acids and aids in degradation of bioplastics. The genes encoding for cellulases, amylases, xylanases, chitinases, proteases, lipases, and esterases have been detected in this strain. This strain can serve as a suitable candidate for bioremediation processes due to the presence of heavy-metal-resistance genes (Martinez et al. 2014). *Haloarcula hispanica* is a halophilic archaean, isolated from a solar saltern, and has been used for isolation of novel haloviruses. The genome sequence of strain N601 can improve our understanding of the physiology, genome organization, and virus-host interactions within different species of the group. Many haloarchaeal species (*Haloferax*, *Haloarcula*, *Natrialba*, and *Haloquadratum*) can synthesize short-chain-length PHAs (SCL-PHAs), a large family of biopolymers with desirable biodegradability, biocompatibility, and thermoplastic features (Ding et al. 2014). Two PHA-producing strains of genus *Erythrobacter* are sequenced recently, *E. longus* strain DSM 6997 and *E. litoralis* strain DSM 8509. They represent aerobic anoxygenic phototrophic bacteria (AAPB), and the draft genomes are available as type strain of *Erythrobacter* genus. These strains are characterized as Gram-negative rods and have the ability to store PHA as a carbon source. Both the strains are slightly halophilic and can play a significant role as potential industrial PHA producers (Wang et al. 2014). Genome sequence of *Bacillus cereus* strain tsu1, isolated on an agar-cellulose plate, contains genes for cellulose degradation and biosynthesis pathways of PHB. *B. cereus* tsu1 can degrade cellulose and can produce valuable biopolymers. Genome sequence of this strain will provide significant strategies for sustainable bioenergy developments and reduced reliance on petroleum-based plastics (Li et al. 2014). *B. cereus* strain A1 is capable of hydrogen production and utilizing starch and starch wastewater. Strain A1 is a facultative anaerobe isolated from anaerobic digestion reactor. This strain can hydrolyze starch and can ferment glucose into hydrogen, and end products are acetate and ethanol (Zhang et al. 2014). *Rhizobium lupini* strain HPC(L) was isolated from saline desert soil. This train HPC(L) belongs to Alphaproteobacteria class, Gram-negative soil-inhabiting organism, and can grow in minimal media supplemented with $CaCO_3$ carbon source. Identification of the PHB synthesis gene cluster supports the carbon-limiting stress under desert conditions (Agarwal and Purohit 2013). *Brevundimonas naejangsanensis* strain B1 is a facultative anaerobic bacterium, can ferment sugars, and is capable of high-efficiency hydrogen production. The genome of this strain can provide insights into mechanisms of high-yield hydrogen production in this strain (Su et al. 2014). *Halanaerobium saccharolyticum* subsp. *saccharolyticum* strain DSM 6643T is a halophilic anaerobic fermentative bacterium capable of efficient hydrogen production (Kivisto et al. 2013). *Clostridium intestinale* strain URNW is a Gram-positive, mesophilic, anaerobic, spore-forming bacterium closely related to the butyrate-producing hydrogen producers, such as *C. intestinale*, *C. acetobutylicum*, *C. perfringens*, *C. butyricum*, and *C. beijerinckii*. This strain is a potential candidate for the production of hydrogen or ethanol (Lal et al. 2013b). *Caloramator celer* strain JW/YL-NZ35 is a strictly anaerobic bacterium capable of producing hydrogen and ethanol and converts sugars to H_2, CO_2, acetate, ethanol, and formate. This strain is able to produce hydrogen at high yields in a natural microbial community and in pure culture (Ciranna et al. 2013). *Clostridium pasteurianum* NRRL B-598 is a heterofermentative, rod-shaped bacterium with versatile sugar-fermenting and proteolytic abilities with hydrogen and ethanol production (Kolek et al. 2014). *Clostridium perfringens* is a Gram-positive strict anaerobe that ferments a vast range of carbohydrates and produces acetate, butyrate, lactate, ethanol, hydrogen, and carbon dioxide of vast industrial applications. The genome sequence will help to identify genes that inhibit and promote hydrogen production in this species (Wong et al. 2014b). *Sulfurospirillum* sp. strain MES was isolated from a metagenome of microbial electrosynthesis system (MES) producing acetate and hydrogen. The reported genome predicts the potential of denitrification in this species. Phylogenetically *Sulfurospirillum* sp. strain MES is closely related to the cultured *S. cavolei* strain Phe91 and uncultured/enrichment culture clones from wastewater-activated sludge and petroleum reservoirs (Ross et al. 2015). *Azotobacter vinelandii* mutant strain CA6 displays different characteristics compared to its originating wild-type parent strain, CA (such as altered molybdate uptake, slow growing, tungstate tolerance, and production of hydrogen gas). The complete genome sequences of these strains may provide a genetic basis for these distinct mutant phenotypes (Noar and Bruno-Barcena 2013). *Clostridium* sp. strain Ade.TY is a new biohydrogen-producing species, accompanied by the production of acetate, butyrate, lactate, formate, ethanol, and butanol of industrial applications. The genome sequence of this strain may provide insights for efficient biohydrogen production and gene interactions, if any, involved in the process (Wong et al. 2014c). *Clostridium bifermentans* strain WYM is an effective biohydrogen producer, and its genome annotation may provide insights into the metabolic pathways involved in efficient biohydrogen production. This strain ferments a wide range of carbohydrates, glucose, fructose, maltose, glycerol, and sorbitol, and produces acetate, lactate, ethanol, hydrogen, and carbon dioxide (Wong et al. 2014a). *Clostridium pasteurianum* strain ATCC 6013 is an anaerobic Gram-positive model organism for the study of nitrogen fixation and clostridial ferredoxins. This strain can ferment waste glycerol and produce biodiesel, bioethanol, and hydrogen gas. The genome sequence of this strain was generated using a modified (hybrid) next-generation sequencing method (Pyne et al. 2014). *Clostridium termitidis* strain CT1112 is a cellulolytic bacterium that can utilize sugars and cellulose and can produce hydrogen, carbon dioxide, acetate, formate, lactate, and ethanol (Lal et al. 2013a). *Thermoanaerobacterium aotearoense* SCUT27 is a thermophilic, strict anaerobe and can utilize xylan, dextran, glucose, cellobiose, xylose, mannose, galactose, and arabinose. This strain has been metabolically engineered as a biocatalyst for the ethanol, hydrogen, and L-lactic acid production (Ai et al. 2014). Recently, the NGS technology using SOLiD system was applied to characterize the biogas-producing microbial community and explore the functional and taxonomical complexity from a composite microbial consortium developing in a biogas fermenter. They identified that both the microbiological diversity and the regulatory role of the hydrogen metabolism appear to be the driving forces optimizing biogas-producing microbial communities. They further suggested a biogas-producing consortium can be determined through the use of metagenomic approach, which can contribute to significant progress in the efficacy and economic improvement of biogas generation (Wirth et al. 2012). Similar studies were carried out to study methane-producing microbial community in solid-state biogas reactor using the Roche/454 GS-FLX Titanium pyrosequencing platform. They identified several novel microbes with varied functional capabilities, which affects the biogas reactor performance (Li et al. 2013). In another study, metagenomes of four parallel biogas reactors digesting fish waste and cow manure was studied, comparing the initial inoculum at day 0 with day 59. During the start phase it operated stably, and important Archaeal and Bacterial species degrading the protein-rich substrate were identified, and in particular microbes involved syntrophic in methane production seemed to be important for the operation of the biogas plant (Solli et al. 2014). Recently, 12 uncultured bacterial near complete genomes with relative abundance as low as 0.06 % were reconstructed from activated sludge metagenomic datasets, thus highlighting the power of metagenomics to discover novel microorganisms (Albertsen et al. 2013).

Metatranscriptomics has offered a new insight into how the microbial community (metagenome) responds to changes in environmental conditions at a functional level. One of the first studies using a metatranscriptome approach of a biogas-producing microbial community from a production-scale biogas plant identified Euryarchaeota and Firmicutes as dominant phyla (Zakrzewski et al. 2012). A metatranscriptomic study has identified that *Micromonospora* species dominates the expression of lignocellulolytic enzymes in the thermophilic community, and this genus is a promising source of lignocellulolytic enzymes for industrial-scale production (Simmons et al. 2014). Metatranscriptomic analysis of *Alviniconcha* (genus of deepwater sea snails) symbionts revealed key differences among symbiont types and in the expression of genes relating to energy metabolism, hydrogen, and sulfur oxidation (Sanders et al. 2013). Metabolic pathways and cellulose-degrading enzymes, endo-β-1,4-glucosidase and β-1,4-glucosidase, were identified by transcriptome analysis (Leonardo et al. 2011). Some studies have coupled metagenomics and metatranscriptomics to complement "microbial cell factories'" abundance to functions. Analyzing metatranscriptomes of microbial cell factories for biofuel production is a powerful technique for discovering potentially robust lignocellulolytic enzymes. Comparative metatranscriptomics have been previously performed on lignocellulose degradation with a focus on termite gut, soil microbiota, and lignocellulolytic enzymes (He et al. 2013). Using these approaches with metagenomic data, novel genes and genomes can be discovered for exploration of "microbial cell factories."

2.7 Conclusion

The culture-independent techniques (metagenomics and metatranscriptomics) coupled with the advanced high-throughput sequencing technologies and data analysis have revolutionized the field of microbiology. It has enabled researchers to explore the microbial genomes and biological process and bioprospecting the "microbial cell factories" for biotechnological applications and human welfare.

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Ravi Ranjan Ph.D. completed his PhD in Molecular Microbiology from University of Delhi and CSIR-Institute of Genomics and Integrative Biology (IGIB), Delhi. He is a life member of the Association of Microbiologists of India (AMI). Presently he is a Research Assistant Professor at the University of Illinois at Chicago. His research interests are in the field of environmental microbiology and genomics, microbiome, and host-pathogen interactions.

Asha Rani Ph.D. received MSc in Biomedical Sciences and PhD in Microbial Biotechnology and Genomics from ACBR University of Delhi and CSIR-IGIB, Delhi. She is presently a Research Assistant Professor at the University of Illinois at Chicago. Her research interests are in the field of environmental and microbial genomics and microbiome in health and disease.

Rajesh Kumar received his MSc and PhD from Department of Botany, University of Delhi. He is presently the Head of the Department of Botany at Hindu College, University of Delhi. His current interests are biological control of *Aspergillus flavus* and identification and characterization of antagonist microbes using 16S rRNA gene sequence analysis.

Ecobiotechnological Approaches: Enrichment Strategy for Improvement of H_2 Production

3

Shantonu Roy and Debabrata Das

Abstract

Hydrogen has been mooted as future fuel on the basis of its carbon neutrality, renewable nature, and highest energy density. In the recent times, its economical production has gained attention. The present chapter deals with a comprehensive insight on dark-fermentative biohydrogen production process. This process is less energy intensive and environmentally benign, and waste materials can be used as substrate. Biochemical insight on hydrogen production via dark fermentation exemplifies the complexity of the process. The maximum H_2 yield of 4 mol H_2 per mol of glucose has been observed when fermentation followed a solely acetate pathway. The potential H_2-producing microorganisms are present in various natural and man-made habitats such as sewage sludge, anaerobically digested sludge, animal waste, compost, hot springs, oceanic sediments, and soil. There are many advantages of working with mixed consortia, viz., presence of different hydrolytic enzymes, better oxygen tolerance, etc. Various pretreatment processes have been explored to enrich H_2-producing microbes. A detailed pretreatment processes, viz., chemical, physical, combined treatments, etc., creates a selection pressure, which could effectively alter the microbial dynamics of the mixed culture. Molecular techniques like polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), terminal restriction fragment length polymorphism (T-RFLP), ribosomal intergenic spacer analysis (RISA), quantitative PCR (qPCR), single-strand conformation polymorphism (SSCP), fluorescence in situ hybridization (FISH), and fluorescence-activated cell sorting (FACS) could be used for advanced and rapid microbial characterization. Use of cheap, renewable, and easily available raw materials could bring down the production cost of bioH_2.

S. Roy • D. Das (✉)
Department of Biotechnology, Indian Institute of
Technology, Kharagpur 721 302, West Bengal, India
e-mail: shantonu20@gmail.com; ddas.iitkgp@gmail.com;
ddas@hijli.iitkgp.ernet.in

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3.1 Introduction

Over the years, the global economy has been powered with fossil fuels. These fuels are nonrenewable in nature, and very limited quantity is available to human population. The reservoirs of these fossil fuels will be exhausted sooner or later. The emission of greenhouse gasses on usage of fossil fuels has posed a serious threat to environment. The accumulation of greenhouse gases has led to increase in global temperatures. Global warming has shown its effect in terms of changes in weather pattern, melting of polar ice, etc. These changes have lead to habitat destruction, raise in sea level, formation of chemical smog, etc. For a healthier future, mankind urgently needs a fuel that would be renewable in nature and would be carbon neutral. Hydrogen as fuel could be a global panacea to fulfill energy demands (Das and Veziroglu 2008). When combusted as a fuel, hydrogen produces only water as a product which makes it an environmentally friendly fuel (Hawkes et al. 2002). At present, hydrogen is produced through various ways such as thermochemical, electrochemical, biological, etc. Biological route of H_2 production has pitched itself as a renewable technology, which not only serves the purpose of energy generation but also helps in organic waste management. Dark-fermentative H_2 production has shown production highest rate among all the biological routes (photofermentation and microbial electrolysis cells). Dark-fermentative H_2 production has been reported by varieties of microbial species. Microbes belonging to obligate/facultative anaerobe growing at mesophilic/thermophilic temperatures have been reported as H_2 producers. Well-known mesophilic species reported for H_2 production are *Enterobacter aerogenes*, *E. cloacae*, *Citrobacter*, *E coli*, *Bacillus coagulans*, *Klebsiella oxytoca*, *Clostridium acetobutylicum*, etc. (Kalia et al. 1994). The overall H_2 yields of these microbes were still less than the theoretical limit (4 mol per mol glucose). Moreover, threat of contamination and competition with non-H_2 producer always lurks on handling these microorganisms in large-scale operation. Such limitation of mesophilic dark fermentation has propelled research toward thermophilic bio-H_2 production. Thermophilic bio-H_2-producing microorganisms are well known for their high yields. Furthermore, these thermophiles have a vast repertoire of hydrolytic enzymes. These hydrolytic enzymes prove very useful in degrading complex carbohydrate sources such as lignocellulosic biomass. Moreover, operation at thermophilic temperatures (55–70 °C) does not allow contamination by pathogens. Extremophiles such as *Thermotoga neoplanita* are well known for its H_2 yield, which is same as theoretical limit. More popular thermophilic bio-H_2-producing microorganism belong to *Thermoanaerobacterium* sp. Another thermophilic bio-H_2-producing microorganism which is well known for its ability to utilize cellulose as feedstock is *Caldicellulosiruptor* sp. Moderate thermophile capable of degrading cellulose and producing H_2 is *Clostridium thermobutyricum*. Thus, thermophilic microbes could be used to utilize high-temperature industrial effluents which have high chemical oxygen demand (COD). The demerit of thermophilic H_2 production is the requirement of energy to maintain high temperature for production. Such problems could be overcome by using insulated reactors, which allow minimal radiation energy losses. Most of the dark-fermentative H_2 productions have been reported on using simple sugars/soluble fermentable sugars. The advent of concept of "organic waste to energy" has driven the concept of development of a mixed microbial consortium that would harbor a symbiotically associated different group of bacteria. A single group of bacteria might not have all the hydrolytic enzymes required for hydrolysis of complex organic compounds like cellulose. The characteristic bacteria of enriched mixed consortium would have the ability to produce hydrolytic enzymes. These hydrolytic enzymes thus help in solubilization of complex carbohydrates present in the organic waste. The soluble fermentable sugars could be then utilized for hydrogen production. The natural microbial flora consists of different types of microbes such as H_2-producing bacteria, H_2-consuming bacteria; methanogenic microorganisms; acetoclastic electrogens; etc. To select H_2-producing microbes among the mixed microbial population is regarded as enrichment of culture. In enrichment process, artificial selection pressure was applied that would selectively promote H_2-producing bacteria and eliminate non-H_2 producers. Various pretreatment processes had been explored for enrichment processes. Different methods, i.e., physical (heat, freezing/thawing, ultrasonic, ultraviolet) and chemical (acid, alkali, organic), were commonly used pretreatment methods (Fang and Lin 2002). One of the advantages of the development of a functional microbial consortia is the presence of symbiotically associated microbes (Kumar et al. 2014). These microbes might produce hydrolytic enzymes which were otherwise absent in the principle H_2-producing microorganisms.

Properties of an enriched mixed consortium could be established with the help of "metagenomics" approaches (José and Thorsten 2007). It is a novel genomics tool that could provide a vital information on the presence of potential H_2 producers in the enriched mixed consortium. Techniques such as ribotyping followed by denaturing gradient gel electrophoresis (DGGE) could help in mining the information regarding the microbial profile of the mixed culture (Fig. 3.1). The present paper would also explore other molecular biology tools to understand the microbial population profile in a mixed consortium. Thus, using suitable enriched culture could prove handy in utilizing complex organic wastes (proteins, lipids, complex cellulosic, and starchy materials) for a maximum energy generation.

3.2 Biochemical Insight via Dark-Fermentative Hydrogen Production

Hydrogen is known to be produced by anaerobic (either obligatory or facultative anaerobes) bacteria (Nandi and Sengupta 1998). Various substrates have been explored for H_2 production, viz., carbohydrates, sugars, proteins, and lipids. The theoretical yields of fermentative hydrogen have been estimated using glucose as substrate. The fate of glucose is that it enters into glycolytic pathway and is converted to acetyl CoA. The fate of acetyl CoA decides the overall theoretical yields. The maximum H_2 yield of 4 mol H_2 per mol of glucose has been observed when fermentation followed solely acetate pathway (Solomon et al. 1995). Equation 3.1 shows that the free energy change on conversion of glucose to H_2 and acetate is spontaneous reaction:

$$\begin{aligned}&C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH\\&+2CO_2 + 4H_2\\&\left(\Delta G^\circ = -206.3\,\text{kJ mol}^{-1}\right)\end{aligned} \tag{3.1}$$

whereas if fermentation follows butyrate pathway, then the maximum theoretical yield of 2 mol of H_2 per mol glucose consumed was observed (Eq. 3.2) (Thauer et al. 1977):

$$\begin{aligned}&C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2CH_2COOH\\&+2CO_2 + 4H_2\\&\left(\Delta G^\circ = -254.8\,\text{kJ mol}^{-1}\right)\end{aligned} \tag{3.2}$$

Equation 3.3 shows that if microorganism follows EMP metabolic path, then the glucose via metabolism can produce 2 mol of hydrogen along with subsequent regeneration of the produced NADH as shown in Eq. 3.4:

$$\begin{aligned}&C_6H_{12}O_6 + 2NAD^+ \rightarrow 2CH_3COCOO^-\\&+4H^+ + 2NADH\\&\left(\Delta G^\circ = -112.1\,\text{kJ mol}^{-1}\right)\end{aligned} \tag{3.3}$$

$$NADH + H^+ \rightarrow NAD^+ + H_2 \tag{3.4}$$

The principle metabolic intermediate associated with H_2 production is acetyl CoA. The fermentable sugar via glycolytic pathway produces pyruvate which is further converted to acetyl CoA. Depending upon the fate of acetyl CoA, the theoretical maximum hydrogen yield would vary from 4 to 2 mol per mol of glucose. The acetyl CoA is produced via two prospecting pathways represented in Eq. 3.5 or 3.6:

$$\begin{aligned}&\text{Pyruvate} + \text{CoA} + Fd_{ox} \rightarrow \text{acetyl CoA}\\&+CO_2 + Fd_{red}\left(\Delta G^\circ = -19.2\,\text{kJ mol}^{-1}\right)\end{aligned} \tag{3.5}$$

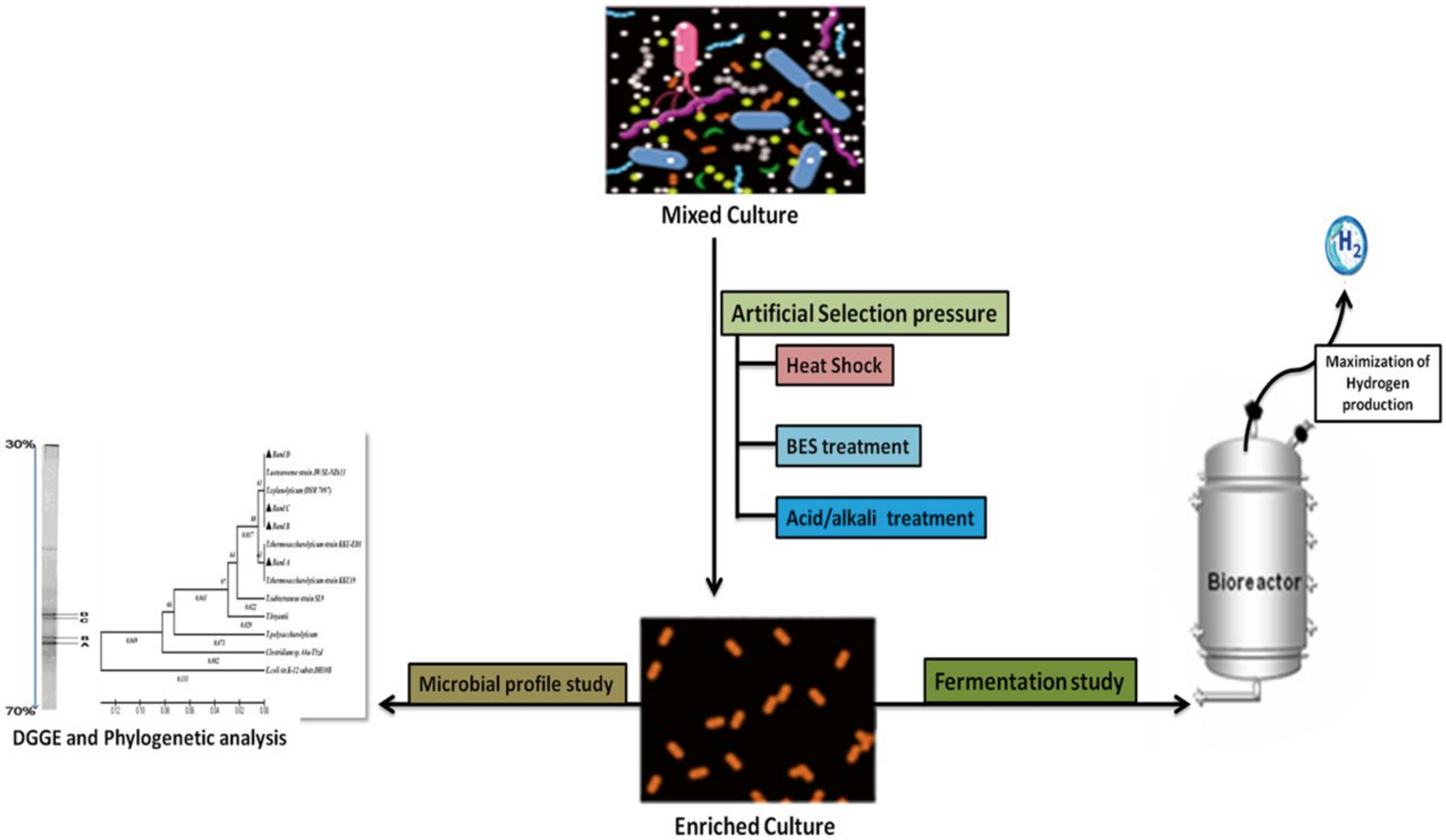

Fig. 3.1 Conceptualization of enrichment process for maximization of H_2 production and molecular approaches for microbial community analysis

$$\begin{aligned}\text{Pyruvate} + \text{CoA} &\rightarrow \text{acetyl CoA} \\ &+ \text{formate} \left(G^{o} = -16.3\,\text{kJ mol}^{-1} \right)\end{aligned} \tag{3.6}$$

Enzyme pyruvate ferredoxin oxidoreductase catalyzes the reaction (Eq. 3.5). The ferredoxin is a coenzyme that functions as electron acceptor (Uyeda and Rabinowitz 1971). The ferredoxin oxidoreductase has been reported in obligate anaerobes, in facultative anaerobes, and also in cyanobacteria (Bothe et al. 1974). The fate of acetyl CoA could lead to either acetate or butyrate production. In both cases, mole of ferredoxin is reoxidized to produce one mole of H_2 catalyzed by hydrogenase enzyme. One extra mole of hydrogen is produced if acetate is the sole metabolic end product. The NADH generated during glycolysis will be used to reduce H^+ ion to H_2, thereby regenerating itself to NAD^+. If the NADH pool is used solely for the oxidation of acetoacetyl CoA to butyrate, then theoretical maximum H_2 yield of 2 mol per mol of glucose has been proposed. But in general, microorganism shows mixed acid fermentation where both acetate and butyrate would be produced. In such case, the H_2 yield would be in between 2 and 4 mol per mol of glucose consumed. Such biochemical pathway is archetypal for *Clostridium butyricum* and *C. pasteurianum* (Daesh and Mortenson 1967; Jungermann et al. 1973). Equation (3.6) shows another pathway of acetyl CoA generation. In this case, there is concomitant production of formate (Neidhardt et al. 1987). This reaction is catalyzed by the enzyme pyruvate formate lyase (Knappe et al. 1974). Microorganisms belonging to group enterobacteria, such as *Enterobacter aerogenes* and *Escherichia coli*, show abovementioned pathway under anaerobic conditions (Nandi and Sengupta 1996).

3.3 Enriched Mixed Culture Development

When large-scale production of H_2 is considered, use of mixed cultures is recommended. The sole reason behind is due to the fact that there is no prerequisite of medium sterilization during operation, thereby decreasing overall cost. Moreover, many wastewater could be used as feedstock for H_2 production (Valdez-Vazquez et al. 2005a, b). The potential H_2-producing microorganisms are present in various natural and man-made habitats. They are found in prominence in sewage sludge, anaerobically digested sludge, enriched sludge, animal waste, compost, hot springs, oceanic sediments, and soil (Ueno et al. 1995; Sparling et al. 1997; Chen et al. 2002; Lin and Lay 2005). The major disadvantage of working with mixed consortia is the chances of dominance of non-H_2-producing microorganisms, methanogens, H_2-consuming microorganisms, homoacetogens, and lactic acid-producing bacteria. Dominance of these microbes thus could lead to decrease in H_2 yields. The need of the hour is to develop strategy to enrich the mixed consortia with H_2-producing microbes. Such enrichment process provides the selection which eventually leads to dominance of H_2-producing microorganisms. Various strategies have been employed for enrichment targeting elimination or suppression of methanogens and H_2-consuming microorganisms. Pretreatment techniques are applied to mixed culture which permits selective enrichment of suitable bacteria. The preparation of H2-producing seed is based on the physiological difference between H_2-producing and H2-consuming bacteria (Zhu and Beland 2006). The properties such as formation of spores under unfavorable conditions (high temperature, extreme acidity, and alkalinity) are the characteristic features of H_2-producing microbes. Methanogens do not posses spore-forming ability. Thus, subjection to adverse condition could be used for selecting H_2 producers as H_2 nonproducers would get eliminated. Therefore, competitive growth and coexistence of other bacteria could be prevented on using such pretreatment methods. The salient features of pretreatment are (Kim et al. 2003; Zhu and Beland 2006)

- Facilitate hydrolysis of complex organic fraction.
- Reduces the effect of rate-limiting step.

The ecological niche where the naturally habituating microbes are present provides all the necessary growth conditions. Mimicking those conditions in laboratory is a challenge. Selection of inoculum from such habitat is critical (O-Thong et al. 2011). The knowledge of nutritional demand, microenvironment, etc., is important to harness the true potential of these organisms (Venkata Mohan et al. 2005). Pretreatment process helps in enriching suitable microflora for improved H_2 production.

The following are the different pretreatment procedures used for enrichment of mixed culture:

- Heat shock treatment
- Chemical treatment
- Acid treatment
- Alkaline treatment.
- Oxygen shock treatment
- Load shock treatment
- Infrared treatment
- Freezing
- Cocktail of treatments

The decrease in start-up time and overall efficiency could be improved by preparing an efficient seed culture (Hawkes et al. 2002).

3.3.1 Heat Shock Treatment

Exposure to high temperature for short period of time and then cooling it to ambient temperature is regarded as heat shock treatment (HST). This technique thus promotes growth of spore-forming bacteria and eliminates non-spore formers (Ueno et al. 1996). The inability to form spores by methanogens and non-spore-forming bacteria at high temperature thereby helps in selective enrichment, whereas H_2-producing microbes such as *Bacillus* and *Clostridium* sp. produce spores in response to HST (Lay et al. 2004). Therefore, the final outcome of HST is a mixed consortium enriched with H_2-producing microbes, whereas methanogens are eliminated. The parameters governing HST depend upon temperatures ranges (80–104 °C) and time of exposure (15–120 min). During HST, the vegetative cells of non-spore-forming microorganisms are killed. These vegetative cells might encompass H_2 consumers, methanogens, non-H_2 producers, etc., but in this process it would also kill H_2-producing microbes who cannot form spores such as *Enterobacter* sp., *Citrobacter* sp., *Bacillus coagulans* sp., etc. (Watanabe et al. 1997). Thus, HST is preferentially suitable for clostridial species as they have the spore-forming ability. The efficiency of H_2 production is even reduced in some reports where HST was used as pretreatment technique (Kraemer and Bagley 2007). HST sludge showed poor stability for H_2 production, and a repeated HST was required to keep reactor performance uniform (Duangmanee et al. 2007). HST has proved to be an effective method for inducing H_2 production in mesophilic batch fermentation (Han and Shin 2004).

3.3.2 Acid Treatment

The pH range (6.8–7.2) has been considered the most favorable for methanogenesis. On the other hand, acetogenic H_2 producers grow over a wide range of pH (Oremland 1988). Methanogenic activity can be effectively suppressed if low pH harboring growth conditions were maintained (Fang et al. 2002a, b). Treatment of native inoculum with acid effectively represses the growth of H_2-consuming microbes. Moreover, harsh/adverse condition such as low pH promotes endospore formation in spore-forming H2-producing bacteria (Chang et al. 2002). During acid pretreatment, pH 3 or low was found suitable (keeping time of exposure 24 h). For pH adjustment, HCl and orthophosphoric acid are employed. Neutralization of pretreated inoculum can be done using NaOH. Formation of salts such as NaCl and Na_2PO_4 during neutralization could also play a crucial role in influencing the microbial profile. Microbes labile to osmotic changes created by accumulated salts might also get repressed during acid pretreatments.

3.3.3 Load Shock Treatment (LST)

Another physical pretreatment employed to enrichment of inoculum is load shock treatment (Fang et al. 2002a, b). In this process the seed culture is subjected to an environment where the volumetric organic load is changed rapidly by increasing the dilution rate. Slow-growing microorganisms will separated out from that of fast growing. Since no physical or chemical treatment was applied, this technique could be more promising than HST as it facilitates presence of diverse group of microbes in the seed culture. Moreover, rapid change in volumetric organic load could also lead to accumulation of organic acids thereby resulting in decrease of pH from 5.5 to 4.6. Thus, LST could eventually eliminate methanogens (Fang et al. 2002a, b). When compared to other pretreatment processes, viz., to base, acid, chemical (BESA), and HST methods, it was reported that LST proved more effective in enriching thermophilic H_2-producing culture (O-Thong et al. 2009).

3.3.4 Chemical Treatment

Many chemicals have been explored as suppressors of methanogens and non-H_2 producers. Iodopropane, acetylene, and 2-bromoethanesulfonic acid (BESA) are few well-known chemical agents used for pretreatment.

3.3.4.1 Treatment with BESA

The mode of action of BESA suggests that it is structurally analogous to coenzyme-M which is required by methanogens to produce methane. Moreover, BESA is chemically inert and do not disturb H_2-producing acidogen (Zhu and Beland 2006). In addition, there are some reports regarding side effects of BESA. It was found that BESA could hamper acetate-producing process. In long-term operation, supplementation of feed with BESA is not feasible, and there is high chance of development of BESA-resistant mutants (Sparling et al. 1997).

3.3.4.2 Iodopropane Treatment for Enrichment

The mode of action of iodopropane is more contrasting as compared to BESA. Iodopropane acts as a corrinoid antagonist which creates hindrance in the functioning of enzymes responsible for methyl group transfer (Kenealy and Zeikus 1981). Vitamin B12 is associated with such enzymes which play a vital role in cellular metabolism of bacteria. Gram-negative microbes are more susceptible to iodopropane when compared to Gram positive. Iodopropane, being hydrophobic in nature, easily enters through the outer cell membrane.

3.3.4.3 Use of Gaseous Acetylene for Seed Culture Enrichment

Acetylene is another chemical inhibitor which causes a nonspecific inhibition of methanogenesis (Chan and Parkin 2002). On exposure to acetylene, the transmembrane pH gradient gets disrupted in methanogens. As the transmembrane pH gradient is disrupted, a decline in ATP synthesis and methanogenesis could be observed (Sprott et al. 1982). Acetylene also shows its inhibitory effects on H_2-producing eubacterial belonging to genus *Enterobacter* sp., but it does not harm the clostridial species and other H_2-producing microbes (Valdez-Vazquez et al. 2005a). The major advantages of using acetylene for pretreatment of seed culture are given below (Valdez-Vazquez et al. 2005a):

- The process is economically cheap as production cost of acetylene is cheap.
- It does not mount up during and after pretreatment.
- No lag time is seen with seed culture treated with acetylene.
- It is more rapid than other physical and chemical methods.

In some reports, 1 %v/v acetylene was used to treat mesophilic seed culture so as to induce H_2 production from model paper mill wastewater in batch reactors.

3.3.5 Other Treatment

3.3.5.1 Alkaline Pretreatment

Alkaline pretreatment was also explored for suppression of methanogens. Subjection of seed culture to extreme alkaline condition (pH 8.5–12) by using NaOH has shown the suppression of growth of methanogens (Cheong and Hansen 2006). If the efficacy of alkaline pretreatment is compared with heat shock treatment, it is observed that HST completely eliminated methanogenic activity, whereas alkaline pretreatment led to partial suppression of methanogens. Thus, lower yields were reported when alkaline treatment has been used for H_2 production (Mu et al. 2007).

3.3.5.2 Oxygen Stress

Being obligate, non-spore-forming anaerobes, methanogens when exposed to oxygen eventually lead to its death. Conversely, spore-forming *Clostridia* could survive such stress. Moreover, facultative H_2-producing anaerobes face no problem when exposed to oxygen (Oremland 1988). Therefore, forced aeration of seed culture could eliminate methanogens. However, a lower H_2 production rate is reported as compared to HST when oxygen stress has been used as a pretreatment technique. Other miscellaneous treatments were also explored such as freezing and thawing, infrared radiation treatment, mild sonication, etc. (Wang et al. 2003a, b). Application of infrared pretreatment to seed inoculum also inhibits bioactivity of H_2 consumers (Fan et al. 2006).

3.3.6 Combined Treatments

Different pretreatment process has unique and contrasting mode of action toward enrichment of mixed consortia. Each process has its own efficacy regarding the nature of parent inoculum and type of feedstock used as substrate and process conditions (Venkata Mohan et al. 2009). HST acid methods show inferior repression methanogens as compared to BESA treatment and thereby low H_2 production (Zhu and Beland 2006). When industrial-/commercial-scale production of H_2 is considered, implementation of both chemical and HST methods could prove to be economically unattractive (Cheong and Hansen 2006). Another strategy of eliminating methanogens is operation of fermentative system at short hydraulic retention time (HRTs). But elimination of methanogens by culturing acidogenic operation is time consuming as it requires a long acclimatization time (10–30 days) (Yu et al. 2002). Requirement of long acclimatization time was not observed in LST and HST processes. On using wastewater as feedstock, the efficacy of different pretreatment methods toward H_2 production showed the following trend: chemical (BESA) treatment > HST > acid methods > untreated inoculum. The combination of HST and acid pretreatment has helped in the development of an effective and enriched microbial consortia (Hawkes et al. 2007). In addition to the above-mentioned fact on using chemical wastewater as substrate, seed culture subjected to HST showed improved H_2 production as compared to acid treatment. This shows that the efficacy of pretreatment process depends upon the characteristics of the feedstock used, viz., wastewater. Cocktail of acid and chemical (BESA) pretreatment methods showed significant improvement in H_2 production when chemical wastewater was used as feedstock. Another combination of pretreatment process, where HST (100 °C, 2 h) and acid (pH 3, 24 h) were applied to seed culture, showed promising improvement in H_2 production (Venkata Mohan et al. 2008). The combination of multiple pretreatment procedures (chemical, HST, and acid) was applied on distillery-based seed culture, and H_2 production improved significantly (Venkata Mohan et al. 2008). All three methods in combination promoted growth of H_2-producing acidogenic spore-forming bacteria and complete suppression of methanogens. Even LST in combination with controlled pH of 5.5 showed improvement in H_2 production (Zhu and Beland 2006).

3.4 Techniques for Studying the Microbial Community in a Mixed Culture

Different pretreatment processes change the microbial population dynamics of the native seed culture. These H_2-producing microbial floras can

Table 3.1 Comparison of different microbial characterization methods

Microbial characterization technique	Advantages	Disadvantages
DGGE and 16S rRNA seq	Taxonomic identification, large database	Cumbersome and time consuming
T-RFLP	Community profiling, taxonomic identification, high sensitivity, high reproducibility	Expensive, time consuming, variation in experimental and theoretical size of fragment, incomplete digestion, formation of pseudo-TRFs
RISA	Taxonomic identification, denaturation, and restriction digestion not required, high sensitivity	Small database, amplification of shorter template, multiple intergenic spacer region
qPCR	Quantify microbial community at lower concentration, fast simultaneous amplification and quantification of target DNA, microbial composition, and dynamics both can be analyzed	PCR biases, community profiles of several species, laborious and expensive
FISH	Fast and simple, direct observation of uncultured microbes, can be used to measure metabolic activity	In mixed culture background, autofluorescence affects the FISH images, optimization of hybridization condition
FACs	Rapid screening of potential microbes	In mixed culture background, autofluorescence affects the qualitative enumeration in mixed culture

be characterized by a number of ways ranging from conventional biochemical, microscopic approaches to highly specific molecular techniques. The efficacy of enrichment of H_2-producing microbes could be visualized with the help of modern molecular biology techniques. The majority of the commonly used methods to identify the microorganisms are dependent on actively grown culture and their production of secondary materials. Molecular techniques could prove a powerful tool for microbial characterization as it is independent of the cultivable nature of microorganisms (Li et al. 2011). Metagenomics techniques could even characterize uncultured bacteria. All the methods have their own pros and cons (Table 3.1), thus more than one method is required for community analysis.

3.4.1 Ribotyping of 16S rDNA and Denaturing Gradient Gel Electrophoresis (DGGE)

The model to study molecular evolution is based on random evolutionary changes occurring in an organism. The molecular clock is based on a constant rate of change in the sequence of a common polymer, a “molecular chronometer” (Woese 1987). The rRNA approach is advantageous because the rRNA molecules are functionally conserved molecules. The primary structures of 16S rRNA and 23S rRNA molecules are composed of sequence regions of higher and lower evolutionary conservation. Following are the attributes of 16S r DNA that make it a suitable candidate marker for understanding evolution:

- Its sequences are highly conserved.
- High copy numbers are present in the genome.
- Required for cellular functioning.

Conserved region undergoes mutation with slow rate, permitting to deduce relationships among the members of three domains: Bacteria, Eucarya, and Archaea (Woese 1987). The most variable regions in the gene help in characterizing the genus and species level (Giovannoni et al. 1988; von Wintzingerode et al. 1997). Denaturing gradient gel electrophoresis (DGGE) technique had been used for determining the microbial profile of the mixed culture. The difference in melting pattern of double-stranded DNA fragments identical in length is the basis of DGGE. The melting pattern varies under a gradient of denaturation condition. The DNA fragments having greater GC content would remain in double-stranded form until it reach suitable denaturant concentrations. The mobility of dsDNA fragments in the acrylamide gel is faster as compared to denatured DNA molecules. Mixed consortia were used in several studies for biohydrogen production. Identification of microorganisms contributing toward hydrogen production in mixed consortia could be done by using DGGE (Roy et al. 2012).

3.4.2 Terminal Restriction Fragment Length Polymorphism (T-RFLP)

It is a PCR-based technique that could be used for characterization of microbial diversity (Liu et al. 1997). The technique relies on the occurrence of restriction site near to a labeled end of an gene. It targets amplification of 16S rRNA gene using fluorescently labeled primer at 5′ end. Different fluorescent tags that can be used are 6-FAM, ROX, TAMARA, and HEX. The information regarding phylogenetic relationship can be inferred by analyzing of sequences of terminal restriction fragment and comparing it with the databases which include Web-based tools such as TAP, T-Align, PAT, and T-RFMA (Li and Fang 2007). The method is highly sensitive with high reproducibility. The major advantage of this technique is the ability to detect or identify atypical or rare organism within a population. However, when compared to DGGE, T-RFLP is relatively time consuming and not cost effective. Techniques such as T-RFLP, FISH, and 16S rRNA cloning library have been used to study microbial community in lab-scale H_2-producing UASB reactor (Castello et al. 2009).

3.4.3 Ribosomal Intergenic Spacer Analysis (RISA)

It is also a technique which is based on PCR. Here the spacer region present in between 16S and 23S rRNA genes has been targeted. Intergenic spacer regions show heterogeneity in terms of length and nucleotide sequence as compared to the flanking genes. Intergenic spacer region could prove to be a suitable candidate for subtyping bacterial strains because of their heterogeneity. The merit of this technique is that it does not require denaturating gradients and restriction digestion steps. One of the demerit of using ribosomal intergenic spacer is the absence of elaborate database for intergenic spacer region for 16S sequences. The preferential amplification of shorter templates could be one more demerit of this process. Moreover, there could be many ribosomal operons in a genome and a single organism, resulting in more than one signal in response to amplification (Tolvanen and Karp 2011). Due to the abovementioned demerits, this technique is not widely used in profiling of hydrogen-fermenting microflora. In one such report, microbial community profiling was performed using RISA where the H_2-producing reactor was operating under two different hydraulic retention times (Iyer et al. 2004). In another study, fermentative H_2 production was observed under organic loading rates of 0.5–19 g COD/L. h in a 2 L continuous flow reactor. The microbial profile at steady state was studied using RISA (Luo et al. 2008).

3.4.4 Quantitative PCR (qPCR)

Most of the techniques used for microbial quantification target either 16S rRNA gene or other functional genes. PCR-based methods can detect DNA/RNA even at lower concentrations. Many factors could be responsible for inefficiency of PCR-based technique, e.g., depletion of reagent and denaturation of polymerase enzyme with advancement of extension cycles. The abovementioned drawbacks could be overcome by using techniques such as quantitative real-time PCR (qRT-PCR). This technique could be used to detect and quantify microbial community even at availability of lower concentrations of genetic material (Zhang and Fang 2006). Simultaneous amplification and quantification of targeted DNA is one of the greatest advantages of qRT-PCR. Thereby, relatively few copies of the gene in the genomic DNA could be easily quantified. The number of amplified genes from genomic DNA represents the quantitative abundance of specific microorganisms in a given community. The most common targeted DNA sequences used in qRT-PCR for microbial identification is either 16S rRNA gene or some other functional gene. Although other DNA sequences such as 5S rRNA gene, 23S rRNA gene, and 16S–23S rRNA gene interspacer region could also be potential targets for studying cladistics, however, their applications are very limited. The functional gene used for microbial community analysis in H_2 metabolism may be [Fe-Fe] hydrogenase and Ni-Fe hydrogenase. Targeting the Fe-Fe hydrogenase and Ni-Fe hydrogenase separately allows the identification and allotment of the microbes to respective functional groups. The Fe-Fe hydrogenase group exclusively includes only hydrogen producers, whereas Ni-Fe hydrogenase group includes both H_2-producing and consuming microbes. In a bioreactor, where bacterial community structure can change dynamically, the abovementioned functional group identification would help in monitoring the stability of the process. One such report is available where H_2 production has been monitored by observing the level of hydrogenase gene (hyd) expression using real-time PCR (Sá et al. 2011).

3.4.5 Single-Strand Conformation Polymorphism (SSCP)

Single-strand conformation polymorphism (SSCP) can also be used for identification of microbial community. The principle behind this technique is the differential mobility of DNA fragments of same-length technique separates according to their secondary structure. Considering functional Fe-Fe hydrogenase genes as marker, capillary electrophoresis-based single-strand conformation polymorphism (CE-SSCP) method has been used to monitor H_2-producing *Clostridia* population in mixed culture (Quemeneur et al. 2010).

3.4.6 Fluorescence In Situ Hybridization (FISH)

FISH is based on microscopic observation where microorganisms of a specific group or species are identified based on use of specific fluorogenic oligonucleotide probes that bind to specific DNA sequence. FISH offers as an excellent mean of microbial identification without DNA extraction and PCR amplification. Cultivation-independent identification of microbes could be done by using fluorescently labelled rDNA-targeted oligonucleotide (Wanger et al. 2003; Loy et al. 2003). The oligonucleotide probes used in FISH are typically labeled either with one or two fluorescent dyes. In addition to microscopic visualization, this technique can also be used for quantitative estimation of microbes during hydrogen production. The major disadvantage associated with FISH is that it a less-sensitive method and requires 10^5 DNA/RNA copies or greater for effective hybridization. In one such report, the spatial diversity of hydrogen-producing bacteria in granules was studied using the FISH technique targeting 16S rRNA gene. Genera such as *Thermoanaerobacterium*, *Caldicellulosiruptor*, and *Thermoanaerobacterium thermosaccharolyticum* were specifically studied using genus-specific probes (O-Thong et al. 2008). In another study, both RFLP and FISH were used to identify *Megasphaera elsdenii* as a prospecting H_2-producing microorganisms (Ohnishi et al. 2012).

3.4.7 Fluorescence-Activated Cell Sorting (FACS)

Flow cytometry technologies could be used extensively for the understanding and investigation of desired microbial community. The modern, powerful techniques like metabolic engineering, synthetic biology, and evolutionary engineering could be complimented with the use of flow cytometry. By targeting fluorescently labeled 16S rRNA or desired mRNA, FC-FISH (flow cytometry fluorescence-activated cell sorting) could help to identify organisms belonging to a specific group, viz., H_2 formers. The major advantage of FC-FISH is that it could also be used for rapid screening of the microorganism (Wanger et al. 2003).

3.5 Organic Wastes as Feedstock for Biohydrogen Production by Mixed Culture

Cheap, renewable, and easily available feedstock/raw materials are required to bring down the production cost of bio-H_2. Wastewater and solid wastes are the potential feedstocks for H_2 production. Use of mixed culture for H_2 production has several advantages:

- A vivid spectrum of enzymatic machinery would be available on working with mixed culture.
- Prerequisite of sterilization would be minimized.
- Decreased start-up time.

For commercial production of biohydrogen, organic wastes should be used. Most of the studies on bio-H_2 are based on utilization of simple carbohydrates, viz., glucose, sucrose, lactose, and maltose. These simple sugars are expensive, and usages of such raw materials are not economically viable. To address this issue, production of bio-H_2 using different organic wastes as substrate is a cheap and promising approach. There is a relatively high abundance of complex sugars (polysaccharides) in nature. Most of these polymeric sugars (cellulose, hemicellulose, amylase, etc.) are inaccessible to microorganisms (Patel et al. 2010). In order to tap the energy bound in these polymeric sugars, a detailed research is required, targeting the pretreatment and saccharification techniques. Bio-H_2 could be considered as renewable and cheap when its production is based on low-value renewable resources. Many high COD-containing wastes have been explored for bio-H_2 production. This includes municipal solid wastes, cheese whey, distillery wastes, food wastes, etc. In most of the cases, mixed cultures were used for H_2 production.

3.5.1 Organic Fractions of Municipal Solid Wastes

It is one of the most abundant wastes that are produced due to anthropological activity. Organic fractions could be considered as a renewable feedstock for H_2 production as it is rich in nutrients such as polysaccharides and proteins. The H_2 yield was still considerably low using raw sewage sludge (0.16 mg of H_2 g^{-1} of dried solids) (Noike and Mizuno 2000). For the improvement of H_2 yield, various pretreatment methods such as ultrasonic treatment, acidification, sterilization, and freezing and thawing were explored. Boiled sludge (heat treatment) lead to solubilization of nutrients present in raw sludge. Usage of boiled sewage sludge gave 15.64 mL of H_2 g^{-1} DS. Pretreatment techniques such as sterilization and freezing and thawing gave H_2 yield of 47 mL of H_2 g^{-1} of DS (Kotay 2008).

3.5.1.1 Food Wastes

Food wastes posses a great environmental threat as they are high in COD. It consists of about 90 % volatile suspended solids. High organic content makes them suitable feedstock for microbial fermentation. In some studies, the food waste generated from residence has been used for both thermophilic and mesophilic hydrogen production. It was found that thermophilic fermentation gave higher H_2 yield (81 mL H_2 g^{-1} VSS) as compared to mesophilic fermentation (63 mL H_2 g^{-1} VSS) (Chen et al. 2012).

3.5.1.2 Dairy Industry Effluents

Dairy industry effluents typically have high chemical oxygen demand (COD) and biochemical oxygen demand (BOD), which makes them hazardous for the environment if discharged untreated. The organic content of wastewater makes them an ideal contender as feedstock for fermentative bacteria (Orhon et al. 1993). One such report where dairy wastewater has been used as feedstock for H_2 production. Maximum hydrogen production of 5.2 mL H_2 g^{-1} COD was observed (Mohan et al. 2008).

3.5.1.3 Distillery or Alcoholic Beverage Industry Wastewaters

It was stated that approximately 12 L of effluent is generated for producing one liter of ethanol. They are rich in biodegradable organic material which includes fermentable sugars, dextrin, hemicelluloses, resins, and organic acids. These wastewaters have high chemical oxygen demand (COD) (80–160 g L^{-1}). Distillery wastewater has been extensively used for biohydrogen production. In one such study, distillery wastewater was fermented in an anaerobic-sequencing batch biofilm reactor to produce H_2. The maximum hydrogen production of 156.7 L H_2 Kg^{-1} COD was observed (Venkata Mohan et al. 2008).

3.5.1.4 Palm Oil Mill Effluent (POME)

These effluents are generally discharged at very high temperature (<60 °C) which make them suitable for thermophilic dark fermentation. Along with high temperature, these effluents also have high organic content. These properties make them ideal substrate for thermophilic dark-fermentative H_2 production. On an average 0.9–1.5 m^3, POME is generated from 1 ton of palm oil being produced. Using UASB reactor, maximum hydrogen production rate of 4.4 L g^{-1} POME d^{-1} was observed (O-Thong et al. 2009).

3.6 Conclusion

Development of a mixed microbial consortium which would have selectively enriched H2-producing microorganisms could help in utilizing complex organic feedstock. A potential isolate might not harbor all the necessary hydrolytic enzymes that would be required for solubilization/saccharification of complex organic compounds like cellulose. An enriched mixed consortium harbors dynamically stable microorganisms which could eventually provide the required enzymatic pool for hydrolysis of complex substrates. The soluble fermentable sugars released after hydrolysis could then be utilized for hydrogen production. Various pretreatment methods have been developed to suppress unwanted microorganisms present along with potential H_2-producing microorganisms. Physical pretreatment such as heat shock and load shock proved to be more suitable for enrichment. Chemical pretreatments were equally effective, but large-scale applications are not cost effective. Moreover, cocktail of pretreatments showed significant improvement. Many molecular and microscopic techniques have been explored to understand the microbial profile of the mixed consortia. Few of the techniques are very sensitive, such as DGGE, qPCR, RISA, etc. that could identify H_2-producing microbes even if they are present in low quantity. Fluorescence techniques such as FISH and FACS proved to be rapid and sensitive techniques for identification of microorganisms. Enriched mixed cultures have been used for H_2 studies where complex organic residues were used as feedstock. Food, starchy wastewater, and distillery effluents are the promising feedstocks that could be used for fermentative H_2 production. Thus, the path of "organic waste to energy" could be truly realized with the help of enriched mixed cultures.

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Shantonu Roy graduated in Microbiology in 2008 from Vidyasagar University. He received his M.Sc. degree in Biotechnology in 2010 from Jadavpur University. He is currently enrolled in Ph.D. with Indian Institute of Technology, Kharagpur. He has been involved in the biohydrogen production process using extremophiles.

Debabrata Das B.Tech. from Jadavpur University and Ph.D. Biochemical Engineering from Indian Institute of Technology, Delhi. He is presently the Professor-in-Charge of P K Sinha Center for Bioenergy; Professor of Department of Biotechnology. He is a Fellow of National Academy of Engineering (FNAE), Fellow of Institute of Engineers (India) (FIE), Fellow of West Bengal Academy of Science and Technology (FWAST), and Fellow of Biotechnology Research Society of India (FBRSI). He is the Editor-in-Chief of the American Journal of Biomass and Bioenergy. His current interests are biohydrogen, algal biorefineries, and microbial fuel cell.

Megasphaera as Lactate-Utilizing Hydrogen-Producing Bacteria

4

Akihiro Ohnishi

Abstract

Hydrogen is likely to become an important energy carrier in the future. Hydrogen fermentation using obligate anaerobes has attracted much attention as a technique to supply inexpensive hydrogen fuel. Ease of use is important for the construction of a practical hydrogen fermentation system. The robustness of the hydrogen producer must be considered also, and a microbe that will not easily be inhibited by saprophytic bacteria and that has good hydrogen productivity should be chosen. *Megasphaera elsdenii* is a lactate-utilizing, hydrogen-producing bacterium (LU-HPB). It can use lactate as a substrate for hydrogen fermentation, and it is not inhibited by the presence of lactic acid bacteria. Thus, heat shock treatment is not required for stable hydrogen fermentation. This is “blind spot” of hydrogen fermentation. LU-HPB shows promise to improve the overall energy budget in hydrogen fermentation. Other species of *Megasphaera* are also linked to the environment, health, and industrial food production. However, the most popular method for detection of *Megasphaera* is conventional culture, which requires a week or more. In this chapter, we will describe rapid methodologies for detection of *Megasphaera* spp., which will contribute to the industry as well as to the development of future hydrogen fermentation systems.

4.1 Introduction

Increasing global consumption of fuel has driven research toward alternative energy sources (Naik et al. 2010; Sims et al. 2010; Scarlat and Dallemand 2011; John et al. 2011; Saidur et al. 2011; Chu and Majumdar 2012). Hydrogen is a promising energy carrier in the future (Murugesan et al. 2009; Yilanci et al. 2009; Blakey et al. 2011). Solar energy is generated by nuclear

A. Ohnishi (✉)
Department of Fermentation Science,
Tokyo University of Agriculture,
1-1 Sakuragaoka 1-chome, Setagaya-ku,
Tokyo 156-8502, Japan
e-mail: a1ohnish@nodai.ac.jp

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fusion reaction of hydrogen in the sun, but control of the fusion reaction is difficult on the surface of the earth (Bradshaw et al. 2011). Hydrogen is not a primary energy source, except in the sun (Abu-Khader 2009; Balat and Kırtay 2010). Hydrogen is the smallest element, with an atomic weight of 1.0, and it is present in both the lithosphere and the hydrosphere in large quantities in compounds such as water and biomass. It requires more energy to generate molecular hydrogen, or H_2 gas, using solar, biological, or electrical energy sources than is obtained by burning it. This limitation is inevitable by the law of conservation of energy.

Hydrogen is an efficient fuel, with a high energy yield that is two- to fourfold greater than the energy yields of other fuels such as methane, gasoline, and coal. Hydrogen has an energy content nearly threefold higher than that of gasoline, by weight. The lower heating value, by weight, for hydrogen is 123 MJ/kg and that for gasoline is 47.2 MJ/kg, although, by volume, the relationship is reversed (Raju and Khaitan 2012). Liquid hydrogen has a density of 0.07 g/cm^3, whereas gasoline has a density of 0.75 g/cm^3. Hydrogen as energy carrier generates electricity and heat via fuel cell. In these cases, hydrogen is combined with oxygen (which constitutes 20.8 % of the volume of air) to form water. One of the major advantages of hydrogen fuel is the absence of significant pollutant (Claassen et al. 1999). Thus, hydrogen is a promising energy carrier that can act as either storage or a transmission medium, analogous to batteries.

The development of hydrogen fuel cells and hydrogen combustion engines is proceeding at a rapid pace (Hwang 2012; Verhelst 2014). In the alternative energy field, hydrogen has been discussed for the basis of energy infrastructure (Balat and Balat 2009; Suh et al. 2011). However, since hydrogen is not present in significant quantities in nature in pure form, the hydrogen production costs is great. An overview of hydrogen source materials and production processes is presented in Fig. 4.1 (Ball and Wietschel 2009). Hydrogen can be produced from various materials (terrestrial resources and underground resources), using various methods (Eltawil et al. 2009). Source materials include underground resources, such as coal and natural gas, as well as terrestrial resources such as biomass (Kalinci et al. 2009) and water (with energy inputs from generation of electricity, e.g., photovoltaics or wind). Hydrogen may be obtained efficiently from underground resources (such as oil and coal) by partial oxidation with steam reformation; this method will probably remain the most economical. Presently, approximately 95 % of the world's hydrogen is produced from natural gas (Lee et al. 2008; Balat and Balat 2009).

However, accessing these underground resources has a significant environmental impact. In addition, if the hydrogen is used to generate electricity, it provides no advantage over direct use of the underground resources. Wind- and solar-generated electrical power can be used to produce hydrogen by electrolysis of water (Delucchi and Jacobson 2011). However, wind and solar resources are limited by climatic constraints and the environmental conditions required for electrical generation. Biomass is biodegradable organic material as terrestrial resources such as plant, animal, and microbe (Saidur et al. 2011). Biomass, on the other hand, is available in many areas and in many forms, and the collection and transportation of biomass materials are comparatively easy (Vamvuka 2011). Energy is released from biomass when it is consumed, burned, or converted into fuel. A variety of techniques is available to convert biomass to hydrogen, including biological, electrolytic, photolytic, and thermochemical methods (Krishna 2013). Especially the low-value biomass resource, such as biowaste, is possibility of useful hydrogen source.

"Biohydrogen" refers to hydrogen that is biologically produced (primarily by bacteria), for example, by conversion of biomass into hydrogen biofuels (Kırtay 2011). Biohydrogen production has been known for over a century. Investigation into practical means of hydrogen fuel production has been carried out for over 30 years. Of the various possibilities for recovering bioenergy, microbial production of biohydrogen has recently gained significant recognition worldwide due to its high efficiency and environmentally friendly nature. Biohydrogen is

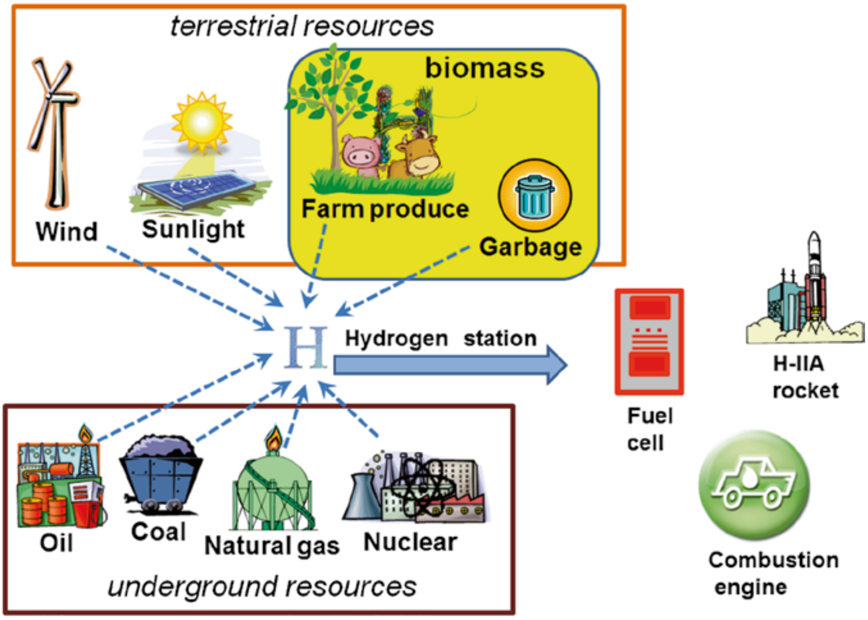


Fig. 4.1 Natural resources and hydrogen production process

hydrogen produced via photo- or dark fermentation process by anaerobic photo- or dark-fermentative microbe (Lee et al. 2010b; Eroglu and Melis 2011; Azwar et al. 2014). Dark- and photo-fermentative microorganisms are metabolically and physiologically diverse, and they therefore generate hydrogen using various mechanisms (Hay et al. 2013). Hydrogen plays a major role in the metabolism of these organisms. Pure cultures have been widely used to study hydrogen producers under dark- and photo-fermentative conditions (Lee et al. 2011; Patel et al. 2012). Out of these possibilities, the greatest possibility for hydrogen generation is through anaerobic dark fermentation. At present, biological hydrogen production is not yet economical, primarily because of the high cost of feed and energy input. The top-ranking technical challenges are identification of industrially cheap raw material, the search for a useful microbe, and development of a stable hydrogen fermentation system (Dovì et al. 2009).

A strain of the genus *Clostridium* has been used as a hydrogen-producing bacterium (HPB) to perform the key fermentative role in a study of hydrogen fermentation. Its ability to generate hydrogen from saccharide is high, and its culture and maintenance are comparatively easy at the laboratory level (Masset et al. 2012). Theoretically, a maximum of 4 mol of H_2 can be generated by dark fermentation of 1 mol of glucose (Hiligsmann et al. 2014). However, the hydrogen yield is primarily affected by the type of metabolite produced as an intermediate during fermentation. To achieve theoretical maximum hydrogen yields, it is important to integrate the dark and light fermentation routes and to exploit various combinations of physiological conditions and substrate utilization (Masset et al. 2012). In dark fermentation, hydrogen and carbon dioxide occur under absence of electron acceptors (Claassen et al. 1999; Kapdan and Kargi 2006). Typical fermentation reactions are

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \qquad (4.1)$$

and

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \qquad (4.2)$$

Compared to pure substrates such as glucose, the use of complex biomass as feed has been considered to be economical (Saxena et al. 2009). The organic compounds provide an electron (and energy) source for the anaerobic bacteria, and hydrogen is a by-product of the anaerobic degradation, effectively maintaining the electron balance involved in anaerobic metabolic processes. The reactions are rapid and do not require sunlight. Therefore, dark fermentation of organic matter by HPB is a biologically efficient and economically feasible process for generating hydrogen (Lee et al. 2011). Thus, microbial hydrogen production may be used to recover unused bioenergy as biohydrogen. Organic waste, in the form of biomass, is an excellent source of hydrogen (Balat and Kırtay 2010). For example, hydrogen fermentation of food waste produces biofuels as hydrogen and simultaneously reduces waste disposal costs. The hydrogen fermentation from industrial waste is attractive interest (Kapdan and Kargi 2006). Microbial conversion of biowaste to hydrogen is a promising area for bioenergy research and development. Thus, generation of hydrogen biofuels by dark fermentation shows potential to have a significant impact on the global economy in future (Balat and Kırtay 2010). However, at present, the conditions and infrastructure required for stable, large-scale hydrogen fermentation are not well established, and new technological developments are required in order to reduce the cost of commercial production of biohydrogen (Show et al. 2012).

Isolating efficient anaerobic microbes from the natural microflora is a promising area of research. And microbial scientists are studying hydrogen fermentation for finding of technical theories (Wong et al. 2014). But experiments are based on hypothetical preconditions, in this field. We think it is a “blind spot.” The most important features are the effects of lactate, lactic acid bacteria (LAB), and the use of pretreatment such as heat shock (Ohnishi et al. 2010).

Some previous studies have shown that lactate as substrate is unsuitable for hydrogen fermentation. Baghchehsaraee et al. (2009) demonstrated that no hydrogen generation was observed with lactate as substrate. From then, lactate has not been used as substrate for hydrogen fermentation (Wang and Wan 2009; Lee et al. 2011). Thus, a hydrogen fermentation system using lactate as the main substrate is no longer used. Reactions of lactate fermentation by LAB are as follows (Montet et al. 2014):

$$\text{Homofermenters; } C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH \quad (4.3)$$

and

$$\text{Heterofermenters; } C_6H_{12}O_6 \rightarrow CH_3CHOHCOOH + C_2H_5OH + CO_2 \quad (4.4)$$

Similarly, mainly *Clostridium* spp. as HPB generate hydrogen from saccharide such as glucose via previous reactions (4.1) and (4.2). Reported phenomenon of reducing hydrogen yield was related with increase of lactate concentration closely. In addition, LAB is present in various places and environmental organic material. (Montet et al. 2014). From these things, it has been thought that LAB is contaminant as hydrogen fermentation inhibitor and substrate competitor. From these conclusions, heat shock of the substrate and/or inoculum have been used for effective hydrogen fermentation. The heat shock treatment for effective hydrogen fermentation is based on the difference of heat tolerance of microbe. Genus *Clostridium* as HPB is spore-forming anaerobic bacteria and their heat tolerance is high. LAB as contaminant of hydrogen fermentation system is mostly nonspore-forming bacteria and their heat tolerance is low. Many researchers reported that heat shock treatment for substrate and/or inocula ensure effective hydrogen fermentation (Table 4.1). Thus, heat shock has been shown to be an ideal pretreatment method for maximizing hydrogen yield. Since LAB and lactic acid are negatively affected for hydrogen fermentation, heat shock treatment is considered essential (Sreela-Or et al. 2011). But, heat shock treatment requires a large energy input. And it is not practical for production of valuable hydrogen fuel.

In this way, the general idea of microbiologic aspect in hydrogen fermentation are as follows: (1) lactate is not a substrate in hydrogen fermentation system, (2) LAB is contaminant in hydro-

Table 4.1 Heat shock treatment condition and inoculum

Temperature (°C)	Time (min)	Inoculum	References
121	30	Waste biosolids	Wang et al. (2003)
105	120	Compost	Khanal et al. (2004)
105	120	Soil	Selembo et al. (2009)
104	120	Compost and soil	Ginkel et al. (2001)
104	120	Soil	Logan et al. (2002)
104	120	Anaerobic sludge	Oh et al. (2003b)
100	120	Anaerobic sludge	Merlino et al. (2013)
100	120	Soil	Tenca et al. (2011)
100	120	Compost	Lay and Fan (2003)
100	60	Anaerobic sludge	Laothanachareon et al. (2014)
100	60	Anaerobic sludge	Sa et al. (2013)
100	60	Anaerobic sludge	Luo et al. (2011)
100	60	Anaerobic sludge	Mohammadi et al. (2011)
100	60	Anaerobic sludge	O-Thong et al. (2009)
100	45	Sewage sludge	Chen et al. (2011)
100	45	Acclimated sludge	Lin and Lay (2004b)
100	45	Acclimated sludge	Lin and Lay (2004a)
100	45	Acclimated sludge	Lin and Lay (2005)
100	30	Anaerobic sludge	Fang, et al. (2006)
100	20	Activated sludge	Zheng et al. (2014)
100	15	Anaerobic sludge	Wang and Wan (2011)
100	15	Dairy manure	Chu et al. (2011)
100	15	Anaerobic sludge	Lu et al. (2009)
100	15	Anaerobic sludge	Lay et al. (1999)
100	15	Anaerobic sludge	Okamoto et al. (2000)
100	15	Anaerobic sludge	Lay (2000)
100	15	Soybean meal	Noike and Mizuno (2000)
100	15	Soybean meal	Mizuno et al. (2000a)
100	15	Soybean meal	Mizuno et al. (2000b)
100	15	Anaerobic sludge	Lay (2001)
100	15	Anaerobic sludge	Han and Shin (2004)
99	90	Anaerobic sludge	Liu et al. (2012)
90	30	Anaerobic sludge	Lee et al. (2010a)
90	20	Anaerobic sludge	Jung et al. (2011)
90	20	Food waste	Kim et al. (2011)
90	20	Without inoculum	Kim et al. (2009)
90	10	Anaerobic sludge	Cavalcante de Amorim et al. (2009)
80	120	Anaerobic sludge	Lakaniemi et al. (2011)
80	10–60	Anaerobic sludge	Noike et al. (2002)
75	60	Sewage sludge	Chang et al. (2002)

gen fermentation, and (3) heat shock treatment, to remove LAB from hydrogen fermentation systems, is essential for stable hydrogen fermentation. In previous hydrogen fermentation systems, these features were essential to the stability of the hydrogen fermentation reaction (Wang and Wan

2009). In a precondition, it was believed that LAB must contaminate hydrogen fermentation and that lactate fermentation should be avoided; further, heat shock treatment is employed as precautions for them (Sreela-Or et al. 2011). However, the advantages of heat shock treatment for energy recovery as hydrogen fuel are not exceeded by the requirement of high energy input. I wonder what they will do next.

We consider these notions to be a blind spot. For construction of a practical hydrogen fermentation system, ease of use is as important as hydrogen productivity. If we can ignore previous notions, many of disadvantages solve. Many advantages may also emerge. We chose robustness, or resistance to contamination, as a primary criterion for identifying an ideal HPB, as it is required for establishing an effective hydrogen fermentation microflora. First, we analyzed the hydrogen productivity of many microflora obtained from different environmental materials, which may or may not include heat shock treatment. The sole carbon source in the medium was lactate. *Megasphaera elsdenii* was identified as a LU-HPB. And the ability of hydrogen production of *M. elsdenii*, metabolites as the end product of hydrogen fermentation, and tolerance of heat shock treatment were analyzed. In the process of these studies, we developed some of useful monitoring tools for genus *Megasphaera*.

The purpose of this chapter is to describe the characteristics of bacteria in the genus *Megasphaera*, primarily the LU-HPB *M. elsdenii*, as well as methods for monitoring *Megasphaera* spp. during the hydrogen fermentation process.

4.2 HPB, Heat Shock Treatment, and LU-HPB

Pure cultures are commonly used in testing microbial hydrogen fermentation. A variety of microorganisms, including members of the Bacteria, Archaea, and Eukarya, across a wide temperature range, are capable of hydrogen production by dark fermentation (Stams and Plugge 2009; Lee et al. 2010b). The highest hydrogen yields are usually achieved by Bacteria. Both obligate anaerobes and facultative anaerobes have been used in studies of dark fermentation of hydrogen (Lee et al. 2011). Among HPB, *Clostridium* spp. and *Enterobacter* spp. have been most widely studied. *Clostridium* spp. are gram-positive, rod-shaped, endospore-forming strict anaerobes, whereas genus *Enterobacter* is gram-negative, rod-shaped, facultative anaerobic bacteria (Vos et al. 2009). *Clostridium* spores are dormant and not metabolically active; spore formation protects DNA from UV radiation, heat, and other stresses (Cerf 1977). Enteric bacteria are oxygen tolerant, gram-negative, nonspore-forming facultative anaerobes. The mechanisms used by *Clostridium* spp. for hydrogen fermentation are well understood, and the expectations for practical continue to grow (Lee et al. 2011). HPB such as genus *Clostridium* generate hydrogen from saccharides via reactions (4.1) and (4.2). The theoretical maximum hydrogen yield through hydrogen fermentation process is 4 mol of H_2/mol of hexose. However, the actual hydrogen yield is around 2 mol of H_2/mol of hexose. Many studies have attempted to improve the hydrogen yield of known HPB or to identify novel species with higher hydrogen yields.

In contrast, environmental engineers have shown more interest in using mixed cultures or complex microflora, for practical reasons (Dong et al. 2009). In operation, control, and handling, a hydrogen fermentation microflora as complex microbe is easier than pure culture (Valdez-Vazquez and Poggi-Varaldo 2009). In this field, the aims and strategies for system development have been based on the study of pure cultures. Some important elements are LAB and lactic acid. LAB do not generate hydrogen from metabolism of sugar, but mainly produce lactic acid via reactions (4.3) and (4.4) (Liu et al. 2011, Castillo Martinez et al. 2013). There are two types of lactate fermentation. One is homolactic fermentation (4.3), and the other is heterolactic fermentation (4.4). Two molecules of lactate are produced from two molecules of pyruvate via glycolysis that is a metabolic pathway of glucose, in homolactic fermentation. One molecule of pyruvate is changed to lactate, and the other is changed to ethanol and carbon dioxide, in heterolactic fermentation.

Various organic materials, such as starch and monosaccharide included in natural biomass, are suitable for hydrogen generation. In contrast, lactate was unsuitable for substrate of hydrogen fermentation (Lee et al. 2011). Baghchehsaraee et al. (2009) found that no hydrogen was produced on lactate as substrate. Currently, lactate-utilizing hydrogen fermentation system that consumes lactate as substrate does not exist. Thus, when LAB contaminate a hydrogen fermentation system, substrate competition for the sugar occurs between HPB and LAB, dramatically reducing the hydrogen yield. LAB are ubiquitous and are thought to inhibit hydrogen production. LAB have been isolated from animals (body surfaces), plants, soils, rivers, and oceans and can multiply at temperatures between 2 and 50 °C (Françoise 2010). Furthermore, LAB produce antibacterial agents, such as bacteriocin, which inhibit hydrogen fermentation (Noike et al. 2002). In a previous study, many researchers have the same opinions about these points. Noike et al. (2002) found that hydrogen generation by genus *Clostridium* was stopped by two species of LAB, genus *Lactobacillus* and genus *Enterococcus*, in a continuous hydrogen fermentation system using organic waste of bean curd production by a complex microflora. They suggested that the bacteriocins produced by LAB are the inhibition of matter of genus *Clostridium* rather than by the decrease in pH. Thus, inhibition of hydrogen production by LAB likely resulted from competition of substrate and production of bacteriocins that inhibit the genus *Clostridium* as HPB.

Based on these data, pretreatment is typically used to obtain or enrich bacteria useful for hydrogen fermentation (Table 4.1). Pretreatments often rely on the spore formation of *Clostridium* spp. as HBP (Tracy et al. 2012). If a complex microflora is exposed to conditions that are difficult for survival, survival potential of *Clostridium* spp. is better than LAB as nonspore-forming bacteria (Valdez-Vazquez and Poggi-Varaldo 2009). Heat shock treatment, acidic/basic treatment, aeration, chemicals, and electrical current of substrate and/or inocula are effective pretreatments for the hydrogen fermentation system (Li and Fang 2007). In particular, it was found that heat shock treatment is a useful pretreatment for maximizing the hydrogen yield. The hydrogen yield is shown based on the amount of hexose sugars, such as glucose, consumed. Many researchers reported various pretreatment methodologies for complex microflora as inocula or substrates to reach optimal hydrogen yield. Heat shock treatment at 100 °C for 15 min, the highest utilization frequency, seems to be simple and effective for screening of HPB (Table 4.1). In addition, it is reported that heat shock treatment also activates clostridial spores to start germination by improving the germination receptor (Hawkes et al. 2007). In the study of heat shock treatment for hydrogen fermentation, temperature was varied from 75 to 121 °C (Li and Fang 2007). The heat shock treatment caused stability in a hydrogen fermentation system at a high probability.

However, heat shock treatment has several disadvantages, also. Heat shock treatment consumes energy, to such a degree that it may cancel out the benefits of the treatment. In addition, some researchers have reported that initial heat shock treatment of the inocula and substrate could not eliminate contaminants such as LAB from the hydrogen fermentation process (Oh et al. 2003a), because LAB are widely distributed and biomass is often used as the substrate for hydrogen fermentation. Based on these preconditions, continuous high energy input is vital for stable or high-yield hydrogen fermentation. In addition, because LAB is considered to be a contaminant for hydrogen fermentation, heat shock treatment is essential during hydrogen fermentation. But the use of heat shock treatment for sustained hydrogen fermentation is not economically viable, because they require substantial energy input, which is a significant problem. Thus, the net amount of fuel obtained will be very small, even if a large quantity of hydrogen was obtained via this hydrogen fermentation system. These problems impede the practical application of hydrogen fermentation. However, several studies have identified LAB that play a useful role in the hydrogen fermentation microflora and activate hydrogen fermentation. Hung et al. (2007) analyzed the yield of hydrogen fermentation from hexose in bioreactor of anaerobic sludge using various substrate concentrations. Fluorescence *in situ* hybridization (FISH) study revealed that LAB was present, inside a mass of complex microflora surrounded by cells of *Clostridium* spp. It was postulated that cells of LAB act as the seeds for flock formation (Hung et al. 2007). Thus, heat shock treatment removes both the disadvantages and advantages resulting from LAB. If stable hydrogen fermentation could be achieved without heat shock treatment, many problems would be solved and many advantages might emerge.

Our strategy for development of a practical hydrogen fermentation system is to search for novel hydrogen fermentation bacteria, which are not inhibited by contamination (Ohnishi et al. 2010). In a previous study, we identified *M. elsdenii* as a LU-HPB (Ohnishi et al. 2012b); the following section summarizes our previous research.

First, we evaluated the hydrogen productivity of some environmental microflora as inocula, with – and without – heat shock treatment. Lactate (15,440 mg/L) was used for substrate as sole carbon source. In the batch test, heat shock-treated inocula did not generate hydrogen. On the other hand, all of the non-heat shock-treated inocula generated hydrogen. Some inocula obtained methane fermentation system showed very high hydrogen productivity. Then, the hydrogen fermentation stability of a hydrogen fermentative microflora, obtained from the acid slurry of a methane fermentation system, was assessed in sequential batch tests. In the heat shock-treated inoculum, no hydrogen generation was observed through the sequential batch tests. In contrast, non-heat shock-treated inoculum showed large quantities of hydrogen generation. Finally, 1600 mL hydrogen/L was recorded.

Further, in hydrogen productivity, the volatile fatty acid (VFA) production, the microbial construction, and the properties of the microbe, HPB were evaluated. Lactate, the substrate for the batch test, was the only VFA after the heat shock-treated inoculum was employed. On the other hand, 3777 mg/L of acetate, 3680 mg/L of propionate, 1978 mg/L of butyrate, and 1260 mg/L of valerate were present in the metabolite as end product when the non-heat shock-treated inoculum was used. Thus, a stable hydrogen fermentation microflora that used lactate was enriched without heat shock treatment.

Next, we analyzed the composition of the microflora, using PCR-denaturing gradient gel electrophoresis (PCR-DGGE). This methodology is a powerful technique for directly determining the genetic diversity of complex microflora without cultivation (Muyzer et al. 1993). This procedure is based on separation of PCR amplicons of the similar length using urea and formamide as denaturants. It is a high-speed assessment method with high accuracy of microflora constructed with complex microbe (Muyzer et al. 1993). With application of a variety of primer sets, PCR can potentially reveal the genetic composition of the microflora as well as taxonomic information. The method for evaluation of microflora diversity was based on electrophoretic mobility of PCR amplicons of partial rRNA gene (approximately 150–500 bp) in polyacrylamide gel with linearly gradient of denaturant (formamide and urea). Species can be identified based on sequence data and their gel banding patterns.

Table 4.2 lists the microflora identified in non-heat shock-treated and heat shock-treated inocula by PCR-DGGE. The similarity of 16S rRNA gene of the isolated strains was analyzed based on the GenBank database, comprehensive public database. In the non-heat shock-treated inoculum, six species belonging to four genera were identified: *Lactobacillus fermentum*, *L. perolens*, *M. elsdenii*, *Pectinatus cerevisiiphilus*, *Clostridium sporogenes*, and *C. lundense*. When the heat shock-treated inoculum was used, two species belonging to two genera were identified: *C. magnum* and *Paenibacillus azoreducens*. The microbial diversity was clearly reduced by heat shock pretreatment of inoculum. The heat shock treatment of inoculum resulted to bias of the microflora of heat-resistant spore-forming bacteria, such as genus *Clostridium*. *Lactobacillus* spp. were clearly eliminated by heat shock treat-

Table 4.2 Construction of microflora after batch test analyzed by PCR-DGGE

Closely related	Similarity (%)	Heat shock treatment inoculum[a]	Non-heat shock treatment inoculum
Clostridium lundense	99		+
Clostridium magnum	100	+	
Clostridium sporogenes	98		+
Lactobacillus fermentum	100		+
Lactobacillus perolens	99		+
Megasphaera elsdenii	98		+
Paenibacillus azoreducens	100	+	
Pectinatus cerevisiiphilus	97		+

[a] Heat shock treatment at 90 °C for 10 min (Ohnishi et al. 2012b)

ment. Thus, only the non-heat shock-treated inocula contained a complex microflora including the LAB, *Lactobacillus*.

However, it is unclear why a high level of hydrogen production could occur stably in non-heat shock-treated inocula, using lactate as substrate. Isolation of the microbes to determine the main hydrogen producer using a cultivation-dependent methodology was required. After using several culture media, the main hydrogen-producing organism was isolated. A lactate-utilizing hydrogen fermentation bacterium was found, and hydrogen productivity and other properties of this bacterium were analyzed. In the analysis of 16S rRNA gene similarity, the isolated strains were belonging to the following three species: *C. sporogenes*, *M. elsdenii*, and *P. cerevisiiphilus*. The hydrogen productivity and VFA composition and concentration of these three strains were evaluated, using non-heat shock treatment and heat shock treatment. When the heat shock-treated isolate was used as the inoculum, hydrogen was not generated. When the non-heat shock-treated isolate was used, hydrogen production was observed by *M. elsdenii* (>1400 mL/L) and *C. sporogenes* (approximately 400 mL/L), but not by *P. cerevisiiphilus* (no hydrogen production). The composition of VFA as metabolite was analyzed. *M. elsdenii* produced acetate (approximately 3200 mg/L); *C. sporogenes* produced acetate (approximately 1600 mg/L). *M. elsdenii* consumed all the lactate present as substrate, but *C. sporogenes* and *P. cerevisiiphilus* did not consume any lactate as substrate. From these things, it was determined that *M. elsdenii* was LU-HPB in the hydrogen fermentation microflora using non-heat shock treated inoculum.

In the pure culture of *M. elsdenii*, around 0.40 mol/mol lactate of hydrogen yield was obtained. The main VFA composition as metabolite was 3200 mg /L of acetate and 3700 mg/L of propionate. This was new finding as Lactate Utilizing-Hydrogen Producing Bacterium. Thus, *M. elsdenii* shows potential as a LU-HPB for use in hydrogen fermentation systems.

Hydrogen fermentation by LU-HPB occurs as follows: based on reactions (4.3) and (4.4), even if such as glucose as substrate was robbed by lactate fermentation; 0.4 mol hydrogen (heterofermenter as LAB) to 0.8 mol hydrogen (homofermenter as LAB) would be recovered per mol glucose. The efficiency of energy of practical hydrogen fermentation systems could be improved on the basis of this theory. When used in cooperation with LAB, LU-HPB likely contribute to improvement of the energy budget in dark-fermentative hydrogen production (Ohnishi et al. 2012b). In addition, combination of lactate fermentation by LAB and hydrogen fermentation by LU-HPB likely promotes organic material consumption and the effective hydrogen generation.

4.3 Characteristics of the Organisms Belonging to the Genus *Megasphaera*

This section describes the morphology, phylogenetic relationships, and biochemical abilities, including hydrogen production, of *Megasphaera* spp., and provides additional data for understanding *Megasphaera* spp. *Megasphaera* spp. have been isolated from food, cattle, environmental samples, and health contexts. *Megasphaera* spp. are strictly anaerobic, nonmotile, mesophilic, and coccal and 0.4–2.0 μm or greater in diameter; they are often found in short chains or in pairs (Haikara and Helander 2006; Vos et al. 2009) (Table 4.3). *Megasphaera* spp. do not form

Table 4.3 Characteristics of genus *Megasphaera*

	M. cerevisiae	*M. elsdenii*	*M. micronuciformis*	*M. paucivorans*	*M. sueciensis*
Hydrogen fermentation ability					
Glucose	nd	+	nd	nd	nd
Lactate	nd	+	nd	nd	nd
Garbage slurry as biomass	+	+	nd	w	w
Cell size (um)	1.5–2.1	1.6–2.6	0.4–0.6	1.2–1.9 × 1.0–1.4	1.0–1.4 × 0.8–1.2
Growth at 45 °C	–	+	nd	–	–
Utilization of:					
Arabinose	±	–	–	–	–
Fructose	+	+	–	–	–
Glucose	–	+	–	–	–
Galactose	–	–	–	–	–
Maltose	–	+	–	–	–
Mannitol	–	+	–	–	–
Sucrose	–	+	–	nd	nd
Mannose	–	–	–	–	–
Lactate	+	+	–	–	–
Gluconate	+	+	–	+	+
Production of:					
Gas	+	+	–	+	+
H_2S	+	+	–	+	+
Acetoin	w	w	nd	–	w
Desulfoviridin	–	–	±	–	–
Susceptibility to:					
5 μg Vancomycin	R	R	S	R	R
10 μg Colistin	S	S	S	R	R
Volatile fatty acids produced[a]	A, P, (iB), B, iV, V, nC	A, (P),(iB), nB, iV, nV, nC	A, P, (iB), nB, iV, (V), PhA	A, (P), iB, B, iV, V, (iC), C	(A), P, iB, B, iV, V, C
DNA G + C content (mol%)	42.4–44.8 (T_m)	53.1–54.1 (Bd)	46.4 (T_m)	40.5 (T_m)	43.1 (T_m)

Data sets for taxa were taken from Marchandin et al. (2009)

Parentheses indicate that production is not constant. Major products are *underlined*

+ Positive, − negative, ± variable, *w* weakly positive, *r* resistant, *s* sensitive, *nd* not determined, *Tm* thermal denaturation, *Bd* buoyant density centrifugation

[a] *A* Acetic acid, *nB* n-butyric acid, *iB* isobutyric acid, *nC* n-caproic acid, *iC* isocaproic acid, *P* propionic acid, *nV* n-valeric acid, *iV* isovaleric acid, *PhA* 2-phenylacetic acid. Major products are *underlined*, while those in *parentheses* are inconsistently produced

spores. Interestingly, although phylogenetic analysis shows that *Megasphaera* belongs to a gram-positive, low-GC-content group, its cell wall composition makes it gram-negative. Electron microscopy has shown that members of the genus *Megasphaera* have a triple-layered cell wall as characteristic of gram-negative group.

This genus includes five species: *M. elsdenii* (proposed in 1971), *M. cerevisiae* (in 1986), *M. micronuciformis* (in 2003), *M. paucivorans* (in 2006), and *M. sueciensis* (in 2006). The individual species was isolated from the various environmental samples, such as clinical specimen of the human health, food, and biofuel production process. All species are nitrate reduction negative, indole formation negative, oxidase activity negative, and catalase activity negative and hydrolysis of aesculin, gelatin, and arginine negative. Utilization of pyruvate is positive in all species. The metabolites as end products are acetate, propionate, butyrate, and valerate. The production of propionate, valerate, caproate, and branched-chain fatty acid such as isobutyrate depends on species and conditions. The optimum cultivation temperature for *Megasphaera* spp. is approximately 30 °C (28–37 °C), but for *M. elsdenii* it is 37–40 °C (25–40 °C). *Megasphaera* spp. show different patterns of resistance to vancomycin and colistin. *M. elsdenii* is resistant to vancomycin but sensitive to colistin (Suihko and Haikara 2001). Very high hydrogen productivity is observed in *M. elsdenii*. The hydrogen productivity of the other members of the genus is currently being studied.

M. elsdenii was the first member of the genus *Megasphaera* to be isolated. Originally named *Peptostreptococcus elsdenii*, it was reclassified by Rogosa (1971) as a new genus, *Megasphaera*, in the family *Veillonellaceae*, which includes the genus *Veillonella*. According to phylogenetic analyses, the genera in this family belong to the phylum *Firmicutes*. *M. elsdenii* has been isolated from the rumens of animals such cattle and sheep, from the feces and intestines of humans and also from human clinical samples, and from anaerobic biowaste treatment processes, including hydrogen fermentation. It was reported that *M. elsdenii* is a common indigenous bacterium of the gastrointestinal tract in human and mammals (Hino and Kuroda 1993; Hino et al. 1994; Hashizume et al. 2003). *M. elsdenii* is considered to be the most important bacterium in the rumen of cattle and in the intestine of other animals (Tsukahara et al. 2006). *M. elsdenii* can generate acetate, propionate, butyrate, and valerate from lactate, depending on the pH, using the acrylate pathway (Counotte et al. 1981). Some culture-based studies have reported *M. elsdenii* to be present in human feces (Hayashi et al. 2002). Regarding its biochemical characteristics, the fermentative metabolism of *M. elsdenii* allows it to utilize carbohydrates and organic acids. In 1989, Marounek et al. reported a large quantity of CO_2 and comparatively little hydrogen productivity (Marounek et al. 1989). However, in 2010, *M. elsdenii* was demonstrated to be a useful producer of hydrogen from garbage waste in a biohydrogen production system (Ohnishi et al. 2010). *M. elsdenii* is a remarkable HPB because it can use glucose and garbage waste as biomass. Furthermore, *M. elsdenii* has been proposed to be important for practical implementation of a hydrogen fermentation system using LU-HPB (Ohnishi et al. 2012b).

Several growth media have been used for cultivation, detection, or isolation of *M. elsdenii*. The most commonly used media are peptone-yeast extract (PY) media supplemented with glucose (PYG) or lactate (PYL) (1 % w/v, final concentration), *Megasphaera* medium (ATCC medium 566), selective medium *Megasphaera*, *Pectinatus* (SMMP), and Reinforced Clostridial Medium (ATCC medium 1503) (Atlas 2010; Vos et al. 2009; Haikara and Helander 2006). In addition, *M. elsdenii* can be selected on some specific media such as *Bifidobacterium*-selective agar and Eugon Agar with maltose and neomycin. A few strains of *M. elsdenii* can grow at 45 °C, but not at 50 °C. On the other hand, *M. elsdenii* cannot cultivate at around room temperature. The pH range for growth of *M. elsdenii* is 4.6–7.8, and the optimum pH is approximately 6.05. *M. elsdenii* is considered to be relatively acid tolerant.

M. elsdenii cannot use xylose, raffinose, trehalose, sucrose, galactose, arabinose, lactose, mannose, rhamnose, cellobiose, glycerol, dextrin, inulin, salicin, or starch for growth and fermentation. Some researchers showed the production of several fatty acids such as branched-chain fatty acid and straight-chain fatty acids (propionate, butyrate, valerate, isobutyrate, isovalerate, and 2-methyl butyrate) from lactate. However, our study yielded different results, especially for branched-chain fatty acids (data not shown). A complete understanding of *M. elsdenii* metabolites requires further study. Many studies on the metabolic pathways of M. elsdenii have shown that propionate is generated and lactate is consumed via the acrylate pathway (Martin 1994). The global image of the metabolic pathways associated with hydrogen production by *M. elsdenii* remains indistinct. The relationships between substrate, end product, and hydrogen production require further study.

In the brewing process, *Megasphaera* contributes considerably to anaerobic beer spoilage (Haikara and Lounatmaa 1987; Sakamoto and Konings 2003). Currently, three of the five known species of *Megasphaera* (*M. cerevisiae*, *M. paucivorans*, and *M. sueciensis*) are understood as notable beer-spoilage gram-negative anaerobic bacterium. Until 2006, *M. cerevisiae* was regarded as the only contaminant (Haikara and Helander 2006; Engelmann and Weiss 1985). However, *M. paucivorans* and *M. sueciensis* were then isolated and characterized from spoiled Italian and Swedish lagers, respectively (Juvonen and Suihko 2006). The spoilage of beer caused by these *Megasphaera* spp. results in extreme turbidity. The metabolites of these *Megasphaera* spp. are similar to genus *Pectinatus* that can produce straight-chain fatty acids such as butyrate, acetate, valerate, caproate, and branched-chain fatty acid such as isovalerate, as well as acetoin (Sakamoto and Konings 2003). When contamination of these species happens in beer, more significant spoilage than LAB (such as genus *Lactobacillus* and genus *Pediococcus*) occurs. This happens because they produce smell of “rotten egg,” from production of hydrogen sulfide. Hydrogen production by these species has not clearly been confirmed. *M. cerevisiae* has lower hydrogen productivity than *M. elsdenii* (Table 4.3). *M. paucivorans* and *M. sueciensis* have weak hydrogen productivity. The effects of fermentation substrate and culture conditions on hydrogen productivity will require detailed investigation in the future.

In contrast with the other described species, very few reports have been published concerning *M. micronuciformis*, isolated from a liver abscess and a pus sample (Marchandin et al. 2003). Further, *M. micronuciformis* has been isolated from some clinical specimens (Zozaya-Hinchliffe et al. 2008). *M. micronuciformis* is the smallest in the genus *Megasphaera*; cells are coccoid and show a convoluted surface after negative staining. Cells of *M. micronuciformis* are mainly single and their diameters are 0.4–0.6 µm. In *M. micronuciformis*, clear hydrogen productivity has not been confirmed to date.

4.4 Rapid Methodology for Detection, Identification, and Monitoring of *Megasphaera* spp.

This section describes specific methodology for detection, identification, and monitoring of *Megasphaera* spp. using FISH and PCR-RFLP. Because of its importance, it is critical to understand the distribution and participation of *Megasphaera* spp. in various environmental samples, not only in terms of hydrogen production but also in terms of industrial fermentation and the potential for clinical infections, for quality assurance in the food industry, human health, and the raising of livestock. The most popular method for detection of *Megasphaera* is the conventional culture method. However, detection of *Megasphaera* is complicated by the strict anaerobic conditions required for its cultivation a week or longer. Therefore, in the beer industry, often the beer of brewers’ product has been sold, before the microbiological judgment becomes clear. Hence, rapid molecular methodologies, such as specific primer sets and real-time PCR, have been developed for beer-spoilage bacteria or rumen bacteria (Juvonen et al. 2008; Methner et al. 2004; Satokari et al. 1998). However, the facilities and equipment required for real-time PCR are very expensive. In the methodology using

Table 4.4 List of oligonucleotides used for FISH probe, PCR primer, and sequencing

Oligonucleotide	Sequence (5′-3′)	*E. coli* position	Purpose
Mega-142F	GATGGGGACAACAGCTGGA	142–160	Genus *Megasphaera-specific* PCR
Mega-X	GACTCTGTTTTTGGGGTTT	1315–1297	Genus *Megasphaera-specific* PCR and FISH probe
20F	AGTTTGATCATGGCTCA	10–26	PCR for all Bacteria
1540R	AAGGAGGTGATCCAACCGCA	1541–1521	PCR for all Bacteria
EUB338	GCTGCCTCCCGTAGGAGT	355–338	FISH probe for all Bacteria
ARCH915	GTGCTCCCCCGCCAATTCCT	934–915	FISH probe for all Archaea

Ohnishi et al. (2011a, b, 2012a) and Manz et al. (1992)

16S rRNA gene sequences, quantitative PCR method has been developed to identify *M. elsdenii* in the microflora of the rumen (Ouwerkerk et al. 2002). However, no methodology capable of detecting all species of the genus *Megasphaera* is currently available.

We developed some methods for rapid detection of *Megasphaera* and identification of *Megasphaera* spp. up to species level The Mega-142F/Mega-X primer set for PCR reaction was developed as a rapid, inexpensive tool for the detection of *Megasphaera* spp. In addition, restriction fragment length polymorphism (RFLP) analysis was optimized for species-level identification of the PCR amplicons (Ohnishi et al. 2011a). Furthermore, a *Megasphaera-specific* FISH method was developed for counting and monitoring *Megasphaera* spp. at the single-cell level (Ohnishi et al. 2012a).

4.4.1 Materials and Method

4.4.1.1 *Megasphaera*-Specific Oligonucleotides

Megasphaera-specific PCR primers and FISH probes were developed. In the designing of a genus-specific PCR primer set for *Megasphaera*, the 16S rRNA gene sequences of *M. elsdenii*, *M. cerevisiae*, *M. micronuciformis*, *M. paucivorans*, *M. sueciensis*, and several closely related species were aligned and compared. Comparative sequence analysis revealed that *Megasphaera* spp. differed in many aspects from other genera, but that a very small number of bases were unique to *Megasphaera*. The oligonucleotides are listed in Table 4.4. The forward primer Mega-142F and the more specific reverse primer Mega-X contain sites complementary to all of *Megasphaera* species. A perfect match for a non-*Megasphaera* bacterium was found for the primer Mega-142F only in the 16S rRNA gene sequence of *Anaeroglobus geminatus*, a member of the family *Veillonellaceae*, shown in Fig. 4.2. However, the more important primer is the reverse primer Mega-X. The target sequence, AAACCCCAAAAACAGAGTC, is unique to *Megasphaera* species. Especially the specific primer Mega-X was not complementary to 16S rRNA gene of any nontarget bacteria, in the public databases and BLAST search (Ye et al. 2012). The primer Mega-X is complementary to the only sequence that is typical of *Megasphaera*.

4.4.1.2 FISH Probes

Oligonucleotides as FISH probes are synthesized with fluorescent dye such as Alexa Fluor 488 (Ex.: 488 nm; Em.: 505–530 nm) or Cy3 (Ex.: 543 nm; Em.: 560–615 nm) and Cy5 (Ex.: 633 nm; Em.: LP 650 nm) (Takara Bio Inc., Japan). Some oligonucleotides were used as FISH probe: EUB338 for all Bacteria-specific FISH probe, ARCH915 for all Archaea-specific FISH probe (Manz et al. 1992), and Mega-X for genus *Megasphaera*-specific FISH probe (Table 4.4). The concentration of FISH probe was adjusted to

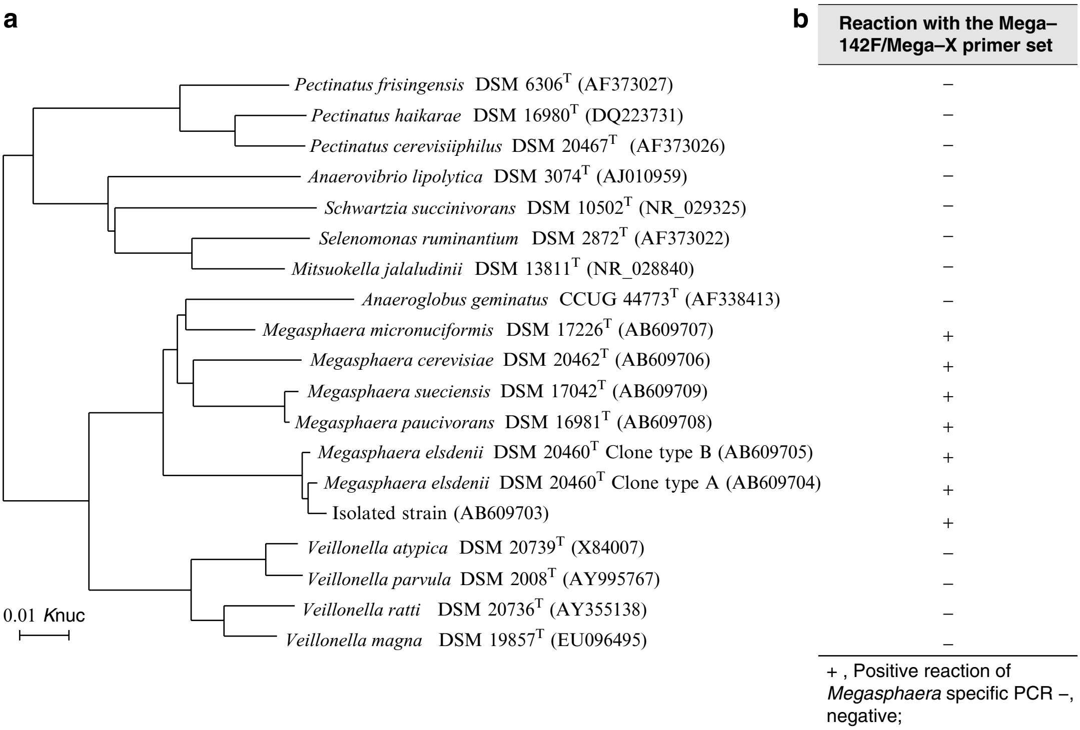


Fig. 4.2 Phylogenetic relationship between the isolates and related bacteria based on 16S rRNA gene sequences (**a**) and Results of the genus *Megasphaera*-specific PCR (**b**). The tree is based on the comparison of approximately 1100 nucleotides of the 16S rRNA gene

50 ng/L in ddH_2O or TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA).

4.4.1.3 DNA Extraction

DNA extraction was performed either by a kit protocol to isolate pure DNA or a rapid protocol to process large numbers of samples. For the kit protocol, DNA is extracted from 1 mL of sample with the FastPure DNA Kit (Takara Bio Inc., Japan). For the rapid protocol, 1 mL of the cultivated medium or samples were centrifuged at 15,000 rpm for 5 min at 4 °C and suspended in 1 mL of TE buffer. This bacterial cell washing process was repeated twice. The sample of suspended cells was then heat treated for 10 min at 100 °C, and the cells were centrifuged at 15,000 rpm for 10 min at 4 °C. The boiled-cell supernatants were used for PCR. Routinely, 1 μL of the DNA solution was used for the PCR analysis. For detection limit determination of the *Megasphaera*-specific PCR, DNA extracted from the target bacterial strains using the rapid protocol was amplified in triplicate PCR.

4.4.1.4 *Megasphaera*-Specific PCR

PCR was performed using PCR kit such as GoTaq Hot Start Green Master Mix (Promega), 1 μM of a PCR primer set (show in Table 4.4; Mega-142F/Mega-X for *Megasphaera*-specific detection) or 20F/1540R for all bacteria (Ohnishi et al. 2010), and 1 μL of extracted DNA solution. Then, PCR reaction was performed using a thermal cycler (Bio-Rad). The amplification profile consisted of 94 °C for 2.5 min, followed by 30 cycles of 15 s at 94 °C, 30 s at an annealing temperature 58 °C, and 30 s at 72 °C. The final extension step was for 7 min. The annealing temperature for *Megasphaera*-specific PCR with Mega-142F/Mega-X as the primer set was 58 °C, and for other bacteria using the primer set 20F/1540R, it was 55 °C. A negative control without template DNA was used in all runs. After the PCR reaction, 5 µL of the PCR solutions were analyzed to the basis of size by 1.5 % agarose gel electrophoresis. And PCR amplicons were detected by ethidium bromide staining and a transilluminator (AE- 6943V-FX; ATTO). The *λHind*III digest (Takara) was used as the molecular size marker. In *Megasphaera*-specific PCR, the presence of an approximately 1,200 bp of detected DNA band was interpreted as a specific PCR amplicon from genus *Megasphaera*.

4.4.1.5 Species-Level Identification of *Megasphaera* by PCR-RFLP Analysis

The lengths of the restriction fragments resulting from use of different restriction enzymes were predicted by in silico analysis program MIKENORA Research (Daiichi Pure Chemicals). As enzyme of restriction, 20 U of *Hae*III and *Msp*I (Takara) were added to 10 µl of the PCR solution. Restriction reaction was performed at 37 °C, 1 h. The reaction was discontinued by adding 3 µl of loading buffer. The restriction fragments were separated electrophoretically on a 4 % agarose gel and detected by ethidium bromide staining and transilluminator (AE-6943V-FX; ATTO). A 20-bp DNA marker (Takara) was used as a standard for size determination of PCR amplicons.

4.4.1.6 FISH Method and Measurement of Bacterial Cell Staining

Fluorescence in situ hybridization (FISH), based on specific function gene or orthologous gene phylogenies, is a useful method for identification and enumeration in single-cell level. The fixation of bacterial cell, cell wall permeabilization, and in situ hybridization methods were performed using a modified method for FISH as described by Manz et al. (1992). After cell wall treatment with lysozyme solution, bacterial cells were dehydrated in a graded ethanol series. Then, 9 µL of hybridization buffer was mounted to each well of the slide. The prewarming, 45 °C for 30 min, for whole-cell hybridization was performed in a moisture chamber. Then, 1 µL of mixed FISH probes, for example, Cy3-labeled Mega-X and Alexa Fluor 488-labeled EUB388, was added, and in situ hybridization was continued for 2 h or overnight. The hybridization buffer and FISH probes on the slide were washed with a piece of prewarmed hybridization buffer without FISH probe. Then, the slide was dipped to 50 mL of prewarmed (2 °C higher than the hybridization temperature) hybridization buffer for 15 min and rinsed with ddH$_2$O. Counter stain was performed for detection of all bacterial cells by 4,6-diamidino-2-phenylindole (DAPI) staining. The slide was dried and covered with low autofluorescence immersion oil and cover glasses.

The observation of slide was performed using a fluorescence microscope or a confocal laser scanning microscope. If the sample contains particle organic matter, the autofluorescence is obtained using other wavelength of the fluorescence labels and is omitted excluded from the FISH image.

4.4.2 Methodological Quality and Application Example

4.4.2.1 Specificity of *Megasphaera-Specific* PCR

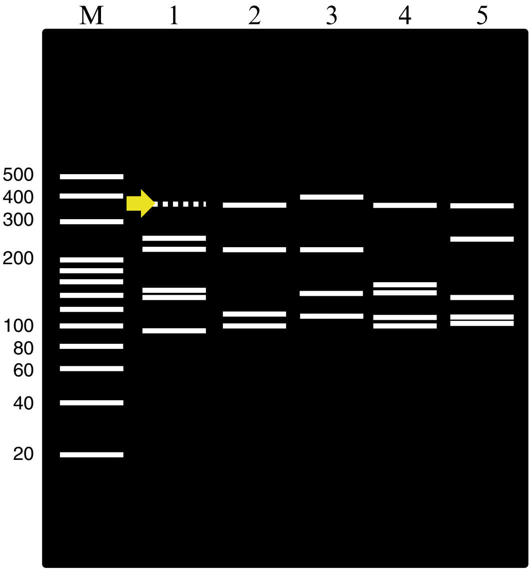
Specific detection of *Megasphaera* spp. was performed with the primer set of Mega-142F/Mega-X. The specificity of the Mega-142F/Mega-X primer set was demonstrated using 17 of phylogenetic relative strains (Fig. 4.2). Optimized annealing temperature for genus *Megasphaera-specific* PCR detection was 58 °C. The limit of detection in 30 cycles of PCR was 1,000 cells/mL.

4.4.2.2 Species Identification by RFLP

RFLP analysis was used to identify the products amplified by *Megasphaera*-specific PCR.

According to the in silico analysis, the highest level of discrimination was achieved by simultaneous digestion with *Hae*III and *Msp*I. This combination is expected to yield species-specific profiles for *Megasphaera* spp., with the following fragment sizes (bp): 346, 151, 139, 107, 90, 48, 45, 39, 35, 27, 24, 25, 20, 19, and 12 for *M. cerevisiae*; 241, 213, 140, 139, 131, 85, 48, 39, 34, 25, 20, and 12 for *M. elsdenii*; 346, 248, 139, 107, 103, 78, 53, 48, 45, 39, 34, 25, 24, 21, 20, and 12 for *M. micronuciformis*; 371, 208, 141, 107, 53, 48, 45, 40, 39, 34, 33, 24, 20, 12, 8, and 1 for *M. paucivorans*; and 346, 209, 108, 93, 48,

Fig. 4.3 The gel image of PCR-RFLP patterns of the genus *Megasphaera* obtained. *Arrow* indicates the unexpected band



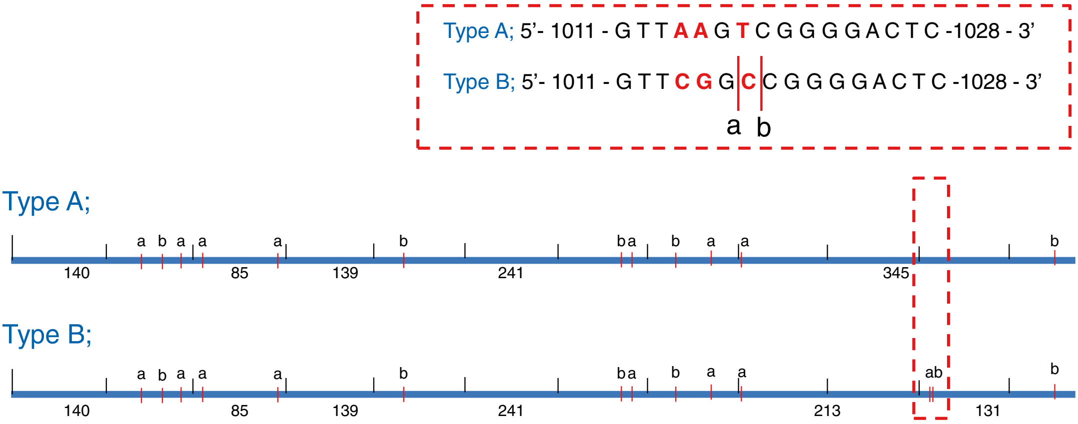


Fig. 4.4 Restriction site and specific sequences on partial 16S rRNA gene clones of *Megasphaera elsdenii* DSM 20460. In a *dashed line*, specific sequences cause a variation in restriction profile. Mutation sites are indicated by *bold-face*. Restriction site on clones were shown in *red solid line*. *a*; restriction site of enzyme *Hae*III(GG/CC), *b*; restriction site of enzyme *Msp*I(C/CGG)

46, 46, 39, 33, 33, 25, 24, 21, 20, 18, 1, and 1 for *M. sueciensis*. The results of the in silico analysis were verified by digesting the PCR products.

The restriction DNA band patterns differed in the species level (Fig. 4.3). The estimated fragment profiles for the four species were matched by an experiment without less than 80 bp of restriction DNA fragments. However, unanticipated DNA fragment of around 350 bp in size appeared in the restriction pattern for *M. elsdenii*. The PCR amplicon of *M. elsdenii* DSM 20460 was cloned and digested, and the typical *Hae*III/*Msp*I restriction patterns for each clone are shown in Fig. 4.4. Thus, two types of restriction profiles were obtained from *M. elsdenii* DSM 20460.

Therefore, the sequences of the two typical clones were determined and analyzed for restriction enzyme sites in silico. The restriction DNA band patterns of type B clone was matched by in silico analysis. The other restriction profile type (Fig. 4.4, Clone type A) included the unanticipated DNA fragment (Fig. 4.3), and in the around 131 bp, the predicted band was absent. The restriction sites and specific sequences of the two clone types of *M. elsdenii* DSM 20460 are shown in Fig. 4.4 (in dashed line rectangle). Mutations at nucleotide region 1015 (A or C), 1017 (A or G), and 1018 (T or C) in type A and B were shown. Clone B included two restriction sites, GG/CC of *Hae*III and C/CGG of *Msp*I, in this location, but these sites were absent from clone A.

From these results, it was shown that the 16S rRNA gene sequence of *M. elsdenii* included two types: 345, 241, 139, 85, 48, 39, 25, 24, 22, 20, and 12 for type A and 241, 213, 140, 139, 131, 85, 48, 39, 34, 25, 20, and 12 for type B. The restriction profile for *M. cerevisiae* generated by *Hae*III/*Msp*I was identifiable on the basis of the 346-, 151-, 139-, 107-, and 90-bp-sized restriction DNA fragments. Similarly, *M. elsdenii* was identifiable on the basis of the 345-, 241-, 213-, 140-, 139-, 131-, and 85-bp-sized restriction DNA fragments. *M. micronuciformis* was identifiable on the basis of the 346-, 248-, 139-, 107-, and 103-bp-sized restriction DNA fragments. *M. paucivorans* was identifiable on the basis of the 371-, 208-, 141-, and 107-bp-sized restriction DNA fragments. *M. sueciensis* was identifiable on the basis of the 346-, 209-, 108-, and 93-bp-sized bands (Fig. 4.3). These profiles permitted us to group all species with the five reference strains belonging to the genus *Megasphaera* (viz., *M. cerevisiae*, *M. elsdenii*, *M. micronuciformis*, *M. paucivorans*, and *M. sueciensis*) (Fig. 4.2). Restriction of the PCR productions by other enzymes such as *Alu*I, *Fok*I, *Bsi*EI, *Apo*I, and *Eae*I was not informative for species-specific identification.

In a previous report, to demonstrate the availability of the primer set Mega-142F/Mega-X, various DNA extract from environmental microflora and isolated strains were analyzed using a rapid protocol. Nine environmental microflora from a two-phase methane fermentation process and a hydrogen fermentation system were subjected to rapid protocol for DNA extraction for genus *Megasphaera*-specific PCR. DNA extracts from all hydrogen fermentation systems and one of the acid generation tanks of a two-phase methane fermentation system showed a positive PCR result. Furthermore, all PCR amplicons were analyzed by RFLP. All results as restriction DNA fragment patterns were matched as *M. elsdenii*. Thus, this primer set can be used for rapid detection of genus *Megasphaera* in environmental samples and species-level identification.

In addition, the samples were used for further isolation of anaerobes. A total of five anaerobic isolates were obtained. One of the isolated strain obtained by anaerobic cultivation from a hydrogen fermentation system demonstrated positive reaction of genus *Megasphaera*-specific PCR. And restriction DNA fragments by RFLP analysis of the PCR amplicon of the isolated strain showed *M. elsdenii* clone type A (Figs. 4.2 and 4.4) (Ohnishi et al. 2011a). Thus, this primer set can be used for rapid detection and species-level identification for genus *Megasphaera* in environmental samples and isolated strains.

4.4.2.3 Single-Cell Detection of *Megasphaera* by FISH Analysis

The Cy3-labeled oligonucleotide Mega-X as FISH probe can be used for direct detection of *Megasphaera* cells. The high specificity for genus *Megasphaera* of this FISH probe was demonstrated. At optimal stringency, with a 10 % formamide concentration, the FISH probe Mega-X cannot detect nontarget bacterium, but can produce a strong signal for *Megasphaera*. The FISH analysis using Mega-X is useful for actual application for enumeration of *Megasphaera* cells. The FISH analysis achieves rapid quantification and single-cell level monitoring of *Megasphaera* spp.

The Mega-X probe was used to test whether *Megasphaera* spp. were the dominant LU-HPB in the simple system of hydrogen fermentation in the previous study. First, to test for the presence of *Megasphaera* spp., *Megasphaera*-specific PCR-RFLP analysis was applied to all fermented slurry. Then, species-level identification by RFLP analysis of all positive PCR amplicons was performed with *Hae*III/*Msp*I. Comparison of the RFLP band profiles with the results of the previ-

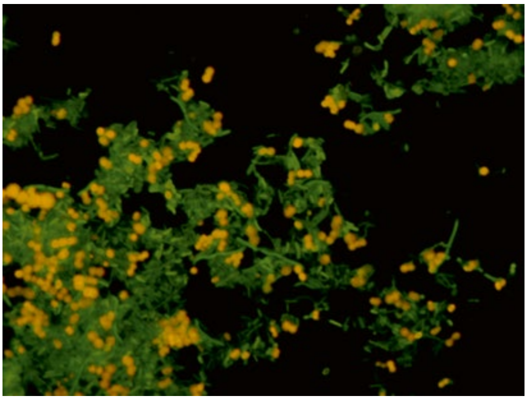


Fig. 4.5 Micrographs of samples from the hydrogen fermented sample with non heat shock treated inoculum. *Megasphaera* cells that hybridized with the FISH probes Mega-X (Cy3 labelled) and EUB338 (Alexa488 labelled) are shown in *pink*; other bacterial cells that reacted with EUB338 (Alexa488 labelled) are shown in *green*. *Bar*, 10 mm

ous study was used to identify species. Unknown band profiles may indicate new species. Finally, the *Megasphaera*-specific FISH method was used to determine the presence of *Megasphaera* in environmental samples (Fig. 4.5).

These methodologies, PCR-RFLP analysis with the primer set Mega-142F/Mega-X, restriction digests using *Hae*III/*Msp*I (Ohnishi et al. 2011a), and FISH analysis using the FISH probe Mega-X (Ohnishi et al. 2012a), are useful for the rapid detection, identification, and single-cell level monitoring of *Megasphaera* spp. in environmental samples. In the future, these methodologies will contribute to the analysis of regional spread of *Megasphaera* spp. in the environment.

4.5 Future Prospects for Use of LU-HPB in Hydrogen Fermentation Systems

Natural biomass is composed of complex organic matter. It is thought that *M. elsdenii* cannot utilize some substrates, such as cellobiose, trehalose, or xylose, shown in Sect. 4.3. Xylose is a main component of natural biomass such as wood and agricultural waste. However, some of LAB can produce lactate from xylose, and *M. elsdenii* is known to generate hydrogen from lactate. Thus, *M. elsdenii* may indirectly produce hydrogen from xylose via LAB in this manner. Yokoyama et al. (2007) showed 0.56 mol/mol xylose as hydrogen yield by complex microflora, and Li et al. (2010) showed 0.96 mol/mol xylose as hydrogen yield by *Thermoanaerobacterium* strain of genetic recombination. However, Abdel-Rahman et al. (2011) showed that *Enterococcus mundtii* QU 25 as one of the LAB can generate lactate as yield of 1.51 mol lactate/mol xylose. Of course *M. elsdenii* cannot consume xylose; however, *M. elsdenii* may generate 0.61 mol/mol xylose of hydrogen via relay of lactate production by *E. mundtii* as LAB. Collaboration of lactate fermentation and hydrogen fermentation likely improves consumption of fermentation materials and the performance of hydrogen fermentation from various biowaste. A model hydrogen fermentation system using lactic acid fermentation could be developed in the future (Fig. 4.6).

A new species of *Megasphaera*, *M. indica*, was identified in 2014 (Lanjekar et al. 2014). Because the genus *Megasphaera*-specific detection methods introduced in this chapter were developed in 2011–2012 (Ohnishi et al. 2011a, 2012a), *M. indica* was not included in this analysis. However, the 16S rRNA gene sequence in *Megasphaera*-specific region in *M. indica* contains no mismatches as the target sites of the PCR primer set or the FISH probe. It is likely that these methodologies can be applied to *M. indica*.

M. indica was isolated in the feces of two healthy human volunteers and was proposed as a new species in 2014. *M. indica* is LU-HPB, similar to *M. elsdenii*, and it has been reported to have similar hydrogen productivity to *M. elsdenii*. The characteristics of the end products of hydrogen fermentation of *M. elsdenii* and *M. indica* are also similar (Fig. 4.7). After cultivation on PYG liquid medium (pH 7.0) at 37 °C for 3 days, these two species produce mainly acetate, butyrate, caproate, and formate from glucose. Likewise, these two species produce mainly acetate, butyrate, and propionate from lactate. Based on our data set, valerate may be a specific end product in *M. elsdenii*. Based on the data published by Lanjekar et al. (2014), succinate is a specific end

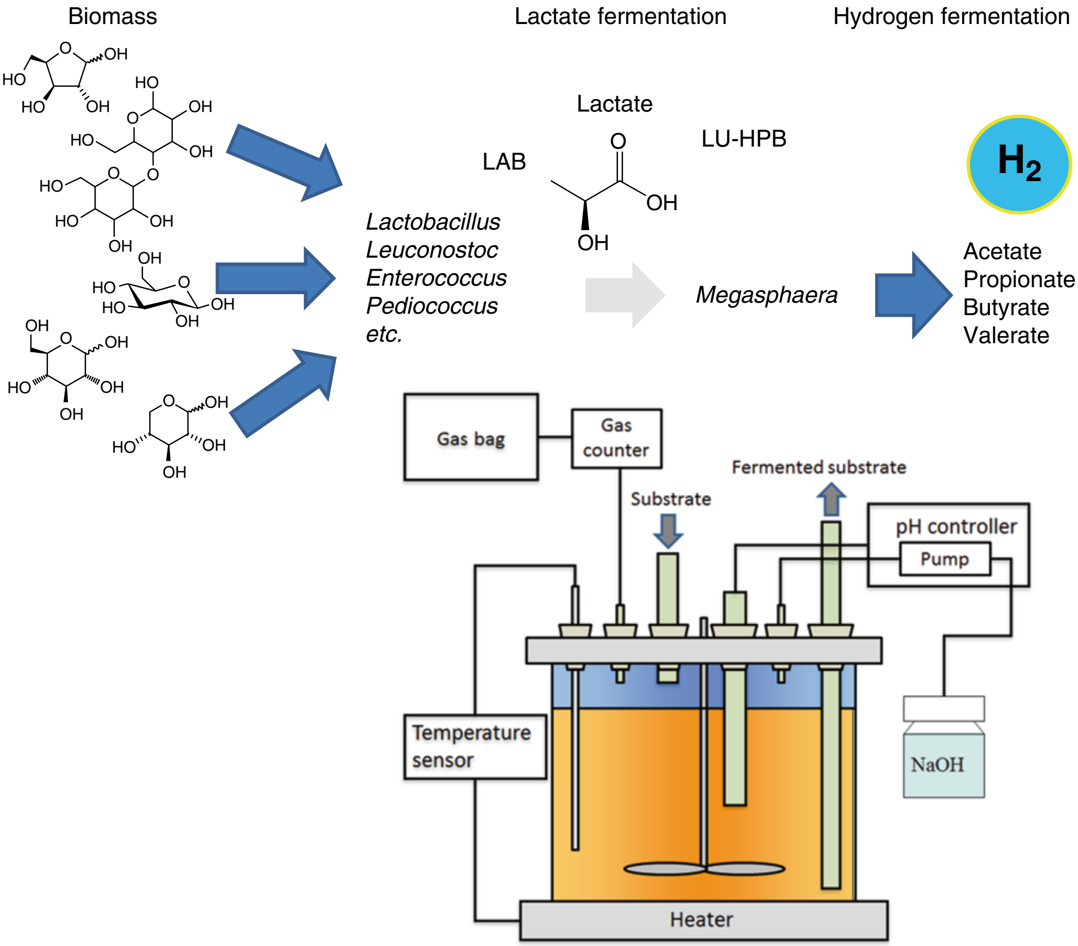


Fig. 4.6 Hydrogen fermentation system model using lactic acid fermentation as pivot

product of *M. indica* but not of *M. elsdenii* when glucose is used as the substrate.

Thus, the genus *Megasphaera* appears to contain a diverse group of species. This leads to the expectation of finding further beneficial species of *Megasphaera* as LU-HPB for implementation of hydrogen fermentation systems. *Megasphaera-specific* detection methods will be useful to achieve this goal.

4.6 Conclusion

Lactate was not recognized as a useful feed for hydrogen production under dark-fermentative conditions, unlike other saccharides. To solve this "blind spot" in the hydrogen production process, we tried minute researches. Using complex microflora with non-heat shock treatment, hydrogen was generated from lactate as substrate. *M. elsdenii* is a lactate-utilizing hydrogen-producing bacterium (LU-HPB). In pure culture, hydrogen productivity of *M. elsdenii* was reached to 0.4 mol/mol lactate. As the interesting point, *M. elsdenii* might be eliminated from hydrogen fermentation system by heat shock treatment of inoculum and/or substrate, because *M. elsdenii* is a non-heat tolerance microbe. This is another "blind spot" in the study of microbial aspect of hydrogen dark fermentation. Even if the glucose as substrate was consumed completely by homotype lactate fermentation, 0.8 mol of hydrogen per mol of con-

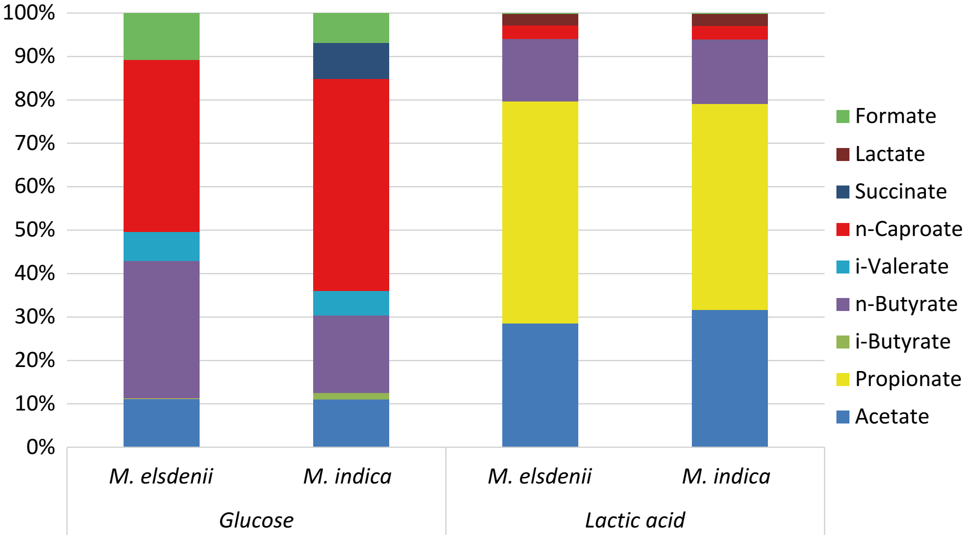


Fig. 4.7 Variations in the types and levels of VFAs detected in *Megasphaera elsdenii* and *Megasphaera indica*

sumed glucose can be recovered. In this way, LU-HPB as new type of hydrogen producer does not require inoculum and/or substrate pretreatments, such as heat shock treatment, and their use may improve the overall energy budget of hydrogen fermentation systems.

In addition, *Megasphaera* species are important both in human health and food production. A *Megasphaera*-specific detection methodology, including specific PCR, RFLP, and FISH, will contribute to rapid detection, identification, and discovery of novel *Megasphaera* spp. in the future.

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Akihiro Ohnishi received the Ph.D. in the field of environment control in biology from Tokyo University of Agriculture. He is Associate Professor of Tokyo University of Agriculture. His current research focuses on the ecology of environmental microflora and highlights "blind spots" in the microbial ecosystems. His current interests are biofuel, uncultivable environmental microorganisms, Japanese sake brewing process, and cocoa fermentation process.

5

Integrative Approach for Biohydrogen and Polyhydroxyalkanoate Production

Sanjay K.S. Patel, Prasun Kumar, Mamtesh Singh,
Jung-Kul Lee, and Vipin Chandra Kalia

Abstract

Carbohydrates especially sugars are employed for the production of different bio-products. Biological waste(s) originating from agricultural, industrial, and municipal sources has been considered as suitable low-cost feed for the production of biofuels and biopolymers. Single-stage production of these bio-products does not lead to complete utilization of organic matter of the biowaste(s) used as feed. Recently, approaches to integrate bioprocesses leading to hydrogen (H_2), polyhydroxyalkanoate (PHA), and methane production are gaining importance to metabolize more than 80 % of the biowastes. Integration of H_2 and PHA has been proposed but has not been widely studied. Here, we are evaluating the feasibility of integrating H_2 and PHA production systems. A further integration of these processes with methanogenesis might be a suitable approach in the near future for overall efficiency.

5.1 Introduction

Mankind has utilized the earth's resources for personal growth and development. Worldwide, the issue of prime concern has been environmental pollution caused by ever-increasing quantum of wastes and rapid depletion of fossil fuels. Hydrogen (H_2) and bioplastic (polyhydroxyalkanoate, PHA) are considered as a clean fuel and biodegradable polymers, which can substitute fossil fuels and petroleum-based non-biodegradable synthetic plastics, respectively (Kalia et al. 2000a, b; Reddy et al. 2003; Kalia and Purohit 2008; Pielke et al. 2008; Jain 2009; Patel et al. 2010, 2011, 2012a, 2015; Rehm 2010; Kumar et al. 2013, 2015a, b, c; Patel and Kalia 2013; Nissila et al. 2014). Almost all activities of our lives are associated with waste

S.K.S. Patel (✉) • J.-K. Lee
Department of Chemical Engineering, Konkuk University, 1 Hwayang-Dong, Gwangjin-Gu, Seoul 143-701, Republic of Korea
e-mail: sanjaykspatel@gmail.com; sanjaykspatel@yahoo.co.in; jkrhee@konkuk.ac.kr

P. Kumar
Microbial Biotechnology and Genomics, CSIR-Institute of Genomics and Integrative Biology (IGIB), Delhi University Campus, Mall Road, Delhi 110007, India

M. Singh
Department of Zoology, Gargi College, University of Delhi, Siri Fort Road, Delhi 110049, India
e-mail: s.mamtesh@yahoo.com

V.C. Kalia
Microbial Biotechnology and Genomics, CSIR-Institute of Genomics and Integrative Biology, Delhi University Campus, Mall Road, Delhi 110007, India

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production to varying extents. Slow and uncontrolled fermentation by microbes is a menace to human health and the environment. The somberness of this problem is exasperated by the release of obnoxious gases by burning of fuels. These have resulted in search for alternative cleaner sources of energy. Although, nature has evolved strong mechanisms for its maintenance, the evolutionary development of living beings is directly organized by environmental conditions. Prospects of depletion of nonrenewable resources have led to a blistering debate on these issues (Hallenbeck and Benemann 2002; Levin et al. 2004; Kalia and Purohit 2008; Manish and Banerjee 2008; Das 2009; Levin and Chahine 2010; Kumar et al. 2013). Predicted estimates for the utilization rate of energy resources show that the coal deposits will be used up within the next 150–200 years and the petroleum deposits in the next few decades. Besides this, the damage being caused to the environment by these fuels is a matter of great concern all over the world (Kalia et al. 1994; Kalia and Joshi 1995; Kalia and Purohit 2008; Levin et al. 2004; Kapdan and Kargi 2006; Jain 2009). The different waste disposable methods exploited at present are (1) burning of waste, (2) composting, (3) landfills, (4) briquetting and recycling of waste material, and (5) microbial treatment, aerobic, anaerobic, etc. Each of these methods has its own advantages and can be used in certain types of wastes. Apart from these conventional methods of waste management, biowaste(s) has been used in the production of biofuels through anaerobic fermentation. It holds the potential for fossil fuel reserves as well as to minimize greenhouse gas discharges. However, the direct role of waste materials as biological is a challenging approach as a feed for the production of important bio-products. Microbial treatment methods have demonstrated the production of biofuels such as H_2 and methane (CH_4) from biowaste materials (Kalia et al. 1992a, b; Sonakya et al. 2001; Raizada et al. 2002; Kalia and Lal 2006; Kalia 2007; Kim and Shin 2008; Xia et al. 2008; Levin and Chahine 2010; Kim and Kim 2013; Kumar et al. 2014a, 2015a).

Recently, integrative approach for the production of H_2 and PHA through the coupling has gained importance to improve the overall process efficiency (Amulya et al. 2014; Ntaikou et al. 2009; Reddy et al. 2012, 2014; Yan et al. 2010; Venkata Mohan et al. 2010; Patel et al. 2011). These two processes have been demonstrated by employing different sets of microbial cultures. Diverse groups of well-known and well-defined H_2 and PHA producers have proved to be more effective than undefined mixed cultures. And these pure cultures can be mixed together to improve the integrative processes (Reddy and Venkata Mohan 2012; Amulya et al. 2014; Patel et al. 2015). In order to develop a robust system, the need is to exploit the available microbial diversity and prepare well-defined microbial consortia. Efficiency of bioconversion of waste(s) to renewable fuels or PHA can be significantly improved through the use of microbes with diverse abilities at each stage of degradation: (1) hydrolysis of the complex organic matter, (2) H_2 production by dark fermentation followed with photosynthetic route, and (3) PHA production process, which utilizes volatile fatty acids produced in the previous stages (Albuquerque et al. 2007; Kumar et al. 2009; Redwood et al. 2009; Patel et al. 2012b, 2015; Arumugam et al. 2014; Rai et al. 2014; Rosa et al. 2014; Yin et al. 2014; Singh et al. 2015). The primary aim of this chapter is to feature integrative production of H_2 and PHA.

5.2 Biological Hydrogen Production

H_2 is recognized as a major alternate energy sources for the twenty-first century (Jain 2009). In view of biowaste(s) utilization, biological H_2 production (BHP) from these renewable sources is a suitable approach. This process has received significant attention for the past few decades. Among the various H_2 production processes, fuel processing, hydrocarbon and gas reforming, desulfurization, pyrolysis, and biomass gasification are energy intensive. BHP can be performed at normal ambient temperature and is relatively less energy intensive (Holladay et al. 2009; Patel et al. 2012a). BHP is carried out either by photosynthetic or non-photosynthetic (dark-fermentative) routes. Presently, these processes are associated with low-H_2-yielding microbes which are still not industrially suitable and higher feed cost. Photosynthetic processes seem sustainable approach, but it is limited by the necessity of light source and high sensitivity of hydrogenases to oxygen (Kalia and Purohit 2008; Manish and Banerjee 2008; Patel et al. 2010, 2012a; Kumar et al. 2013).

In prokaryotic organisms, H_2 evolution is carried out through two enzymes: hydrogenase and nitrogenase to release excess of protons formed during the metabolic processes. Hydrogenases are present in variable sizes and structure, which are classified into three groups: (1) NiFe-, (2) FeFe-, and (3) Fe-containing enzymes. Based on their activities, these can be divided into four subgroups as (a) uptake; (b) cytoplasmic H_2 (sensors) and cyanobacterial; (c) bidirectional, heteromultimeric, or cytoplasmic; and (d) H_2-producing, conserving energy and associated with the cell membrane. Among these, NiFe hydrogenases are more dominant and widely distributed. FeFe and NiFe hydrogenases are involved primarily in H_2 production and consumption, respectively (Kumar et al. 1998, 2013; Atta and Meyer 2000; Kalia et al. 2003a; Kalia and Purohit 2008).

Very few new H_2 producers have been reported in the past decades with little improvement in the H_2 production yields (Kalia and Purohit 2008; Patel et al. 2012a). Recently, screening and genomics approaches have been evaluated to search for novel H_2 producers (Kalia et al. 2003a; Rittmann et al. 2008; Porwal et al. 2008). In the dark-fermentative BHP process, maximum theoretical H_2 yields of 2 or 4 mol/mol of hexose can be achieved, when butyrate and acetate are generated as intermediate metabolites of sugar metabolism (Eqs. 5.1 and 5.2). In addition to this, the H_2 production yields are also influenced by feedback inhibition caused by high partial pressure (Levin et al. 2004; Kalia and Purohit 2008; Patel et al. 2012a). Dark-fermentative H_2 production yields (mol/mol hexose) have been reported by organisms belonging to (1) Firmicutes – *Bacillus* spp. (*B. cereus*, *B. thuringiensis*, *B. coagulans*, and *B. licheniformis* (1.5–2.6)), *Caldicellulosiruptor saccharolyticus* (up to 3.6), *Ethanoligenes harbinese* (up to 2.85), and *Clostridium* spp.(*C. beijerinckii*, *C. butyricum*, and *C. pasteurianum* (up to 2.4–3.2)); (2) *Thermotogales* (*Thermotoga neapolitana*) (up to 3.8); and (3) *Proteobacteria* – *Enterobacter aerogenes*, *E. cloacae* (2.85–3.8), and *Escherichia coli* (up to 3.12) (Kotay and Das 2007; Porwal et al. 2008; Patel et al. 2012a, 2014; Kumar et al. 2013, 2015c).

$$C_6H_{12}O_6(\text{hexose}) + 2H_2 \rightarrow 2C_2H_4O_2(\text{Acetate}) + 2CO_2 + 4H_2 \quad (5.1)$$

$$C_6H_{12}O_6 \rightarrow C_4H_8O_2(\text{Butyrate}) + 2CO_2 + 2H_2 \quad (5.2)$$

In the photo-fermentative process, organisms utilize direct sunlight to initiate anaerobic photosynthesis. During this process, H_2 is evolved by nitrogenase under nitrogen-deficient conditions. An overall theoretical maximum H_2 yield of 12 mol/mol of hexose sugar can be produced (Eq. 5.3). Photo-fermentation of different VFAs can evolve up to 10 mol of H_2 (Eqs. 5.4, 5.5, 5.6, and 5.7). Under photo-fermentative conditions, H_2 yields (mol/mol of substrate) have been reported by organisms belonging to α-proteobacteria: (1) *Rhodobacter* – *R. capsulatus* and *R. sphaeroides* (3.6–7.2) and (2) *Rhodopseudomonas* – *R. faecalis* and *R. palustris* (3.5–6.2) (Patel et al. 2012a).

$$C_6H_{12}O_6(\text{Glucose}) + 6HO \rightarrow 12H_2 + 6CO_2 \quad (5.3)$$

$$C_2H_4O_2(\text{Acetate}) + 2H_2O \rightarrow 4H_2 + 2CO_2 \quad (5.4)$$

$$C_4H_8O_2(\text{Butyrate}) + 6H_2O \rightarrow 10H_2 + 4CO_2 \quad (5.5)$$

$$C_4H_6O_5(\text{Malate}) + 3H_2O \rightarrow 6H_2 + 4CO_2 \quad (5.6)$$

$$C_4H_6O_4(\text{Succinate}) + 4H_2O \rightarrow 7H_2 + 4CO_2 \quad (5.7)$$

For improvement in the BHP from biowaste(s) as primary feed, H_2-producer's acclimatization, selective enrichment, and defined mixed cultures should be evaluated from different biowastes or effluents. Although mixed cultures are largely demonstrated as suitable inocula for the H_2 production from the complex feed, these processes can result in uncontrolled fermentation, when unsterilized feed is used. This might lead to a significant variation in H_2 yields from mixed cultures: 1.0–2.5 mol/mol of hexose (Kleerebezem and van Loosdrecht 2007; Venkata Mohan et al. 2010; Patel and Kalia 2013). Search for hydrolytic and H_2 producers was found to be effective from effluents of industrial effluents (Porwal et al. 2008; Rani et al. 2008; Patel et al. 2014). Immobilization of microbes by different methods has also helped in increasing H_2 production under continuous culture conditions. It was used to overcome the problem of washout of cultures during this process (Kumar et al. 1995; Ivanova et al. 2008; Patel et al. 2010). Both natural and synthetic polymers as supports were used for H_2 production (Yokoi et al. 1997; Kumar and Das 2001; Ivanova et al. 2008). Fermentative H_2 production with immobilized cultures on lignocellulosic supports resulted in variable yields due to differential loading of cells under similar sets of experimental conditions. These variations may be minimized with selection of suitable supports for each type of H_2 producer. These immobilization reports suggested that use of nonpolluting, renewable wastes as biocompatible support seems to significantly support long-term H_2 production (Kumar and Das 2001; Patel et al. 2010, 2014, 2015).

5.3 Polyhydroxyalkanoates Production

PHAs are natural polymers, which are stored as food reserve by many prokaryotic organisms. Mostly, they are synthesized under excess of carbon (C) in the environment and limitations of essential nutrients such as nitrogen, phosphorus, or iron, which are essential for their growth (Kumar et al. 2009, 2013, 2014b; Singh et al. 2009, 2013, 2015; Wu et al. 2012; Arumugam et al. 2014). It serves primary role as a reserve food source and is used by organisms under stress environmental conditions for their survival. PHA accumulation by organisms varies in the range of 50–90 % of their total dry cell mass (DCM) (Singh et al. 2009; Kumar et al. 2013, 2015b). Currently, industrial scale production of PHA is limited by the costly feed material. These are synthesized either as homo- or copolymers. Polyhydroxybutyrate (PHB) is a homopolymeric form of PHA, which is widely reported from phylogenetically diverse organisms. Biosynthesis of PHB involves three enzymes in the serial order: (1) β-ketothiolase (*phaA*), (2) acetoacetyl-CoA dehydrogenase (*phaB*), and (3) PHB synthase (*phaC*) (Kalia et al. 2003b, 2007; Reddy et al. 2003; Singh et al. 2009; Rehm 2010). A wide variation in PHA polymers synthesis is due to variation in the *phaC* gene in these organisms. On the other hand, organisms can also use different secondary pathways such as methylmalonyl CoA, de novo fatty acid, or metabolic pathway involving stereospecific 2-enoyl-CoA hydratases (Kumar et al. 2013).

PHA has been recognized as the best candidate for use as biodegradable polymers. Homopolymer such as PHB is not suitable for the broadest range of applications due to its low strength and high crystalline properties (Singh et al. 2009, 2015; Rehm 2010; Kumar et al. 2013). In comparison, of homopolymers (PHB), copolymers are a more suitable choice with nearly similar properties as synthetic polyethylene. Both Gram-negative (*Alcaligen*es, *E. coli*, *Pseudomonas*, and *Ralstonia*) and Gram-positive (*Bacillus*, *Clostridium*, and *Rhodococcus*) groups of organisms have been reported for PHA production. Thus, various approaches involving a suitable complementation of organism and biowaste(s) as feed are required for producing PHAs with different properties such as high molecular weight and compositions for large-scale biotechnological applications.

5.4 Integrative Hydrogen and Polyhydroxyalkanoate Production

BHP from the biomass is considered as the most environment-friendly process. But, the major challenges associated with lower yields of H_2 and removal of only 30–40 % of total organic matter during this process (Reddy et al. 2014). It is thus imperative to club this process with other systems, which will enable achievement of high yields and complete utilization of biowaste(s). In view of the objective of attaining efficient utilization of feed, the production of different bioproducts has been demonstrated through the integration of two- or multi-stage system (Vincenzini et al. 1997; Kim and Kim 2013; Saraphirom et al. 2013; Patel et al. 2012a, b, 2015). The feasibility of integrating the two stages of producing H_2 and CH_4 has been shown as (1) dark-fermentative H_2 production followed photo-fermentation, (2) dark-fermentative H_2 production followed by CH_4 production, and (3) photo-fermentative H_2 production followed by CH_4 production (Hallenbeck and Ghosh 2009; Patel et al. 2012a, b; Kim and Kim 2013; Xia et al. 2008; Rai et al. 2014; Yin et al. 2014). The feasibility of a new integrative approach to couple the H_2 production was observed recently (Ntaikou et al. 2009; Venkata Mohan et al. 2010; Yan et al. 2010; Patel et al. 2011, 2015; Reddy et al. 2014). H_2 and PHA production processes are competing with each other. These have been widely studied in photo-fermentative organisms *R. palustris*, *R. sphaeroides*, *Rhodospirillum rubrum*, and *Thiocapsa roseopersicina* (Hustede et al. 1993; Kim et al. 2011; Chen et al. 2012; Fulop et al. 2012; Ye et al. 2013). This correlation was supported by the comparison made between wild type and its PHB-negative mutants, where the latter resulted in high H_2 production. Recently, different dark-fermentative organisms with abilities to produce both H_2 and PHA have been reported. These belonged to organisms identified as *Bacillus* sp., *B. licheniformis*, *B. subtilis*, *B. cereus*, *B. thuringiensis*, *E. aerogenes*, *Proteus mirabilis*, and *Pseudomonas stutzeri* (Porwal et al. 2008). This work was followed by a few reports on *Bacillus* for integrative two stages – H_2 and PHB production (Patel et al. 2011; Singh et al. 2013). In general, H_2 production occurs during anaerobic and PHA production under aerobic conditions. PHAs can be mobilized and utilized for a survival of organisms to overcome environmental stress conditions such as carbon or nutrient limitation, UV light irradiation, temperature, and pH fluctuation (Wu et al. 2012).

5.4.1 With Different Cultures in Two-Stage Process

Different sets of H_2 and PHA producers are used for an integrative study of two-stage system. For the H_2 production stage, different undefined consortia and defined mixed cultures were used (Table 5.1). As undefined cultures, inocula were prepared by selective enrichment of H_2 producers through heat treatment of anaerobic sludge (Ntaikou et al. 2009; Venkata Mohan et al. 2010; Yan et al. 2010; Saraphirom et al. 2013; Reddy et al. 2014). Integrative production using defined or undefined mixed cultures of H_2 producers and aerobic consortia or pure cultures for PHA have been reported to be highly variable and depend upon the type of feed (Table 5.1). A synthetic or acidogenic wastewater prepared by adding pure glucose resulted in maximum H_2 yields of 280 L/kg of chemical oxygen demand (COD) by anaerobic consortia with slightly variable PHA contents of total DCM by aerobic consortia (25 %), *Bacillus tequilensis* (40–59 %), and *Pseudomonas otitidis* (54–58 %) (Venkata Mohan et al. 2010; Reddy et al. 2012; Amulya et al. 2014). Among these, PHA producers in a second step, *B. tequilensis*, resulted in maximum PHA contents of 59 % of DCM. H_2 production yields from biowastes are lower than synthetic wastewater as feed. The different biowaste resulted in H_2 yields of 24–70 L/kg of total solids (TS) from pea shells (Patel et al. 2015), 118 L/kg COD from food waste (Reddy et al. 2014), 196 L/kg COD from olive mill (Ntaikou et al. 2009), and 105 L/kg COD from Taihu blue algae (Yan et al. 2010). In addition, PHA contents (%) in second stage were up to 64.7 by *B. cereus* EGU43 from pea shells (Patel et al. 2015), 36 by *B. tequilensis* from food waste (Reddy et al. 2014), 8.9 by *Pseudomonas* sp. from olive mill (Ntaikou et al. 2009), and 43.3 by *B. cereus* from Taihu blue algae (Yan et al. 2010). The high H_2 yields from synthetic wastewater may be explained by pure glucose as a main component as compared to mixed sugars and some inhibitory substrates in cases of biowastes as feed. Combinations of anaerobic sludge and *Cupriavidus* sp. KKU resulted in low H_2

Table 5.1 Integrative biohydrogen (H_2) and polyhydroxyalkanoate (PHA) production in two stages with separate cultures

	Organisms		Process parameters (in H_2/PHA stage)					
Feed	H_2 stage	PHA stage	Working capacity (L)	pH	Temp. (°C)	IP[a] (Days)	Yields[b]	References
Food waste	Anaerobic consortia	Aerobic consortia	0.82/0.11	6.8/7.0	29/29	3/1	9.3/39.6	Reddy and Venkata Mohan (2012)
		Bacillus tequilensis	0.84/0.10	6.0/7.0	29/28	2/1.5	118/36.0	Reddy et al. (2014)
Olive mill waste	Anaerobic sludge	*Pseudomonas* sp.	0.50/0.75	5.5/7.0	35/25	98/3.5	196/8.9	Ntaikou et al. (2009)
Pea shells	Defined mixed culture (MMC4)	*Bacillus cereus* EGU43	0.25/0.20	7.0/7.0	37/37	4/2	70/19.1	Patel et al. (2015)
			0.80/0.20	7.0/7.0	37/37	40/2	24/22.2	
	MMC4 (Immobilized)		0.80/0.20	7.0/7.0	37/37	40/2	54/64.7	
Synthetic wastewater	Anaerobic consortia	*B. tequilensis*	18.0/0.10	6.0/7.0	29/29	3/1.5	142/40.0	Amulya et al. (2014)
			0.84/0.10	6.0/7.0	29/28	2/1.5	280/59.0	Venkata Mohan et al. (2010) and Reddy et al. (2014)
		Aerobic consortia	1.40/0.12	6.0/7.0	29/29	1/1	280/25.0	Venkata Mohan et al. (2010)
		Pseudomonas otitidis	18.0/0.25	6.0/7.0	29/28	3/1.5	280/58.0	Venkata Mohan et al. (2010) and Reddy et al. (2012)
Synthetic acids			18.0/0.25	6.0/7.0	29/28	3/1.5	280/54.0	
Sweet sorghum	Anaerobic sludge	*Cupriavidus* sp. KKU	1.00/0.10	5.0/7.0	30/30	12/3	0.68[c]/71.4	Saraphirom et al. (2013)
Taihu blue algae	Anaerobic sludge	*Bacillus cereus*	3.00/2.00	7.5/7.0	35/30	–[d]/2	105/43.3	Yan et al. (2010)

[a] Incubation period

[b] L H_2/kg COD or TS per PHA (%) of DCM

[c] mol H_2/mol of hexose

[d] Values not given

yields of 0.68 mol/mol of hexose and highest PHA contents of 71.4 % of DCM. A single report on defined mixed culture (MMC4), a combination of six strains belonging to *Bacillus* spp. – *B. cereus* EGU43, *B. pumilus* HPC464, *B. thuringiensis* EGU45, *Bacillus* sp. HPC459, *E. aerogenes* EGU16, and *P. mirabilis* EGU21, has shown dark-fermentative H_2 production integrated with pure cultures of PHA producers (Patel et al. 2015). These results suggest efficient PHA production at the second stage than most previous works. Thus, selective design of the H_2 producers will be a more promising approach during the near future.

5.4.2 With Same Culture

Dark- and photo-fermentative organisms have abilities to produce both H_2 and PHA. Photo-fermentative organisms *R. palustris*, *R. sphaeroides*, and *R. rubrum* have been well studied for the production of H_2 and PHA and their overall yields could be improved through selective mutagenesis (Hustede et al. 1993; Vincenzini et al. 1997; Kim et al. 2011; Chen et al. 2012). Previous studies suggested that these two processes are significantly dependent on each other and a negative correlation was observed between them with pure cultures under the same conditions. However, two-stage systems are adapted for improving the overall yields of H_2 production followed with PHA production. On the other hand, *Bacillus* has been recently reported to produce H_2 and PHA in two-stage systems (Patel et al. 2011; Singh et al. 2013). In general, sugar is used for dark fermentation and volatile fatty acids for photo-fermentative conditions productions. The H_2 and PHA production yields of these organisms were highly variable with different types of feed (Table 5.2). The optimum temperatures for the H_2 and PHA production were about 30 and 37 °C for photo- and dark-fermentative organisms with the narrow range of pH 6.8–7.0, respectively. These productions occurred up to 11 days for pure cultures. Interestingly, lower pH value and incubation of 5.1 and 3 days was observed with co-culture of *E. aerogenes* and *R. sphaeroides* (Arumugam et al. 2014). From glucose as feed, *Bacillus* spp. – *B. cereus* and *B. thuringiensis* strains – have produced up to 1.92 mol H_2/mol of glucose, whereas H_2 yields were 0.91 mol/mol of glucose by *R. sphaeroides* ATCC17023 (Hustede et al. 1993; Patel et al. 2011; Singh et al. 2013). Among these strains, PHA content was observed in the range of 4.9–31 % of DCM. A negative correlation was observed in *B. cereus* and *B. thuringiensis* for overall H_2 and PHA production. In a shift in feed from glucose to fructose, *R. sphaeroides* ATCC17023 improved H_2 yields from 0.91 to 1.70 mol/mol hexose but slightly decreased the PHA content from 31 to 27 %. These results suggested that both H_2 and PHA production are significantly influenced with both feed and type of organism (dark or photo-fermentative).

Different volatile fatty acids – acetate, propionate, pyruvate, lactate, butyrate, malate, fumarate, and succinate – were used by photo-fermentative organisms for H_2 and PHA production (Table 5.2). *R. palustris* WP3-5 resulted in H_2 in the range of 2.13–3.65 mol/mol of substrates and PHA contents in the range of 0.1–17.1 % of DCM from acetate, propionate, lactate, butyrate, and malate as pure feed (Chen et al. 2012; Wu et al. 2012). Maximum H_2 yields of 3.65 mol/mol substrate and PHA contents of 17.1 % were observed on lactate and acetate as feed, respectively (Wu et al. 2012). Even on same substrate such as malate, two strains WP3-5 and 42OL of *R. palustris* have shown significant differences in their PHA contents. *R. palustris* 42OL accumulated PHA, which was about 180-fold higher than the *R. palustris* with contents of 0.10 % of DCM (Vincenzini et al. 1997; Chen et al. 2012). However, slightly lower H_2 yield of 1.48 mol/mol of malate was observed with *R. palustris* 42OL strain for incubation of 3 days in comparison with 2.13 mol/mol of malate by *R. palustris* WP3-5 for longer incubation period of 11 days. Similarly, between two *R. sphaeroides* strains KD131 and ATCC17023, the latter strain is reported for H_2 and PHA production from different VFAs (Hustede et al. 1993). This resulted in H_2 yields (mol/mol of substrate) and PHA contents (%) – (1) 2.20 and 11.0 from pyruvate, (2)

Table 5.2 Integrative biohydrogen and polyhydroxyalkanoate production with same cultures

		Process parameters (in hydrogen/polyhydroxyalkanoate stage)					
Feed	Organisms	Working capacity (L)	pH	Temp. (°C)	IP[a] (Days)	Yields[b]	References
Acetate	*Rhodopseudomonas palustris* WP3-5	0.10/0.20	6.8	32	11	1.84/5.30	Chen et al. (2012)
					6	2.82/17.1	Wu et al. (2012)
	Rhodobacter sphaeroides KD131	0.40/0.50	7.0	30	3	0.80/21.0	Kim et al. (2011)
	Rhodospirillum rubrum Ha	0.10/0.25	7.0	30	7	nd[d]/63.0	Hustede et al. (1993)
	Rhodospirillum rubrum S1	0.10/0.25	7.0	30	7	nd/50.0	
Propionate	*R. palustris* WP3-5	0.10/0.20	6.8	32	6	2.48/11.8	Wu et al. (2012)
Pyruvate	*Rhodobacter sphaeroides* ATCC17023	0.10/0.25	7.0	30	7	2.20/19.0	Hustede et al. (1993)
Butyrate	*R. palustris* WP3-5	0.10/0.20	6.8	32	11	3.57/8.40	Chen et al. (2012)
Lactate	*R. sphaeroides* ATCC17023	0.10/0.25	7.0	30	7	3.67/24.0	Hustede et al. (1993)
	R. palustris WP3-5	0.10/0.20	6.8	32	6	3.65/3.75	Wu et al. (2012)
Malate	*R. sphaeroides* ATCC17023	0.10/0.25	7.0	30	7	3.08/16.0	Hustede et al. (1993)
	Rhodopseudomonas palustris 42OL	5.00/5.00	6.8	30	3	1.48/18.0	Vincenzini et al. (1997)
	R. palustris WP3-5	0.10/0.20	6.8	32	11	2.13/0.10	Chen et al. (2012)
Fumarate	*R. spreads* ATCC17023	0.10/0.25	7.0	30	7	3.15/15.0	Hustede et al. (1993)
Succinate	*R. sphaeroides* KD131	0.40/0.50	7.0	30	3	1.87/5.90	Kim et al. (2011)
	R. sphaeroides ATCC17023	0.10/0.25	7.0	30	7	1.89/4.00	Hustede et al. (1993)
	R. rubrum Ha	0.10/0.25	7.0	30	7	3.85/2.00	
	R. rubrum S1	0.10/0.25	7.0	30	7	3.11/1.00	
Glucose	*Bacillus cereus* EGU3	0.25/0.20	7.0	37	5	0.96/4.90	Patel et al. (2011)
	B. cereus EGU43	0.25/0.20	7.0	37	5	1.20/8.10	
	B. cereus EGU44	0.25/0.20	7.0	37	5	1.92/8.80	
	Bacillus thuringiensis EGU45	0.25/0.20	7.0	37	5	0.58/18.6	Singh et al. (2013)
		0.25/0.20	7.0	37	5	1.67/11.0	Patel et al. (2011)
	R. sphaeroides ATCC17023	0.10/0.25	7.0	30	7	0.91/31.0	Hustede et al. (1993)
Fructose		0.10/0.25	7.0	30	7	1.70/27.0	
Oil cake	*Enterobacter aerogenes* + *Rhodobacter sphaeroides*	7.95/na[c]	5.1	30	3	7.95[e]/60.3	Arumugam et al. (2014)

[a] Incubation period of whole process

[b] mol H_2/mol substrate per PHA (%) of DCM

[c] Not applicable

[d] H_2 production not observed

[e] L H_2/L of medium

3.67 and 24.0 from lactate, (3) 3.08 and 16.0 from malate, (4) 3.15 and 15.0 from fumarate, and (5) 1.89 and 4.00 from succinate (Hustede et al. 1993). On the other hand, *R. sphaeroides* KD131 has shown H_2 yields and PHA contents in the range of 0.80–1.87 mol/mol of substrate and 5.9–21.0 %, respectively (Kim et al. 2011). Interestingly, *R. rubrum* strains S1 and Ha could not produce H_2 from acetate as sole carbon source, but resulted in high contents of PHA about 50 and 63 %, respectively (Hustede et al. 1993). In contrast to acetate, these strains have shown high H_2 production on succinate with yields of 3.11 and 3.85 mol/mol of substrate, whereas significant decrease in the PHA contents from 50–63 % to as low as 1–2 % (Hustede et al. 1993). In a simultaneous production approach, co-cultures of *E. aerogenes* (dark fermentative) and *R. sphaeroides* (photo-fermentative) demonstrated H_2 and PHA under dual dark and light phase of 16 and 8 h from *Calophyllum inophyllum* oil cake (Arumugam et al. 2014). The H_2 yields and PHA contents were 7.95 L/L medium and 60.3 % over a short incubation period of 3 days, respectively.

5.5 Future Perspectives

Many reports have suggested that both dark- and photo-fermentative organisms have abilities to synthesized H_2 and PHA. A very few reports are available on the integrative H_2 and PHA production by both dark- and photo-fermentative organisms. Among these strains, dark-fermentative organism – *Bacillus* – seems a potential candidate, which is a facultative organism having abilities to produce both H_2 and PHA. It has a significant advantage over the competitive photosynthetic organism, where light requirement is a must and long period of incubation is needed during overall fermentative process. In addition, it has ability to synthesize bioactive molecules (acyl-homoserine lactonases), which may selectively inhibit a wide range of organisms, which may accompany biowaste as contaminants during fermentation of unsterilized feed (Kalia and Purohit 2008, 2011; Kalia 2013; Kumar et al. 2013). *Bacillus* can synthesize more than 60 % content of PHA of DCM within 48 h, whereas photosynthetic organisms *R. palustris* and *R. sphaeroides* require up to 11 days to accumulate less than 10 % of PHA of DCM (Hustede et al. 1993; Chen et al. 2012; Amulya et al. 2014; Patel et al. 2015). For the reduction in high cost of feed (sugars), biowaste materials can be used as substrate. It seems a suitable integrative approach for efficient utilization of total organic matter present in the waste. Thus, a suitable complementation of these organisms or defined set of multiple organisms could be more beneficial to overcome the problems associated with (1) limited and selective utilization of feed by pure cultures, (2) broad range of tolerance towards changes in physiological conditions such as pH and temperature, and (3) bacterial population shift in cases of undefined nature of anaerobic sludge or mixed consortia as enriched inoculums. In addition, these processes can be further integrated to methanogenesis for the almost complete degradation of biowaste.

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Sanjay K.S. Patel received M.Sc. degree in Biotechnology from Himachal Pradesh University, Shimla, and Ph.D. degree from University of Pune, India. He is presently an Assistant Professor at Konkuk University, Seoul, South Korea. He is a life member of Association of Microbiologists of India (AMI) and the Biotech Research Society of India (BRSI). His research interests are in the areas of bioenergy and nano-technology.

Prasun Kumar received his M.Sc. degree in Biotechnology from KIIT University, Bhubaneswar, India. He is currently working as a senior research fellow at CSIRIGIB, Delhi, India. His current interests are biopolymers, biofuels and quorum sensing. His main theme of research is "production of polyhydroxyalkanoates from biowastes". He is a life member of the Association of Microbiologists of India (AMI) and the member of American Society for Microbiology (ASM).

Jung-Kul Lee M.Sc. in Biochemistry from Seoul National University and Ph.D. in Biological Science from KAIST, Daejeon, South Korea. He is presently Co-head of Institute of SK-KU Biomaterials and Professor of KU. He is Associate Editor of Journal of Microbiology and Biotechnology and Editor of Enzyme and Microbial Technology. His current interests are protein engineering and nanobiotechnology.

Mamtesh Singh received the M.Sc. degree in Zoology from University of Delhi, India, and Ph.D. in Biotechnology from CSIR-IGIB, Delhi, India. She is currently working as Assistant Professor in Gargi College, University of Delhi, India. Her current interests are microbial production of polyhydroxyalkanoates as biopolymers from biowaste.

Vipin Chandra Kalia M.Sc. and Ph.D. Genetics from IARI, New Delhi. He is presently the Chief Scientist at CSIR-IGIB and Professor of AcSIR. He is a Fellow of Association of Microbiologists of India and Fellow of National Academy of Sciences (FNASc). He is the Editor-in-Chief of the Indian Journal of Microbiology (India). His current interests are bioenergy, biopolymers and quorum sensing. Google Scholar: http://scholar.google.co.in/citations?hl=en&user=XaUw-VIAAAAJ. Website: http://www.igib.res.in/?q=V.C.Kalia

6

Recent Advances in Feedstocks and Enzyme-Immobilised Technology for Effective Transesterification of Lipids into Biodiesel

Madan L. Verma and Colin J. Barrow

Abstract

There are several technological and economic challenges that need to be addressed to make biodiesel production profitable. Among the technological obstacles in enzyme-catalysed transesterification process for biodiesel production, the selection of feedstock and robust biocatalyst are the critical factors for developing a cost-effective bioprocess. Feedstocks, mainly second and third generations, have been used recently to economise biodiesel production. Nanotechnology has revolutionised the enzyme immobilisation technology by providing versatile nanomaterials. Biocompatible nanomaterial is emerging as a novel immobilisation support for lipase enzyme to advance biodiesel production. Nanomaterials possess excellent properties such as higher surface area-to-volume ratios, lower mass transfer resistance, and quick separation from the reaction mixture using magnetic field. Utilising the cheap renewable feedstock such as waste oil and microalgae oil, nanomaterial-immobilised enzyme can be reused thus economising the process of enzymatic transesterification for biodiesel production. The chapter provides an insight of recent progresses in improving immobilised lipase technology, focusing on innovation in feedstock and nanomaterial processing such as synthesis, functionalisation, and characterisation with regard to biodiesel production. This chapter concludes that synergies between nanotechnology and industrial biotechnology will become an integral part of sustainable biodiesel production.

6.1 Introduction

Due to the environmental impacts and declining supplies of fossil fuels, there is growing interest in renewable energy sources, such as solar, hydro, wind, and biofuels (Brennan and Owende 2010; Pugh et al. 2011). In the present scenario, the source of 80 % energy consumption and

M.L. Verma (✉) • C.J. Barrow
Centre for Chemistry and Biotechnology, Faculty of Science Engineering & Built Environment, Deakin University, Geelong Waurn Ponds Campus, Geelong, VIC 3217, Australia
e-mail: madan.verma@deakin.edu.au; madanverma@gmail.com; colin.barrow@deakin.edu.au

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Table 6.1 Overview of biodiesel-feedstock types

Class	Feedstock	References
First-generation	Edible oil: soya bean oil (*Glycine max*), rapeseed oil (*Brassica napus*), palm oil (*Elaeis guineensis*), coconut oil (*Cocos nucifera*), sunflower oil (*Helianthus annuus*), peanut oil (*Arachis hypogaea*), safflower oil (*Carthamus tinctorius*), sesame oil (*Sesamum indicum*), safflower oil (*Carthamus tinctorius*), rice bran oil (*Oryza sativa*), corn (*Zea mays*), cotton oil (*Gossypium hirsutum*), passion fruit seed oil (*Passiflora edulis*), moringa oil (*Moringa oleifera*)	Karmakar et al. (2010), Singh and Singh (2010), Ahmad et al. (2011), Shahid and Jamal (2011), Lin et al. (2011) and Kumar and Sharma (2015)
Second-generation	Nonedible oil: jatropha oil (*Jatropha curcas*), neem oil (*Azadirachta indica*), polanga oil (*Calophyllum inophyllum*), Karanja oil (*Pongamia pinnata*), babassu oil (*Orbignya martiana*), rubber seed oil (*Hevea brasiliensis*), mahua oil (*Madhuca indica* and *Madhuca longifolia*), tobacco oil (*Nicotiana tabacum*), jojoba oil (*Simmondsia chinensis*), croton oil (*Croton megalocarpus*), tall oil (*Carnegiea gigantea*), castor oil (*Ricinus communis*)	Kafuku and Mbarawa (2010), Karmakar et al. (2010), Singh and Singh (2010), and Lin et al. (2011), Shahid and Jamal (2011), Ashraful et al. (2014) and Kumar and Sharma (2015)
	Animal fats: Pork lard (*Sus scrofa domesticus*), beef tallow (*Bos taurus*), poultry fat (*Gallus gallus domesticus*), fish oil (*Salmo salar*), etc.	
Third-generation	Oleaginous microorganisms: microalgae (*Chlorella protothecoides, Chlorella vulgaris, Scenedesmus* sp., *Nannochloropsis* sp., *Isochrysis galbana*, *Pithophora, Spirogyra, Microcystis*), yeasts (*Lipomyces starkeyi, Rhodosporidium toruloides, Rhodotorula glutinis, Yarrowia lipolytica*), moulds (*Cunninghamella echinulata, Aspergillus terreus*, *Claviceps purpurea*, *Tolyposporium*, *Mortierella alpina*, *Mortierella isabellina*, *Mucorales*), bacteria (*Mycobacterium*, *Nocardia*, *Rhodococcus*, *Streptomyces*, *Gordonia* sp., *Rhodococcus opacus*)	Chisti (2007, 2008), Gouda et al. (2008), Singh and Singh (2010), Lin et al. (2011), Shahid and Jamal (2011), Ageitos et al. (2011), Singh et al. (2011), Liand and Jiang (2013) and Kumar and Sharma (2015)

98 % carbon emissions results from three fossil fuels, such as petroleum, coal, and natural gas (Escobar et al. 2009). Therefore, reducing the use of non-renewable energy sources would considerably lessen the amount of greenhouse gases. Such issues can be minimised by using renewable energy sources. Hence, a renewable energy source, clean and environmentally safe, is a promising alternative solution. In fact, processing of renewable energy sources is the research priority area in many countries for fulfilling the growing energy demands. Among the renewable energy sources, liquid biofuel is the only alternative to fossil diesel (Nigam and Singh 2011).

Liquid biofuels are categorised on the basis of feedstock processing (Table 6.1). First-generation biofuels were based on edible vegetable oils, such as soya bean and sunflower oils (Karmakar et al. 2010; Ahmad et al. 2011; Shahid and Jamal 2011; Lin et al. 2011; Kumar and Sharma 2015). Second-generation biofuels were based on non-food sources, such as jatropha oil, jojoba oil, waste oils (grease, recyclable), and animal fats (Kafuku and Mbarawa 2010; Karmakar et al. 2010; Singh and Singh 2010; Shahid and Jamal

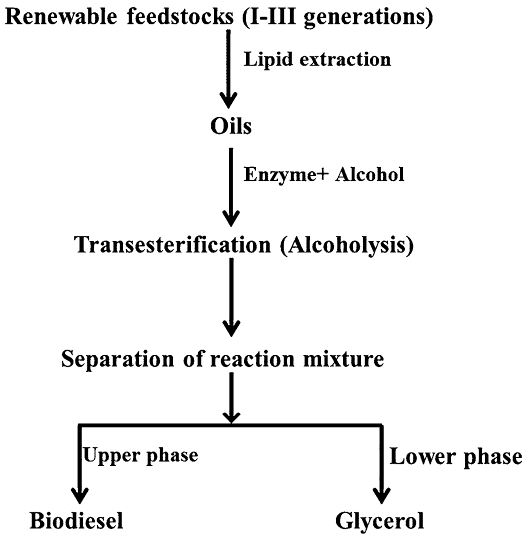

Fig. 6.1 Flow diagram showing enzyme-catalysed biodiesel process

2011; Ashraful et al. 2014; Kumar and Sharma 2015). Nonfood feedstocks were intended to reduce dependence on edible vegetable oils. In comparison to first-generation feedstock, there is no requirement of arable land or freshwater, thereby eliminating competition with agricultural land (Pinzi et al. 2014). Third-generation feedstocks were based on oleaginous microorganisms including microalgae (Chisti 2007, 2008; Gouda et al. 2008; Patil et al. 2008).

Biodiesel has attracted considerable interest due to environmentally benign and renewable sources. Biodiesel is a non-toxic and biodegradable fuel with the lowest emissions of greenhouse gases (Kralova and Sjooblom 2010; Tan et al. 2010). Biodiesel is fatty acid alkyl esters, which is produced by transesterification reactions between lipids (like animal fats and vegetable oils) and alcohols. It is catalysed by chemical (acids, alkalis) or enzymatic (lipase enzymes) routes. The production of biodiesel consists of two steps: (1) the oil extraction from feedstock and (2) further conversion of oil to biodiesel (Fig. 6.1). The enzymatic process for biodiesel production is a good alternative to chemical process because of its cleaner and more efficient route; the cost of the process can be minimised by enzyme immobilisation modification (Du et al. 2005; Kumari et al. 2009). Additionally, biodiesel can be used in current diesel car infrastructure without any major modifications (Luque et al. 2010). Therefore, biodiesel is emerging as the most promising nonpolluting transportation fuel (Lu et al. 2007).

The primary enzymes, lipases from different animal, microbial, and plant sources, have been used extensively for producing biodiesel (Verma et al. 2008, 2013a; Hasan et al. 2009; Tan et al. 2010; Kanwar and Verma 2010; Stergiou et al. 2013). Lipases as enzymes also provide catalytic capability in processes such as hydrolysis, aminolysis, transamidation, and transesterification (Verma et al. 2008, 2009, 2011, 2012, 2013b; Verma and Kanwar 2010; Wang et al. 2012). Among these, microbial lipases have been primarily used for enzyme-catalysed biodiesel production (Kanwar and Verma 2010). Protein engineering and enzyme-immobilised technology have played a significant role in making a feasible biodiesel process at the industrial setting (Verma et al. 2013a, 2014; Misson et al. 2015).

Transesterification yield for enzymatic-catalysed biodiesel process is regulated by several parameters such as feedstock quality, choice of enzyme, substrate-molar ratio, temperature, and water content (Tan et al. 2010). However, the major factor limiting development and use of biodiesel is high production cost. High production cost heavily relies on the prices of the feedstock and enzyme. The feedstock expense accounts for ca. 75 % (Lim and Teong 2010). Feedstock prices are a critical parameter in strategies to make biodiesel process more competitive with fossil fuels (Huang et al. 2015). Biocatalyst cost is the second concern which is quite high as compared to acid/alkali catalyst. Enzymatic biodiesel production can be made economically attractive by enzyme-immobilised technology. Immobilised enzyme can be separated and reused many times to alleviate the enzyme cost. With the advanced functional nanomaterial, nanotechnology has made tremendous impact in industrial biotechnology (Kim et al. 2008; Ansari and Husain 2012). Recently, enzyme-immobilised technology has used many advanced functional material for bioenergy production (Verma et al. 2013a, 2014; Misson et al. 2015).

This chapter provides an insight of enzyme-immobilised technology for transesterification of lipids into biodiesel, including advances in enzyme immobilisation materials, feedstock types, and the impact of nanomaterials for the bioenergy production.

6.2 Biodiesel Feedstock Types

The biodiesel feedstock is a critical factor that has enhanced the production cost (Robles-Medina et al. 2009). The trend for biodiesel-feedstock selection is being changed from costly edible oil to cheap microbial oils including waste oil. The selection of feedstock is a technical challenge and depends on the agro climatic region (Hama and Kondo 2013). A variety of feedstock have been employed for the biodiesel production. Generally, the feedstock types include vegetable oils, animal fats, waste oils, and oleaginous microorganisms.

6.2.1 Vegetable Oils

The most common feedstock used in biodiesel production is vegetable oils derived from edible plants. The edible oils such as rapeseed (*Brassica napus*), soya bean (*Glycine max*), palm oil (*Elaeis guineensis*), coconut (*Cocos nucifera*), sunflower (*Helianthus annuus*), peanut (*Arachis hypogaea*), safflower (*Carthamus tinctorius*), and sesame (*Sesamum indicum*), etc. are used for biodiesel production in many countries depending on the agro climatic region (Karmakar et al. 2010). Edible oil-based biodiesel production has been criticised due to its low sustainability and potential conflict with the primary resources (for instance, food, fibre, land, water, and fertiliser) (Pinzi et al. 2014). Due to the recent debates on "food versus fuel," nonedible oils are emerging as one of the main contenders for biodiesel production (Abdulla et al. 2011). The nonedible oils, such as jatropha (*Jatropha curcas*), neem (*Azadirachta indica*), polanga (*Calophyllum inophyllum*), Karanja (*Pongamia pinnata*), babassu (*Orbignya martiana*), rubber seed (*Hevea brasiliensis*), mahua (*Madhuca indica* and *Madhuca longifolia*), and tobacco (*Nicotiana tabacum*), are the most attractive alternative to edible oils (Demirbas 2009). Among these nonedible oil sources, *Jatropha curcas*, a multipurpose plant with many attributes and considerable potential, is gaining importance for biodiesel production (Divakara et al. 2010).

6.2.2 Animal Fats

Animal fats such as tallow (beef tallow from domestic cattle and mutton tallow from sheep), pork lard (rendered pork fat), chicken fat, and grease have been used as biodiesel feedstock (Bankovillic et al. 2014). The nature of animal fat differs from plant oil due to the very high fatty acid content and various distribution of fatty acid. Animal fat use is very limited for biodiesel production. Waste animal fats from meat processing industries are a good source of animal fats; it can be an opportunity to make cost-effective biodiesel process. Additionally, the uses of this biodiesel feedstock have also eliminated the waste disposal issue (Janaun and Ellis 2010).

6.2.3 Waste Oils

The usage of the refined oil for biodiesel production is not economically feasible. Cooking oil waste and soapstock are available and offer an attractive alternative feedstock for biodiesel production (Hama and Kondo 2013). The conversion of this waste oil to value-added products also provides a solution to the environmental concerns. The vegetable oils underwent oxidation, hydrolysis, and polymerisation reactions at high temperature; thus, this waste oils need a process optimisation for considering an ideal feedstock (Lam et al. 2010). Azocar et al. (2010) warranted the 16 million generation of the waste oil, a sufficient amount of biodiesel feedstock for biodiesel production. The cost of processing can be easily compensated due to the abundance of the waste oil. With proper research focus and development, the waste oils can indeed become the next ideal feedstock for biodiesel (Lam et al. 2010).

6.2.4 Oleaginous Microorganisms

Oleaginous microorganisms for biodiesel production have been the subject of research for lipid bioprospecting as an alternative to agricultural commodities (Ratledge 1991; Ageitos et al. 2011; Sitepu et al. 2014). Oleaginous microorganisms such as yeasts, fungi, bacteria, and microalgae have shown the high lipid accumulation. Some microorganisms contain 80 % oils of the cell dry weight. Therefore, oleaginous microorganisms are available for substituting conventional oil in biodiesel production (Meng et al. 2009).

6.2.4.1 Algae

Microalgae are the foremost feedstock for biodiesel production because of high lipid content and non-requirement of arable land and water (Chisti 2007; Wijffels and Barbosa 2010). Microalgae such as *Chlorella protothecoides*, *C. vulgaris*, *Scenedesmus* sp., *Nannochloropsis* sp., *Isochrysis galbana*, *Pithophora*, *Spirogyra*, and *Microcystis* etc. have been used as feedstock for biodiesel. Various modes of microalgae growth (autotrophic, mixotrophic, and heterotrophic) in liquid media and high photosynthetic activity for converting CO_2 into carbon-rich lipids have provided tremendous opportunity for lipid bioprospecting (Chisti 2007, 2008; Gouveia and Oliveira 2009). Most of the algal strains possess good oil content; however, environmental stresses such as nutrient deficiency and high temperature can induce maximum algal oil content (Solovchenco et al. 2008). Moreover, the physical property of algal biodiesel is in good agreement with the diesel fuel (Miao and Wu 2006).

However, drying of wet algal biomass is a major bottleneck in viable commercial production of the biodiesel; this needs an efficient drying protocol to improve the lipid extraction method by significantly lowering the power consumption and the drying time (Bagchi et al. 2015).

6.2.4.2 Yeasts and Moulds

Hyper-lipid-producing yeasts possess characteristics such as fast growth, high cell density, and consequently high oil accumulation (Ageitos et al. 2011). The optimisation of fermentation media can further increase the lipid content (Sitepu et al. 2014). The oleaginous yeasts include *Lipomyces starkeyi*, *Rhodosporidium toruloides*, *Rhodotorula glutinis*, and *Yarrowia lipolytica* (Ageitos et al. 2011). Recently published review has discussed the potential oleaginous yeast species suitable for lipid extraction (Sitepu et al. 2014). The use of oil from fungi is very limited for biodiesel production. Many mould species, such as *Cunninghamella echinulata*, *Aspergillus terreus*, *Claviceps purpurea*, *Tolyposporium*, *Mortierella alpina*, *Mortierella isabellina*, and *Mucorales*, have significant level of lipid accumulation (Liand and Jiang 2013).

6.2.4.3 Bacteria

Oleaginous bacteria are considered as a potential feedstock for biodiesel production because of the well-understood pathway for fatty acid synthesis and amenability to the metabolic engineering for enhanced lipid productivity (Alvarez and Steinbuchel 2002; Wentzel et al. 2007). Bacteria can accumulate oil under specific environmental conditions; this lipid composition is different from other microbial oils (Kurosawa et al. 2010). Oleaginous bacteria such as *Mycobacterium*, *Nocardia*, *Rhodococcus*, *Streptomyces*, *Gordonia* sp., and *Rhodococcus opacus* have been known for the substantial lipid accumulation (Gouda et al. 2008). *Gordonia* sp. has demonstrated a high quantity of oil accumulation (80 %) under specific conditions (Gouda et al. 2008). Kalscheuer et al. (2006) engineered *Escherichia coli* for biodiesel production. This metabolically engineered *Escherichia coli* produced the fatty acid ethyl ester concentrations using renewable carbon sources.

6.3 Lipid Extraction from Biodiesel Feedstock

The organic solvent and supercritical flow extraction methods are commonly employed for lipid extraction from the agricultural feedstock. Generally, organic solvent extraction is the most commonly used method; this extraction methodology has been optimised by Bligh co-workers (Bligh and Dyer 1959). Efficient lipid extraction from microalgae is not feasible due to the interference of the rigid cell wall (Wiltshire et al. 2000). The extraction dynamic study was investigated in order to improve extraction efficiency (Cho et al. 2012). The most commonly used organic solvents such as hexane, acetone, benzene, and chloroform have shown efficiency in lipid extraction from microalgae (Harun et al. 2010). The ideal organic solvent penetrates into the algal cells efficiently and matches the polarity of the desired compounds for lipid extraction (e.g. hexane for nonpolar lipids). The feedstock material is mechanically disrupted prior to solvent extraction in order to improve the physical contact between solvent and feedstock for maximising the lipid extraction (Cooney et al. 2009). The algal cell disruption followed by solvent extraction has improved significantly the lipid extraction yield (Prabakaran and Ravindran 2011; Zheng et al. 2011).

Supercritical fluid extraction is an environmentally friendly technology because of the green methodology employed for lipid extraction from biodiesel feedstock (Halim et al. 2011, 2012). This extraction method is considered most suitable due to the following advantages: the production of pure extracts, free from harmful chemicals, safe to thermally sensitive products, and quick separation (Mendiola et al. 2007; Sahena et al. 2009). Supercritical fluids are substances that behave like liquid and gas with increased solvating capability above their critical points of temperature and pressure. For example, carbon dioxide is the most commonly used fluid in the extraction of lipid from microalgae due to the above cited advantageous properties. The efficiency of lipid extraction depends on parameters such as pressure, temperature, CO_2 flow rate, and extraction time (Andrich et al. 2006; Xu et al. 2008; Sahena et al. 2009; Harun et al. 2010). Optimised fluid extraction can be achieved by modifying the co-solvent, pressure, temperature, flow rate, and extraction time. Moreover, removal of carbon dioxide is very easy after the completion of extraction; it is in gaseous form at ambient temperature (Mendes et al. 2006; Halim et al. 2011).

6.4 Nanotechnology Delivers Biodiesel Production

Nanotechnology has become an integral part of the bioenergy production (Serrano et al. 2009). This section deals with an overview of nanomaterial synthesis and characterisation with respect to enzyme-catalysed biodiesel production. In addition, the salient feature of nanomaterials as the most suitable enzyme immobilisation support is also discussed.

6.4.1 Synthesis and Characterisation of Functionalised Nanomaterials

Nanotechnology has provided a variety of nanomaterials with a plethora of applications (Misson et al. 2015). Top-down and bottom-up approaches were used to synthesise nanomaterials (Biswas et al. 2012). Nanoparticle was synthesised by the co-precipitation method (Wu et al. 2008; Kalantari et al. 2012). Nanofibre was synthesised by electrospinning, self-assembly, and phase separation techniques (Bhardwaj and Kundu 2010). Nanoporous gold with different pore sizes was obtained by a combination of simple dealloying and thermal annealing methods (Qiu et al. 2008). Carbon nanotube was primarily produced by chemical vapour deposition method (Volder et al. 2013). Graphene nanosheet was prepared by thermal exfoliation of graphite oxide (Kishore et al. 2012).

The pristine nanomaterial is surface functionalised to improve the efficiency of nanomaterials for enzyme immobilisation (Wu et al. 2008; Johnson et al. 2011; Pavlidis et al. 2012a, b; Verma et al. 2013a, b). The modification process involves grafting of desirable functional groups onto the nanomaterial's surface (Shim et al. 2002). The surface-modified nanomaterial renders stable dispersion and improves biocompatibility for high enzyme activity (Pavlidis et al. 2010). The chemicals used for nanomaterial surface modification include organic and inorganic materials such as silica, natural polymers (chitosan, dextran, starch, gelatine), synthetic polymers, biopolymers, and dendrimers (Jiang et al. 2013).

The immobilised enzymes have been assessed for the biocatalytic retention based on stability and reusability studies. The apparent enzyme activity assay can be measured easily to see the biocatalyst functionality. However, in situ stability of the enzyme on the nanomaterial surface can be studied using biophysical tools (Ganesan et al. 2009). Nanomaterial-immobilised enzymes have been characterised primarily using microscopic and spectroscopic techniques with two main objectives: to find out (1) the suitability of the nanomaterial for a particular study and (2) need for further modification of the nanomaterial to improve the functionality (Cruz et al. 2010; Pavlidis et al. 2012a, b). Electron microscopic (both transmission and scanning) and atomic force microscopic techniques have been used for nanomaterial-immobilised enzyme studies with regard to shape, size, and dispersion behaviour. Spectroscopic techniques such as circular dichroism, UV-vis, Raman, Fourier transform infrared, and X-ray photoelectron have been employed to elucidate the structure of biomolecules loaded onto the nanomaterials. The conformation stability of the biomolecules on the nanomaterial surface has led to the discovery of the best immobilisation material (Lee et al. 2009; Andrade et al. 2010).

6.4.2 Advances in Nanomaterial-Based Enzyme Immobilisation Technology for Biodiesel Production

The application of industrial enzymes has a tremendous scope in the era of energy crisis and environmental degradation because of the green methodologies employed to achieve more sustainable chemical product (Illanes et al. 2012). However, the enzyme is often very facile and denatured entity in vitro milieu. A slight change in the working condition of the enzyme renders loss of catalytic activity. This problem of high cost and instability of the enzyme for the industrial application can be minimised to a quite extent by using an immobilisation technology. Enzyme immobilisation is an old and mature technology for the use of the enzymes at the commercial level (Mateo et al. 2007; Jochems et al. 2011). Immobilisation techniques such as adsorption, encapsulation, cross-linking, and covalent binding have been used for industrial application. Although adsorption is associated with enzyme leaching, the covalent method is the most commonly employed robust technique for enzyme stabilisation (Misson et al. 2015).

The nanomaterial-enzyme interactions via covalent immobilisation method have provided strong bonding and protection from the extreme environments. Nanomaterial-immobilised enzyme possesses thermal and pH stabilities with recycling capabilities. Enzyme immobilisation techniques have been improved with the advances in material as these techniques solely relied on the property of the carrier material (Kim et al. 2008). Advanced functional materials improve the property of the enzymes with respect to industrial application (Misson et al. 2015). In recent years, one of the significant advances in the enzyme immobilisation material is the capability to prepare the advanced functional material with well-defined sizes and shapes in the nanoscale regimes. With the rapid development in nanotechnology, the enzyme immobilisation was successfully done on various nanomaterials, such as nanoparticle, nanofibre, nanotube, nanoporous, nanosheet, and nanocomposite (Kim et al. 2006a, b; Gupta et al. 2011; Verma et al. 2011, 2012, 2013a, b, 2014; Ansari and Husain 2012; Misson et al. 2015). Now, nanomaterial-immobilised enzymes have found applications in the biodiesel production (Fig. 6.2). Nanomaterial endows the following advantageous properties of an ideal enzyme immobilisation support:

(a) *Surface area-to-volume ratios:* Nanomaterials have relatively larger surface area-to-volume ratios as compared to bulk materials (Wang 2006; Gupta et al. 2011). Nanomaterial offers high enzyme loading

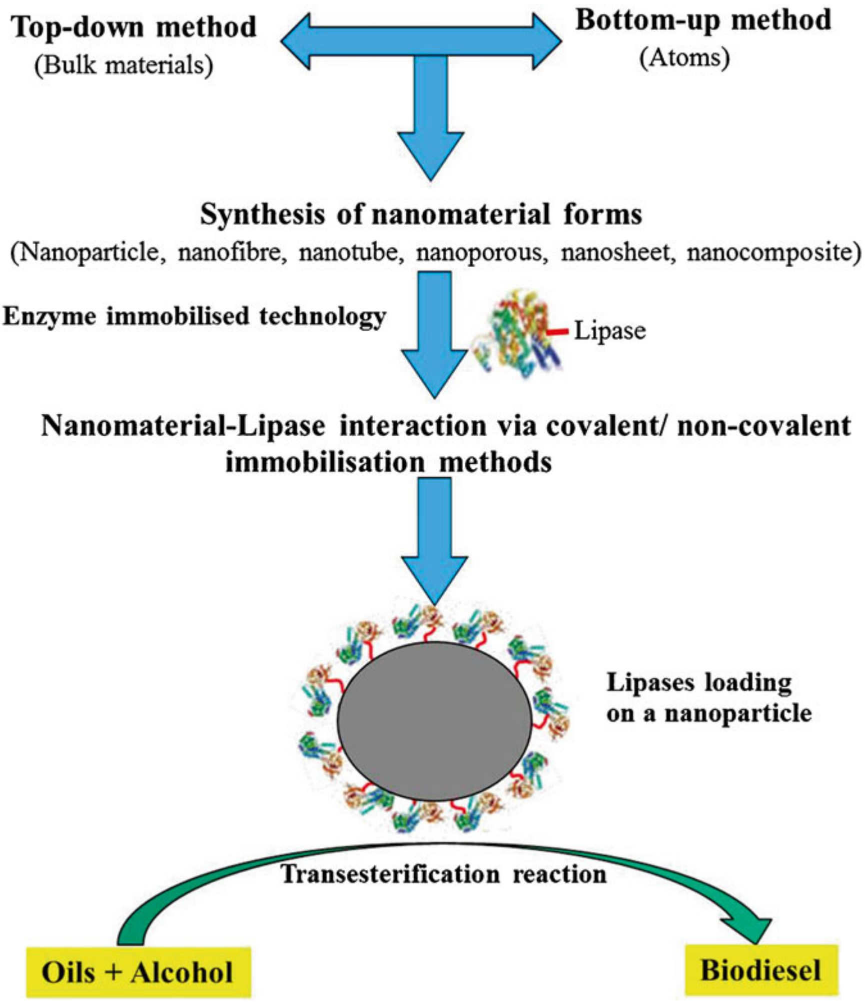

Fig. 6.2 Flow diagram showing an integrated process of using different nanomaterial forms for lipase-catalysed biodiesel production

due to large surface area and subsequently leads to better catalytic activity. This makes the nanomaterial as an ideal support for enzyme immobilisation as compared to conventional bulk materials. Enzyme loading on the nanoparticle can be easily counted based on the well-defined shape, density, and molecular weight of the nanomaterial. Kumar et al. (2013) demonstrated the high enzyme loading on single nanoparticle (17 enzyme molecules per nanoparticle).

(b) *Mass transfer:* Nanomaterial has low mass transfer resistance that improves the catalytic activity of an immobilised enzyme. Nanomaterial-immobilised enzymes have shown high enzyme activity and stability (Kim et al. 2006b). Additionally, the nanoscale nature of both the materials and enzymes makes the immobilised enzyme to behave as a free enzyme.

(c) *Separation ease:* Nanomaterials such as magnetic nanoparticle make the separation of product and immobilised enzymes quite easy by using external magnetic field and render a time-efficient process (Safarik and Safarikova 2009; Ren et al. 2011; Borlido et al. 2013; Kumar et al. 2014). This provides more reusability and stability to the bound enzyme as compared to bulk materials where centrifugation and/or filtration is only the option to separate the immobilised enzyme from the product. Separation operation may lead to enzyme leaching/instability due to mechanical shear while mixing the immobilised enzymes aggregates with appropriate buffer for start a new reaction (Yiu and Keane 2012).

Additionally, nanofibre-immobilised enzyme can easily separate from the reaction mixture without the use of any separation (centrifugation, filtration, and magnetic) techniques. The non-magnetic nanomaterial can be easily tailored to magnetic nanocomposite with the inclusion of magnetic nanoparticle, to ease the separation step in any bioprocess. Nanomaterials offer a quick separation method for isolating immobilised enzyme and product from the bioprocess, thus lowering the production cost (Borlido et al. 2013).

(d) *Flow rate:* The nanomaterial behaves as a stable monodispersed particle in aqueous suspension, showing Brownian motion (Wang 2006). The stability of the enzyme-immobilised nanomaterial suspension can be easily checked by zetasizer (Wang et al. 2012). It has been demonstrated from the Stokes-Einstein equation that the mobility and diffusivity of the nanoparticles have to be smaller than those of relatively larger size macroparticle. It has been shown that this Brownian motion may be responsible for high activities obtained when enzymes are immobilised on nanoparticles (Jia et al. 2002).

(e) *Reactor design:* Nanomaterial, especially nanofibre, offers larger flexibility in reactor design as they are easier to prepare and easier to handle (Nair et al. 2007). Wang et al. (2011a) developed a packed-bed reactor for effective transesterification of soya bean oil into biodiesel. Enzyme-immobilised nanofibre reactor was employed to achieve a continuous steady substrate hydrolysis (Huang et al. 2008). The feasibility of nanomaterial-immobilised enzyme at reactor scale has shown the commercial potential (Wang et al. 2011a).

6.5 Impact of the Nanomaterials to Improve Enzymatic Transesterification for Biodiesel Production

Recently, different types of nanomaterials have been used as a novel support for the immobilisation of lipases to accomplish enzymatic transesterification reaction for biodiesel production (Table 6.2). This section deals with the application of different forms of nanomaterial for biodiesel production.

6.5.1 Application of Nanoparticle-Immobilised Lipase for Biodiesel Production

Lipases sourced from porcine pancreas lipase, *Candida rugosa* and *Pseudomonas cepacia*, were covalently immobilised onto the amino-functionalised magnetic nanoparticles for biodiesel production at a reactor scale (Wang et al. 2009). Consequently, lipase of *Pseudomonas cepacia*, being resistant to methanol, was selected for enzymatic transesterification reactions using soya bean oil and methanol substrates. High transesterification activity was reported with the immobilised lipase in a reactor. A hundred percent efficient transesterification was observed in the first three cycles. High protein load rendered high transesterification activities of immobilised enzyme (95 %) than the free enzyme.

Lipase sourced from *Thermomyces lanuginosus* was covalently immobilised to the amino-functionalised magnetic nanoparticles using either glutaraldehyde or EDC as a cross-linker (Xie and Ma 2009, 2010). A high yield of biodiesel production (90 %) was reported from immobilised lipase to the amino-functionalised magnetic nanoparticles using soya bean and methanol substrates. Transesterification efficiency of immobilised lipase using glutaraldehyde cross-linker was higher than immobilised lipase using an EDC cross-linker. The efficient chemistry of glutaraldehyde as compared to EDC chemistry for stabilising the nanomaterial-enzyme interaction has been demonstrated.

Table 6.2 Nanomaterial-immobilised lipase for biodiesel production

Enzyme (lipase) source	Substrate (+ methanol)	Nanomaterial	Immobilisation method	Biodiesel yield (%)	References
Burkholderia sp.	Olive oil	Ferric silica nanocomposite	Adsorption	90	Tran et al. (2012)
		Hydrophobic magnetic particle	Adsorption	70	Liu et al. (2012)
Pseudomonas cepacia	Soya bean oil	PAN nanofibre	Covalent	90	Li et al. (2011)
		Fe_3O_4 nanoparticle	Covalent	95	Wang et al. (2009)
		Fe_3O_4 nanoparticle	Covalent	75	Wang et al. (2011a)
		Nanoporous gold	Adsorption	90	Wang et al. (2011b)
	Rapeseed oil	Polyacrylonitrile fibre	Adsorption	94	Sakai et al. (2010)
	Waste oil	Magnetic nanoparticle	Covalent	79	Yu et al. (2013)
	Soya bean oil	Magnetic silica nanocomposite	Covalent	54	Kalantari et al. (2013)
Thermomyces lanuginosus	Soya bean oil	Fe_3O_4 nanoparticle	Covalent	90	Xie and Ma (2010)
T. lanuginosus and *Candida antarctica*	Waste grease	Fe_3O_4 nanoparticle	Covalent	99	Ngo et al. (2013)
Pseudozyma sp.	Sunflower oil	Fe_3O_4 nanoparticle	Covalent	71	Alex et al. (2014)
Rhizopus miehei	Triolein	Silica nanoparticle	Encapsulation	86	Macario et al. (2013)

Lipase sourced from *Pseudomonas cepacia* was covalently immobilised on a functionalised magnetic nanoparticle for biodiesel production in a packed-bed reactor (Wang et al. 2011a). Enzymatic transesterification for biodiesel production was achieved using soya bean oil and methanol substrates. Emulsification of soya bean oil before enzymatic transesterification process improved the biodiesel yield. Immobilised lipase showed high transesterification efficiency (75 %). The same researchers scaled up the biodiesel production and used a four-packed-bed reactor system (Wang et al. 2011a). High biodiesel yield was achieved in four packed-bed reactors in comparison to the single-packed-bed reactor. High transesterification efficiency (88 %) was attributed to the longer residence time, robust nanoparticle-immobilised lipase system, and resistance to solvent inactivation.

The magnetic nanoparticle was modified with a hydrophobic moiety to synthesise a novel immobilisation material (Liu et al. 2012). Lipase sourced from isolated *Burkholderia* sp. was immobilised onto the modified magnetic particles via an adsorption method. Enzymatic transesterification reaction conditions using olive oil and methanol substrates were optimised by response surface methodology for biodiesel production. The immobilised lipase was used six times for biodiesel production without significant loss of catalytic activity. The transesterification efficiency achieved by immobilised lipase was also comparable with a commercial lipase (Novozym® 435). The transesterification yield was 70 %. High enzyme loading (4619 U/g) based on the Langmuir adsorption isotherm and response surface methodology optimisation for transesterification reaction parameters demonstrated the feasibility of using the immobilised lipase in practical applications.

Lipases from *T. lanuginosus* and *Candida antarctica* were covalently immobilised to the novel magnetic nanoparticle aggregates (Ngo et al. 2013). The immobilised enzyme suspension was freeze-dried to achieve high enzyme loading per gram nanoparticles. The transesterification reaction was achieved using the substrates of waste oil (grease) and methanol. The immobilised *T. lanuginosus* lipase showed the best performance among immobilised enzymes known thus for the production of biodiesel, giving high transesterification yield (99 %) in 12 h. The nanoparticle-immobilised lipase retained high catalytic activity (88 %) even after 11 cycles.

The lipase sourced from *Pseudomonas cepacia* was covalently immobilised onto magnetic nanoparticle (Yu et al. 2013). The transesterification reaction was achieved using waste cooking oil and methanol substrates. Enzymatic transesterification reaction conditions were optimised by response surface methodology. This study reported the significant yield of biodiesel production (79 %). Additionally, the use of low-cost feedstock may lower the production cost of biodiesel as well as facilitate the disposal of waste oil.

A solvent-tolerant esterase from *Pseudozyma* sp. was covalently immobilised to the amino-functionalised magnetic nanoparticles using glutaraldehyde cross-linker (Alex et al. 2014). The nanoparticle-immobilised enzyme was used for biodiesel production using substrates of sunflower oil and methanol. The enzymatic reaction was carried out at 50 ° C for 72 h. The immobilised biocatalyst was retained 100 % activity even after prolonged storage at 4 ° C. The transesterification yields for biodiesel production after the 2nd and 3rd cycles were 71 and 40 %, respectively. This study demonstrated the applicability of magnetic nanoparticle-immobilised *Pseudozyma* sp. lipase for biodiesel production.

6.5.2 Application of Nanofibre-Immobilised Lipase for Biodiesel Production

Sakai et al. (2010) studied the immobilised lipase of *Pseudomonas cepacia* for biodiesel production at reactor level. The enzyme was immobilised on electrospun polyacrylonitrile nanofibres via physical adsorption method. High transesterification yield (94 %) for biodiesel production was reported using immobilised enzyme with rapeseed oil and butanol substrates. The nanofibre-immobilised enzyme was robust and demonstrated the initial reaction rates 65-fold higher than the commercial immobilised lipase (Novozym® 435) on a total catalyst mass basis. Batch and continuous reactors were used for biodiesel production by nanofibre-immobilised lipase. The immobilised lipase showed no loss in enzyme activity during a long 20 day continuous operation in a reactor. The excellent catalytic activity of the nanofibre-immobilised lipase may be the result of a conformational change of lipase that enables free access of substrates to the active centres of the enzymes.

Polyacrylonitrile nanofibre was used as an immobilisation material for *Pseudomonas cepacia* lipase (Li et al. 2011). The nanofibre was activated via amidination reaction for covalently immobilisation of enzyme. Enzymatic transesterification reaction was achieved using soya bean oil and methanol substrates. The immobilised lipase (9 U/mg proteins) showed good transesterification yield. High transesterification yield (90 %) achieved with only slight loss (10 %) of original conversion recorded even after ten times recycled use. High catalytic efficiency for biodiesel production was attributed to an excellent immobilisation strategy that led to only slight changes in the immobilised enzyme kinetic parameters.

6.5.3 Application of Nanocomposite-Immobilised Lipase for Biodiesel Production

Tran et al. (2012) studied the reversibly immobilised lipase of *Burkholderia* sp. on a nanocomposite. The nanoparticles were synthesised by coating Fe_3O_4 core with silica shell. The immobilised enzyme was employed for biodiesel production using olive oil and methanol substrates. Maximum loading of enzymes (29.45 mg per g nanoparticles) and subsequent high biodiesel production (90 %) were reported. The biodiesel production achieved was higher than 90 %. This immobilised lipase demonstrated high solvent tolerance and reusability (Tran et al. 2012).

The biodiesel was produced using lipase immobilised on the magnetic silica nanocomposite particles of various structures (Kalantari et al. 2013). Nonporous and mesoporous silica coating was done to synthesise a novel nanocomposite support for the enzyme immobilisation. Lipase sourced from *Pseudomonas cepacia* was covalently immobilised to the amino-functionalised particles using glutaraldehyde as a cross-linker. The immobilised enzymes retained approximately 55 % of the initial conversion capability following five times of reuse. The lipase immobilised onto the mesoporous silica structure with an optimised pore size range (1–15 nm) showed higher stability and better biocatalytic potential for biodiesel production than the enzyme immobilised on nonporous silica.

Silica nanoparticle was synthesised to immobilise *Rhizomucor miehei* lipase by an encapsulation method (Macario et al. 2013). Enzyme was stabilised by encapsulation in a liposome hybrid nanosphere. The liposomal phase coated with porous silica stabilised the internal liposomal phase and, consequently, protected the lipase. The transesterification reaction for biodiesel production was achieved using triolein and methanol substrates. The nanoparticle-immobilised lipase performed a high biodiesel production as compared to the mesoporous-bound lipase (Macario et al. 2013). This study demonstrated that the biocompatible microenvironment inside the liposome enabled immobilised lipase to preserve its free and stable conformation. The transesterification efficiency for biodiesel production by the immobilised enzyme was higher than the free enzyme.

6.5.4 Application of Nanoporous-Immobilised Lipase for Biodiesel Production

Lipase sourced from *Pseudomonas cepacia* was immobilised via an adsorption method onto the nanopores of a porous gold material (Wang et al. 2011b). The immobilised enzyme achieved high transesterification yield for biodiesel production (90 %) as compared to the free lipase (74 %). The immobilised lipase also improved the catalytic potential during extreme reaction conditions of high temperatures and organic solvents. High catalytic activity of immobilised enzyme might be explained by the physical confinement inside the relatively small pores. Specifically, the matching of molecular diameter of *Pseudomonas cepacia* lipase and nanopore dimension of the gold as an immobilisation support are key factors in achieving high transesterification activity.

The advantages of nanomaterial supports have been demonstrated with the above cited recent studies of immobilised enzymes used to process the efficient enzymatic transesterification reaction for biodiesel production.

6.6 Conclusions

Transesterification of renewable feedstock using nanomaterial-immobilised lipase is a promising process for biodiesel production. The valorisation of biodiesel feedstock is still very much an ongoing area of research due to the fact that it has the tremendous potential to make competitive biodiesel process. Recent advances in functional nanomaterial supports for enzyme immobilisation have led to several benefits when compared to immobilisation on bulk materials or free enzymes. Nanomaterial-immobilised lipases have improved the yield of biodiesel production because of higher enzyme loading, multiple recycling, and protection from denaturation of enzymes. Some nanomaterial-immobilised enzymes have been employed for biodiesel production. Nanotechnology has shown the potential to promote the generation of sustainable bioenergy production.

6.7 Opinion

The main hurdles in biodiesel-feedstock processing needs to be revisited due to high water content, longer drying period, and maximum energy consumption. Further bioprospecting for hyperlipid producing oleaginous microbial strain should be continued for sustainable biofuel production. Only limited study for biodiesel production using nanomaterial-immobilised enzyme has been done at commercial scale. Also, the use of recently discovered graphene nanosheets for potential application in biodiesel production needs investigation. This requires extensive efforts to explore the other potential nanomaterials for bioenergy production. However, more studies are required to understand the technical bottlenecks such as toxicity issues and development of safety evaluation guidelines. It is opined that the recent advances in molecular biotechnology and nanotechnology and the development of robust nanomaterial-immobilised enzyme for biodiesel production appear to be promising. Thus, nanotechnology has the potential to tremendously impact the biodiesel research field.

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Madan L. Verma received his M. Tech. in biotechnology from Anna University, Chennai, India, and PhD in microbial biotechnology from HP University, Shimla, India. He is a researcher at Deakin University, Australia. He is actively serving as a reviewer for many reputed journals. He is also a member of many international and national professional bodies. His current interests are nanobiotechnology, bioenergy, and bioprocessing.

Colin J. Barrow received his PhD in natural products chemistry from the University of Canterbury, New Zealand. He is the Director of the Centre for Chemistry and Biotechnology at Deakin University, Australia. His current interests are omega-3 biotechnology and nanobiotechnology. He has many publications and several patents. He is a founding member of the International Society for Nutraceuticals and Functional Foods.

Biotechnology in Aid of Biodiesel Industry Effluent (Glycerol): Biofuels and Bioplastics

7

Prasun Kumar, Sanjeet Mehariya, Subhasree Ray,
Anjali Mishra, and Vipin Chandra Kalia

Abstract

Crude glycerol produced by the biodiesel industries as a waste has gathered significant attention as a cheap carbon source. It was recently realized that this forthcoming problem of waste glycerol may be circumvented through biological routes. The role of glycerol to serve as start material for a plethora of chemicals has been well recognized. The lead of science in the field of biotechnology has broadened the application range of glycerol using microorganisms. Here, we are dealing with the potential of the glycerol for the production of third-generation fuels and polymers. The reduced nature of the glycerol molecule makes it a suitable substrate for these biological processes. It would be worth observing that the rapidly thriving biodiesel industry will certainly assist in offering a low-cost glycerol feed for producing other valuable bioproducts. An integrative approach to merge all these procedures could possibly assist in growing and managing a sustainable energy production.

7.1 Introduction

The escalating energy crisis worldwide is questioning our capability to generate cheap and sustainable energy and fuels. This has created a state of affairs having an alarming depletion of petroleum reserves and rapidly increasing problems due to environmental pollution. Thus, the overall focus has now shifted to look for prospective fuels that should be cheap, energy efficient, and based on renewable resources (Kotay and Das 2007; Kalia and Purohit 2008). In continuation to bioethanol and biomethane as alternative sources of bioenergy, efforts have brought another equivalent energy-efficient fuel, biodiesel, into use. Being of

P. Kumar (✉) • S. Mehariya • S. Ray • A. Mishra
Microbial Biotechnology and Genomics,
CSIR-Institute of Genomics and Integrative
Biology (IGIB), Delhi University Campus,
Mall Road, Delhi 110007, India
e-mail: prasun.mcr@gmail.com;
subhasreeray141@gmail.com;
anjalimishra2608@gmail.com;
anjali.mishra@igib.in;
smehariya@gmail.com

V.C. Kalia
Microbial Biotechnology and Genomics,
CSIR-Institute of Genomics and Integrative Biology,
Delhi University Campus, Mall Road,
Delhi 110007, India

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biological origin and clean in nature, it has been produced on an industrial level. However, it leaves CG as a major by-product. One tenth of the total biodiesel produced comes out as effluent. Consequently, the quantum of waste glycerol has risen to unmanageable levels. Recent advancements in biotechnology have equipped us with several ways to valorize glycerol waste (Maru et al. 2012, 2013; Kumar et al. 2015a), which may increase up to sixfold by 2020 (Christoph et al. 2006; Vlassis et al. 2013). Furthermore, the effluent containing 70–75 % glycerol leaves a large scope to utilize it without the need to pretreat. The effluents also possess minor quantities of methanol, oils, salts, and other organic compounds, which may prove harmful to bio-treatment process (Tan et al. 2013).

Glycerol is a sugar alcohol which is nontoxic, hydrophilic, and odorless compound. Structurally, it is very much similar to triglycerides, which are central to lipid metabolic pathways. The reduced nature of glycerol makes it suitable for easy uptake by microbes and produces value-added bioproducts (Fig. 7.1). A large number of biotechnological applications include production of succinic acid (Lee et al. 2001), citric acid, 1,3-propanediol (Papanikolaou et al. 2004), ethanol, acetate, lactate, omega-3 polyunsaturated fatty acids, hydrogen (H_2) and polyhydroxybutyrate (PHB) (Dharmadi et al. 2006; Yang et al. 2012; Maru et al. 2013; Sharninghausen et al. 2014; Kumar et al. 2015b, c), and methane (CH_4) (Vlassis et al. 2013). Since H_2 has been recognized as the future fuel and PHAs as an alternative to plastics, we have focused on these two bioproducts in this write-up. However, as 65 % of the total energy present in the organic matter of the bio-waste remains unutilized at the end of these processes, it is desirable to subject their effluents to biomethanation. Since it leads to complete digestion (up to 95 %), we have also made a presentation on this aspect as well. This integrated process can be defined as a biorefinery (Luo et al. 2011).

7.2 Biological Hydrogen Production from Glycerol

Microbes can metabolize different organic matter-rich biowastes into H_2 under dark and photosynthetic conditions. Among the dark fermentative bacteria, *Bacillus* and *Clostridium* (Gram-positive), *Citrobacter*, *Enterobacter*, recombinant *Escherichia*, and *Klebsiella* (Gram-

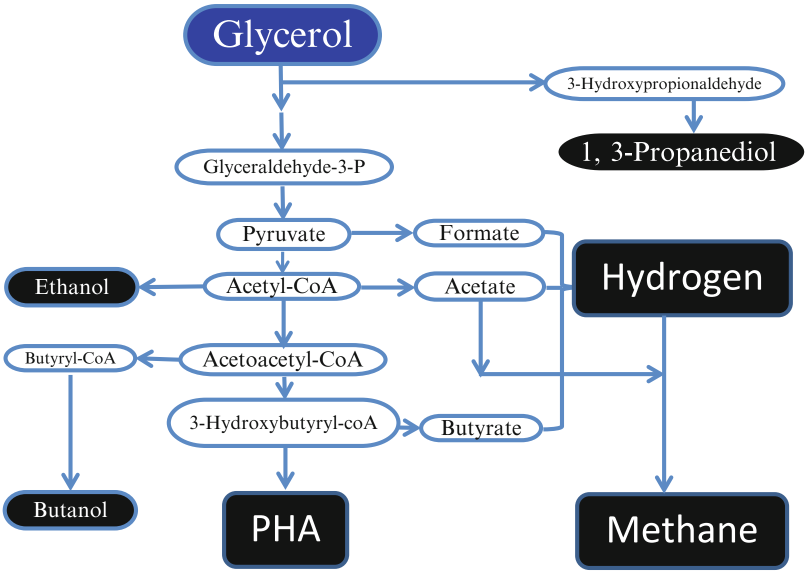

Fig. 7.1 Bioconversion of glycerol into biofuels and biopolymers

negative) have been quite effective in producing H_2 (Kalia et al. 1994; Kumar et al. 1995; Kalia and Purohit 2008; Yasin et al. 2013; Kumar et al. 2013, 2015a, c; Patel et al. 2012a, b, 2014; Patel and Kalia 2013). Although most studies have focused either on dark or photosynthetic routes, the average yields have been in the range of 0.16–3.8 or 0.23–7.2 mol H_2/mol sugar, respectively. It has been realized that an integrative approach will be better, as it has been possible to get up to 8.3 mol H_2/mol sugar (Patel et al. 2012b, 2015). This yield is much more than the theoretically expected yield of 4 mol H_2/mol sugar (Kalia and Purohit 2008; Patel et al. 2012a, b, 2015). Since 60 % of the total hydrogen production cost is incurred toward feed, CG, which is emerging as a cheap biowaste and is available in plenty, has become an attractive substrate for biological hydrogen production (BHP) (Sanchez-Torres et al. 2013).

7.2.1 Batch Culture Digestion

Digestion of glycerol to produce H_2 has been carried out by different researchers (Table 7.1). Glycerol is proving as a better substrate in comparison to the frequently used glucose-containing feeds. It has been revealed that glycerol being more reduced than glucose is able to produce higher quantities of NAD(P)H, which consequently can account for its higher H_2 yielding capacity (Maru et al. 2012). Under batch culture conditions, different bacteria are able to produce H_2 on different media supplemented with 0.1–5.0 % (v/v) glycerol under mesophilic conditions as well as under thermophilic temperatures. Depending upon the bacteria being used as inoculum, H_2 yields (mol/mol glycerol) vary as follows: (1) *Anaerovibrio glycerini*, 0.8 at 1 % feed; (2) *Bacillus*, 0.25–2.2 at 2 % feed; (3) *Clostridium butyricum* and *C. pasteurianum*, 0.23–0.93 at 0.5–2 % feed; (4) *Enterobacter aerogenes*, *Enterobacter* sp., and *Escherichia coli*, 0.8–2.9 at 0.33–2.5 % feed (pure glycerol, PG); (5) *Halanaerobium saccharolyticum* strains DSM 6641 and DSM 7379, 0.35–0.6 and 0.89–1.6 at 0.25–1 % feed consumed, respectively; (6) *Klebsiella* spp., 0.05–0.19 at 0.25–1 % feed; and (7) *Thermotoga maritima* and *T. neapolitana*, 2.79 and 0.28–2.42 at 0.65 % and 0.1–1.3 % feed, respectively (Schauder and Schink 1989; Ito et al. 2005; Kotay and Das 2007; Seifert et al. 2009; Selembo et al. 2009a; Kivistö et al. 2010; Hu and Wood 2010; Ngo et al. 2011; Wu et al. 2011; Maru et al. 2012; Varrone et al. 2012; Lo et al. 2013; Sarma et al. 2013; Kumar et al. 2015c). In contrast to pure cultures as inocula, different studies have employed mixed bacterial cultures, where H_2 yields (mol/mol glycerol) have been as follows: (1) 0.28–2.96 at 0.3–3 % feed under mesophilic conditions and (2) 0.3 at 2 % feed under thermophilic conditions (Sittijunda and Reungsang 2012a). With immobilized bacterial culture on UASB granules or self immobilized cells, the H_2 production rate was 1.37 mmol/L/h (Sittijunda and Reungsang 2012b) and 80 mmol/L/h (Ito et al. 2005), respectively. Heat-pretreated activated sludge could generate 11.5–38.1 mL H_2/g COD (Akutsu et al. 2009). In an innovative approach, microbial electrolysis cells have been employed to use domestic wastewater to produce 1.8–3.9 mol H_2/mol glycerol fed (Selembo et al. 2009b). *Enterobacter* and *Bacillus* are among those organisms which have the ability to utilize pure and CG with equal efficiency (Marques et al. 2009; Markov et al. 2011; Kumar et al. 2015c). In general, most of the bacteria are able to transform glycerol in the range of 0.1–1 %; however, *Bacillus*, *Enterobacter* spp., and *Klebsiella* spp. have better tolerance to glycerol. These bacteria could produce H_2 at higher glycerol levels of up to 5 % (Ito et al. 2005; Wu et al. 2011; Kumar et al. 2015c).

7.2.2 Continuous Culture Digestion

Digestion of glycerol as feed under continuous culture has been studied in a continuous stirred tank reactor (CSTR) and packed bed reactor (PBR) modes (Table 7.2) (Ito et al. 2005; Lo et al. 2013; Kumar et al. 2015c). *Clostridium* is a well known organism for producing H_2 in an effective manner (Heyndrickx et al. 1991; Fountoulakis

Table 7.1 Hydrogen production by bacteria using glycerol in batch culture

Bacterial culture	Medium; conditions [pH, temp. (°C), rpm]	Glycerol		Yield (mol/mol)	References
		Type	g/L		
Anaerovibrio glycerini ATCC33276	Mineral; 7.2, 25, –	Pure	0.92	0.63^{a}	Schauder and Schink (1989)
Bacillus coagulans IIT-BT S1	MYG; 6.5, 37, –	Pure	15	2.2	Kotay and Das (2007)
Bacillus thuringiensis EGU45	Mineral + $NaNO_3$; 7.0, 37,–	Pure	10	0.75	Kumar et al. (2015c)
Clostridium pasteurianum	HMG; 7.5, 35, –	Pure	16.4	0.23–0.93^{a}	Lo et al. (2013)
C. butyricum DSM 2478	HM100 + 1 % NaCl; 7.0, 37, –	Pure	5	0.54^{a}	Kivistö et al. (2010)
Enterobacter sp. H1	MYG; 6.34, 37, 200	Pure	20	0.85	Maru et al. (2012)
Enterobacter aerogenes HU-101	YE 0.5 g/L + tryptone 0.5 g/L; 6.8, 37, 120	Pure	10	0.89	Ito et al. (2005)
			25	0.82	
		Crude	3.3	0.90	
			25	0.71	
E. aerogenes NBRC 12010	NBRC702; 6.0, 30, 200	Crude	9.9	0.77	Sakai and Yagishita (2007)
E. aerogenes NRRL B407	Complex + slaughter house waste (10 mg/L); 6.0, 30, 150	Crude	10	0.92	Sarma et al. (2013)
	Complex + brewery waste (10 mg/L); 6.0, 30, 150			0.98	
E. aerogenes	Mineral; 7.0, 37, 50	Pure	20	0.89	Markov et al. (2011)
	Synthetic + YE + tryptone; –, 37, –	Crude	20	710.0^{b}	Marques et al. (2009)
Escherichia coli	Minimal; –, 37, 120	Pure	10	0.88	Tran et al. (2014)
	Minimal; 6.3, 37, –	Pure	10	0.68^{c}	Hu and Wood (2010)
Halanaerobium saccharolyticum DSM 6641	HM100 + 15 % NaCl; 7.0, –, –	Pure	2.5	0.6^{a}	Kivistö et al. (2010)
			5	0.46^{a}	
			10	0.39^{a}	
			20	0.35^{a}	
H. saccharolyticum DSM 7379	HM100 + 15 % NaCl; 7.0, 37, –	Pure	2.5	1.6^{a}	Kivistö et al. (2010)
			10	0.89^{a}	
Klebsiella sp. HE1	Mineral; 5.5–7.0, 35, 200	Pure	50	0.056–0.19	Wu et al. (2011)
K. pneumoniae DSM2026	YE; 6.5, 37, –	Crude	20.4	0.53	Liu and Fang (2007)
Thermotoga neapolitana DSM 4359	M-3; 7.3, 80, 200	Pure	12.9	2.42	Maru et al. (2012)
	TMB; 7.5, 75, –		1–9	0.28–1.0^{a}	Ngo et al. (2011)
T. maritima DSM 3109	M-3; 6.9, 80, 200	Pure	6.5	2.79	Maru et al. (2012)

(continued)

Table 7.1 (continued)

Bacterial culture	Medium; conditions [pH, temp. (°C), rpm]	Glycerol		Yield (mol/mol)	References
		Type	g/L		
Enterobacter spH2: *Enterobacter* spH1: *Citrobacter freundii* H3 (1:1:1)	MYG; 6.3, 37, 200	Pure	20	0.76	Maru et al. (2012)
Anaerobic sludge	Minimal; 6.8, 37, 120	Pure	10	0.41	Seifert et al. (2009)
		Crude	30	0.71	
Mixed cultures (wheat soil)	MES buffer; 6.2, 30, –	Pure	3	0.28	Selembo et al. (2009a)
		Crude	3	0.31	Selembo et al. (2009a)
Mixed cultures	Synthetic; 6.8, 37, 120	Crude	15	0.96	Varrone et al. (2012)
Mixed culture mesophilic	Minimal; 6.8, 37, 120	Pure	3	0.28	Selembo et al. (2009a)
		Crude	3	0.31	
Mixed cultures thermophilic	Sludge + endoenutrient; 5.5, 55, –	Crude	20.3	0.3	Sittijunda and Reungsang (2012a)
UASB granules of beer wastewater	Sludge + endoenutrient; 5.5, 35, –	Crude	22.2	1.37[c]	Sittijunda and Reungsang (2012b)
Heat-pretreated microflora	Mineral media + oil + peptone; –, –, –	Pure	–	11.5–38.1[d]	Akutsu et al. (2009)
Microbial electrolysis cells	Domestic wastewater; 7.0, 37, –	Pure	1	3.9	Selembo et al. (2009b)
		Crude	0.69	1.8	

[a] Consumed basis
[b] mL L^{-1}
[c] mmol H_2L^{-1} h^{-1}
[d] mL-H_2 g^{-1} COD

Table 7.2 Hydrogen production by bacteria using glycerol in continuous culture

Inoculum	Mode of exp.	Glycerol		Feeding time	H_2 yield (mol/mol)	References
		Type	g/L			
Heat-treated anaerobic sludge	Continuous electrolytic microbial cell	Pure	0.74	HRT 8 h	5.4	Escapa et al. (2009)
Anaerobic sludge	PBR	Crude	3675[a]	HRT 0.5 h	200[b]	Fernandes et al. (2010)
Bacillus thuringiensis EGU45	Continuous	Pure	20	HRT 2d	0.386	Kumar et al. (2015c)
	Fed batch	Crude	20	HRT 2d	0.393	
Clostridium butyricum LMG 1212 t2	CSTR	Pure	10	HRT 12 h	0.38	Heyndrickx et al. (1991)
Clostridium spp. biofed anaerobic sludge	CSTR	Crude	10	HRT 25 d	0.27	Fountoulakis and Manios (2009)

(continued)

Table 7.2 (continued)

Inoculum	Mode of exp.	Glycerol		Feeding time	H_2 yield (mol/mol)	References
		Type	g/L			
Clostridium pasteurianum CH4	CSTR	Crude	10	HRT 12 h; 35 °C; pH 7.5	0.77	Lo et al. (2013)
Enterobacter aerogenes HU-101	PBR	Pure	10.1	HRT 10 h	80	Ito et al. (2005)
		Crude	10.1	HRT 10 h	63	
Thermotoga maritima	CSTR	Crude	0.25	0.017 dilution rate/h	2.41[c]	Maru et al. (2012)
			0.25	0.025 dilution rate/h	2.27[c]	
			0.25	0.035 dilution rate/h	2.23[c]	
			0.25	0.050 dilution rate/h	2.30[c]	

[a] gCOD/L

[b] mL-H_2 g^{-1} COD

[c] H_2 yield on consumed basis

and Manios 2009; Lo et al. 2013). *Clostridium* operates under strictly anaerobic conditions. *Clostridium butyricum* in CSTR mode fed with 1 % glycerol could produce 0.38 mol H_2/mol at a hydraulic retention time (HRT) of 12 h. Under similar conditions, *C. pasteurianum* has been found to convert CG into 0.77 mol H_2/mol (Lo et al. 2013). *Clostridium* sp. along with anaerobic sludge was not very effective as H_2 producer (Fountoulakis and Manios 2009). *Enterobacter aerogenes* under continuous culture conditions in PBR was equally effective with pure and CG as feed (Ito et al. 2005). Recently, *Bacillus thuringiensis* EGU45 immobilized on lignocellulosic support materials has been shown to efficiently utilize both PG and CG to produce H_2 in a continuous culture conditions. Here, the H_2 yield varied from 0.204 to 0.386 mol/mol of PG and 0.273 to 0.393 mol/mol of CG (Kumar et al. 2015c). *T. maritima* proved to be suitable for upscaling as the yield in batch culture could be realized under continuous culture conditions as well, which varied from 2.23 to 2.41 mol H_2/mol glycerol consumed (Maru et al. 2012). A few other combinations have been able to yield which is comparable to that recorded with *Enterobacter* sp. (Ito et al. 2005; Escapa et al. 2009; Fernandes et al. 2010).

7.3 Biotransformation of Glycerol to Biomethane

Anaerobic digestion of biological materials has been attracting environmental biologists because of its unique characteristics. The foremost advantage is the nearly complete degradation of organic matter to the extent of around 95 % in CH_4 and CO_2. The enormous amount of efforts has gone into exploiting this process for waste treatment and its potential to produce energy. Although the range of biogas generation varies from around 200 to 400 L/kg TS on an average, with a CH_4 content of 55–70 %, however, the energy yields are not high enough for commercial production (Kalia et al. 1992a, b; Kalia 2007). It has obliged researchers to go in for value addition by transforming the different intermediates into other bioproducts such as biohydrogen and biopolymers, among other bioactive molecules (Kalia and Joshi 1995, Kalia et al. 2000, 2007; Sonakya et al. 2001; Raizada et al. 2002). Biomethane production from wastes has been dealt in other chapters in this book. Here, we are focusing on biodiesel industry waste, which is rich in glycerol for its potential to produce CH_4 either as an individual feed or as a co-substrate (Table 7.3).

Table 7.3 Co-digestion of glycerol and biowastes for methane production

Feed	Digester mode	Glycerol (g/L)	Conditions		CH_4 yield	References
			pH	°C		
Waste frying oil	Batch	3.0	7.2	37	283 L CH_4/kg COD	Oliveira et al. (2014)
Nutritional medium	Batch	1	7	25	180 L CH_4/kg COD	Peixoto et al. (2012)
Sewage sludge	CSTR[a]	20	6.8	37	1.1 L/L/d	Jensen et al. (2014)
Olive mill waste: Slaughter house waste water:: 1:4	CSTR	10	7.5	35	2.1 L/L/d	Fountoulakis and Manios (2009)
Raw rapeseed straw	CSTR	30	7.5	55	314 L CH_4/kg VS	Luo et al. (2011)
Raw rapeseed straw	CSTR	30	7.5	55	322 L CH_4/kg VS	
Sewage sludge	CSTR	10	7.5	35	2.3 L/L/d	Fountoulakis et al. (2010)
Cattle manure	CSTR	60	7.5	55	348 L CH_4/kg COD	Castrillón et al. (2011)
Cattle manure	CSTR	40	7.5	35	144 L CH_4/kg COD	
Cattle manure	IBR[b]	60	7.6	55	590 L CH_4/kg VS	Castrillón et al. (2013a)
Cattle manure (CM): food waste (FW) (94:2)	IBR	40	7.8	55	2.43 L CH_4/L/d	Castrillón et al. (2013b)
CM:FW (87:10)	IBR	30	7.3	55	2.57 L CH_4/L/d	
CM:FW (83:15)	IBR	20	7.3	55	0.65 L CH_4/L/d	
CM:FW (82:15)	IBR	30	7.3	55	1.52 L CH_4/L/d	
CM:FW (94:0)	IBR	60	7.2	55	2 L CH_4/L/d	
Pig manure (PM)	CSTR		7.4	35	215 L CH_4/kg COD	Astals et al. (2011)
Pig manure	CSTR	30	7.7	55	4.96 L CH_4/d	Astals et al. (2013)
Mixed waste (MW) (maize silage 31 % + corns 15 %, PM 54 %)	CSTR	60	6.7	40	439 L/kg VS	Amon et al. (2006)
MW + 10 % rapeseed meal	CSTR	60	6.7	40	432 L/kg VS	
Feeding medium	CSTR	0.25[d]	7.6	35	0.074 L CH_4/L/d	Vlassis et al. (2013)
Feeding medium	PABR[c]	3.0[d]	7.6	35	0.993 L CH_4/L/d	

[a] Continuous stirred tank reactor
[b] Induced bed reactor
[c] Periodic anaerobic baffled reactor

Many biowastes as feed are not found suitable for efficient anaerobic digestion. In order to improve their digestion, they can be supplemented with wastes which may contain easily hydrolyzable carbohydrates, fats, and proteins. Glycerol can be added as a co-substrate to assist the digestion process (Costa et al. 2011; Vlassis et al. 2013; Oliveira et al. 2014). Co-digestion of biowastes offers the following advantages over single feed: (1) dilutes toxic materials, (2) higher organic matter to help in reducing the reactor size and finally the digestion, (3) stabilization of the slurry, (4) better management of mixed wastes, and (5) makes the process cost effective (Vlassis et al. 2013; Kumar et al. 2014b). Anaerobic digestion of PG under continuous culture conditions produced (1) 74 mL CH_4/L/d in a continuous stirred tank reactor at 0.25 g COD of feed/L/d and (2) 993 mL CH_4/L/d in a periodic anaerobic baffled reactor at 3 g COD of feed/L/d (Vlassis et al. 2013). Supplementation of nutrient medium with 1 % glycerol was effective in achieving 180 mL CH_4/g COD (Peixoto et al. 2012).

Animal manure has a low-specific CH_4 yield of 148–185 L/kg volatile solids (Amon et al. 2006). However, co-digestion of 3–6 % glycerin with mixed wastes (maize silage, pig manure, and rapeseed waste) resulted in a CH_4 yield of 570–680 L/kg VS (Amon et al. 2006), whereas animal manure and CG (5–10 %, w/w) could produce 0.82 L/g in CSTR. Under batch culture conditions, co-digestion of animal (pig) manure (20–80 %) and PG showed that for optimum biogas yields, 80 % animal waste is desirable, with a CH_4 yield of 215 mL/g COD (Astals et al. 2011, 2013). It was realized in a later study that 4 % PG can lead to a fourfold enhancement in biogas yield (Astals et al. 2013). Animal manure mixed with a range (4–8 %, w/w) CG revealed that 4 % CG is the optimum level as co-substrate, where biogas production of 5.9 m^3/tonne of wet waste was higher than the feed alone (Castrillón et al. 2011). However, co-digestion of animal manure with glycerol (6 %) under continuous culture resulted in 590 L CH_4/kg VS (Castrillón et al. 2013a). Animal manure along with food waste and glycerol (2–6 %) under thermophilic conditions yielded 0.65–2.57 L CH4/L/d (Castrillón et al. 2013b). Digestion of potato processing wastewater along with glycerol resulted in an additional production of 740 mL biogas for each mL of glycerol fed (Fountoulakis and Manios 2009). CH_4 yield from a mixture of CG (1 %, v/v) and sewage sludge was improved by twofold (Fountoulakis and Manios 2009; Fountoulakis et al. 2010). CG (2 %, v/v) and sewage sludge mixture produced 1.0 L CH_4/L/d and enabled 50 % enhancement in CH_4 yields (Fountoulakis et al. 2010; Jensen et al. 2014). Mixtures of rapeseed straw with 3 % glycerol could be digested to produce 322 mL CH_4/g VS (Luo et al. 2011).

Biomethanation of macroalgae *Gracilaria vermiculophylla* was enhanced by 18 % by adding 2 % glycerol. Co-digestion of *Sargassum* sp. (a brown macroalgae) with 0.3 % glycerol resulted in up to 56 % enhancement in CH_4 yield and 38 % higher CH_4 production rate (Oliveira et al. 2014). Higher concentrations (>10 %) of glycerol proved inhibitory to bacteria due to the following reasons: (i) unable to withstand the osmotic stress and (ii) presence of toxic compounds such as salts and methanol (Fountoulakis et al. 2010; Astals et al. 2011).

7.4 Biotransformation of Glycerol to Polyhydroxyalkanoates

Plastic being nonbiodegradable is likely to get accumulated in the environment. These add to the waste management problems. Another obvious issue linked to plastics is the use of petroleum products as feed. The limited fossil fuel reserves are a constant worry on the minds of environmentalists and government agencies. At the beginning of the twentieth century, microbiologists found that bacteria can accumulate food reserves, which have properties similar to plastics. Since then bioplastics such as PHB were thoroughly studied to replace synthetic plastics (Singh et al. 2009; Kumar et al. 2013; Naranjo et al. 2013). In view of the fact that PHB is quite brittle in nature, a search for more sturdy biopolymers such as polyhydroxyalkanoates (PHAs) was initiated (Reddy et al. 2009a, b; López et al. 2012; Singh et al. 2015). It has been realized that many bacteria are able to produce PHA under stressed environmental conditions. Their composition varies with the type of feed and the producer organism (Kumar et al. 2014a). However, commercial production is still not economically feasible. The major cause of this high price is linked to the cost of the feed and the recovery process. Copolymers of PHA produced by bacteria are more desirable than PHB. In order to reduce costs, an obvious solution is to use cheap raw materials, and biowastes are an obvious choice. It can help to reduce at least 45 % of the production cost (Singh et al. 2009). So far, a wide range of biowastes has been shown to get metabolized to PHAs: agricultural wastes, dairy products, oily wastes, palm oil mill effluents, wheat straw, used cooking oil, etc. (Singh et al. 2009, 2015; Gasser et al. 2014; Martino et al. 2014)

As the cost of glycerol is going down, this waste is being used for generating novel bioproducts including PHAs (Table 7.4). *Cupriavidus necator*, Gram-negative bacteria

Table 7.4 Polyhydroxyalkanoate production by microbes on glycerol

	Feed			Polyhydroxyalkanoate			
Microbes	Type	Conc. (%, w/v)	Cell dry weight (g/L)	Content (g/L)	Yield (%)	Molecular weight (M_w)	Reference
Ralstonia eutropha	Pure	2	27	17.5	65	–	Bormann and Roth (1999), Gözke et al. (2012) and Mothes et al. (2007)
	Crude	1	37	20	55	3.5×10^5	
Cupriavidus necator	Pure	2	82	51	62	–	Cavalheiro et al. (2012)
	Crude	1	50	24	48	6.2×10^5	
	Crude	4	45	17	38	5.5×10^6	
Escherichia coli	Pure	3	9.6	1.6	17	1.9×10^6	Almeida et al. (2010)
	Pure	2.2	8.3	3.5	42	–	Nikel et al. (2008)
Methylobacterium rhodesianum	Pure	2	21	8.8	42	–	Bormann and Roth (1999)
Pseudomonas oleovorans	Pure	1	3.0	1.2	40	3.14×10^5	Ashby et al. (2011)
	Crude	1	2.9	1.1	38	2.61×10^5	
	Crude	5	1.4	0.38	27	1.07×10^5	Ashby et al. (2004)
P. corrugata	Crude	5	1.7	0.68	40	6.56×10^5	
Paracoccus denitrificans	Crude	1	50	25	50	7.5×10^5	Mothes et al. (2007)
Vibrio sp. M20	Pure	3	0.44	0.19	42.8	–	Chien et al. (2007)
Zobellella denitrificans	Crude	15	81.2	54	67	–	Ibrahim and Steinbüchel (2009)
Bacillus sp.	Pure	0.2–2	2.5–7.3	1.4–4.4	52–60	$5.1–6.3\times10^5$	Reddy et al. (2009b), Full et al. (2006), and Sangkharak and Prasertsan (2012)
B. thuringiensis	Pure	1	6.1	3.9	64	1.0×10^5	Rohini et al. (2006)
	Pure	1	2.7	1.54	57	–	Kumar et al. (2015b)
	Crude	5	4.8	3.6	74	3.8×10^5	
B. cereus	Pure	2	3	2	60	–	Sangkharak and Prasertsan (2012)
B. licheniformis	Pure	2	10	6.5	68	–	Sangkharak and Prasertsan (2012)

(continued)

Table 7.4 (continued)

Microbes	Feed		Cell dry weight (g/L)	Polyhydroxyalkanoate		Molecular weight (M_w)	Reference
	Type	Conc. (%, w/v)		Content (g/L)	Yield (%)		
B. megaterium	Pure	2–5	5–25	2.4–15	31–62	5.2–6.3 × 10^5	Naranjo et al. (2013), Reddy et al. (2009a), and López et al. 2012
B. sphaericus	Crude	2	20	6.3	31	–	Sindhu et al. (2011)
Mixed microbial consortia	Crude	1	5	2.5	50	3.0 × 10^5	Dobroth et al. (2011)
Unidentified osmophilic organism	Pure	1	21.3	16.2	76	2.5 × 10^5	Koller et al. (2005)

(previously known as *Ralstonia eutropha*), has been one of the most widely studied organisms for PHA production. It can produce PHAs from various substrates. These organisms have been equally effective at metabolizing both pure and crude form of glycerol, where these could produce 17–51 g/L, with a yield in the range of 38–65 % of cell dry weight (CDW) (Bormann and Roth 1999; Mothes et al. 2007; Cavalheiro et al. 2012; Gözke et al. 2012; Naranjo et al. 2013). *Escherichia coli* have been widely reported as a host for expression of genes for PHA production (Kalia et al. 2007). This organism could tolerate 1–3 % glycerol and convert it to 1.6–19 g/L equivalent to 17–51 % PHA yield (Nikel et al. 2008; Almeida et al., 2010). *Methylobacterium rhodesianum* produce 42 %, w/w of PHA on 2 % PG (v/v) (Bormann and Roth 1999). Another high PHA-producing Gram-negative bacteria, *Pseudomonas* spp., is well known for mcl-PHA production. With glycerol as feed (1–5 %, v/v), *Pseudomonas oleovorans* 0.3–1.2 g/L with a yield in the range of 27–40 % of CDW (Ashby et al. 2011). *Pseudomonas corrugata* proved to be a better PHA producer compared to *P. oleovorans*, as it could PHA with a much higher M.Wt. of 6.56 × 10^5 (Ashby et al. 2004). Similarly, few other Gram-negative bacteria, *Paracoccus denitrificans*, *Vibrio*, and *Zobellella denitrificans*, have been able to metabolize CG into 42–67 % PHA (Chien et al. 2007; Mothes et al. 2007; Ibrahim and Steinbüchel 2009). Among Gram-positive bacteria, so far only *Bacillus* spp. have been able to metabolize 1–5 %, v/v glycerol into PHA: (1) *B. megaterium* produced 31–62 %, w/w of CDW (Full et al. 2006; Reddy et al. 2009a, b; López et al. 2012; Naranjo et al. 2013); (2) *B. cereus and B. thuringiensis* had a capacity of 60–74 %, w/w (Rohini et al. 2006; Sangkharak and Prasertsan 2012; Kumar et al. 2015b); (3) *B. sphaericus* produced 31 %, w/w; and (4) *B. licheniformis* resulted in 68 %, w/w (Rohini et al. 2006; Sindhu et al. 2011; Sangkharak and Prasertsan 2012) (Table 7.4). Mixed microbial consortia could utilize pure as well as CG to yield 50–76 %, w/w PHA which accounted for 2.5–16.2 g/L of the total CDW (Koller et al. 2005; Dobroth et al. 2011).

Interestingly, it has been observed that the N limitation is not crucial for higher PHA and its copolymer production when glycerol is being used as feed, especially in case of *Bacillus* spp. (Nikel et al. 2008; Kumar et al. 2013, 2015b; Singh et al. 2013). This leaves an open scope to exploit the abilities of various bacteria to produce PHA under minimal fermentative checkpoints at large scale. Further, integrative approach may be adopted for sequential production of other biologically important compounds through using well-designed consortium of *Bacillus* (Kumar et al. 2015a, b).

7.5 Perspectives

Glycerol as a waste has the desired potential of being transformed by a wide range of bacteria into H_2, PHA, and CH_4. Since *Bacillus* sp. have been shown to have properties for degrading wastes and converting the intermediates into H_2 and PHA, it will be desirable to carry out studies for exploiting glycerol on a commercial scale (Porwal et al. 2008; Patel et al. 2010, 2012a, b, 2015; Kumar et al. 2013, 2015a, b, c; Patel and Kalia 2013; Singh et al. 2013). Although CG still poses a few problems due to the presence of toxic materials, however, these can be overcome through innovative strategies such as the eco-biotechnological approaches and synthetic biology.

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Prasun Kumar received his M.Sc. degree in Biotechnology from KIIT University, Bhubaneswar, India. He is currently working as a senior research fellow at CSIR-IGIB, Delhi, India. His current interests are biopolymers, biofuels and quorum sensing. His main theme of research is “production of polyhydroxyalkanoates from biowastes”. He is a life member of the Association of Microbiologists of India (AMI) and the member of American Society for Microbiology (ASM).

Sanjeet Mehariya graduated in Zoology honours in 2011 and Master’s in Biomedical Sciences in 2013 from University of Delhi. She is working as Project-JRF at CSIR-IGIB, Delhi, India. She enjoys exploring genomic shuffling in genes possessing quorum quenching ability, bacterial biofilm inhibition and 16S *rrs* gene based identification of novel bacterial strains from diverse backgrounds.

Subhasree Ray did Master’s in Biotechnology from KIIT University, Bhubaneshwar, Odisha, India. She is currently working as a junior research fellow at CSIR-IGIB, Delhi, India. Her current research interests lies in biofuels and biopolymers from biological wastes. Her main focus of research is “polyhydroxyalkanoate copolymers production from crude glycerol”. Her work has been published in various peer-reviewed international journals.

Anjali Mishra graduated in Zoology honours in 2011 and Master’s in Biomedical Sciences in 2013 from University of Delhi. She is working as Project-JRF at CSIR-IGIB, Delhi, India. She enjoys exploring genomic shuffling in genes possessing quorum quenching ability, bacterial biofilm inhibition and 16S *rrs* gene based identification of novel bacterial strains from diverse backgrounds.

Vipin Chandra Kalia M.Sc. and Ph.D. Genetics from IARI, New Delhi. He is presently the Chief Scientist at CSIR-IGIB and Professor of AcSIR. He is a Fellow of Association of Microbiologists of India and Fellow of National Academy of Sciences (FNASc). He is the Editor-in-Chief of the Indian Journal of Microbiology (India). His current interests are bioenergy, biopolymers and quorum sensing. Google Scholar: http://scholar.google.co.in/citations?hl=en&user=XaUw-VIAAAAJ. Website: http://www.igib.res.in/?q=V.C.Kalia

Recent Achievements in the Production of Biobased 1,3-Propanediol

8

Noura Raddadi, Stefania De Giorgi,
and Fabio Fava

Abstract

The increasing concerns related to the high prices and limited availability of fossil fuels have led to a remarkable growth of biodiesel industry in recent years. This, in turn resulted in the accumulation of high quantities of crude glycerol as a by-product of the transesterification reaction. It is indeed estimated that the production of 100 kg of biodiesel results in the production of 10 kg of crude glycerol. The obtained crude glycerol by-products could be purified and further used in several processes, but this purification process is costly. The biorefinery concept, i.e. the exploitation of wastes or by-products for the obtainment of high-value products, has been then considered as an interesting alternative strategy for the valorisation of this by-product. In this context, several fine chemicals, among which is 1,3-propanediol, are produced from biodiesel-derived raw glycerol feedstock. In this chapter, we report on recent achievements in the production of 1,3-propanediol from biodiesel-derived crude glycerol including a detailed literature review on the biotechnological processes developed and the strategies employed for yield improvement. The bottlenecks of these biorefinery processes are also discussed.

8.1 Introduction

In recent years the growing environmental and economic concerns have led to an increasing interest in green chemistry. An interesting approach is the use of industrial by-products/wastes for producing 'high-value' chemicals through microbial fermentation, i.e. biotechnological processes, generally referred to as biorefinery. Several studies (Festel Capital 2007) have showed how biotechnological processes have gained a considerable market share in the last 10

N. Raddadi (✉) • S. De Giorgi • F. Fava
Department of Civil, Chemical, Environmental
and Materials Engineering (DICAM),
via Terracini 28, 40131 Bologna, Italy
e-mail: noura.raddadi@unibo.it

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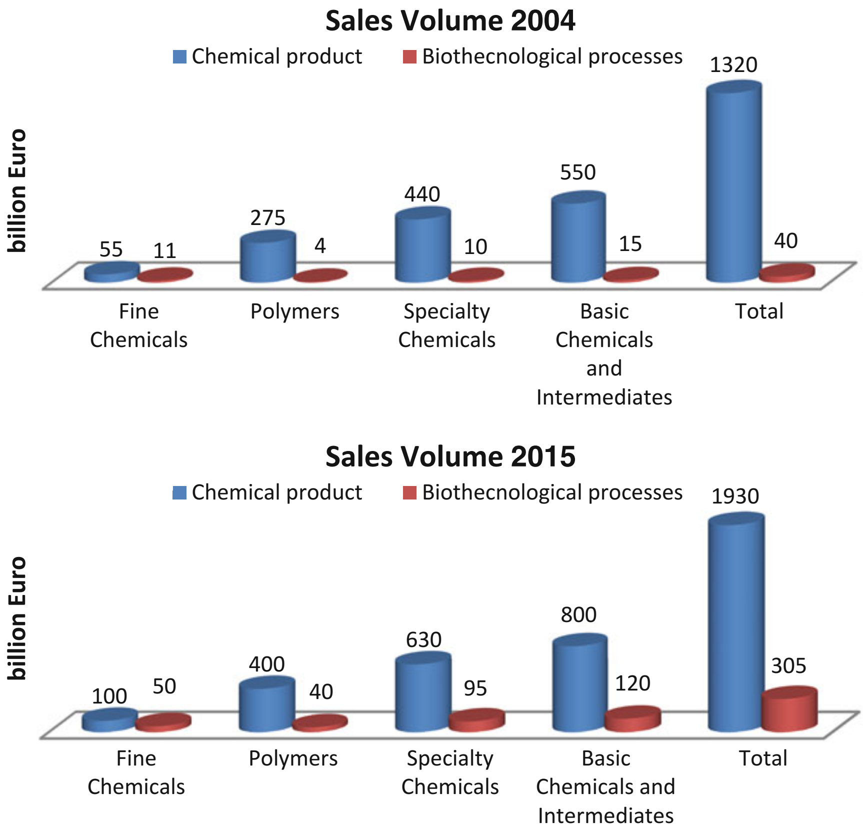

Fig. 8.1 Development of biotechnological processes shares with chemical products in 2004 and in 2015 (Modified according to Saxena et al. 2009)

years (Saxena et al. 2009) (Fig. 8.1). This is owing to the advantages they have compared to their chemical counterparts. Biotechnological processes are indeed characterised by eco-sustainability since they can use renewable raw material as substrate for microbial growth and biobased molecule production and generally without extreme operating conditions and toxic by-products. Moreover, green chemistry can reduce the strictly dependence of chemical industry from common fossil fuels.

Among the biobased compounds, 1,3-propanediol (1,3-PDO) is getting increasing interest. The 1,3-PDO worldwide market is growing rapidly, and actually it achieves over 100 million pounds annually. The relevance of this market is highly associated with the increasing number of 1,3-PDO-based products. In 2012, the global demand for 1,3-PDO was 60.2 kt and is estimated to reach about 150 kt in 2019 (Lee et al. 2015). In this chapter, a careful literature review on recent achievements in its production is reported. Before going into detail in the biotechnological processes developed for obtaining this biobased chemical and the strategies employed for yields improvement, a short description of the characteristics of 1,3-PDO and the fields of application are provided.

8.2 1,3-PDO Properties and Applications

1,3-PDO is an organic compound, with formula $CH_2(CH_2OH)_2$, which has been considered in the past as a 'speciality chemical' due to its high price (about 30 US$/kg compared to 2 US$/kg for other petrochemical feedstocks) and low availability

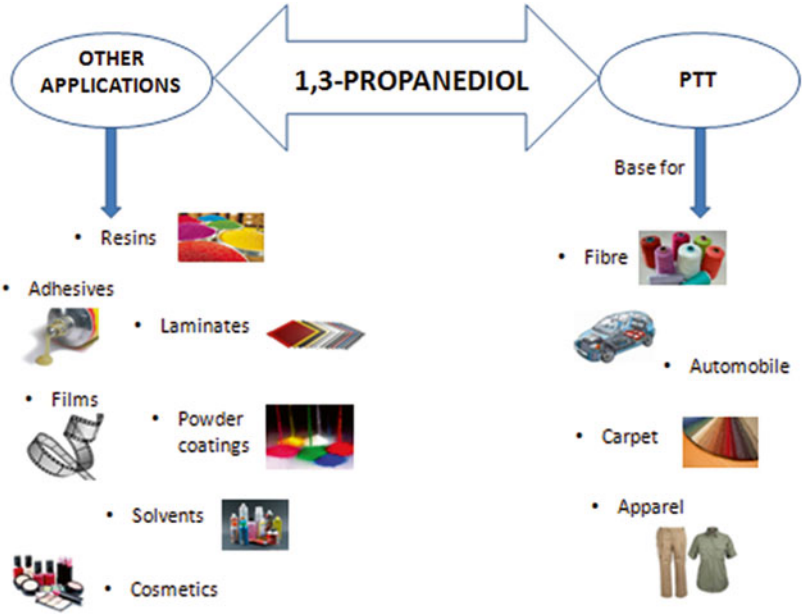

Fig. 8.2 1,3-PDO industrial applications (Modified according to Kaur et al. 2012)

(Biebl et al. 1999). It is a non-flammable diol with low toxicity and a good solubility in many common solvents like water, alcohols and ethers (Sullivan 1993). These characteristics, in addition to a particular structure with two hydroxyl groups on C1 and C3 that makes it an ideal platform for polycondensation reactions, give its many potential industrial applications (Fig. 8.2). Among the plastics based on PDO, the polytrimethylene terephthalate (PTT) polyesters obtained based on 1,3-PDO and either dimethyl terephthalate (DMT) or terephthalic acid (TPA) polycondensation are the most known polymers (Kurian 2005) . PTT polyesters found applications in carpeting, textiles and apparel, engineering thermoplastic, films and monofilaments (Saxena et al. 2009). In thermoplastic polyurethanes (TPU), the 1,3-PDO use, as a building block or chain extender, can improve thermal stability and abrasion resistance, in addition to a linear shape that increases mechanical properties (Rashmi et al. 2013). In addition to being used as biodegradable plastic feedstock (Umare et al. 2007), 1,3-PDO also finds interesting applications in chemical industry: in solvent system, it increases the flexibility without compromising the stability properties; in laminates, adhesives and resins, it reduces intrinsic viscosity; in cosmetic products it promotes long-lasting effects and weathering resistance. 1,3-PDO can also be used in high-strength glasses or in engine coolant formulations (low corrosion effects and lower toxicity than ethylene glycol) as industrial biocide for biofouling prevention in cooling systems. Its stability and low toxicity properties make it suitable even in products for personal and home care. Finally it has a vector role in different kinds of medicines (vitamin H and immunosuppressive drugs) (Saxena et al. 2009; Leja et al. 2011; Kaur et al. 2012; Metsoviti et al. 2013).

8.3 Production of 1,3-Propanediol

8.3.1 Chemical Processes

The 1,3-PDO has been produced for several years mainly through two chemical processes (Kraus 2008). DuPont company developed an acrolein-based process, which consists in a first hydration to 3-hydroxypropionaldehyde (3-HPA) that is then hydrogenated in the presence of nickel to form 1,3-PDO (Fig. 8.3).

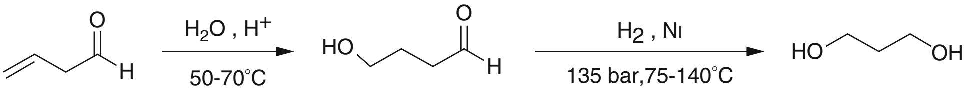

Fig. 8.3 DuPont process for 1,3-PDO synthesis (Modified according to Saxena et al. 2009)

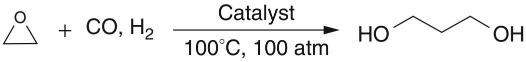

Fig. 8.4 Shell process for 1,3-PDO synthesis (Modified according to Saxena et al. 2009)

A different 1,3-PDO production process based on ethylene oxide hydroformylation has been developed by Shell company (Fig. 8.4). In a first step ethylene oxide reacts with carbon monoxide in catalyst organometallic presence to produce hydroxyl aldehyde, which is subsequently reduced by hydrogenation into PDO. The catalyst used may be homogeneous (such as cobalt) or heterogeneous (copper), using syngas (a blend of H_2 and CO) as hydrogen source which reduces the catalyst's selectivity and stability (Lange 2001).

Moreover, a patent of 2007 reported on PDO production process from ethylene oxide in a single step using cobalt-ruthenium catalysts ligated with a phosphoalkanoate ligand, soluble in methyl tert-butyl ether, at high temperatures (80–100 °C) and pressures (500–1500 psi), in syngas presence. PDO's yields are about 70 mol%, but the process is overall considered unsustainable (Powell and Weider 2007).

8.3.2 Biotechnological Production of 1,3-PDO

Complications encountered in chemical processes, together with a growing global interest in 'green chemistry', have led to the development of alternative strategies for 1,3-PDO production, i.e. the development of biotechnological processes based on microbial bioconversion of glycerol to 1,3-PDO.

8.3.2.1 Glycerol Metabolism and 1,3-PDO Production

Glycerol is the substrate naturally converted by several microorganisms to1,3-PDO (Saxena et al. 2009). All microorganisms defined as 'natural producers' are able to metabolise glycerol through reductive and oxidative parallel processes (Fig. 8.5), catalysed by different enzymes encoded by *dha* operon: glycerol dehydratase (GDHt), 1,3-prodanediol oxidoreductase (PDOR), glycerol dehydrogenase (GDH) and dihydroxyacetone phosphate kinase (DHAK).

In an ideal anaerobic process, the highest 1,3-PDO yield can reach 0.875 mol/mol glycerol, but during fermentation process, pyruvate competes with 3-HPA for oxidoreductase, thus leading to a different product composition depending on the microorganism type and process conditions. For example, in the case of *Klebsiella pneumoniae*, 1,3-PDO production was promoted at a pH range between 6.5 and 7.5, while 2,3-butanediol production requires lower 6.5 pH (Lee et al. 2015). In *Clostridium butyricum*, where GDHt is vitamin B12 independent, major by-products are acetic and butyric acids (Papanikolaou et al. 2004); butanol is produced by *Clostridium pasteurianum* (Biebl 2001). Along with these, also ethanol, lactic and succinic acids and butanediol are produced as by-products of *Enterobacter* (Biebl et al. 1999; Drozdzynska et al. 2011; Kaur et al. 2012; Zhang et al. 2006). The different studies reporting on the production of 1,3-PDO from pure glycerol by pure bacterial strains (free cells) are summarised in Table 8.1.

8.3.2.2 Biorefinery Approach for 1,3-PDO Production

The microbial production of 1,3-PDO using glycerol has gained much more interest with the development of the biodiesel industry. Indeed, glycerol is the main by-product of biodiesel production process that should be properly managed before disposal (Almeida et al. 2012; Maervoet et al. 2012; Yang et al. 2012). However, this would result in added costs that could be avoided through the use of this by-product as an alternative to pure glycerol for the production of different glycerol-

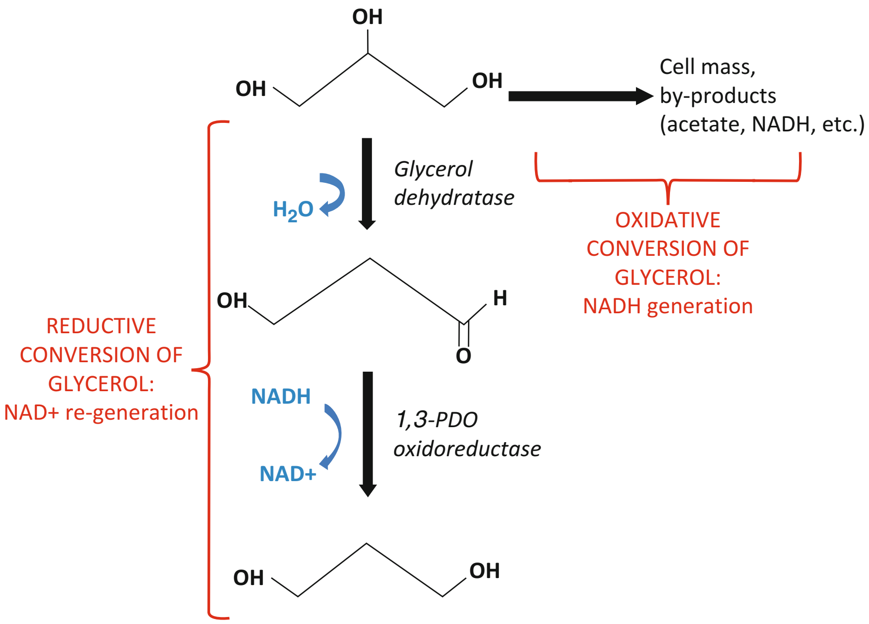

Fig. 8.5 1,3-PDO anaerobic production from glycerol (Modified according to Nakamura and Whited 2003)

Table 8.1 List of microorganisms used for 1,3-PDO production from pure glycerol

			1,3-PDO			
Microorganism	Process	Aeration	(g/L)	a	(g/L/h)	References
K. pneumonia XJPD-L	Fed-batch	Aerobic	65.26	0.56	NA	Ma et al. (2009)
K. pneumonia TUAC01	Batch	Aerobic	22.5	NA	NA	Hao et al. (2008)
K. pneumonia HR526	Fed-batch	Aerobic	102.06	0.65	NA	Xu et al. (2009)
C. freundii	Batch	Anaerobic	25.36	NA	NA	Anand and Saxena (2012)
C. butyricum AKR102a	Fed-batch	Anaerobic	93.7	NA	NA	Wilkens et al. (2012)
C. butyricum VPI 1718	Batch	Anaerobic	13.8	NA	NA	Chatzifragkou et al. (2010)
C. werkmanii DSM 17579	Batch	Anaerobic	NA	0.62	2.84	Maervoet et al. (2012)
Halanaerobium saccharolyticum D6643^T	Batch	Anaerobic	NA	0.61–0.63	NA	Kivisto et al. (2012)

a: $\mathbf{mol_{PDO}/mol_{GLY}}$
NA not available

based fine chemicals. This would, indeed, allow achieving two goals: by-product valorisation and sustainable production of high-value chemicals. Moreover, the emphasis on developing environmentally friendly processes has shifted the focus in the last years on using by-products/wastes as renewable raw materials (da Silva et al. 2009; Saxena et al. 2009; Dobson et al. 2012; Metsoviti et al. 2012; Yang et al. 2012).

Up to date, several studies have been reported on the use of raw glycerol by-product of the biodiesel industry for the production of 1,3-PDO by different bacteria under different process conditions (Table 8.2).

Table 8.2 List of microorganisms used for 1,3-PDO production from crude glycerol and characteristics of the process

Microorganism	Process	Aeration	Cells	1,3-PDO			References
				(g/L)	a	(g/L/h)	
Clostridium butyricum VPI 1718	Continuous	Anaerobic	Free	14.2	NA	NA	Chatzifragkou et al. (2010)
C. butyricum VPI 1718	Fed-batch	Anaerobic	Free	67.9	0.55 (g/g)	NA	Chatzifragkou et al. (2011)
C. butyricum AKR102a	Fed-batch	Anaerobic	Free	76.2	NA	2.3	Wilkens et al. (2012)
C. pasteurianum DSM 525	Batch	Anaerobic	Free	NA	0.211	0.91	Jensen et al. (2012)
C. beijerinckii NRRL B-593	Continuous	Anaerobic	Immobilised (pumice stones)	NA	0.79 (g/g)	NA	Gungormusler et al. (2011)
C. diols DSM 15410	Fed-batch	Anaerobic	Free	85	NA	NA	Otte et al. (2009)
Citrobacter freundii	Batch	Anaerobic	Free	25.63	NA	NA	Anand and Saxena (2012)
C. freundii ATCC 8090	Batch	Anaerobic	Free	4.35	28 %	NA	Ferreira et al. (2012)
C. freundii FMCC-B 294 (VK-19)	Fed-batch	Anaerobic	Free	68.1	0.4 (g/g)	0.79	Metsoviti et al. (2013)
Klebsiella pneumoniae DSM 4799	Fed-batch	Anaerobic	Free	80.2	0.55	1.16	Jun et al. (2010)
			Immobilised (polyurethane)	NA	NA	1.61	
K. pneumoniae isolate	Continuous	–	Immobilised (glass raschig rings)	17.9	NA	NA	Gungormusler et al. (2013)
P. agglomerans DSM 30077	Fed-batch	Microaerobic	Immobilised (polyurethane foam)	NA	NA	3.6	Casali et al. (2012)

A: **mol_{PDO}/mol_{GLY}**
NA not available

8.3.2.3 Approaches for Improving Biotechnological Production of 1,3-PDO

Although biotechnological processes are highly attractive from an environmental and economic sustainability point of view, they still present some drawbacks: low yields, substrate and product inhibition and by-product formation to be subsequently separated from the product of interest. Specifically, in the case of 1,3-PDO high glycerol concentrations, intermediates such as 3-HPA in addition to by-products have been reported to cause growth inhibition and 1,3-PDO production (Saxena et al. 2009). In order to overcome these bottlenecks, different strategies have been designed to optimise biotechnological processes for 1,3-PDO production (Hartlep et al. 2002; Gonzalez-Pajuelo et al. 2006), with particular attention to those employing biodiesel-derived raw glycerol as substrate, for its low cost and high availability, but with cell toxic effects (Chatzifragkou et al. 2010).

Pretreatment of crude glycerol using nonpolar solvents (like hexane or petroleum ether) (Anand and Saxena 2012) could be a strategy; however, this would increase the industrial process costs. The following alternatives have then been considered:

(a) *Fed-Batch Fermentation*

Citrobacter freundii FMCC-B 294 (VK-19) has been tested by Metsoviti et al. (2013) for its capability to tolerate different concentrations of biodiesel-derived raw glycerol under sterile and non-sterile bioprocess conditions, in batch and fed-batch configurations. The authors found that a better performance of the strain was observed under fed-batch process conditions achieving 68.1 g/L of 1,3-PDO in the presence of 170 g/L raw glycerol as substrate, after 90 h. Also *K. pneumoniae* DSM 4799 shows better performance under fed-batch conditions, as has been reported in a study by Jun et al. (2010) where a 1,3-PDO concentration of 80.2 g/L was obtained after 69 h with 35–40 g/L of raw glycerol as substrate (27 % higher than that obtained with pure glycerol) (Jun et al. 2010).

High 1,3-PDO production has been achieved with a *C. butyricum* AKR102a strain in fed-batch fermentation. In 1 L fermentation, 93.7 g/L of 1,3-PDO was obtained from pure glycerol and 76.2 with crude glycerol (30 g/L initial glycerol concentration). A 200 L-scale fermentation has been also tested where a concentration of 61.5 g/L 1,3-PDO was obtained (Wilkens et al. 2012). *C. butyricum* VPI 1718 fermentation with non-sterile crude glycerol (purity 81 % w/w) has been performed by Chatzifragkou et al. (2011) under different process conditions: in batch cultures the strain had similar behaviour with crude glycerol feedstock from different origins, producing final 1,3-PDO concentrations between 11.5 g/L (with 20 g/L initial crude glycerol after 12 h) and 41.9 g/L (with 80 g/L initial crude glycerol after 52 h). Under fed-batch condition, better performances have been shown by the strain producing 67.9 g/L 1,3-PDO with 20 g/L initial crude glycerol concentration after 87 h.

(b) *Co-Fermentation*

Maervoet et al. (2012) have tested a new strategy for improving 1,3-PDO yield by the strain *Citrobacter werkmanii* DSM 17579 using co-substrate fermentation process in which another carbon source is used for biomass growth, while glycerol is only used for 1,3-PDO production. Using the sugar alcohols D-mannitol and D-galactose, the authors reported that they were able to obtain 30 % higher yields compared to using glycerol alone (Maervoet et al. 2012). In another study, co-fermentation of glucose and glycerol for enhanced 1,3-PDO production by *Lactobacillus diolivorans* DSM 14421 was also carried out. In batch fermentations with 70 g/L of glycerol and 0.2 g/L of glucose, a concentration of 41.7 g/L of 1,3-PDO after 139 h was obtained, while in a fed-batch fermentation with 1:1 glucose and glycerol molar ratio, productivity improved up to 73.7 g/L after 190 h (Pflugl et al. 2015). Co-fermentation of glucose and glycerol has been also analysed by Vieira et al. (2015) in batch, fed-batch and continuous cultures, for enhanced 1,3-PDO production by *Lactobacillus reuteri* ATCC 23272, under anaerobic and microaerobic conditions. Although 1,3-PDO production was found to be quite similar under all three fermentation conditions (about 10 g/L with 0.7 g 1,3-PDO/g glycerol) in anaerobiosis, the continuous process resulted in higher1,3-PDO productivity with 4.92 g/(L h) compared to 1.42 g/(L h) obtained under batch condition (Vieira et al. 2015).

(c) *Immobilised Cells Under Continuous Fermentation Process Conditions*

The production of 1,3-PDO from raw glycerol with *Clostridium beijerinckii* NRRL B-593 used as immobilised and suspended cells under continuous process conditions has been evaluated by Gungormusler et al. (2011). Using ceramic materials and pumice stone as immobilisation supports in packed bed reactor (97 % immobilisation rate for both materials) and 40 g/L glycerol concentration, conversion yields of 0.23 and 0.79 were obtained, with the two supports, respectively. The study reported an important influence of hydraulic retention time (HRT) on fermentation performance. The highest yield was indeed obtained after an HRT of 12 h, and although lower HRT conditions have limited by-product presence, it resulted in lower 1,3-PDO molar yields (Gungormusler et al. 2011).

Also Casali et al. (2012) reported on the improvement in 1,3-PDO productivity and yield with immobilised bacterial cells under continuous fermentation conditions. Using Vukopor S10 or polyurethane foam (PUF) as immobilisation supports of *C. freundii* DSM 15979 and *Pantoea agglomerans* DSM 30077 in packed bed bioreactors, they obtained the best performance with *P. agglomerans* in PUF reactor with HRT of 2 h. Specifically, a 1,3-PDO productivity of 3.6 g/(L h) that is 3.5 times higher than suspended cells was obtained (Casali et al. 2012).

Finally, cultures of *K. pneumoniae* isolate (GenBank no. 27F HM063413) immobilised on stainless steel wire, glass raschig ring and Vukopor supports have been employed in continuous production of 1,3-PDO from waste. Although glass raschig ring is better in immobilisation ration terms (78 %), Vukopor was the best solution for glycerol conversion (50.4 %) and 1,3-PDO yield (17.9 g/L) (with HRT of 4 h) (Gungormusler et al. 2013).

(d) *Mixed Cultures*

Bizukojc et al. (2010) have proposed a model for improving 1,3-PDO production from raw glycerol fermentation in a syntrophic process where *C. butyricum* and methanogenic archaea *Methanosarcina mazei* are cocultured. In this process, the archaea would act as a scavenger for the by-products acetic and formic acids used for methanogenesis, reducing by the way their inhibitory effect on *C. butyricum.* They reported that, by this syntrophic process and in the presence of methanol as co-substrate, removal of up to 70 % acetate and formate would be achieved (Bizukojc et al. 2010).

(e) *Aerobic Conditions*

The ability of some 1,3-PDO natural-producer strains to grow on glycerol and produce the chemical was also investigated. The studies were performed using bacteria from the *Enterobacteriaceae* family that are facultative anaerobic microorganisms. In this context, *Citrobacter* was reported to produce 1,3-PDO also under aerobic conditions (Hao et al. 2008; Anand and Saxena 2012). Also, Chen et al. (2003) reported on the improvement of 1,3-PDO production by *K. pneumoniae* when the fermentation was performed under microaerobic conditions compared to the anaerobic ones.

(f) *Genetic and Metabolic Engineering Strategies*

1. *Natural Producers*
   3-HPA accumulation, microbial and GDHt and PDOR activity inhibition may be one of the low-yield causes in 1,3-PDO production fermentation process. It has been observed that 3-HPA increase is strictly connected to glycerol level and pH of the medium. An aerobic 1,3-PDO producer, *K. pneumoniae* TUAC01, has been subjected to an overex-

pression of *dhaT* gene, which encodes the PDOR, maintaining 3-HPA consumption rate higher than formation one allowing to reduce its concentration in fermentative broth (from 7.55 to 1.49 mmol/L and from 12.57 to 2.02 mmol/L with 30 g/L and 50 g/L glycerol substrate concentrations, respectively) and consequently its inhibition effect (Hao et al. 2008).

In the case of *K. pneumonia* HR526, a mutant without D-lactate dehydrogenase (LDH) activity and hence deficient in lactate production was constructed by Xu et al. (2009). In aerobic fed-batch fermentation process, the mutant showed 1,3-PDO fermentation and conversion yields of 102.06 g/L and 0.65 mol/mol compared to 95.39 g/L and 0.55 mol/mol obtained by the wild type (Xu et al. 2009).

Genome shuffling approach has been applied by Otte et al. (2009) for improved 1,3-PDO production in *Clostridium diolis* DSM 15410 and limited substrate and product inhibition. Through chemical mutagenesis mutants with better tolerance to high substrate concentrations have been isolated and then used for protoplast fusion. After several genome shuffling rounds, it was possible to select a mutant able to produce up to 85 g/L 1,3-PDO which corresponds to an 80 % improvement compared to the wild-type yield, from pure glycerol (Otte et al. 2009).

2. *Genetically Engineered Microorganisms*
   The biotechnological production of 1,3-PDO from different carbon sources like glucose, sugar cane molasses or corn hydrolysate using genetically engineered microorganisms has also been reported (Kaur et al. 2012). High yields have been obtained by Tang et al. (2009), using a recombinant strain of *E. coli* with 1,3-PDO production genes from *C. butyricum* SYU 20108, *dhaB1* and *dhaB2*, codifying for. A two-stage pure glycerol anaerobic fermentation has allowed to obtain 104.4 g/L 1,3-PDO with 90.2 % (g/g) conversion rate (Tang et al. 2009). Also, DuPont developed an interesting biotechnological process for 1,3-PDO production from glucose as carbon source with a recombinant *Escherichia coli* able to first convert glucose into glycerol and further to 1,3-PDO achieving a concentration of 135 g/L with a bioconversion yield of 0.6 mol 1,3-PDO/mol glucose (Nakamura and Whited 2003; Drozdzynska et al. 2011). Although highly competitive, this process has however the limitations of being in competition with human food on one side and the necessity of adding expensive vitamin cofactors on the other.

8.3.3 Downstream Processes

Particular attention should be paid to downstream process aimed to 1,3-PDO recovery and purification, in order to make biotechnological processes more competitive in the industry for producing a monomer whose purity is fundamental for 1,3-PDO-based polymer quality. Separation of 1,3-PDO, characterised by high boiling temperature (214–219 °C) and hydrophilicity (thanks to hydroxyl groups' presence), from a fermentation broth full of by-products and not pure feedstock residues, is very difficult, and various methods have been studied (Saxena et al. 2009; Kaur et al. 2012). Simple approaches like evaporation and vacuum distillation (which allows to reduce 1,3-PDO boiling point) have been revealed unsuitable for high energy amount demands and low yields, respectively. The presence of salts and denaturation of macromolecules make broth very viscous; therefore, desalination and deproteinisation can increase evaporation efficiency, but it would not be economically sustainable (Ames 2002). Hao et al. (2006) employed a reactive extraction that allows to solve 1,3-PDO hydrophilicity problems in common liquid-liquid extraction. The flow scheme, based on flocculation, counter-current reactive extraction and distillation, is presented in Fig. 8.6.

Flocculation (E) is an important step for removing soluble proteins and cellular debris which may produce emulsion during extraction process. The combined use of chitosan and polyacrylamide (150 and 70 ppm, respectively) could decrease impurity concentration with a supernatant recovering ratio higher than 99 %. Liquid-

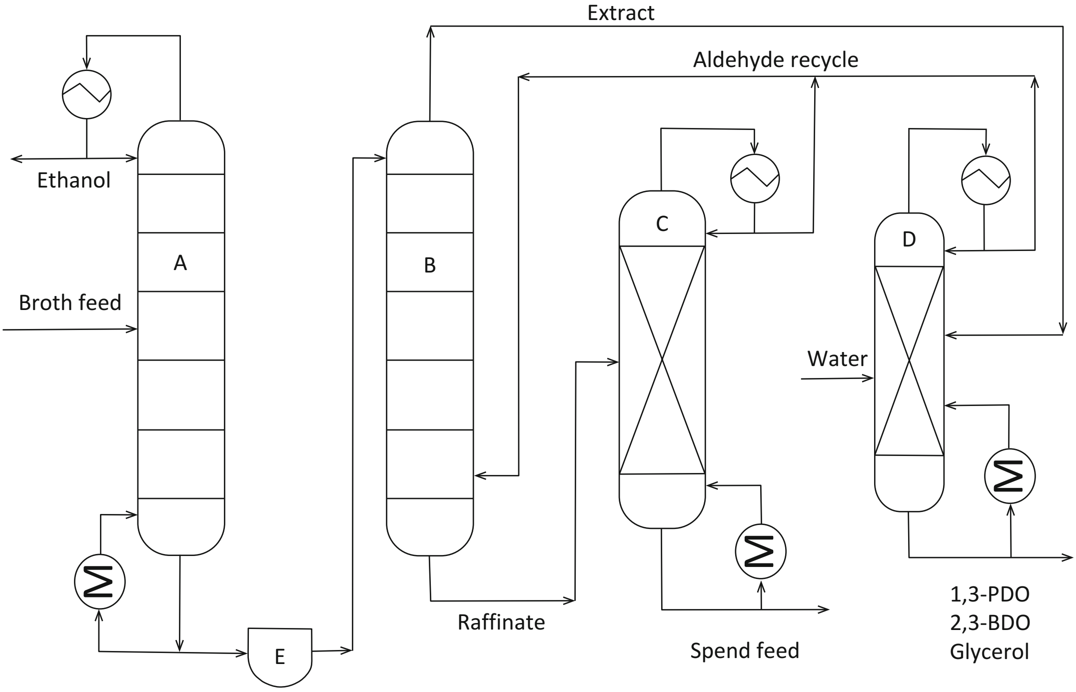

Fig. 8.6 Flow scheme of downstream process for 1,3-PDO recovery from fermentation broth. A flocculation taker (*E*) and four columns are used for distillation (*A*), reactive extraction (*B*), aldehyde recovery (*C*) and reactive distillation (*D*) (Modified according to Hao et al. 2006)

liquid extraction with butyraldehyde (B) has been carried out to form highly hydrophobic 1,3-PDO acetals, miscible with aldehydes; only ethanol acetal is hydrophilic, so it has been removed from broth before reactive extraction (A). In reactive distillation column (D) acetals have been hydrolyzed using a strongly acidic cation exchange resin. The bottom of the column is a mix of 1,3-PDO, 2,3-butanediol, glycerol and glycerol acetals that can be separated by vacuum distillation thanks to their different boiling points (Hao et al. 2006). Nevertheless the problems related to desalination, for avoiding catalyst inactivation (strong acidic resin), and energy expenses could not be overcome with the previous approach (Kaur et al. 2012).

An alternative technique has been based on the use of an aqueous two-phase system (ATPS) for 1,3-PDO extraction separation from fermentative broth. An ATPS composed of methanol/phosphate has been proposed by Li et al. (2011), where methanol has the double role of extractant for 1,3-PDO and solvent for salt crystallisation. Using 35 % (v/v) methanol, saturated phosphate solution and pH 10.7, the partition coefficient of 1,3-PDO has achieved 38.3 %, and the diol recovery has reached 98.1 %. Also cell, protein and organic salt removal has been investigated, and the results showed a good performance (again with 35 % (v/v) methanol and pH 10.7): 99.85 % (cell removal ratio), 92.4 % (protein removal ratio) and from 76 to 90 % (final organic salt removal ratio after recovering methanol) (Li et al. 2011).

A novel downstream process divided into four steps (biomass removal, protein removal, broth concentration and 1,3-PDO separation by silica gel chromatography) has been proposed by Anand et al. (2011) (Fig. 8.7). In an initial step, efficient removal of cells (98.8 % by polypropylene hollow fibre cartridge microfiltration) and proteins (96 % with 30 g/L activated charcoal, which also removed coloured impurities) was performed. Afterwards, the clarified broth has

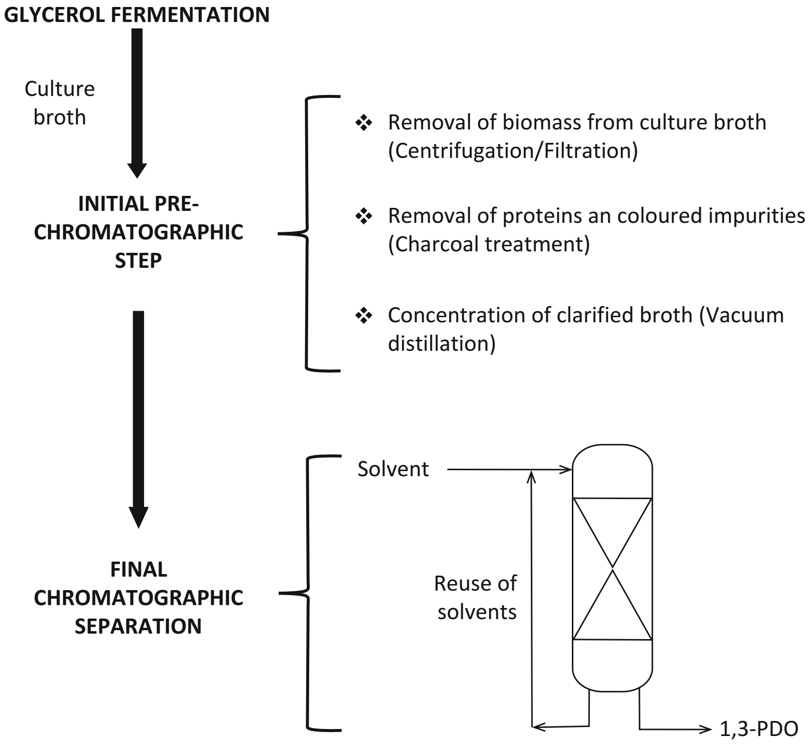

Fig. 8.7 Schematic representation of 1,3-PDO purification from glycerol fermentation broth (Modified according to Anand et al. 2011)

been concentrated ten times via vacuum distillation, resulting in complete removal of ethanol and acetic acid. Inorganic salts (K_2HPO_4, KH_2PO_4, Mg_2CO_3) could be removed via precipitation and crystallisation at 4 °C. In the final step, a 98 % purity grade 1,3-PDO is obtained by chromatography using silica resin and methanol and chloroform as stationary and mobile phases, respectively (Anand et al. 2011).

8.4 Conclusion and Future Perspectives

Biotechnological production of a highly versatile and valuable molecule like 1,3-PDO, through waste valorisation, was one of the many achievements of green chemistry in the last decade. Growing drawbacks related to environmental pollution, decrease of fossil feedstock availability and the increasing demand for an eco-sustainable industry represent deep gaps in the traditional concept of chemical industry. Marketing low-cost bio1,3-PDO is a key point for the development of a competitive and environmentally sustainable industry.

Microorganisms' use for the direct conversion of crude glycerol into a high value-added molecule allows to achieve two goals at the same time: dispose a waste valorising a resource, which means lower process costs. However, although very advantageous from process conditions' point of view (bland operating conditions, absence of expensive and harmful metal catalysts, decrease of undesirable by-products), the biotechnological process still presents bottlenecks related to very low yields and use of a not pure raw material, potentially toxic for microorganisms (Chatzifragkou et al. 2010). Several studies have been carried out in the aim of improving production yields. These include genetic and metabolic engineering and process improvement (immobilisation cells, fed-batch and continuous configurations, use of co-substrates). These aspects, along with research improving downstream process for obtaining a pure biobased product and the increasing policies for supporting bio-industry, represent tools that will lead to the end of fossil fuel dependence.

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Noura Raddadi Ph.D. Currently assistant professor of AgroFood Biotechnology and Microbiology at the University of Bologna. She got her Ph.D. in Agricultural Microbiology at the University of Milan (Italy) and was a visiting researcher at the University of Tromsø (Norway) and postdoc at the University of Turin (Italy). She has been interested in developing biorefinery processes for the valorisation of agro-food by-products/wastes and in ecology and biotechnology (production of extremozymes/biosurfactants, biodegradation of synthetic plastics) of microorganisms from nonconventional and marine environments.

Stefania De Giorgi Ph.D. Student of Agricultural, Environmental and Food Science and Technology at *Alma Mater Studiorum*, Bologna (Italy). She is currently working on the development and optimisation of biotechnological processes for the valorisation of agro-industrial by-products and wastes. Her activity is focused on packed bed bioreactor configurations and downstream processes.

Fabio Fava Ph.D. Full professor of Industrial and Environmental Biotechnology. He earned his Ph.D. in Applied Microbiology from the Institute of Chemical Technology at the University of Prague (Czech Republic). He was a visiting scientist at New Jersey Institute of Technology (NJ, USA), Hazardous Substance Management Research Center (NJ, USA) and Rutgers University (NJ, USA). He has expertise in development and optimisation of biotechnological processes for the integrated valorisation of agro-industrial wastes and for bioremediation of contaminated soils/water.

9

Role of Microorganisms in Microbial Fuel Cells for Bioelectricity Production

Ravinder Kumar, Lakhveer Singh,
and Zularisam Ab. Wahid

Abstract

The catalytic microorganisms oxidise the organic matter to produce electrical energy in microbial fuel cells (MFCs). The microorganisms that can shuttle the electrons exogenously to the electrode surface without utilising artificial mediators are referred as exoelectrogens. The microorganisms produce specific proteins or genes for their inevitable performance towards electricity generation in MFCs. Multiple studies have confirmed the expression of certain genes for outer membrane multiheme cytochromes (e.g. OmcZ), redox-active compounds (e.g. pyocyanin), conductive pili, and their potential roles in the exoelectrogenic activity of various microorganisms, particularly in the members of *Geobacteraceae* and *Shewanellaceae* family. This chapter explores the various mechanisms of microorganisms that are advantageous for the technology: biofilm formation, metabolism, electron transfer mechanisms from inside the microorganisms to the electrodes and vice versa.

9.1 Introduction

Microbial fuel cells (MFCs) are fascinating biological fuel cells that typically contain two compartments, i.e. the anode and the cathode, and use biological catalysts (mostly bacteria) to produce electric energy from organic matter present naturally in the environment or in waste (Wang and Ren 2013). General principle of a microbial fuel cell is presented in Fig. 9.1. The microorganisms that act as biocatalysts oxidise organic and inorganic substrate to carbon dioxide and generate electrons at the anode. It requires transferring these electrons from inside the cells to the anode (surface) in anoxic conditions to produce electric current (Logan and Rabaey 2012). The bacteria can transfer these electrons to the anode by producing electron shuttles (e.g. flavins, phenazines, etc.) or by using electron mediators generally found in extracellular environment (e.g. humic substances) (Brutinel and Gralnick 2012, Kotloski and Gralnick 2013). Alternatively, the

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R. Kumar • L. Singh (✉) • Z. Ab. Wahid
Faculty of Engineering Technology,
Universiti Malaysia Pahang (UMP),
LebuhrayaTunRazak, 26300 Gambang, Kuantan,
Pahang, Malaysia
e-mail: raviump@gmail.com; raviump@yahoo.com;
lucki.chem09@gmail.com; lakhveer@ump.edu.my;
zularisam@ump.edu.my

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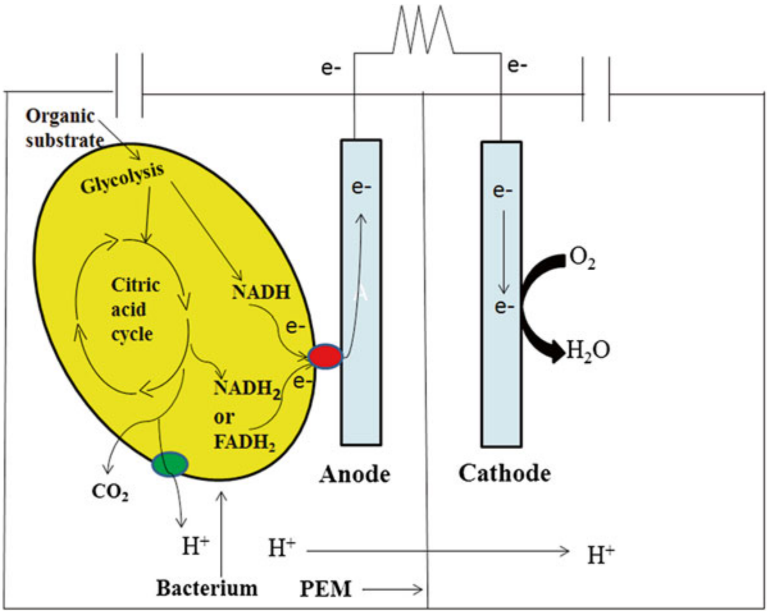
Fig. 9.1 General principle of a microbial fuel cell



electrons can be transferred via electrically conductive proteinaceous filaments, referred as ‘microbial nanowires’ produced by the bacteria (Malvankar and Lovley 2012). The electron shuttles further may be reduced by outer surface redox-active molecules, such as c-type cytochromes (Inoue et al. 2010a; Voordeckers et al. 2010; Orellana et al. 2013). In earlier MFC studies, chemical mediators (e.g. neutral red) were added to the system to carry the electrons from inside the cell to the electrode for electricity production (Park and Zeikus 1999; Bond et al. 2002). Electrons from the anode surface are passed through a resistor or another type of electrical device to the cathode surface and protons through a proton exchange membrane (PEM) or cation-selective membrane (commonly used Nafion, Ultrix and Salt Bridge) where they combine with oxygen to form water (Huang et al. 2012).

With the instantaneous increase in the global energy demand every year, overconsumption and dwindling of nonrenewable sources of energy, microbial electricity production may become a pivotal form of bioenergy because MFCs offer effective opportunities of extracting current from a wide range of biodegradable organic matter and renewable biomass from simple molecules such as carbohydrates and proteins to complex mixtures of organic matter present in animal, human and food processing wastewaters. The versatility and availability of different microorganisms to use wide range of organic matter makes MFC an exemplary and quirky technology for renewable bioelectricity production. The MFC technology is not a new technology, but it is only recently MFCs are in the limelight of research for bioelectricity production (Rabaey et al. 2003, 2004; Schroder et al. 2003; Liu et al. 2004). MFC is a promising technology for harvesting energy and can be advantageously combined with various applications, such as bioremediation, sensors and powering electronic monitoring devices (Patil et al. 2012; Ren et al. 2012).

The diverse microbes (mostly bacteria) from different phylogenetic groups have been reported to generate electricity in MFCs without using a mediator. Five classes of Proteobacteria, Firmicutes and Acidobacteria phyla have shown electrical current generation, but also, some microalgae, yeast and fungi have been reported in MFCs, being used as substrate or assist the anode or the cathode. The nomenclature of such microorganisms is not standardised yet; however, some terms have been given for microorganisms that can transfer electrons exogenously to the anode without using any artificial mediator. These terms include exoelectrogens, electrogenic microorganisms, electrochemically active bacteria, anodophiles, anode-respiring bacteria and electricigens. Moreover, microorganisms can also be termed according to their functions in the MFC, e.g. sulphate-reducing bacteria and iron-reducing bacteria can be referred as sulphate reducers and iron reducers, respectively. The microorganisms that donate electrons to the electrode (anode) in MFCs can be referred to as electrode reducers, while those that accept electrons from the electrodes are referred to as electrode oxidisers. The prevalent bacterial species known to produce electricity in MFCs include dissimilatory iron-reducing *Geobacter spp*. (Bond and Lovley 2003), *Shewanella* spp. (Gorby et al. 2006) *Rhodoferax ferrireducens* (Chaudhuri and Lovley 2003), *Aeromonas hydrophila* (Pham et al. 2003), *Pseudomonas aeruginosa* (Jayapriya and Ramamurthy 2012), *Clostridium butyricum* (Park et al. 2001) and *Enterococcus gallinarum* (Chisti 2007). Alternatively, microalgae have been used as a substrate or biocathode in MFC (Wang et al. 2012). In yeast, besides *Saccharomyces cerevisiae*, *Hansenula anomala* also showed current production successfully in MFC (Prasad et al. 2007). However, use of yeasts for electricity generation using MFCs in general does not seem to have been considered further very deeply. An oxygenic phototrophic cyanobacterium *Synechocystis sp*. which produces nanowires has been discovered to generate electricity in MFC (El-Naggar et al. 2010). The microorganisms can contribute effectively for power generation those able to oxidise organic compounds completely and transfer the electrons with accelerated rates to the anode. Biofilms on the anode have been demonstrated to increase the current density due to the direct electron transfer between the microbes and the surface of the anode. Earlier studies have shown that biofilms of mixed cultures have more capability to produce higher current density than the biofilms of pure cultures (Dumas et al. 2008). For example, a bacterium *Brevibacillus sp*. produced little power as a pure culture in MFC but produced comparatively high power when a *Pseudomonas sp.* was added in MFC (Pham et al. 2008). The bacteria capable of dissimilatory metal reduction can effectively produce electricity in a mediatorless MFC. Such bacteria transfer electrons either by excreting electron shuttles or by direct contact via outer membrane cytochromes. Later, another mechanism for electron transfer was revealed providing the evidence that bacteria synthesise appendages known as microbial nanowires that are capable of transferring electrical current. In a study, a bacterium *Pelotomaculum thermopropionicum* was found connected to the methanogen *Methanothermobacter thermautotrophics* by an electrically conductive appendage, promoting the interspecies electron transfer (Gorby et al. 2006). Multiple studies suggest that quorum-sensing chemicals (e.g. fatty acyl-homoserine lactones) play an important role in the communication between the bacteria of different species within the biofilm (Schaefer et al. 2008). *Pseudomonas aeruginosa* produces pyocyanin that acts as an electron shuttle and signalling molecule to upregulate the transcription of quorum-sensing genes (Dietrich et al. 2006).

This chapter describes the different microbial mechanisms that are advantageous to the MFC technology, including formation of biofilm by the microorganisms, different mechanisms of electron transfer from microorganisms to electrode and vice versa, followed by the description of high current-producing microorganisms used for electricity production.

9.2 Biofilm Formation and Its Regulation

Bacteria prefer to live in polymeric matrix (contains proteins, lipids, carbohydrate, etc.) produced by the bacteria attached to a surface, which is known as a biofilm. In MFCs, it is highly significant to produce electroactive biofilms to generate electricity more efficiently. Biofilm formation is regulated via different pathways depending on the microbe used in the MFC, the substrates, electrode material and the operating conditions of the MFC. The physiological and morphological properties of electrode surface also influence biofilm formation. Some particular studies demonstrated that microorganisms favour to adhere on hydrophobic surfaces in rival to hydrophilic materials (Patil et al. 2012). The earlier studies suggest that the bacteria unable to form biofilms on the electrode can't generate substantial current densities in MFCs. However, the bacteria able to form thick biofilms on the anode generate higher current densities in rival to bacteria adept to form thin biofilms. For example, confocal microscopy revealed that *Thermincola ferriacetica*, Gram-positive bacteria which form thick biofilms (~38 μm), generated a sustained current density 7–8 Am^{-2} (Prathap et al. 2013), while *Thermincola potens*, which form monolayer biofilms, produced comparatively lower current densities (Wrighton et al. 2011).

The process of biofilm formation is triggered by the transport of microbes to a surface, followed by their attachment to the surface (in MFC, on anode or cathode), formation of microcolonies and biofilm maturation (Sauer et al. 2002). The bacterial cells produce some adhesins, and carbohydrates (polysaccharides), nucleic acids and proteins interconnect and encase the bacteria in the form of a biofilm (Pamp and Nielsen, 2007). The most distinguished feature of electroactive biofilms is their ability to respire terminal electrons from metabolism onto electrode surfaces or soluble electron acceptors (Bond et al. 2002). It has been demonstrated that outer membrane c-type cytochromes are crucial for biofilm formation in *Geobacter spp.* and *Shewanella spp.* (Bond and Lovley 2003; Gorby et al. 2006). While, type IV pili protein composed of PilA monomers are chiefly responsible for *Geobacter spp*. and *Aeromonas spp*. conductive biofilm formation (Pham et al. 2003; Malvankar and Lovley 2012). *G. sulfurreducens* deficient in *omcZ* and *pil*A genes inhibited biofilm formation and consequently, the current production, suggesting the role of c-type cytochromes and the protein pilin in biofilm formation (Inoue et al. 2010a). While in *Shewanella spp.* other redox-active components such as flavins mediate the exocellular electron transport through biofilm (Kotloski and Gralnick 2013). In *P. aeruginosa* biofilm formation, bacteria transfer to the surface with the movement of flagella. Cellular aggregation and microcolony formation is driven by type IV pili, and the subsequent formation of mushroom-shaped biofilm occurs via a maturation process that requires cell-to-cell signalling (Stoodley et al. 2002; Merritt et al. 2010; Malone et al. 2012). The mechanism known as quorum sensing (QS) allows bacterial population to communicate and coordinate group behaviour. QS regulates the expression of biofilm-related genes and is pivotal for structural development of biofilm in *P. aeruginosa* and other microorganisms as well (Diggle et al. 2003; Holm et al. 2006).

The biofilms of mixed culture generate high-power densities than pure culture. For example, a mixed culture-inoculated MFC produced ca. 20 % more power in rival to pure culture in the similar MFC (Ishii et al. 2008). However, the role of non-exoelectrogens (the microorganisms when used as pure cultures were not able to generate electric current) in power generation is not known. In monolayer biofilms, bacterial cells remain in the close proximity with anodic surface and transfer the electrons directly to the anode either via c-type cytochromes or electron shuttles. While in thick multilayer biofilms, it has been found that biofilms produce pili that mediate the electron transfer from the distant cells to the anode surface (Reguera et al. 2006). The role of pili and its role in electron transfer are discussed in the later section of the chapter. The use of microorganisms at the cathode to catalyse oxygen reduction has increased the interest in cathodic biofilm studies. In rival to anodic biofilms, it has been observed that power generation slowly decreases with increase in thickness of cathodic biofilms (Behera et al. 2010).

9.3 Microbial Metabolism and Bioelectrogenesis

Many microorganisms have been experimented in MFCs for electricity generation, bioremediation and other manifold applications. Besides, several nutrients (acetate, glucose, starch, sucrose, ethanol, lactate and xylose, etc.) and wastewaters (beer brewery wastewater, chocolate industry wastewater, swine wastewater, paper recycling wastewater and protein-rich wastewater, etc.) from various sources have been used as substrate for microbial growth in MFC technology (Liu et al. 2004). Despite the availability of wide range of substrates and microorganisms, only restricted and specific microorganisms are known to produce electricity in MFCs. Exoelectrogens from various categories such as Gram-positive bacteria, Gram-negative bacteria, yeast, cyanobacteria, algae and even fungi have already been utilised in different kinds of MFCs. Those organisms are substantially efficient for electricity generation that can completely oxidise complex organic substrates into their respective components in the anodic chamber. But, a particular exoelectrogen can oxidise specific substrates or a specific type of substrate for its growth and energy production. Moreover, depending on the type of substrate, every exoelectrogen has different pathways and genes, enzymes or proteins for its degradation or oxidation. Therefore, selection of a suitable bacterial consortia and preferred substrate determine the output of MFC. For example, a MFC fed with aerobic-anaerobic sludge inoculum and glucose, when operated for 3 months, increased the bacterial substrate to electricity conversion rates sevenfolds (Rabaey and Verstraete 2005).

In MFC, organic substrates containing carbohydrates, lipids and proteins serve as electron donors for redox reactions at the anode to produce energy. These complex organic molecules further undergo through glycolysis and other respective processes to yield acetyl Co-A, which then participate in citric acid cycle. Three equivalents of reduced NADH are generated from three nicotinamide adenine dinucleotide (NAD^+), one flavin adenosine dinucleotide (FAD) reduces to $FADH_2$, and CO_2 is released as by-product in single turn of citric acid cycle. These metabolic pathways (glycolysis and Krebs cycle) occur in cytoplasm in both prokaryotes (bacteria) and eukaryotes (yeast). NADH and $FADH_2$ act as electron carriers, which then transfer their electrons to electron transport chain (ETC) to produce energy carrier molecule, adenosine triphosphate (ATP). In bacteria, respiratory reaction occurs in the cell membrane (constituting outer cell membrane, inner cell membrane and periplasm), the machinery containing all the proteins or enzymes required for the electron transfers (the basis of MFC). While in yeast, ETC resides on the inner mitochondrial membrane. The ETC typically contains four intermediary proteins, NADH dehydrogenase, ubiquinone, coenzyme Q and cytochromes (however, these intermediary proteins may vary with species). The electrons are passed through these proteins to the final electron acceptor, and the protons (reduced) are pumped out of the cell, in the anode which is then transferred to the cathode through PEM. Prior to the prominence that bacteria can facilitate electron transfer, chemical mediators were utilised to catalyse electron transfer from inside the bacterial cell to the anode surface. These mediators react with ETC components and get reduced, release out of the cell and transfer their electrons to the anode.

Moreover, metabolism of the bacteria can switch from oxidative phosphorylation (metabolism) to fermentative metabolism depending on the anode potential. At low anode potential, in the presence of electron acceptors (sulphate, nitrate, etc.), bacteria adapt to oxidative metabolism, and the electrons are deposited on electron acceptors. But, when electron acceptors are not present, bacteria prefer the fermentation metabolism. During the fermentation process, e.g. of glucose, one-third of electrons can be used for electricity generation, while the rest of electrons reside in the fermentation products, which can be further oxidised by anaerobic bacteria such as *Geobacter sp.* in MFC for current generation (Logan 2004; Rabaey et al. 2005; Reguera et al. 2005). Beyond electricity generation, many bacteria (*Clostridium sp., Enterococcus sp.*) have been inoculated anaerobically in MFCs to produce fermentation products (Logan 2009). Like *Geobacter sp.* is the most efficient exoelectrogen known in MFC, *Clostridium sp.* is the most efficient hydrogen producer in MFC (Singh et al. 2013; Singh and Wahid 2015). No doubt, biofilms of mixed consortia studied so far in MFC have showed higher-power densities than pure cultures, and it can be conceived due to the networks of metabolisms between the bacteria in biofilms, but it needs a complete elucidation and experimental corroboration over the matter. The potential of anode plays an important role to determine the bacterial metabolism. Negative anode potential influences the bacteria to deliver the electrons through more reduced complexes (Logan 2009). As a result, the bacteria extract less energy and greater is the energy recovery in MFC and thus the power output. Evidently, microbial community of sulphate-reducing bacteria at negative anode potentials produced higher-power density, 45 mA m^{-2} at −0.6 V than 15 mA m^{-2} at −0.2 V (Chou et al. 2014). Also, setting the cathode potential has shown to improve the performance of MFC. A study demonstrated that MFC for Cr (VI) reduction with set cathodic potentials at -300 V increased the maximum power density from 4.1 W/m^3 (control, no set potential) to 6.4 W/m^3, and the start-up time was reduced to 19 days from 26 days as compared to control (Huang et al. 2011).

9.4 Mechanisms of Electron Transfers

In MFC, electron transfer chiefly occurs in two directions: at the anode, from microorganisms to electrode, and at the cathode, from electrode to microorganisms when biocathodes are used to catalyse oxygen reduction.

9.4.1 Electron Transfer from Microorganisms to Electrode

Microorganisms can transfer electrons to an electrode directly by three mechanisms (see Fig. 9.2) known till date: (1) short-range electron transfer via redox-active proteins such as cytochromes present on the outer surface of bacterial cell membrane; (2) electron transport via microbial-secreted soluble electron shuttles, for example, flavins and pyocyanin; and (3) long-range electron transfer through conductive pili.

9.4.1.1 Direct Electron Transfer via Cytochromes

Geobacter sulfurreducens has been studied most extensively to comprehend the mechanisms for direct electron transfer. *G. sulfurreducens* contains the enzymes for the central metabolism to anaerobically oxidise carbon (effectively acetate) completely to carbon dioxide and water and can transfer electrons to different electron acceptors (Kiely et al. 2011). The genetic studies of *G. sulfurreducens* genome unveiled the presence of many c-type cytochromes containing heme groups in their motifs, exposing on the outer surface of cell (Leang et al. 2010; Inoue et al. 2010a). The abundance of cytochromes is an advantageous characteristic for the organism that ameliorates electron transport across cell/electrode interface. The other compounds or proteins that help in electron transport include quinones, iron-sulphur proteins and b-type cytochromes. The electron transport proteins are present in the periplasm or on outer membrane of *G. sulfurreducens*. Besides, many studies including gene deletions demonstrated that c-type cytochromes transfer electrons to diverse extracellular electron acceptors in vitro as well as in vivo (Leang et al. 2003; Inoue et al. 2010a; Voordeckers et al. 2010). The immunogold labelling of *G. sulfurreducens* biofilms validated the accumulation of profuse OmcZ at biofilm and anode interface (Inoue et al. 2010a), while OmcZ mutant strain halted the electron transfer between biofilm and the anode. Hence, all the results confirmed the vital role of OmcZ in direct electron transfers. Nevin et al. compared the gene expression in cells of *G. sulfurreducens* biofilms growing on different electron acceptors, between cells grown on graphite and graphite with fumarate. The microarray studies revealed the genes *omcB*, *omcT*, *omcE*, *omcS* and *omc*Z encode c-type cytochromes. OmcZ and OmcE cytochromes were most abundant in current harvesting cells, while OmcS was least abundant. Further, the cells deficient in *omcZ* inhibited the current production and biofilm formation, showing the

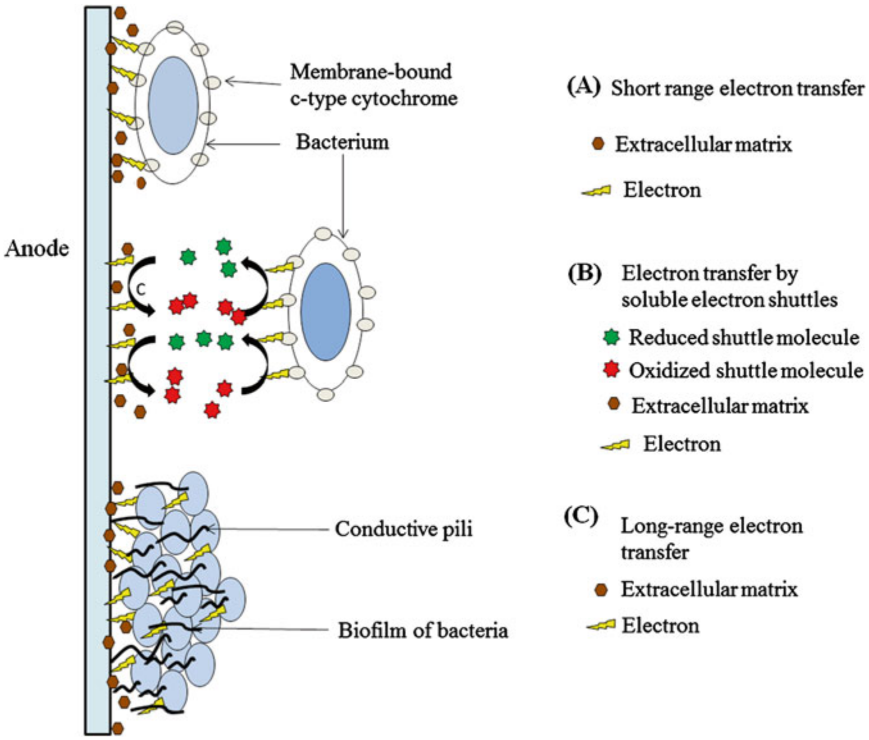


Fig. 9.2 Different mechanisms of electron transfers from microorganisms to electrode

importance of the cytochrome in the electron transfer. The cells deficient in other genes didn't show any impact on current generation as well as on biofilm formation (Leang et al. 2003; Inoue et al. 2010a; Smith et al. 2013). Multiple evidences suggest that OmcZ is the most important cytochrome in high current-producing biofilms and is an octaheme hydrophobic protein which occurs in two forms, one large (OmcZ$_L$) and one short (OmcZ$_S$, the predominant form) (Inoue et al. 2010a, b). It has been suggested that in *G. sulfurreducens* biofilms, OmcZ mediates the electron transfer through the biofilm, while OmcB mediates the electron transfer across the biofilm/electrode interface. The cytochromes OmcS and OmcE also play a secondary role in electron transfer through the biofilm (Richter et al. 2009). A study demonstrated that OmcF mutant strain of *G. sulfurreducens* showed low current density (Kim et al. 2008). Further, the results suggested that the OmcF is either directly or indirectly involved in electron transfer process, and hence OmcF is a pivotal role in electricity production (Kim et al. 2008).

In *G. sulfurreducens* proteins other than the outer membrane c-type cytochromes, the outer-membrane multicopper protein OmpB and OmpC are also required for Fe (III) oxide reduction (Mehta et al. 2006; Holmes et al. 2008). However, it's not clear how these multicopper proteins affect the electricity production in MFCs, therefore, will be a highly interesting topic for future research. *Desulfovibrio alaskensis* G20, a sulphate-reducing bacteria studied to identify the components involved in electron flow, revealed a new model for electron transfer and showed that type I tetraheme cytochrome c_3 (TpIc_3) and the transmembrane complexes (QrcA) also play a key role to transfer the electrons across the cell membrane for sulphate reduction (Keller et al. 2014).

Gram-positive species of the genus *Thermincola potens* has also been studied to elucidate the direct electron transfer mechanism. Surface-enhanced Raman spectroscopy evinced the expression of profuse multiheme c-type cytochromes (MHCs) on the cell wall or cell surface during *T. potens* growth on hydrous ferric oxides or AQDS, an analogue of the redox-active components of humic substances. The results unveiled unique evidence for cell wall-associated cytochromes and involvement of MHC in transporting the electrons across the cell envelope of a Gram-positive bacterium (Wrighton et al. 2011). A better understanding of genes or proteins involved in direct electron transfer along with genetic manipulation can amend increases in current production and efficiency of MFCs.

9.4.1.2 Electron Transfer via Electron Shuttles Secreted by Microorganisms

Some microorganisms have been identified that can mediate the electron transfer to soluble or insoluble electron acceptors or electrodes by secreting soluble electron shuttles, for example, *Shewanella oneidensis*, *Pseudomonas aeruginosa* and *Geothrix fermentans*, etc. *G. fermentans* releases a soluble electron shuttle which promotes reduction of Fe (III) oxides (Bond and Lovley 2005). *G. fermentans* secreted two different soluble redox-active electron shuttles to reduce Fe (III); first was riboflavin at redox potential of −0.2 V and the other, still unknown at redox potential of 0.3 V (Mehta and Bond 2012). *P. aeruginosa* produces pyocyanin and phenazine-1-carboxamide that are very important for electron transfers. A mutant strain of *P. aeruginosa*, deficient in the synthesis of pyocyanin and phenazine-1-carboxamide, achieved only 5 % power output as compared to wild type's strains (Baron et al. 2009). Further, the study demonstrated that pyocyanin promotes substantial electron transfer, not only used by *P. aeruginosa* but also by other bacterial species (Baron et al. 2009; Shen et al. 2014). Moreover, overexpression of phzM (methyltransferase-encoding gene) in *P. aeruginosa*-phzM-inoculated MFC increased the pyocyanin production by 1.6-folds and consequently the exocellular electron transfer efficiency and power output (Yong et al. 2014).

Shewanella species produces flavin mononucleotide and riboflavin as the extracellular electron shuttles to reduce of Fe (III) oxides coupled with anoxic growth of the species (Von et al. 2008; Chaudhuri and Lovley 2003). Fluorescence emission spectra showed an increase in concentration of quinone derivatives, and riboflavin in the cell-free supernatant of *Shewanella loihica* PV-4 strain grown on graphite electrode, responsible for direct electron transfer and mediated electron transfer, produced maximum anodic current density of 90 μAcm^{-2} (Jain et al. 2012; Marsili et al. 2008). Kotloski and Gralnick (2013) identified a flavin adenine dinucleotide transporter in *S. oneidensis*, responsible for the export of flavin electron shuttles to further the electron transfer to insoluble substrates. In *S. oneidensis* MR-1, decaheme c-type cytochromes MtrC and OmcA present on the outer surface of the cell; part of multiprotein complex helps in hopping the electrons cell membrane (Baron et al. 2009). The cytochrome OmcA also plays an important role in the attachment of bacteria to the electrode surface during biofilm formation (Coursolle et al. 2010). Electron transfer complex MtrCAB responsible for direct and mediated exocellular electron transport in *S. oneidensis* (Baron et al. 2009) was introduced in *E. coli* with a more tunable induction system. The strains showed limited control of MtrCAB expression and impaired cell growth, and the results demonstrated that maximum current densities was produced not by the strains that expressed more MtrC and MtrA but by the strains with improved cell growth and fewer morphological changes (Goldbeck et al. 2013).

Lactococcus lactis produces variegated membrane-associated quinones which mediate electron transfer to extracellular electron acceptors such as Fe (III) and Cu (II) (Fuller et al. 2014). The bacterium transfers electrons to the anode via soluble redox mediators. The study suggested that one of these two mediators was 2-amino-3-dicarboxy-1,4-naphthoquinone (Freguia et al. 2009). *Klebsiella pneumoniae* strain L17 also studied in MFC produces a recycle electron shuttle 2, 6-di-tert-butyl-p-benzoquinon to transfer electrons to the anode (Lifang et al. 2010; Torres et al. 2010).

9.4.1.3 Electron Transfer via Microbial Nanowires

Long-range electron transfer is mediated by dense network of conductive pili produced by the microorganism, responsible for the conductive biofilms of high current production. Though, diverse microorganisms are known to produce pili, only *Shewanella sp.* (Leung et al. 2013; Pirbadian and El-Naggar 2012) and *Geobacter sp.* (Malvankar et al. 2012; Snider et al. 2012; Bonanni et al. 2013) are competent to produce conductive pili that account for electricity production.

The role of conductive pili in long-range electron transfer in biofilms was demonstrated earlier in *Geobacter sulfurreducens*, and the study revealed that these electronic networks contributed for more than tenfold increase in electricity production (Reguera et al. 2006). *G. sulfurreducens* pili are type IV pili composed by the monomers of PilA protein (Craig et al. 2004). Type IV pili are small structural proteins of molecular weight ca. 7–20 kDa, 10–20 μm long and 3–5 μm broad with a conserved N-terminal domain forming α-helix with a transmembrane domain and a protein-protein interaction domain (Craig et al. 2004). Moreover, C terminus of PilA contains a conserved sequence of aromatic amino acids (Trp, Phe, Tyr, His and Met) responsible for overlapping of pi-pi orbitals in the pili structure and consequently for metal-like conductivity and lacks in nonconductive biofilms (Vargas et al. 2013). The function of PilA is directly regulated by PilR which functions as an RpoN-dependent enhancer-binding protein. Further, the study revealed that a strain deficient in *pilR* gene showed waned insoluble Fe (III) reduction as well as soluble Fe (III) reduction (Juárez et al. 2009). The hypothesis that cytochromes are associated with *G. sulfurreducens* pili and serve a key role in electron transfer along with pili was ruled out with the publication of Malvankar et al. (2011); the study unveiled that conductivity of *G. sulfurreducens* nanowires don't attribute to cytochromes because the spacing between cytochrome to cytochrome was ca. 200 times greater than required for electron hopping. It was further clarified by Liu *et al.*, who demonstrated a *G. sulfurreducens* strain PA, that *pilA* gene was replaced with *pilA* gene of *Pseudomonas aeruginosa* PAO1, expressed the pili subunits and *c*-type cytochrome OmcS similar to control strain, but showed waned current production and Fe (III) oxides reduction. Further, the results suggested that *c*-type cytochrome OmcS on pili don't confer for the conductivity of pili (Liu et al. 2014a, b; Smith et al. 2014). Fanghua et al. revealed that magnetite can facilitate microbial extracellular electron transfer. The study demonstrated that magnetite compensated for the extracellular electron transfers for OmcS-deficient strain in Fe (III) oxide reduction (Liu et al. 2014a).

Conducting probe atomic force microscopy technique and gene deletion studies of MtrC and OmcA suggested that *S. oneidensis* MR-1 nanowires are conductive in nature (El-Naggar et al. 2010). Electronic transport characteristics of *S. oneidensis* MR-1 nanowires was further studied and exhibited p-type, tunable electronic behaviour with a field-effect mobility (Leung et al. 2013). In an alternative study, deletion of the structural pilin genes (mshA-D) which encode for extracellular Msh (mannose-sensitive hemagglutinin) structural proteins in *S. oneidensis* MR-1 produced 20 % less current compared to control strain, indicating extracellular electron transfer ability of intracellular- and membrane-bound Msh biogenesis complex in *S. oneidensis* MR-1 (Fitzgerald et al. 2012). A multistep hopping mechanism has been proposed for extracellular charge transfer in *S. oneidensis* MR-1 biofilms, suggesting that redox components are associated with each other at less than 1 nm distance, forming a chain along extracellular appendages, responsible for electron hopping or electron tunnelling (Polizzi et al. 2012). However, the actual organisation of cytochromes on *S. oneidensis* MR-1 nanowires and their exact role in electron transfer mechanism is yet to be clarified.

The pilus-associated *c*-type cytochrome OmcS and pili have also been associated with electron transfer via direct interspecies electron transfer (DIET). Gorby et al. provided the first evidence that nanowire production is not limited to dissimilatory metal-reducing bacteria; further the study demonstrated that an oxygenic phototrophic cyanobacterium *Synechocystis* and thermophilic, fermentative bacterium *Pelotomaculum thermopropionicum* produced electrically conductive nanowires that established connections with the methanogen *Methanothermobacter thermautotrophicus* for efficient electron transfer and energy distribution (Gorby et al. 2006). The mechanism DIET has also been seen within aggregates of *G. metallireducens* and *Methanosaeta harundinacea* in anaerobic digesters (Rotaru et al. 2014). Granular-activated carbon (GAC) has been hypothesised to stimulate DIET between bacteria and methanogens (Liu et al. 2012). GAC simulates the role of pili and associated *c*-type cytochrome involved DIET (Liu et al. 2012). The molecular mechanism of DIET and its contribution towards energy production is not understood well and, therefore, demands a deep investigation into the matter.

9.4.2 Electron Transfer from Electrodes to Microorganisms

Many microorganisms have already been used as biocathodes in the technology, but only limited information is available on electron transport mechanisms from electrode to microbes. Though, it's clear that microorganisms use different mechanisms to accept electrons from the cathode (see Fig. 9.3) than to donate electrons to the anode, Gregory et al. provided the first evidence that *Geobacter species* can accept electrons directly from an electrode (Gregory et al. 2004). Alternatively, *Shewanella oneidensis* MR-1 in the aerated cathode produced riboflavin, an electron shuttle mediator to transfer electrons to Cr (VI) (Xafenias et al. 2013). *Acinetobacter calcoaceticus* and *Shewanella putrefaciens* as pure cultures excrete redox compound similar to pyrroloquinoline quinone (PQQ) that further use outer membrane-bound redox compounds for extracellular electron transfer (Freguia et al. 2010). An acidophile microorganism, *Acidithiobacillus ferrooxidans*, used as biocathode demonstrated that the redox species, an outer membrane-bound cytochrome c (Cyc2), is associated to microbial-catalysed O_2 reduction (Carbajosa et al. 2010). Transmission electron microscopy of immunogold-labelled *Leptospirillum* group II bacterium-dominated biofilm (acidophilic microbial communities) revealed that Cyt_{579} (structurally, 70 % α-helical) is localised in periplasmic space (Jeans et al. 2008) and helps in accepting the electrons derived from Fe (II) oxidation (Jeans et al. 2008). Similarly, another unusual membrane protein, Cyt_{572} (structurally, β-helical), isolated from acidophilic microbial communities showed the abil-

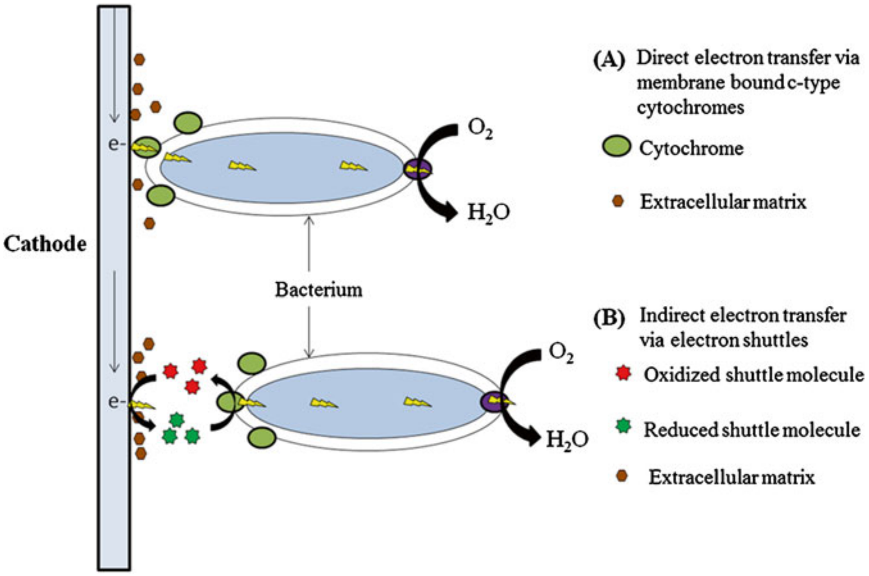


Fig. 9.3 Mechanisms of electron transfers from electrode to microorganisms

ity for Fe (II) oxidation (Jeans et al. 2008), but it's still elusive that the protein participates in electron transfer mechanisms. Recently, cyclic voltammetry scanned an unidentified redox-active molecule secreted from *P. aeruginosa*, involved in the electron transfer from the electrode to targeted azo bonds, leading to decolorisation of azo dye (Wang et al. 2014). A biocathodic microbial community predominated by *Proteobacteria*, *Bacteroidetes* and *Firmicutes* during dechlorination of pentachlorophenol (PCP) in MFC transferred the electrons directly, as cyclic voltammetry characterisation of the medium didn't confirm any redox mediator secreted by the bacteria (Liu et al. 2013). Besides, many Gram-negative and Gram-positive bacteria utilised as biocathodes such as *Dechlorospirillum anomalous*, *Acinetobacter calcoaceticus*, *Staphylococcus carnosus*, *Streptococcus mutans*, *Enterococcus faecalis*, *Shigella flexneri*, *Kingella denitrificans* and *Lactobacillus farciminis* have shown the ability to transfer electrons directly or accept the electrons indirectly from different electrodes through redox-active compounds for manifold applications of the technology (Thrash et al. 2007; Aulenta et al. 2010; Cournet et al. 2010). Unfortunately, the molecular mechanism of accepting electrons from the electrodes in any microorganism is yet not understood well and can be taken as a future aspect.

9.5 High-Power-Producing Microorganisms in MFC

The power density produced by a particular microorganism such as *Geobacter sp.* can't be compared to other microorganism, e.g. *Shewanella sp.*, unless the MFC structure, operating conditions, nutrients and chemical solutions used for the study will be indistinguishable. Till date, many microorganisms used in different MFCs produced electrical energy in unalike conditions. Although this chapter describes only the prevalent microorganisms studied (in anode and cathode) that produced efficient power densities in MFC technology, also, some novel microorganisms were discovered recently. The microorganisms with known and unknown natural electron mediators are given in Tables 9.1 and 9.2, respectively.

9.5.1 Microorganisms in Anode

The most studied and efficient exoelectrogens in MFC technology belong to *Geobacteraceae* family of bacteria. *G. sulfurreducens*, δ-proteobacteria, can reduce acetate with ca.100 % electron recovery to generate electricity. The organism has successfully produced the current density of 3147 mA/m^2 in a MFC with gold electrodes, acetate as the electron donor and fumarate as the electron acceptor (Richter et al. 2008). However, *G. metallireducens* (pure culture) could produce only 40 mWm^{-2} power output in MFC using wastewater as inoculum (Min et al. 2005). *Shewanella spp.*, γ-proteobacteria, can reduce iron and manganese and can use them as electron acceptors. *Shewanella oneidensis* DSP10 in a miniature MFC using lactate as the anolyte and ferricyanide as catholyte produced power density of 3000 mW/m^2 which is quite appreciable (Ringeisen et al. 2006). Recently, *S. putrefaciens* in a single-chamber microbial fuel cell (sMFC) produced maximum power density of 4.92 W/m^{3}using $CaCl_2$ as anolyte (Pandit et al. 2014). *Rhodopseudomonas palustris*, α-proteobacteria and a photosynthetic purple non-sulphur bacterium, can utilise volatile acids, yeast extract and thiosulphate and produce power density of 2720 mW/m^2 higher than mixed cultures in indistinguishable MFCs (Xing et al. 2008). A thermophilic, Gram-positive, metal-reducing bacterium, *Thermincola ferriacetica*, is able to generate current from acetate and exhibited maximum current density 12 Am^{-2} (Prathap et al. 2013). *Pseudomonas aeruginosa*, γ-proteobacteria, in MFC produced power density of 4310 mWm^{-2} using glucose as electron donor and graphite electrodes as the electron acceptor (Rabaey et al. 2004). A sulphate-reducing bacterium, *Desulfovibrio desulfuricans*, in MFC with surface-treated graphite felt electrodes generated maximum current density of 233 mA/m^2 which was ca. 50 % higher than with untreated elec-

Table 9.1 Microorganisms with known electron transfer intermediaries

Microbes	Current density/power density	Proteins/compounds involved in electron transfers	References
Geobacter sulfurreducens	3147 mA/m^2	Type IV pili c-Type cytochrome Z	Inoue et al. (2010a)
Geobacter metallireducens	40 mW/m^2	c-Type cytochromes I.e. OmcB and OmcE	Min et al. (2005)
Shewanella oneidensis	3000 mW/m^2	flavins, riboflavin	Ringeisen et al. (2006)
Shewanella putrefaciens	4.92 W/m^3	c-Type cytochromes I.e. MtrC and OmcA,	Pandit et al. (2014)
Rhodopseudomonas palustris	2720 mW/m^2	c-Type cytochromes	Xing et al. (2008)
Thermincola ferriacetica	12000 mA/m^2	Anthraquinone 2, 6 disulfonate	Prathap et al. (2013)
Pseudomonas aeruginosa	4310 mW/m^2	Pyocyanin Phenazine-1-carboxamide	Rabaey et al. (2004)
Desulfovibrio desulfuricans	233 mA/m^2	c-Type cytochromes	Kang et al. (2014)
Desulfovibrio alaskensis	–	Tetraheme cytochrome C_3 Transmembrane complexes (QrcA)	Keller et al. (2014)
Klebsiella pneumonia	199 mA/m^2	2, 6-Di-tert-butyl-p-benzoquinone	Lifang et al. (2010)

Note: Units of surface power density are given in mW/m^2, volume power density in W/m^3 and units of current density in mA/m^2

Table 9.2 Microorganisms with unknown electron transfer intermediaries

Microbes	Current density/power density	References
Bacteria		
Escherichia coli	3390 mA/m^2	Qiao et al. (2008)
Saccharomyces cerevisiae	282 mA/m^2	Raghavulu et al. (2011)
Lysinibacillus sphaericus	85 mW/m^2	Nandy et al. (2013)
Citrobacter sp.	205 mA/m^2	Xu and Liu (2011)
Ochrobactrum sp.	2625 mW/m^3	Xin et al. (2014)
Algae		
Scenedesmus	1926 mW/m^2	Cui et al. (2014).
Arthrospira maxima	10 mW/m^3	Inglesby et al. (2012)
Cyanobacteria 1	14 mW/m^2	Yuan et al. (2011)
Chlorella vulgaris	2485 mW/m^3	González et al. (2013)
Coriolus versicolor	320 mW/m^3	Wu et al. (2012)

Note: Units of surface power density are given in mW/m^2, volume power density in mW/m^3 and units of current density in mA/m^2

trodes (Kang et al. 2014). *E. coli*, Gram-negative bacteria in MFC successfully achieved power density of 1300 mW/m^2 at 3390 mA/m^2 current density (Qiao et al. 2008).

Saccharomyces cerevisiae in sMFC (open-air cathode) fed with synthetic wastewater using noncatalysed graphite as electrodes without the use of artificial mediators generated maximum current density 282.83 mA/m^2 (Raghavulu et al. 2011). Other yeast *Hansenula anomala* using Pt electrode and ferricyanide as catholyte produced power density of 2.9 W/m^3 (Prasad et al. 2007). Moreover, *Candida melibiosica* in a MFC of modified carbon felt electrode with surface nickel nanostructures produced significant power output of 720 mW/m^2 (Hubenova and Mitov 2010). Some microorganisms not used commonly in MFC and a few novel exoelectrogens discovered recently have also shown the ability to produce electricity.

Analysis of 16S rRNA gene sequences has unveiled a new exoelectrogen; *Geobacter anodireducens* showed 98 % similarity to *Geobacter sulfurreducens* but cannot reduce fumarate as the electron acceptor (Sun et al. 2014a, b). Another novel strain, *Ochrobactrum sp.* 575 isolated recently from the anodic chamber of a xylose MFC, produced maximum power density of 2625 mW/m^3. Further, the results suggested that xylose digestion in *Ochrobactrum sp.* 575 was different to other electroactive bacterial strains, which depends on the succinate oxidation respiratory chain instead of traditional NADH oxidation respiratory chain (Li et al. 2014). *Klebsiella pneumonia*, Gram-negative, nonmotile, lactose-fermenting bacteria in a cubic air-chamber MFC, generated 199.2 mA/m^2 current density and maximum voltage output of 426.2 mV (Lifang et al. 2010). A Gram-positive bacterium *Lysinibacillus sphaericus* in MFC using graphite felt as electrode generated a maximum current density of ca. 270 mA/m^2 and power density of 85 mW/m^2 (Nandy et al. 2013). Further, *Citrobacter sp.* SX-1 can utilise diverse simple substrates like acetate, glucose, sucrose, glycerol and lactose in MFCs but produced the highest current density of 205 mA/m^{2}from citrate (Xu and Liu 2011; Kimura et al. 2014; Zhang et al. 2013). Besides bacteria and yeast, microalgae have been also used in MFC technology either as bioanode or a substrate assisting the anode for the prevalent application. *Scenedesmus*, green algae in powder form as substrate, was used in anode and *Chlorella vulgaris* as a biocathode in MFC produced maximum power density of 1926 mW/m^2 (Cui et al. 2014). In another study, *Arthrospira maxima* was used as a substrate as well as a carbon source for the metabolism and growth of *R. palustris* in a micro-MFC and exhibited volumetric power density of 10.4 mW/m^3, the highest in rival to other substrates used in the study (Inglesby et al. 2012). Furthermore, blue-green algae (cyanobacteria) in a sMFC produced maximum power density of 114 mW/m^2 at 0.55 mA/m^2 current density (Yuan et al. 2011). In microalgae-assisted MFCs, algae degradation produces intermediate compounds like acetate and lactate which can be further used by exoelectrogens such as *G. sulfurreducens* for bioelectricity production.

9.5.2 Microorganisms in Cathode

Geobacter spp. highly efficient as bioanodes in MFC also evidenced to be prelusive biocathodes to accept the electrons from cathodic electrodes (Gregory et al. 2004). The study revealed that *G. metallireducens* reduced nitrate to nitrite and *G. sulfurreducens* reduced fumarate to succinate with the electrode as the sole electron donor (Gregory et al. 2004). Furthermore, *G. sulfurreducens* reduced fumarate in a reactor with stainless steel electrodes producing the current density of 20.5 Am^{-2} (Dumas et al. 2008). *Shewanella oneidensis* MR-1 as biocatalyst in the air-cathode MFC and lactate as electron donor showed increase in Cr (VI) reduction rate with maximum current density of 32.5 mA/m^2 (Xafenias et al. 2013). The study demonstrated the expression of riboflavin in the electron transport. In an alternative investigation, *Shewanella putrefaciens* and *Acinetobacter calcoaceticus* showed the ability to reduce the oxygen to water with increased rate by utilising outer membrane-bound cytochromes and self-excreted PQQ respectively (Freguia et al. 2010). An acidophile microorganism, *Acidithiobacillus ferrooxidans*, fed as a biocathode in MFC, up to 5 Am^{-2} of current densities, were obtained for O_2 reduction at low pH (Yuan et al. 2011). In a study, *Enterobacter and Pseudomonas spp.* demonstrated for the catalysis of acetate oxidation actually resulted to catalyse the electrochemical reduction of oxygen-producing maximum current density of 145 mA^{-2} (Parot et al. 2009). Cyclic voltammetry unveiled that *Micrococcus luteus* and other Gram-positive (*Staphylococcus spp., Lactobacillus farciminis*) and Gram-negative bacteria (*Pseudomonas fluorescens, Escherichia coli, Acinetobacter sp.*) are able to catalyse the electrochemical reduction of oxygen on the carbon electrode (Cournet et al. 2010). Seawater-formed aerobic biofilms coated on stainless steel electrodes have shown significant ability to catalyse oxygen reduction and achieved current densities up to 460 mA/m^2 at different set potentials (Bergel et al. 2005).

An acetate-fed MFC utilising *Chlorella vulgaris* as a biocathode produced maximum power density of 1926 mW/m^2. CO_2 produced at the anode was used by *C. vulgaris* as a carbon source for its growth. Further, the study demonstrated that *C. vulgaris* could not grow in acetate-fed MFC without anodic CO_2 supply (González et al. 2013; Cui et al. 2014). The immobilisation of *C. vulgaris* into the cathode chamber turned the MFC highly efficient, consequently producing the power density 2485.35 mWm^{-3} at a current density of 7.9 Am^{-3}, while the MFC with suspended *C. vulgaris* achieved 1324.68 mWm^{-3} power density (Zhou et al. 2012). A strain of white-rot fungus, *Coriolus versicolor* (secretes laccase to reduce oxygen at the cathode), inoculated in the cathode chamber of a MFC to catalyse the cathodic reaction generated the maximum power density 320 mWm^{-3} (Wu et al. 2012).

9.6 Future Directions

It is unfortunate for the MFC technology that the studied applications of the technology are still confined to the four walls of the laboratory. In other words, the technology is not commercialised yet. The electron transfer mechanisms from exoelectrogens to electrodes are well understood only in *Geobacter spp.* and *Shewanella spp.*; hence, the investigations about electron transfer mechanisms in other microorganisms are also intended. Further, the efficiency of the exocellular electron transfer rates can be increased by genetic manipulations. The microorganisms exhibiting conductive pili are proposed to be discovered, though such microorganisms can generate higher-power densities. The electron transfer mechanisms from electrodes to microorganisms are still not known. The microorganisms that can accept the electrons from the electrode will have a great significance in cathode compartment. The outer membrane multicopper proteins, OmpB and OmpC, showed their key role in Fe (III) oxide reduction, but a deep investigation is needed to explore their functions in electron transfer mechanisms.

9.7 Conclusions

In MFCs, the microorganisms act as the power houses of the MFC, and those that can form conductive biofilms is of great importance in MFCs. The bacteria produce specific proteins such as c-type cytochromes, pili and QS that play important roles in the formation of a conductive biofilm. Moreover, line of multiple studies suggests that c-type cytochromes, OmcZ and OmcB, are crucially required in electron transfer mechanisms. So far, only *Geobacter spp.* and *Shewanella spp.* are able to perform the long-range electron transport through pili. The other exoelectrogens like *Pseudomonas spp*. secretes pyocyanin, and *Shewanella spp.* prefers to use flavins to transfer electrons to the electrodes. The latter exoelectrogen has shown to accept electrons from the electrodes using riboflavin as electron mediator. Further, some unusual cytochromes, Cyt_{579} and Cyt_{572}, have been reported to mediate the electron transfers from the electrode to bacterial cells. The use of biocathodes has made the technology more economic. Moreover, MFC has become the only technology towards renewable energy production and other manifold applications.

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Ravinder Kumar He finished his bachelor degree in Life Sciences from Himachal Pradesh University, Shimla, in 2009. He postgraduated in Biotechnology from CCS University Meerut in 2011 and currently is a Ph.D. student in Universiti Malaysia Pahang working on microbial fuel cells.

Lakhveer Singh He received his M.Sc. in Chemistry from Punjab University Chandigarh, India, in 2009, and Ph.D. in Industrial Chemistry from University Malaysia Pahang (UMP), Malaysia, in 2013. He is currently a senior lecturer at the faculty of Faculty of Engineering Technology, UMP Malaysia. He is the editor of Advance in Chemical and Biological Engineering Journal (China) and guest editor for International Journal of Energy Engineering (USA). His main areas of interest are bioenergy, bioreactors development, wastewater treatment, MFC, and green technology.

Zularisam Ab. Wahid He finished his M.Sc. and Ph.D. in Environmental Engineering from UTM, Malaysia. He is presently the Dean Professor of Faculty of Engineering Technology at UMP, Malaysia. He is a member of Young Scientists Network – Academy Sciences Malaysia and Institution of Engineer Malaysia (IEM). He is the editor in chief of International Journal of Engineering Technology and Science and editor/reviewer of Journal of Civil Engineering and Geo-environmental. His current interests are bioenergy and advanced material development.

10

Biological Electricity Production from Wastes and Wastewaters

Jai Sankar Seelam, Deepak Pant, Sunil A. Patil,
and Balasaheb P. Kapadnis

Abstract

Attributed to their multifaceted abilities, microorganisms have been constantly explored for several applications ranging from product synthesis, energy recovery to waste treatment. Biological production of electricity has been an important area of research in the past decade and half. Bioelectrochemical systems (BESs) offer a promising solution in aiding the energy development sector due to its supplementing ability to generate electricity from wastes and wastewaters. This chapter lays focus on the mechanisms and applicability of microorganisms to tap the potential in the wastes and wastewaters to function as active substrates for bioelectricity generation. Simultaneous bioenergy recovery is an added advantage in the BESs along with waste treatment. The main emphasis is on the electron-transfer mechanisms across microorganisms and electrodes, reactor architecture, and operating conditions. A brief overview on the potential of various solid wastes and wastewaters from domestic, agricultural, and industrial sectors is also included. The advancements in the field of microbial electrocatalysis have been highlighted under various sections which shed some light on the possibilities of active integration of

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J.S. Seelam (✉)
Department of Biotechnology, Indian Institute of Technology Kharagpur, Kharagpur 721302, India

Separation and Conversion Technologies, Flemish Institute for Technological Research (VITO), Boeretang 200, Mol 2400, Belgium
e-mail: shankar.jaishi@gmail.com; seelam@iitkgp.ac.in

D. Pant
Separation and Conversion Technologies, Flemish Institute for Technological Research (VITO), Boeretang 200, Mol 2400, Belgium
e-mail: pantonline@gmail.com; deepak.pant@vito.be

S.A. Patil
Laboratory of Microbial Ecology and Technology, Ghent University,
Coupure Links 653, B-9000 Ghent, Belgium
e-mail: sunilmicro12@gmail.com; sunil.patil@ugent.be

B.P. Kapadnis
Department of Microbiology, Savitribai Phule Pune University, Pune 411 007, India
e-mail: kapadnisbp@gmail.com; bpkap@unipune.ac.in

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BESs with other existing bioprocesses. Further technical and technological advancements can supplement the capability of waste to bioenergy conversion concept of BESs to tackle the energy sustainability and waste management issues.

10.1 Introduction

The quest to find sustainable ways for producing electricity has become a challenging task for the scientific and industrial world. Modernization and industrialization may have eased the living style but have massively increased our dependence on electricity for its regular maintenance and functioning. To meet this high energy demand from the over burgeoning population, many unsustainable resources are being extensively exploited which not only lead to their rapid depletion but also have a negative toll on the environment. Consequently, this has led to the development of clean and green alternate technologies for electricity generation during the past few decades. This advancement looked one step ahead by tapping the potential of wastes and wastewaters to serve as a potential feedstock for energy recovery thereby curtailing the dependence on conventional carbon-based fuels. This also serves the purpose of reducing the costs incurred in waste treatment systems. One such promising technology that has been developed over the last decade is the bioelectrochemical systems (BESs) such as microbial fuel cells (MFCs) which employ microbial communities for the conversion of chemical energy present in the wastes and wastewaters into the electrical energy (Oh and Logan 2005; Min and Logan 2004; Liu et al. 2004; Moon et al. 2006; ElMekawy et al. 2014a, b). An MFC is a bio-catalyzed system which harnesses bioenergy in the form of electrical energy through microbial oxidation of biodegradable organic matter present in the wastes or wastewaters under mild reaction conditions (ambient temperature and pressure) (Logan 2004a, b; Aelterman et al. 2006; Moon et al. 2006). The energy recovery is usually accompanied by or linked to simultaneous waste treatment (Patil et al. 2009; Pant et al. 2013; Sevda et al. 2013a). In this chapter, emphasis is laid on the mechanism of biological generation of electricity by microorganisms by degrading the organic matter present in waste particulates and wastewaters. In subsequent sections, a brief description on various feedstocks, technical, and technological advancements in the BESs is included.

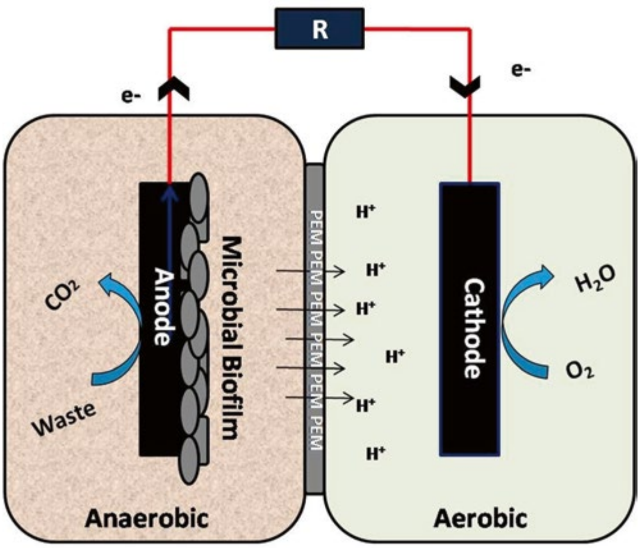
10.2 MFCs: Fundamentals and Technology

10.2.1 MFC: Principle

Electrocatalytic conversion of chemical form of energy stored in the chemical bonds of organic matter, wastes, or wastewaters to electricity using microorganisms is the principle mechanism of a MFC. A typical MFC comprises of anode and cathode chambers, which are separated by an ion exchange separator (Fig. 10.1). Anodic oxidation and cathodic reduction reactions govern the electrocatalytic activity in MFCs (Mohanakrishna et al. 2015). In the anode chamber, microorganisms oxidize the organic matter present in the wastes to produce carbon dioxide, electrons, and protons. The diffusion of protons from anodic to cathodic chamber through an ion exchange membrane generates a potential difference between the anode and the cathode which leads to the flow of electrons, i.e., current from the anode to the cathode through an external circuit. Conventionally, oxygen functions as a terminal electron acceptor which combines with the electrons and protons at cathode surface to form water as an end product.

General electrode reactions considering glucose as an electron donor and O_2 as a terminal electron acceptor are presented below:

Fig. 10.1 Schematic diagram of a double-chambered microbial fuel cell. *R* resistance and *PEM* proton exchange membrane. The oxygen reduction reaction at the cathode can be abiotic or catalyzed by the microorganisms



Anode reaction: $C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 24H^+ + 24e^-$
Cathode reaction: $24H^+ + 24e^- + 6O_2 \rightarrow 12H_2O$
Overall reaction: $C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + \mathit{Electrical\ energy}$

The overall reaction is breakdown of the substrate into carbon dioxide and water with electrical energy as a main product.

10.2.2 Electron-Transfer Mechanisms from Microorganisms to the Anode in MFCs

The key mechanisms involved in microbial electron transfer (ET) to the anode are illustrated in Fig. 10.2. These include the ET via (a) membrane-bound cytochromes and/or electrically conductive cell appendages such as pili or nanowires, (b) self-excreted (by microorganisms) or exogenous redox mediators, and (c) via the oxidation of reduced primary metabolites such as hydrogen (Patil et al. 2012). Broadly, these can be categorized into direct electron transfer (DET) and indirect or mediated electron-transfer (IET/MET) mechanisms.

The DET occurs when the microorganisms are attached to the electrode surface. This mechanism is prominent in two well-studied microorganisms, viz, *Geobacter sulfurreducens* (Bond and Lovley 2003; Reguera et al. 2006) and *Shewanella oneidensis* MR-1 (Kim et al. 2002; Gorby et al. 2006; Biffinger et al. 2007). In these microbial strains, involvement of terminal reductases such as c-type outer-membrane cytochromes in ET to the anode has been well documented (Patil et al. 2012). In particular, in the case of *G. sulfurreducens*, outer-membrane cytochromes such as OmcS (Mehta et al. 2005), OmcB (Leang et al. 2003), and OmcZ are involved in the DET process. Among these, OmcZ is prominent in establishing and promoting the DET to the anodes. Similarly, in *S. oneidensis* MR-1, membrane spanning and outer-membrane cytochromes help in establishing rapid connection between cytoplasm and extracellular electron acceptor. Specifically, the role of outer-membrane MtrC-OmcA complexes in EET to anodes has been established in the case of the *S. oneidensis* MR-1 strain (Baron et al. 2009). *G. sulfurreducens* are shown to have electrically conductive pili referred to as nanowires, (Reguera et al. 2006), which assist long-range ET across anodic biofilms. Similarly, in *S. oneidensis* MR-1, the nanowires have been reported to play a role in DET to the electrodes (Gorby et al. 2006).

In the case of IET, direct contact of microbial cells to the electrode is not necessary. In one of the IET mechanisms, electrochemically or meta-

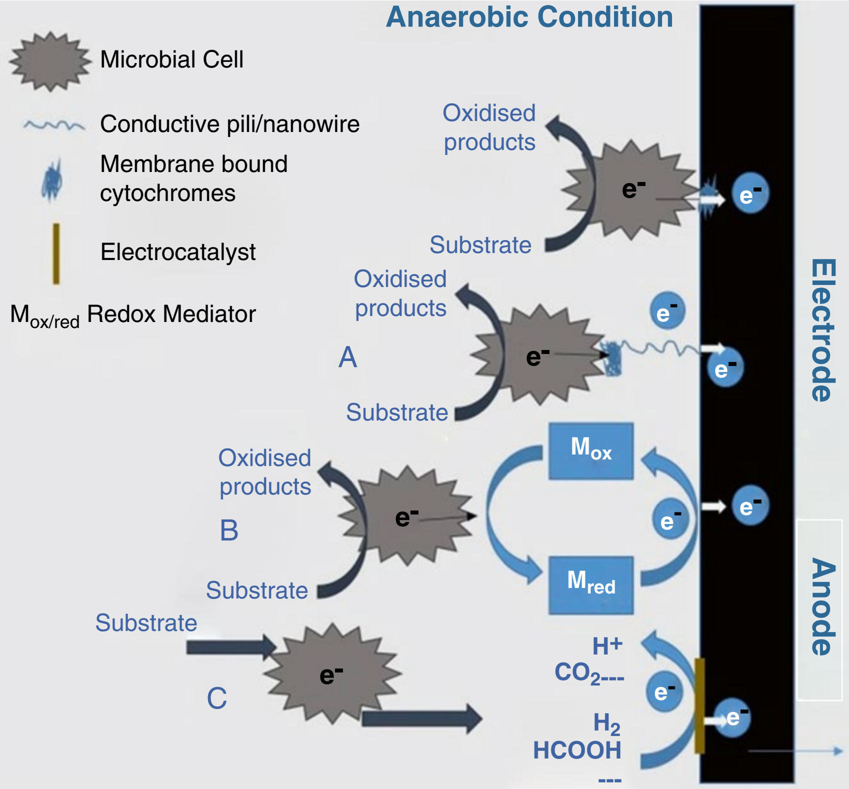


Fig. 10.2 Electron transfer (ET) mechanisms from microorganisms to the electrode (anode) in MFCs. *A*. Cell-membrane-bound cytochromes and/or electrically conductive pili (nanowires) mediated ET. *B*. Self-secreted (by microbes) or exogenous redox mediated ET, and *CET*. via oxidation of reduced primary metabolites

bolically produced hydrogen and formate can mediate and boost the ET to anodes. In another IET mechanism, soluble redox mediators that can be added externally (Biffinger et al. 2007; Rozendal et al. 2006; Lee and Rittman 2010) or produced by microbes (Cheng et al. 2009; Clauwaert and Verstraete 2009) facilitate the ET process. These mediators promote electrogenic activity of biofilm and are secreted specially by species of *Shewanella* and *Pseudomonas*. Flavin mononucleotide (FMN) and riboflavin are the mediators that are identified in *Shewanella* species (Rabaey et al. 2005b). The ET is mediated by compounds like phenazines in *Pseudomonas* sp. (Rabaey et al. 2005b). Riboflavin encourages efficient ET at EAB-electrode interface as it accumulates at the interface during sustained incubations (>72 h) (Marcus et al. 2011).

10.2.2.1 Electron Transfer Across Electrocatalytically Active Anodic Biofilms

Microorganisms are the core elements of the MFCs, which determine the performance in terms of current production by forming a slime layer on the anode surface, technically referred to as electrocatalytically active biofilm (EAB). The diffusion of wastes and nutrients to the microbes is governed by the natural chemical gradient which in turn modulates the biological activity of biofilm both spatially and temporally (Marcus et al. 2007; Logan et al. 2006). The process of ET across the biofilm is a mechanically and spatially heterogeneous pathway (Torres et al. 2010) and occurs through varied mechanisms such as DET and/or MET. The position of cells within the biofilm affects the mechanism of ET with DET dominating in those resting at electrode surface, and indirect mechanisms are required to transport the electrons across the biofilm thickness. The cells on the outer rim of the biofilm are farther from the electrode surface and are readily exposed to the waste and nutrients, whereas the cells in direct contact with electrode surface are prone to many physical and chemical limitations. Redox-active components on the outer membrane of the bacteria like c-type cytochromes (Shi et al. 2009) assist in the DET when they come in contact with electron acceptor. Indirect mechanisms involve intermediary synthesis of either electron shuttles, redox mediators (Gralnick and Newman 2007), or appendages like nanowires (Gorby et al. 2006; Reguera et al. 2005). Additionally, IET depends either on solid conductive matrix of variable composition or produced soluble and mobile electron carriers (Torres et al. 2010).

10.2.3 Electrocatalytically Active Anodic Biofilms

Microorganisms are present either as planktonic cells or EABs in BESs (Borole et al. 2011). Biofilms can be monolayered or multilayered collection of microorganisms on the electrode surface which facilitate ET and subsequent power generation. Both biofilm and planktonic microorganisms coexist and work in unison to produce electricity. The EABs house both electrochemically active and inactive microorganisms with the former contributing actively to the generation of electricity from waste organics or inorganics. The latter supplement the electricity production process by degrading the complex organics through mechanisms like fermentation or utilizing other electron donors or acceptors. The electrochemically active microorganisms primarily help in efficient ET and enhance the energy output and treatment efficiency. The majority of the electroactive microbial communities are generally either enriched or adapted from mixed microbial inoculum sources before using in BESs (Borole et al. 2011). The power output depends on the activity of electrode-associated biofilms. Eliminating non-electroactive microbial communities like methanogens, nitrate reducers, hydrogen scavengers, and, potentially, aerobic organisms in EABs is critical for getting better power output with MFCs. Therefore, flow-over or flow-through anodes are currently being preferred in BESs because high substrate concentration and pH gradients help in the washout of suspended, non-electroactive microorganisms. Operational, biological parameters and system design are the critical parameters which contribute toward high performance of a biofilm. The structure and the composition of the biofilm are dependent on the operational conditions which are well associated with electroactivity and performance of the system. The composition and activity of the biofilm depend on the type of inoculum and mode of operation.

The biological parameters which influence the formation of an efficient EAB are the source and nature of inoculum (mixed or consortia) and substrate, type (gram positive or negative), and the nature of enrichment. The inoculum affects biofilm parameters like growth and ET rates and mechanisms, abundance, film thickness, conductivity, and substrate uptake, all of which contribute to the activity of the biofilm and the performance of the system (Borole et al. 2011; Gimkiewicz and Harnisch 2013; Erable et al. 2010; Dulon et al. 2006). The use of pure or mixed culture affects the electroactivity and at the same time also influences the power output. Studies found that gram-negative bacteria generate higher current compared to gram-positive bacteria as the presence of cell envelope structures (thick cell wall) in gram-positive bacteria inhibits easy transfer of electrons from the cell to electrode (Milliken and May 2007). However, an investigation found that *Thermincola* spp., a gram-positive microbe, isolated from a thermophilic system, were able to transfer electrons directly to electrode which might be with the help of c-type cytochromes of the cell envelope (Wrighton et al. 2008). EABs are complex in nature and are susceptible to minute changes in the system (Borole et al. 2011). In waste treatment system, biofilm-forming electroactive bacteria capable of DET are crucial in generating high current densities. Formation and sustenance of a conductive biofilm matrix which can ably facilitate high ET rates between electrodes and microorganisms is the critical need for commercial and large-scale applications.

10.2.3.1 Pure or Mixed Culture Inoculum Sources

The nature of the microbial inoculum plays an important role in contribution toward the internal resistance of the BESs. Pure as well as consortium of microorganisms have been employed by several research groups in MFCs. Very few pure cultures of bacteria help in DET from the cell membrane to the electrode. Exoelectrogenic bacteria such as *Geobacter* spp. (Bond and Lovley 2003; Dumas et al. 2008a, b), *Shewanella* sp. (Kim et al. 1999a, b; Ringeisen et al. 2006; Biffinger et al. 2007; Liang et al. 2009; Yang et al. 2011b), and *Rhodoferax* sp. (Liu et al. 2007a, b) showed promising ability to generate bioelectricity. The major challenge with pure cultures is the possibility of microbiological contamination and low growth rate. To overcome this problem, a wide variety of mixed consortia of bacteria or waste streams such as domestic wastewater (Min and Logan 2004), soil (Niessen et al. 2006), fresh as well as marine sediments (Zhang et al. 2006), activated sludge (Ki et al. 2008; Patil et al. 2009), and anaerobic digester sludge (Kim et al. 2004; Chae et al. 2010) have been used. The mixture of different microorganisms assists in the transfer of electrons derived from the metabolism of organic wastes in this case.

The bacteria in pure culture systems are highly substrate specific compared to their mixed culture counterpart. Studies found that the power density using mixed cultures is relatively higher than those using pure cultures in same MFC (Logan et al. 2006; Ishii et al. 2008). Mixed microbial communities are preferred mainly due to their stability, robustness, nutrient adaptability, and stress resistance. Additionally, the ease of availability and tolerance to environmental changes makes the use of mixed cultures highly promising for bulk-scale systems. The mixed culture systems adapt slowly to generate stable power output which is evident from the reports that the start-up time ranges from a few days to 3 months. The use of complex substrates such as wastewaters and mixed inoculum sources leads to the growth of a diverse group of microbes at anode and in bulk of MFCs (Min et al. 2005b; Venkata Mohan et al. 2007).

10.2.4 MFC Configurations

A wide variety of MFC designs and configurations have been employed by several research groups for simultaneous waste treatment and bioelectricity generation. Based on the architecture and operation, they are broadly classified into double-chambered, single-chambered air-cathode, up-flow, and stack MFCs.

10.2.4.1 Two- or Double-Chambered MFC

A typical double-chambered MFC comprises of anodic and cathodic chambers separated by a cation or proton exchange membrane (CEM or PEM). These membranes facilitate the flow of cations or protons to the cathode chamber and limit the diffusion of oxygen or other oxidants to anode chamber. The two-chambered MFCs can have various practical shapes. H-type MFC is the most extensively used MFC configuration, which consists of two bottles or units connected by a tube containing a PEM as separator (Min et al. 2005b; Oh and Logan 2006; Hou et al. 2009; Picot et al. 2011). The power generated in this system is quite low due to high internal resistance (Logan et al. 2006). This configuration primarily finds its application in basic research such as evaluating power generation using new electrode or separator materials or for microbial community analysis that develops during the degradation of specific pollutants (Sevda et al. 2013b). Nowadays, parallel-plate configurations are more frequently used. These offer better membrane to working volume ratio, low resistance, and better symmetry (Fuentes-Albarrán et al. 2012).

10.2.4.2 Single-Chambered Air-Cathode MFC

The scale-up of double-chambered MFCs has been found to be tough task due to its complex design. Hence, single-chambered MFCs are developed to minimize the costs and construction issues. A typical single-chambered cell consists of only an anode chamber with cathode in direct contact with air widely referred to as air cathode. The cost-effective single-chambered air-cathode MFCs have been found to be more advantageous over the double-chambered MFCs (Park and zeikus 2003; Liu and Logan 2004; Sukkasem et al. 2008; Lorenzo et al. 2009; Tugtas et al. 2011; Zhang et al. 2011a). This is mainly due to following reasons: (1) no aeration in cathode chamber is needed and passive air can be used; (2) ease of operation, no recycling, or chemical regeneration of catholyte is required; and (3) higher volumetric power density can be obtained because of smaller cell volume (Fan et al. 2007). In this configuration, the PEM is bonded with the cathode material into a single entity.

In order to further minimize the costs, membrane-less MFCs have been developed. These MFCs were easier to construct and generated relatively high power density (Liu and Logan 2004). However, before the columbic efficiency was much lower than the system with membrane mainly due to the consumption of substrate by the oxygen diffused across the cathode (Liu and Logan, 2004). Other major challenge in this system is the distance between the anode and the cathode. It is confined to range of 1–2 cm because of higher risk of short circuit and potential negative effect of oxygen on the activity of biocatalysts on the anode (Liu et al. 2005; Cheng et al. 2006a). Researchers have tried many configurations in order to overcome these obstacles and generate high current density (Min et al. 2005b; Oh and Logan 2006; Hou et al. 2009; Picot et al. 2011).

10.2.4.3 Up-Flow MFC

In the initial attempts to scale up MFC technology, up-flow MFCs (UMFCs) seemed to be a promising configuration for bulk-scale wastewater treatment and simultaneous electricity generation (Jang et al. 2004). This hybrid design combining the features of MFC and up-flow anaerobic sludge blanket (UASB) reactor minimizes the power consumption during agitation/mixing. In this design, increased rate of electrochemical reactions and quick biofilm formation is well facilitated due to nonmechanical mixing or agitation. When the reactor is fed from the bottom of the anodic chamber with anolyte along with simultaneous discharge of effluent from cathodic chamber to anodic chamber, an up-flow hydraulic pattern is created. This pattern ensures proper mixing of the anolyte. To avoid any biogas accumulation, the PEM in UMFC is inclined at an angle of 15° to the horizontal plane. Being a low-power consuming and continuously fed MFC, UMFC is considered to be an encouraging design for large-scale treatment of wastewaters. Scalable and commercial UMFCs – multiphase UMFC and U-shaped cathode UMFC – have been developed by researchers showcasing the ability of MFCs to generate high power output (Yang et al. 2011a).

10.2.4.4 Stack MFC (Scalable MFCs)

The voltage or current output can be enhanced using a uniquely structured MFC architecture known as stack MFCs. In this system, several MFCs are connected either in series or parallel based on the operational requirements. The MFCs are connected head-to-tail using insulated pipes. The current gets summed up when the cells are connected in parallel and the voltage remains same. While in series connection, common current flows through the cells and the voltage gets added (Larminie and Dicks 2000). In both series and parallel connections, the performance of individual cells is interindependent on each other. A study found that current production in series connection is six times lower compared to that in parallel connection when operated at same volumetric flow rate. Due to relatively high short-circuit current in parallel connection, high rate of biochemical reaction is achieved than fuel cells in series (Aelterman et al. 2006). Stack MFC with parallel connection is the most appropriate configuration for rapid substrate degradation and high current densities. Electrode separators are essential in this type of configuration limiting their use in open environment (Kim et al. 2012; Cheng and Logan 2007).

10.2.5 Factors Affecting the MFC Performance

In self-sustaining systems like MFCs, the research focus has been always on enhancing the efficiency of waste treatment and optimizing the bioelectrocatalytic performance of these systems. Several critical parameters such as microbial inoculum and its concentration, substrate composition and concentration, pH of the feed, feeding rate, temperature, electrode materials, ion exchange separators, and reactor configuration influence the performance of MFCs. In general, mixed microbial communities offer to be better biocatalysts for electricity generation with wastewaters. Loading pH affects current density and coulombic efficiency in a waste treatment system. Metabolic activities like proton translocation, amino acid degradation, adaptation to acidic or basic conditions, and virulence (Olson 1993) are dependent on the pH of the system. The influent pH affects the start-up time of biofilm formation and also the maximum current outputs (Patil et al. 2011). Higher current density and coulombic efficiency were observed in acidophilic conditions. Effective electron discharge at higher resistance was observed in acidic conditions compared to neutral and alkaline conditions (Veer Raghavulu et al. 2009). In small-scale systems, the pH gradients across the electrode hinder the growth of microorganisms and lead to reduced performance of microorganisms in contact with electrode surface. At low pH, microorganisms are prone to higher stress levels. Investigations found that enriched bacteria have improved pH tolerance compared to normal cultures (Borole et al. 2011). Acidic pH facilitates efficient proton transfer to the cathode chamber and at the same time also minimizes the proton gradient. A study found acidic pH of 6.0 proved to be ideal for mixed consortia to form biofilm with simultaneous electricity generation (Veer Raghavulu et al. 2009).

The impact of temperature on the MFC performance is crucial for long-term and commercial operations. The growth and electrocatalytic properties of the biofilm vary with temperature. In tropical weather conditions, the performance of bacteria enriched at higher temperatures showed great promise in MFC applications. In such cases high current density is observed at temperature of 40 °C (Liu et al. 2010). Additionally, the temperature used during the enrichment phase also affects the bioelectrocatalytic performance of the system (Patil et al. 2010). Bacteria enriched at lower temperatures produced higher current densities when operated at low temperatures. The thermophilic bacteria are found to be generating higher current densities at high temperatures like 60 °C. Substrate concentrations and loading also affect the MFC performance. The metabolism might shift toward other metabolic pathways like acetogenesis or methanogenesis when there is excess of substrate and absence of low-resistance path to an electron sink. Further, the performance of MFCs depends on many other factors like electrode spacing, anolyte conductivity, and membrane type (Liu et al. 2005; Cheng et al. 2006b).

Electrode materials and their properties are the key factors which influence the performance of MFCs. High electrical conductivity, biocompatible surface, chemical stability, inert nature (non-oxidative), non-self-destructive and electrocatalytic activity, and sustainability of its properties with time are some of the important properties of the electrodes, which need to be considered (Srikanth et al. 2011; Rosenbaum et al. 2007; Guo et al. 2014b). Both electron propagation and electron-transfer characteristics vary with properties of electrode (Aelterman et al. 2008; Larrosa-Guerrero et al. 2010; Liu et al 2010). In open circuit condition, the biofilm formation is affected by nature of electrode but not affected in closed conditions (Larrosa-Guerrero et al. 2010). Current densities vary with surface roughness or microbially accessible surface of the electrode (Dumas et al 2008a). Biofilm growth and diffusion of substrate is maximized by either increasing the pore properties or nano-modification of electrodes. This modification maximizes the true surface area for swift electron exchange. The influence of substrate composition, waste, and wastewaters on the production of bioelectricity will be explained in the upcoming sections.

10.2.6 Electrode Materials Used in MFCs

A wide variety of materials have been investigated to function as electrodes over the past few decades. Several noble metals like Pt, Au, Ag, and Pd and other metals like Rh, Ir, Ni, and Cu have been used mainly due to their properties like high conductivity, broad working potential range, and specificity for sensing and detection applications (Kumar et al. 2013). But high costs and weak adhesion of inoculated bacteria restrict the utility of these electrodes in MFCs. Later on, researchers turned to other materials like, stainless steel, aluminum, and carbon based materials to function as anodes (Ouitrakul et al. 2007). High ohmic and activation losses were reported when nickel was used and weaker adhesion of inoculated bacteria on stainless steel limited the application of these materials as anodes.

Several advantages associated with carbon-based electrodes prompted researchers to use these materials as a potential substitute to previously employed metal-based electrode materials. Many forms of carbonaceous materials with/without modifications have been tried as anode materials in MFCs. These include mainly carbon cloth (Liu et al. 2005), carbon felt (Logan et al. 2007), carbon foam (Chaudhuri 2003), reticulated vitreous carbon (RVC) (He et al. 2005), graphite sheets (Srikanth et al. 2011; Venkata Mohan et al. 2007, 2009a, b), graphite rods (Rabaey et al. 2004), graphite granules (Rabaey et al. 2005a), graphite fiber brushes (Logan et al. 2007; Zou et al. 2010; Liu et al. 2005; Cheng et al. 2006b), and carbon fiber mats (Chen et al. 2011; Patil et al. 2013). Relatively high physical strength, enhanced conductivity, eco-friendly nature, low cost, roughness, biocompatibility, etc. made carbon-based materials more suitable as anodes.

These carbonaceous electrodes are grouped broadly into flat, packed, and brush electrodes based on their configuration. Materials like carbon cloth and paper, graphite plates, glassy carbon, carbon mesh, and fibers fall under the category of flat electrodes, whereas carbon felt, reticulated vitreous carbon, granular activated carbon, granular graphite, and graphite disks fall under the stuffed electrodes. Graphite fiber, a brush electrode, possesses fibrils which are structures formed by winding finely cut slices of carbon fibers in the form a brush. These fibrils enhance the sustenance of microorganisms on the surface of anodes. Fiber brush electrodes have higher surface area compared to flat and stuffed configurations (Kumar et al. 2013). Carbon nanostructures are being used in several studies because they exhibit excellent electron-transfer characteristics with a high surface area to volume ratio and also provide a viable support for biofilm growth (Sharma et al. 2008). Further, impregnated and immobilized nanostructures are being extensively explored due to their positive impact on conductivity and charge transfer (Wei et al. 2011).

Generally, the cathode material is the same as that of anode. In wastewater-fed MFCs, carbon fibers linked with conducive and noncorrosive materials like nickel and titanium proved to function as good cathodes (Hasvold et al. 1997, 1999). Previously, precious metal like pt has been used as a catalyst when oxygen is used as the electron acceptor. But recent advancements in electrode development found new cathodes where pt is held on the electrode supporting material using a binder like Nafion (perfluorosulfonic acid) or polytetrafluoroethylene (PTFE). Studies found that though density of pt loading can be reduced to minimize the costs, cheaper alternates like cobalt- and iron-organic mixture catalysts should be used (Cheng et al. 2006b; Zhao et al. 2005) mainly due to the high costs incurred in electrode development. Materials like carbon paper and graphite rods are not quite suitable for scale-up because of their inherent lack of durability, structural strength, and high costs. Design of new electrodes like activated carbon air cathodes is quite essential for large scale and longer use of MFCs for wastewater treatment (Zhang et al. 2011a, c, 2014; Pant et al. 2010b).

10.3 Potential Waste and Wastewater Feedstocks for MFCs

10.3.1 Potential of Wastes and Wastewaters as Substrates for MFCs

A diverse array of waste and wastewaters offers to be a rich and renewable substrate for bioenergy, biofuels, and value-added chemical generation (Rozendal et al. 2008a, b). The use of negative or low-value waste streams helps in simultaneous tackling of globally and environmentally critical issues like sustainable energy sources, pollution reduction, and wastewater treatment (Pant et al. 2012). Abundance, cheaper costs, and sustainability of wastes make them an economic commodity to be used as a potential substrate source for MFC technology for the production of sustainable, renewable, and eco-friendly power with simultaneous accomplishment of waste treatment. Major constituents of wastes/wastewaters act as active electron donors to promote growth, metabolic activity, and functioning of electrogenic bacteria.

10.3.2 Influence of Wastewater Nature or Composition on the Performance of MFCs

The substrate is the most integral component in any biological system principally because it serves as carbon (nutrient) and/or energy source (Pant et al. 2012). The composition, nature, and characteristics of the wastes are the driving factors which determine the efficiency and commercial viability of the waste to energy conversion systems. The concentration of individual components of waste/wastewaters that can be transformed into energy is of special interest in BESs (Angenent and Wrenn 2008). The composition of the microbial assemblage and its integrity on the electrode surface as a biofilm varies drastically with the nature of the waste stream. Equally, critical performance parameters like coulombic efficiency, power density and treatment efficiency, or COD removal of waste treating MFCs depend on the influent constituents and concentration (Chae et al. 2009).

10.3.3 Types of Wastes Feedstocks

The primitive/first usage of wastewaters in MFCs for bioelectricity generation dates back to 2004 (ElMekawy et al. 2015). Since then, a broad spectrum of soluble or dissolved complex organic wastes/wastewaters and renewable biomass emerging from domestic to industrial sectors have been employed for simultaneous bioenergy generation and waste remediation (Pant et al. 2012; ElMekawy et al. 2015). Several solid wastes like food wastes, cattle manure, wheat straw, corn stover, etc. and various domestic, industrial, and agricultural wastewaters have been studied for bioelectricity generation. Tables 10.1 and 10.2 showcases an overview of the performance of MFCs fed with various wastes and wastewaters.

10.4 Electricity Production and Waste/Wastewater Treatment Using MFCs

The wastes and wastewaters are the most obvious potential substrates to operate MFCs due to their high organic content. Various electroactive and non-electroactive microorganisms aid in transforming the chemical energy stored in chemical compounds in biomass or wastes to electrical energy. Due to direct conversion of chemical energy into electricity instead of heat in MFCs, Carnot cycle with a limited thermal efficiency is avoided and theoretically a higher conversion efficiency system (>70 %) can be developed, similar to a conventional fuel cell (Du et al. 2007). Besides renewable production of electricity, biofilms of electroactive bacteria in MFCs facilitate proficient removal of organic carbon from wastewaters (Pant et al. 2012). By doing so, MFCs potentially reduce the energy requirement by over 50 % compared to the energy required for conventional treatment technologies where huge amount of energy is spent on aerating the activated sludge (Du et al. 2007). Comparatively, they produce 50–90 % lesser disposable solids during the treatment process (Holzman 2005). Most importantly, they enhance and sustain the growth of bioelectrochemically active microbes during the treatment ensuring operational stability (Du et al. 2007). The most striking advantage of bioelectricity over bioproducts production using BESs is that it can be utilized in situ or on

Table 10.1 An overview of performance of MFCs with different wastes

Feedstock	MFC configuration	Microbial inoculum source	Maximum power density (mW/m^2)*	Treatment efficiency	Energy recovery OR coulombic efficiency	References
Vegetable waste	Single chamber	Enriched mixed culture	172.86	COD – 62.86 %	COD-144.83 J/Kg	Venkata Mohan et al. (2010)
Cattle manure	Three chambers (dual anode)	Enriched *E. coli* and manure leachate culture	93	**	49 KJ/Kg of dry manure CE-5.2 %	Zheng and Nirmalakhandan (2010)
Corn stover hydrolysate	Single-chambered air cathode	Enriched wastewater	331	**	CE-20-30 %	Wang et al. (2009b)
Wheat straw	Two chambers	Enriched wastewater	123	**	CE-37.1 %	Zhang et al. (2009c)
Blue green algae	Single chamber	Pre-enriched microbial community from waste water	PD-144 CD-550 mA/m^2	COD – 78.9 %	CE-28.2 %	Yuan et al. (2011)
Food wastes	Two chambers	Anerobic fermentor effluent	240.3	**	**	Choi et al. (2011)
Food wastes	Two chambers	Ostrich dung enriched with acetate	101	**	**	Choi et al. (2011)
Marine sediments	Two chambers	*Desulfuromonas acetoxidans*	16	**	**	Bond et al. (2002)
Lignocellulosic biomass	Single-chambered air cathode	Sequentially enriched mixed culture	**	COD-83 %	CE-21 %	Catal et al. (2011)
Yogurt waste	Two chambers	Lixiviated composite leachate waste	CD -1450 mA/m^2	**	**	Cercado-Quezada et al. (2010)
Potato waste	Three chambers with central cathodic chamber	Methanogenic activated sludge	**	COD-87 %	0.3 %	Durruty et al. (2012)
				COD-91 %	7.6 %	
				(Anaerobic treatment + MFC)	(Anaerobic treatment + MFC)	

PD Power density, *CD* Current density *CE* Coulumbic efficiency

*with respect to the projected surface area of the anode

**not reported

Table 10.2 A comparative overview of performance of MFCs with different wastewaters

Feedstock	MFC configuration	Microbial inoculum source	Power density (mW/ m^2)*	Treatment efficiency	Energy recovery or coulombic efficiency	References
Chemical wastewater	Two chambers	Anaerobic mixed consortia	CD – 747.96 mA/ m^2	62.90 %	**	Venkata Mohan et al. (2007)
Synthetic wastewater	Two chambers	Selectively enriched anaerobic mixed consortia	222.59 mA/ m^2	74.20 %	**	Venkata Mohan et al. (2007)
Chemical wastewater	Two chambers	Selectively enriched anaerobic mixed consortia	67.48 mW/ m^2	61 %	**	Venkata Mohan et al. (2007)
Agricultural wastewater	Single chamber	Mixed culture	0.015 mW/cm^2	2.9 %	**	Nimje et al. (2012)
Domestic wastewater	Single chamber (air cathode)	Enriched mixed culture	422 mW/ m^2	25.80 %	**	Ahn and Logan (2010)
Winery wastewater	Four single-chambered, cubic-shaped MFC	Wastewater served as both inoculum and substrate	**	65 ± 7 %	18 ± 4 %	Cusick et al. (2010)
Urine	Two chambers	Microflora from activated sludge	8 mA/ m^2	22-30 %	**	Ieropoulos et al. (2012)
Domestic wastewater	Single chamber	Anaerobic sludge	300 mW/ m^2	**	**	Liu et al. (2011a)
Urban wastewater	Single chamber (air cathode)	Effluent from a parent MFC treating synthetic wastewater	1.42 W/m^3	80 %	**	Puig et al. (2011)
Mixed volatile fatty acids	Single chamber	Anaerobic sludge collected from a local wastewater treatment plant	PD = 0.69 W/m^3 CD = 2.62 A/m^3	**	20 ~ 39 %	Teng et al. (2010)
Bad wine	Two chambers	Mixed culture	3.82 W/m^3	**	45 %	Rengasamy and Berchmans (2012)
Palm oil mill effluent (POME)	Two chambers	Mixed culture	622 mW/ m^2	23 ± 1 %	32 %	Jong et al. (2011)
Beer brewery wastewater	Single chamber	Anaerobic mixed consortia	264 mW/ m^2	40-43 %	19.75 %	Wen et al. (2009)

Swine wastewater	Single/two chamber(s)	Wastewater was used as the inoculum	Two chambers: 45 mW/ m^2	92 %	8 %	Min et al. (2005a)
			Single chamber: 261 mW/ m^2			
Chemical wastewater	Two chambers	Selectively enriched mixed consortia	222.59 mA/ m^2	74.2 %	**	Venkata Mohan et al. (2007)
Domestic wastewater	Single chamber	Anaerobic mixed consortia	325.51 mA/ m^2	**	**	Venkata Mohan et al. (2009c)
Chocolate industry wastewater	Two chambers	Activated sludge	CD 3020 mA/m^2	COD – 75 %	**	Patil et al. (2009)

PD Power density, *CD* Current density
*with respect to the projected surface area of the anode
**not reported

site without any purification or isolation steps (Kang et al. 2010). Both solid wastes and liquid waste streams can be potentially treated using MFCs as further elaborated in subsequent sections.

10.4.1 Solid Wastes

The origin of various solid waste feedstocks is dispersed over many domains ranging from marine environment (Bond et al. 2002) to agriculture and domestic sectors (Pant et al. 2010a; ElMekawy et al. 2015). Agricultural residues like corn stover, cattle manure, wheat straw, etc. have been tried as substrate in BESs. The carbon and nitrogen compounds present in cattle manure help in proliferation of microbial communities in MFCs (ElMekawy et al. 2015). Agricultural particulate matter like wheat straw and corn stover is majorly composed of cellulose and hemicellulose which can be actively utilized for bioelectricity generation. The challenge of using such lignocellulosic biomass rests on the inability of the electroactive microorganisms to degrade them directly. Conversion of cellulose into monosaccharides or other low-molecular-weight compounds through hydrolysis (Ren et al. 2007) and hemicellulose into soluble sugars using cellulolytic enzyme treatment or steam explosion process (Zuo et al. 2006) becomes a necessary step to boost the degradation and activity of microorganisms in this case. These hydrolyzed compounds are ideal substrates to support bioelectricity generation. Microbial communities with both cellulolytic and exoelectrogenic activities are necessary to maximize the power output with such agricultural wastes (Rezaei et al. 2009b).

Highly biodegradable food wastes in the form of vegetable waste, yogurt waste, and other edibles are available in surplus due to daily routine of mankind. These can be readily used to tap bioelectrochemical energy due to their rich organic content (Digman and Kim 2008; Li and Yu 2013). Pre-fermentation of these food wastes before feeding them in MFCs can lead to better performances. Studies performed under different operational and experimental conditions using multiple solid residual wastes highlight their promising use in MFCs (Table 10.1). The power densities achieved with such wastes vary from 16 to 331 mW/m^2 accompanied by COD removal efficiencies lying between 62 % and 91 %.

10.4.2 Wastewater Sources

10.4.2.1 Industrial Wastewaters

The effluents from slaughter houses, chemical, brewery, food processing, and other industries are the most sought out substrates in BESs (Katuri et al. 2012; Li et al. 2013a, b). The ease of availability and the necessity to treat these high organic matter-containing high strength effluents have made these wastewaters an ideal fuel source to generate bioelectrochemical energy. Wastewater streams from food processing and beverage industries like brewery, winery, dairy, vegetable, meat, and other food-processing industries are abundant in availability, rich in organic content, and possess high biodegradability (Digman and Kim 2008; Li and Yu 2013; Guo et al. 2014a). The absence of microbial growth inhibiting agents in these wastewaters adds up to an additional advantage. In the frequently used wastewaters in MFCs, COD concentration ranges from 3000 to 5000 mg/L (Zhang et al. 2013a, b; Zhuang et al. 2012). Integrated treatment systems coupled with MFCs have also been employed to treat wastewaters such as palm oil mill effluents to pull down the costs incurred in conventional treatment of the mill wastewaters (Ahmad et al. 2011; Cheng et al. 2010; Leaño et al. 2012). MFCs have been proved to be better treatment systems for animal and chemical industrial wastewaters with COD removal efficiencies ranging from 65 to 92 % (Table 10.2).

10.4.2.2 Domestic and Agricultural Wastewaters

The domestic and agricultural wastewaters are relatively less strong in terms of organic content compared to industrial counterparts (Pant et al. 2010a). The necessity to treat household and sanitary let-offs led researchers and public environmental bodies to focus on treatment systems like BESs. These low-strength wastewaters have been well exploited by several research groups to obtain better energy output than industrial wastewaters. Manure wash water and agricultural effluents are more efficient than agricultural particulate and residual wastes for electricity production in MFCs (Zheng and Nirmalakhandan 2010). Power densities obtained using domestic wastewaters are greater than agricultural and industrial counterparts (Table 10.2). However, the treatment efficiencies obtained using these wastewaters are relatively lower due to lesser COD levels. Table 10.2 gives a comparative overview of the most commonly used wastewaters employed for bioenergy generation in MFCs.

10.5 Challenges of Employing MFCs for Waste/Wastewater Treatment

Though MFC technology seems to be a promising bioprocess system, its utility is limited by various operational and economic challenges. From application's point of view, power density of about 1 kW/m^3 or equivalent current density of 5000 A/m^3 of total anolyte volume or 50 A/m^2 of projected anode surface area, if an average voltage output of 0.2 V is expected to be reachable under load conditions, would be sufficient for long-term and commercially viable applications (Clauwaert et al. 2008). Thus far, maximum current densities ranging from 10 to 25 A/m^2 for milliliter-scale systems and 6 A/m^2 or lower for liter- or higher-scale systems have been achieved with wastewater-fed MFCs (Rabaey et al. 2010a). The challenges associated with the use of MFCs for wastewater treatment are discussed in the upcoming sections.

10.5.1 Scale-Up

The commercialization of bioelectricity production using MFCs has always been a curious and challenging attempt for many research groups. The notable attempts of pilot testing of MFCs were made by three research groups during the last decade. A reactor with total volume of 1 m^3 comprising of 12 modules, each 3 m high, was tested at Foster's brewery in Yatala, Queensland (Australia), by the Advanced Water Management Center at the University of Queensland (Logan 2010). This MFC generated maximum current of 2A/cell at 400 mV voltage with power density of 0.5 W/m^2 of membrane area and 8.5 W/m^3 of reactor volume. Additionally, COD removal of 0.2 kg COD/(m^3 d) was reported in this pilot study (Keller and Rabaey 2011). Researchers at University of Connecticut and their collaborators (Fuss and O'Neill and Hydroqual Inc.) set up a system at a site in the USA (Jiang and Li 2009) treating wastewater, removing up to 80 % of the chemical oxygen demand present at 300–600 mg/L. A large-scale setup for biohydrogen production using MEC technology was constructed at the Napa Wine Company, in Oakville, CA, USA, by Penn State researchers with engineering services by Brown and Caldwell (Walnut Creek, CA, USA). It consisted of 24 modules, each with six pairs of electrodes, and is approximately treating 1 m^3 of wastewater (Logan 2010).

The scale-up of this technology is limited by many factors such as the cost of electrodes, low power densities, and potential losses in long-term operation. Extensive tests including pilot-scale studies are necessary to know the performance of materials at larger scale and their longevity, and at the same time examination of BESs with variations in fuel (wastewater) composition, temperature, and as a function of maintenance (e.g., to control fouling on electrodes) are highly critical (Logan 2010). The economic and operational limitations in bulk-scale systems are discussed in the forthcoming sections. Stacks cells are found to be useful in enhancing the performance, but the problem of voltage reversal due to differences in resistances between stack cells and substrate starvation in cells during operation (Oh and Logan 2007) limits their chance of operating on large scale. It has been found that the voltage reversal can be minimized by avoiding low substrate concentrations (that occur in fed-batch cycling) using continuous flow and by closely matching internal resistances among cells in the stack (Logan 2010).

10.5.2 Operational Limitations

The major operational limitations of pilot-scale MFCs include operating under natural environment; exposure to sun, wind, rain, and insects; low temperature during night time; uneven flow and composition of wastewater; clogging of feed line due to biofilm growth; and lastly difficulty in online potentiometric measurement at large scales (Keller and Rabaey 2011). Partial utilization or degradation of waste in large-scale continuous reactors is another major point of concern. The growth of excessive and unwanted biomass in cathode chambers and biofouling on cathode also affects the long-term performance in pilot-scale MFCs.

10.5.3 Economic Limitations

The major obstacle in bulk-scale production systems is the high costs of electrodes, installation, and operation. Though introduction of current collectors into electrodes (Zhang et al. 2009a; Zuo et al. 2008), chemical treatments, and use of precious metals (Cheng and Logan 2007; Liu et al. 2007b) enhance the power output, the economic constraints restrict their utility. For example, fuel cell grade materials can cost approximately \$1,000/m^2 which is quite expensive (Logan 2010). Cheaper electrode treatment techniques like simple heat treatment of the carbon mesh offer to be economical and sufficient for good energy generation (Wang et al. 2009a) compared to expensive high-temperature ammonia gas treatment which facilitates bacterial adhesion and increases power densities (Cheng and Logan 2007). For scale-up, graphite fiber brush anodes (Feng et al. 2010; Logan et al. 2007; Nielsen et al. 2007) are promising electrode materials. In waste treatment systems, aeration in cathode chamber is a costly affair. Alternate materials like activated carbon with metal mesh current collector are used for oxygen reduction. Cathodes impregnated with metals like iron and nickel displayed power densities ranging from 23 W/m^3 to 36 W/m^3 (Aelterman et al. 2009; Zhang et al. 2009a). But usage of cheaper metals like stainless steel and cheaper binders protects the cathode from corrosion and at the same time improves power densities in bulk-scale systems. Interestingly, biocathode research is catching pace to help developing BESs into large-scale energy-producing units. In attempts to pull the cost further down, removal of membrane separators improved the performance in treatment systems. But, the use of membrane separator is beneficial in scale-up systems as it allows closer electrode spacing and prevents short circuiting which in turn improves power densities on a volumetric basis (Logan 2010).

10.6 MFCs Toward a Sustainable Technology Development

Some strategies that can be used to improve the performance of MFCs are discussed below.

10.6.1 Waste/Wastewater Pretreatment

The nature of substrate and applied organic load affects power generation as well as substrate degradation in MFCs. Majority of solid wastes are highly heterogeneous in nature which hinder rapid metabolic degradation by electroactive microorganisms. Pretreated wastes/wastewaters generated either through fermentation or hydrolysis offer optimum organic content compared to raw substrates. Pretreatment minimizes the activation losses which further enhances the bioelectrochemical activity of the biocatalyst and the process efficiency. It has been reported that pre-fermented food wastes show high catalytic activity and decent current density with added advantage of effective electron transfer. With pre-fermented waste, 47 % higher current density has been obtained than that with untreated waste (Goud and Mohan 2011).

10.6.2 Bioaugmentation

Bioaugmentation majorly finds its application in treatment systems as it accelerates the treatment efficiency of hazardous waste sites or bioreactors for the effective removal of undesired compounds.

Studies found that bioaugmentation was beneficiary in improving the start-up of a bioreactor (Wilderer et al. 1991), to boost reactor performance (Stephenson and Stephenson 1992), to protect the existing microbial community against adverse effects (Venkata Mohan et al. 2009a), to accelerate the onset of degradation process (Bathe et al. 2005; Hu et al. 2008; Park et al. 2008), or to compensate for organic or hydraulic overloading (Chong et al. 1997). It offers a promising strategy to improvise the working of wastewater treating MFCs. It spins around the concept that the active inter-special interactions in a biological system like MFC could aid in efficient electron transfer (Lovley 2006) and consequently enhance the bioelectricity generation. Augmentation with robust and catabolically relevant organisms having specialized and desired characteristics improves the bioprocess efficiency. Nowadays, indigenous wild type or genetically modified organisms are employed as augmenting catalysts (Veer Raghavulu et al. 2012). The alliance between *Brevibacillus* sp. and *Pseudomonas* sp. enhanced the energy outcome and highlighted the higher and effective electron transfer (Pham et al. 2008). The synthesis of mediators by *Pseudomonas* sp. helped its counterpart to achieve extracellular electron transfer. On similar grounds, *Shewanella* sp. has been identified to function as an augmenting agent (Veer Raghavulu et al. 2012) under diverse environmental conditions to assist electron transfer due to its ability to synthesize redox mediators (Fredrickson et al. 2008; Richter et al. 2007; Lower et al. 2007). A stable and higher electrogenic activity throughout the operation was reported in a system augmented with *S. haliotis* (Veer Raghavulu et al. 2012). High potential difference is maintained for a longer period enabling higher electron discharge and reduction in activation losses. Augmented systems have greater functioning than those using individual and mixed consortia (Veer Raghavulu et al. 2012). Syntrophically associated bioaugmented systems can be commercially viable mainly due to its ability to supplement performance parameters like higher power output for longer periods, stable electron discharge throughout operation, and high substrate degradation.

10.6.3 Bioprocess Integration

For a sustainable technology development, neutral-energy operation, cost-effective process, stable performance, high effluent quality to meet water reclamation and reuse requirement, less resource consumption, a low environmental footprint, and good social equity are quite essential (Muga and Mihelcic 2008; Levine and Asano 2004). In the present technological scenario, it is quite challenging to achieve these traits concurrently. Bioprocess integrations offer a fascinating concept to visualize an efficient and sustainable technology for electricity generation and simultaneous waste treatment. Attempts to integrate MFC technology with other bioprocesses generated higher current densities compared to conventional reactors. Incorporating processes like forward osmosis (Zhang et al. 2011b) and activated sludge process (Liu et al. 2011b) with MFC showcased better performance in terms of power/current densities. The use of forward osmosis membrane separator facilitates better proton diffusion with water flux and more electricity. This technology helps in simultaneous wastewater treatment, water extraction from wastewater, and bioelectricity generation. Development of a sophisticated reactor design like anaerobic fluidized bed MFC and bioelectrochemical membrane reactor holds great promise for sustainable and green energy recovery and waste treatment process. Simultaneous integration of multiple treatment processes like an up-flow anaerobic sludge blanket reactor–MFC biological aerated filter (UASB–MFC–BAF) highlights the commercial ability of integrated MFC technology (Zhang et al. 2009a).

10.7 Microbial Electrocatalysis: Latest Advancements and Other Applications

Over the past few years, MFC technology paved way for developing advanced and alternate bioprocesses. Several research groups showed keen interest in investigating its possible integration with other processes mainly due to its innovative features and environmental benefits (Logan et al.

2006; Rabaey and Verstraete 2005; Rozendal et al. 2009). Microbial electrocatalysis has been explored to develop applications for the production of hydrogen (Logan et al. 2008) and other chemicals (Rozendal et al. 2009), resource recovery (Xie et al. 2014), desalination (ElMekawy et al. 2014a), and bioelectrochemical treatment. A brief discussion explaining these advancements is given in the upcoming sections.

10.7.1 Microbial Electrolysis Cells: H_2 Production

Biohydrogen is projected to be crucial player in the nonfossil fuel-based future economy. This green fuel was initially produced using two bioprocesses: converting carbohydrates by fermentative bacteria (dark fermentation) and converting organic acids by photosynthetic bacteria (photofermentaion) (Liu et al. 2010). Microbial electrolysis cells (MECs) are developed to curtail the limitations and challenges of biohydrogen production using these two processes. In this modified MFC system, hydrogen is produced by electrohydrogenesis from acetate or fermentation end products. Bacteria referred as exoelectrogens help in the oxidation of substrate to transfer electrons to anode. The basic difference between MFC and MEC lies in the cathodic reaction. In a MFC, current is produced by the oxygen reduction under aerobic condition at cathode, whereas in a MEC, due to anaerobic condition at cathode, no spontaneous generation of current is possible. Current is spontaneously produced in MFCs due to the higher redox potential of oxygen compared to that of a microbial anode which facilitates easy flow of electrons from anode to cathode. But in MECs, there is no spontaneous flow of electrons because the redox potential of hydrogen reduction, protons to hydrogen, at cathode is lower compared to that of reaction at anode. Thus, a small external voltage is required to the circuit for the reaction to proceed (Logan et al. 2006). MECs were initially referred to as bioelectrochemically assisted microbial reactors (BEAMR) (Logan et al. 2006). Owing to higher hydrogen recovery and wider substrate diversity, biohydrogen production through MECs gained a lot of interest compared to that of the fermentative counterparts. But, the production has been low with domestic wastewaters feeds (Ditzig et al 2007; Wagner et al. 2009). Simultaneous production of methane is one of the major limiting factors in extensive use of MECs for hydrogen production. Several studies have focused on evaluating the simultaneous wastewater treatment capacity of MECs. Treatment efficiencies ranging from 19 to 72 % have been reported when swine wastewaters were used as substrate (Wagner et al. 2009). Reported pilot-scale studies using winery wastewaters displayed 44 % lesser performance compared to lab-scale setups (Cusick et al. 2011). This shows the need for further improvements in developing efficient MEC technology.

10.7.2 Microbial Electrosynthesis

Besides electricity generation and waste treatment, BES has garnered great interest in the field of microbial electrosynthesis. This concept aims at reducing carbon dioxide or waste gases and organic substrates to multicarbon compounds using different terminal electron acceptors (Nevin et al. 2010). The first report of reducing carbon dioxide to acetate and 2-oxobutyrate was exhibited by biofilms of *Sporomusa ovata* growing on graphite cathode surfaces. This field addresses the use of microorganisms to function as biocatalysts on cathodes (i.e., biocathodes) to perform electricity-driven synthesis of chemicals and fuels compounds (Rabaey et al. 2010b; Sharma et al. 2013). Other hydrocarbons such as methane and ethanol have been synthesized using this mechanism (Pant et al. 2012). Extensive research is yet to be done to establish this field on lab and then pilot to commercial scales.

10.7.3 Microbial Desalination

The major limiting factors in large-scale treatment of wastewaters using MFCs are (1) lower conductivity (1–2 mS/cm) and alkalinity (100–

300 mg $CaCO_3$/L) of wastewaters (Rozendal et al. 2008a; Ter Heijne et al. 2006; WEF 2007) and (2) pH variations during operation. The main reason for the pH drop is the relative slower diffusion of protons compared to transfer of electrons which subsequently inhibits microbial activity (Luo et al. 2012). Recent advancements found a way to curtail these limiting factors in the form of a microbial desalination cell (MDC). MDC is a modified version of a MFC with a central desalination chamber separated from anode and cathode chambers with the help of an ion exchange membrane. In MDC, a potential gradient is generated due to typical anodic and cathodic reactions. This gradient generated by the bacteria helps in the diffusion of ions from the central chamber to the adjoining cathode and anode chambers (Cao et al. 2009) thereby increasing the ionic strength and the conductivity of the wastewater. For example, the effluents of oil and gas production industries are a rich source of bicarbonate ions (Benko and Drewes 2008). The transfer of these bicarbonate ions during desalination to anode chamber increases the alkalinity of wastewater which further enhances the treatment efficiency and bioelectricity production. A study found that an MDC can improve the power density by four times, COD removal by 52 %, and coulombic efficiency by 131 % (Luo et al. 2012) clearly highlighting an integrative and promising approach for wastewater treatment with simultaneous energy production and desalination.

10.7.4 Bioelectrochemical Treatment of Pollutants

In an advancing approach, BESs are being explored for bioelectrochemical treatment of various organic, inorganic, and aromatic compounds alongside conventional COD reduction.

The shift of focus toward these systems from conventional treatment methodologies is primarily attributed to the synthesis of toxic by-products, operational, and economic constraints of existing physicochemical remediation approaches. Moreover, biological treatment systems are relatively 5–20 and 3–10 times cheaper in terms of capital and operational costs, respectively, than advanced oxidation methods (Marco et al. 1997). In these systems, several chemotrophic and heterotrophic species, primarily present in mixed culture, actively degrade pollutants and environmentally hazardous compounds by both cathodic reduction and anodic oxidation reactions (Mohanakrishna et al. 2015). The anaerobic treatment at cathode requires an organic co-substrate (electron donor) to create reductive conditions in the cathodic chamber. The resultant-reduced products are found to be eco-friendly and harmless. Several investigations showcased the ability of BESs to treat perchlorates (Thrash et al. 2007), sulfides (Rabaey et al. 2006), nitrates (Clauwaert et al. 2007; Virdis et al. 2008), nitrobenzene (Mu et al. 2009a), azo dyes (Mu et al. 2009b; Ding et al. 2010), and chlorinated organic compounds (Aulenta et al. 2007). These compounds, in the absence of oxygen, perform the role of electron acceptors to accomplish terminal electron reduction which further facilitates their remediation in BESs (Mohanakrishna et al. 2015). Other compounds like sulfur and estrogens are treated at the anode (Chandrasekhar and Venkata Mohan 2012; Kiran Kumar et al. 2012). Nitrogen-rich effluents are treated using simultaneous nitrification-denitrification technique (Zhang and He 2012). In azo dye degradation process, the thick color is removed due to the formation of colorless amines (Frijters et al. 2006).

10.8 Conclusions and Future Prospects

Waste to bioenergy conversion offers a promising way to tackle the energy sustainability and waste management issues. This chapter summarized the ability of BESs such as MFCs to generate bioelectricity from different wastes and wastewaters with simultaneous waste treatment. Though the technology seems advantageous in terms of waste treatment, the magnitude of energy recovery is still the major point of concern. It is challenged by various constraints and limitations for large-scale applications. Investigations aimed at enriching desirable electroactive microbial communities could help developing MFCs into a competitive technology. Complexity of wastes, expensive electrode materials, and complex reactor architectures are the domains which seek further technological advancements for better outcome. Waste pretreatment, bioaugmentation, and bioprocess integration are viable options for improvising wastewater treatment using MFCs. To achieve a commercially viable and eco-friendly technology, research should be carried on integrating MFCs with other bioprocess like fermentation, desalination, bio-product recovery, and metal recovery. Research focuses in the direction of operating MFCs at higher loading rates with cheaper electrode materials, and simplified reactor designs are important in order to realize the practical applications of MFCs for tapping energy from the wastes and wastewaters. The future for BESs seems promising in wastewater treatment sector as some of these technologies are currently under trial or operational at larger scales. These include production of methane from wastewater using MECs (Cambrian Innovation Inc., 2013) and energy-efficient wastewater treatment with MFCs (www.emefcy.com).

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Jai Sankar Seelam Jai Sankar Seelam (B.Tech, 2014) graduated with a Bachelor's degree in Biotechnology and Biochemical Engineering from the Indian Institute of Technology (I.I.T), Kharagpur. He is currently enrolled in MSc – Water Technology (joint degree) at Wetsus Academy, Leeuwarden, the Netherlands for the academic session 2015–2017. He worked as a guest researcher at VITO NV, Belgium, in summers of 2013 and 2014. His research interests include wastewater treatment and energy recovery from wastes and wastewaters.

Deepak Pant Deepak Pant (Ph.D., Environmental Biotechnology, 2007) is a senior scientist at the Flemish Institute for Technological Research (VITO), Belgium, currently working on bioenergy, specifically, the design and optimization of bioelectrochemical systems for energy recovery from wastewater and microbial electrosynthesis for production of value-added chemicals and fuels through electrochemically driven bioprocesses. He has one book (published by Springer), 52 peer-reviewed publications with 2300 citations (h-index 24), and 15 book chapters to his credit. More details: be.linkedin.com/pub/deepak-pant/b/b44/942/

Sunil A. Patil Sunil A. Patil (PhD, 2011) is currently working as a postdoctoral researcher at the Laboratory of Microbial Ecology and Technology (LabMET), Ghent University, Belgium. His main research areas include microbial bioelectrochemical systems for energy recovery from wastewater, microbe-electrode interactions, and electricity-driven bioproduction of value-added chemicals and fuels from CO_2. He has 24 peer-reviewed publications and five book chapters to his credit.

Balasaheb P. Kapadnis Balasaheb P. Kapadnis (PhD, 1981) is currently working as an emeritus professor at the Department of Microbiology, Savitribai Phule Pune University, Pune, India. His main research areas include environmental microbiology with reference to biological management of pathogens and value addition to waste. He has 75 peer-reviewed publications and 10 book chapters to his credit.

11 Regulation of Lignin Biosynthesis Through RNAi in Aid of Biofuel Production

Archana Kumari, Vinod Kumar Nigam,
and Dev Mani Pandey

Abstract

The plant cell walls, which contain lignocelluloses, are one of the major sources for biofuel production. However, the process of conversion of lignocellulose to biofuel is very costly due to the high cost of biomass pretreatment for removal of lignin. Although, there is huge information about the lignin synthesis, their major roles in plant biology, specifically for the lignocellulose metabolism, need to be understood. Through various biotechnological processes, lignin content could either be reduced or the modification of its composition as well as structural arrangement could be made. Genetically modified plants can have a great advantage to decrease their recalcitrance without affecting their product, indicating a successful cell wall modification that may eventually lead to cost-effective biofuel production. Here, we have tried to summarize our information with respect to lignin biosynthesis and also discussed how lignin can be altered through RNAi for cost-effective biofuel production.

11.1 Introduction

Biotechnology has a major role in plant improvement and it also plays a pivotal role in biofuel production. Demand of energy has tremendously increased due to rapidly growing world population (Ragauskas et al. 2006; Gressel 2008) and also due to the high fluctuation in the global market, which makes it necessary to improve or develop new sources of energy to compensate the high energy demands. From last few decades, biofuels are replacing the demand on fossil fuels and are considered as renewable energy sources (Sánchez and Cardona 2007; Li et al. 2008). Up to the present time, the production of biofuel in the most part of the world, especially in developed countries, has been mainly dependent on some crops like maize and other crops. Biofuels, produced using these sources, are known as lignocellulosic biofuels, which are also represented as a substitute source of fuel for the future (Koonin 2006). Actually, lignocellulosic biomass has a

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A. Kumari • V.K. Nigam • D.M. Pandey (✉)
Department of Bio-Engineering, Birla Institute of Technology, Mesra, Ranchi 835215, Jharkhand, India
e-mail: archana.bioinfo87@gmail.com; vnigam.bisr@gmail.com; dmpandey@bitmesra.ac.in

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huge potential for biofuel production on a very large scale, where complete biomass would be changed to biofuels. Some crop plants for lignocellulosic biomass production are *Panicum virgatum* L. (switchgrass), *Miscanthus*, and *Populus* spp. (poplar), and these candidate crops have various eco-friendly and other advantages over nonrenewable fossil fuels along with annual crops (Gray et al. 2006; Perlack and Stokes 2011).

The major advantage of perennials is their capacity to re-grow every year after harvest, which helps for proper timing, and also their dynamism used for replanting each and every season. Also, they have the capacity to translocate carbohydrates as well as minerals into belowground for the storage purpose at the dormancy stage (Propheter and Staggenborg 2010). The secondary cell walls (SCW) of vascular plants are made up of three major components, i.e. cellulose, hemicellulose, and lignin, also known as lignocellulosic biomass. For efficient biofuel production, it is necessary to break down this complex network. There are three major steps involved in cellulosic biofuel production: (1) biomass pretreatment, (2) hydrolysis process and saccharification, and (3) fermentation of sugars into ethanol. Mostly, pretreatment of biomass is processed using acid or steam, which helps to release the polysaccharides, using various enzymes (especially cellulase and hemicellulose). These multifaceted polysaccharides are easily transformed into modest sugars, and finally these sugars are converted into ethanol using microbial fermentation process. During the pretreatment process, lignin has capacity to restrict the release of polysaccharides and also restrict the enzymatic action in the course of alteration. High use of acids during saccharification decreases its effectiveness (Keating et al. 2006; Novo-Uzal et al. 2012); this leads to the costly cellulosic biofuel production (Sticklen 2006). The best solution for this problem can be achieved by reducing the lignin content in plant biomass for biofuel production (Bouton 2007). Definitely, this goal can be achieved, but it may take a long time; whereas the modern era of biotechnology provides various techniques for cheap biofuel production (Gressel 2008).

Various biotechnological approaches mostly target the upregulation of cellulose pathway and hemicellulose pathway enzymes, which are involved in increasing plant biomass (Mosier et al. 2005; Ransom et al. 2007). Also, some approaches will directly or indirectly help to decrease the saccharification efficiency only because of lightning, or it may help to diminish the enzymatic processes during saccharification (Chapple et al. 2007; Chen and Dixon 2007; Sticklen 2008). The conversion process is very costly for lignocellulose to sugar because it includes the costs of biomass refinement, its collection, transportation process, storage, etc. The process of pre-treatment itself can be considered to cost about ~21 % of the overall investment (Chen and Dixon 2007). For the cost-effective generation of biofuels, researchers have mainly considered the optimization process of pretreatment, hydrolysis processes, and fermentation. The various aspects for regulation of lignin biosynthesis through RNAi in aid of biofuel production will be discussed one by one (Fig. 11.1).

11.2 Biosynthesis of Lignin

Various scientific reports have confirmed that the adaptation processes of numerous plants (especially vascular plants) to terrestrial habitats were promising only due to the lignin, which gives mechanical support and other associated characters (Jones et al. 2001). Previously, it was said that only vascular plants contain lignin, but recently it has been revealed also in the bryophyte (Espineira et al. 2011). In nature the second most abundant polymer is lignin. This lignin works as intramolecular and intermolecular bonding agent, as well as plays an essential role in providing rigidity to SCW and the plant body (Novo-Uzal et al. 2012). It is especially designed by oxidative coupling. In these oxidative couplings, 3 p-hydroxyphenylpropanoids result in the formation of p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units (Novo-Uzal et al. 2012). During the last few decades, the lignin biosynthetic pathway has become an important field for researcher especially for genetic and

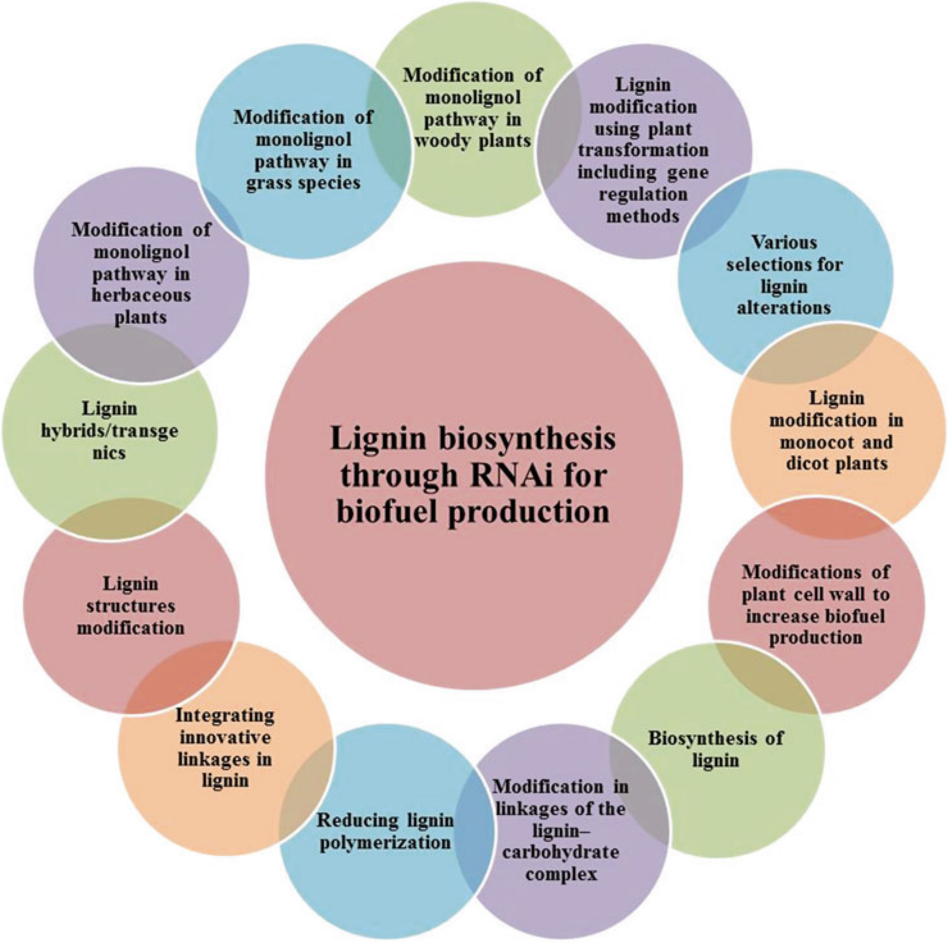

Fig. 11.1 Various aspects of regulation of lignin biosynthesis through RNAi in aid of biofuel production

transgenic studies. The lignin biosynthetic pathways in higher plants have been widely studied (Hisano et al. 2009). Various research works have suggested that the mentioned biocatalysts are essential, especially for monolignol biosynthesis (Fig. 11.2). Enzymes are as follows: phenylalanine ammonia-lyase, C4H, 4CL, CCR, hydroxycinnamoyl-CoA-shikimate, HCT, C3H, CCoAOMT, F5H, COMT, and CAD (Boerjan et al. 2003; Rogers and Campbell 2004). Genes responsible for lignin regulation have been studied in many plants including dicot as well as monocot plants. Due to availability of huge amounts of information regarding the genes which control the lignin biosynthesis and by using newly developed plant transformation methods, nowadays numerous methods are used to alter lignin content in biofuel crops. Alteration can be done by overexpression or down regulation of genes which controls the lignin synthesis, its regulation, and its polymerization (Chen et al. 2011). Generally, decrease or increase in lignin content depends on the type of gene which is involved in its regulation. For instance, genes having up-regulation like C3H and some other genes showed the down regulation to reduce the lignin content, whereas S/G ratio is affected due to the down regulation of F5H and COMT (Weng et al. 2008; Zhou et al. 2009). Numerous discoveries stated that some model plants like tobacco and some other plants like *Arabidopsis* (Weng et al. 2008; Zhou et al. 2009) can be useful for

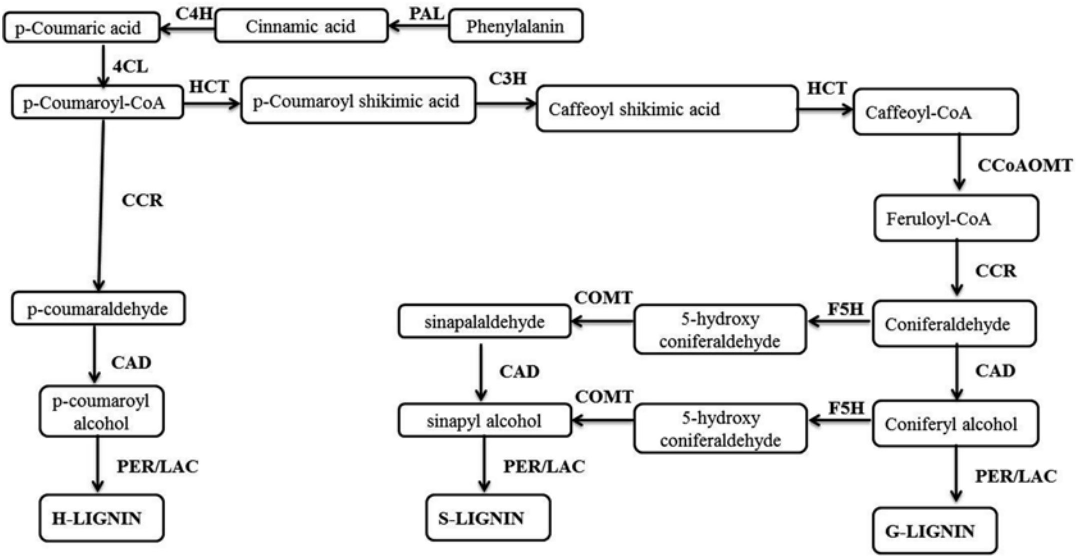

Fig. 11.2 Overview of lignin biosynthetic pathway. The key enzymes involved in this pathway are as follows: PAL-L-tyrosine ammonia-lyase; C4H-trans-cinnamate 4-monooxygenase; 4CL-p-hydroxycinnamic acid:CoA ligase; CCR-p-hydroxycinnamoyl coenzyme A reductase; HCT-4-coumaroyl-CoA:shikimate O-(hydroxycinnamoyl) transferase; C3H-coumarate 3-hydroxylase; CCoAOMT-caffeoyl coenzyme A methyltransferase; F5H-ferulate 5-hydroxylase; COMT-S-adenosyl-L-methionine:caffeic acid-O-methyltransferase; CAD-cinnamyl-alcohol:NADP+ oxidoreductase; PER-peroxidase; and LAC-laccase

cellulosic feedstock crops because as we know the lignin pathway is conserved in various plants.

In dicot plants, G and S monolignol units compose the lignin polymer with a little amount of H units. Similarly, in monocot lignins, H units are in higher proportion as compared to the dicot (Boerjan et al. 2003; Chen et al. 2014). There are two major steps involved in lignin biosynthesis: first is biosynthesis of monolignols and the second is their polymerization. In angiosperm taxa the monolignol biosynthesis pathway is highly conserved. The deamination process of phenylalanine to cinnamic acid by L-tyrosine ammonia-lyase (PAL) and subsequent conversion into p-coumaric acid through trans-cinnamate 4-monooxygenase (C4H) are the preliminary stages in the monolignol biosynthesis. During this bioconversion, p-coumaric acid goes through a progressive hydroxylation and ethylation reactions which initiate the final synthesis of the monolignols (Humphreys and Chapple 2002; Boerjan et al. 2003). A number of monolignols are the key factors of the lignin structure, and these factors are directly associated with the species, their cell type, and their developmental stages.

11.3 Modifications of Plant Cell Wall to Increase Biofuel Production

Generally development of cell wall in plants involves the deposition of polysaccharides and also involves the production of complex network of polysaccharide (Boerjan et al. 2003; Cosgrove 2005). For providing mechanical support and to form new sclerenchyma cells, SCWs are formed by some particular type of cells (Evert 2006). Mostly, SCW is a key resource of biomass for biofuel production due to the high content of cellulose, hemicelluloses, and lignin. For the better understanding about the mechanism of SCW formation, various approaches have been made (Boerjan et al. 2003). Using various biotechnological approaches, especially by RNAi method, the monolignol biosynthesis pathways can be modified.

11.4 Lignin Modification in Monocot and Dicot Plants

Plants have potential for biofuel production, including monocot along with the dicot species, e.g. perennial grasses such as switchgrass and sugarcane in monocot and in dicot poplar and alfalfa, respectively. The cell wall composition is different in both monocot and dicot species which differ from each other with respect to the types and structures of polysaccharides, which are present, and how they are interlinked in the presence of great quantities of phenolics and amino acids (Shedletzky et al. 1992; Vogel 2008). The comparative arrangements of the non-cellulosic constituents (particularly pectin and hemicellulose) have a propensity for two. Both dicot and monocot species cell walls are made up of Type I CWs, whereas microfibrils of cellulose are interlinked with xyloglucans and enclosed through the complex network of pectins and proteins. On the basis of a cross-linking pattern of glucoarabinoxylans, Type II cell walls are characterized, which hold huge amounts of phenolic acids (McCann and Carpita 2008; Vogel 2008). In spite of the differences in composition and structure, the lignin biosynthesis pathway is said to be preserved in various plants, and their subsequent growth in lignin alteration can be perceived in monocots as well as in dicots (Yuan et al. 2008; Fu et al. 2011). Some examples are best to explain these things, for example, the manipulation process of lignin in alfalfa (Jung et al. 2012) has also been able to transfer to other plants like switchgrass and sugarcane.

11.5 Various Selections for Lignin Alterations

Various genes responsible for the lignin biosynthesis have been recognized. Numerous enzymatic conversion processes are responsible for monolignol synthesis (Weng and Chapple 2010). In these processes, each and every stage is demonstrating targets for manipulation of lignin due to down regulation of genes by some enzymes responsible for the catalyzation. Modifying the process of monolignol polymerization is another way for modification of lignin (Weng et al. 2008; Vanholme et al. 2008, 2012). An alternative method for modifying lignin synthesis is modification of regulators of lignin biosynthetic genes. Numerous TFs during SCW formation control the differential expression of monolignol biosynthesis genes (Weng et al. 2008; Zhong et al. 2008, 2010), where some can be modified in such a way that they help to decrease the flow of carbon into lignin biosynthesis (Vanholme et al. 2012). MicroRNAs or miRNAs have been considered the most modern approach for the regulation of plant development and regulation of lignification (Fu et al. 2012; Rubinelli et al. 2013). miRNA approach can also help to increase bioenergy-related traits. Clearly, there are lots of biotechnological methods that can be utilized for the alteration of lignin in cell walls. Currently, modern technology provides a brief idea about the lignin biosynthesis (Fornale et al. 2010; Shen et al. 2012; Rubinelli et al. 2013). Using RNAi gene silencing method, a number of monolignol biosynthetic genes have been identified and show down regulation in commercially significant bioenergy harvests. This is also helpful for the better understanding of their consequence on lignin biosynthesis and saccharification (Table 11.1). Improvement has been made for the identification and characterization of those genes which regulate or might influence essential bioenergy features, for instance, biomass yield and biofuel production (Bonawitz and Chapple 2010; Rubinelli et al. 2013).

11.6 Lignin Modification Using Plant Transformation including Gene Regulation Methods

The advanced plant transformation method and gene regulation shows an important role in lignin modification. It also controls the organization of a well-structured and effective transformation system for unique crops, which is very potent for manipulating lignin biosynthetic genes in order to alter biomass quality or quantity (Wang and

Table 11.1 RNAi modification in lignin biosynthesis pathway in plants

Plant	Gene	Lignin manipulation		References
		Lignin content	S/G ratio	
Switchgrass	4CL	↓ ~18–32 %	↓	Xu et al. (2011)
Switchgrass (greenhouse grown)	COMT	↓ ~ 6–15 %	↓	Fu et al. (2011)
Switchgrass (field grown)	COMT	↓ ~ 7–15 %	↓	Baxter et al. (2014)
Sugarcane (greenhouse grown)	COMT	↓ ~ 4–15 %	↓	Jung et al. (2012)
Sugarcane (field grown)	COMT	↓ ~ 6–13 %	↓	Jung et al. (2013)
Switchgrass	CAD	↓ ~ 14–21 %	↓	Fu et al. (2012)
Maize	CAD	↓ ~ 6.4 % in midribs	↓	Fornale et al. (2010)
Switchgrass	MYB4	↓ ~ 40–52 %	↓	Shen et al. (2012)

Ge 2006). Up to the present time, genomic changes in plants have been implemented using two key approaches: a) *Agrobacterium*- mediated alteration and b) particle bombardment (Ishida et al. 1996). The first method, i.e. *Agrobacterium* method, is mostly used for dicot plants, for example, alfalfa, poplar, and tobacco, as these plants are natural hosts for *Agrobacterium*. Subsequently wide research confirmed that the *Agrobacterium* technique can also be used for various monocot plants (Somleva et al. 2002; Gressel 2008). Previous studies showed that grass transformation was based on particle bombardment. Further researches proved the benefits of *Agrobacterium-mediated* transformation; it is one of the best methods of choice for transforming biofuel crops (Wang and Ge 2006; Wang et al. 2012).

Specifically, for the lignin gene modification knockdown and other methods can be used (Lu et al. 2003). At first, decreased lignin content in plants was recognized from various natural mutants. But, further due to improvement of genomic alteration modus operandi, RNA interference (RNAi), virus-induced gene silencing (VIGS), and some other methods have been used for gene silencing (Capell and Christou 2004; Chen et al. 2006). RNAi is also known as antisense method. RNAi is a direct method of alteration in a particular gene makeup. In this case, the expressed gene gives a small dsRNA homologous of the target gene sequence (Wesley et al. 2001; Miki et al. 2005). This method has been successfully used for altering many plant characters by targeting down regulation of the given gene (Wesley et al. 2001). Sometimes, overexpression of target genes helps in cosuppression leading to the endogenous gene silencing. Therefore, antisense or RNAi method possibly will not able to completely eradicate gene expression; sometimes this procedure is stated as "knockdown" to discriminate it from "knockout" processes wherein transcription of a particular gene is totally abolished. RNAi is one of the modern approaches in today's science, also known as one of the major tools for studying gene functions in plants using genetic engineering (Lu et al. 2003; Dixon et al. 2007).

11.7 Modification of Monolignol Pathway

11.7.1 In Woody Plants

Lignin biosynthesis process is very particular with respect to woody plants, and it also provided essential lignification information based on the genetic as well as biochemical characteristics (Li et al. 2006). Differentiation of xylem mainly due to the regular cell division in the vascular cambium is considered as a basic process of wood formation. The daughter cells, which are obtained from mother cells of xylem, further go for various processes, including cell division, subsequent cell expansion as well as thickening of the cell wall (CW), and programmed cell death (Evert 2006).

Lignin constitutes about to 21 % of the dry weight of wood as a major SCW component. The S/G ratio in *Populus* is nearly two, and also various studies supported lignin biosynthesis by woody species only because they have socioeconomic value as raw materials (Hu et al. 1999). Manipulation of monolignol biosynthetic genes has allowed the amendment of lignin content and its composition (Wagner et al. 2009; Min et al. 2012). 4CL acts as an intermediate for the activation of hydroxyl-cinnamates for monolignol biosynthesis (Fig. 11.2). 4CL gene shows the down regulation in *Populus tremuloides* which resulted in approximately ~46 % decrease in lignin, with S/G subunits decreased. Consequently cellulose content improved by approximately 15 % using RNAi in the gymnosperm (*Pinus radiata)* (Wagner et al. 2009). 4CL showed the repression which causes a decrease in lignin content, nearly about 37 %; on the other hand, an increase of the polysaccharide was detected for galactan (Lu et al. 2010). Scientists showed 4CL gene silencing using RNAi in *Populus trichocarpa*, where hydrolysis done byseveral enzymes which indicates the rise of the saccharification efficiency to nearly about 65 % of the transgenic wood where 4CL gene still downregulated (Lu et al. 2010). CAld5H acts as a catalyst for the hydroxylation and is one of the main enzymes for S monolignol production (Franke et al. 2000; Osakabe et al. 1999; Li et al. 2003). The RNAi technique is used for antisense gene of 4CL and CAld5H, and overexpression in unsteady aspen gives about 53 % decrease in lignin content and nearly 65 % rise of lignin S/G ratio (Fig. 11.2) (Stewart et al. 2009).

Cinnamoyl-CoA reductase acts as a catalyst for the alteration of cinnamoyl-CoA esters to their consistent cinnamaldehydes during the monolignol biosynthesis pathway. In some hybrid plants, CCR gene shows the down regulation because of RNAi; therefore, the lignin content was also decreased by nearly 51 %, and this hybrid had well-developed pulping efficiency (Franke et al. 2000; Lu et al. 2013). Due to the down regulation of CCR, cellulose in poplar was not increased, but it helps to improve the saccharification yield because of the huge amount of cellulose conversion (Leple et al. 2007; Wadenback et al. 2008). Again, due to the CCR down regulation, the field-grown poplar had an approximately 160 % higher ethanol yield as compared to the wild type (Wagner et al. 2013). On the other hand, in *P. radiata*, reduction of lignin content to nearly about 47 % was found in a CCR RNAi line (Wagner et al. 2013; Van Acker et al. 2014). The former phase of monolignol biosynthesis is catalyzed by cinnamyl alcohol dehydrogenase, which converts hydroxycinnamyl aldehydes to alcohols.

11.7.2 In Grass Species

Grasses like *P. virgatum*, *Miscanthus sinensis*, *Oryza sativa*, and *Zea mays* are considered as cellulosic bioenergy crops (Parrish and Fike 2005; Rubin 2008), whereas other grasses like switchgrass have the capacity to grow well on marginal land (Heaton et al. 2008), and, as a result, it does not contend for cropland (Schmer et al. 2008). COMT and other related genes have also been categorized in switchgrass (Parrish and Fike 2005), and researchers also recognized some other genes which play an essential role in the regulation of monolignol biosynthesis (Shen et al. 2013).

Down regulation of COMT by RNAi helps to decrease the lignin content about 25 % and also it decreases the lignin S/G ratio from 0.70 to 0.38, in transgenic switchgrass lines (Fu et al. 2011). As compared to the wild type, ethanol yield in switchgrass was nearly about 39 % higher due to down regulation of COMT (Fu et al. 2011). It has been shown that 2-year-old, field-grown COMT RNAi switchgrass plants had interconnected release of sugar and ethanol compared to those which were grown in a greenhouse. And they did not confirm any undesirable effects on yield of biomass or vulnerability to disease, which confirmed that the COMT gene with RNAi effect in switchgrass may be exploited in bioenergy synthesis at very low expenditure (Fu et al. 2011; Xu et al. 2011). There was about 80 % reduction of plant protein extract due to RNAi gene silencing of the 4CL in switchgrass; it also leads to decrease in lignin content by nearly about 33 % in a severe knockdown line (Saathoff et al. 2011; Baxter et al. 2014).

Another process, i.e. saccharification, was studied using two *Brachypodium distachyon* mutants at different positions; it also has point mutations in the CAD gene and is found to have the capacity to increase the saccharification efficiency (Tu et al. 2010). Also, one of the good examples is *Lolium perenne*, a type of grass, in which alterations of lignin synthesis pathways by RNAi control the down regulation of the CCR and COMT genes, increasing the enzyme digestibility (Sattler et al. 2010; Trabucco et al. 2013). The leaves and other plant parts of sorghum, rice, and sugarcane can be used as feeds for producing biofuels. Vascular bundles are present in stalks which are made up of fibre cells and several thick-walled vessels. Some brown *midrib* (*bm*) like sorghum mutants and maize mutants have decreased the lignin content in which several have been characterized as COMT or CAD mutations (d'Yvoire et al. 2013). As a consequence of mutation of sorghum, COMT and CAD increase the conversion efficiency, i.e. cellulose to ethanol, to about 23 and 22 %, respectively. The increase was very high in the double mutant. In sugarcane saccharification efficiency improved by ~29–31 % due to COMT RNAi gene silencing method. Due to the presence of this gene transgenic plants can easily grow in a glasshouse or in the ground (Petrik et al. 2014). The grass has significant amounts of p-coumarate (pCA), whereas p-coumaroyl-CoA:monolignol transferases (PMTs) have been recognized as biocatalyst having the ability for acylating monolignols with p-CAs in *B. distachyon* and *O. sativa* (Saballos et al. 2008; Ralph 2010; Marita et al. 2014). Consequently, the inactivation of BdPMT in *B. distachyon* initiated a decrease of p-coumarate yield, whereas in *Z. mays*, RNAi of p-coumaroyl-CoA:hydroxycinnamyl alcohol transferase (pCAT) accounts for the creation of pCA-monolignol conjugates, which also caused the reduction of pCA levels (Dien et al. 2009; Jung et al. 2012, 2013). They have the capacity to produce lignin with very low quantity of pCA and higher amount of monolignol conjugates, which could be chopped by some chemical procedures like acidolytic or alkaline methods, and responsible for a great approach for developing saccharification efficiency especially in grasses (Withers et al. 2012).

11.7.3 In Herbaceous Plants

High biomass can be produced by perennial herbaceous plants (Reddy et al. 2005). Alfalfa plant can also be used for biofuel production. This plant has four enzymes, i.e. CAD, CCR, C4H, and COMT, which control the regulation of monolignol pathway (Bhattarai et al. 2013). The connections among lignin content including their chemical structure as well as enzymatic saccharification for generating bioenergy were inspected via recombinant alfalfa lineages that were down-regulated individually for six lignin biosynthetic enzymes C3H, CCoAOMT, COMT, C4H, F5H, and HCT (Guo et al. 2001; Zhao et al. 2013). There was a very high negative correlation between lignin and released carbohydrates using enzymatic degradation. In HCT the maximum saccharification efficiency was observed, where it ensured the minimum lignin content. Xylan contained extra sugars in transgenics as compared to the wild type, which indicates that lignin alteration too alleviates the convenience of residual hemicellulose to hydrolytic enzymes (Shadle et al. 2007; Dien et al. 2011). Additionally, the alfalfa COMT hybrid, which has the capacity to reduce S lignin, was also examined for ethanol production (Vermerris and Mclntyre 1999; Zhao et al. 2013).

11.7.4 Lignin Hybrids/Transgenic

As we know, lignin controls the growth and development of the plant (Pilate et al. 2002; Novaes et al. 2010; Gallego-Giraldo et al. 2011a, b). Genes are responsible for controlling the monolignol pathway as down regulation of C3H, CCR, and HCT resulted in a dwarf phenotype apart from CAD gene. The decreased biomass produced less ethanol, e.g. down regulation of CCR in hybrid poplar had approximately 161 %

more ethanol production as compared to the wild type (Voelker et al. 2010). The retardation in growth may be only caused by the variation of the salicylic acid (SA) level or may be because of the agitation of the auxin signal (Van Acker et al. 2014). In *bm2* mutant of *Z. mays*, low levels of lignin were observed, where flowering, for instance, is longer as compared to the wild type. Newly developed map-based cloning confirmed that concerned gene (*bm2*) governs the expression of a methylene tetrahydrofolate reductase, which is responsible for the biosynthesis of SAM (Gallego-Giraldo et al. 2011a, b; Bonawitz et al. 2014). This research delivers one of the best examples to circumvent lignin biosynthetic genes for producing new recombinant plants with low lignin; however, these transgenic plants are not disturbing the agronomic performance of the field (Sticklen 2006).

In transcriptional coregulatory complex, some genes like MED5a and MED5b control the homeostatic suppression of phenylpropanoid biosynthesis in a model plant (Bonawitz et al. 2012; Tang et al. 2014). Modification of these genes in a model plant leads to C3H missense mutant, which ultimately decreased epidermal fluorescence 8-1 and also helping to save the underdeveloped growth and lignin insufficiency, as well as it also provided fresh lignin which consists totally of p-hydroxyphenyl lignin subunits. Modification of the intermediates in the transcriptional level and in signalling pathway level resulted in a strong response with respect to cell wall defects which may be responsible for an additional prospective for reducing biomass recalcitrance.

11.7.5 Lignin Structure Modification

Even though various scientific approaches for lignin reduction are due to gene silencing, monolignol pathway genes in a number of species confirmed a well-defined methodology for increasing the biofuel production and also to enhance the saccharification efficiency. A large number of strategies have been tested for modifying the lignin structure affecting the growth of plant and also to support those materials which are more easily digested (Wilkerson et al. 2014).

11.7.6 Integrating Innovative Linkages in Lignin

For producing novel linkage in lignin, it may be polymerized via isolated CW of maize and added lignin precursors, such as the combination of ferulate is well matched with usual lignification reactions where it allows its integration into lignin polymers (Elumalai et al. 2012). CWs which contain coniferyl ferulate esters have the capacity to give ester inter-unit connections which help to enhance alkaline delignification (Grabber et al. 2008) and also enzymatic degradation, due to the fact that ester linkages are easily chopped by alkali. Increased delignification was detected as soon as epigallocatechin gallate (EGCG) was integrated into lignin, so EGCG-lignified walls produced ~35 % extra glucose and overall complete sugars (Ralph 2010). Another example of chemically labile ester bonds; when these were brought together for the lignin support in crossbreed poplar, i.e. *P. alba* x *P. grandidentata*, develop a new conjugate. Reason behind that is due to the overexpression of an M. sinensis gene, which is responsible for encoding feruloyl-coenzyme A: monolignol transferases (FMT), catalyze the development of new conjugate. The advanced scientific approach to develop these types of transgenic plants shows a new line of attack to increase plant biomass for biofuel production (Wilkerson et al. 2014).

11.8 Reducing Lignin Polymerization

The enzyme hydroxycinnamoyl-CoA hydratase-lyase (HCHL) has a capacity to cut the propanoid side chain of HC lignin precursors and as a result it provides monomers. These monomers, combined with lignin polymers, lead to the non-existence of propanoid side chains and conjugated double bonds. It also helps for incapacitating them from additional polymerization by means of other monomers (Eudes et al. 2012). Further, the overexpression of *Pseudomonas fluorescens* HCHL in plants triggered the growth of a side chain monomer, which leads to reduction in lignin polymerization (Mayer et al. 2001). SCW-specific promoter derives a HCHL gene in *Arabidopsis* plants which regulates the overexpression in normal phenotype in comparison to tobacco plants stating CaMV-35S promoter determined HCHL, which were dwarfed and germ-free. In model plants, overexpression of HCHL helps to moderate lignin polymerization and had more saccharification efficiency, nearly about ~71 % rise over wild type (Mayer et al. 2001; Eudes et al. 2012).

11.9 Modification in Linkages of the Carbohydrate Complex and Lignin

The carbohydrate complex and lignin follow the similar processes as lignin polymerization (Capanema et al. 2004). For the formation of a complex in flexible environment, carbohydrate complexes and lignin tie the xylan and polymers of cellulose to combine with lignin in the SCW of angiosperms. As a result, SCW formed a multilayered arrangement which is a complex of adjacent cell walls to produce an inflexible matrix of the three polymers (Schniewind and Berndt 1991; Balakshin et al. 2007). Its intense flexibility and very compressive power act as key obstacles for biofuel production and affect the enzymatic hydrolysis of cellulose. There are some unique linkages in xylan with respect to lignin i.e. the primary hydroxyl group of esters are present side by side with respect to the uronic acid of the MeGlcA in xylan (Bromley et al. 2013). Mutations of GUX1 and GUX2 lead to reduced MeGlcA, which also affects the connection between xylan and lignin (Watanabe and Koshijima 1988; Mortimer et al. 2010). Disruption of the ester bond of the ferulate arabinoxylan composite revealed the increased CW destruction, whereas the overexpression of an *Aspergillus niger* ferulic acid esterase in the apoplast and endoplasmic reticulum of tall fescue improved sugar discharge too much as compared to wild type (Buanafina et al. 2010).

11.10 Aftermath of Lignin Modification

As described above, there are a large number of genes that can be modified in such a way that it can alter lignin biosynthesis pathway. Also, it helps to increase the saccharification efficiency and, in this manner, provides huge amount of biofuel per mass of feedstock. On the other hand, some negative effects which are connected with disturbing lignin levels in plants, for example, in hybrid switchgrass overexpression of PvMYB4, lead to low lignin (Bi et al. 2011). In vascular plants, due to cell wall collapse, water conductance gets affected and this might be one of the major reasons of dwarfism in lignin altered plants. But when it is linked to improved saccharification, reasonable growth was observed as a consequence, which might be tolerable. On the other hand, increased pest susceptibility or extreme dwarfism would not be valuable in the context of agricultural fields (Hammond-Kosack and Jones 1996; Reddy et al. 2005; Vermerris et al. 2010). The key outcome of lignin down regulation is also related to the stress posed by plant pathogens. It is well known that plants store lignin and some phenolic compounds that help in defence mechanism against pathogens in plants (Reddy et al. 2005; Shen et al. 2012). It also affects the biotic and abiotic stress mechanism of plants (Bi et al. 2011). In a recent study, it has been found that the plant defence mechanism is controlled by the monolignol genes (Moura et al. 2010). One of the best examples given by Tronchet and co-workers revealed that the primary CAD genes (CAD-C and CAD-D), which play a major role in model plant lignification (Hoffmann et al. 2004; Tronchet et al. 2010), show an essential function in the reaction of *Arabidopsis* against bacterial infections.

However, various research works propose that the negative impact on disease resistance is due to lignin gene silencing, but not in all cases. Genes which encode for HCT showing down regulation were linked with higher tolerance against fungal infection and drought, possibly as a result of cross talk concerning lignin and salicylic acid biosynthesis. Clearly, we can say that still there is a need to gain more knowledge for the better understanding of the role of lignin in whole plant physiology with respect to its field locations. A new generation of hybrid plants is using RNAi, where the extensive lignin variety related transgenes will permit improved understanding of synergistic transgene effects.

In a recent study, it was demonstrated that the yield penalty can be side-stepped using a tissue-specific promoter intended to diminish lignin level only in the fibre cells, which ultimately help for inhibiting vessel collapse. Similar approaches could be streamlined and used for biofuel generation using perennial grasses. Clearly, RNAi technology contains much potential to increase biofuel production per hectare, and then the modification of lignin biosynthesis will be desirable to understand its capacity (Boudet et al. 2003; Yang et al. 2013).

11.11 Conclusions

The plant CWs contain the whole energy of plant biomass. The key difficulty for biofuel production from plants is their high cost of obtaining sugars from CWs. Due to the presence of lignin and other multifaceted structure of CWs, cellulosic biomass is quite challenging as compared to starch which further breaks down into sugars. On the other hand, hybrid/transgenic down regulation of major lignin genes developed by RNAi controlled the reduction of lignin content, improved the availability of cellulases, and also improved dry matter degradability for cellulose degradation. Due to the development of new hybrid plants using RNAi, the wall polysaccharides showed the down regulation since simple hydrolyzed sugars in the transgenic matter were considerably higher. Therefore, lignin modification can be achieved by RNAi, an approach of redesigning the features of the feedstocks. A large number of reports have been found which confirmed the down regulation of lignin biosynthesis in monocots; however, only limited information is available in dicot species. In current scenario, there is no specific information available on lignin conversion in the context to key biofuel crops.

The lignin biosynthetic pathways are conserved across species; in addition information obtained from dicots species should be relevant to monocots species. In this modern era, still there is a serious requirement to scientifically distinguish molecular factors of lignin biosynthesis in monocot and dicot and also to develop novel approaches to increase biofuel production from the major biofuel crops. Decreased lignin content with the help of RNAi method is possibly one of the most effective approaches for cost reduction, which is also associated with pretreatment as well as hydrolysis of lignocellulosic feedstocks. Though several probable issues which are negative in nature should also be addressed, future research works are desirable to disintegrate the pessimistic association amid a reduction in lignin content and large productivity of biomass. Probably these problems appeared as answerable if we concentrate on the approaches that could help on the modification of these important complex carbon sources, like management of cellulose and hemicellulose biosynthesis and its accumulation. Importantly, it is also essential to assess the effect of CW modification on plant structure and their tolerance capacity for abiotic and also biotic stresses. Using RNAi new cultivars can be developed with optimized biomass quality and overall production that could ultimately benefit the biofuel industry with a huge amount of biofuel production.

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Archana Kumari received the M.Sc. degree in Bioinformatics from BIT, Mesra, Ranchi, India. She is a Ph.D. student and working as an Institute Fellow at BIT, Mesra, Ranchi. She is working on expression and networking of some least-studied TF genes in rice. Her current research interests are biofuel and biopolymers derived from plant sources as well as understanding the role of RNAi for biofuel production.

Vinod Kumar Nigam is an Associate Professor at the Department of Bioengineering, BIT, Mesra, Ranchi, India. He did his M.Sc (Biochemistry) and Ph.D. in Biochemical Engineering from IIT, BHU, Varanasi, India. He was associated with an industry for 2 years in production and downstream processing of penicillins and xylanase. He was a Scientist for 2 years at BISR, Jaipur, India. He is working in the area of process biotechnology, enzyme technology, and bioprospecting.

Dev Mani Pandey is an Associate Professor at the Department of Bioengineering, BIT, Mesra, Ranchi, India. He completed his Ph.D. in Plant Physiology from CCS HAU, Hisar, India. He did his postdoctoral studies in South Korea and worked in Plant Molecular Biology and Functional Genomics. He was a Consultant to PBGB, IRRI, Los Baños, Philippines, in 2008. His research interest is on plant physiology, biotechnology, and bioinformatics.

Microbial Cellulose Synthesis

12

Nivedita Nagachar and John McManus

Abstract

Cellulose, the most abundant biopolymer on earth, has served mankind in countless applications from crude building resources to feedstock for advanced synthetic materials. As interest grows in alternative fuel resources, cellulose is among the primary contenders as a feedstock for generating these fuels. In recent years, interest in plant-derived, photosynthetically fixed cellulose has intensified, as this remains the major untapped natural source of stored solar energy in the form of carbon-carbon bonds. Although not usually considered in the biofuel sector, the ubiquity of microbial cellulose and the engineering of photosynthetic microbes for cellulose production make microbial cellulose a viable candidate. Not only does the remarkable versatility of microbial cellulose make its study worthwhile, but the microbes responsible for its synthesis serve as a simpler model for understanding the complex biological processes underlying plant cellulose synthesis.

12.1 Introduction

Cellulose is a naturally occurring polymer produced by organisms in all domains of life including plants, animals, fungi, and bacteria (Chawla et al. 2009). It is the major cell wall component of plants and the largest repository of photosynthetically fixed carbon on earth. Although the content of cellulose varies with plant types, it is typically around 45 %. Interest in cellulose has a rich history; its benefits to humans range from the most basic such as clothing and writing to newer technologies including feedstocks for synthetic polymers and biofuels. When chemically modified, cellulose can be used to make plastic, rayon, etc. Although humans have been exploiting cellulose in different forms for centuries, we have only understood its structure and composition since 1838. Cellulose was first reported by a French chemist, Anselme Payen, who isolated it from wood and determined its chemical formula. He described it as the solid

N. Nagachar (✉) • J. McManus (✉)
Department of Biochemistry and Molecular Biology,
Pennsylvania State University,
306 South Frear Building, University Park,
PA 16803, USA
e-mail: nun3@psu.edu; jbm209@psu.edu

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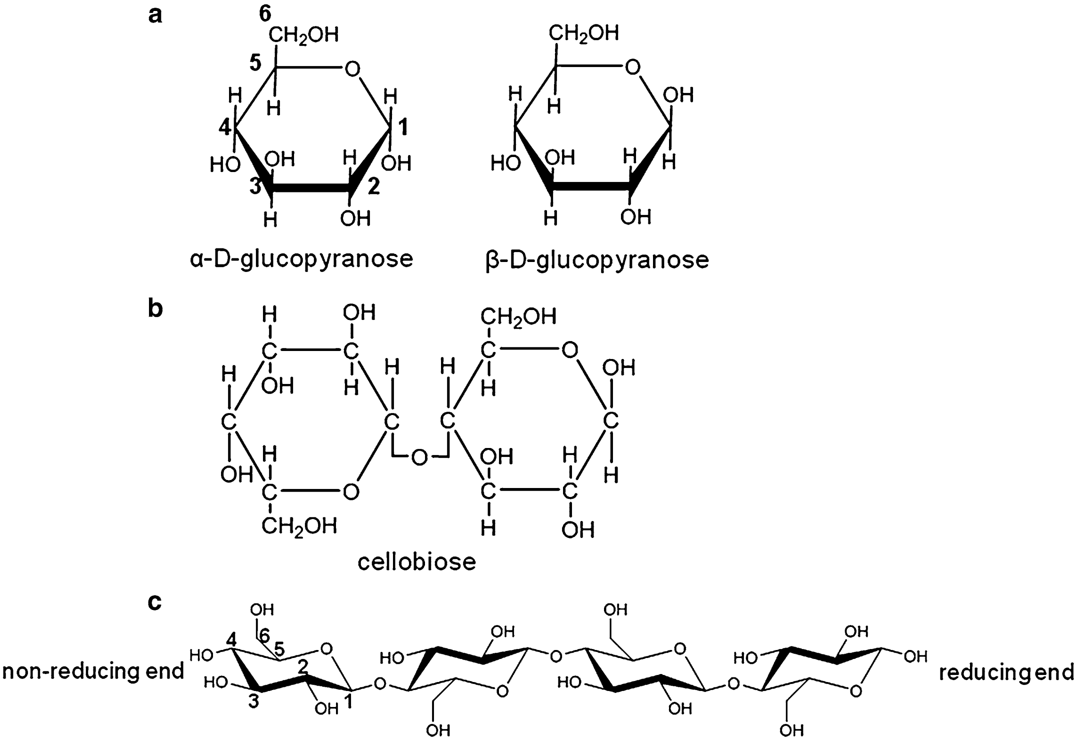

Fig. 12.1 (**a**) Cellulose is made up of alternating glucose units, a six-carbon sugar. (**b**) The repeating unit of cellulose is cellobiose. (**c**) Cellulose is synthesized by adding glucose units to the 4-carbon hydroxyl of the nonreducing end

fibrous material left after harsh treatments with acids and ammonia and subsequent extraction with water, ether, and alcohol.

12.1.1 Cellulose Structure

Cellulose is comprised of polymers of β-1,4-linked glucose residues (Fig. 12.1a), resulting in a linear configuration with each glucose residue rotated 180° relative to its neighbor (Carpita 2011). Thus, the basic repeating unit of cellulose is considered to be β-1,4-glucose dimer, cellobiose (Fig. 12.1b). In contrast with the helical α-1,4-linked glucan polymer, starch, or the α-1,4-linked, α-1,6-branched glucan polymer, glycogen, which plays an important role in carbon storage, cellulose's straight, unbranched structure leads to interpolymer hydrogen bonding and van der Waals interactions such that water is excluded. This imparts high flexibility and tensile strength (Nishiyama et al.2003) and makes it an excellent structural component for plant cell walls and bacterial biofilms. Cellulose is synthesized from membrane-bound enzyme complexes by adding new glucose units to the nonreducing, 4-carbon hydroxyl end of the glucan polymer. At the other end of the growing glucan polymer, the reducing, 1-carbon hydroxyl end extends into the extracellular space and associates with other β-1,4-glucan polymers to form the macroscopic cellulosic structure (Fig. 12.1c) (Brown 2004).

The nascent β-1,4-glucan polymers aggregate into bundles, called microfibrils, historically defined as the smallest visible strands recognized by electron microscopy. The cellulose microfibril is poorly defined as the size can vary depending on the organism from which the microfibril originates (Saxena and Brown 2005). In nature, nearly all isolated microfibrils bear the crystalline structure expected when β-1,4-glucan polymers are arranged laterally and unidirectionally with no disorganized randomness or tangled mass (Saxena and Brown 2005). This parallel arrange-

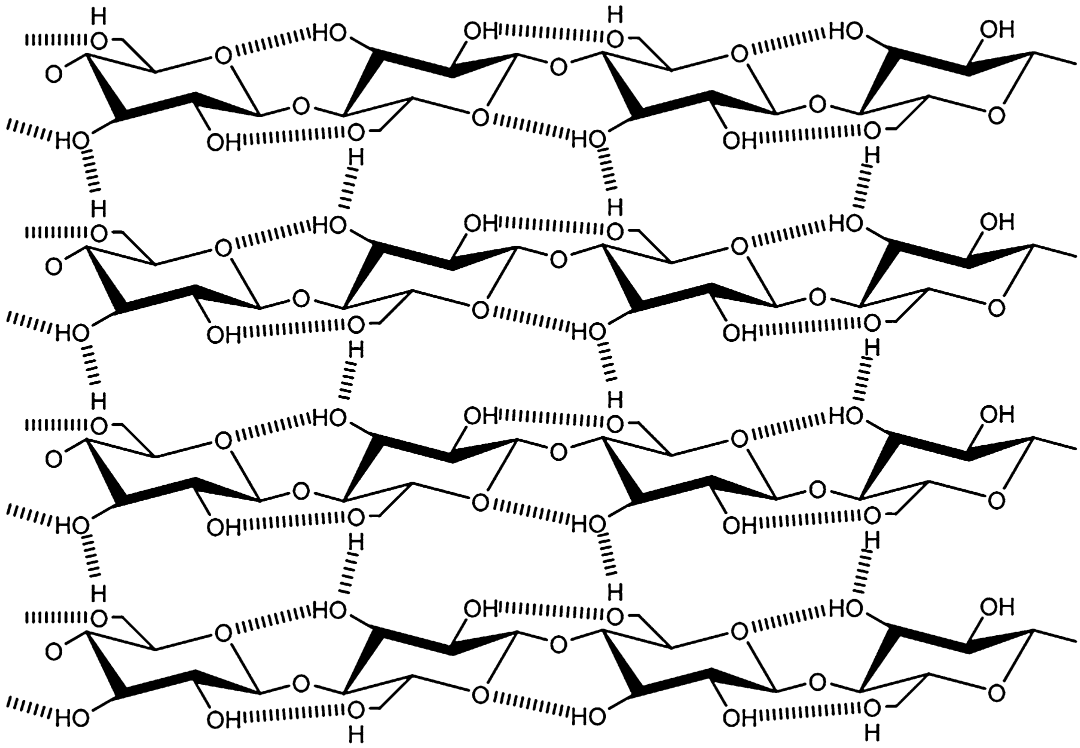

Fig. 12.2 Cellulose I is comprised of parallel glucan polymers associating through van der Waals forces and hydrogen bonding (shown by *hashed lines*)

ment is termed cellulose I (Fig. 12.2). In contrast, polymers with the thermodynamically more stable, amorphous, antiparallel arrangement are referred to as cellulose II. The presence of an additional hydrogen bond per glucose unit explains the thermodynamic favorability of cellulose II. However, cellulose I is more prevalent in nature due to the directionality in β-1,4-glucan synthesis, that is, with all reducing ends extending outward.

Cellulose microfibrils associate in a hierarchical manner to form the visible macroscopic cellulose structure particular to each organism, and several factors are implicated in this hierarchical ordering. Organization of cellulose microfibrils from the nascent glucan polymers may be partially dictated by the arrangement of the cellulose synthase complexes (CSCs) embedded in the cell membrane. For example, the CSCs of *Arabidopsis thaliana* adopt a round rosette-like arrangement, while the bacterial CSCs of *Gluconacetobacter hansenii* are arranged into a linear array, forming a barrel-like microfibril and a sheet-like microfibril (Jarvis 2013). The "decoration" of the cellulose microfibrils with various proteins and polysaccharides plays an important part in morphology and crystallinity. Plant cell walls, in addition to the crisscrossing matrix of the cellulose microfibrils, include interspersed hemicelluloses, lignin, and pectins, imparting cellulose crystallinity between 30 and 50 % (Fig. 12.3a) (Wiedemeier et al. 2002). By contrast, bacterial cellulose (BC), lacking these intercalating compounds, exhibits a higher degree of purity and crystallinity (~60–90 %) (Fig. 12.3b) (Harris and DeBolt 2008; Park et al.2010).

Mutagenesis studies on cellulose-synthesizing organisms have identified a number of accessory proteins important in cellulose synthesis. These accessory proteins are not directly responsible for the synthesis of the β-1,4-glucan polymer, but they play an integral part in the proper crystallization and deposition of the polymers. Many are included, along with the β-1,4-glucan-

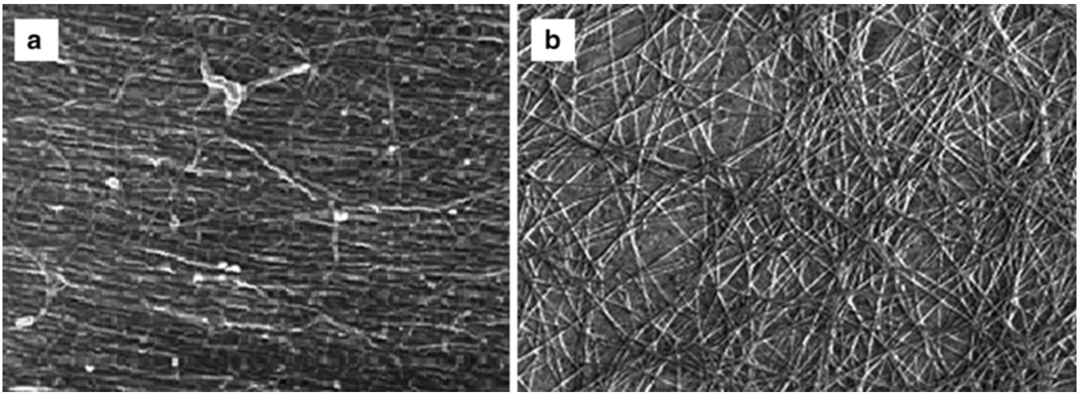

Fig. 12.3 FESEM images reveal the morphological differences between (**a**) plant cellulose from *A. thaliana* and (**b**) bacterial cellulose from *G. hansenii* (Courtesy: Publisher – The Company of Biologists Limited)

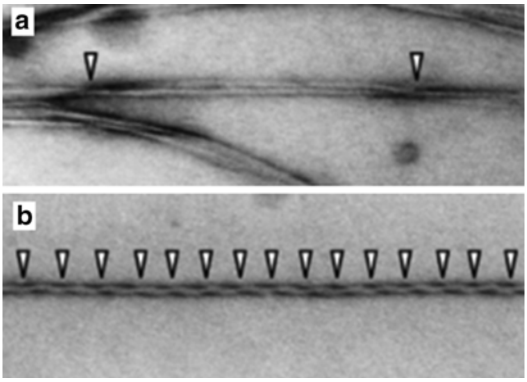

Fig. 12.4 (**a**) Wild-type BC microfibrils under TEM (indicated by *arrows*) as compared to (**b**) the hyper-twisted fibers from mutants lacking endoglucanase, $\mathrm{Cmc}_{\mathrm{Ax}}$ (Courtesy: Publisher – ASM journals)

synthesizing core, as part of the CSC. For example, in Gram-negative cellulose-synthesizing bacteria, additional proteins have been identified which are required for the passage of the glucan polymers through the periplasm and into the extracellular space. Interestingly, in both plants and bacteria, several cellulose-degrading enzymes have been identified as playing a key role in the proper formation of the higher-order cellulose structure. One possible reason for the necessity of cellulose-degrading enzymes is to trim away abnormal β-1,4-glucan polymers, which disrupt the higher-order structure of the cellulose (Fig. 12.4) (Zuo et al. 2000; Yasutake et al. 2006; Nakai et al. 2013).

In nature, cellulose is degraded by cellulases. These protein complexes consist of β-1,4-endo and β-1,4-exo glucanases, which release one cellobiose per turnover, and β-glucosidases, which are responsible for breaking the cellobioses into their constituent glucoses. Cellulases secreted by bacteria associate with a large non-catalytic central protein (scaffoldin) to form a multicomponent, secreted, cellulose-degrading complex called the cellulosome. Cellulases also contain an aromatic amino acid-rich carbohydrate-binding domain attached to the catalytic domain by a serine-rich linker region, allowing the enzymes to associate with the β-1,4-glucan substrate (Sadhu et al. 2013).

12.2 Bacterial Cellulose

Cellulose is produced by various genera of both Gram-positive and Gram-negative bacteria, including *Aerobacter*, *Achromobacter*, *Agrobacterium*, *Azotobacter*, *Cyanobacteria*, *Escherichia*, *Gluconacetobacter* (previously, *Acetobacter*), *Klebsiella*, *Rhizobium*, *Rhodobacter*, *Salmonella*, and *Sarcina* (Nobles et al. 2001; Zogaj et al. 2001; Römling 2002; Shoda and Sugano 2005). Of these, *G. hansenii* and *A. pasteurianus* (Park et al. 2003; Jung et al. 2005) produce the most useful solid extracellular bacterial cellulose (BC). This cellulose plays an important role in nature by conferring chemical, mechanical, and biological protection. In symbiotic or infectious interactions, cellulose assists in the adhesion process (Römling 2002).

The production of BC is a complex process and involves (1) the polymerization of glucose residues into linear β-1,4-glucan polymers, (2) extracellular secretion of these linear polymers in the form of microfibrils, and (3) the crystallization of these microfibrils into hierarchically assembled ribbons (Mohite and Patil 2014). This ribbon grows to become a visible reticular and regular structure. Biodegradability of BC is excellent, and it holds water to a larger capacity than plant cellulose, and it is also known to have a very good biological affinity. The unique properties exhibited by BC have sparked interest as an alternative biodegradable material available for medical, food, and chemical industry (Keshk 2014).

12.2.1 Cellulose and *Gluconacetobacter hansenii*

Due to its capacity to generate an abundance of high- quality crystalline cellulose, ease in culturing, and speedy growth, *Gluconacetobacter hansenii* (formerly *Acetobacter xylinum)* has served as a model organism for the study of cellulose production, since the 1880s. *G. hansenii* is a Gram-negative, obligate aerobe ubiquitous in the environment and is particularly found on fruits and vegetables and in fruit juices, alcoholic beverages, and vinegar. Its extensive cellulose production abilities were reported as early as the 1880s and were later confirmed by several research groups. Chemical and structural analyses were carried out later, which concluded that bacterial cellulose was chemically identical to plant cellulose (Valla et al. 2009). Light microscopy studies discovered that the cellulose produced was fibrillar and not an outgrowth of cell wall, that is, the fibers are formed by an extracellular process of crystallization. The ability of *G. hansenii* to produce cellulose from a variety of carbon sources was also documented. In vitro cellulose synthesis was achieved in the 1980s from membranes isolated from *G. hansenii*, which led to the discovery of the ubiquitous bacterial regulatory molecule cyclic-di-GMP (Valla et al. 2009). The catalytic core was isolated and identified from detergent-solubilized membranes through a partial purification technique known as product entrapment (Valla et al. 2009). Then, in the 1990s, through the use of genetic manipulation techniques, additional accessory proteins were discovered and continue to be identified (Deng et al.2013; Sunagawa et al.2013).

Fig. 12.5 (**a**) BC produced in shaking cultures forms a pellicle at the *bottom* of the medium, while (**b**) BC formed in static cultures is on *top* of the medium

12.2.2 Culturing of *G. hansenii*

Cellulose is produced by *G. hansenii* under both static and shaking conditions in the form of a pellicle at the top and bottom of the media, respectively (Fig. 12.5). Static cultures require longer culturing periods resulting in lower productivity, while shaking cultures produce higher amounts of cellulose in less time due to the greater diffu-

Table 12.1 Comparison of bacterial cellulose produced by *Gluconacetobacter* under different culture methods

Culture method	Carbon source	Supplement	BC production (g/L)	Culture time	References
Batch reactor	Fructose	Agar, oxygen	14.1	72 h	Bae et al. (2004)
Batch reactor	Fructose	Agar	12	56 h	Bae et al. (2004)
Fed-batch reactor	Glucose	Ethanol, oxygen	15.3	50 h	Shoda and Sugano (2005)
Fed-batch reactor	Molasses	None	7.8	72 h	Bae and Shoda (2004)
Batch airlift reactor	Fructose	Oxygen	10.4	52 h	Chao et al. (2000)
Batch airlift reactor	Fructose	Oxygen, agar	8.7	44 h	Chao et al. (2001)
Batch rotating disk	Glucose	None	3.5	7 days	Krystynowicz et al. (2002)
Shaking flask	Glucose	Ethanol	15.2	8 days	Son et al. (2001)

sion rates of oxygen into the medium. The cellulose-producing wild-type strains appear small and rough on agar media; by contrast, strains that do not produce cellulose are large and smooth. As a result, it is easy to identify cellulose-producing strains by simple visual inspection on agar medium (Kim et al. 2007).

The typical medium used for *G. hansenii* growth is SH. This medium is rich in glucose (2 %) allowing for high cellulose production; however, due to the cost, SH media are not suitable for commercial BC production. Economical feasibility of BC is dependent on its productivity, and in turn the productivity is dependent on culture conditions, such as method of cultivation, carbon and nitrogen sources, temperature, pH, and dissolved oxygen. Alternatively, molasses and corn steep liquor can be used as carbon and nitrogen sources, respectively (Bae and Shoda 2004), in fermentation industry. Different methods of cultivation and the resulting cellulose yield have been well documented in bacteria (Table 12.1) (Shoda and Sugano 2005).

12.2.3 Purification of Cellulose

For purification, the *G. hansenii* cellulose pellicle is boiled with 0.1 M sodium hydroxide at 80 °C with gentle stirring, which eliminates bacterial cells and components of culture medium integrated within the cellulose network. The pellicle is then washed several times with water, until the pH returns to 7 (Deng et al. 2015). The pellicles can be freeze-dried and stored at room temperature, allowing for use in later studies.

12.2.4 Mechanism of Cellulose Biogenesis

The mechanism of formation and structure of BC microfibrils has been comprehensively studied (Iguchi et al. 2000). Both forms of cellulose, I and II, are produced by *G. hansenii* in the interior of their cells, spun out of CSCs on the cell membrane surface, and accumulate in the medium. The CSCs are arranged into linear arrays, shown to be around 50–80 nm in length (Fig. 12.6a–d) (Kimura et al. 2001; Sunagawa et al. 2013), and appear to show polarity, being localized to one side of the bacterium. The *G. hansenii* cellulosic macrostructure is formed in a stepwise hierarchical manner characterized by (1) sub-elementary fibrils, (2) elementary fibrils, (3) microfibrils, and (4) ribbons. First, each of the pore-like extrusion sites secretes 1.5 nm wide sub-elementary fibrils composed of 10–15 glucan polymers (Deng et al. 2015). Then these sub-elementary fibrils produced by different extrusion sites will aggregate

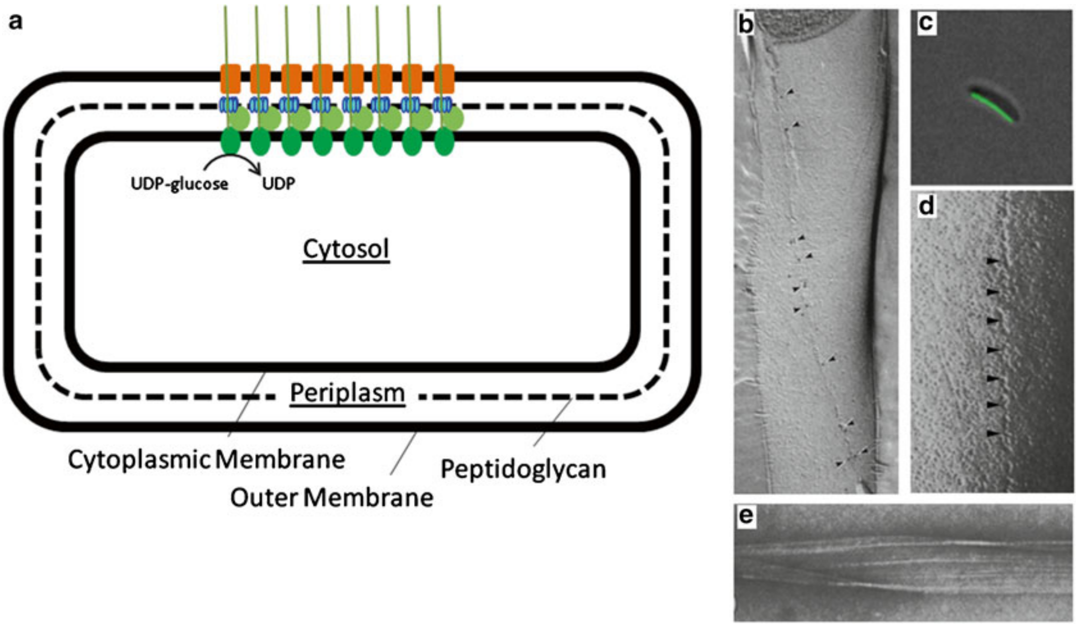

Fig. 12.6 (**a**) A schematic diagram of the *G. hansenii* cell shows the association of the CSCs to form the cellulose-synthesizing array. Freeze-fracture TEM images of the *G. hansenii* cell show (**b**) AcsB immune-gold labeled and (**d**) unlabeled particles arranged in a linear array (*black arrows*). (**c**) Fluorescence microscopy reveals a linear complex composed of GFP-tagged AcsD. (**e**) These arrays result in the ribbon-like cellulose morphology revealed by freeze-fracture TEM (Courtesy: Publisher – ASM journals, Elsevier, National Academy of Science)

to form 3.5 nm-wide elementary fibrils, and the elementary fibrils in close proximity crystallize to form 6–7 nm wide microfibrils, which further organize into bundles. Twisted cellulose ribbons of 40–60 nm in width (Fig. 12.6e) are formed by fasciations of bundles. Aggregation leads to the cellulose pellicle which forms at the top of static cultures. The macroscopic cellulose sheets formed in *G. hansenii* have high Young's modulus, 15–35 GPa, highest of all two-dimensional organic materials, and a tensile strength in the range of 200–300 MPa.

12.2.5 The Cellulose Synthase Complex

Genes involved in cellulose biosynthesis in *G. hansenii* are the most extensively characterized among bacterial strains with respect to cellulose synthesis. Several genes are shown to be involved in *G. hansenii* cellulose synthesis, three of which make up the *acs* (acetobacter cellulose synthase) operon, namely, *acsA*, *acsB*, and *acsC*. A fourth gene, *acsD*, originally thought to be part of the *acs* operon, has since been shown to contain its own promoter (Deng et al. 2013). The resulting protein products, AcsAB (168 kDa), AcsC (138.7 kDa), and AcsD (17.3 kDa), likely associate to form the cellulose synthase complex (CSC) in *G. hansenii.*

AcsAB is synthesized as a single peptide but is subsequently cleaved into two proteins, AcsA and AcsB, with molecular masses of 85 and 100 kDa, respectively (McManus et al. 2015). The cleavage site, between residues 757 and 758, occurs after an alanine-glutamine-alanine triplet, indentified in Gram-negative bacteria as a signal peptidase cleavage motif. The 85 kDa AcsA peptide, representing the cytoplasmic and membrane spanning component, contains conserved DxD, QxxRW, and TED motifs typical of cellulose synthases and a PilZ domain, responsible for c-di-GMP binding. The 100 kDa AcsB peptide represents the primarily periplasmic component. Together these proteins comprise the core catalytic domain, called AcsA-AcsB, of the CSC, and mutants lacking either protein are unable to generate cellulose.

Homology studies suggest that AcsC is an outer membrane protein, and based on its sequence, hydrophobicity, and membrane localization, it is predicted to form a pore-like structure which facilitates the extrusion of cellulose synthesized inside the cell (Iyer et al. 2013). Mutants lacking AcsC show no accumulation of cellulose in the media; however, they appear to retain functioning catalytic cores, further bolstering the claim of function as a glucan porin.

AcsD is localized to the periplasmic space forming a homo-octamer, which holds four glucan polymers in its central pore. It is the only Acs protein from *G. hansenii* whose crystal structure is resolved (Hu et al. 2010). It might function as a glucan chaperone by channeling the newly synthesized glucan polymers to AcsC and thereby prevent the accumulation of rogue strands in the periplasm. Mutants lacking AcsD show reduced accumulation of cellulose in the medium as well as cellulose morphology differences when compared with wild type. Two other genes, cmc_{Ax} and ccp_{Ax}, involved in the process of cellulose synthesis share an operon located upstream of *acs* operon (Kawano et al. 2002; Deng et al. 2013). cmc_{Ax} encodes a secreted β-1,4-endoglucanase (Kawano et al. 2002). Addition of anti-$\mathrm{Cmc}_{\mathrm{Ax}}$ antibodies to the culture medium inhibited the accumulation of cellulose in the medium. Cellulose production was regained when small amounts of purified $\mathrm{Cmc}_{\mathrm{Ax}}$ are added or $\mathrm{Cmc}_{\mathrm{Ax}}$ is overexpressed in *G. hansenii* (Kawano et al. 2002). Furthermore, mutants lacking cmc_{Ax} show reduced amounts of cellulose in the medium characterized by hyper-twisted fibers (Fig. 12.4), suggesting that cmc_{Ax} may be responsible for alleviating hyper-twisting of cellulosic microfibrils. ccp_{Ax} codes for cellulose-complementing protein ($\mathrm{Ccp}_{\mathrm{Ax}}$), which interacts with AcsD and co-localizes in the linear cellulose synthase array along one side of the cell (Sunagawa et al. 2013) (Fig. 12.6c). Its absence leads to the dispersal of the array and no accumulation of cellulose in the media. Additionally, these mutants do not retain active catalytic cores due to degradation of AcsB, leading to the speculation that $\mathrm{ccp}_{\mathrm{Ax}}$ plays an important role in CSC and array integrity. For these reasons, $\mathrm{Ccp}_{\mathrm{Ax}}$ is also included as a component of the CSC. Figure 12.7 shows a schematic diagram of components believed to associate to form the *G. hansenii* CSC.

Downstream of *acsD* is bgl_{Ax}, which is predicted to encode a 79 kDa β-glucosidase (Tajima et al. 2001). $\mathrm{Bgl}_{\mathrm{Ax}}$ is classified into the family 3 glycoside hydrolases, based on amino acid sequence similarities (Tajima et al. 2001). This enzyme has exo-1,4-β-glucosidase activity, which cleaves nonreducing end of cellotriose and larger cello-oligosaccharides. It also has been shown to exhibit glucosyltransferase activity. This enzyme is not essential for the BC accumulation in the medium, but its disruption causes a reduction in cellulose accumulation. The function of $\mathrm{Bgl}_{\mathrm{Ax}}$ in connection with cellulose synthesis remains to be discovered. Figure 12.8 shows a schematic arrangement of the genes required for proper cellulose synthesis and formation. Formation of c-di-GMP, the activator of cellulose synthesis, is catalyzed by a diguanylate cyclase, Dgc1. As expected, mutants lacking Dgc1 do not accumulate cellulose in the medium; however, these mutants retain active catalytic core domains. Crp-Fnr, a transcriptional regulator, is also involved in cellulose synthesis, as a mutant of *crp-fnr* does not produce cellulose and regulates the expression of bgl_{Ax} (Deng et al. 2013).

12.2.6 Cellulose Production in Other Prokaryotes

Although cellulose biosynthesis has been extensively studied in *G. hansenii*, the production of cellulose is widespread among prokaryotes. The plant pathogen *Agrobacterium tumefaciens* produces cellulose, and the two operons identified in the process are called *celA-C* and *celD-E*. In addition, two other genes, *celG* and *celI*, were identified, negatively affecting cellulose synthesis (Matthysse et al. 2005). The organization of genes required for cellulose synthesis in *A. tumefaciens* shows limited similarity when compared with *G. hansenii* CSC genes. Homologues of

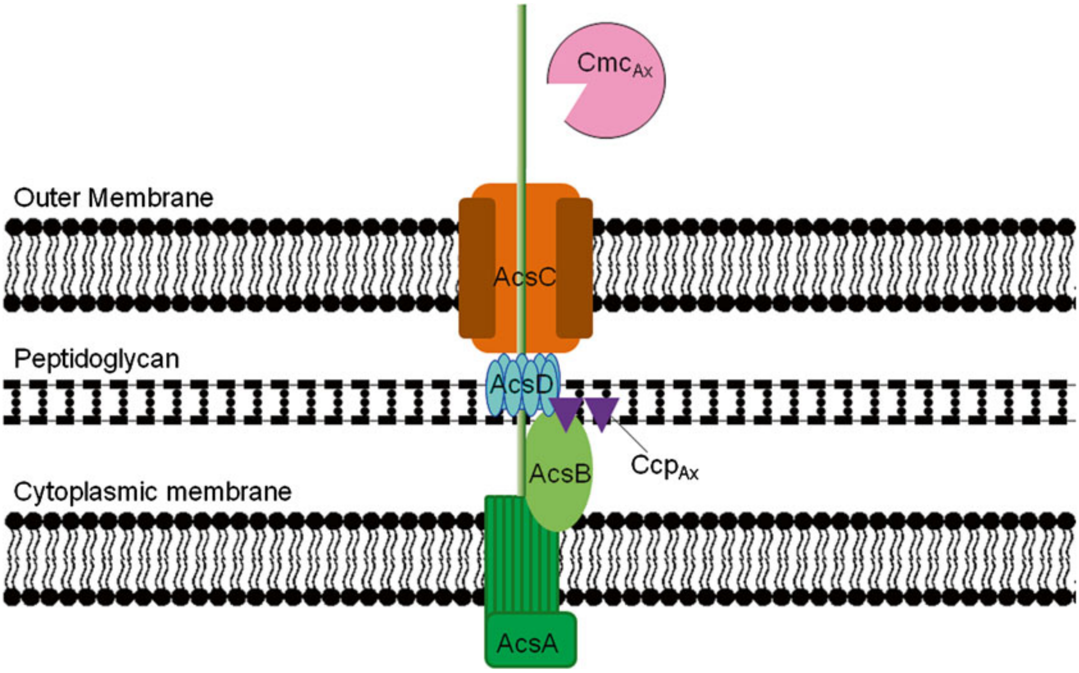

Fig. 12.7 A schematic diagram of a single CSC shows the catalytic core, composed of AcsA and AcsB, spanning the cytoplasmic membrane into the periplasm. The core associates with Ccp_{Ax} and AcsD to assist in guiding the glucan polymer to AcsC, responsible for secretion into the extracellular space. The glucan polymers can associate to form higher-order morphology, and rouge polymers are removed by Cmc_{Ax}

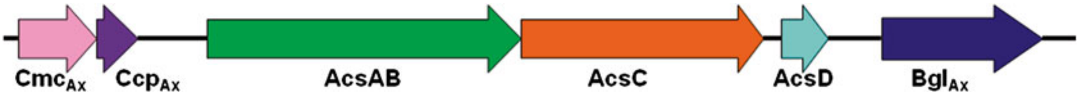

Fig. 12.8 A schematic diagram of the genes necessary for normal cellulose synthesis

celA, *celB*, *celC*, and *celG* of *A. tumefaciens* were found in the nitrogen-fixing *R. leguminosarum* (Laus et al. 2005). The capacity of cellulose production by *Escherichia coli*, *Salmonella typhimurium*, *Salmonella enteritidis*, and *Klebsiella pneumoniae* has been confirmed (Zogaj et al. 2001; Römling and Lunsdorf 2004). The genes encoding the putative cellulose synthases are called *bcsA*, *bcsB*, *bcsZ*, and *bcsC* to indicate their similarity to corresponding genes in *acs* operon of *G. hansenii*. Although genetic studies regarding cellulose synthesis have been performed in a variety of bacterial species, the biochemical nature of these proteins and the CSCs they form remains unknown, leaving *G. hansenii* the best understood system for microbial cellulose synthesis.

12.3 Cellulose Synthase Enzymology

A benefit to the field of cellulose synthesis was the 2013 resolution of the crystal structure of the cellulose synthase catalytic core domain from *Rhodobacter sphaeroides* (Morgan et al.2013). In just a short time, the structure has provided a great deal of insight into the chemical mechanism of cellulose synthesis, the translocation of the glucan polymer, and the mechanism of regulation by cyclic-di-GMP. However, the bulk of what can be understood about cellulose deposition and formation in bacteria still leans heavily on the historical model, *G. hansenii*. This is due to two major distinctions: (1) *R. sphaeroides* does not secrete cellulose extracellularly, and (2) *it* is

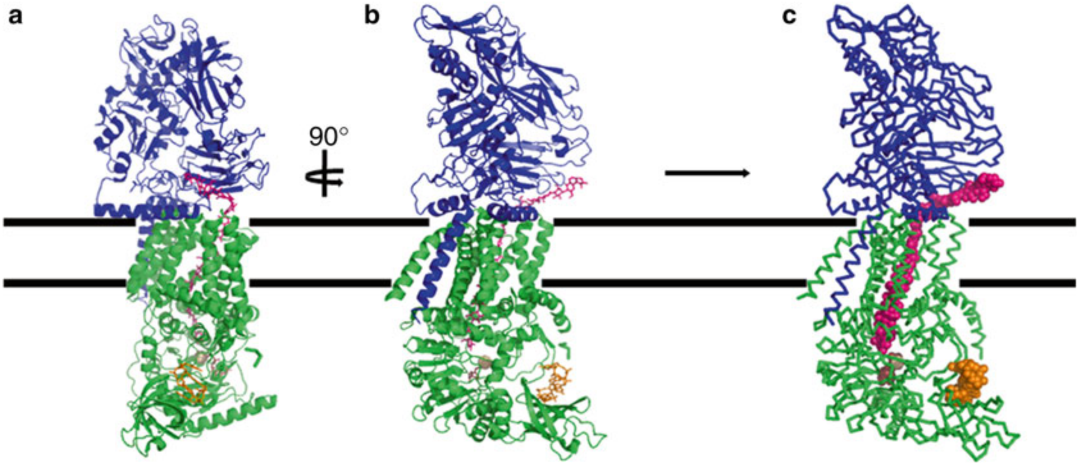

Fig. 12.9 (**a**) BcsA (*green*) and BcsB (*blue*) form a 1:1 heterodimer. UDP (*brown*) sits in the catalytic site. Cyclic-di-GMP (*orange*) bind 2:1 in the PilZ domain. The glucan polymer (*magenta*) traverses the cytoplasmic membrane and exits into the periplasm. (**b**) BcsA-BcsB rotated 90°. (**c**) A ribbon representation of BcsA-BcsB with space-filling models of UDP, cyclic-di-GMP, and the glucan polymer

not known to contain the accessory proteins found in *G. hansenii* important in deposition and hierarchical cellulose crystallization. For comparison with *G. hansenii's* AcsA-AcsB, the *R. sphaeroides* cellulose synthase heterodimer, called BcsA-BcsB, is encoded by two separate genes and shares only 30 % sequence identity with AcsA-AcsB. Furthermore, the enzyme turnover number for both systems differs by nearly two orders of magnitude (90s^{-1} for *R. sphaeroides* and 1s^{-1} for *G. hansenii*) (Omadjela et al.2013). This may be linked to differences in crystallization of the glucan polymers, shown previously to have rate effects on cellulose synthesis. Despite this, there are several conserved motifs (explicated in the previous section) which suggest that the chemical mechanism of cellulose synthesis is identical in both systems and likely shared between all cellulose-synthesizing enzymes.

12.3.1 Cellulose Synthase Structure

The crystal structure in Fig. 12.9 shows BcsA-BcsB forms a 1:1 heterodimeric integral membrane complex (Morgan et al.2014). BcsA associates with the cytoplasmic membrane through eight transmembrane helices. These helices form a 33 Å long by 8 Å wide channel which traverses the bilayer to accommodate a maximum of ten glucose residues opening toward the periplasmic space. The large cytoplasmic side of BcsA contains both the catalytic domain, giving it access to the intracellular pool of UDP-glucose, and the regulatory PilZ domain.

The periplasmic BcsB associates with the membrane through a single alpha helix and contains two carbohydrate-binding domains. As the β-1,4-glucan polymer exits the BcsA subunit, it bends at a near 90° angle and appears to associate with BcsB. While little is known about the function of BcsB, truncation studies of BcsB have shown that all that are required for activity are the C-terminal transmembrane helix and amphipathic helix. Therefore, it may play a role in the stabilization of the BcsA transmembrane region.

12.3.2 Chemical Mechanism of Cellulose Synthesis

Addition of glucose residues to the 4-carbon hydroxyl of the nascent glucan polymer is thought to proceed through an SN_2-like single displacement mechanism, similar to other invert-

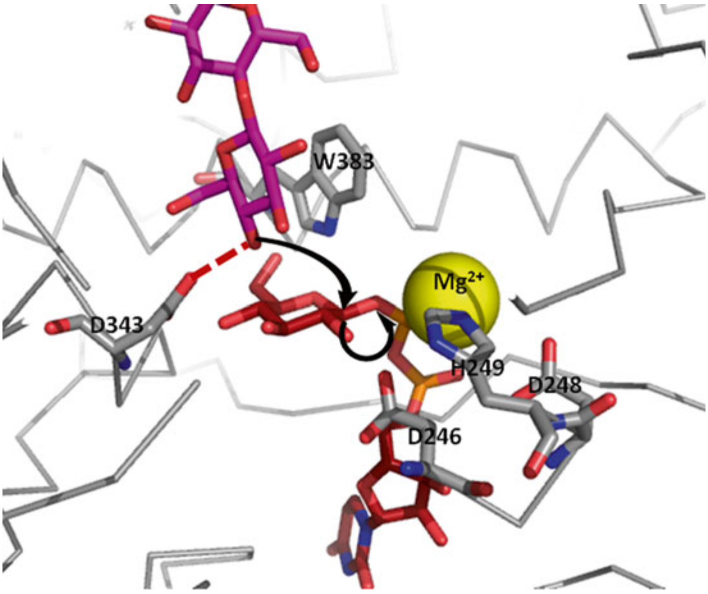

Fig. 12.10 Addition of glucose to the nonreducing end of the nascent glucan polymer occurs first, by deprotonation of the 4-carbon hydroxyl by aspartate 343. The deprotonated hydroxyl makes a nucleophilic attack on the 1-carbon of the UDP-glucose substrate, displacing the UDP and forming the new glycosidic bond

ing glycosyl transferases (Lairson et al. 2008). A conserved threonine-glutamate-aspartate is present in bacterial cellulose synthase enzymes, with the aspartate assuming the role of the general base, required to deprotonate the 4-carbon hydroxyl (Morgan et al.2014). The formation of the glycosidic bond proceeds through a nucleophilic attack, by the deprotonated hydroxyl, on the 1-carbon of the UDP-glucose, which is stabilized by a divalent cation, coordinated by two conserved aspartates (Fig. 12.10). This displaces the UDP and leads to inversion of stereochemistry about the anomeric carbon. The currently vague mechanism of glucan polymer translocation must then occur before the addition of the next glucose.

The above mechanism assumes a preexisting glucan polymer; what remains unclear is initiation of cellulose synthesis in a glucan-free cellulose synthase. This may occur through a primer intermediary, similar to initiation in glycogen synthesis, or through a self-priming mechanism. Earlier work suggested this primer may exist as sitosterol-β-glucoside (Peng et al. 2002). The *R. sphaeroides* crystal structure discredited this hypothesis and suggests that even a single glucose molecule is sufficient to initiate cellulose synthesis (Morgan et al.2014), giving ground to the self-priming hypothesis, which may involve water-mediated UDP-glucose hydrolysis, allowing the freed glucose to initiate cellulose synthesis.

12.3.3 Mechanism of Regulation by Cyclic-di-GMP

The ubiquitous bacterial regulator, cyclic-di-GMP, binds the PilZ domain of BcsA in a 2:1 stoichiometry. Binding of the two cyclic-di-GMPs leads to several conformational changes. The most apparent is the opening of a gating loop domain which allows access to the active site for substrate diffusion to occur. Additionally, the TED motif shifts the aspartate into position to act as the catalytic base. Upon binding UDP-glucose, the gating loop adopts another conformation, burying itself in the active site, coordinating with the UDP, thus excluding water, thereby preventing water-mediated hydrolysis of UDP-glucose.

12.4 Perspective

BC provides structural integrity to biofilm architecture and a surface suitable for the adherence and accumulation of cells (Sutherland 2001). BC has a high degree of commercial versatility as a potential resource for biofuels, chemicals, and synthetics (Himmel et al. 2007). Current products derived from BC include food additives, acoustic diaphragms for audio instruments, paper, textiles, wound dressings, fiber glass filter sheets, medical supplies, cosmetics, and optically transparent composites (Czaja et al. 2007; Yano et al. 2005). The greater purity and crystallinity of BC, as compared to plant cellulose, make it a more suitable substance for various applications (Römling 2002). New materials with desirable properties are being pioneered either by chemical treatment of extant BC or the direct genetic manipulation of various genes involved in the cellulose biosynthesis. Hence, a better understanding of the biochemical and molecular mechanisms of cellulose biosynthesis and its regulation will assist in developing new strategies for the eradication of biofilm-forming bacteria and the optimization of cellulose production for industrial applications and biofuel feedstocks.

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Nivedita Nagachar is a Ph.D. from the University of Hull, United Kingdom. She is currently working as a Research Associate at the Pennsylvania State University, USA. Her interests include cellulose synthesis, cellulose and lignin degradation, iron metabolism, bacteriology, molecular biology, enzymology, and protein technology.

John McManus is currently enrolled in Ph.D. program at the Pennsylvania State University, USA, where he studies the cellulose synthase complex in *Gluconacetobacter hansenii*. Prior, he was employed in the renewable energy sector working with algae-based biofuels. His interests include cellulose synthesis and degradation, protein expression and purification, enzyme kinetics, microbial ecology, and renewable energy.

Technological Advances for Treating Municipal Waste

13

Prabhakar Dattatray Pandit,
Madhuri Kisanrao Gulhane,
Anshuman A. Khardenavis, and Atul N. Vaidya

Abstract

Municipal solid waste management (MSWM) is a challenging task, which has grown immensely due to the changes in lifestyle throughout the world. In developed countries, appropriate norms and adequate administrative and financial resources clubbed with innovative technologies are some driving force, which is proving helpful in MSWM. However, an ever-changing waste composition and some techno-environmental issues still exist. On the other hand, in developing countries in the absence of these provisions, MSWM is becoming a key of socioeconomic development. Due to variations in waste composition, there is a need to adopt multiple means of waste disposal. It thus demands revisiting the available know-how and making the required corrections to suit the grossly ill-classified wastes. In this chapter, characteristic components of MSW and possibilities of their disposal through thermal and biological treatment technology are reviewed. The generation of renewable energy while treating waste makes disposal an organized and attractive option.

13.1 Introduction

Population growth and rapid industrialization have resulted in massive migration of people from rural to urban areas. As a consequence, tons of municipal solid waste are generated daily, whose management is becoming a challenging task, especially for developing countries (Raizada et al. 2002; Kalia 2007). Increased solid waste quantity creates burden on available treatment and disposal facilities. In developing countries, inappropriate ways of waste disposal have an adverse impact on environment and human health. Efficient solid waste manage-

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P.D. Pandit • M.K. Gulhane • A.A. Khardenavis (✉)
Environmental Genomics Division, CSIR-National Environmental Engineering Research Institute (CSIR-NEERI), Nehru Marg, Nagpur 440020, India
e-mail: p_pandit@neeri.res.in; prabhakarbt999@gmail.com; mk_gulhane@neeri.res.in; madhurigulhane@yahoo.in; aa_khardenavis@neeri.res.in; anshuman.neeri.egd@gmail.com

A.N. Vaidya
Environmental Genomics Division, CSIR-National Environmental Engineering Research Institute (CSIRNEERI), Nehru Marg, Nagpur 440020, India

Solid and Hazardous Waste Management Division, CSIR-National Environmental Engineering Research Institute (CSIRNEERI), Nehru Marg, Nagpur 440020, India
e-mail: an_vaidya@neeri.res.in

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ment system is needed in order to overcome these inadequacies in waste management (Kaushal et al. 2012). In general practice, lack of proper segregation leads to serious health issues. Leachates of heavy metals and other toxic compounds from disposal site into the ground water add to these issues. Waste reduction and recycle and reuse policies promote waste management to some extent (Jha et al. 2011). Management of MSW requires proper infrastructure and maintenance of facilities for collection and storage, the safe transport, processing, and disposal of solid wastes. Incidentally, it is lacking in most cities. In this chapter, we have reviewed the present scenario of municipal solid waste management (MSWM) in developing countries with special reference to processing and disposal practices, their limitations, and possible solutions for sustainable management of this misplaced resource.

13.2 MSW Management Scenario

13.2.1 Generation Rate

Average waste generation (kg/capita/day) in various regions of the world varies from 0.6 million tons in the USA to 0.52 million tons per day in China. Among the developing nations, Brazil, Mexico, China, and India lead the rest in terms of waste generation, which increase at the rate of 1.33 % per year. It is attributed to increased urbanization and changing lifestyle. In India, MSW generation has increased eightfold since 1947 (Cherian and Jacob 2012).

In Indian cities, the average per capita per day MSW production is estimated to be around 0.21–0.50 kg (Kumar et al. 2009a). At the above per capita generation capacity, the quantity of MSW is expected to increase from 34 million tons in 2000 to 83.8 million tons by 2015 and further to 221 million tons by 2030 (Kaushal et al. 2012). In comparison, average per capita per day MSW generation (kg/capita/day) in other developed economies in Asia is higher as observed in the case of Thailand (0.66), Malaysia (0.80), and Singapore (1.10). Though developed nations have higher per capita MSW generation, however, it has also drastically increased in developing nations primarily due to lack of appropriate technological management options.

13.2.2 Characteristic Components

Depending upon the source of generation, MSW consists of residential, commercial or institutional, and municipal service waste. The components of MSW can be classified into the following major classes:

(a) Domestic and hazardous: batteries, containers, and chemicals from paints, fertilizer, and pesticide industry
(b) Biodegradable: paper and kitchen and green waste
(c) Recyclable: plastic, metal, paper, and glass
(d) Composite: clothes and plastic goods
(e) Inert: building waste and debris, rocky material, etc.

Different aspects like economic growth, geography, resources of energy, climatic conditions, and culture contribute to MSW composition. In typical Indian cities, MSW contains 40 % organic fraction, 37 % combustible fraction, 8 % recyclables, and 15 % inert material. A comparison with world status of MSW composition indicates that the composition in regions of Europe and Central Asia matches that of Indian cities with nearly 40 % of the MSW composed of organic fraction. On the other hand, the proportion of organic fraction in MSW in remaining parts of the world varies around 50–55 % with the remaining being composed of recyclable (25–30 %) and inert material (5–10 %).

13.2.3 Management Practices

MSWM includes discrete steps such as storage at source, segregation at storage site, collection, transportation, treatment, reuse, recycle, and ultimate disposal. Appropriate strategies in exploitation of these steps decide the route of sustainable management of MSW. Cost-effective ways of collection, processing, and disposal of MSW in developing countries are limited compared to developed countries which is attributed to lower MSWM awareness, financial constraints, ineffective legislations, lack of reduction, and reuse and recycle policies.

In the developed countries, landfilling is the principal waste disposal method since 1960; gradually MSWM is shifting away from landfilling toward incineration and reuse and recycling policy. Controlled release of gases and leachates in landfills and dioxins and furans came into focus from 1980 to 1990 (Marshall and Farahbakhsh 2013). As waste composition gradually changed, the developed nations shifted policies for MSWM toward more sustainable technologies.

13.3 Management Technologies Adopted for Processing

13.3.1 Landfilling

Landfilling stands as a cost-effective way of biological waste disposal. Comparing with various methods available for managing organic fraction of MSW (OFMSW), anaerobic digestion of municipal solid waste is one of the most promising by which such waste can be efficiently managed throughout the world by conversion into valuable products such as in bio-power generation (Lee et al. 2009). MSW is subjected to following steps during anaerobic digestion: hydrolysis, acidogenesis, acetogenesis, and methanogenesis under controlled conditions in the absence of oxygen, leading to the formation of biofuel in the form of biomethane, organic amendment in the form of liquid leachate, and solid residue with a corresponding decrease in environmental pollution.

13.3.2 Incineration

Thermochemical treatment systems for MSWM include incineration and gasification (Arena 2012). Incineration leads to 80–90 % reduction in the volume of solid waste by complete combustion at a temperature range of 980–2000 °C, leading to destruction of toxic waste and energy recovery. Earlier, it was considered that incineration was responsible for GHG emission; however, the present view is that energy is recovered in the form of heat resulting in reduction of GHG emission (Habib et al. 2013). The incineration energy recovery now focuses on waste-to-energy technology leading to lower pollution load on nearby areas as compared to other anthropogenic sources (Kadir et al. 2013). The heat generated is converted to steam for driving the turbine for generating electricity. Different thermal degradation methods are available for the treatment of waste including (1) pyrolysis at 400–1000 °C and (2) gasification at 1000–1400 °C under limited availability of oxygen. Pyrolysis results in production of gas (syngas), liquid (pyrolysis oil), or solid (char, mainly ash and carbon), while gasification leads to the formation of syngas, which is combustible and can be used as a fuel like natural gas.

Gasifier leads to improved quality of solid residues and significant pollution reduction especially in dioxins, furans, and NOx in addition to easier handling, metering, control, and burning of fuel syngas than solid MSW and lower CO_2 emission (Ruiz et al. 2013). Gasification plants are economical in medium- or small-scale applications compared with direct combustion plants. Treated syngas is efficiently used in turbines, otto engines, and quality fuels like diesel, gasoline, etc.

Integrated waste management options are currently being applied in developing countries for recovery and recycle of resource and energy generation from the solid waste. Thermal and biological conversion methods are prominent technological options for MSW management. Biological techniques for conversion of organic material into energy include aerobic composting and anaerobic digestion, while thermal techniques involve thermal breakdown of solid materials into gaseous constituents by using an indirect external source of heat.

13.4 Existing Scenario of MSW Management Technologies

13.4.1 Landfilling

In India, nearly 60 % of MSW was disposed on poorly managed dumping grounds rather than sanitary landfilling which is most evolved practice (http://thinkoutsidethebin.com). Such dumping has serious environmental implications including surface water contamination. Other hazards of landfilling include leaks in landfills and release of greenhouse gas (Themelis and Ulloa 2007). According to USEPA (United States Environmental Protection Agency), there has been a decrease in the total amount of MSW going to landfills in the USA from 142.3 to 137.2 million tons during 1990–2007 while the number of landfills declined steadily from 7924 to 1754 (Adeolu et al. 2011). In the UK, nearly 48 % of the municipal waste is still disposed of in landfill sites. The amount of waste landfilled has declined from 141 to 95 million tons in European countries. The landfilling rates have further dipped by 4.4 % per year since the year 2002 resulted in a drop in landfilling share of EU from 68 % in 1995 to 38 % in 2008 (http://epp.eurostat.ec.europa.eu.). In developed Asian country like Japan, landfilling is the secondary treatment process of waste disposal after incineration with only 16 % of waste being sent to the landfill in comparison to 70 % in the USA.

13.4.2 Incineration

Among the developed countries, MSW disposal through incineration with energy recovery was followed at 115 waste-to-energy facilities for treating 11.7 % of MSW. European countries show great variation in the distribution of number of incineration plants and quantity of incinerated waste like Germany, Italy, and France have greater than 60 % incinerators in comparison with other EU countries. There are no incineration plants in Greece and Ireland, whereas Denmark and Sweden lead in higher incineration rate in terms of per capita waste generation since law restricts the disposal of combustible waste into landfill, with about half of all MSW being incinerated. Slovenia, Poland, and Estonia have no incineration plants, while in Hungary small amount of waste is incinerated compared with landfilled indicating lower rate of waste incineration in EU member countries (Pires et al. 2012). Among the developed Asian countries, incineration is a well-practiced activity in Japan with about 80 % waste incinerated in Tokyo, which is home to 20 MSW incineration plants located all over the city. Similarly in Singapore, 41 % of the MSW generated was incinerated in four incineration plants having a total capacity of 8200 tons/day (Zhang et al. 2010). In spite of dominance of incineration plants in the EU countries, it has been considered to be an inappropriate technology for developing nations in Africa owing to the high capital and operating cost relative to other management techniques such as open land sites available for landfilling.

In India, there are very few gasifiers which treat agro-residues and forest wastes along with MSW. Incineration is not a preferred option for managing MSW in India owing to high organic and inert matter and very low calorific value (800–1100 kcal/kg). Currently plasma gasification plants are in operation in Japan, Canada, the USA, and India in which a plasma arc is used to create a high-temperature plasma (~10,000 °C) using air, oxygen, steam, or other feed. The MSW is heated to a gasification temperature of 2000–3000 °C resulting in the formation of syngas and melting the inorganic components to form a glassy, nonhazardous slag. Plasma arc gasification shows higher potential for conversion to electricity than conventional gasification with plasma gasification projects being pursued by at least 15 companies in the USA and Canada for production of electricity as well as ethanol, methanol, hydrogen, and diesel from MSW. Since 2001, Utashinai (Japan) produces electricity by gasifying municipal solid waste with more than 100 gasification plants producing energy continuously in Japan, Europe, and Korea (www.gasification.org). In various European countries, fluidized bed-type gasification facilities have been built over the last 10 years in order to circumvent the rising costs of landfills (Nixon et al. 2013), while Fischer-Tropsch fuels from coal gasification have been produced for many years in South Africa.

13.4.3 Bio-power

Global bio-power capacity has been increasing notably which includes LFG, biogas, and synthetic gas (syngas). Average bio-power generation of some of countries annually in between 2010 and 2012 shows the USA leading in bio-power generation followed by Germany, Brazil, and China, respectively. Majority of the countries included in the list of bio-power-generating countries are developed nations with developing Asian nations such as India and Thailand lagging at the 18 and 19 positions, respectively. This indicates that the gap in the bio-power generation between the developed and developing nations is quite large and there is ample scope for improving this scenario by application of appropriate technologies suited to the developing world.

Among the various technologies available, anaerobic digestion (AD) is best option for MSWM in developing countries which besides solving the challenge of energy crisis can simultaneously lead to waste reduction. The organic content of waste in developing countries is higher in comparison to developed countries, which can serve as major substrate for bioenergy formation (http://thinkoutsidethebin.com).

Among the developed countries, the USA has an annual electricity generation between 14 to 331 million kilowatt-hours (kWh). On the other hand, the nonelectric energy production from anaerobic digesters has increased from 1 million kWh in 2000 to 54 million kWh in 2009 (http://www.epa.gov). In Europe, 244 anaerobic digestion facilities dealing with the OFMSW have been constructed or are being setup, having a cumulative capacity of 7,750,000 tons per year of organics which represents 25 % of all biological treatment and around 20 % of all municipal solid waste disposed in Europe. Spain and Germany have an installed capacity of about 1–2 million tons/year. Though small-scale facilities for anaerobic digestion are being implemented in South Africa, economics of the process are governed by scale of the plants which should be of at least 30,000 tons/year capacities with 40,000–50,000 tons/year preferred for better economics (Greben 2009).

13.5 Limitations

13.5.1 Landfill Technology

Production of leachate and landfill gas (LFG) from landfill disposal of MSW poses numerous risks to the environment. Degradation of landfilled material leads to generation of liquid leachate. Release of leachate from landfill operation site after closure is a critical issue. Leachate may contain suspended materials, by-products of degradation including harmful and toxic compounds such as heavy metals, POPs (persistent organic pollutants), and organic material exerting high COD and BOD, which may adversely affect plant growth, percolate through the soil and contaminate the drinking water, and disturb groundwater ecosystem leading to adverse impacts on humans living in the vicinity (Carpenter et al. 2008). MSW landfilling emits landfill gas (LFG) which is considered as greenhouse gas as it contains methane which has higher potential for global warming compared with CO_2. The presence of recyclable material in municipal waste further compounds the problems associated with landfilling by increase in the volume of waste and additional requirement of land area for disposal. The pressure on such municipal landfills can be alleviated by the development of improved recovery methods for recyclables at household level (www.southafrica.info). Owing to these problems associated with landfilling operations, it is a less preferred option for achieving the goal of sustainable development.

13.5.2 Incineration

Incineration of MSW may lead to generation of acidic gases, polychlorinated biphenyls (PCBs), and volatile organic carbon (VOCs), which are major sources of air pollution. In addition, incineration generates huge quantity of ash consisting of major elements such as Al, Si, Na, Mg, K, Ca, Fe, and Cl. Further, the presence of common oxides is likely to result in harmful effects on environment and human health. Dioxin which is constituent of fly ash is considered as hazardous waste (Zhang et al. 2012; Lu et al. 2013).

13.5.3 Gasification

Controlled equipments for sulfide and alkali chloride removal are necessary which otherwise increases toxicity and explosion ability of syngas. Plants are designed to convert the feedstock in two steps: (1) gasification and (2) syngas combustion and conversion, thus making them more complex and difficult to operate and maintain. In the small-scale waste treatment plants, efficiency of internally fired systems is low. The actual nature of pollutants is dependent on the processing of syngas downstream of the gasifier, and eventual oxidation of syngas leads to formation of dioxins, furans, and NOx which may be potentially hazardous. Synthesis of quality chemicals and energy resources from syngas requires costly treatments which is prohibitive to this application.

13.5.4 Anaerobic Digestion

Efficiency of biomethanation depends on growth of microorganisms, which in turn is affected by many operational parameters like pH, temperature, carbon/nitrogen (C/N) ratio, organic loading rate (OLR), reactor design, inoculum, and hydraulic retention time (HRT) (Krishania et al. 2013). Methanation is faster under mesophilic and thermophilic conditions, while the efficiency decreases at lower temperatures in winter since microbes grow faster under thermophilic temperature conditions, thereby resulting in lower HRT of the substrate in the digester (Weiland 2010). In order to avoid lower gas production during anaerobic digestion of MSW, it is essential to optimize the conditions of pretreatment and reactor operation in line with the composition of feedstock. Owing to the complex composition of MSW, it may require some pretreatment or supplementation of additives for enhancing the rate of degradation which in turn will lead to higher biogas production (Feng et al. 2012). Long retention time and low efficiency of organics removal limit the performance of anaerobic digestion since longer HRTs result in high accumulation of volatile fatty acids (VFAs), leading to drastic decline in pH and high production of butyric acid in VFA mixture, which affects the biogas efficiency by inhibiting methanogenesis (Kim et al. 2008; Molino et al. 2013). Level of nitrogen in the feedstock also affects the rate of digestion by changing the C/N ratio, which if low may lead to toxicity and at higher ratio may inhibit rate of digestion. Microbial biomass is disrupted under excessive mixing conditions, thus reducing the rate of fatty acid oxidation and hence the overall biogas production (Padoley et al. 2012).

13.6 Modification of Technologies and Road Map for Sustainable MSW Management

13.6.1 Landfill Technology

Treatment of landfill leachate includes numerous methods which may employ either indirect or direct approach. Indirect way consists of transfer of leachate via piping from landfill to local municipal sewage treatment plant which is the implemented practice in most of the Asian countries (Johari et al. 2012). Among the direct methods, physical processes like coagulation, stripping, adsorption, and chemical processes like chemo-oxidation are used for nonbiodegradable organic matter reduction (Boonyaroj et al. 2012). Waste pretreatments like leachate recirculation and aeration considerably reduce leachate and landfill gas quantity which is characteristic operational improvement of landfill technology (Mahar et al. 2009). Application of calcium hydroxide and alum has been shown to remove up to 69 % and 54 % of COD from the landfill leachate by coagulation, in addition to reduction in turbidity from the leachate by 99.9 % and 94 %, respectively.

Thermal treatments of leachates for separation of organic and inorganic constituents consist of oxidation, incineration, stripping, and evaporation. In spite of the disadvantage of generating volatile exhaust gases, thermal treatment is still preferred for treating leachates with toxic components (Chou et al. 2013). Biological treatment is an effective option for treating organic content of leachate, but emission of organochlorine like toxic substances into air presents a major obstacle for this option. Efficient reduction of soluble organic matter in anaerobic landfill is achieved after aerobic treatment of MSW (Salati et al. 2013). Flux chamber method can be used to estimate methane gas emission from landfill sites which indicates faults in landfill capping and abnormal gas emissions in landfill area (Di Trapani et al. 2013). Carbon isotope analysis in leachate can serve as an effective tool for monitoring biodegradability and stability of MSW in landfill (Wimmer et al. 2013). Application of advanced waste recycling technologies (membrane and advanced oxidation technologies) for improving waste recycling in developed countries has led to a reduction in volume of landfilled MSW, thus causing a decline in emission of landfill gas (Renou et al. 2008; Johari et al. 2012). Methane produced from landfill gas can be used in incinerators, which may reduce the need for combustion (Ray et al. 2012).

13.6.2 Incineration

The hot, acidic exhaust containing oxides of sulfur and hydrogen chloride produced during incineration can be sprayed with lime powder by scrubbers, thereby neutralizing the gases. Alternatively, the gases could be passed through an activated carbon column for absorbing heavy metals and pollutants – polychlorinated biphenyls (PCBs) and volatile organic compounds (VOCs) (Nzihou and Stanmoreb 2013). Nitrogen oxides can be removed by a selective noncatalytic system consisting of urea or ammonia with nitrogen, carbon dioxide, and water followed by removal of the fine particulates and dust particles by bag filter system.

Ashes can be treated by thermal treatments after differential separation techniques. Reaction with HCl, chelation with EDTA, and leaching have been proposed to be effective for promoting the use of fly ash from MSW and recover metals (Fedjea et al. 2010). Electrochemical processing in combination with washing greatly reduces heavy metals from fly ash (Kirkelunda et al. 2013). In addition, end-of-pipe treatments are effective for minimization of dioxins in the atmospheric emission of flue gas and include techniques such as bag filtration and lime scrubbing (Cloirec 2012). Water extraction removes soluble salts from fly ash resulting in more stable synthetic material production from ash. Nonhazardous material results from fly ash due to application of milling process for stabilization of heavy metals (Chen et al. 2013).

13.6.3 Gasification

The problems associated with wide-scale application of syngas technology could be overcome by using different reactor systems like entrained, fluidized, and fixed bed including vertical shaft and plasma rotary kiln, each of which is suited for particular application. One example for such a system specific for gasification-based waste to energy is a modular system combining pyrolysis, gasification, and combustion (Arena et al. 2011). In comparison with different gasifiers, only entrained flow and dual fluidized bed gasifiers have been shown to produce high-quality syngas suitable for liquid fuels at pilot or field scales. Advanced technologies such as gas turbines and fuel cells result in higher efficiency of gasification. Gasifiers having large capacity are efficient for treatment of MSW for better carbon conversion as it provides uniform process temperatures and better solid-gas interactions. Fuel for gas can be obtained from gasification of biomass waste with high energy efficiency achieved through integration of gasification into combined cycle power plant (Minguez et al. 2013).

Heat and power can be simultaneously generated from syngas by "heat gasifier" as used in case of ammonia production in Japan in a "power gasifier" configuration (Calvo et al. 2012). The fuel can be dried on the top of the gasifier, thereby enabling the use of high moisture-containing wastes. Fuel flexibility of MSW gasifiers has improved using grate furnace in the UK and Germany (Margallo et al. 2012). Plasma gasifiers can be used in waste-to-energy units where plasma torches fire to melt inorganic matter present in the MSW. Combination of oil material adsorption in scrubber and catalytic reforming in gasifier combination can further result in tar removal which is considered as an important step in biomass gasification (Shen and Yoshikawa 2013).

The fact that MSW is largely available in developing countries in unsorted form, the variations in size and composition of constituent materials in unsorted MSW make it unsuitable for treatment by thermal technologies. Such materials may have adverse effect on the process or emission control systems, thereby leading to reduction in efficiency of the thermal treatment technologies. Efficiency of thermal technologies like gasification can be improved through pretreatment processes of MSW which converts this waste into consistent chemical and physical properties necessary for feed used in gasifier. Preprocessing of waste includes mechanical sorting, manual separation, pellet formation, and shred and blend with other materials. Essentially, the feedstocks need to be pretreated by any one of the following means before gasification in order to increase process integration and lower the cost of gasification, and this includes three main options: pelletization, torrefaction, and fast pyrolysis. However, plasma gasifiers can provide good syngas quality along with added advantage of using feedstocks directly without pretreatment.

13.6.4 Anaerobic Digestion

The efficiency of biogas production from lignocellulosic MSW can be increased by application of various pretreatment methods including chemical pretreatment with alkali like NaOH and pretreatment with liquid ammonia at relatively high temperature called ammonia fiber explosion (AFEX) which is also effective for wastes with lower lignin and high cellulose content (Esfahani and Azin 2011; Menon and Rao 2012). Pretreatment by hydrolyzing with dilute acid is the most common chemical pretreatment used for high lignocellulosic substrate. Enzymatic pretreatments have been also employed for this process (Sonakya et al. 2001). Additives such as nickel have been shown to improve the anaerobic digestion efficiency by inducing the F430 enzyme of methanogenic bacteria (Brulé et al. 2013). Operational conditions for anaerobic reactor need to be optimized for effective methanogenesis with thermophilic temperature, proper C/N ratio, and co-digestion of different wastes favoring high rates of anaerobic digestion (Kalia et al. 2000b; Lo et al. 2010). Optimum organic loading rate helps in maintaining proper VFA concentration during the digestion process. Application of reactors of different configurations can be beneficial for enhanced recovery of biogas from MSW which include single-stage continuous fed system (all biochemical reactions in one reactor) and two-stage or multistage continuous fed system (hydrolysis, acetogenesis, and methanation occur separately) (Ward et al. 2008). Among the single-stage and two-stage reactors, high efficiency of biogas production is achieved in two-stage reactors owing to separation of phases (Fezzani and Cheikh 2010). Different constituents of MSW can be effectively treated using bioreactor systems like anaerobic filter, sludge blanket, and tubular and stirred tank reactors (Bouallagui et al. 2005). Of these, the batch reactor configuration is simple and suitable for quick digestion of MSW (Khalid et al. 2011).

13.7 Conclusion

No single solution can be applied to all different types of wastes generated, which needs to be devised in waste-specific manner. Overcoming the major issues faced by various technologies and application of different possible solutions can lead to sustainable management of MSW. Innovative applications of ash are being investigated due to environmental and technological problems associated with incineration and ash disposal and also for application of syngas to improve gasification technology. The lowering of application of landfilling in most countries of the world, owing to problems associated with leachate control and landfill gas, is a challenge faced by treatment technologies based on landfilling for managing MSW. Anaerobic digestion (biomethanation) is emerging as a promising technology for effective utilization of misplaced resource in the form of MSW (Kalia et al. 1992a, b; Kalia and Joshi 1995). The conversion of waste through modern biotechnological advancements can help to develop effective degradation strategies and novel methods for producing bioenergy, biopolymers, and bioactive molecules (Kalia et al. 2003a, b; Reddy et al. 2003; Selvakumaran, et al. 2008, 2011; Verma et al. 2010, 2011). It is possible to generate biofuels like hydrogen from biowaste (Kalia et al. 1994; Kalia and Purohit 2008) and bioplastics (Kalia et al. 2000a; Porwal et al. 2008; Kumar et al. 2009b; Singh et al. 2015). Ecobiotechnological and coculture approaches and integration of different processes are effective in case a large number of organisms are involved during digestion (Singh et al. 2013). This approach has been effective in producing hydrogen, biopolymers, and methane from a pure or mixed biowaste (Kumar et al. 1995; Singh et al. 2009; Kumar et al. 2013, 2014a, b, 2015a, b; Patel et al. 2010, 2011, 2012a, b, 2014, 2015; Patel and Kalia 2013). More recently, metagenomic methods have also been studied for improving ETPs (Rani et al. 2008). This paper can help in deciding upon appropriate choice of MSW treatment technology and further development of future treatment technologies.

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Prabhakar Dattatray Pandit He received his M.Sc. degree in Biotechnology from Dr. Babasaheb Ambedkar Marathwada University subcenter, Osmanabad, India. He is currently working as DST-INSPIRE SRF and doing Ph.D. under AcSIR program at CSIR-NEERI, Nagpur, India. His current interests are “biofuels, sustainable municipal solid waste treatment and technologies, and metagenomic analysis in anaerobic digestion system.”

Madhuri Kisanrao Gulhane She received her M.Sc. degree in Microbiology from SGBAU, Amravati. She is presently working as CSIR-SRF and doing her Ph.D. under AcSIR program at CSIR-NEERI, Nagpur, India. Her current research focuses on “the metagenomic study of lab-scale anaerobic bioreactor-treating vegetable waste.” She is the life member of Biotechnology Research Society of India (BRSI).

Anshuman A. Khardenavis He received his Ph.D. in Microbiology from RTM Nagpur University. He is presently working as a scientist, at CSIR-NEERI. His current interests include sustainable management of organic fraction of municipal solid waste through biofuel production, industrial enzymes, and feed applications. He is a life member of Association of Microbiologists of India.

Atul N. Vaidya He is a chemical engineer working as a head at Solid and Hazardous Waste Management Division, CSIR-NEERI. His current interests include developing strategies for sustainable management of municipal solid waste, biotechnological processes, and cleaner technologies for wastewater treatment and environmental restoration with recourse to recovery of value-added chemicals. He is a life member of Indian Institute of Chemical Engineers (IIChE).

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Waste Remediation Integrating with Value Addition: Biorefinery Approach Towards Sustainable Bio-based Technologies

C. Nagendranatha Reddy, J. Annie Modestra,
A. Naresh Kumar, and S. Venkata Mohan

Abstract

The perpetual increase in the environmental pollution and the diminution of fossil fuels are forcing mankind towards the usage of sustainable and eco-friendly technologies to build a green and global future. Similarly, increase in human population is eventually resulting in the discharge of huge quantities of waste that need serious attention. If the waste is managed aptly, the negatively valued waste would absolutely result in the generation of a definite value-added product. A multifaceted approach is needed to alleviate the energy crisis in an interdisciplinary way by integrating waste remediation towards bioenergy generation. Diverse forms of energy, viz. biohydrogen, bioelectricity, biodiesel and bioplastics could be produced by utilizing waste/wastewater as substrate by the catalytic action of bacteria. The inherent potential of the diverse bacteria present in wastewater can be effectively exploited for the generation of bioenergy along with the recovery of value-added products in a green and integrated approach. In this context, sustainable, green and eco-friendly technologies were described in this chapter to exploit the potential of waste/wastewater in the framework of biorefinery.

14.1 Introduction

The excessive usage of fossilized fuels in various industries, automobiles and other sectors has led to its depletion, forcing the mankind to look for alternative fuels like solar, wind, hydro, thermal, etc (Noori and Saady 2013; Cao et al. 2014). Another potential alternative energy in which there is much scope for energy generation is "the waste" that is generated from various industries, household activities, municipalities, etc., throughout the world (Li and Yu 2011; Ghimire et al. 2015). Management and treatment of large quantities of waste and wastewater generated worldwide is particularly challenging due to the problems being faced during its treatment and disposal. This waste requires treatment, prior to their disposal into natural water and environmen-

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C.N. Reddy • J.A. Modestra • A.N. Kumar •
S.V. Mohan (✉)
Bioengineering and Environmental Sciences (BEES),
CSIR-Indian Institute of Chemical Technology
(CSIR-IICT), 500007 Hyderabad, India
e-mail: vmohan_s@yahoo.com

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tal bodies. Utilization of waste and wastewater in a proper way plays a crucial role in the treatment and energy industry, thus generating various forms of bioenergy along with the recovery of value-added products (Venkata Mohan 2010; Brentner et al. 2010; Venkata Mohan et al. 2009a, 2014d). Biodegradable waste could be converted to various forms of energy which has potential to meet world's growing energy demand (Rittmann 2008). Apart from waste remediation, several sustainable technologies were developed to produce industrially important chemicals, solvents, materials and fuels that not only reduce our dependency on fossil fuels and products but also help in mitigating environmental pollution (Amulya et al. 2014, 2015; Ghimire et al. 2015). Biofuel production from renewable sources is a viable means for environmental and economic sustainability and is widely considered as one of the most sustainable alternatives to petroleum-based fossil fuels (Venkata Mohan et al. 2014a; Dragone et al. 2010). Various energy forms like biohydrogen, bioelectricity, bioplastics and biodiesel were generated by treating various wastes and wastewater as substrate. H_2 has been recognized as a promising energy carrier for the future because of high efficiency, energy yield (122 kJ/g), renewable nature and potential sustainability (Mohanakrishna et al. 2011). Currently, global H_2 production is majorly from fossil source and electrolysis of water. In recent times, biological routes of H_2 production gained promising attention among the research fraternity around the globe (Venkata Mohan et al. 2009b). Broadly biological process of H_2 production can be classified into bio-photolysis (direct and indirect) and fermentation (dark and photo) processes or combination of these processes (Chen et al. 2008). The acid-rich effluents (comprising of carboxylic acids) produced through acidogenic fermentation process could be used with an integrated approach for the synthesis of valuable by-products (eg: bioplastics/biofuels). Polyhydroxyalkanoates/polyhydroxybutyrates (PHAs/PHBs) are high molecular weight (mass of 50–100 kDa), carbonaceous, cellular reserve storage products that guard against starvation in bacteria and occur in many types of Gram-positive and Gram-negative bacteria, under excess carbon and nutrient-deprived conditions (Madsen 2008). They feature many traits similar to modern synthetic plastics viz., a high degree of polymerization, highly crystalline, optically active and insoluble in water (Madsen 2008). Apart from the bioplastics, microbial fuel cell (MFC) is another alternative systems for generating bioenergy in terms of bioelectricity by utilizing microbial membrane potential differences. It is a bio-electrochemical system that converts chemical into electrical energy via redox reactions (Ahn and Logan 2010, Franks and Nevin 2010; Venkata Mohan and Srikanth 2011, Srikanth and Venkata Mohan 2012). Apart from bioelectricity generation, MFC is classified into bio-electrochemical treatment (BET) and bio-electrochemical system (BES) based on the application of technology and the focus of the study. In BET, the main focus would be on treatment and waste remediation by removal of various toxic pollutants (Venkata Mohan et al. 2009a; Mohanakrishna et al. 2010a). BET has gained prominence in the research community as it not only generates bioelectricity but also removes toxic compounds considering various industrial waste and wastewater as substrates and is being applied for various treatment technologies. BES technology can be extended to the production of other value-added products by attenuating the carbon dioxide (CO_2) to various viable fuel alternatives, viz. ethanol, butanol, organic acids, etc., in its terminal reduction reactions along with the power generation, contributing its part in reducing the carbon footprints on the Earth (Venkata Mohan et al. 2013a; Ahn and Logan 2010; Rabaey and Rozendal 2010). Crop-based terrestrial sources of biomass require finite area of land for its cultivation, whereas microalgae are deemed to be potential alternative for generating biomass. Microalgae offers several advantages over conventional terrestrial crops like high productivity, non-competitive for food and higher CO_2 sequestration compared to terrestrial plants. Moreover it is highly feasible for oil production and conversion efficiency (Venkata Mohan et al. 2014b; Duan and Savage 2010).

Atmospheric CO_2 sequestration by microalgae is a possible approach for harnessing renewable energy and is considered to be more beneficial than oil-cultivating crops (Chisti 2008). A paradigm shift from waste remediation towards energy generation has been of great interest in the scientific community and the positive aspects of waste/wastewater as a feedstock for many biological processes thus enabling the reduction of greenhouse gas emissions with simultaneous waste remediation. In a biorefinery approach, the waste generated can be effectively recycled and reused by integrating the waste coming from one industry/sector that becomes the raw material for another and thus making the technology sustainable. Realizing even a tiny proportion of the benefits anticipated will motivate us to move forward in this direction and transform waste treatment towards sustainability. In this perspective, an attempt was made in this chapter to elucidate the various forms of biofuels, bioenergy and other value-added products that can be generated by waste remediation in a biorefinery approach.

14.2 Biohydrogen

Hydrogen is a carbon-free clean fuel and has been attracting great interest as a clean, renewable and effective energy carrier which can minimize the fossil fuel-derived energy dependence and global economy increment with subsequent attenuation in environmental pollution. Studies were carried out on H_2 energy including production, storage, use and safety issues (Reddy et al. 2011; Venkata Mohan et al. 2009b). Additionally, hydrogen can be directly used either in combustion engines because of its highest energy per unit weight, i.e. 143 GJ per ton among known gaseous biofuels, or to produce electricity via fuel cell technologies. On the other hand, 96 % of hydrogen is being produced (conventionally) via thermochemical processes using fossil fuel as an energy source. These processes are energy intensive and unsustainable causing CO_2 emissions, which leads to environmental pollution (Venkata Mohan et al. 2009a, 2011). On the contrary, microbial hydrogen production by using renewable carbon sources like organic wastes and sunlight works at normal temperatures and pressures (Nikhil et al. 2014). Therefore, biological hydrogen production is considered as an important step to a sustainable biofuel process and has the significant potential to replace fossil fuels (Venkata Mohan 2009; Mohanakrishna and Venkata Mohan 2013). Hence, various technologies were developed to harness biohydrogen from clean renewable sources. These renewable biohydrogen-producing technologies have potential to become cost competitive as they can utilize low-cost waste biomass as feedstock, viz. organic fractions of municipal solid waste along with agricultural, industrial organic waste and wastewater.

14.2.1 Biohydrogen Production Methods

Basically, biological H_2 production process is classified into light-dependent photosynthesis and light-independent dark-fermentation process. In case of light-dependent process, it occurs via direct and indirect bio-photolysis of water using green algae and cyanobacteria, which utilize inorganic carbon and water in the presence of sunlight to produce H_2 (Chandra and Venkata Mohan 2014; Uyar et al. 2009). The light-dependent process is of two types, viz. oxygenic and anoxygenic, where green algae follow oxygenic while photosynthetic bacteria (PSB) follow anoxygenic process (Dasgupta et al. 2010). Compared to other processes, dark fermentation is rapidly studied and is a promising technology for biohydrogen production owing to its higher production rates and treatment capacity for organic wastes (Venkata Mohan et al. 2009a, 2011; Hallenbeck et al. 2012). The process proceeds via mineralization of carbon-rich substrates under anaerobic microenvironment. Theoretically, 1 kg COD can produce 20.83 moles of H_2 (466.6 l of H_2/41.6 g of H_2) through dark-fermentation process. Dark-fermentation process comprised of different phases, viz.

hydrolysis, acidogenesis and acetogenesis, with the involvement of physiologically diverse groups of microorganisms to generate H_2 along with the accumulation of volatile fatty acids (VFAs) also known as carboxylic acids (Dahiya et al. 2015: Venkata Mohan et al. 2012). Initially, hydrolysis proceeds through hydrolytic microorganisms, which convert the complex organic matter to monomers. Thus, with the course of time and fermentation process (acidogenesis step), monomer sugars are converted into biohydrogen and low molecular weight organic acids (Venkata Mohan et al. 2007, 2009a; Mohanakrishna et al. 2011). The major advantage of dark fermentation is the direct utilization of complex and challenging organic waste as substrate, which significantly enhances their economic viability. On the contrary, with aerobic respiration, oxygen is reduced and water is the final product. Biohydrogen (H_2) is produced in the process of disposing excess electrons through the activity of hydrogenase enzyme (Venkata Mohan 2012b; Silva et al. 2013). Under anaerobic environments, protons (H^+) can act as electron acceptors to neutralize the electrons generated by oxidation of organic substrates, consequently by producing H_2. Therefore, hydrogen production via a mixed dark-fermentative culture is a complex microbial system, influenced by a number of parameters such as substrate type, inoculum type, inoculum enrichment method and bioreactor design and operation. Therefore, studies were evaluated with the influence of substrate type, substrate loading, inoculum pretreatment methods, reactor design, etc. (Venkata Mohan et al. 2014c; Chandra et al. 2014) (Fig. 14.1).

Mixed microbial cultures can be directly used as inoculum for biohydrogen production, which is practically viable with minimal maintenance and more productivity than single strains. Some adverse microorganisms which lower the total H_2 yield might be present in mixed cultures of fermentative microorganisms, either by consuming the H_2 produced or by altering the biochemical pathways of the H_2 synthesis (Kannaiah and Venkata Mohan, 2012; Venkata Mohan 2009). The major H_2 consumers include methanogens which could be controlled by various pretreatment methods to the parent inoculum, such as shocks of heat, acid, alkaline, oxygen, load, chemical, infrared irradiation, freezing and thawing, microwave irradiation, etc. (Venkata Mohan 2008; Sarkar et al. 2013). The major challenges in dark-fermentative H_2 production systems are their low energy conversion efficiency due to the accumulation of carboxylic acids (acetate (C2), propionate (C3) and n-butyrate (C4), etc.). Additionally, these lead to a decrease in pH, which limits microbial H_2 production (Matthew et al. 2010: Venkata Mohan et al. 2008d, 2010b, 2012). With the integration approach, carboxylic acids (carboxylate platform) could be used for the production of bioplastics (polyhydroxyalkanoates/polyhydroxybutyrates: aerobic bacteria), biofuels (bioethanol/bioelectricity: anaerobic bacteria) and as substrate for biological nutrient removal processes (Li and Yu 2011; Matthew et al 2010; Dahiya et al. 2015). The co-produced hydrogen from the acidification process could be used for the synthesis of biofuels. In this, 1 mol of VFA needed 2 mol of hydrogen (Khardenavis, et al. 2007; Li and Fang 2007; Hollmann 2012). During excess of carbon and nutrient limiting conditions such as due to N and P stresses, bacteria produce PHA, and it accumulates as reserve storage material (Reddy et al. 2014). Moreover, VFAs can act as a good substrate for biological nutrient removal processes and bioelectricity generation in microbial fuel cells (MFCs) (Wei et al. 2008; Lenin babu et al. 2013a). This will alleviate the dark fermentation by not only avoiding the end-product inhibition (acids accumulation), but also prevents pH drop resulting through VFA's accumulation. Thus, the creation of a hydrogen economy with an integrated approach accelerates the production and use of hydrogen as an energy carrier that could lead to sustainable energy systems with simultaneous attenuation in the environmental pollution (Pasupuleti et al., 2014) (Table 14.1)

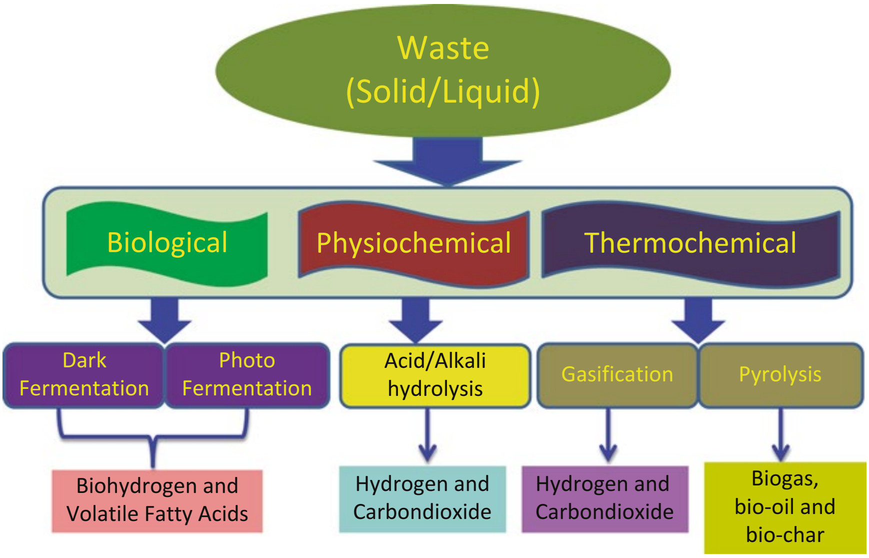

Fig. 14.1 Various processes of producing biohydrogen-utilizing waste as substrate

Table 14.1 Fermentative biohydrogen production yields from diverse waste materials

Feedstock (Waste/wastewater)	Culture type	H_2 yield	References
Rice winery WW (36 g COD/L)	Anaerobic mixed culture	389 mL/g VSS h	Yu et al. (2002)
Potato starch (1 g COD/L)	Anaerobic mixed culture	0.59 mol/mol starch	Logan et al. (2002)
Sucrose-containing wastewater	Anaerobic sludge+PSB	0.39 m^3-H_2/kg COD	Tao et al. (2007)
Olive mill wastewater	Anaerobic sludge+PSB	0.66 m^3-H_2/kg COD	Eroglu et al. (2006)
Molasses wastewater	Anaerobic sludge+ARB	1.34 m^3-H_2/kg COD	Lu et al. (2009)
Wheat starch	Anaerobic mixed culture	0.83 mol/mol starch	Hussy et al. (2000)
Sucrose (20 g COD/L)	Anaerobic mixed culture	3.47 mol/mol sucrose	Chen et al. (2001)
Food waste (3 % VS)	Anaerobic mixed culture	111 mL/g VSS h	Kim et al. (2004)
Vegetable waste (3 g COD/l)	Anaerobic mixed culture	70 ml L^{-1} h^{-1}	Venkata Mohan et al. (2009c)
Distillery/spent wash	Anaerobic mixed culture	83 ml L^{-1} h^{-1}	Venkata Mohan et al. (2008e)
De-oiled microalgae	Anaerobic mixed culture	5.12 ml L^{-1} h^{-1}	Venkata Subhash and Venkata Mohan (2014a)
Dairy wastewater	Anaerobic mixed culture	12 ml L^{-1} h^{-1}	Venkata Mohan et al. (2007)

14.3 Microbial Fuel Cells (MFCs)

MFCs are the bio-catalyzed electrochemical systems that use bacteria as catalyst to generate bioelectricity by the degradation of organic matter. Through the occurance of a series of redox reactions, MFCs have the ability to convert energy from chemical form to electrical by linking the microbial metabolism via electron-donating and electron-accepting conditions (Venkata Mohan et al. 2014e). The use of MFCs for power generation was first documented in 1915 by Potter and his co-workers (Potter 1910). Many years later in 1980, studies were conducted to improve the performance of MFCs, and nearly after two decades, extensive studies were carried out in the development of MFCs (Kim et al. 2002). MFCs offer dual benefits of bioelectricity generation with simultaneous waste remediation that makes the process eco-friendly, by utilizing simple and complex wastewater/waste as a carbon substrate (Venkata Mohan 2014; Gil et al. 2003; Orta et al. 2011; Pant et al. 2012).

14.3.1 Mechanism of Bio-electrogenesis

Microorganisms/bacteria accomplish metabolic pathway in aerobic (presence of oxygen) or anaerobic (absence of oxygen) microenvironments depending on the nature of metabolism (Venkata Mohan 2012a). Microorganisms utilize the available substrate and generate reducing equivalents (e^- (electrons) and H^+ (protons)). These redox carriers generated, viz. nicotinamide adenine dinucleotide (NAD), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), etc., helps in generating energy (adenosine triphosphate (ATP)) during respiration. In MFCs, the electrodes introduced in to the cell act as artificial electron acceptors that induce the generation of potential difference. Oxidation occurs at the anode, generating protons (H^+) and electrons (e^-), while reduction takes place at the cathode. The electrons remain at the anode, generating negative anodic potential, while H^+ move towards the cathode via the proton exchange membrane, generating positive cathode potential. The difference between positive cathodic and negative anodic potentials is considered as cell voltage/electromotive force that drives the electron flow (Fig. 14.2). The redox reactions occurring at anode and cathode can be represented as:

$$C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 24H^+ + 24e^- (Anode) \quad (14.1)$$

$$4e^- + 4H^+ + O_2 \rightarrow 2H_2O \quad (\text{Cathode}) \quad (14.2)$$

$$C_6H_{12}O_6 + 6H_2O + 6O_2 \rightarrow 6CO_2 + 12H_2O \quad (\text{Combined}) \quad (14.3)$$

The anodic oxidation reaction and terminal cathodic reduction reaction are separated by a selectively permeable ionic membrane that allows the passage of protons and capture of e^- through the circuit (Venkata Mohan et al. 2012; Venkata Mohan et al. 2008a; Mohanakrishna et al. 2010a; Lenin et al. 2012). Various factors, viz. substrate, reactor configuration, electrode materials, distance between the electrodes, catholyte, anolyte, biofilm growth, membrane, anodic and cathodic metabolic environment, etc., are found to influence the MFC performance. MFC is a sustainable process because of its potential to generate bioelectricity by utilizing waste as carbon source (Venkata Mohan et al. 2008b). Various waste waters were used as substrates to evaluate the power generation capability of MFC. Electron transfer from bacterial membrane to the electrode is an important factor that influences the power generation capability of MFC. There are two feasible mechanisms by which the electron transfer takes place, viz. (1) mediated electron transfer (MET) and (2) direct electron transfer (DET). MET occurs either by the addition of artificial mediators or by the secretion of soluble mediators resulting from bacterial metabolism, and DET occurs through the bacterial cell wall and electrode contact without the involvement of any redox mediators (Schroder 2007). Nanowires, biofilm or membrane-bound proteins facilitate direct transfer of electrons from the bacterial membrane towards anode. In few species, electrons released during the bacterial metabolism get transferred through soluble shuttling compounds, i.e. mediators, which are essential in promoting electron transfer by minimizing the losses. Many bacteria are capable of forming a biofilm that regulates the electron discharge onto the anode. Many Gram-positive bacteria participate in direct electron transfer through the formation of biofilm by the presence of teichoic acids that aid in the bacterial surface adherence to the electrode surface (Liu et al. 2012, Annie Modestra and Venkata Mohan 2014). Electrochemically active bacteria present in the biofilm facilitate effective electron

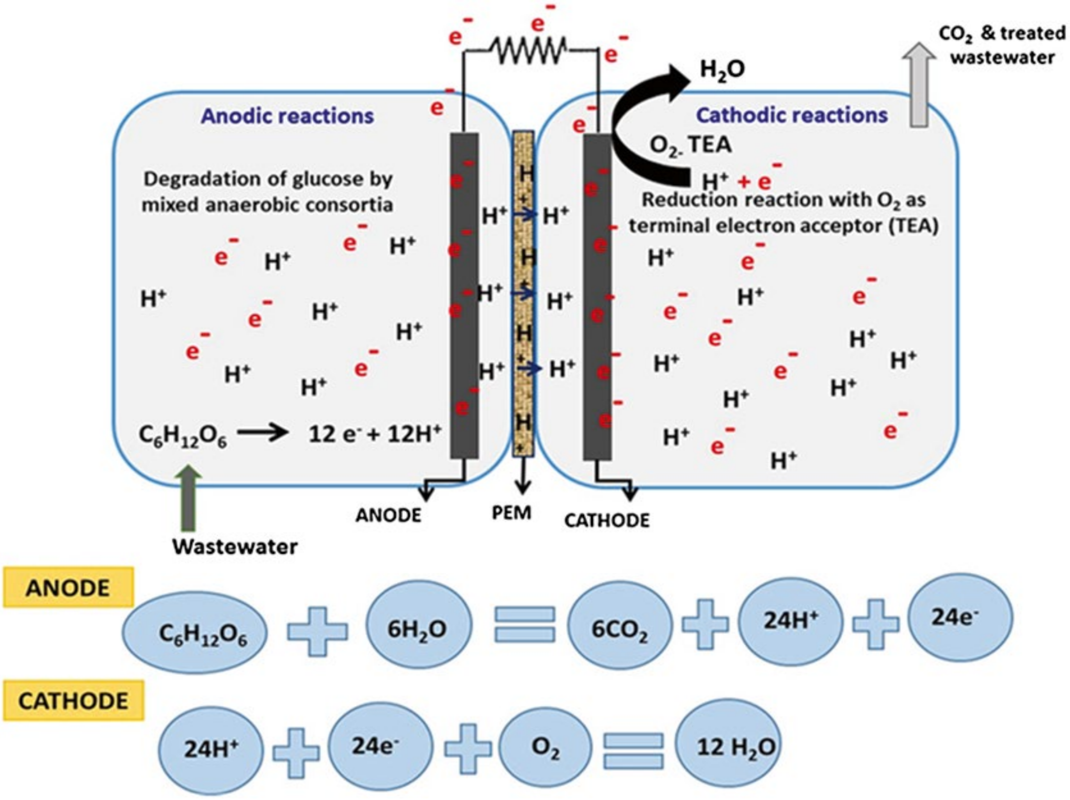

Fig. 14.2 Mechanism of bioelectricity generation along with the redox reactions at anode and cathode in a microbial fuel cell

transfer from the anode, thereby contributing to enhanced electrogenesis. Bacterial community present in the biofilm has a synergistic interaction which is essential in regulating the electron transfer (Venkata Mohan et al. 2008c). Interaction between MFC components and microorganisms should be studied to optimize and explore the potential of fuel cell and biocatalyst. This optimization helps in escalating the treatment efficiency through which the bioenergy production and recovery of products are maximized (Venkata Mohan et al. 2012; Velvizhi and Venkata Mohan 2011). Power generation and electron discharge in MFC are mainly based on the potential difference between physical, chemical and biological components of the fuel cell. MFCs primarily make use of anaerobic bacteria, but other types of organisms like photosynthetic bacteria have also been used in MFCs for tapping the scope of solar energy, thus widening the MFC research towards applicability (Chandra et al. 2012; Srikanth et al. 2009a, b; Raghavulu et al. 2013; Venkata Mohan et al. 2013b). These fuel cells can also be designed with different configurations, viz. benthic MFCs, sediment type and plant-based and stacked MFC. In benthic MFCs, natural native habitation like aquatic ecosystem is used for harnessing bioelectricity (Chiranjeevi et al. 2013a), but solar energy (indirect) is used for renewable energy generation by placing electrodes at the rhizosphere (roots of a living plant) (Chiranjeevi et al. 2013b). Various strategies, viz. stacking of fuel cells, multiple electrode assemblies, etc., can improve the efficiency of microbial fuel cells in terms of power generation (Kim et al. 2012).

14.3.2 Bio-electrochemical Treatment System (BET)

Bio-electrochemical treatment (BET), an alternative form of MFC, is an emerging interest in the current research scenario that can be utilized for waste remediation, more specifically towards the removal of complex pollutants and heavy loaded organic content present within the wastewaters. When wastewater treatment is considered as prime motto, apart from power generation, MFC can be also called as BET. Many physical, oxidation, bio-, physico-, and electrochemical reactions can be triggered in the anodic chamber of BET operation for the treatment of complex pollutants (Venkata Mohan et al. 2009a; Velvizhi and Venkata Mohan 2015). The anode chamber of MFC can be equated to anaerobic bioreactor and thus behaves like a conventional electrochemical cell which is used for treating wastewater. The redox reactions occurring during the BET operation help in degrading organic toxic/xenobiotic pollutants/complex wastewaters (Venkata Mohan et al. 2009a, 2010b; Mohanakrishna et al. 2010b). The principle of BET lies on the electron transfer mechanism of electrochemically active organisms associated with redox reactions that help in power generation with simultaneous waste reduction. Coupling of bio-anode to a counter-electrode (cathode) will definitely help in improving the efficiency of the wastewater treatment, which needs to be exploited (Venkata Mohan and Chandrasekhar 2011, 2012). Direct and indirect anodic oxidations (DAO and IAO) are the two mechanisms that facilitate the effective removal of pollutants (Kiran kumar et al. 2012). Pollutants such as elemental sulphur, azo dyes and estrogens/endocrine-disrupting compounds (EDC) act as mediators for electron transfer in the anode chamber of BET (Dutta et al. 2009; Kumar et al. 2012). In the near future, we can expect MFC/BET to improve the current energy status and the processes of treating wastewater (Venkata Mohan 2012a).

14.3.3 Microbial Electrolysis Cell (MEC)

An alternative route for biological H_2 production from renewable resources is the microbial electrolysis cells (MECs) (Venkata Mohan and Lenin Babu 2011; Lenin Babu et al. 2013b; Venkata Mohan et al. 2013a, 2014e). MEC differs from MFC in the aspect of application of external potential which helps in providing the reducing equivalents for H_2 formation at cathode by overcoming the thermodynamic barrier. H^+ reduction to H_2 occurs at the surface of cathode by the electrons liberated from anode. MECs offer comparatively higher H_2 yields than the conventional anaerobic fermentation and water-splitting (electrolysis) processes (Cheng and Logan 2007). Single-chamber membrane-less MEC was proved to achieve high H_2 recovery, despite being more economical. Enhanced hydrogen production accompanied by wastewater treatment was achieved by integrating dark fermentation with MEC process (Lenin Babu et al. 2013a). Similarly, dark fermentation and electrohydrogenesis together result in high H_2 yield and low energy consumption in comparison to the conventional water electrolysis, thereby making the process economically feasible (Cheng and Logan 2007).

14.3.4 Bio-electrochemical Systems (BES)

MFC has additional benefits of recovering value-added chemicals, viz. acetate, butanol, ethanol, etc., through the terminal reduction reactions from waste by bio-electrochemical synthesis (Rabaey and Rozendal 2010). Depending on the redox conditions for electron donation and accepting at the cathode, different value-added chemicals can be synthesized by their reduction at cathode. Thus, BES can also be used for the recovery of products apart from the treatment. Redox potential plays a critical role in the

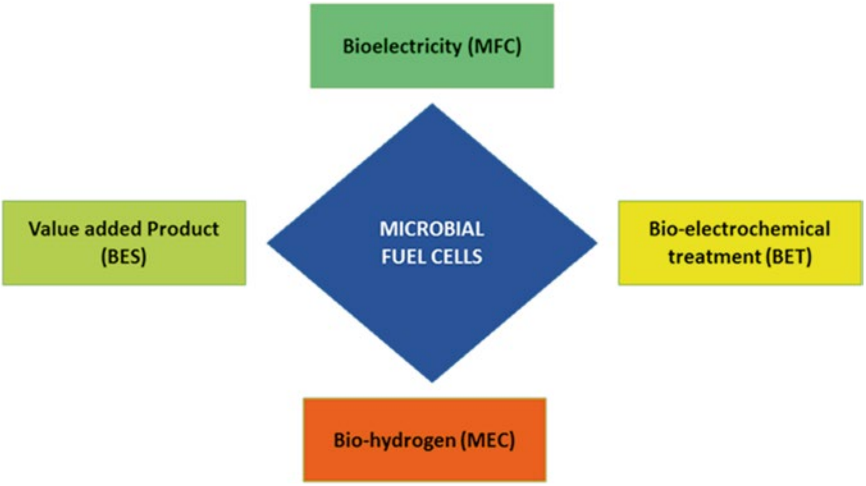

Fig. 14.3 Multidimensional functions of microbial fuel cell (MFC) as bio-electrochemical system (BES), microbial electrolysis cell (MEC) and bio-electrochemical treatment (BET)

product formation, as the fate of chemicals synthesis or carboxylic acids depends on the reduction potential (Gong et al. 2013; Rabaey and Rozendal 2010). Few biological reactions can aid in the product synthesis by the *in situ* potential, while few requires additional potential to get reduced into a value-added product. During such reactions, the redox potential can be maintained by applying the potential externally to cross the thermodynamic energy barrier towards the product formation. In BES, biocathode is also employed for the microbially catalyzed electrosynthesis of various value-added chemicals that forms a stable enriched community towards the synthesis of specific product.

MFC with multidimensional utility offers dual benefits of value addition and simultaneous waste remediation (Fig. 14.3). Though MFC research offers multiproduct recovery, the output achieved either in terms of power output or product synthesis is still very less. Hence, strategies should be designed and implemented effectively in the MFC research towards the enhancement in product recovery and power output. The performance of these systems is highly relied on the electron transfer machinery in the organisms. Future research focusing on improving the electron transfer mechanisms in bacteria is presumed to have significant influence in augmenting of power/product recovery in MFC.

14.4 Bioplastics

The vast usage of synthetic plastics by mankind has become the intensified problem of solid waste disposal over the past few years due to their non-biodegradable property (Swift 1993). Environment issues like global warming and green house effect are caused due to short-time usage and dumping in landfills or are incinerated which will result in release of toxic gases. In order to minimize the problems associated with synthetic plastics, the thirst for developing bio-based polymeric materials is increasing. Polyhydroxyalkanoates (PHAs), a type of bioplastics, are deemed to be an alternative to synthetic plastics that has the complete biodegradable and biocompatible nature (EPA 2000; Anderson and Dawes 1990; Reddy et al. 2003). Bioplastics synthesis was initiated in the 1920s by Maurice Lemoigne, who discovered the intracellular granules in Gram-positive bacterium *Bacillus megaterium*, which are polyesters (poly(3-hydroxybutyrate), P(3HB)) belonging to polyhydroxyalkanoates (PHAs) (Lemoigne 1926; Singh et al. 2009). Nearly after a decade, 11 varieties of PHAs with linear and branched repeating units of four to eight were reported. PHA are high molecular weight (mass of 50–100 kDa) carbonaceous, nontoxic, cellular reserve/storage products produced by both Gram-positive and Gram-negative bacteria under excess carbon and nutrient-deprived conditions (Madsen 2008; Porwal et al. 2008). The properties of PHA that make them outstanding when compared to synthetic polymers are their high biodegradability, their production from biological and renewable sources and their being pure enantiomers, elastomers, thermoplastic, biocompatible and nontoxic. Other common forms of bioplastics are poly-b-hydroxyvalerate (PHV) and poly-b-hydroxybutyrate (PHB) (Luengo et al. 2003). PHAs are synthesized under certain nutrient-deprived growth conditions such as carbon load shock (excess) and put short of one or more essential nutrients, viz. nitrogen, oxygen, sulphur, phosphorus and trace elements (iron, calcium and magnesium) (Hazer and Steinbuchel 2007). PHA usually appears in granular form having a diameter of 0.2 to 0.7 μm consisting of a hydrophobic core containing the polymer (97.7 %) surrounded by a membrane coat made of proteins (1.8 %) and lipids (0.5 %). PHAs are classified into three major groups, viz. short chain (scl; 3–5 C), medium chain (mcl; 6–14 C) and long chain length (lcl; ˃15 C), depending on the carbon atom number present in the monomer.

Usage of wastewater acts as an alternative to expensive substrates like oils, hydrocarbons, carbohydrate, etc., and will lower the costs involved in investment and operation (Albuquerque et al. 2011; Bengtsson et al. 2008). Pure substrates, microorganism cultures, high production cost, recovery of bioplastics, etc., will influence the costs (4–9 times) of biological plastics production compared to conventional plastics production apart from biodegradability and the other major concerns (Kasemsap and Wantawin 2007; Choi and Lee 1997). For PHA producton, wastewaters like biohydrogen reactor effluents (Venkata Mohan et al. 2010a, Amulya et al., 2014), food waste (Reddy and Venkata Mohan 2012), olive oil mill effluent (Beccari et al. 2009), sugar cane molasses (Albuquerque et al. 2013), tomato cannery wastewater (Liu et al. 2008), paper mill wastewater (Bengtsson et al. 2008), pea shells (Patel et al. 2012) and municipal wastewater (Chua et al. 2003) are utilized. Genetically engineered bacteria are used to produce PHA of 90 % dry cell weight (DCW) (Johnson et al. 2009).

14.4.1 Bacterial PHA Synthesis

PHA synthesis by bacteria can be either scl-PHAs or mcl-PHAs which are confined only to specific organisms such as *Ralstonia eutropha* and *Azohydromonas lata* that synthesize scl-PHAs, while *Pseudomonas* spp. produce mcl-PHAs (Koller et al. 2010). PHA synthesis occurs under certain stress conditions rather than the growth conditions. During stress conditions, NADH accumulation occurs, which inhibits citrate synthase causing the accumulation of acetyl CoA. This acetyl CoA will ultimately result in the initiation of bioplastics/PHA synthesis. In terms of wastewater for PHA synthesis, the organic matter present in wastewater will be hydrolyzed to simple molecules such as glucose which will be further degraded to generate VFAs, the key precursors for PHA production. PHA synthesis is initiated by bacteria through series of reactions, viz. condensation, reduction and conversion, which are catalyzed by the enzymes – β-ketothiolase, NADPH-dependent acetoacetyl CoA reductase and PHA synthase, respectively. The combination of two molecules of acetyl CoA results in the formation of acetoacetyl CoA, which gets reduced to (*R*)-3-hydroxybutyryl-CoA and is finally converted to form PHA and free coenzyme-A is released in this process. Acetyl CoA concentration intracellularly and free coenzyme-A play a significant role in the PHA synthesis which is regulated at the enzymatic level (Venkata Mohan et al. 2013a). PHA production which is eco-friendly and sustainable that utilizes the renewable feedstock is gaining considerable attention. Various fermentation strategies like batch, particularly fed-batch, and continuous fermentations have been investigated (Wang and Lee 1997). However, aerobic dynamic feeding strategy is the most preferred strategy for

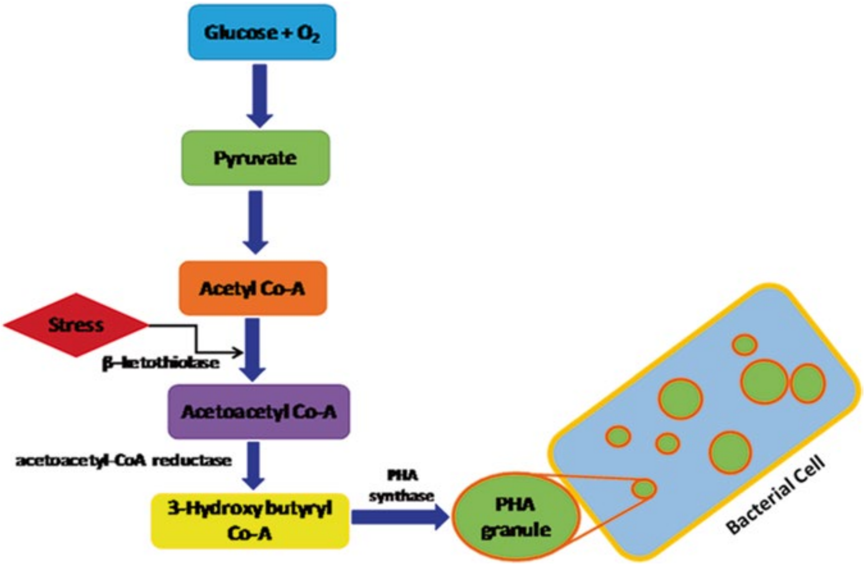

Fig. 14.4 Biosynthetic pathway of bioplastics production in bacterial cell

PHA production, wherein the 'feast' and 'famine' provided will enhance the synthesis/storage of PHA granules (Villano et al. 2010) (Fig. 14.4).

PHA applications are not only limited to packaging, but also can be used for wide variety of purposes like medical, pharmaceuticals, agro-industrial products, etc. (Saharan et al. 2011; Akaraonye et al. 2010). Oxidative breakdown of PHAs yield water and CO_2 as end products. Though extensive research is being carried on PHA synthesis, commercial production of PHA becomes economically feasible by considering two aspects. Enrichment of highly productive strains capable of using a diverse range of wastes as substrates and lowering the cost are the crucial aspects that need considerable attention towards large-scale production.

14.5 Biodiesel

Biodiesel produced from various sources is one of the renewable biofuels that is being explored by researchers in current scenario to stop the utilization of depleting fossil fuels which emit green house gases and are responsible for ozone depletion and climate change (pollution). First-generation fuel sources (edible sources: soybean, rapeseed, sunflower, safflower, etc.) and second-generation fuel sources (nonedible oil sources: frying oil, grease, tallow, lard, karanja, jatropha, mahua oils, etc.) are expensive to produce and require manpower, potentially compete with food-producing plants for land and utilize minerals and nutrients (Alcantara et al. 2000; Dorado et al. 2002; Francis and Becker 2002; Chisti 2008; Marsh 2009). Therefore, research is now deviated towards the usage of renewable (CO_2, sunlight, wastewater, etc.), cheap and reliable resources, which are more promising and economically viable (Venkata Mohan et al. 2014b). Environmental sustainability and economic feasibility are the two main motivations that microalgal biofuel production is gaining acceptance when compared to other agro- and terrestrial-based fuels (Harrison et al. 2012).

14.5.1 Biodiesel from Microalgae

Microalgae, the current forerunners of renewable sources, shall effectively meet the global demand for transport fuels by producing biodiesel and eliminating the problems associated with utilization of terrestrial plants for biodiesel production. Several aspects of microalgal biodiesel that have gained notable interest by many research organizations and entrepreneurs worldwide are non-competition for food crops, high productivity per acre, use of less non-cultivable land, potential for scalability, use of waste and wastewater resources, production of valuable coproducts, more biofuel conversion efficiency, utilization of minerals and nutrients present in the wastewater, CO_2 sequestration from atmosphere and fast growth when compared to terrestrial plants (Varfolomeev and Wasserman 2011; Venkata Mohan et al. 2011, 2014a). All the above-mentioned factors constitute to the merits and advantages of using microalgae as potential feedstock for biofuel production. Moreover, microalgae when compared to conventional biofuel producers do not show much impact on the world's food supply and environmental pollution. Currently, microalgae-derived biofuels are being encouraged and promoted as third-generation biofuel feedstock as it has potential for scalability and does not compete with food crops (Venkata Mohan et al. 2014b). Microalgal cultivation accounts for more than half the primary productivity of the food chain and provides required higher biomass, and in addition, the microalgal fatty acids best suit the biodiesel production (Gouveia and Oliveira 2009; Hoek et al., 1995). According to Weissman, the maximum dry biomass of 365 t per hectare per year is possible theoretically (Weissman 2008) . Algae are photosynthetic microorganisms that require water for survival and are capable of carbon assimilation (heterotrophic and mixotrophic modes) (Prathima Devi and Venkata Mohan, 2012; Venkata Subhash and Venkata Mohan 2014a; Venkata Subhash et al. 2014; Chandra et al. 2014). The biggest challenge associated with biodiesel production over the next few years is to minimize the overheads on algal farming and develop technologies to improve oil production.

14.5.2 Carbon Assimilation in Microalgae

Lipid productivity and carbon assimilation are notably influenced by the nutritional mode undertaken by the microalgae (Xu et al. 2006). According to the type of carbon source, microalgae are classified into three groups, namely, autotrophs, heterotrophs and mixotrophs (Venkata Mohan et al. 2014c). In photoautotrophic mode, which is the usual condition that prevail in environment, microalgae use sunlight and carbon dioxide as sources of energy through photosynthesis pathway (Huang et al. 2002; Chen et al. 2011). The heterotrophs utilize sunlight and organic carbon for energy (Kaplan et al. 1986), but the carbon source acts as energy source in dark heterotrophic growth condition, which is the unique condition that is observed in many microalgal species (Venkata Mohan et al. 2014a; Perez-Garcia et al. 2011). Mixotrophic mode of nutrition is depicted by combination of both autotrophic and the heterotrophic mechanisms in which the facility of utilizing atmospheric CO_2 and utilizing organic carbon and micronutrients present in the surrounding microenvironment and hence the light energy does not influence the biomass growth (Chandra et al. 2014; Chang et al. 2011, Venkata Mohan et al. 2014a). This mode of operation is often observed in ecological water bodies in which nutrients and organic carbon act as integral parts and microalgae symbiotic functioning with other living constituents is visualized (Venkata Mohan et al. 2014a). Microalgae are flexible enough to switch their nutritional mode from one another based on environmental conditions, substrates (Carbon) and sunlight availability (Kaplan et al. 1986).

14.5.3 Substrates for Microalgae Growth and Lipid Synthesis

The promising technology of algal cultivation shall be integrated to several other processes for optimum utilization of atmospheric CO_2 and nutrients present in wastewater for its growth (Wang et al. 2008). For microalgae growth and lipid synthesis, the mode of nutrition plays a significant role in the metabolic pathways. The property of microalgae as photosynthetic microorganism and its ability to assimilate carbon in both heterotrophic and mixotrophic modes provide the advantage of integrating various wastewater treatment for algal biodiesel production (Venkata Mohan et al. 2014d; Prathima Devi et al. 2012, 2013). Wastewaters and atmospheric CO_2 are the abundantly available waste substrates utilized for microalgal growth and lipid production.

In photoautotrophic mechanism, production of biodiesel by utilizing atmospheric CO_2 is considered as sustainable and unswerving approach for the generation of algal biofuels (Graham and Wilcox 2000; Takagi et al. 2000; Yoo et al. 2010; Ge et al. 2011). Microalgae have the ability to utilize carbonates from wastewater and efficiently fix CO_2 emitted either from industries or by automobiles than the terrestrial plants (Brennan and Owende 2010; Venkata Subhash et al. 2013; Chiu et al. 2008; Indra et al. 2010). During the process of CO_2 fixation, microalgae utilize CO_2 as an inorganic carbon source, while water acts as an electron donor for the storage of reserve food material such as carbohydrates, which are further transformed to lipids under certain stress conditions (Venkata mohan et al. 2014b; Prathima Devi and Venkata Mohan 2012) (Table 14.2)

By taking environmental sustainability and economical feasibility into contemplation, wastewater can be used as a potential source of substrate in algae-based biodiesel production (Venkata Mohan et al. 2014b; Huntley and Redalje 2007; Stephens et al. 2010) because algae have the ability to utilize nutrients present and help in removing heavy metals (Mallick 2002; De-Bashan and Bashan, 2010; Hoffmann, 1998). When compared to chemical-based treatments, algal-based treatment systems are efficient in removing nutrients from wastewater as it has the ability to remove nutrients in higher concentrations (Zhang et al. 2008; Martinez et al. 2000; Ruiz-Marin et al. 2010), and it is used as a tertiary treatment system for removal of toxins and heavy metals other than accumulated nutrients (Ahluwalia and Goyal 2007). Because of the

Table 14.2 Different types of wastewaters used for different cultivation modes

Cultivation mode	Inoculum	Type of wastewater	References
Heterotrophic	*Chlorococcum* sp.	Dairy effluent	Beevi and Sukumaran (2014)
Heterotrophic	*Scenedesmus*	Designed synthetic waste (fructose, glucose and acetate)	Ren et al. (2013)
Heterotrophic	*Botryococcus braunii*	Secondary treated sewage	Orpez et al. (2009)
Heterotrophic	Mixed inoculum	Carpet mill effluent	Chinnasamy et al. (2010)
Heterotrophic	Mixed inoculum	Domestic wastewater	Prathima Devi et al., (2013)
Heterotrophic	Mixed inoculum	Acid-rich effluents from hydrogen-producing reactor	Prathima Devi and Venkata Mohan (2012b)
Heterotrophic	Mixed inoculum	Volatile fatty acids	Boyle and Morgan (2009)
Heterotrophic	*Chlorella vulgaris*	Agro-industrial coproducts, ethanol thin stillage and soy whey	Mitra et al. (2012)
Heterotrophic	*Chlorella vulgaris*	Synthetic wastewater	Perez-Garcia et al. (2010)
Heterotrophic	*Auxenochlorella protothecoides*	Municipal wastewater	Zhou et al. (2012)
Autotrophic	*Chlamydomonas reinhardtii*	Artificial wastewater	Kong et al. 2012
Autotrophic	Mixed inoculum	Designed synthetic waste	Prathima Devi and Venkata Mohan (2013)
Mixotrophic	*Chlorella pyrenoidosa*	Piggery wastewater	Wang et al. (2012)

(continued)

Table 14.2 (continued)

Cultivation mode	Inoculum	Type of wastewater	References
Mixotrophic	*Chlorella pyrenoidosa*	Soybean process wastewater	Hongyang et al. (2011)
Mixotrophic	Mixed inoculum	Domestic wastewater of different water bodies	Venkata Mohan et al. (2011c)
Mixotrophic	*Scenedesmus* sp.	Artificial wastewater	Voltolina et al. (1998)
Mixotrophic	*Chlorella* sp.	Dairy manure wastewater	Johnson and Wen (2010)
Mixotrophic	*Scenedesmus* sp.	Fermented swine wastewater	Kim et al. (2007)
Mixotrophic	Mixed inoculum	Designed synthetic waste	Prathima Devi et al. (2013)
Mixotrophic	Mixed inoculum	Domestic waste water	Venkata Mohan and Prathima Devi (2014)
Mixotrophic	Mixed inoculum	Diary and municipal wastewaters	Woertz et al. (2009)

above said reasons, microalgal wastewater treatment seems to be a probable method for cleaning wastewater with simultaneous value-added product generation (Venkata Mohan et al. 2014c)

Microalgae are capable of synthesizing triacylglycerols (TAG) in the form of storage lipids for biodiesel production by utilizing several wastewaters, viz. diary (Beevi and sukumaran 2014), piggery (Wang et al. 2011), domestic (Venkata Mohan et al. 2014b), designed synthetic wastewater (Chandra et al. 2014), municipal (Li et al. 2012; Zhou et al. 2012), fermented swine (Kim et al. 2007) and acid-rich effluents from hydrogen-producing bioreactor (Prathima Devi and Venkata Mohan 2012) wastewaters as potential carbon sources. Integration of both growth and starvation phases in a sequential order and nutrient limitation strategies will enhance the cell growth and the amount of lipid formation in microalgae (Venkata Mohan and Prathima Devi 2012; Prathima Devi and Venkata Mohan 2012). The nutrient-limiting conditions (nitrates and phosphates) during the starvation phase cause decrement in the thylakoid membrane content as the enzyme acyl hydrolase is activated and phospholipid hydrolysis stimulation (Prathima Devi et al. 2012).

14.5.4 Mechanism for Biodiesel Production

The de novo synthesis of fatty acids in algae occurs primarily in the thylakoid (via photophosphorylation) and stromal (Calvin cycle) region of the chloroplast (Liu and Benning 2012). In microalgae, lipid synthesis pathway generally occurs in a series of four steps: accumulation of carbohydrates, formation of acetyl CoA and malonyl CoA, palmitic acid synthesis and chain elongation for higher fatty acid synthesis (Venkata Mohan et al. 2014c).

Carbohydrates are synthesized through photosynthesis (autotrophs) and assimilate from outside the cell (heterotrophs). In photoautotrophic mode, an endogenous source of acetyl CoA was provided by photosynthates, whereas in heterotrophic mode of nutrition, the carbon uptake will be through an inducible active hexose symport system outside the cell (Tanner 1969; Komor 1973; Komor and Tanner 1974). In heterotrophic mode, energy is invested in the form of ATP which is further divided into light-dependent and light-independent systems. Depending on the species of algae and specific culture conditions, the produced carbohydrate is converted into various products, including TAGs (Liu and Benning 2012). The glucose transport in the cell is decreased in light-independent heterotrophic mode (dark) as the expression of the hexose/Hþ symport system is inhibited by light (Kamiya and Kowallik 1987; Perez-Garcia et al. 2011). The pyruvate formed in the cytosol by the glycolytic conversion of glucose is generated into acetyl CoA which is then transported to plasmid for conversion into fatty acid and TAG subsequently. The formed TAG is formed into lipid bodies after it is transported back to cytosol (Amulya et al. 2015).

During the process, seven molecules each of acetyl CoA and CO_2 form malonyl CoA (7 molecules). The formed malonyl CoA synthesizes long carbon-chain fatty acids, and the acyl group (saturated) produced becomes the substrate for subsequent condensation through multistep sequencing process (Ohlroggeav and Browseb 1995). The formation of palmitic acid, the principal product of fatty acid synthase systems and precursor of other long-chain fatty acids, occurs after condensation, reduction, dehydration and reduction reactions (Alban et al. 1994; Fan et al. 2011). The formed palmitic acid modifies further and increases its length by addition of acetyl groups to form stearate and other saturated long fatty acids, viz. oleiceate, linealate, etc. (Smith et al. 2009; Thelen and Ohlrogge 2002).

14.5.5 Microalgae Cultivation

Microalgal cultivation increases biomass growth and lipid production. The requirements for microalgae cultivation are light as source for photosynthesis, macronutrients (nitrates and phosphates) and micronutrients (sulphur, potassium, magnesium) dissolved in liquid medium (Venkata Mohan et al. 2014b; Mata et al. 2010). Photobioreactors are generally used configurations to cultivate microalgae on commercial scale. The amount of biomass and lipid production can be enhanced in this closed systems by utilizing resources present in the form of waste/wastewater (Chisti 2007; Campbell 2008; Robert et al. 2012). For reaching higher biomass concentrations in a short time, fed-batch cultivation is the most effective technique and this is achieved in heterotrophically grown cultures by controlling the rate of addition of organic carbon and energy source (Venkata Mohan et al. 2014b). The steps involved in overall microalgal cultivation include pre- and postharvesting processes. The key aspects involved in preharvesting step are mode of cultivation and selection of appropriate inoculums. Followed by preharvesting process, the series of sequentially integrated steps involved in postharvesting for conversion of algal biomass to biodiesel are harvesting, drying, cell disruption, extraction and transesterification. Based on the type of strain, substrate used (nutritional mode) and extraction method employed, the postharvesting steps vary accordingly and energy-demanding step is the only limitation faced in the harvesting step. Drying of biomass is a prerequisite step prior to extraction process as the moisture present in the biomass interferences with the solvents used in the extraction and transesterification processes. The various extraction processes used are solvent (Folch and Sloane-Stanley 1957; Additions and Revisions, 2002), Soxhlet (Additions and Revisions, 2002), wet lipid (Sathish and Sims 2012), hydrothermal liquefaction (Brown et al. 2010; Biller et al. 2012; Peterson et al. 2008), ultrasonic (Harun et al. 2010; Wei et al. 2008; Cravotto et al. 2008), supercritical carbon dioxide extraction (SC-CO_2) (Sahena et al. 2009; Mendiola et al. 2007; Cooney et al. 2009; Eller 1999), pulsed electric field technologies (Guderjan et al. 2007), enzymatic treatment (Mercer and Armenta 2011; Halim et al. 2011, Fajardo et al. 2007) and osmotic shock (Fajardo et al. 2007, Mario 2010). Of these technologies, solvent extraction is being widely used for extracting lipids from the biomass.

The production of glycerol and mono-alkyl fatty acid esters by the reaction of alcohol with triglycerides is called the process of transesterification (Venkata Mohan et al. 2014b; Harrison et al. 2012). There are different types of transesterification, namely, direct (Sathish and Sims 2012), acid-catalyzed (Zhang et al. 2003), base-catalyzed

(Vargha and Truter 2005) and enzyme-catalyzed transesterification (Bisen et al. 2010). Based on the type of catalyst used, the process of transesterification process is manipulated (Canakci and Gerpen 1999). The fatty acid methyl esters (FAME), the most prevalent alkyl esters in the current biodiesel market, are produced by the transesterification of lipids using methanol (alcohol) and glycerol is formed as by-product in this process (Gong and Jiang 2011; Knothe et al. 1997).

14.5.6 Other Biodiesel Producers

Apart from algae, bacteria and fungi can produce a high content of lipid (>20 %) and are termed as 'oleaginous' (Ratledge and Wynn 2002) and serve as potential alternative oil resources for biodiesel production (Meng et al. 2009). Oleaginous bacteria such as *Mycobacterium, Streptomyces, Rhodococcus* and *Nocardia* can accumulate TAG at high concentrations in case of excess amount of carbon and nutrient-limiting conditions. The accumulation takes place mostly during the stationary phase of growth (Olukoshi and packter 1994) and during nitrogen-limited conditions. The organisms continue to assimilate the carbon source, but cell proliferation is hampered as nitrogen is required for protein and nucleic acid synthesis (Alvarez and Steinbuchel 2002). These conditions encourage carbon flux towards the accumulation of triacylglycerols (TAG) within discrete lipid bodies in the cells. Bacterial TAG structure and composition varies depending on the microorganisms and carbon source. The isolated filamentous fungus *Aspergillus sp.*, using waste as substrate (corncob liquor), depicted good lipid accumulation (Venkata Subhash and Venkata Mohan 2011). *Mortierella alliacea*, a filamentous fungus, stores arachidonic acid in the mycelia (Venkata Subhash and Venkata Mohan 2014b).

14.5.7 Biorefinery

Wastewater is a potential source for microalgal growth and if all the factors are optimized for biomass and lipids, it turns out to be a practical and feasible technology (Venkata Mohan et al. 2014b). In biorefinery approach, the microalgal wastewater treatment can be combined with biodiesel production to make the process sustainable. Microalgae can be used in light-independent heterotrophic (utilize nutrients present in wastewater) and autotrophic (utilize CO_2 emitted from industries and automobiles) modes of treatment, making the whole process highly sustainable and reliable.

The process of biomass and lipids production by mixed microalgae utilizes sunlight, nutrients present in wastewater and atmospheric CO_2 and thereby releases O_2 into the environment. Based on the type of process opted, biomass and lipids can be converted to biodiesel, H_2 and de-oiled algal cake, which in turn are utilized for production of bio-oil, charcoal, fuel gas and bioelectricity (thermochemical conversion: pyrolysis, gasification, hydrothermal liquefaction and direct combustion), biogas and bio-H_2 (biochemical conversion: anaerobic digestion, fermentation and photobiological H_2 production) and biodiesel (chemical conversion: transesterification) (Groom et al. 2008). The waste or wastewater obtained from this processes can again be recycled and reused as a source of nutrients for microalgae cultivation (Fig. 14.5).

14.6 Future Perspectives

The problems faced by mankind for the survival in current society with tremendous environmental pollution has brought a significant change to build a sustainable and eco-friendly market for the generation of bio-based products. Since two decades, many innovations were made to realize the potential of waste as wealth. In addition, the perception of humankind on bacteria as harmful agent is now turned as a biocatalyst for the generation of many value-added products. The recent advances made in the field of wastewater and environmental engineering were environmentally sustainable in developing various bioenergy forms. Biohydrogen production via dark and photo-fermentation has gained significant impact in the field of renewable bioenergy. Similarly, bioelectricity generation, biodiesel and bioplas-

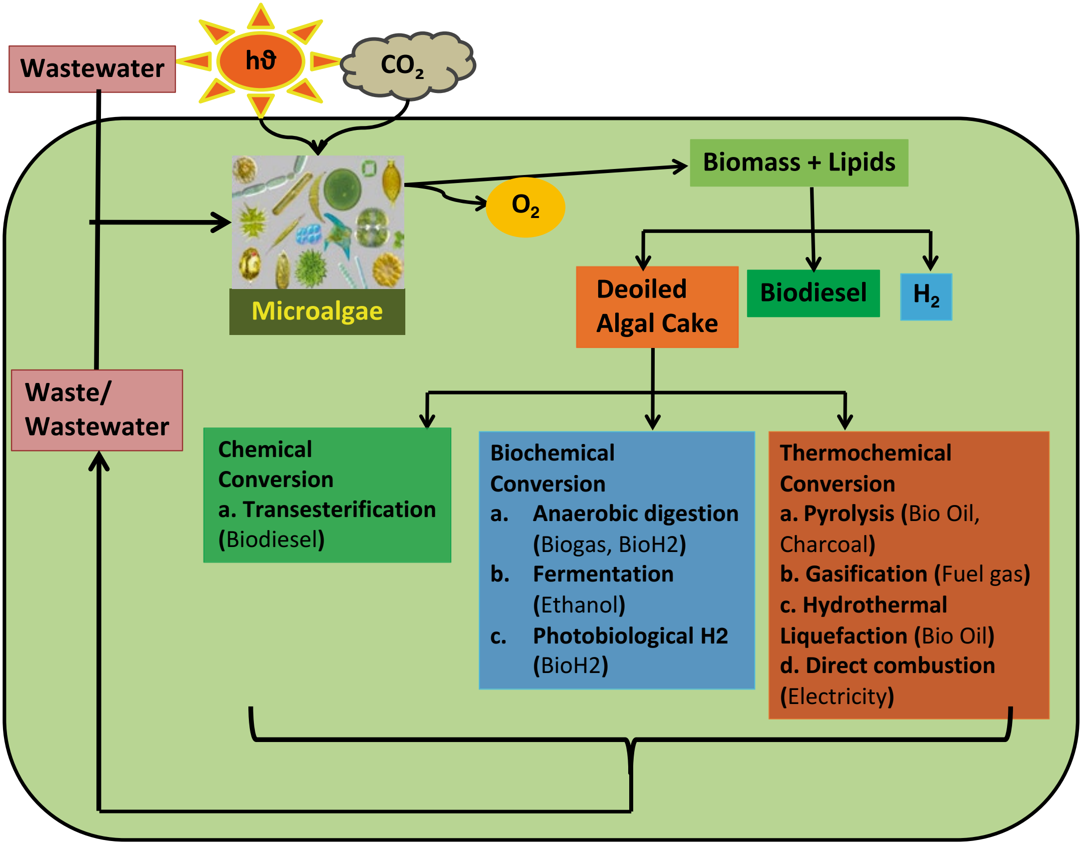

Fig. 14.5 Biorefinery approach of waste and wastewater utilized by microalgae for generation of value-added products

tics production in an environmentally viable way were discussed in this chapter. Though many technological interventions are made, there are still few challenges to be addressed to make the lab scale processes towards industrial and practical implementations. Bio-process engineering, optimization of operational factors and low yield of the end products are the stumbling blocks that need to be overcome prior to scaling up. Besides this, fundamental understanding with a multidisciplinary and integrated approach would help in the establishment of sustainable waste remediation technologies with simultaneous bioenergy generation. As said by Einstein, ‘the whole of science is nothing more than a refinement of everyday thinking’; can make us think about resolving the issues associated with green technologies towards practical implementation in an economical way.

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C. Nagendranatha Reddy received Masters (M.Tech) degree in Biotechnology from SRM University, Chennai, India. He is currently working as a Senior Research Fellow at CSIR-IICT, Hyderabad, and enrolled in Ph.D. (Biological Sciences) with Academy of Scientific and Innovative Research (AcSIR). His current interests are the evaluation of bioremediation process for azo dye based wastewater in bio-electrogenic and non bio-electrogenic systems with simultaneous harnessing of value added products. He is a life member of The Biotech Research Society, India (BRSI).

J. Annie Modestra received Masters (M. Tech) degree in Biotechnology from Jawaharlal Nehru Technological University (JNTU), Anantapur, India. She had qualified GATE and is currently working as CSIR-Senior Research Fellow. She is pursuing Ph.D in Engineering Sciences, registered with Academy of Scientific and Innovative Research (AcSIR) at CSIR-IICT. Her interests are the bioelectrochemical reduction of CO_2 into value-added products using bio/microbial electrochemical systems. She is also experienced in microbial electrochemical technologies for bioelectricity generation and waste remediation.

A. Naresh Kumar received the M.Sc. degree in Biotechnology from Osmania University, Hyderabad, India. He is currently working as a Project Senior Research Fellow (SRF) at CSIR-IICT, Hyderabad. He has enrolled in Ph.D with Academy of Scientific and Innovative Research (AcSIR), CSIR-IICT.His current interests are the evaluation of bioprocess for the production of biofuels by using biodegradable portion of municipal solid waste through acidogenic fermentation and microbial electrochemical technologies.

S. Venkata Mohan received M.Tech and Ph.D. (Environmental Engineering) from Sri Venkateswara University, Tirupati, India. He is currently working as a Principal Scientist at CSIR-IICT, Hyderabad, and an Associate Professor of AcSIR. He is recipient of coveted "Shanti Swarup Bhatnagar (SSB) Prize 2014" in Engineering Sciences. His main research interests are in the domain of environmental bioengineering and biofuels specifically in the field of biohydrogen, microbial fuel cell, bio-electrochemical systems and algal-based fuels. He also received several awards and honours, such as "National Bioscience award 2012", "Prosper.net-Scopus Young Researcher Award in Sustainable Development 2010" under Energy Category by United Nations University and Elsevier, "NASI-Scopus Young Scientist Award 2010", etc. Dr Mohan is elected fellow of National Academy of Engineering (FNAE), Biotech Research Society of India (FBRSI), Andhra Pradesh Akademy of Sciences (FAPASc), etc. He is subject editor for the Journal of Energy, Associate Editor for Frontiers in Energy Research and is serving as an editorial board member of several journals viz. Bioresource Technology, The Open Waste Management, Advances in Energy Research, etc.

15 Renewable Energy Derived from Food Waste and Co-digestion of Food Waste with Waste-Activated Sludge

Esra Uçkun Kiran and Yu Liu

Abstract

Food waste (FW) is a big environmental and social problem worldwide and its amount continues to increase. Traditionally, FW is currently landfilled or incinerated for possible energy recovery. However, both landfilling and incineration are leading economic and environmental stresses. On the other hand, due to its nutrient-rich composition, theoretically FW can be utilized as a feedstock for the production of renewable energy through various fermentation processes. In this chapter, the state of the arts of bioconversion technologies of FW for renewable energy generation are reviewed. The hydrolysis of FW is considered as the rate-limiting step for the production of biofuel and biogas. To enhance the performance of bioconversion, different pretreatment strategies, process configurations, and key process parameters are discussed. Lastly, anaerobic co-digestion of FW with bio-sludge, which is a promising approach to improve the AD performance, was also presented.

15.1 Current Practices of Food Waste Management

Food waste (FW) is originated from many various sources, e.g., households, cafeterias, restaurants, etc. One third of food produced for human consumption (i.e., nearly 1.3 billion tons) is lost or wasted throughout the food supply chain (FAO 2012). The amount of FW continues to increase due to rapid economic and population growth, particularly in Asia. The annual amount of FW in Asia could increase from 278 to 416 million tons from 2005 to 2025 (Melikoglu et al. 2013).

Landfilling, incineration, composting, animal feed use, and anaerobic digestion are the main

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E. Uçkun Kiran
Advanced Environmental Biotechnology Centre,
Nanyang Environmental and Water Research Institute,
Cleantech One, Clearntech Loop, #06-08 637141,
Singapore
e-mail: esrauckun@ntu.edu.sg; esrauckun@yahoo.com

Y. Liu (✉)
Advanced Environmental Biotechnology Centre,
Nanyang Environment and Water Research Institute,
Nanyang Technological University,
Singapore 637141, Singapore

School of Civil and Environmental Engineering,
Nanyang Technological University,
50 Nanyang Avenue, Singapore 639798, Singapore
e-mail: cyliu@ntu.edu.sg

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FW handling strategies. In many developing countries, FW is landfilled. Unfortunately, the capacity of landfills is not able to handle the increasing volume of FW (Ngoc and Schnitzer 2009). Moreover, landfilling is an unsustainable approach as it creates uncontrolled green house gas emission (Kosseva 2009). In some developed countries, FW is incinerated with other municipal solid waste in order to reduce its volume and generate energy. The incineration of FW is a favorable option against landfilling as it provides controlled energy recovery (Othman et al. 2013). Still, incineration may not be feasible in low-income countries due to its high capital and operating costs (Ngoc and Schnitzer 2009). Moreover, incineration leads to the production of dioxins and greenhouse gas emission (Katami et al. 2004).

Composting is another approach for handling FW, converting FW to valuable soil conditioner and fertilizer (Gajalakshmi and Abbasi 2008). It is more environmental friendly and has higher economic efficiency compared to landfilling and incineration. However, the high moisture content of FW leads to harmful leachate (Cekmecelioglu et al. 2005). In fact, compost produced is more costly than commercial fertilizers, and commercial demand for compost produced from FW is limited (Aye and Widjaya 2006). FW can be also used as animal feed. The main disadvantage of FW to be used as animal feed is its high moisture content, which makes it unstable to microbial contamination (Esteban et al. 2007). To prevent this, FW is generally dried, but greenhouse gas emission increases depending on the energy usage during drying of FW, which is related to the moisture content of FW (Takata et al. 2012).

Anaerobic digestion (AD) is another option which yields methane and carbon dioxide as the main products (Othman et al. 2013). AD of FW has been widely studied for biogas generation, which is a viable option for volume reduction of and energy recovery from FW (Uçkun Kiran et al. 2014). It has been reported that AD process prior to incineration could help to reduce global warming potential, due to the controlled recovery of methane and carbon dioxide (Hirai et al. 2001). On the other hand, FW can be a potential resource for the production of high-value chemicals (Lin et al. 2013). In general, the production of organic acids, biodegradable plastics, and enzymes from FW creates higher value than converting it into biogas, animal feed, etc. (Sanders et al. 2007). However, the market demand for these high-value chemicals is lower than that for renewable energy (Tuck et al. 2012). Therefore, this chapter attempts to review the biological valorization techniques of FW that have been developed for the production of renewable energy, i.e., ethanol and biogas, from FW.

15.2 Characteristics/Composition of FW

The compositions of FW vary regionally, depending on the diet habits. Table 15.1 shows the compositions of FW reported in different studies. FW has high moisture content (61.3–87.6 %); therefore, it could be considered as an easily biodegradable biomass. The solid content of FW is primarily composed of carbohydrate (starch, cellulose, and hemicelluloses), proteins, and lipids (Uçkun Kiran et al. 2014). Total carbohydrate and protein contents of FW are in the range of 35.5–69 % and 3.9–21.9 %, respectively (Table 15.1).

15.3 Ethanol Production from FW

Global demand for ethanol has been increasing due to its wide industrial applications and utilization as biofuel. Ethanol is mainly used as starting material to produce ethylene, a key substrate for the synthesis of polyethylene and many other plastics, which has a market demand of more than 140 million tons annually (Uçkun Kiran et al. 2014). The global production of bioethanol has been projected to continue its rapid increase and to reach 155 billion liters by 2020 mainly due to the renewable energy goals and policies (OECD/FAO 2011). Traditionally, bioethanol produced starch and cellulose-rich feedstocks, e.g., corn, potato, and sugar cane (Yan et al. 2012). However, these are major food sources,

Table 15.1 Characteristics of mixed FW

Origin	pH	MC*	TS	VS/TS	Total sugar	Starch	Cellulose	Lipid	Protein	Ash	Reference
Dining hall	NR	79.5	20.5	95.0	NR	NR	NR	NR	21.9	NR	Han and Shin (2004)
Cafeteria	5.1	84.1	15.9	15.2	NR	NR	NR	NR	NR	NR	Kim et al. (2008a)
Cafeteria	5.1	80.0	20.0	93.6	NR	NR	NR	NR	NR	1.3	Kwon and Lee (2004)
MSW	NR	85.0	15.0	88.5	NR	NR	15.5	8.5	6.9	11.5	Rao and Singh (2004)
Cafeteria	4.6–5	79.1	20.9	93.2	NR	NR	NR	NR	NR	NR	Ramos et al. (2012)
Cafeteria	NR	75.9	24.1	NR	42.3	29.3	NR	NR	3.9	1.3	Ohkouchi and Inoue (2006)
Residents	4.9	80.8	19.2	92.7	NR	15.6	NR	NR	NR	NR	Pan et al. (2008)
Dining hall	NR	80.3	19.7	95.4	59.8	NR	1.6	15.7	21.8	1.9	Tang et al. (2008)
Dining hall	NR	82.8	17.2	89.1	62.7	46.1	2.3	18.1	15.6	NR	Wang et al. (2008)
Restaurant	3.9	80.0	20.0	95.0	70.0	NR	NR	10.0	13.0	NR	Zhang et al. (2005)
Dining hall	5.6	82.8	17.2	85.0	62.7	46.1	2.3	18.1	15.6	NR	Ma et al. (2008)
Cafeteria	NR	61.3	38.7	NR	69.0	NR	NR	6.4	4.4	1.2	Uncu and Cekmecelioglu (2011)
Food court	NR	64.4	35.6	NR	NR	NR	NR	8.8	4.5	1.8	Cekmecelioglu and Uncu (2013)
Canteen	NR	81.7	18.3	87.5	35.5	NR	NR	24.1	14.4	NR	He et al. (2012)
Restaurant	NR	81.5	18.5	94.1	55.0	24.0	16.9	14.0	16.9	5.9	Vavouraki et al. (2014)
Restaurant	NR	81.9	14.3	98.2	48.3	42.3	NR	NR	17.8	NR	Zhang and Jahng (2012)

Total Solid, total sugar, starch, cellulose, lipid, protein, and ash contents were given in wt% on the basis of dry weight. Volatile solid contents were given as the %VS ratio on total solid basis. *MC* moisture content, *VS* volatile solid, *TS* total solid, *NR* not reported

Table 15.2 Ethanol production from FW

Waste	Method	Vessel type	Pret.	Microorganism	Time (h)	Y_{RS} (g/g FW)	$Y_{Ethanol}$ (g/g FW)	$Y_{Ethanol}$ (g/g RS)	P (g/Lh)	References
FW	Repeated batch Simultaneous	1 L fermenter with 0.8 L working vol.	None	*S. cerevisiae* ATCC26602	264	0.12	0.06	0.5	3.7	Ma et al. (2007)
Fruit waste	Simultaneous	500 mL flask	Drying, steam explosion	*S. cerevisiae, Pachysolen tannophilus*	24	0.25	0.11	0.4	NR	Sharma et al. (2007)
FW	Separate	500 mL flask 100 mL working vol.	None	*S. cerevisiae* KA4	16	0.23	0.12	0.49	NR	Kim et al. (2008a)
FW	Simultaneous	Flask with 100 g FW	None	*S. cerevisiae*	48	0.11	0.08	NR	NR	Ma et al. (2007)
FW	Separate continuous	Tower-shaped reactor, 0.45 L working vol.	LAB spraying	*S. cerevisiae* strain KF-7	15	0.12	0.03	0.26	24	Tang et al. (2008)
FW	Simultaneous	Flask with 100 g FW	None	*S. cerevisiae*	67.6	0.35	0.23	NR	NR	Wang et al. (2008)
FW	Continuous simultaneous	Fermenter with 4.3 kg FW	LAB spraying	*S. cerevisiae* KF7	25	0.36	0.09	0.24	17.7	Koike et al. (2009)
FW	Simultaneous	1 L fermenter with 0.8 L working vol.	None	*S. cerevisiae* KRM-1	48	0.89	0.06	NR	10.08	Ma et al. (2009a)
FW	Repeated batch simultaneous	250 mL flask 150 mL working vol.	None	*Zymomonas mobilis* GZNS1	14	0.15	0.07	0.49	10.08	Ma et al. (2008)
FW	Simultaneous	250 mL flask 200 mL working vol.	None	*S. cerevisiae*	48	0.60	0.36	0.22	NR	Hong and Yoon (2011)
FW	Separate	5 L fermenter with working volume of 3 L	None	*S. cerevisiae*	24	0.27	0.16	NR	1.18	Kim et al. (2011a)
FW	Simultaneous	Fermenter with 200 g FW	None	*Saccharomyces italicus* KJ	352	0.13	NR	NR	2.24	Li et al. (2011)

Mandarin waste	Simultaneous	100 mL baffled flasks	Drying	*S. cerevisiae*	15	0.52	0.34	NR	3.5	Oberoi et al. (2011a)
Banana peels	Simultaneous	100 mL baffled flasks	Drying	*S. cerevisiae*	15	0.37	0.32	0.43	2.3	Oberoi et al. (2011b)
FW	Separate	250 ml flask 100 mL working vol.	None	*S. cerevisiae*	96	0.50	0.2	0.39	NR	Uncu and Cekmecelioglu (2011)
FW	Separate	250 mL flask 100 mL working vol.	None	*S. cerevisiae*	48	0.65	0.23	0.36	NR	Cekmecelioglu and Uncu (2013)
Waste bread	Separate	300 mL flask 80 g waste bread	Drying	*S. cerevisiae* ethanol red	72	0.37	0.27	NR	NR	Kawa-Rygielska et al. (2012)
FW	Separate (fb)	500 mL flask 200 g FW	None	*S. cerevisiae* H058	48	0.29	0.14	0.47	NR	Yan et al. (2011)

NR not reported, *pret.* pretreatment, *LAB* Lactic acid bacteria, *RS* reducing sugar, *Y* yield, *P* productivity, *Simultaneous* Simultaneous saccharification fermentation, *Separate* Separate saccharification fermentation, *fb* fed-batch thus their usage as a source for fuel production could lead to the consequence of insufficient food supply. To prevent this situation, while to decrease the production cost, the utilization of carbohydrate-rich, abundant, and cheap substrates, such as agro-industrial residues and food waste, has been explored for bioethanol production. Starch can be simply saccharified by commercial enzymes and afterward fermented to ethanol particularly by *Saccharomyces cerevisiae*. However, the hydrolysis of cellulose is more complicated. Hence, FW hydrolysis becomes more challenging if its cellulose content is high.

15.3.1 Pretreatment Strategies of FW

In order to improve the process performance and ethanol yield, different pretreatment strategies, including mechanical, thermal, and chemical methods, have been be explored. Physical pretreatments by grinding, blending, and homogenizing are usually applied to reduce the particle size of FW and create a homogeneous medium for saccharification and fermentation. Thermal pretreatment is generally applied for improving production yield, but at the cost of energy and water consumption. It should be realized that heat pretreatment may lead to side reactions, such as Maillard reaction through which the amount glucose and amino acids are reduced (Sakai and Ezaki 2006). Moreover, inhibitory substances, e.g., furfural and hydroxymethyl furfural, can be generated during the thermal pretreatment. Therefore, the thermal pretreatment, in general, is not recommended as long as microbial contamination is tolerable. Instead of thermal pretreatment, acidic conditions can be created to control undesirable microbial contamination and putrefaction (Koike et al. 2009; Ye et al. 2008). In this case, acid-tolerant ethanol-producing microorganisms (e.g., *Zymomonas mobilis*) can be employed for ethanol production from FW (Tao et al. 2005). In most cases, harsh chemical pretreatments are not required for the bioconversion of FW to ethanol (Tang et al. 2008). Dilute acids or alkali can be also used for FW pretreatment, as long as the release of inhibitory substances (e.g., furfural) is controlled during the pretreatment.

15.3.2 Saccharification

The efficiency of ethanol fermentation depends on the degree of saccharification as yeasts cannot ferment starch or cellulose directly into bioethanol. As such, different types of commercial enzymes have been used for an effective saccharification. For example, a mixture of α-amylase, β-amylase, glucoamylase, and β-glucosidase of various origins was used for the effective saccharification of starch. Hemicellulases, cellulases, and xylanases including endoglucanase, exoglucanase, and β-xylosidase can also be used to improve the saccharification degree by hydrolyzing cellulose and hemicellulose components of FW (Tomasik and Horton 2012).

Table 15.2 summarizes the glucose and ethanol yields obtained from FW. The highest glucose concentration (65 g reducing sugar/100 g FW) with 70 % conversion was obtained after 6 h of enzymatic hydrolysis using α-amylase (120U/g dry substrate), glucoamylase (120U/g dry substrate), cellulase (8 FPU/g dry substrate), and β-glucosidase (50 U/g dry substrate) (Cekmecelioglu and Uncu 2013). In a study of Hong and Yoon (2011), a mixture of commercial enzymes containing α-amylase, glucoamylase, and protease resulted in 60 g reducing sugar/100 g FW.

Use of commercial enzymes for hydrolysis of FW has the disadvantages of high cost and low efficiency because no tailored multienzyme cocktails are available for the hydrolysis of FW. In addition, each commercial enzyme has different optimum ranges for the hydrolysis. Therefore, the saccharification process would either be operated suboptimally with a mixture or take a long time to complete each enzymatic step one after the other. In order to make the enzymatic hydrolysis of FW more cost-effective, the enzymes should be produced in situ from a cheap feedstock without complex and costly downstream separation and purification. In a study of Uçkun Kiran et al. (2015), a fungal mash rich in hydrolytic enzymes was produced by solid-state fermentation of FW and was applied for the saccharification of FW. Using this fungal mash, 90–95 % of starch in FW could be hydrolyzed within 24 h.

15.3.3 Process Configurations

Process optimization is crucial for achieving high-glucose yield, which can be realized by optimizing the enzymes' dosage, temperature, solid loading, mixing, and reaction time required for saccharification (Ado et al. 2009; Sharma et al. 2007; Shen et al. 2009; Zhang et al. 2010). It should be noted that high-glucose concentration may eventually result in catabolite repression of the enzymes as well as low ethanol yields (Oberoi et al. 2011a). To prevent this, fed-batch and simultaneous saccharification and fermentation (Ssf) processes have been applied (Ma et al. 2009b; Oberoi et al. 2011a).

The fed-batch culture has been frequently used for the saccharification of biomass which is then fermented to ethanol (Ballesteros et al. 2009). According to Yan et al. (2012), saccharification and following ethanol fermentation were both improved significantly using fed-batch fermentation compared to batch fermentation. Ssf is another option to mitigate the catabolite repression. It combines saccharification and ethanol fermentation into a single process in order to maintain the glucose concentration at a low level so as to prevent possible catabolite repression. This combined process can be conducted in a single bioreactor. Hence, higher ethanol productivity can be obtained by lower energy consumption in shorter time using less enzyme (Ballesteros et al. 2009). Optimization of process parameters is essential for the Ssf process as enzymes and microorganism may have different optimum pH and temperature ranges. In a study by Hong and Yoon (2011), about 60 g reducing sugar and 36 g ethanol were produced from 100 g of FW after 48 h fermentation. Koike et al. (2009) investigated the ethanol fermentation from FW in a continuous Ssf process and reported the highest ethanol productivity of 17.7 g/Lh. Ma et al. (2009b) studied the Ssf of FW by acid-tolerant *Zymomonas mobilis* without any sterilization, and they obtained 15.4 g sugar per 100 g FW and 0.49 g ethanol per g sugar within 14 h, with an ethanol yield of 10.08 g/Lh.

15.3.4 Other Strategies for Improving Ethanol Yield

Some other strategies such as use of engineered strains (He et al. 2009; Wang et al. 2012a) and cell recycling either by sedimentation or membrane retention were investigated to improve the ethanol yield (He et al. 2012). Recombinant strains with amylase-expressing gene and also some engineered strains with improved ethanol tolerance have also been employed for ethanol fermentation (Li et al. 2011). However, the stability of the recombinant strains has not been verified yet. Cell recycling has been known to enhance the performance of the continuous fermentation considerably (Wang and Lin 2010).

15.3.5 Large-Scale Ethanol Production from FW

There are some pilot and full-scale plants producing ethanol from various wastes. Kumamoto University and Hitachi Zosen Company developed a pilot plant for ethanol production from municipal solid waste. They could produce 60 l of ethanol from one ton of municipal solid waste and use the by-products for biogas production (Japan-for-Sustainability 2013). In Finland, ST1 Biofuel established a system between 7 ethanol biorefineries converting different kinds of waste to ethanol with a total annual capacity of 11 ML (Energy-Enviro-Finland 2013; ST1 2013). In Spain, citrus wastes have been used to produce ethanol with a yield of 235 L/ton dry biomass (BEST 2013; Citrotechno 2013). E-fuel developed a home ethanol system for homeowners and small businesses to convert sugar-/starch-rich liquid waste into ethanol in site (E-fuel 2009).

15.4 Biogas Production

Anaerobic digestion (AD) is a frequently used biochemical process to reduce the volume of waste biomass and recover energy in the form of biogas. It is performed by a group of microorganisms which degrade the organic substrates into gases (mainly methane and carbon dioxide) in the absence of oxygen. AD is a promising approach for FW management due to obvious reasons (Morita and Sasaki 2012; Nasir et al. 2012).

AD is a complex multistep process which is performed by several types of anaerobic microorganisms. The overall AD process can be separated into four main steps: hydrolysis, acidification, acetogenesis, and methanogenesis. A simplified AD process is presented in Fig. 15.1. The complex organic polymers found in FW such as starch, cellulose, proteins, and lipids are first hydrolyzed into their monomers and oligomers (Fig. 15.1, Group 1). These bioconversion processes are performed by the extracellular enzymes of the microorganisms to facilitate the nutrient transport through the cell membrane. When these mono- and oligomers are transported into the cell, they are mainly used as energy resource and also for the production of cellular components, and they are further hydrolyzed into dicarboxylic acids, short chain fatty acids, carbon dioxide, ammonia, and hydrogen by acidogenic bacteria (Fig. 15.1, Group 2). At this acidogenesis step, large amount of carbon dioxide and hydrogen are produced. If the carbohydrate content of the substrate is high, hydrogen production will be high and can be harvested directly to use it as biofuel. The acidogenic bacteria has high growth rate and are able to tolerate low pH (5–6). As a result of their rapid growth, the acids may inhibit the AD if they are not metabolized eventually. In the next step, fatty acids are metabolized to acetate, carbon dioxide, and hydrogen by obligate hydrogen-producing acetogenic bacteria (Fig. 15.1, Group 3), while carbon dioxide and hydrogen are used to produce acetate by hydrogen-oxidizing acetogens (Fig. 15.1, Group 4). Lastly, carbon dioxide-reducing hydrogen-oxidizing methanogens produce methane (Fig. 15.1, Group 5). Methane can also be generated from acetate by acetoclastic methanogens (Fig. 15.1, Group 6).

Obviously, the AD of FW is a complex process because all the nutrients are simultaneously digested in a single system by a variety of microorganisms. Therefore, it is challenging to maintain the long-term process stability and efficiency of the AD process (Uçkun Kiran et al. 2014).

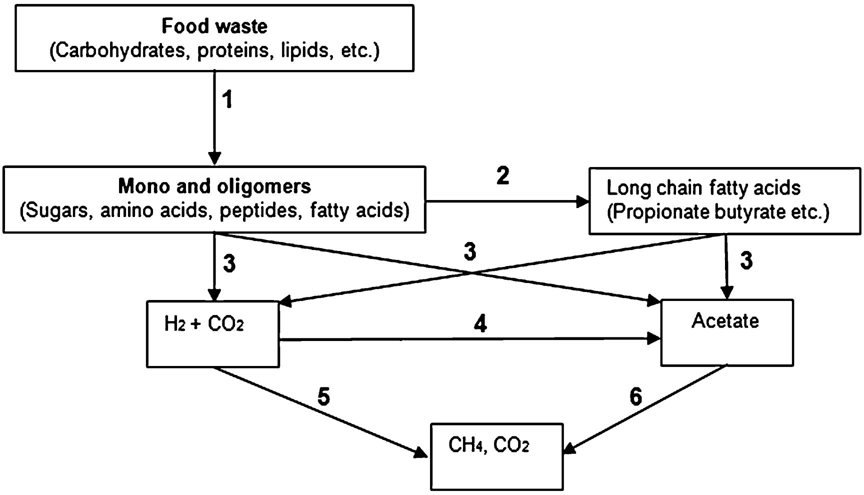

Fig. 15.1 A simplified AD process from Parawira (2012)

15.4.1 Factors Affecting AD of FW

15.4.1.1 Temperature

Temperature is one of the key parameters affecting the AD because it influences the microbial growth, enzymes activities, substrate characteristics, and consequently the methane yield. Although conventional AD is performed at mesophilic temperatures of 35–37 °C (Forster-Carneiro et al. 2008), thermophilic AD has the advantages of higher specific growth rate, a faster metabolism, and higher methane yield (Kim et al. 2011b). FW is rich in proteins which might cause some problems during thermophilic AD. The free ammonia concentration may increase faster at high temperatures and became inhibitory for AD. However, this problem can be solved by use of acclimatized inoculum.

15.4.1.2 pH, Free Ammonia and Volatile Fatty Acids (VFA)

Due to the complexity of FW and inoculum, it is difficult to maintain stable operation of AD process. The imbalances in pH are created especially during the bioconversion of proteins and lipids due to the accumulation of free ammonia and VFA. This in turn affects the functions of the extracellular enzymes and the rate of FW hydrolysis. Nutrient-rich FW can be easily acidified into VFA by fermentative bacteria, causes a decrease in pH, and subsequently inhibits methanogenic activity. Moreover, production of excessive free ammonia can also inhibit methane-synthesizing enzymes, leading to proton imbalance and potassium deficiency and ultimately cell lysis. The inhibitory ammonia concentration for methanogenesis is still ambiguous. According to Mata-Alvarez (2003), inhibition occurred at total ammonia concentrations of 1200 mg/L and above, while Hartmann and Ahring (2005) reported an inhibitory threshold of 650 mg/L. The efficiency of methanogenesis in full-scale AD plants co-digesting organic waste and animal manure was found to decrease at the total ammonia concentration greater than 4000 mg/L in the digestate (Angelidaki et al. 2005). However, it has also been recognized that microorganisms can adapt to high free ammonia concentration, implying that it would not be easy to determine the exact ammonia concentration at which the instability of AD system may happen (Banks et al. 2011a; Forster-Carneiro et al. 2008). In order to mitigate the inhibitory effect of excess ammonia, many different strategies including ammonia stripping, biological nitrogen removal, and electrochemical conversion have been extensively explored (Ahn 2006; Park et al. 2010; Walker et al. 2011).

15.4.1.3 Feedstock Composition

FW is a carbohydrate-rich feedstock with protein, lipids, and minerals. In order to have a well-balanced medium for microbial growth as well as process stability, the C and N contents in the feedstock should be well balanced. A C/N ratio of 20–30 has been reported to be ideal for AD (Gomez et al. 2005). High carbohydrate content of the FW indeed is helpful for mitigating ammonia inhibition, but the high C/N ratio of FW may generally cause operational problems during AD of FW (Dai et al. 2013). To tackle this problem, FW might be co-digested with some other waste biomass (e.g., cattle manure and sewage sludge) for better control of the C/N ratio for long-term stable operation of AD (El-Mashad and Zhang 2010). The concept of co-digestion has been discussed in detail in Sect. 4.5.

The high lipid content of FW (6.4–24.1 %, db) can also cause operational problems of AD. Although the biomethane potential of lipids (e.g., 1014 L/kg VS) is more than that of carbohydrates (e.g., 370 L/kg VS), long chain fatty acids (LCFA) have been identified as inhibitory to AD, causing system failure at high VFA concentrations. In addition, cell transport mechanism may be spoiled by adsorption of LCFA onto cell surface (Zonta et al. 2013). In general, the LCFA inhibition can be mitigated by diluting its concentration through the addition of inoculum or co-digestion with lipid-poor feedstocks (e.g., cattle manure and sludge) (Palatsi et al. 2009). It should be noted that the deficiency of essential trace elements in FW suppresses methane production and ultimately causes failure of AD (Zhang and Jahng 2012). Trace elements, such as Na, K, Mg, Ca, Al, Cr, Co, Cu, Zn, Ni, etc., are essential especially for the synthesis of various enzymes and their activities (Facchin et al. 2013). Still, at elevated concentrations, they are toxic (Chen et al. 2008). FW generally contains high concentration of Na and K, while it is deficient in heavy metals like Cu, Co, and Zn (El-Mashad et al. 2008). To improve the AD performance, insufficient trace elements can be supplemented, or FW can be co-digested with other biomass for preventing Na and K inhibition (El-Mashad and Zhang 2010; Zhang and Jahng 2012).

Table 15.3 summarizes the conversion yields of various types of FW to methane. The highest biogas yield of 850 L/g VS was achieved during AD of FW in a two-stage process, and approximately 85 % of the energy in FW was converted to renewable energy in this system (Koike et al. 2009). Nowadays, intensive effort has been dedicated to the bioconversion of FW to biogas for energy recovery, and operation strategies have been developed to improve the energy recovery and volume reduction of FW.

15.4.2 Process Configurations

15.4.2.1 Single-Stage AD

Single-stage AD is usually used for municipal solid waste treatment. This system has advantage of less frequent technical failures and has a smaller investment cost, as all the reactions (i.e., hydrolysis, acidogenesis, acetogenesis, and methanogenesis) take place simultaneously in a simple digester (Zhang et al. 2007). AD can be realized either wet or dry (Nasir et al. 2012). Compared to wet AD, dry AD yields lower methane production as well as lower VS reduction because of the transport limitation of VFA (Nagao et al. 2012). It had been demonstrated that a single reactor digesting FW was not stable due to the VFA accumulation and low pH, leading to low biogas production (El-Mashad et al. 2008). In fact, the stability of single-stage AD is a serious issue when easily biodegradable FW is dealt with.

15.4.2.2 Two-Stage AD

Two-stage AD has been used for producing both hydrogen and methane in two separate reactors (Chu et al. 2008). In the first reactor, hydrogen-producing bacteria and acidogens convert FW to hydrogen and volatile fatty acids (VFAs), while VFAs are converted to methane and carbon dioxide by slow-growing acetogens and methanogens in the second digester. Park et al. (2008) compared the performance and stability of single- and two-stage thermophilic AD systems using synthetic FW. In both systems, the highest methane yield of 90 % (based on COD) was obtained at the OLR of 15 g COD/Ld. Still, the propionate concentration in the single-stage reactor showed variation and was higher than that of two-stage system, demonstrating less stable digestion. Massanet-Nicolau et al. (2013) also evaluated the performance of single- and two-stage AD of FW, and they found that the methane production yield in two-stage fermentation system was enhanced by 37 % and could be run at much shorter HRTs and higher loading rates. Similar observations were also reported by Lee and Chung (2010).

15.4.3 Reactor Configurations

In order to stabilize methanogenesis, use high substrate loading and immobilize the inoculum; packed bed reactors (PBR) and fixed bed systems have been developed (Kastner et al. 2012). Parawira et al. (2005) compared the performances of two different systems: one consists of a solid-bed reactor for hydrolysis/acidification connected to an upflow anaerobic sludge blanket methanogenic reactor (UASB), while the other was a solid-bed reactor connected to a methanogenic reactor packed with wheat straw as carriers for mesophilic AD of solid potato waste. Although PBR hydrolyzed the substrate faster than UASB, the methane yields and the cumulative methane productions were found to be comparable in both systems. Among the high rate anaerobic digesters, UASB reactor has been frequently used for the digestion of different organic wastes. UASB provide the immobilization of anaerobic bacteria by granulation, resulting in

Table 15.3 Methane production from FW

Waste	Pretreatment	Process type	Vessel type	Duration (days)	HRT (days)	OLR (kg VS/m^{3}days)	OLR (kg COD/m^{3}days)	Y_{Biogas} (mL/gVS)	Y_{CH4} (mL/g VS)	Efficiency (VS, %)	References
FW	None	Single stage	Bioreactor with 8 L working vol.	30	12	NR	NR	NR	NR	NR	Kim et al. (2006a)
FW	None	Single stage	Batch	28	10–28	NA	NA	600	440	81	Zhang et al. (2007)
FW	None	Single stage	Batch 5 L	60	20–60	NR	NR	490	220	NR	Forster-Carneiro et al. (2008)
FW	None	Single stage	Digester with 900 m^3 tank vol.	426	80	2.5	NR	643	399	90	Banks et al. (2011)
FW	None	Single stage	Bioreactor with 150 mL working vol.	368	20–30	2.19–6.64	NR	NR	352–450	NR	Zhang and Jahng (2012)
FW	FW liquidized at 175 °C for 1 h	Single stage	UASB with 2 L working vol.	72	4–10	NR	2–12.5	NR	NR	93.7	Latif et al. (2012)
FW	None	Single stage	CSTR with 3 L working vol.	225	16	NR	9.2	NR	455	92.2	Nagao et al. (2012)
FW	None	Single stage	Digester with 800 mL working vol.	30	batch	NA	NA	621	410	NR	Zhang et al. (2013)
Fruit and veg. waste	None	Single stage	Serum bottles with 135 mL vol.	100	NA	NA	NA	NR	180–732	NR	Gunaseelan (2004)
FW and SS	None	Single stage	Bioreactor with 3.5 L working vol.	250	13	2.43	4.71	NR	321	55.8	Heo et al. (2004)

(continued)

Table 15.3 (continued)

Waste	Pretreatment	Process type	Vessel type	Duration (days)	HRT (days)	OLR (kg VS/m^{3}days)	OLR (kg COD/m^{3}days)	Y_{Biogas} (mL/gVS)	Y_{CH4} (mL/g VS)	Efficiency (VS, %)	References
FW	None	Two stage	Bioreactor with 12 L working vol.	60	20	8	NR	NR	NR	86.4	Youn and Shin (2005)
FW	None	Two stage	CSTR with 10 L working vol.	150	5	6.6	16.3	NR	464	88	Chu et al. (2008)
FW	None	Two stage	Bioreactor with 4.5 L working vol.	200	1–27	NR	15	578	520	NR	Park et al. (2008)
FW	LAB pretreatment and SsF	Two stage	Bioreactor with 5 L working vol.	98	7	NR	NR	850	434	NR	Koike et al. (2009)
FW	None	Two stage	Rotating drum with 200 L working vol.	30	NR	4.61	NR	769	546	82.2	Wang and Zhao (2009)
FW	Heat pretreatment (100 °C, 30 min)	Two stage	UASB with 2.3 L working vol.	60	3.9–6.4	NR	NR	NR	NR	80	Lee et al. (2010)
FW	None	Two stage	Gas-sparging type reactor with 40 L working vol.	96	15.4	NR	4.16	NR	NR	88.1	Lee and Chung (2010)
FW	Enzymatic pretreatment	Two stage	UASB with 2.7 L working vol.	75	2.2	NR	2.2	NR	NR	61	(Moon and Song 2011)
FW	Homogenized using blender	Two stage	Bioreactor (10 L) and MBR (3 L)	19	23	10	NA	NR	357	81	Trzcinski and Stuckey (2011)

Fruit and veg. waste	None	Two stage	Bioreactor with 0.5 L working vol.	29	1	1–9	NR	NR	530	95.1	Mtz. Viturtia et al. (1989)
Potato waste	None	Two stage	PBR with 1 L working vol.	38	NR	NR	1 to 3	NR	390	NR	Parawira et al. (2005)
FW	None	Three stage	UASB with 4800 L working vol.	NR	12	54.5	ND	ND	254	90.1	Kim et al. (2008b)

SS seed sludge, *Y* yield; UASB, upflow anaerobic sludge blanket reactor, *SsF* simultaneous saccharification fermentation, *MBR* membrane bioreactor, *LAB* lactic acid bacteria, *NR* not reported, *veg* vegetable, *pret.* pretreatment, *NA* not applicable, *NR* not reported high microbial growth and good settling characteristics (Moon and Song 2011). These in turn allow for high OLR and long SRT. Latif et al. (2012) investigated the mesophilic and thermophilic AD of liquidized FW in UASB reactor by stepwise increasing OLR and temperature and observed 93.7 % of COD removal in UASB reactor, together with a high methane production of 0.912 L/g COD due to low VFA accumulation under controlled temperature and pH. The highest biogas production of 1.37 L/g COD was recorded at the temperature of 55 °C and OLR of 12.5 g COD/L with a HRT of 4 days. Continuously stirred tank reactor (CSTR) and fluidized bed reactor (FBR) have also been explored for methanogenesis (Kastner et al. 2012). 670 normalized liters (NL) biogas/kg VS with the CSTR and 550 NL biogas/kg VS with the FBR were reported, while the average methane content in biogas was about 60 % in both reactor systems, but FBR exhibited higher stability than CSTR.

15.4.4 Pretreatments

Although FW is readily biodegradable with a volatile fraction of up to 90 % of total solids, the hydrolysis of solid FW into soluble organics has been known as the rate-limiting step of AD (Zhang et al. 2014). As a result, AD of FW often suffers from long solid residence time and low conversion efficiency, indicating that a large anaerobic reactor is required (Quiroga et al. 2014). Therefore, different pretreatment methods, such as mechanical, thermal, thermochemical, and enzymatic pretreatments, have been developed for enhancing the hydrolysis of polymers in FW.

15.4.4.1 Mechanical Pretreatments

Mechanical grinding using bead milling, screw presses, or press extruders decrease the particle size of FW, i.e., this helps to increase the surface area, resulting in increased food accessibility for the bacteria. Kim et al. (2000) reported that the substrate utilization was doubled when the average particle size decreased from 2.14 to 1.02 mm, demonstrating that particle size is affecting the AD performance. Therefore, the mechanical pretreatment should be optimized in order to minimize the excessive grinding that has negative impact on AD performance and energy consumption (Cesaro and Belgiorno 2014). It had been found that the solubility of FW and methane yield increased by 40 and 28 %, respectively, when the particle size of FW was reduced from 0.843 to 0.391 mm, while over-reduction in particle size would lead to VFA accumulation and a decreased FW solubility and methane production (Izumi et al. 2010).

There are also some reports on the pretreatment of FW by microwave and ultrasonication. The microwave treatment of FW can lead to temperature increase up to 175 °C which significantly improves the solubility of FW, but only 5–16 % increment was obtained in methane yield (Marin et al. 2010). Shahriari et al. (2013) found that the liquor produced after the microwave pretreatment of FW can generate threefold more methane than untreated ones. The ultrasonication of FW was also investigated. For example, Elbeshbishy et al. (2011) reported that 67 % VSS removal could be accomplished after the AD of ultrasonicated FW, whereas Quiroga et al. (2014) evaluated the effects of ultrasonication on co-digestion of FW with cattle manure and sludge, e.g., at a lower HRT, 31–67 % increase in methane yield was obtained. On the contrary, it had also been reported that ultrasonicated fat, grease, oil, and kitchen waste would not help to improve biogas production (Li et al. 2013). These different observations might be due to various moisture contents of different types of FW and the operation conditions of the processes. In addition, detailed cost-benefit analysis of these pretreatment methods should be assessed in a holistic manner prior to large-scale applications.

15.4.4.2 Thermal and Thermochemical Pretreatments

Thermal pretreatment can improve the hydrolysis of FW. The soluble COD concentration of FW was found to be doubled, with 29 % increase in the biogas yield after pretreatment at 150 °C for 1 h (Liu et al. 2008). Wang et al. (2006) reported that hydrolytic and acidogenic processes were improved after the pretreatment of FW at 70 °C for 2 h and at 150 °C for 1 h, respectively. However, it should be noted that the degree of solubilization of FW may not be proportional to biogas yield. This is due to the fact that aggressive thermal pretreatments can increase the FW biodegradation, but inhibit the biogas production (Tampio et al. 2014). Inhibitory Maillard reactions may take place between the carbohydrates and amino acids at temperatures above 100 °C, which slows down protein hydrolysis (Vavouraki et al. 2014). It had been shown that autoclaved FW at 160 °C and 6.2 bar had lower ammonium and hydrogen sulfide concentrations than untreated ones, most likely due to decreased protein hydrolysis because of the formation of Maillard compounds (Tampio et al. 2014). Consequently, untreated FW generated 5–10 % more methane than pretreated FW.

Thermochemical pretreatment of FW has also been explored. Vavouraki et al. (2014) investigated the thermochemical pretreatment of FW using dilute acids at different temperatures of 50, 75, and 120 °C for 30–120 min. It was found that soluble sugar concentration was increased by 120 % under the optimized thermochemical conditions (i.e., 1.12 % HCl for 94 min or 1.17 % HCl for 86 min at 100 °C) compared to untreated FW. Although the solubilization of FW can be improved significantly using acids at elevated temperature, the release of carboxylic acids, furans, and phenolic compounds could be possible during the pretreatment, resulting in inhibition of methanogens and less biogas production (Zhang et al. 2014). Some studies showed that the enhanced methane production could offset the energy requirement of the thermal pretreatment (Ariunbaatar et al. 2014), whereas Liu et al. (2008) reported no net energy gain when the energy necessary by the pretreatment process was counted in the overall energy balance of the treatment system.

15.4.4.3 Enzymatic Pretreatments

Enzymatic pretreatment can improve the solubilization of FW without producing any inhibitory compounds. Commercial enzymes including carbohydrases (e.g., glucoamylase, arabinase, cellulase, β-glucanase, hemicellulase, xylanase, proteases, and lipases) have been used for the hydrolysis of FW (Moon and Song 2011). The pretreatment of FW with multiple commercial enzymes appeared to be more efficient than that with a single one (Kim et al. 2006b; Moon and Song 2011). However, it should be realized that commercial enzymes are costly (about USD120 to treat one ton of FW with glucoamylase and alpha-amylase at 10U/g FW) and generally available in single-type form. In order to make the enzymatic hydrolysis of FW more cost-effective, enzymes should ideally be produced on-site from a cheap feedstock. In a study of Uçkun Kiran et al. (2015), a fungal mash rich in glucoamylase and protease was produced from cake waste and then applied directly for enzymatic hydrolysis of mixed FW. The enzymatic pretreatment using this fungal mash was shown to be more efficient than commercial enzymes. The biomethane yield and production rate on FW pretreated with this fungal mash were found to be, respectively, 2.3 and 3.5 times higher than those without pretreatment, while 80.4 ± 3.5 % of the overall volatile suspended solid destruction of FW was achieved. These results showed that direct use of the fungal mash without any further separation and purification is a promising approach for high-efficiency FW treatment.

15.4.5 Co-digestion of FW

AD of FW has a great potential for methane generation (Zhang et al. 2014). However, the inhibition of the biogas production is a big challenge when FW is used as sole substrate in long-term AD operations due to the imbalance of the nutrients. The C/N ratio of FW is generally out of the optimum range defined for the AD process (Sosnowski et al. 2003). Moreover, some heavy metals such as zinc, iron, molybdenum, etc. are insufficient, while sodium, potassium, etc. are excess in FW (El-Mashad and Zhang 2010). The high lipid content of FW also inhibits its AD. In order to balance the nutrients and mitigate inhibition, co-digestion of FW with some other organic wastes (e.g., cattle manure, green waste, and

Table 15.4 Co-digestion of FW with other organic substrates for improving performance of AD

Feedstock	Action of co-digestion	Influencing factor	Reference
FW and DS	Enhance system stability	Less inhibition from sodium	Dai et al. (2013)
FW and DS	Improve methane yield	Less inhibition from lipids and potassium	Carucci et al. (2005)
FW and SS	Afford high organic loading rate	High buffering capacity from ammonia	Kim et al. (2011b)
FW and SS	Improve methane yield and production rate	High BMP potential	Koch et al. (2015)
FW and SS	Allow higher organic loadings	Trace elements supplement	Parry and Evans (2012)
FW and WW	Improve biogas productivity and process stability	Trace elements supplement	Zhang et al. (2011)
FW and WW	Improve methane yield and TOC utilization	High buffering capacity	Wang et al. (2013)
FW and CM	Improve methane yield and system stability	High buffering capacity and trace elements supplement	Zhang et al. (2013)
FW and CM	Improve biogas production	High buffering capacity from ammonia	Marañón et al. (2012)
FW and CM	Improve methane yield	Nutrient balance	El-Mashad and Zhang (2010)
FW and CM	Increase energy returns and reduce GHG emission	Nutrient balance	Banks et al. (2011b)
FW and livestock waste	Improve methane yield and VS reduction	High buffering capacity	
FW, CM and oil	Improve methane yield	High buffering capacity	Neves et al. (2009a)
FW, CM and fat	Improve methane yield	Lipid supplement	Neves et al. (2009b)
FW, CM and card packaging	Allow higher organic loadings and gave a more stable process	Trace elements supplement	Zhang et al. (2012)
FW and yard waste	Improve methane yield	Less VFA accumulation	Brown and Li (2013)
FW and distiller's grains	Increase biogas production	High buffering capacity from ammonia	Wang et al. (2012b)
FW and green waste	Improve VS reduction	C/N ratio	Kumar et al. (2010)

Revised from Zhang et al. (2014). *SS* sewage sludge, *DS* dewatered sludge, *CM* cattle manure, *WW* wastewater

sewage sludge) has been investigated extensively as summarized in Table 15.4.

The co-digestion of FW with sewage sludge has gained increasing interest recently due to huge amount of sewage sludge generated from wastewater treatment plants globally. AD of the sewage sludge has been practiced as one of the most feasible options for sludge stabilization, volume reduction, and energy recovery. However, the sewage sludge has high water content leading to a low methane yield during AD (Dai et al. 2013). Therefore, dewatering of sewage sludge prior to AD is necessary. Even so, methane yield could not be improved much probably due to low VS/TS ratio and high ammonia concentration of the dewatered sludge. Existing evidence shows that co-digestion of sludge or dewatered sludge with high organic content FW is a feasible approach of improving the methane yield because of a complementary and synergistic effect and mixed FW and sewage sludge during AD (Kim et al. 2011). For example, FW provides extra carbon source, while sewage sludge is a good source of ammonia and helps to dilute the harmful and excessive substances contained in FW, which may inhibit microbial growth during AD. This may explain growing interest in co-digestion of sewage sludge and FW for enhanced energy recovery and waste volume reduction.

It should be noted that the composition of mixed FW and sewage sludge has a significant effect on the performance of co-digestion. In order to obtain high methane yield and production rate, the ratio of 35 % by VS of sewage sludge to FW had been recommended for co-digestion (Koch et al. 2015). In addition, sludge acclimation can help to mitigate the inhibition, while shorten the lag phase of microbial growth during co-digestion. It had been reported that AD of FW was inhibited by potassium and lipid at a respective content of 55 g/kg dry FW and 13 % in FW with unacclimated inoculum (Carucci et al. 2005). After a long acclimation period, a higher methane yield of 53 % was obtained during the AD of a mixture of FW and sludge at the ratio of 60 % versus 40 %. Most of the co-digestions were conducted at mesophilic conditions due to the fact that thermophilic digestion is more energy intensive. However, Kim et al. (2011b) developed a temperature-phased anaerobic sequencing batch reactor, and a faster metabolism was observed at high organic loading rate of 5.9 % TS with a VS/TS ratio of 71.9 % as compared to conventional two-stage mesophilic systems. This might be due to the enhanced stability of thermophilic methanogens and alleviated alkalinity through enhanced protein degradation. The long-term operations showed 44.2–76.5 % of VS removal during the co-digestion of sewage sludge and FW, while producing 0.2 m^3 CH_4/kg VS_{added} at the organic loading rate of 6.1 g VS/L/day and a short HRT of 7 days through the synergy of, co-digestion, sequenced operation and temperature phasing. Lastly, it should be noted that the greenhouse gas emission can be reduced significantly by co-digestion of organic wastes. However, the collection and transportation of FW would challenge the effectiveness of co-digestion.

15.5 Remarks

FW has posed a big challenge on environment and society. The development of an economical, sustainable, and environmental friendly integrated FW management approach is crucial and urgently needed. One should have to change mindset first towards this goal. That is, FW can no longer be seen as a waste; instead, it represents a very important resource of carbon and nutrients which can be used as feedstock for production of various types of renewable energy, e.g., biofuels and methane. Nowadays, ethanol fermentation from FW is straightforward and does not require harsh and complex pretreatments. However, the main drawback is associated with the high production cost due to use of commercial enzymes for saccharification of FW. The possible solution is to use in situ produced highly active crude multienzymes without further separation and purification. Biogas production via AD of FW is another option for energy recovery in the form of methane gas and volume reduction. AD of FW can be realized in single- and two-stage mesophilic or thermophilic systems. In general, pretreatment of FW is necessary prior to AD. Nowadays, co-digestion of FW with other waste biomass including sewage sludge has attracted more and more attention, with the focus on enhancing the stability and efficiency of AD. Moving forward, FW should not be regarded as waste, but a resource for biorefinery.

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Esra Uçkun Kiran M.Sc. and Ph.D. Biotechnology from Middle East Technical University, Turkey. She is presently working as a research fellow in Advanced Environmental Biotechnology Centre at Nanyang Technological University, Singapore. She is a Fellow of American Chemical Society and Bioenergy Society of Singapore. Her main areas of research are clean and renewable energy, enzyme technologies and biofuels.

16 Metabolism of Long-Chain Fatty Acids (LCFAs) in Methanogenesis

Parinita Sharma, Anshuman A. Khardenavis, and Hemant J. Purohit

Abstract

Anaerobic digestion (AD) is an important process for generating third-generation fuel in the form of methane from a variety of organic wastes. Efficiency of AD is dependent on inhibitory effect exerted by the substrate on methanogenic pathway. Here we assess the potential of utilizing lipid-rich waste as a suitable substrate for methane production. Anaerobic digestion of lipids leads to production of long-chain fatty acids (LCFAs) which are known to inhibit acetoclastic methanogens. The problems faced during AD of lipid-rich waste, strategies for overcoming the problems, and application of genomic tools for characterization of microbial community involved in biomethanation of this substrate are also discussed.

16.1 Introduction

Anaerobic digestion (AD) is a process of decomposition of organic matter by mixed microbial inoculum in the absence of oxygen in four steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Among various substrates, significant amount of lipid-rich waste produced each year from different industries such as food processing, dairy, olive oil mills, slaughterhouses, and edible oil processing can serve as alternative source for anaerobic conversion to methane. The utilization of lipid-rich wastes as suitable substrates for biomethane production, the associated problems which negatively affect the AD process, and the methods for overcoming these problems have been reported by many researchers (Table 16.1). Wastes from these industries contain fats, oil, and grease (FOG) which together represent the lipid-rich layer. The tendency of FOG layer to float on the surface of water leads to clogging within the discharge pipes due to its accumulation. In anaerobic treatment systems, hydrolysis of such lipids by extracellular lipases leads to formation of glycerol along with long-chain fatty acids (LCFAs), followed by β-oxidation resulting in formation of acetate and hydrogen. LCFAs in FOG are mainly of saturated (37–48 %) and unsaturated (51–58 %) nature whose reduced state makes FOG an ideal

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P. Sharma • A.A. Khardenavis (✉) • H.J. Purohit
Environmental Genomics Division, CSIR-National Environmental Engineering Research Institute (CSIR-NEERI), Nehru Marg, Nagpur 440 020, India
e-mail: aa_khardenavis@neeri.res.in; anshuman.neeri.egd@gmail.com; hj_purohit@neeri.res.in; hemantdrd@hotmail.com

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Table 16.1 Methane production from various sources of long chain fatty acids (LCFAs)

Lipid-rich waste	Lipid/LCFA composition/conc	HRT/strategy of operation	Time	Lag phase	Methane/biogas potential	Methane/biogas yield	Inhibitory conc.	Reference
Simulated lipid waste	Triolein: 5–47 % (COD basis) Abundant palmitate followed by oleate, myristate, and stearate	Batch	120 days	5–40 % = 10 days; 47 % = 60 days	93–100 % of theoretical yield	800–900 ml/g of VS added	31 %	Cirne et al. (2007)
(i) Landfill leachates	19 % (VS basis)	Semicontinuous pilot plant	47 days	–	23.5 % increase over period without co-digestion	12.4 Nl/kg	–	Pastor et al. (2013)
(ii) Used oil						970.6 Nl/kg		
(i) Cattle manure	–	Batch BMP	17 days	–	78 % of theoretical yield	509 mL CH_4/g VS added	–	Kougias et al. (2014)
(ii) Antifoam agents (oils)	0.05–0.5 % (v/v_{feed})	Batch BMP	25 days	–	70–83 %	623–837 mL CH4/g VS added	Complete inhibition for tributyl phosphate	
	Rapeseed oil	CSTR	110 days	–	–	284–377 mL CH4/g VS added		
	Oleic acid							
	Octanoic acid							
	Tributylphosphate							
(iii) Co-digestion of antifoam with cattle manure	0.05–0.5 % (v/v_{feed})	Batch BMP	25 days		89–91 %	498–655 mL CH4/g VS added	–	
	Rapeseed oil				82–94 %	528–600 mL CH4/g VS added	0.5 %	
	Oleic acid				14–92 %	92–192 CH4/g VS added	0.5 %	
	Octanoic acid				0	0	0.05 %	
	Tributyl phosphate							
	Individually with cattle manure							
Co-digestion of sewage sludge with trapped grease waste	12–37 %; OLR: 2.2–2.8 kg COD/m^3.d (COD basis)	Bench-scale CSTR	550 days	–	1.2 and 2.2 times increase over sewage sludge alone	–	37 %	Silvestre et al. (2014)

(i) Simulated OFMSW	Diluted pet food; OLR: 0.55–2.57 kg TVS/m^3.d (VS basis)	17 days; Pilot scale semicontinuous, semicontinuous, completely mixed liquid reactor	~275 days	10 days	–	0.8 m^3 biogas or 0.5 m^3 CH_4/kg TVS removed	—	Fernández et al. (2005)
(ii) Co-digestion of OFMSW with	0.97 kg TVS/m^3.d						–	
(a) Animal fats	4–28 % of OLR (predominant palmitic, stearic, oleic, and linoleic acids)					0.55–0.75 m^3 biogas or 0.35–0.5 m^3 CH_4		
(b) Vegetable fats (coconut oil)	28 % of OLR (predominant lauric, myristic, palmitic acids)					0.7 m^3 biogas or 0.45 m^3 CH_4	–	
(i) Cow manure and food waste	4.6 g COD/ $L_{reactor}$·d	15 days; CSTR	148 days	–	–	–		Neves et al. (2009)
(ii) Co-digestion of cow manure and food waste with increasing pulses of canned fish industry oily waste	4.6 g COD/ $L_{reactor}$·d + 9–18 g COD/ L.d		203 days	–	70 % increase over cow manure and food waste alone	Offline BMP 0.4–0.9 g COD-CH_4/g VS	18 g COD/ $L_{reactor}$·d	
			224 days	–		0.6-0.9 gCOD-CH4/g VS		
Oleate acclimatized sludge with LCFAs	OLR: 6 kg COD/m^3/ day	1.18–2.7 days; EGSB	~800 h	500 h	–	5200 mg COD–CH_4 g VSS measured at end of assay	–	Pereira et al. (2005b)
(i) Oleic acid								
(ii) Palmitic acid		1.18–2.7 days; EGSB	~800 h	50–60 h		4570 mg COD–CH4 g VSS measured at end of assay	–	
(i) Waste- activated sludge (WAS)	Substrate/inoculum (S/I) = 0.08	BMP	750 h	81.8 h	–	117 mL CH_4/g TVS	–	Liet al. (2011)
(ii) Co-digestion of WAS with	3–12 g (S/I = 0.46–1.59)			3.9 h		124–134 mL CH4/g TVS	12 g (S/I = 1.59)	
(a) Synthetic kitchen waste (KW)								
(b) Fat, oil, and grease (FOG)	0.2–1.4 g (S/I = 0.3–1.61)			28.3 h		31.9–418 mL CH4/g TVS	0.7–1.4 g (S/I = 0.85–1.61)	

substrate for anaerobic digestion with a potential for increased biogas production owing to the abundant carbon content (Suto et al. 2006; Canakci 2007). The LCFAs so formed have also been suggested to inhibit the anaerobic digestion system (Pereira et al. 2005a, b).

Though the basic degradation pathway of saturated LCFAs involving β-oxidation has been known for a long time, various studies report different pathways for degradation of unsaturated LCFAs. Some studies have suggested saturation of the unsaturated LCFAs prior to β-oxidation, while other studies proposed β-oxidation of unsaturated LCFAs before they were completely saturated (Lalman and Bagley 2000, 2001). Irrespective of the type of LCFAs (saturated or unsaturated), the primary route followed for microbial degradation of LCFAs consisted of the following steps: (1) adsorption of LCFA onto the cell surface, (2) uptake of LCFA, and (3) β-oxidation and formation of lower molecular weight products (Sousa et al. 2010). One unique feature of LCFA decomposition was demonstrated by Sousa et al. (2010), which suggested that bacteria with capacity for degrading unsaturated fatty acid also possessed the ability to degrade saturated fatty acids, but not vice versa.

16.2 LCFA Detection Methods

One of the problems encountered during biomethanation studies with LCFAs is the lack of appropriate methods for detection of the LCFAs which is attributed to the low solubility of LCFAs, especially the saturated ones. Most of the methods for quantification of LCFAs involve extraction in suitable solvents, followed by sample preparation by derivatization. The processed samples could then be subjected to LCFA quantification by various methods, majority of which are based on chromatography.

Tarola et al. (2012) quantitatively determined the composition of fatty acids produced by the action of lipase on drying oils. The procedure involved extraction of fatty acids with n-heptane and derivatization with ɠ-bromoacetophenone followed by quantification by HPLC. The analytical enzymatic procedure provided advantage of separation of analytes with greater efficiency and sensitivity for <0.5 μg/mL of free fatty acids (FFAs).

Free fatty acids (FFAs, C_4–$C_{18:3}$) were quantified from bovine milk by Amer et al. (2013). A novel GC–MS (gas chromatography–mass spectrometry)-based method was developed, which consisted of derivatization of FFAs with ethyl chloroformate and validation by using appropriate standards. The major advantage of this method was the absence of solvent extraction and evaporation steps which reduced the loss of short-chain free fatty acids (SCFFAs) during sample preparation.

Salimon et al. (2014) compared two methods for analysis of FAs and *trans* fatty acids in bakery products with the help of gas chromatography. Methods were based on the use of KOCH3/HCl and trimethylsilyl diazomethane (TMS-DM) as reagents for derivatization which showed that KOCH3/HCl method required shorter time and was more convenient than TMS-DM method for analysis of *cis/trans* fatty acid samples. However, the use of highly polar capillary column and flame ionization detector can provide the benefit of analyzing the LCFAs without the need for derivatization step as was demonstrated by Jiang et al. (2012) in the case of palmitic, stearic, and oleic acids.

Capillary gas chromatography method was developed by Neves et al. (2007) in which linear calibration curves for C12–C18 LCFAs were constructed in the range 25–1270 mg/L. Pentadecanoic acid (C15:0) was used as internal standard for quantifying all the acids with response factors ranging from 0.79 to 1.09. Owing to the time-consuming and tedious nature of conventional methods of sample processing, Kang and Wang (2005) devised a rapid and simple method for the analysis of long-chain polyunsaturated fatty acid contents by combining the two steps of conventional analysis, i.e., extraction and methylation, into a single step.

16.3 Mathematical Modeling and Kinetics of LCFA Degradation

A number of studies have evaluated the improved methane production observed under varying operational conditions with application of linear regression models for indicating the first-order production rates (Hansen et al. 2004; Heo et al. 2004; Carucci et al. 2005). Angelidaki et al. (1999) developed a dynamic model for prediction of process performance of AD of complex material and co-digestion of different wastes. Detailed description of physiological conditions was incorporated in the model along with other primary modulating factors such as free ammonia, acetate, volatile fatty acids (VFAs), and LCFAs to describe the effect of co-digestion on methane production.

Various configurations of reactors could also be used to study the mathematical model of LCFA degradation. One such model was developed by Knobel and Lewis (2002), wherein mathematical modeling was used for discussing all reactions occurring before sulfate including hydrolysis of solid substrates, β-oxidation of long-chain fatty acids, acidogenesis, and acetogenesis. Using this model, prediction of the dynamic and steady state behavior of different types of reactors was arrived at for simulation using both simple and complex carbon sources.

In addition to linear models, nonlinear regression models were applied for describing the co-digestions of FOG and kitchen waste (KW) for demonstrating amelioration of the methane production along with shortening of lag phases of biodegradation (Li et al. 2011). However, accumulation of LCFAs on the sludge during digestion of FOG induced a delay in initial methane production by creating a physical barrier which hindered the transfer of substrates and products. This phenomenon was shown to be reversible which could be eliminated after biomass-associated LCFA was completely mineralized (Pereira et al. 2005b). The decomposition of LCFAs is dependent on the enthalpy-driven energy released from methanogenesis by release of protons and electrons which are used during spontaneous methanogenesis for methane production. Thus, LCFAs could be efficiently decomposed into methane, carbon dioxide, and SCFAs when sufficient thermal energy was supplied (Oh and Martin 2010).

Zonta et al. (2013) applied mathematical modeling for studying the dynamics of LCFA inhibition on AD in the presence of synthetic adsorbent (bentonite) and synthetic substrate (sodium oleate). Validation of the model provided an insight into the biophysics of the inhibitory process. The model also confirmed the higher sensitivity of the acetoclastic population than the acidogenic population to the LCFA inhibition and highlighted the significance of distribution of saturated/unsaturated LCFAs degraders in the evolution of the system.

16.4 Inhibition of Methanogenesis by LCFAs

Detailed studies on the pathways involved in inhibition of AD process by LCFAs can provide deeper insight into the mechanism of inhibition. β-oxidation, the rate-limiting step in LCFA degradation, initially reaches a maximum with increase in LCFA concentration, followed by a decrease thereafter, indicating that LCFA acts as a substrate inhibitor for β-oxidation. Shin et al. (2003) compared the inhibitory effects of LCFAs (16–18 carbons) on β-oxidation and VFA degradation using acclimated granular sludge. Assessment of VFA degradation rates and β-oxidation by applying Gompertz equation indicated maximum methane production of 86–90 % of the theoretical values for VFA and 60–70 % for LCFAs. Acetate degradation was affected to a greater extent in the presence of unsaturated LCFAs (oleate, linoleate) than saturated (stearate, palmitate) ones, while the effect of LCFA inhibition on propionate degradation was less pronounced than on acetate degradation. Degree of saturation of LCFAs was shown to effect the AD process, and the observation that unsaturated LCFAs contributed to additional solids removal in comparison to saturated LCFAs was attributed to higher solubility of unsaturated LCFAs than the saturated LCFAs (Shin et al. 2003).

There are different opinions on the rate-limiting step in formation of methane from LCFAs. Cavaleiro et al. (2013) suggested β-oxidation to be the limiting step in the above pathway by demonstrating that the first steps of unsaturated LCFA degradation were not obligatory and syntrophic. Different reactors were fed with saturated and unsaturated C16 and C18 LCFAs in the presence or absence of selective inhibitor of methanogens, bromoethanesulfonate, followed by analysis of bacterial community composition by denaturing gradient gel electrophoresis (DGGE). Degradation of oleate (C18:1) resulted in the higher accumulation of myristate (C14:0) and palmitate (C16:0) in the bioreactors in which methanogenesis was inhibited than compared to non-inhibited methanogenic bioreactors.

The inhibitory effect of LCFAs on AD process was reportedly caused by limitations for transport of LCFA accumulated onto anaerobic sludge (Pereira et al. 2005b). Comparison of specific methanogenic activity of two sludges before and after mineralization of LCFAs showed accumulation of palmitic acid. White spots of precipitated palmitic acid were observed between the nonencapsulated sludge, and very high initial methanogenic activity was observed in the case of all tested substrates except butyrate. In the encapsulated sludge, it was adsorbed into the surrounding, and methane production was reported only from ethanol and H_2/CO_2 after a lag phase of 50 h. Thus, it was concluded that LCFA caused hindrance in the transfer of substrates by creating physical barrier, thereby leading to a delay in initial methane production.

16.5 Effects of LCFAs on Anaerobic Digestion Process

Lipid-rich wastes are generated from different sources including slaughterhouse, meat-processing plants, dairy, edible oil, grease trap, and food wastes from restaurants. Inhibition of anaerobic process by lipids is known to be caused due to sludge flotation and washout in the presence of LCFAs which are hydrolyzed products of lipids. FOG is a major component of lipid-rich surface layer of wastewater generated by food processing and cooking, which is primarily composed of LCFAs (palmitic, stearic, oleic, and linoleic acids) (Suto et al. 2006; Canakci 2007). Problems associated with AD of excessive FOG include inhibitory effects such as lag phase for decomposition, cessation of biogas production, and digester washout.

Though excessive addition of LCFAs causes inhibition in the AD process, Zhu (2013) observed an increase in the production of biogas in the reactors fed with high concentration of LCFAs. Among all the LCFAs added in same dosage, highest increase in digester performance was achieved with linoleic acid, while a mixture of oleic acid and stearic acid was reported to be least effective. From the study, the authors suggested that reactor stability could be improved and LCFA accumulation could be avoided by keeping the dosage of oleic acid below 30 %. The finding that although methane production was not completely inhibited by either of the LCFAs, oleate was more inhibitory to methanogens than palmitate was confirmed by Sousa et al. (2013). The authors studied the effects of saturated (palmitate [16:0]) and unsaturated (oleate [18:1]) LCFAs on hydrogenotrophic methanogens by adding *Methanospirillum hungatei* and *Methanobacterium formicicum* to oleate- and palmitate-degrading enrichments. The survival of the two cultures in enrichments was monitored by DGGE analysis which showed higher resistance and presence of *M. formicicum* in both oleate and palmitate enrichments. On the other hand, *M. hungatei* was detected only in palmitate enrichment and viability tests, confirmed the presence of higher percentage of damaged cells of *M. hungatei* indicating higher sensitivity of this culture to oleate than *M. formicicum*.

The inhibitory effect exerted by LCFAs is reported to be stronger for gram-positive organisms even at low concentrations than gram-negative organisms. The inhibition was linked to the adsorption of the LCFAs on the cell wall of anaerobic consortium, thus causing interference in the transport of substrate and products. Cell wall composition of methanogens played a role in sensitivity of the methanogens to LCFAs. Thermophiles were more sensitive to LCFAs than mesophiles owing to their variable cell wall composition. Zeitz et al. (2013) studied the inhibitory effects of saturated fatty acids on methanogenesis by *Methanosarcina barkeri, M. mazei, Methanococcus voltae* (at 37 °C), and *Methanothermobacter thermoautotrophicus* (at 65 °C). The effect of the methanogenic coenzyme M (HS-CoM) on inhibition by LCFAs showed that methanogens were susceptible to C10 and C11 and less to C14 LCFAs. C18 LCFA affected *M. thermoautotrophicus*, while in *M. mazei* cultures, the action of C14 was not prevented by HS-CoM, thereby suggesting that the SFA-induced effect on methanogenesis was not due to the inhibition of HS-CoM.

16.6 Strategies for Overcoming Inhibitory Effect of LCFAs

In an anaerobic environment, extracellular lipases produced by acidogenic bacteria hydrolyze lipids to generate glycerol and free LCFAs of which glycerol is further converted in the bacterial cell to acetate by acetogenesis and acetate and hydrogen were produced from LCFAs through β-oxidation pathway (syntrophic acetogenesis). The process efficiency depends on the utilization of generated hydrogen by hydrogenotrophic methanogens. Along with hydrogen, low solubility of saturated LCFAs creates limitation in the degradation of 16 and 18 carbon-saturated acids, while five times higher degradation has been observed in case of unsaturated fatty acids. These process limitations have been shown to be overcome by application of co-digestion strategy which is considered to be an effective, cheap, and suitable method for reducing the process limitations and improving methane yields (Alatriste-Mondragón et al. 2006).

Fernández et al. (2005) demonstrated the advantage of co-digestion strategy during AD of fats of different origins with organic fraction of municipal solid waste (OFMSW) in a semicontinuous reactor. Under mesophilic conditions, 88 % total fat removal occurred at corresponding biogas yield of 0.8 m^3/kg total volatile solids (TVS) removed and 60 % as methane, with neither LCFAs nor VFAs being detected irrespective of the source of the fat (animal or plant origin).

Improved process efficiencies have also been shown to be achieved by co-digestion of FOG with 25 % reduction in biosolids being reported in addition to 60–70 % biogas production (Bailey 2007; York et al. 2008). In addition to co-digestion, effect of co-substrates was also investigated on the degradation of LCFAs in single- and two-stage upflow anaerobic sludge blanket (UASB) digesters (Kuang 2002). Glucose was found to be better co-substrate than cysteine for promoting granule formation in a single-stage UASB resulting in decreased toxicity of sodium oleate. On the other hand, granule formation was severely affected on addition of cysteine and sodium oleate in combination, while different microbial communities were recovered from LCFA-inhibited digester on addition of combination of glucose and cysteine. However, similar strategy of using co-substrates consisting of two LCFAs led to conflicting results as was demonstrated by Cavaleiro et al. (2008) who incubated two anaerobic sludges with oleate and palmitate in batch mode. An initial decrease in methane production was noted due to the presence of biomass-associated LCFAs, which improved significantly when sufficient time was given for the degradation of this LCFA. This created a lag phase for methane production and simultaneously increased the tolerance of the acetotrophic methanogens to LCFAs. Alternatively, the authors suggested that pulsed addition of lipid-rich waste (dairy wastewater) containing 53 % fat could improve the efficiency of cumulative methane production and COD removal in each new pulse, along with significant decrease in VFA levels.

The lipid concentration in lipid-rich waste was shown to have a strong correlation with efficiency of methane production. An initial lag phase of 6–10 days is detected before degradation of lipids starts. Enzymatic hydrolysis of lipids could reduce the lag phase and enhance the hydrolysis, but the intermediates produced during the hydrolysis have been shown to be inhibitory to latter steps of the degradation process (Cirne et al. 2007). This drawback could be overcome and methane production could be enhanced by controlled, intermittent input of an increasing concentration of fat as was demonstrated in the co-digestion of manure and food waste by controlled, intermittent inputs of oil (Neves et al. 2009).

In another study of co-digestion, Pastor et al. (2013) did a comparative evaluation of biogas production from sludge generated in wastewater treatment plant (WWTP), landfill leachates, and used oil. The biogas production was highest (970.6Nl/kg) in the case of used oil during co-digestion with sludge in a pilot plant operated under semicontinuous conditions. Silvestre et al. (2014) co-digested sewage sludge with different doses of grease waste in the bench-scale CSTR under thermophilic conditions. Addition of grease waste up to 27 % resulted in increased methane yield, but higher doses of grease waste led to instability and LCFA accumulation. Thus, though, the addition of grease waste promoted acetoclastic activity by inhibiting the hydrogenotrophic activity, it suggested that tolerance to LCFAs could be enhanced by slow addition of lipid-rich waste.

Inhibition of biogas production has been observed in the presence of LCFAs even under varying physiological conditions such as digestion in batch and semicontinuous experiments under thermophilic conditions at LCFA concentration exceeded 1.0 g/L. However, recovering strategies such as adsorbing the LCFAs, increasing the biomass/LCFA ratio by diluting with active inoculum, and reducing the bioavailable LCFA concentration could be used for overcoming the inhibition (Palatsi et al. 2009). Alternatively, anaerobic biomass could be acclimatized to an inhibitory concentration of LCFA by repeated exposure leading to enhancement in degradation rates.

In spite of various reports on the sensitivity of methanogens to LCFAs, acclimation strategy could result in increased resistance as observed in case biofilm formed in the presence of oleic acid in comparison to control biofilm produced in the absence of lipids (Alves et al. 2001).

Qian (2013) studied the effect of four different LCFAs (stearic, oleic, linoleic, and linolenic acids) and showed improved degradation efficiency when a mixture of stearic and oleic acid (1:2 by mass ratio) was fed in the digester compared to stearic acid alone due to lower metabolic capacity of bacteria for saturated LCFAs on account of their lower solubility. Increase in solubility of stearic acid by dissolving it in oleic acid could result in enhanced degradation efficiency of stearic acid.

Foam formation is an important factor in the degradation of lipid-rich waste. Lienen et al. (2013) investigated the microbial community in a full-scale biogas plant fed with sewage sludge and FOG as substrate together with *Microthrix parvicella* (which promoted the formation of foam) over a 15-month period. QPCR indicated the presence of higher number of *M. parvicella* following an excessive foaming in comparison to reference digesters. Shift in the number of *M. parvicella* was observed corresponding to its seasonal abundance in the sludge of the WWTP.

In addition to foaming promotion by lipid-rich waste, different oils have also been known to serve as agents for foam control/reduction as demonstrated by Kougias et al. (2014). The study compared the role of oils on foam reduction in manure-based biogas reactors which indicated that though all the oils studied (octanoic acid, oleic acid, rapeseed oil, and tributyl phosphate) efficiently suppressed foaming in reactors, only rapeseed oil had a synergistic effect on methane yield, while tributyl phosphate was inhibitory to the biogas process.

16.7 Characterization of LCFA Degrading Methanogenic Community by Genomic Tools

Combination of enrichment studies with molecular approaches has revealed the classification of LCFA-degrading microbes in phylogenetically diverse bacterial groups (Hatamoto et al. 2007a;

Sousa et al. 2007a, b). Enrichment, isolation, and SIP [RNA-based stable isotope probing] have enabled characterization of bacterial community involved in AD of diverse LCFAs as sole energy source (Hatamoto et al. 2007a). Bacteria associated with the family *Syntrophomonadaceae* were found to be dominant along with evidence for existence of the bacteria belonging to the phylum *Firmicutes* in the enrichment cultures (Hatamoto et al. 2007a). SIP has also been employed for determining the microbial diversity in methanogenic sludges involved in LCFA degradation using ^{13}C-labeled palmitate (Hatamoto et al. 2007b). The study revealed the bacterial diversity actively involved in the degradation of LCFA including members from families *Syntrophaceae* and *Syntrophomonadaceae*, phyla *Bacteroidetes* and *Spirochaetes*, and clone cluster from class *Deltaproteobacteria*.

Dominance of uncultured bacteria belonging to the family *Firmicutes* and *Proteobacteria* phyla was reported from stable oleate and palmitate enrichment cultures by DGGE (Sousa et al. 2007a, b). Syntrophic fatty acid-oxidizing bacteria belonging to *Syntrophomonas* were found to be prevalent in the identified, predominant DGGE bands which also showed the ability of oleate enrichment culture to utilize palmitate without any change in DGGE profile. However, reverse study with palmitate-specialized culture demonstrated a lag phase of 3 months for degradation of oleate after which a change in DGGE profile was observed.

Immunological probes have been used for targeting saturated fatty acid-β-oxidizing syntrophic bacteria which included three mesophilic *Syntrophomonadaceae* species. The methanogenic rRNA was found to be comprised of *Methanomicrobiales* which were found to be the main hydrogen-utilizing microorganisms. 0.2–1 % rRNA belonged to family *Syntrophomonadaceae*, but majority belonged to the genus *Syntrophomonas* (Hansen et al. 1999).

Microbial diversity of methanogenic sludges degrading LCFAs has been characterized by SIP (Hatamoto et al. 2007b). Sludge was incubated with ^{13}C-labeled palmitate (1 mM), and ^{13}C-labeled bacterial rRNA was detected after 8–19 days of incubation. Sequencing of clones carrying reverse transcribed 16S rRNA suggested the occurrence members of *Syntrophaceae*, *Deltaproteobacteria, Clostridium*, *Bacteroidetes*, *Spirochaetes*, and *Syntrophomonadaceae* showing the involvement of varied bacterial groups in the anaerobic degradation of LCFAs. Other molecular tools such as cloning of 16S rRNA gene and in situ hybridization were also used by the authors for analyzing LCFA-degrading microbes (Hatamoto et al. 2007a). Palmitate, stearate, oleate, and linoleate were used as substrates, and predominant bacteria belonging to *Syntrophomonadaceae* were detected in addition to members belonging to phylum *Firmicutes* and class *Deltaproteobacteria*. The authors were also able to detect and characterize a strain from family *Syntrophomonadaceae*, which possessed the capacity of generating acetate and methane from palmitate in syntrophic association with *Methanospirillum hungatei* along with a strain from the phylum *Firmicutes*.

Strain OL-4^{T} was isolated from anaerobic expanded granular sludge bed reactor treating an oleate-based effluent, which was found to degrade oleate in co-culture with *Methanobacterium formicicum* Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM) 1535^{NT} (Sousa et al. 2007a, b). Phylogenetic analysis of 16S rRNA revealed the identity of this isolate which was found to be closely related to *Syntrophomonas erecta* DSM 16215 T, *Syntrophomonas wolfei* subsp. *wolfei* DSM 2245 T, and *Syntrophomonas sp.* TB-6; hence, it was named *Syntrophomonas zehnderi* sp. nov.

Kazakov et al. (2009) explored the ability of bacteria for utilizing branched-chain amino acids and fatty acids as the sole carbon sources and converting them into acetyl-coenzyme A, propanoyl-CoA, and propionyl-CoA by comparative genomics approach. DNA motifs and transcriptional factors that controlled the FA and ILV utilization pathways in *Proteobacteria* were identified by this approach, which revealed existence of remarkable variability in the regulatory systems controlling genes associated with the fatty acid degradation pathway.

Though numerous molecular tools have enabled characterization of LCFA-degrading community, the rarity of complete genome sequences available in database has hindered protein identification due to which studies on metaproteomes of complex microbial communities have remained a big challenge (Sousa et al. 2013). Still, proteomic analysis can play a key role in understanding the cellular responses to stimuli during degradation applications. Han et al. (2008) studied gene expression changes in *Escherichia coli* in response to LCFA (oleic acid) which revealed an altered expression level of proteins and synthesis of nine new proteins (AldA, Cdd, FadA, FadB, FadL, MalE, RbsB, Udp, YccU) compared to glucose.

The significance of fadBA5 operon of *Pseudomonas aeruginosa* in utilization of LCFAs as sole carbon source was demonstrated by Kang et al. (2008). The regulation of fadBA5 by LCFAs was validated by constructing a P_{fadBA5}–lacZ fusion using the promoter region of fadBA5, which led to the identification of *PsrA* as regulator for derepressing fadBA5 operon during binding of LCFA.

The presence of *Desulfovibrionales* and *Syntrophobacteraceae* groups in sulfate-reducing enrichment culture was identified by Sousa et al. (2009). Authors studied the diversity of the palmitate- and oleate-degrading anaerobic bacteria in the presence of sulfate as electron acceptor, by DGGE. Inhibition of methanogenic and acetogenic syntrophic bacteria was observed when the LCFA-degrading methanogenic communities were subcultured in the absence of sulfate. Since archaea were not detected by real-time PCR, it was concluded that the bacterial degradation of LCFAs was also influenced by the substrate.

The significance of understanding the microbial interactions essential for optimizing methane formation from LCFA-containing waste streams in bioreactors was highlighted by Sousa et al. (2010). It was proposed that interspecies hydrogen transfer played a key role in the LCFA conversion to methane for which the syntrophic cooperation of acetogenic bacteria and methanogenic archaea was required.

Salvador et al. (2013) used a combination of metaproteomics approach with 16S rRNA gene pyrosequencing for revealing the microbial composition of the sludge incubated with stearate and oleate. Analysis of proteins by LC-MS/MS showed that archaeal proteomes were better identified than bacterial ones. Organisms from the class *Deltaproteobacteria* were reported to be abundant and dominant being the *Syntrophobacter fumaroxidans*, while the methanogenic population was dominated by *Methanosaeta concilii*.

16S rDNA DGGE profiling has also been used for characterizing the eubacterial and archeal community structure at the start and at the end of operation of thermophilic anaerobic digesters fed with manure (Palatsi et al. 2010). Exposure of the reactor biomass to inhibitory pulses of LCFA resulted in improvement in hydrogenotrophic and acidogenic activity though no change in the microbial community upon exposure to LCFA was detected. DGGE profiles revealed several uncultured ribotypes from β-oxidation bacterial genera (*Clostridium* and *Syntrophomonas*) and syntrophic archaeae related to genus *Methanosarcina*. Physiological nature of biomass adaptation was tested by mathematical model IWA IDM1 which the explained mechanism of the LCFA inhibition by considering inhibitory concentration of substrate and specific biomass content.

16.8 Perspectives

Lipid-rich waste from different industries provides an alternative feed source with potential in biomethanation. However, the inhibitory effect of LCFAs generated during lipid hydrolysis limits the wide-scale use of this feed source for methane production. Biotechnological approach can aid in circumventing the problems associated with anaerobic digestion of LCFAs, and enhancement in efficiency of AD process can be achieved by application of genomic tools.

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Parinita Sharma received her M.Sc. degree in Biotechnology in 2013 from Hislop School of Biotechnology, Nagpur University, Nagpur, India. She is currently working as Research Fellow at CSIR-NEERI, Nagpur, India. Her current research focuses on “biomethane production from long chain fatty acids (LCFAs)”.

Anshuman A. Khardenavis, Ph.D. in Microbiology from RTM Nagpur University. He is presently working as scientist at CSIR-NEERI. His current interests include sustainable management of organic fraction of municipal solid waste through biofuel production, industrial enzymes and feed applications. He is a life member of Association of Microbiologists of India.

Hemant J. Purohit a Microbiologist with training in molecular biology working as Head, Environmental Genomics Division, CSIR-NEERI. His current interests include developing deep insight through gene expression and their networks into biofuel production, bioremediation and stress tolerance through application of omics tools. He is editor of Indian Journal of Microbiology, associate editor of Applied Biochemistry and Biotechnology and academic editor of Plos One.

Bioremediation of Pesticide-Contaminated Soil: Emerging Options

17

Pooja Bhardwaj and Atya Kapley

Abstract

The increasing demand on agricultural produce has increased the usage of pesticides that in turn have increased environmental pollution, leading to serious health concerns. Most of these chemicals are recalcitrant and hence accumulate in the environment. A large number of remedial measures have been proposed over the years, but bioremediation still remains the green route. This chapter discusses the various bioremediation options available and the difficulties in taking lab-scale studies to the field.

17.1 Introduction

Pesticides brought in a revolution in the agricultural field and are being used to increase crop yield, suppress growth of weeds and animal pests, and protect agricultural produce (Fehd 2013). They can be classified on the basis of target organism, chemical group, environmental toxicity, environmental persistence, or other features (Fig. 17.1) (Eldridge 2008). Most of the pesticides fall under the major classes of chlorophenoxy acids, organochlorines, organophosphates, carbamates, and s-triazines (Kuhad et al. 2004). Organochlorinated pesticides, organophosphate pesticides, and carbamates have widespread application as insecticides in agricultural crops for controlling pests in vegetables, cereals, grains, fruits, rangelands, forests, and wetlands (Smith 1992). Chlorophenoxy acid and s-triazines were used as herbicides to control broad-leaved annual and perennial weeds and grassy weeds in a range of agricultural crops, for example, corn and *Sorghum* (Cravotta 1982).

Although pollutants and organisms vary a lot, however, their extensive usage has generated too much of pollution that it is becoming difficult to manage. In principle, pesticides are allowed to be used only in case they are not persistent after a certain duration. However, pesticide residues are found universally in the nature (Fenner et al. 2013). Some pesticides are still found in the ecosystem in spite of their use being banned for more than a decade (Muñoz-Arnanz and Jiménez 2011; Park et al. 2011; Jin et al. 2013; Byard et al. 2015). Detrimental health effects, not only to the human system but to the ecosystem at large, are widely reported. For example, continuous exposure to the herbicide atrazine causes

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P. Bhardwaj • A. Kapley (✉)
Environmental Genomics Division, CSIR-National Environmental Engineering Research Institute (CSIR-NEERI), Nehru Marg, Nagpur 440 020, India
e-mail: a_kapley@neeri.res.in

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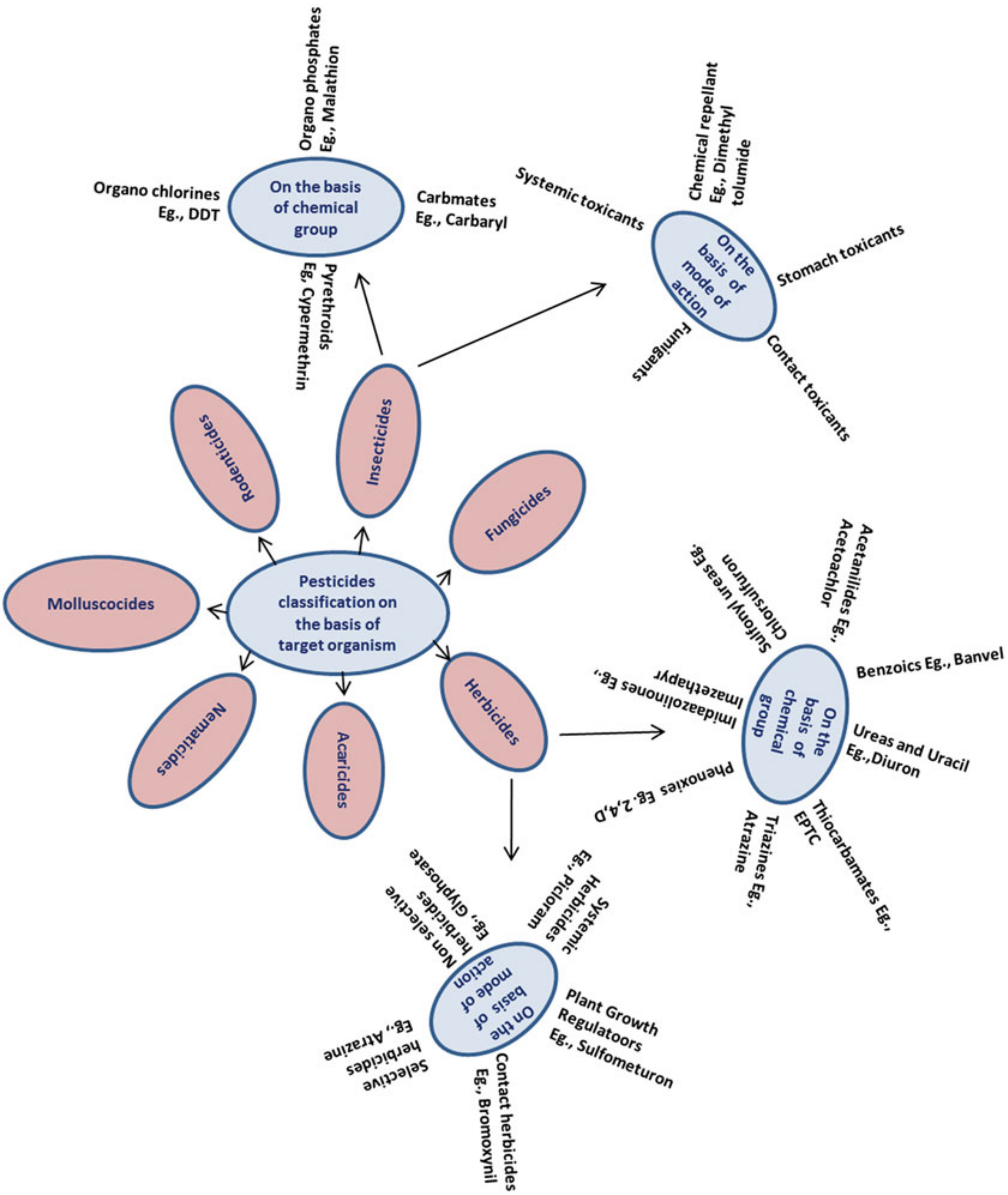

Fig. 17.1 Classification of pesticides on the basis of chemical group and mode of action

demasculinization and feminization in frog and induces endocrine disruption and oxidative stress in mice (Hayes et al. 2011). HCH isomers have been categorized as possible human carcinogens and endocrine disruptors with proven teratogenic, mutagenic, and genotoxic effects (Nayyar et al. 2014). Endosulfan, an organochlorinated pesticide, is primarily neurotoxic but has also been associated with developmental and endocrine disruption, nervous system impairment, as well as immunological and reproductive dysfunction. Diuron, a herbicide, inhibits the photosynthesis of phytoplankton by reducing the dissolved oxygen and nitrogenous waste and diminishing zooplankton population (Barrett and Jaward 2012). Poisoning with organophosphates may induce status epilepticus leading to severe brain damage (Shrot et al. 2014). With adverse effects seen across multiple levels of biological organization, remediation protocols dominate the research scenario. Several physicochemical and biological methods have been developed. Physicochemical methods involve photooxidation, hydrolysis, volatilization, adsorption, incineration, catalytic destruction, use of adsorbent, and evaporation (Pereira 2014), while biological methods include the use of microorganisms and plants that effectively degrade the pollutant. Both methods have their merits and demerits; while chemical methods are faster, they often lead to conversion of one form of pollutant to another. Biological methods are slower but yield to complete utilization of the pesticide by the microorganism or plant and are hence also referred to as the “green route” of pollution control. It has been reported that a biological route for soil remediation was estimated to cost £ 5–170 per ton of soil as compared to £ 12–600 per ton of soil under chemical treatment (Juwarkar et al. 2010).

Many bacterial isolates are known to degrade pesticides. A short overview of the major pesticides used and the bacteria reporting their biodegradation is listed in Table 17.1. Bacteria have enormous catabolic potential that can be used in bioremediation. However, laboratory scale studies do not usually demonstrate success in the field. There are a variety of reasons for this. Laboratory conditions do not mimic the stress of the environment, where uncontrolled temperatures and varying nutrient levels control the biodegradative capacity of the microbial flora. A new concept of ecosystem biology that includes identification of factors that can aid in the bioremediation process along with monitoring and identifying the microbial capacities can help in effective management of pesticide pollution (Paliwal et al. 2012).

This chapter reviews the current tools and processes being used for bioremediation of pesticides and also addresses the problems in taking lab-scale research to the field.

17.2 Bioremediation: A Complex Network of Microbial Cooperation

Bioremediation is defined as the use of microorganisms including bacteria and fungus for cleanup of xenobiotics in a contaminated site. The organic pollutants are either mineralized by the microorganisms and converted to carbon dioxide and water or biotransformed into less toxic products or converted into substrates used as energy sources. The drawback arises when biotransformed products are more toxic than the pesticide, and this aspect need to be studied in depth.

Bioremediation can be broadly classified under two categories, in situ (contaminants can be treated on-site) and ex situ (the contaminated soil is treated after being removed from the site). In situ treatments involve bioattenuation, bioaugmentation, biostimulation, and bioventing, while the most popular ex situ treatments are landfarming, composting, and reactors.

17.3 Bioattenuation

Bioattenuation, also called natural attenuation, utilizes the inherent capacity of the natural microflora present in the contaminated niche. Either, the catabolic genes required for biodegradation are already present in the niche or are acquired over a period of time. Under the right conditions, microorganisms will utilize the pollutant as a source of energy, and hence, natural attenuation occurs at most polluted sites. However, the rate of degradation is dependent on a variety of factors that do now allow natural attenuation to proceed at rates required for bioremediation. Hence, scientists need to monitor these conditions to make sure that natural attenuation is working.

During course of time with exposure to contaminants, microorganism started to adapt themselves and start utilizing contaminants as food to keep them alive. In 1973, bacteria to degrade an organophosphorus (OP) pesticide were first isolated from a rice field in the Philippines. It thus

Table 17.1 Representative examples of bacteria reported in biodegradation of major pesticides

Pesticide	Class	Isolation site	Bacteria reported	Gram staining	Reference
Endosulfan	Organochlorine	Coffee plantation soil	*Alcaligenes faecalis JBW4*	−ve	Kong et al. (2013)
Lindane	Organochlorine	Organochlorine pesticide-contaminated sediments	*Streptomyces* sp. *M7*	+ve	Fuentes et al. (2011)
Chlorpyrifos	Organochlorine	Soil	*Pseudomonas* sp. *IRLM.1*, *IRLM.2*, *IRLM.3*, *IRLM.4*, *IRLM.5*	−ve	Latifi et al. (2014)
		Wastewater	*B. subtilis Y242*	+ve	El-Helow et al. (2013)
Malathion	Organophosphate	Egypt soil	*Acinetobacter* spp.	−ve	Hamouda et al. (2013)
			Bacillus spp.	+ve	
Diazinon	Organophosphate	Soil	*Pseudomonas peli BG1*	−ve	Mahiuddin et al. (2014)
			Burkholderia caryophylli BG4	−ve	
			Brevundimonas diminuta PD6	−ve	
Phorate	Organophosphate	Sugarcane field	*Bacillus aerophilus strain IMBL 4.1*	+ve	Jariyal et al. (2014)
			Brevibacterium frigoritolerans strain IMBL 2.1	+ve	
			Pseudomonas fulva strain IMBL 5.1	−ve	
Fenamiphos	Organophosphate	Soil	*Pseudomonas putida*	−ve	Chanika et al. (2011)
Oxamyl	Carbamate				
Carbofuran	Carbamate				
Fenobucarb	Carbamates	Rice paddy field	*Sphingobium* and *Novosphingobium*	−ve	Kim et al. (2014)
Fenpropathrin	Pyrethroid	Soil	*Rhodopseudomonas* sp. *The strain PSB07-8 (first reported genus)*	−ve	Zhang et al. (2014)
Cyfluthrin	Pyrethroids	Sewage sludge	*Lysinibacillus sphaericus strain, FLQ-11-1*	+ve	Hu et al. (2014)
Cypermethrin	Pyrethroids	Soil	*Catellibacterium* sp. *strain CC-5*	−ve	Zhao et al. (2013)
Fenvalerate	Pyrethroids	Agricultural field	*Pseudomonas viridiflava*	−ve	Selvam et al. (2013)

Deltamethrin	Pyrethroids	Soil	*Serratia marcescens DeI-1 and DeI-2*	−ve	Cycon et al. (2013)
Atrazine	Chlorinated triazine herbicide	Soil	*Frankia alni ACN14a*	+ve	Rehan et al. (2014)
			Arthrobacter sp. TC1	+ve	Strong et al. (2002)
Sulcotrione	β-triketone herbicide	French agricultural soil	*Pseudomonas* sp. *1OP (first reported)*	−ve	Calvayrac et al. (2014)
Acetochlor	chloroacetamide herbicide	Soil	*Rhodococcus* sp. *T3-1*	+ve	Hou et al. (2014)
			Delftia sp. *T3-6*	−ve	
			Sphingobium sp. *MEA3-1*	−ve	

highlighted the ability of natural attenuation of soil. Several phylogenetically distinct bacteria were isolated from around the world, which can degrade OPs either by co-metabolism or by employing OPs as a source of energy (Singh 2009). Triazine herbicides used heavily in the latter half of the twentieth century constituted a nitrogen-rich source for microbes. As a result of bioattenuation, *atz*A, a dechlorinase enzyme active against both atrazine and simazine was isolated from various soil bacteria from diverse locations in the mid-1990s (Noor et al. 2014). Indication of strong self-remediation potential of agricultural soil is its ability to completely clean up simazine (100 mg kg^{-1}) in 10 days after herbicide application (Wan et al. 2014). Indian soils contained a large number up to 10^4–10^5 copies (g^{-1} dry weight (dw) soil) of atrazine degradation genes activated only after the first expose to contaminants. Probably, the target genes get triggered to gear up for site cleanup (Nousiaine et al. 2014). Natural attenuation occurs at most polluted sites; however, for quick and complete proper site cleanup, the right conditions must exist in the niche. Monitoring these conditions or monitored natural attenuation (MNA) is a better option to make sure that natural attenuation is effective (Joutey et al. 2013; Jørgensen et al. 2010).

17.4 Biostimulation

This process involves stimulating the natural microflora present in the contaminated niche by addition of rate-limiting nutrients. At times, even though the soil bacteria contain the genes required for biodegradation of target pollutants, substrate utilization does not occur. This could be due to the lack of nutrients required for bacterial growth. Hence, amendment of the soil with carbon or nitrogen sources kick-starts their metabolism and results in substrate utilization. For example, addition of citrate as carbon source enhanced microbial atrazine-degrading efficiency from 78 to 87 % forcing microbes to utilize atrazine as nitrogen source (Lima et al. 2009). The removal of total petroleum hydrocarbon was found to be increased by 49 % when biostimulated with inorganic nutrient addition (Asquith et al. 2012). Similarly, organophosphate pesticide dichlorvos was efficiently removed when amended with NPK (20:10:10) fertilizer and NH_4NO_3 as nitrogen source by bacterial consortium (Agarry et al. 2013). Carbofuran degradation was biostimulated by amending hydrogen (HY), ethanol (ET), and methane (ME) as organic amendment. HY was the most effective amendment to biostimulate carbofuran degradation by ET and ME (Pimmata et al. 2013). When *Azospirillum lipoferum* (Beijerinck) was biostimulated with peat moss, it was found to degrade chlorpyrifos and cyanophos in soil more rapidly, confirming that the niche was lacking required nutrients, but the soil contained a well-adapted hydrocarbonoclastic microbial community (Romeh and Hendawi 2014).

17.5 Bioaugmentation

The main role of bioaugmentation is the addition of a new gene pool that will increase the rate of biodegradation. A number of success stories describing improved efficiency of bioremediation using bioaugmentation are known (Cycon et al. 2009; Sniegowski et al. 2011). For example, bioaugmentation of atrazine-contaminated soil with *Pseudomonas* sp. ADP ($9 \pm 1 \times 10^7$ CFU g − 1) resulted in rapid 99 % atrazine removal within 8 days (Lima et al. 2014). Spiking simazine-contaminated soil with *Pseudomonas* sp. M1HP4 cells enhanced degradation and increased the number of simazine-degrading microorganism in the soil (Morgante et al. 2010). Accelerated removal of methyl parathion and p-nitrophenol from soil was achieved when bioaugmented with *Pseudomonas* sp. strain WBC-3, which resulted in complete removal of 0.536 mg g^{-1} dry soil treatment within 15 days and without accumulation of toxic intermediates (Wang et al. 2014). Besides using individual bacteria for bioaugmentation, a consortium would help better survival rates in natural environments (Chen et al. 2014; Polti et al. 2014; Zaki et al. 2014). *Paenibacillus* (*Bacillus*) *polymyxa* (*Prazmowski*) and *Azospirillum lipoferum* (*Beijerinck*) were found to degrade the organophosphorus insecticides, chlorpyrifos, chlorpyrifos- methyl, cyanophos, and malathion in mineral salts media as a carbon and phosphorus source. The same when inoculated together improved the rate of degradation of chlorpyrifos and cyanophos in soil (Romeh and Hendawi 2014). Sagarkar et al. (2013) compared the bioremediation efficiency of natural attenuation, biostimulation, and bioaugmentation strategies and demonstrated bioaugmentation to be the fastest method of pollutant removal.

17.6 Bioventing

According to USEPA, bioventing is an in situ remediation technology where the inherent microflora is used to degrade organic matter which is adsorbed to different layers of the soils. The degradation can be enhanced by supplying oxygen or adding nutrients enhancing activity of indigenous bacteria. In this technology, air is supplied to the deeper layers of soil by using high-pressure pumps to aid in the biodegradation process (Shukla et al. 2010). Bioventing can be applied for wide area of soil and remediation of contaminants present at deeper level of soil surface (Shiomi 2013). Bioventing has its own limitations, that is, it can only be applied to the compounds that can be degraded aerobically and it is highly affected by soil texture like it requires low clay content and high soil permeability for its effectiveness (Frazar 2000).

17.7 Landfarming

Landfarming is a process commonly practiced in agricultural field for biotreatment. It can either be performed in situ or ex situ and is done for upper zone soil up to 10–35 cm. It involves the periodically turned over soil to aerate the mixture. This technique has been reported successfully for the biodegradation of oily sludge or petroleum refinery waste (Lin et al. 2010; Sanscartier et al. 2009; Prasad et al. 2012). The limitations of this technique are space requirement, chances of leaching of toxic metal ions to ground, and increases in time length for degradation of recalcitrant products. These problems could be overcome by integrating landfarming with bioaugmentation, biostimulation, and molecular monitoring microarray (Sagarkar et al. 2013; Lin et al. 2010; Paudyn et al. 2008).

17.8 Composting: Organic Amendments to Soil for Bioremediation

Compost is a decayed organic material, plant, or animal, like leaves and manure that are used to improve structure of the soil and provide nutrients. Composting is a technique that involves combining contaminated soil with compost thereby providing nutrients that support the growth of soil microorganism, thus helping in biodegradation (Prasad et al. 2012). For instance, compost of Oliver cake was used to bioremediate a field contaminated with four pesticides, terbuthylazine, prometryn, cyanazine, and simazine (Moreno and Pena 2009). 89 % of PAHs – anthracene, benzo(a)anthracene, chrysene, fluoranthene, fluorene, phenanthrene, and pyrene – was degraded during composting in comparison to bioaugmentation (Sayara et al. 2011). However, if composting is not designed correctly, it could lead to methane production, odor emissions, and heavy metal buildup in the compost (Taiwo 2011). Composting efficiency depends on the contaminants and wastes used for composting (Megharaj et al. 2011).

17.9 Bioreactors

An ex situ process of bioremediation for contaminant removal from soil is to run bioreactors. Bioreactors refer to a manufactured engineered system that supports a biologically active environment (IUPAC). Slurry bioreactors (SB) have been run to bioremediate soil contaminated with recalcitrant pesticides, explosive substances, aromatic hydrocarbons, and chlorinated organic compounds either at lab-scale or commercial scale. Slurry bioreactors allow us to control several environmental parameters such as concentration of organic carbon and nutrients (biostimulation) and bioaugmentation – addition of inocula – and/or help increase the availability of pollutants for biodegradation, either by employing surfactants or inducing their production within the reactor (Robles-González et al. 2008). Use of SB along with electron acceptor is an interesting area. Here, single electron acceptor and sucrose as co-substrate was successfully demonstrated in the bioremediation of hydrocarbons and organochlorinated compounds (such as lindane) (Robles-González et al. 2012). Similarly, 93 % of 2,4-dichlorophenoxyacetic acid was removed from mineral soil in aerobic slurry bioreactor within 14 days (González et al. 2006). Using activated biomass as source of microorganism, the pesticides belonging to different classes, viz., organophosphorus and synthetic pyrethroids (chlorpyrifos, cypermethrin, fenvalerate, and trichlopyr butoxy ethyl ester), have been taken at a concentration of 25–100 mg L^{-1}, amended with surface soil (alluvial soil) in a bioreactor. This converted toxic levels into environmentally friendly compounds which in longer duration in nature would convert into nutrient biomass (Geetha and Fulekar 2008). In a report, 96.97 % of carbofuran was removed in slurry phase batch bioreactor when augmented with *Burkholderia cepacia* PCL3 immobilized on corn cob. Following 82.23 % was removed when bioaugmentation and biostimulation (addition of molasses) combined (Plangklang and Reungsang 2010). Several problems are associated with bioreactors, like soil has to be excavated from site and has to be further processed like crushing, removal of coarse particles. The installation of slurry bioreactors and their maintenance is cost ineffective, which is the major disadvantage of the SB. Further studies like the characterization of microbial communities and their role in improving reactor operation and design optimization are required before this can be a viable option.

17.10 Addressing the Problem of Bioavailability

An important question that needs to be asked is, despite all biological parameters being in place, why do pesticides persist in the environment? This could probably be because their concentration is too low for bacterial action or that they are not bioavailable for biodegradation. Most pesticides are hydrophobic in nature, hence remain insoluble or are soluble in minute amounts and remain unavailable to the bacteria. For example, pesticides insoluble in water like dichlorodiphenyltrichloroethane (DDT), hexachlorocyclohexane (HCH), endosulfan, and benzene hexachloride (BHC) persist in soil and sediments having half-life of 100–200 days due to less bioavailability (Odukkathil and Vasudevan 2013). Pesticides barely soluble in water, for example, atrazine with 0.030 g L^{-1}solubility, chlordane with 0.0001 g L^{-1} (USEPA, technical fact sheet), chlorpyrifos with 0.002 g L^{-1} (APVMA), and dieldrin solubility of 0.186 g L^{-1} (Steven, USEPA), are difficult to degrade.

To allow these compounds to be available to bacteria for utilization, biosurfactants can be used that help increase their water solubility and thus make them available for biodegradation (Vander Gast et al. 2003; Gentry et al. 2004; Mukherjee et al. 2006; Mrozik and Seget 2010). Biosurfactants enhance microbial growth, lower surface and interfacial tension, and allow wetting and penetrating, resulting in shortening of microbial adaptation and degradation time (Kosaric 2001). For example, a biosurfactant-producing bacterium, *Rhodococcus* sp., was developed by a team of scientist, as biosurfactant is effective and ecologically safe for petroleum-affected sand or soil (Synergy services, http://www.greenesenergy.com/Images/Interior/download%20center/synergy/bioremediationbioaugmentation.pdf).

The degradation of tetradecane, hexadecane, and pristine was enhanced when bioaugmented with a biosurfactant-producing bacterial isolate, *Pseudomonas aeruginosa* UG2 (Jain et al. 1992). Degradation of the chlorinated pesticide endosulfan was enhanced by 30–45 % after bioaugmentation with a biosurfactant-producing isolate, *Bacillus subtilis* MTCC1427 (Awasthi et al. 1999; Paul et al. 2005). Bioremediation of environmental niches would thus greatly benefit by including biosurfactant-producing isolates in their consortium.

17.11 The Use of Genetically Engineered Microorganism (GEM)/Genetically Modified Microorganism (GMO) for Bioremediation

The first report of a GEM to enhance biodegradation of petroleum hydrocarbons in oil spill was the superbug *Pseudomonas putida* engineered by A.K. Chakraborty (Chatterji 2007). Two strains were patented in 1981, *P. aeruginosa* (NRRL B-5472) and *P. putida* (NRRL B-5473), that contained naphthalene, salicylate, and camphor degradation genes. The possibilities for using GEMs in bioremediation are vast. These include (1) modification of enzyme to increase its specificity, (2) synthetic constructs for the designing of a new metabolic pathway, (3) introduction of a marker gene for identification of recombinant in contaminated environment, and (4) construction of biosensor for the detection of target chemical compounds (Wasilkowski et al. 2012). GEM, *Pseudomonas fluorescens* HK44, was the first isolate approved for field trials for the bioremediation of naphthalene (Sayler and Ripps 2000). A number of isolates have been reported that were genetically modified to enhance biodegradation. For example, simultaneous degradation of pesticides – organophosphate and organochlorine – was possible by fusing the organophosphorus hydrolase with INPNC (ice nucleation protein) of *Pseudomonas syringae* onto the cell surface of an HCH-degrader *Sphingobium japonicum* UT26 (Cao et al. 2013). Recently, several developments in the field of recombinant DNA technologies such as development of "suicidal-GEMs" (S-GEMs) have also been carried out to achieve safe and efficient bioremediation of contaminated sites (Kumar et al. 2013). List of some GEM/GMOs is provided in Table 17.2.

However, there is considerable controversy relating to the use and release of genetically modified organisms into the environment. Concerns of safety and ecological damage need to be addressed, and a large number of countries, including India, do not permit the use of GEMs in bioremediation or their release in uncontrolled conditions. For this reason, the use of free or immobilized enzymes gained popularity. Bioremediation of triazine herbicides in contaminated water bodies has been demonstrated using free enzymes (Scott et al. 2010). This technique is however more difficult to apply in soil. Immobilized enzymes have demonstrated greater success.

Cellulose-containing carriers (straws) were applied for hexahistidine-tagged OP hydrolase His6-OPH immobilization, which imparted stability to the enzyme, ensuring its activity in the soil contaminated with OP pesticides. Using this method, 630 mg Paraoxon/850 mg Diazinon/185 mg parathion per kg soil was decomposed in less than 10 days, where the half-life of active biocatalyst in sand was estimated in 130 days (Sirotkina et al. 2012).

17.12 Factors Controlling Bioremediation

Figure 17.2 describes the biotic and abiotic factors affecting bioremediation. Even though routes of degradation are known and analytical methods are available for the assessment of biodegradation, it is not possible to accurately predict bioremediation unless the biotic and abiotic factors that control the behavior of microorganisms are considered.

Factors influencing rate of biodegradation can be summarized as follows:

17.12.1 Soil pH

Growth of microbial community as well as the bioavailability of pesticides depends on the soil pH, particularly for attaching ionic pesticides – glyphosate or sulcotrione (Calvet 1989; Mamy and Barriuso 2005). Adsorption increases or

Table 17.2 List of genetically engineered microorganism/genetically modified organism developed for soil pesticide remediation

GEM/GMO	Gene inserted	Function	References
Anabaena and *E. coli*	*LinA2*	Lindane degradation	Chaurasia et al. (2013)
Pseudomonas aeruginosa (NRRL B-5472)	Naphthalene, salicylate, and camphor degradation genes	Naphthalene, salicylate, and camphor degradation genes	Wasilkowski et al. (2012)
Pseudomonas putida (NRRL B-5473)	Naphthalene, salicylate, and camphor degradation genes	Naphthalene, salicylate, and camphor degradation genes	Wasilkowski et al. (2012)
Rhodococcus strain RHA1	*Co-expression of bphC* and *etbC*	Increase the spectrum of PCB substrates	Hauschild et al. (1996)
Rhodococcus corallinus to Rhodococcus TE1	s-Triazine hydrolase	Atrazine degradation	Chen and Mulchandani (1998)
P. putida BH to Escherichia coli	Phenol hydroxylase	Trichloroethane degradation	Fujita et al. (1995)
Burkholderia cepacia PR1 *to Pseudomonas fluorescens*	Toluene *o*-monooxygenase	Trichloroethane degradation	Yee et al. (1998)
Alcaligenes eutrophus JMP134	Phenol hydroxylase and catechol 2,3-dioxygenase	Phenol degradation and oxidative removal of TCE	Hauschild et al. (1996)
Deinococcus radiodurans	Tod gene, toluene dioxygenase	Oxidation of toluene, chlorobenzene, 3,4-dichloro-1-butene, and indole	Lange et al. (1998)
D. radiodurans	–	Bioremediation of mixed wastes containing both radionuclides and organic solvents	Lange et al. (1998)

decreases with pH depending on the charges present on pesticides. For instance, the retention of glyphosate increases with decrease in the soil pH since negative charges decrease. This allows the adsorption of pesticides on negatively charged materials such as organic matter or clay (Chaplain et al. 2011). Biodegradation of pesticides depends whether the given pesticide is susceptible to hydrolysis mediated under alkaline or acid conditions (Chowdhury et al. 2008).

17.12.2 Organic Matter

The organic matter fraction in agricultural soils plays the role of the principal sorbent in the case of sparingly soluble pesticides and in the transport and bioavailability of pesticides in the soil. Organic matter present in the soil is due to crop residues, microbial biomass, and organic amendments. This contains heterogeneous group consisting of hydrophobic and hydrophilic groups (Calvet et al. 2005). The organic matter is the major sorbent in soil which leads to high reactivity on the surface of the minerals and organic molecules. It enables various types of interactions with soil pesticides, thus affecting bioavailability (Chaplain et al. 2011). Certain level of organic matter is necessary to ensure the survival of an indigenous microbial population for pesticides degradation (Chowdhury et al. 2008).

17.12.3 Minerals

Minerals also act as adsorbents to pesticides helping in their bioavailability. Silicates in clay, oxides, and hydroxides enhance pesticides adsorption (Barriuso et al. 1994; Chaplain et al. 2011). Oxides and hydroxides which are linked to clays have a high surface activity, which depends on the soil pH.

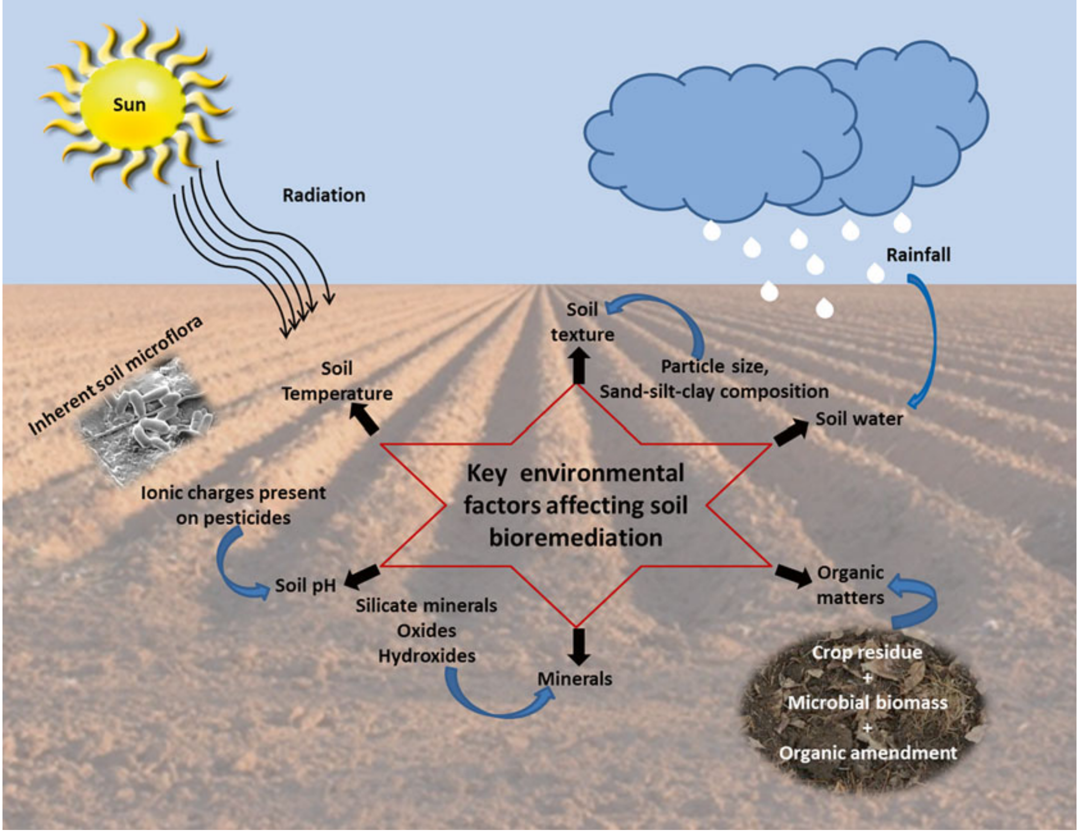

Fig. 17.2 Key soil parameters playing an important role in controlling bioremediation efficiency

17.12.4 Temperature

The molecular structure of any pesticide decides the influence of temperature on its degradation. The adsorption of pesticides is inversely proportion to temperature as it is an exothermic reaction, while the desorption processes are endothermic (Hulscher and Cornelissen 1996). Fluctuation in temperature may enhance or suppress microbial growth, thus affecting the bioremediation process. Pesticides' solubility enhances with temperature – mesophilic temperature in the range of 25–40 °C is optimal for pesticide degradation. Persistence of contaminants increases at lower temperature and higher concentration of pesticides (Chowdhury et al. 2008).

17.12.5 Soil Water Content/ Moisture

Water is one of the most important factors supporting microbial growth and its functioning. Water acts as a solvent, which regulates pesticides movement and diffusion. The adsorption of pesticides increases with water content. It helps in diffusion of pesticides to the sorption regions. At high water content, OM shows enhanced hydrophilic behavior, and this has a greater sorption potential toward hydrophilic pesticides or vice versa for hydrophobic pesticides. 25–40 % soil moisture favors bioremediation, whereas 10–25 % moisture resists bioremediation process (Cho et al. 2000). Generally, transformation rate of pesticide gets enhanced with water content;

hence, moist soil favors pesticide degradation as compared to pesticides in submerged soil. However, oxygen diffusion is limited and anaerobic degradation is preferred rather than aerobic in paddy soil (Chowdhury et al. 2008). Pesticide degradation in submerged soils is higher than those in unsaturated soils (Krishna and Philip 2011).

17.12.6 Soil Texture

Soil texture is defined on the basis of physical texture, particle size distribution, and sand-silt-clay composition. The amount of water and air permeability in the soil is decided by its texture. Thus, oxygen circulation is controlled, regulating the microbial growth and effecting bioremediation. Leaching is also governed by soil texture, hence regulating the bioavailability of the pesticide versus the transfer into ground water. Soil with high clay content prevents oxygen and nutrients from reaching the soil microbes, and moisture cannot be controlled in fine-textured soil, thus effecting aerobic biodegradation of contaminants as in bioventing. Hence, a prior knowledge of soil texture is very crucial before formulating a bioremediation strategy.

17.12.7 Inherent Soil Microflora

Soil microbial community plays a critical role in cycling soil elements and in turn affects soil fertility and agriculture productivity (Ahemad and Khan 2011). Insecticides accumulate to an undesirable level with repetitive use and effect soil enzyme activity and physiological characteristics of soil microbiota. But with exposure to xenobiotics, they themselves develop a strategy to utilize it as a food source for energy referred to as natural attenuation. In case of bioaugmentation, microbial community may behave normally and work in coordination with the augmented bacteria or may inhibit the augmented microbes.

17.13 Problems of Field Trials for Bioremediation

The high success rates of bioremediation trials in the laboratory flounder at field-scale trials. A large number of factors are responsible. The main factor being that the controlled conditions of the laboratory cannot be replicated in the environment. The biotic and abiotic characters discussed earlier control microbial response and hence control bioremediation. Scale-up is another important factor that needs to be considered carefully before taking lab-scale research into field trials. The differences in degradation rates between the lab and field demonstrate the significance of scale-up factors, which are important for technology transfer and application in the field. An example highlighting this case is of bioventing. Comparison of microcosm studies with mesocosm scale resulted in scale-up factors, which varied from 1.9 to 2.7 depending upon the soil type (Khan and Zytner 2013).

In case of bioaugmentation, it is essential to consider the natural microbial population existing in the soil and the survival of bioaugmented consortia. Many factors may lead to their failure, such as the strain, ecology, contaminant, environmental constraints, and the protocols for introducing culture (Mrozik and Piotrowska-Seget 2010). For instance, organic matter is one soil parameter that influences the efficiency of bioaugmentation. It plays a critical role in bioavailability of pollutants. It also affects the survival of bioaugmented microbes and their degradation potential. Survival of pentachlorophenol (PCP) degraders – *Rhodococcus chlorophenolicus* and *Flavobacterium* sp. – in natural peaty soil revealed that degrading activity by *R. chlorophenolicus* remained detectable for 200 days without any additional carbon source, whereas that of *Flavobacterium* sp. declined rapidly in a short period of 60 days. *R. chlorophenolicus-like* cells were even detected after a year (Briglia et al. 1990). In some cases, bioaugmentation did not affect the biodegradation efficiency significantly (Silva et al. 2009).

Whenever the soil is supplemented with xenobiotics, the population dynamics of the microbes inhabiting the soil changes. Microbial mixed cultures are more suitable for bioremediation of recalcitrant compounds compared to pure cultures. Biodiversity helps to enhance environmental survival and increase the range of catabolic pathways available for the biodegradative capacity of the pollutant (Fuentes et al. 2014). This was demonstrated by Sebiomo et al. (2013) who showed the decrease in microbial population when soil was treated with herbicides (atrazine, prime extra, paraquat, and glyphosate). It was observed that herbicides introduced into soil cause the reduction in percentage of organic matter. Shift in genetic structure depends on the prolonged exposure of microbiota to pesticides (Crouzet et al. 2010).

17.14 Successful Trials

Research is being focused on taking lab-scale work to the field. Table 17.3 lists some success stories. Krishna and Philip (2011) carried out a study to check degradation efficiencies for individual pesticides, lindane, methyl parathion, carbofuran, and their mixtures in Indian soils. Soil bioaugmented with mixture of pesticide-enriched cultures demonstrated that methyl parathion degrades at maximum and lindane at minimum (Krishna and Philip 2011). Lab-scale, i.e., soil microcosm study (250 g soil) carried out by Sagarkar et al. (2013), was taken to field scale (100 kg mesocosm) for atrazine bioremediation (Sagarkar et al. 2014). The study compared the efficiency of three modes of bioremediation, bioaugmentation (BA), biostimulation (BS), and natural attenuation (NA), for herbicide removal. Results demonstrated that BA was more efficient followed by BS then by NA for atrazine removal from the soil. Chen et al. (2011) carried out a microscale study (200 g) for biodegradation of pyrethroids, fenvalerate, and the product of its hydrolysis – 3-phenoxybenzoic acid (3-PBA). Soil was bioaugmented with *Stenotrophomonas* sp. ZS-S-01 having capacity to degrade fenvalerate and 3-PBA, along with deltamethrin, beta-cypermethrin, beta-cyfluthrin, and cyhalothrin work in a high range of pH and temperature, without forming any accumulative by-product. BA showed remarkable decrease in half-lives as compared to NA. Endosulfan mineralization by *Halophilic bacterium* JAS4 had potential to degrade up to 1000 mg L^{-1} of endosulfan by catabolic activity and transform them into simpler compounds. DT50 for a-endosulfan, b-endosulfan, and endosulfan sulfate was efficiently decreased when *H. bacterium* JAS4 was inoculated in sterile soil with nutrients (BA + BS) as compared to BA. This study demonstrated the increased efficiency of bioremediation by combining BA with BS. A successful field trial was demonstrated by using bioaugmentation in the form of a product in powder form, thus proving to be cost effective and easy to handle (Silambarasan and Abraham 2014).

An integrated strategy must be developed in order to remediate soil contaminated with heavy metals and POPs. For instance, Zhu et al. (2012) carried out a vessel study for bioremediation of

Table 17.3 A few success stories where bioremediation was carried out on mesocosm scale/field trials

Pollutant	Treatment strategy	Scale of treatment	Reference
Lindane, methyl parathion, carbofuran	BA with mixed culture	Mesocosm	Krishna and Philips (2011)
Atrazine	BA, BS, NA	Mesocosm	Sagarkar et al. (2013)
Cd, DDT, and its metabolite	BA with *Sedum alfredii* DDT-1	Vessel study	Zhu et al. (2012)
Endosulfan	BA with *halophilic bacterium* JAS4 and BS	Agriculture field	Silambarasan and Abraham (2014)
Petroleum hydrocarbon	Bioventing	Mesocosm	Khan and Zytner (2013)
Chlorpyrifos	BS with straw compost	Agriculture field	Laine and Jørgensen (1996)

**BA* bioaugmentation, *BS* biostimulation, *NA* natural attenuation agricultural field which was contaminated with cadmium (Cd), DDT, and its metabolites such as DDE and DDD using a Cd-hyperaccumulator plant – *Sedum alfredii* (SA) – and DDT-degrader strain DDT-1 and noticed that the level of both the pollutants decreased by 31.1 % and 53.6 %, respectively. Khan and Zytner (2013) took a lab bioventing experiment from microscale to mesoscale holding 4 kg of three different soil types: loamy soil, silty soil, and mixed soil for the removal of petroleum hydrocarbon. Scale-up factors varied from 1.9 to 2.7 while comparing the degradation rates of the microscale and mesoscale experiments. The variation of degradation rates in micro- and meso-level studies indicated the significance of scale-up factors (Khan and Zytner 2013). Bioremediation of chlorophenol-contaminated soil was demonstrated in a composting experiment, carried on full-scale for 3-years. Here, the contaminated soil was mixed with bark chips and nutrients only, without the addition of any inocula. The laboratory experiments carried out for chlorophenol mineralization used the remediated soil as inocula with straw compost and observed 56 % mineralization. No harmful side reactions like enhanced methylation were observed using straw compost during the development of a composting system to accelerate the bioremediation process in a soil contaminated with chlorophenol (Laine and Jørgensen 1996). Bioremediation on-site therefore requires a thorough understanding of nature and concentration of contaminants to be dealt with. As in a mixture of pesticides, degradation of one may affect the degradation of the other either by supporting or inhibiting microbial action (Swarcewicz et al. 2013). A priori knowledge is useful on choosing the best bioremediation strategy for field applications.

17.15 How to Overcome the Limitations: Converting Failure into Success

Figure 17.3 lists the questions that need to be answered to overcome the limitations in bioremediation and increase the success rate of field trials. A detailed analysis of the site to be remediated is crucial, where the analysis of soil parameters will indicate the availability of nutrients and energy sources required for bacterial growth and suggest the requirement for biostimulation. Similarly, assessing the natural microbial population and the gene pool present on-site will indicate if bioaugmentation is required or not. Other than analyzing the input parameters, it is also very essential to analyze the progress of bioremediation using analytical and genomic tools. Analytical tools will help understand the degradation/biotransformation products which not only indicate the bioremediation efficiency but also indicate the presence of any toxic intermediate, if formed. Quantification of target genes is necessary to understand the role of the microbial community and their performance in the bioremediation process. Hence, bioremediation cannot be addressed as a universal treatment package but as a site-specific problem with custom designed solutions.

Combining bioaugmentation and biostimulation methodologies is an effective strategy to address the problem of insufficient nutrients as well as increased gene pool responsible for degradation. This was successfully demonstrated in a microcosm study for simazine bioremediation. Contaminated soil bioaugmented with *Arthrobacter* sp. SD3-25 demonstrated 39 % degradation, but efficiency increased to 90 % when bioaugmentation was coupled with biostimulation by the addition of external carbon source (Guo et al. 2014). Similarly, in the case of cis- and trans-dichloroethene (DCE) bioremediation, the biodegradation rate was significantly enhanced, i.e., by 14 % for cis-DCE and 18 % for trans-DCE degradation within two weeks as compared to that of bioaugmentation or biostimulation alone (Olaniran et al. 2006). Another example was seen in the case of atrazine degradation where biostimulation with citrate or succinate increased cell survival of bioaugmented *Pseudomonas* sp. ADP (Silva et al. 2004). Bioaugmentation in combination with biostimulation has proven to be an affective technology for bioremediation (Qureshi et al. 2009; Pimmata et al. 2013).

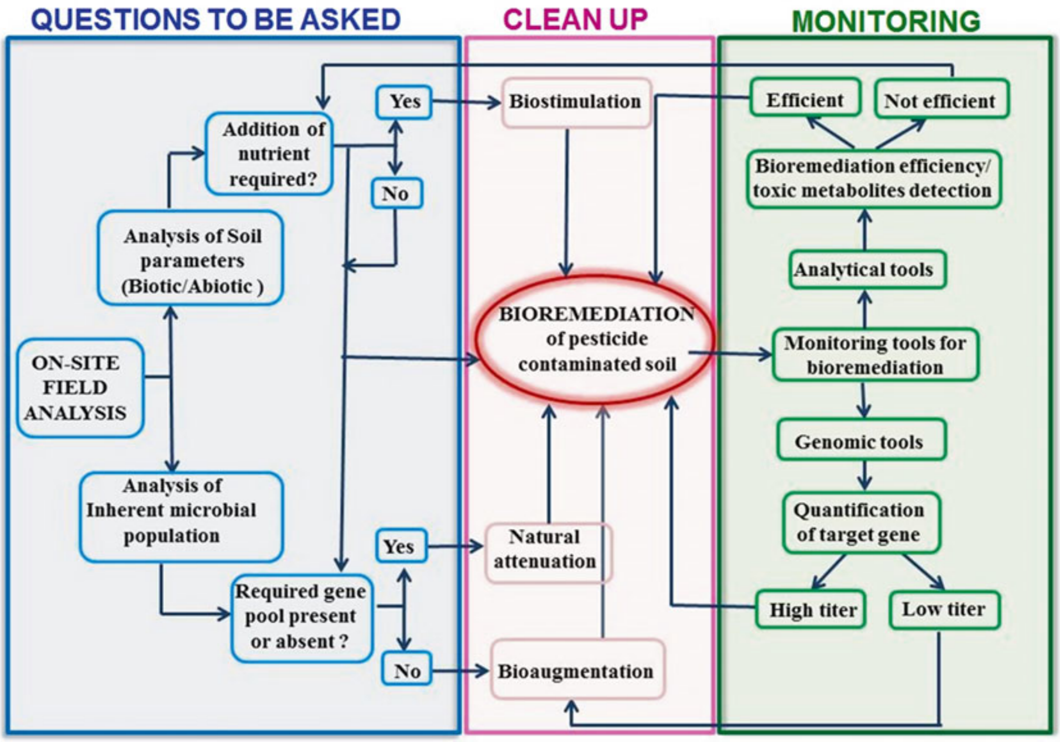

Fig. 17.3 Questions to be answered and steps to be taken to ensure efficient bioremediation

Another factor that can be considered to improve the efficiency of bioremediation is the use of bacteria having broad substrate range for degradation. Since field conditions do not ever contain one substrate that requires bioremediation, as in the laboratory studies, potential microbial candidates having capacity to utilize broad range of pesticides as a substrate could be better candidates to bioremediate the field contaminated with multiple xenobiotics at a time. For example, *Bacillus* sp. DG-02 that degrades fenpropathrin, along with synthetic pesticides pyrethroids (deltamethrin, λ-cyhalothrin, β-cypermethrin, β-cyfluthrin, bifenthrin, and permethrin), can be an efficient strain to be used in bioremediation (Chen et al. 2014). A number of multiple substrate-degrading strains are reported in literature: *Sphingobium japonicum* UT26, a GEM constructed by Cao et al., to degrade a mixture of parathion and c-HCH (Cao et al. 2013); *Serratia marcescens*, a diazinon-degrading bacteria, able to remove organophosphorus pesticides (OPPs), i.e., chlorpyrifos, fenitrothion, and parathion (Cycon et al. 2013); and *Stenotrophomonas* sp. HPC383, with a capacity to degrade various aromatic compounds: catechol, cresols, 4-methylcatechol, phenol, and hydroquinone in addition to crude oil (Verma et al. 2011).

17.16 The Importance of Keeping Track: Monitoring the Progress of Bioremediation

As discussed, bioremediation involves the use of the catabolic potential of the microbial community in degradation of pesticides in the soil. This community is affected by a number of biotic and abiotic factors. Hence, monitoring the progress of bioremediation is important so midterm corrections can be made in case of any failure. There are a number of analytical and genomic tools that can be used to monitor progress (Martin-Laurent et al. 2003; Purohit et al. 2003; Kapley and Purohit 2009; Monard et al. 2013). Application of these tools for site-specific characterization, risk assessment of polluted sites, and the selection of proper bioremediation methods will make it feasible to find environmentally benign and economical solutions that would be impossible to obtain using the conventional methods.

17.17 Conclusion

The use of the microbial community in removal of pesticides from contaminated soil is a complex and multistage process with potential to restore contaminated environments. However, it can proceed only in favorable conditions. The blind spot in this process are mainly the lack of information about factors which are crucial for microbial growth and metabolism in highly polluted environment. Future research needs to be focused in these areas to successfully convert lab-scale know-how into technologies that can be applied in the environment.

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Pooja Bhardwaj graduated in Biotechnology in 2009 from Barkatullah University, Bhopal. She received her M.Sc. degree in Biotechnology in 2011 from Barkatullah University, Bhopal. She is currently enrolled in Ph.D. in Biotechnology with Rashtrasant Tukdoji Maharaj Nagpur University, Nagpur, and works at CSIR-National Environmental Engineering Research Institute. She is studying comparative genomics of different atrazine degrading pathway in soil bacteria.

Atya Kapley Ph.D. from University of Hyderabad, Hyderabad, Principal Scientist at the National Environmental Engineering Research Institute, Nagpur, works in the field of environmental genomics. She uses a multi-disciplinary approach, combining conventional microbiology tools with bioinformatics and molecular tools to address rising levels of environmental contamination. She has worked on the bioremediation of pesticide contaminated soil and uses metagenomics approach to study microbial communities in activated biomass of wastewater treatment plants with the aim to understand and improve biological treatment capacity.

18

Biotransformation of Arsenic in Environment Mediated by Microorganisms

Supriya Kore

Abstract

The health of the people worldwide is at risk due to the drinking of water contaminated with arsenic (As). Occurrence of As in drinking water is due to the natural processes and rarely by man-made activities. Mobilization of As from natural or anthropogenic sources in the drinking water is the first crucial step responsible for human health implication. Exposure to As can damage body parts leading to diseases such as cancer of the bladder and the skin, diabetes, cardiovascular diseases, and developmental, neurological, and metabolic disorders. Evidences from scientific studies suggest that mobilization of As is a microbiological phenomenon. All living organisms show resistance or sensitivity to As depending on the concentration of As to which they are exposed to. But few groups of microorganisms utilize As for their growth. In the environment, microorganisms interact with As through a variety of mechanisms, including sorption mobilization, precipitation, and redox and methylation reaction. The microbial activities in the environment may be beneficial or detrimental affecting the fate and mobility of As in the biogeochemical cycle. This review highlights the different systems which have evolved in microorganisms to resist the high concentration and to participate in environmental As cycles.

18.1 Introduction

Historically, humans get exposed to arsenic (As) as a poisonous compound either intentionally or unintentionally. Health of millions of people is at risk due to the toxicity caused by exposure to As. Drinking water contaminated with As is one of the major routes of exposure. Some of the geological areas that contain As in drinking water are of great concern, which contain more than permissible limits of As. Management of

S. Kore (✉)
Dr. D. Y. Patil Biotechnology and Bioinformatics Institute, Tathawade, Pune-33, Maharashtra, India

Dr. D. Y. Patil Vidyapeeth, Pune, Maharashtra, India
e-mail: supriya.kore@dpu.edu.in

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Fig. 18.1 Occurrence of Arsenic in three different forms: (**a**) gray (**b**) yellow (**c**) black

As-contaminated water is difficult because of its distribution and abundance in the environment. All living cells, from bacteria to humans, have detoxification mechanisms. In microorganisms, some microbes show As resistance, while others may utilize or require As for their normal physiological functions.

18.2 Properties of As

The element As and its compounds are known to exist for centuries. These compounds are considered as potent poisonous metalloid in the environment. As a rare crystal element, about 1 % of As contribute to the Earth's crust (Mandal and Suzuki 2002). Geological origin of the metal is rocks and minerals, where principal reservoirs are the rocks. The most common ores of As include pyrites, realgar, and orpiment (Fig. 18.1). Due to the ubiquitous nature, As ranks 12th, 14th, and 20th in human beings, in seawater, and in Earth's crust, respectively (Jomova et al. 2011). Since the discovery of arsenic sulfide (As_2S_3) by Albertus Magnus, it is used as homicidal agent. Later, its use was extended in medicine, agriculture, electronics, and metallurgy (Oremland and Stolz 2003). With its wide distribution in nature, its association was always observed with the ores and metals like Cu, Pb, and Au. In nature, As exists in inorganic as well as organic forms and differs with its oxidation states. Four different oxidation states, (+ 5) arsenate, (+3) arsenite, (0) elemental As, and arsenide (−3), exist. Among all these, trivalent (+3, AsIII) and pentavalent (+5, AsV) oxidation states are more toxic after exposure. The toxic, gaseous forms of elemental As (0, As0) and arsines (−3 AsIII) originate from anoxic environment, and both forms occur rarely in nature (Cullen et al. 1989). Arsenate detected in water under aerobic environment and arsenite trivalent As are prevalent in anoxic environment. Pentavalent As adsorbed strongly to the commonly occurring minerals like ferrihydrite and alumina; due to this ability, its mobility in the water gets reduced (Smedley and Kinniburgh 2002). Both inorganic and organic forms have been determined in the water (IPCS 2001). Compared with organic As compound, inorganic As forms exhibit higher toxicity. Methylated organoarsenic compounds are found in the natural environments, primarily as a breakdown metabolic product excreted by animals and humans (Oremland and Stolz 2003). The methylated species formed due to metabolism reflect that every organism has capacity to biotransform or generate energy from the metalloid.

18.3 Sources of As

18.3.1 Soil

Concentration is higher in the soil than rocks from which they are originated. The most important factor for its higher concentration in soil is due to parental rock, human activities, climate, and organic and inorganic compound present in soil. In most of the rocks like fine-grained argillaceous sediments and phosphorus, As concentration is higher, and it may vary between 0.5 and 2.5 mg kg^{-1} (Kabata-Pendias and Pendias 1992). Marine sediments might contain 3000 mg kg^{-1}

concentrations of As. The presence of As in the soil of various countries vary from 0.1 to 40 mg kg^{-1} (Mandal and Suzuki 2002). Inorganic species mostly occur in soil, but is bound to organic material. Arsenate is a stable species in aerobic environments and is strongly adsorbed onto clay, iron, or manganese. The speciation of As in soil depends on the adsorbing organic compound amount in the soil, the pH and the redox potential. In iron-rich soil, arsenate precipitates as ferric arsenate. Arsenates of Al and Fe ($AlAsO_4$–$FeAsO_4$) are present in acidic soils (Mandal and Suzuki 2002).

18.3.2 Air

Natural activities in environment such as volcano, low temperature biomethylation, and microbial reduction release As in air (Gomez-Caminero et al. 2001). Atmospheric As contamination is due to industrial processes, smelting, and nonferrous metals and fossil fuel combustion.

18.3.3 Water

As in water is in very low concentration. The presence of As depends upon the geothermal areas of the natural water system. Natural and anthropogenic activities in the environment contribute to the presence of As in water. Table 18.1 summarizes the concentration of As in groundwater in different countries. Some of the mechanisms of release of As in water from the rocks are the following:

1. Oxidation of As-bearing pyrites by O_2, Fe^{3+}, NO_3, or other electron (e^-) acceptors; as a result As is released in the groundwater (Acharyya et al. 1999). Oxidation of arsenopyrites reaction is due to excessive withdrawal of the groundwater (Hossain 2004).

$$\mathrm{FeAsS} + 3.5\mathrm{O_2} + 4\mathrm{H_2O} \rightarrow \mathrm{Fe(OH)_3} + \mathrm{H_3ASO_4} \\ +2\mathrm{H^+} + \mathrm{SO_4}^{-2}$$

2. Reductive dissolution of iron hydroxides and oxide metals subsequent to the release of adsorbed As mobilizes it in water. The speciation of iron is controlled by Eh, pH, and surrounding environment conditions. Reductive dissolution is a key mobilization process under a wide range of condition (McArthur et al. 2001).
3. Competitive anions present in fertilizers exchange with adsorbed As (V) (Nordstrom 2000).

The proposed mechanisms of As release due to oxidation and reduction reaction are probably by the microorganisms present in the environment. Provisions of the groundwater for the drinking purposes contain the metal in a concentration range above the permissible drinking water (Mandal and Suzuki 2002). Ironically, it is these sources that have significant impacts on human health around the world (Wharton 2010).

18.3.4 Anthropogenic

Anthropogenic point sources in the environment exceed three times the natural sources. These sources always distribute As as localized pollution, whereas natural sources were distributed broadly (Nordstrom 2000, 2002). Utilization of natural resources such as smelting, pigment and dyes, processing of the pressure-treated wood, mining, and combustion of coal all contribute to the release of As into the environment. Accumulation of As in soil is due to the As-containing pesticides, fertilizer usage, fossil fuels after burning, and disposal of wastes from animal industry. As-producing countries account for a total of about 97 % of the world's total production (Nelson 1977). As use in dyes and pesticides has been reduced by replacing them with synthetic compounds, but still these are used in some agriculture processes. Gross contamination of As >900 mg/kg resulted in Eastern Europe due to storage of chemical weapons. Roxarsone (4-hydroxy-4-nitrophenylAs acid), an organoarsenic, is used as intestinal palliative for swine and increases growth in poultry farm

Table 18.1 Global groundwater arsenic contamination and human population at risk

Population affected	Conc. of arsenic (mgl^{-1})	Natural source	Affected area	Toxic effects	Year of incidence	References
140,000	0.4–0.6	Well water	Taiwan	Hyperpigmentation, keratosis, cancer	1961–1985	Thornton and Farago, (1997)
130,000	0.8	Tocance river	Antofagasta, Chile	Raynauds syndrome, ischemia of tongue, myocardial ischemia, lung and bladder cancer	1959–1970	Smith et al. (2000)
6 million	–	Groundwater sediments	West Bengal	Asosis	1978	Chakraborti et al. (2001)
200,000	0.008–0.624	Volcano sediment	Mexico	Cutaneous manifestation, skin cancer	1963–1983	Nelson (1977)
10,000	0.1	Groundwater	Argentina	Skin cancer	1955	De Sastre et al. (1992)
250	0.18–0.21	Well water	Millard County	Hypersensitive heart diseases, nephritis, prostate cancer		Lewis et al. (1999)
	0.05–1.7	Colombia River	Lane County		1962–1963	Goldblatt et al. (1963)
	0.05–1.4	Well water	Lassen County	Increase as body burden		Goldsmith et al. (1972)
	0.10–0.41	Well water	Ontario	As dermatitis	1937	Wyllie (1937)
50 families	3	Bedrock groundwater	Nova Scotia		1976	Boyle et al. (1998)
Few thousand	0.06–4.00	Well water	Hungary	Melanosis skin cancer, internal cancer hematologic abnormalities	1941–1983	Nagy and Korom (1983)
2 million	4.4	Well water and natural occurrence	China	Keratosis, melanosis neuritis, gastroenteritis, hypertrophy, cancer and gangrene, DNA damage in buccal epithelial cells	1980	Jin et al. (2003)
	0.05–0.545	Well and spring water	Northern India	Cirrhosis, non-cirrhotic portal fibrosis		Datta and Kaul (1976)

(Czarnecki et al. 1982). These compounds resulted in excess in the manure than accumulating in the flesh, meat, or eggs (Morrison 1969).

18.4 History of As

As is known to humans from the ancient times as a poisonous compound. As is used as homicidal and suicidal agent, known as the king of poisons in Greece and Rome. Later, it got a place in medical practice (Scheindlin 2005). Paul Ehrlich in 1910 introduced Salvarsan, a new As-based drug used against syphilis. Salvarsan, the magic bullet, was used until the use of penicillin (Riethmiller 2005). Over the last few decades, researchers have demonstrated the efficacy of As trioxide for treating relapsed acute promyelocytic leukemia (Pott et al. 2001). Toxicity of As depends on its species and the type exposure to the organisms. The exposure may be accidental, intentional, or occupational from different environmental sources such as ingestion through As-contaminated food and water. Study of the kinetic and metabolism of different forms of As in an organism depends on the physiological properties and type of metabolism in the organism. Exposure to As in human affects different organs and systems; acute exposure is characteristics of gastrointestinal disorders, vomiting, diarrhea, blood in urine, shock, and death. Chronic As exposure leads to skin lesion (hyperpigmentation) and respiratory symptoms (cough and bronchitis) and also leads to cancer, As-induced genotoxicity, modified DNA methylation, oxidative stress, carcinogenesis, and inhibition of DNA repair. Table 18.1 refers to the incidences of global groundwater As contamination and human population at risk.

18.5 As Metabolism in Humans

As does not have any nutritional value in humans, but metabolic reaction for the element has been evolved. Like other toxic metals, metabolic processes convert the metal from a toxic form to less toxic end products. Metabolism of inorganic As to methylated As compounds is known as biotransformation reaction. Methylated As compounds appear to excrete as a faster form from the body. Metabolism involves reduction of arsenate to trivalent state and oxidative methylation to form trimethylarsine oxide, a pentavalent organic end product. Reduction by two e^- converts arsenate to arsenite, which is catalyzed by reductase glutathione-S-transferase enzyme. Methylation of As requires S-adenosylmethionine and methyltransferases (Hughes 2002). The metabolism of As in humans varies with species and even in population as well as the rate of the methylation reaction (Vahter 2000). Glyceraldehyde-3-phosphate dehydrogenase is found to be major intracellular arsenate reductase (Németi et al. 2006), which requires glutathione, and NAD as substrate (Németi and Gregus 2005). The enzyme-specific activity is much higher in malignant cells, due to the enzyme activity, which contributes to the rapid reduction of arsenate in their cytosol into more toxic As(III).

18.6 Mechanism of Action of Arsenate Toxicity in Human

Pentavalent As is a molecular analog of phosphate that can replace phosphate in some of the biochemical reactions in humans. In human's red blood cell instead of phosphate, arsenate replaces the sodium pump even in the anion exchange system of transport (Kenney and Kaplan 1988). Arsenate replaces phosphate which results in the generation of unstable by-products. The unstable molecules interfere with normal phosphorylation leading to the uncoupling of adenosine-5′-triphosphare (ATP) in the presence of arsenate termed as arsenolysis. Substrate level arsenolysis occurs in the glycolytic pathway where arsenate is metabolized to form 1-arsenato-3-phospho-D-glycerate. The anhydrous form is unstable and further forms arsenate and 3-phosphoglycerate. Mitochondrial level arsenolysis occurs during oxidative phosphorylation leading to the formation of adenosine-5′-diphosphate-arsenate, and ADP arsenate hydrolyzes faster than ADP phosphate. In both the phosphorylation, the levels of the ATP get reduced substantially (Hughes 2002).

Table 18.2 Microbial isolates involved in biotransformation of arsenic

Type and name of microorganisms	References
Methylation of arsenic	
Rhodopseudomonas palustris	Qin et al. (2006); Yuan et al. (2008)
Alcaligenes	Bentley and Chasteen (2002)
Pseudomonas	
Mycobacterium	
Burkholderia sp. MR1	Yoshinaga et al. (2011)
Streptomyces sp.	
Arsenic-resistant organisms	
Synorhizobium meliloti	Yang et al. (2005)
Frankia alni	Wu and Song Beitz (2010)
Salinispora tropica	
Arsenate-reducing organisms	
Pisciarelli solfatara	Huber et al. (2000)
Pyrobaculum arsenaticum	
Shewanella sp. strain ANA-3	Malasarn et al. (2008)
Sulfurospirillum barnesii	Malasarn et al. (2004)
Desulfosporosinus sp. strain Y5	Perez-Jimenez et al. (2005)
Wolinella succinogenes	
Alkaliphilus metalliredigenes	Stolz et al. (2006)
Clostridium sp. strain OhILAs	
Alkaliphilus oremlandii	Fisher et al. (2008)
Sulfurospirillum barnessi	Stolz and Oremland (1999)
Sulfurospirillum arsenophilum	
Arsenite-oxidizing microorganisms	
Rhizobium sp. strain NT-26	Santini et al. (2000)
Herminiimonas asoxydans	Muller et al. (2003)
Alcaligenes faecalis	Anderson et al. (1992)
Ralstonia sp. strain 22	Lieutaud et al. (2010)
Agrobacterium tumefaciens	Kashyap et al. (2006)
Thiomonas arsenitoxydans	Arsene Ploetze et al. (2010)
Achromobacter sp. strain SY8	Cai et al. (2009)
Pseudomonas sp. TS44	
Ochrobactrum tritici	Branco et al. (2009)
Hydrogenobaculum sp. strain 3684	Clingenpeel et al. (2009)
Alkalilimnicola ehrlichii sp.	Hoeft et al. (2007)

18.7 Mechanism of Action of Arsenite Toxicity in Human

As has a specific affinity for sulfur in enzymes, where sulfhydryls lead to toxicity. Increase of reactive oxygen species reduces the cellular antioxidant glutathione and consequently causes cell damage (Dopp et al. 2010). Arsenite also has an affinity for the diothiol groups present on some of the enzymes. These enzymes are involved in the redox homeostasis synthesis of DNA and repair protein folding. Nonspecific binding to the enzyme sites may be considered as detoxication mechanism. Also, in vitro studies indicate the interaction of arsenite to thiol-containing molecules such as GSH and cysteine (Miller et al. 2002). Impact of arsenite living cells depends on the cellular respiration state and the production of free radicals which affects the speciation of As.

18.8 Mechanism of Action of Methylated Arsenate and Arsenite in Humans

Methylated species are conversion of inorganic As forms in the mammalian liver (Roy and Saha 2002). The long methylation of As is considered as the formation of less toxic product, but recent studies have proved the products of methylation to be more toxic (Petrick et al. 2000). Monomethylarsonous acid (MMA^{III}) is comparatively a more potent inhibitor of the enzyme pyruvate dehydrogenase (PDH) than arsenite. Inhibition of PDH by arsenite shows depletion of the ATP and carbohydrates (Hughes 2002). The methylated product will not be absorbed by the cell, so it can be easily excreted in the body, but if it enters and gets absorbed in the cell, it shows cytotoxicity (Thomas et al. 2007).

18.9 Environmental As Cycle

Like the natural carbon, nitrogen, oxygen, sulfur, and other elemental cycles, there are cycles of toxic metals as well. Natural activities such as weathering of rocks, biological metabolic reaction, volcanic eruption, and anthropogenic processes contribute to As cycling in the environment. Rocks are the principal reservoirs for As; the distribution of the metal from rocks into soil, water, and air continues its flux in the environment. Human activities like burning of coal, smelting industry, and mining also contaminate the environment. The soluble As concentration is largely dependent upon redox conditions, pH, adsorption reactions, and microbial activities in the environment. Living cells specifically microbes carry redox reaction, whereas higher organisms bioaccumulate the metal at a concentration higher than that of the environment (Roy and Saha 2002).

Conversion of arsenate to arsenite is a redox reaction controlled by available oxygen in the environment. The presence of the major and minor species of As depends on the pH. As in soil is controlled both by the physical and chemical properties of the soil, which thus influence adsorption-desorption processes. Biological reaction, such as methylation of As to its derivative monomethyl arsonic acid or dimethylarsinic acid, also contribute to the As cycling. Solubility of the different forms in water depends upon the groundwater levels and shallower depth of water. The concentration of As varies in the water. Many geothermal waters contain high As concentration (Paez-Espino et al. 2009). Many more factors other than the leaching of As from rocks in sediments and to water control the groundwater contamination. Additional contributors are concentration of phosphate, bicarbonate, silicate, and organic matter in the water. Natural water environments such as closed basins in arid as well as semiarid climates and strongly reducing aquifers also lead to high As concentration in water. The natural biogeochemical cycle of As in the environment involves various reactions such as oxidation, reduction, methylation, and demethylation reactions (Fig. 18.2). Microorganisms are known to influence the natural reaction and affect the speciation and the toxicity (Islam et al. 2004). In recent years, the toxicity of As has been reported from exposure to natural sources, but it is not clear as to which specific mechanisms are responsible for the mobilization of As. It seems that mobilization is due to a combination of

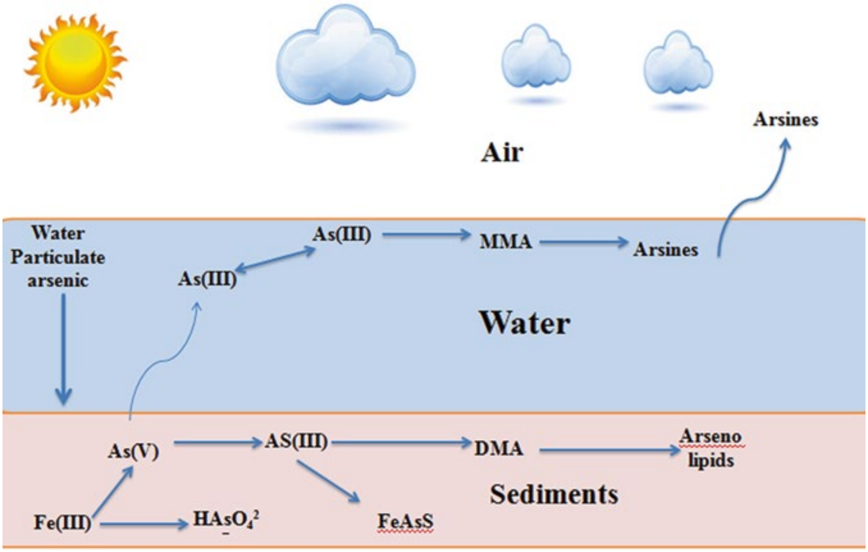


Fig. 18.2 Environmental arsenic cycle

chemical, physical, and microbial activities (McArthur et al. 2001).

18.10 Microbial Role in Elemental Cycle

Microbes require metals in small quantities referred to as essential metals and serve as micronutrients. Many other metals have non-biological role and are nonessential, so potentially toxic to the microbes. Biotransformation of metals is either for redox conversion to stabilize molecules or conversion of inorganic to organic or vice versa. Microorganisms respond to the metal in the environment in multiple ways. The response depends on the species of microorganisms and the type of metal. The interaction of the metal with microorganisms leads to damage cell membrane, modification of enzyme function, and damage of the genetic material, especially if exposed to high levels of essential or nonessential metals. In response to the As, microbes develop different mechanisms as chelation, compartmentalization, exclusion, and immobilization (Di Toppi and Gabbrielli 1999). Microbiological processes can either solubilize metals, thereby increasing their bioavaibility and potentially toxicity, or immobilize them to reduce the toxicity. Metal biotransformation by microorganisms plays a crucial role in biogeochemical cycles, which can be exploited for bioremediation of metal-contaminated environments (Lloyd and Lovley 2001).

18.11 Biotransformation of As by Microorganisms

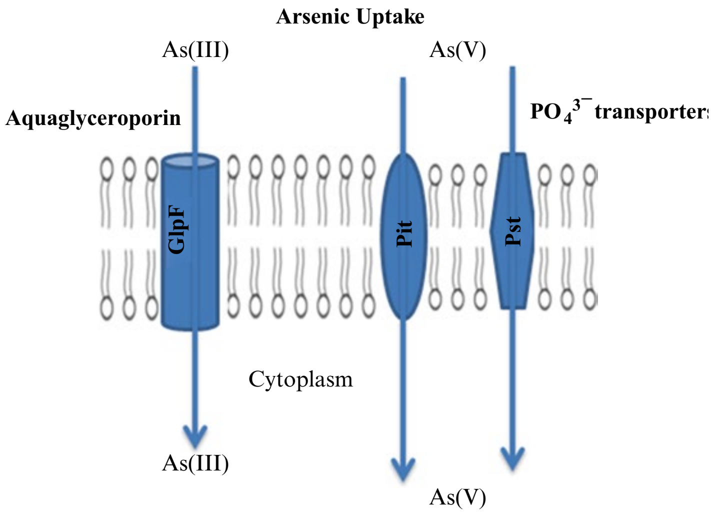
Primordial atmosphere is reducing, so As in the environment might be present in arsenite form. Hence, early microorganisms evolved with detoxification for arsenite. Global occurrence of As in the environment enforced As resistance mechanisms or used As as energy source in different microorganisms (Silver and Phung 2005; Stolz et al. 2006). As the atmosphere changes and becomes oxidizing, microorganisms detoxify the arsenate in the environment.

Microorganisms oxidizing a variety of organic or inorganic e^- donors are referred to as dissimilatory arsenate-respiring prokaryotes (DARPs). Arsenite to arsenate is an oxidation reaction carried by chemoautotrophic-oxidizing bacteria which can be referred to as CAOs. These bacteria use oxygen or nitrate as terminal e^- acceptor to fix carbon. Heterotrophic arsenite-oxidizing bacteria (HAOs) also oxidize arsenite to arsenate and use it for growth by using organic carbon as an energy source. Arsenate to arsenite, the reductive reaction in anaerobic respiration, uses arsenate as e^- acceptor. Microorganisms resistance to arsenate, referred to as arsenate resistance microorganisms (ARMs), reduce As(V) to As(III) or precipitate the metal outside the cell. As the dissimilatory arsenate-respiring prokaryotes, arsenate-resistant microorganisms do not gain energy from the conversion reaction, but are used for coping to the presence of high As in the environment. This type of microbial resistance for arsenate has been known since 1918 (Oremland and Stolz 2003).

18.12 As Uptake Pathway in Microorganisms

In the environment, As is found in extreme high concentration which leads to toxicity, but still the cell does not have specific uptake transporters (Gourbal et al. 2004). However, As transported through exiting transporters in microorganisms has similarity to the chemical analog to other known molecules (Rosen and Liu 2008). In *E. coli* cell, two phosphate transporters exist, Pit (phosphate inorganic) and Pst (phosphate specific), for uptake of phosphate (Rosenberg et al. 1977). Arsenate is an analog of phosphate; both the transporters catalyze the arsenate as oxyanions uptake, but the phosphate inorganic transporter system appears to be dominant compared to other system for arsenate and chemically very close to life essential phosphate (Styblo et al. 2000; Wolfe-Simon et al. 2010; Kalia 2010). Arsenite, which is in uncharged form, can be taken up into cells by the transporter (GlpF) specific for glycerol (Fig. 18.3) (Sanders et al. 1997). GlpF is an aquaglyceroporin, which is a member of the

Fig. 18.3 Uptake of arsenate and arsenite by microorganisms



aquaporin superfamily. It has the unique characteristic of having multifunctional channels that transport neutral organic solutes such as glycerol and urea (Rosen 2002).

18.13 Arsenate Reduction by Microorganisms

Microbial reduction of arsenate mobilizes the arsenite, which is more toxic than arsenate, and contaminates the groundwater. Microbes take up the arsenate and reduce to arsenite sequestration or extrusion. Recent studies realized the impact of environment on arsenate-reducing prokaryotes (Hoeft et al. 2002). Dissimilatory arsenate-reducing prokaryotes in anaerobic condition use arsenate as their respiratory oxidant. Energetically, the reaction is balanced when coupled with organic matter oxidation (Ahmann et al. 1994).

Cultivation of arsenate-respiring bacterial cultures in enrichment medium indicates that these are widespread and metabolize actively in nature. Their distribution is recognized using incubation of sample of anoxic environment sediments from 1 to 5 millimolar arsenate. Determination of the most probable number (MPN) of sediments from lake containing arsenate suggests that the number of cultivable arsenate-reducing bacteria present are in the range from 10^3 to 10^5cells per gram in the environment (Ahmann et al. 1994). In the first report of an As-respiring strain MIT-13, the microbial cell appears to be like vibrio. Lactate utilization from the medium was observed to be proportional to the reduction of arsenate to arsenite. MIT-13 strain reduces arsenate and dissolves the solid iron arsenate. These microorganism-respiring arsenates belong to different phylogenetic groups (Stolz and Oremland 1999; Silver and Phung 2005). There are 16 species of arsenate reducer in pure culture, including representative from each group and considerable increase in the novel species, low GC, Gram-positive bacteria, thermophilic eubacteria, and crenoarchea. Different scientific works have reported the diversity of location like freshwater sediments, estuaries, soda lake, hot spring, and gold mines (Newman et al. 1998; Stolz and Oremland 1999; Oremland and Stolz 2003). Even reports of gastrointestinal tracts of animals (Herbel et al. 2002) and subsurface aquifer materials show the presence of arsenate-reducing organisms. The DARPs are highly diverse phylogenetic assemblage of natural ones. These sources include not only garden varieties of microbes, physiologically suited for living at neutral pH, mesophilic temperatures, and low salinity, but also several extremophiles were able to adjust to high temperature, pH, and/or salinity for construction of their assemblage (Huber et al. 2000; Gihring and Banfield 2001).

DARPs are ubiquitous in nature and can be easily enriched in uncontaminated soils or sediments after addition of the arsenate under anaerobic condition. The organic matter normally present in these soils and sediments serves as the biological substrate to fuel the reduction of reaction of arsenate. Few reports indicate the arsenate-reducing microbial isolates from the As prevalence areas in the geochemical conditions. Microorganisms utilizing the arsenate, selenate, or molecular oxygen as e⁻ acceptor were isolated from Japan gold mines, and these were found to belong to Aquificales (Takai et al. 2002). Another obligatory anaerobic isolate, *Pyrobaculum arsenaticum*, from the hyperthermophilic hot spring precipitates the As_2S_2/As_4S_4 when arsenate and thiosulfate or L-cysteine are present in the medium. Another strain also reported to be *P. aerophilum* grows on arsenate as e⁻ acceptor (Huber et al. 2000).

In the terrestrial As-rich geothermal environment, the presence of a *Thermus* species able to oxidize arsenite and reduce arsenate was reported. In the presence of oxygen, the species oxidize arsenite a hundredfold greater than abiotic rates. In the absence of the oxygen, the same strain utilizes e⁻ acceptor arsenate with lactate oxidation (Gihring and Banfield 2001). Two *Bacillus* species – *B. asoselenatis* and *B. selenitireducens* – were isolated from an alkaline, hypersaline, As-rich water body, the Mono Lake, California. Malasarn (2008) reported the *Shewanella* sp. strain ANA-3, which is a respiratory arsenate reducer that shows reductase activity (Malasarn et al. 2008). Arsenate reducers can use different e⁻ donors such as hydrogen, acetate, formate, pyruvate, butyrate, citrate, succinate, fumarate, malate, and glucose (Niggemeyer et al. 2001). Even some strains recently isolated degrade more complex aromatic molecules such as benzoate and toluene (Liu et al. 2004). Recent studies realized the impact of environment on arsenate-reducing prokaryotes (Hoeft et al. 2002).

18.14 Arsenate Reductase Enzymes

Reduction of arsenate by two different mechanisms has been described, the one by cytoplasmic arsenate reductase coupled with arsenite efflux pump. The information was encoded by *ars* operon which regulates As resistance. A novel arsN gene, which encoded for a protein which was reported to have high resemblance to acetyltransferases, was linked to arsenic resistance (Chauhan et al. 2009). The second type of reduction is dissimilatory reduction process that includes the utilization of arsenate as a terminal (Paez-Espino, et al. 2009).

18.15 Dissimilatory Arsenate Reductases in DARPs

Arsenate reductase in DARPs is carried out by terminal reductase, which is different from the ARMs reductase. The reductase enzyme has been characterized from only few of microbial strains: *Chrysiogenes arsenatis* (Krafft and Macy 1998), *Bacillus selenitireducens* (Afkar et al. 2003), and *Shewanella trabarsenatis* strain ANA-3 (Malasarn et al. 2008). There are differences in the enzymes characterized from these microorganisms. *C. arsenates* and *S. trabarsenatis* enzymes reduce arsenate and present in periplasm, whereas *B. selenitireducens* reduces arsenite selenite and is associated with the periplasm via protein interaction (Afkar et al. 2003). The enzyme is composed of large and small subunits of about 100 kDa and 30 kDa, respectively. A heterodimeric structure of enzyme contains an iron sulfur cluster, classified as dimethyl sulfoxide (DMSO) reductase family. The enzyme contains arsenate-binding site and bis-molybdoprotein guanine dinucleotide and one cluster [4Fe-4S] cluster.

18.16 As Resistance Microorganisms

Arsenate-resistant microorganisms were discovered earlier and were studied longer than DARPs. *Shewanella* strain ANA-3 has been studied for both the activities as reduction of arsenate and resistance mechanisms (mNewman 2003; Saltikov et al. 2003). These organisms can reduce arsenate in aqueous or solid phase; CN8 strain is a fermentative arsenate-resistant microorganism, which is capable of reducing aqueous but not the solid phase of arsenate (Langner and Inskeep 2000). Bacteria can oxidize the Fe II by nitrate reduction to get energy for the growth under anoxic condition forming a solid ferric oxide to which arsenate and arsenite get adsorbed. The extracellular polymeric substances outside the bacterial cell can also accumulate the metallic ions capacity to resist the same (Slyemi 2012). Better understanding of arsenate-reducing prokaryotes and arsenate resistance microorganisms with the molecular tool is required to find an individual microbial role in the mobilization of the As in environment.

18.17 Detoxifying Arsenate Reductases in ARMs

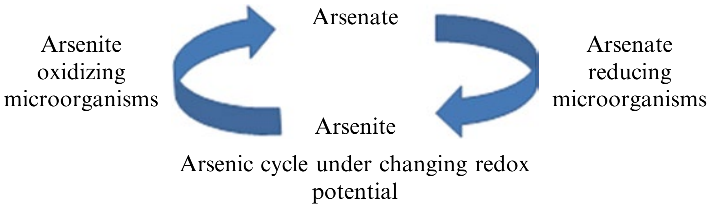
Detoxification and resistance are the most extensively studied mechanisms for As controlled by *arsc* genes. The cytoplasmic arsenate reductase is a small monomeric enzyme present in the cytoplasm. It reduces the arsenate by three cysteine thiol involving cascade of nucleophilic attack. In prokaryotes, the thioredoxin and glutaredoxin, two distinctly related families that evolved, were identified to be coupled with As reductases. These two enzymes differ in their structure, the mechanism of reduction, and the location of cysteine residues (Mukhopadhyay and Rosen 2002). Reductases arc genes are located in *ars* operons and chromosomal as well as plasmid DNA locus. In *Eukarya*, the third type of reductases Arr2P was detected (Mukhopadhyay et al. 2003). Studies in *Staphylococcus aureus* found the arsenate reductase ArsC protein encoded on pI258 plasmid (Mukhopadhyay and Rosen 2002). The enzyme reaction thioredoxin is a reductant and requires the NADPH. This enzyme is similar in structure to protein tyrosine phosphate (Zegers et al. 2001). The arsenate covalently bind to cysteine, which then reduces arsenate by nucleophilic attack.

In *E.coli* studies for the second family of reductase, glutaredoxin-coupled reductase is encoded by *arsC* gene present on R773 plasmid *ars* locus. The enzyme requires glutathione and glutaredoxin, a small thiol for the reduction activity. Arsenate reduction is catalyzed by three residues – ArsC, GSH, and glutaredoxin – which form a covalent bond with the single catalytic cysteine residue. It results in the formation of disulfide bond, between Cys12 and a glutathione cysteine. In the last step, glutaredoxin reduces the arsenate releasing the arsenite by disrupting disulfide bind and regeneration of reduced ArsC (Mukhopadhyay and Rosen 2002).

18.18 Arsenite Oxidation by Microorganisms

Microorganisms convert arsenite to arsenate by oxidation reaction, which is important for mobilization and speciation of As in the environment. These microorganisms are physiologically diverse and occur in two types of microbes, i.e., heterotrophic arsenite oxidizers referred to as HAOs and chemolithoautrophic arsenite oxidizers referred to as CAOs. Heterotrophic oxidation in cell's outer membrane converts arsenite, which is considered as detoxification process. The oxidation reaction coupled with oxygen or nitrate reduction produces CO_2 from organic matter in the cell. In the heterotrophic arsenite-oxidizing bacteria, organic carbon is used as a source of energy for cell growth. Isolation of arsenite-oxidizing microorganisms, both the CAOs and HAOs aerobic As(III) oxidizers from As-rich environment, is of importance in bioremediation of the pollutant (Salmassi et al. 2002; Santini et al. 2002). Thirty more strains of microorganisms representing nine genera, including γ, β, and α-Proteobacteria, have been reported. A fast-growing CAOs strain NT-26, member of the *Rhizobium*, evolved from α-*Proteobacteria* (Santini et al. 2000). The strain NT-26 can grow as autotroph as well as heterotrophy using arsenite and an organic compound as energy source, respectively. An arsenite-oxidizing strain HR13 from hot spring has been also reported to be used in the bioremediation of aquatic systems (Battaglia Brunet et al. 2002; Lièvremont et al. 2003). Aerobically, the isolate could oxidize As(III) for detoxification purposes (Gihring and Banfield 2001). Under anaerobic conditions, the strain HR13 uses arsenate as e^- acceptor growing on lactate (Fig. 18.4).

Fig. 18.4 Arsenic reduction and oxidation by microorganisms



Characterization of the microbes involved in these oxidations has been mostly confined to investigations of HAOs (Salmassi et al. 2002). Some of the HAOs identified by molecular techniques collected from various hot springs of Yellowstone National Park (Jackson et al. 2001). In geothermal streams with high arsenite concentration, biologically driven oxidation occurs, which thus becomes aerated (Wilkie and Hering 1998; Langner et al. 2001). A fast-growing chemolitotrophic arsenite oxidizer strain belonging to *Agrobacterium*/*Rhizobium*, found in gold mine from Northern Territory of Australia, was able to utilize arsenite (Santini et al. 2000). *Ectothiorhodospira*, a novel species (MLHE-1), grows under anaerobic conditions, where it uses arsenite as e^- donor and nitrate as the e^- acceptor. The strain also grows as an autotroph with sulfide or H_2 gas as substitutes for arsenite. MLHE-1 grew in heterotrophic conditions on acetate with oxygen or nitrate as the e^- acceptor (Oremland et al. 2002). MLHE-1 could not grow on or oxidize As(III) under aerobic conditions suggesting the tight coupling between the arsenate respiratory reduction at expense of e^- donors such as organic compound and H_2.

18.19 Arsenite Oxidase Enzyme in As-Oxidizing Microorganisms

Microbes oxidize arsenite by arsenite oxidase (Aox) enzyme to produce arsenate. The oxidase enzyme present in periplasm is different from the arsenate reductase present in the *aox* operon. The oxidized enzyme is a heterodimer containing two subunits: catalytic subunit (AoxB) and beta subunit (AoxA). The catalytic subunit works as an e^- shuttle as risk type, and smaller (AoxA) subunit contains the molybdopterin center-like [3Fe-4S] cluster. Study of the enzymes in microbial cells showed homolog of arsenite oxidase in genomes of *Aeropyrum pernix*, *Burkholderia*, *Chloroflexus aurantiacus*, *Chlorobium limicola*, *C. phaeobacteroides*, *Nitrobacter hamburgensis*, *Rhodoferax ferrireducens*, and *Sulfolobus tokodaii* (Stolz et al. 2010). Oxidized molybdenum cofactor undergoes a direct nucleophilic attack with arsenite in this reaction, leading to the formation of reduced molybdenum with arsenate. In an operon, the arsenite oxidase enzyme subunit-encoding gene *aoxA* is upstream to the gene catalyzing for the subunit aoxB (Slyemi 2012).

18.20 Methylation of As in Microorganisms

Methylation, originally thought as detoxification step, is a widespread phenomenon in nature. Methyl group addition results in methylated species of As: methyl arsenite, dimethyl arsenate, dimethyl arsenite, and trimethylarsine oxide as like in humans. Methylation reaction forms of As are gaseous state and are easily liberated into the environment after the oxidation of methylated form is converted back to the oxidized form of arsenate. In the methylation pathway, the series of reactions which reduce arsenate involve oxidative methyl group addition to the metal (Dombrowski et al. 2005). Methylation reaction requires S-adenosylmethionine (SAM) for methyl groups. S-adenosylmethionine gene *arsM* is identified in 120 microbial species (Qin et al. 2006). Earlier in 1951, Challenger proposed the methylation reaction in fungal species *Scopulariopsis brevicaulis* (Challenger 1951). Archaea and aerobic eubacteria (Honschopp et al. 1996) produce the methylated forms of arsines. Metal As can also be converted to arsenobetaine and As-containing sugars. There is an abundance of these compounds in marine and terrestrial plant and animals (Francesconi and Kuehnelt 2002). Considering that the methylated form of As is less toxic than inorganic As compounds, these microorganisms involved in the methylation are potential sources for bioremediation. Demethylation of monomethyl and dimethylarsenic compounds were reported, and use of methylated As compounds – ASALs as a carbon source – is possible (Maki et al. 2004).

18.21 As Efflux Machinery

Two possible basic mechanisms of arsenite extrusion in bacteria are first by arsenite carrier protein and second by an As efflux pump ArsB. In the arsenite carrier protein, the use of the membrane potential is used for generating energy required for the function. In the second approach, the energy is provided by the ATPase and ArsA via ATP hydrolysis. In the prokaryotes, ArsA/B system is working in majority of bacteria, while in some only the ArsB system works. Activation of ArsA by arsenite occurs using the formation of thiolate complex with the three cysteine molecules and the arsenite (Silver and Phung 1996). The gene *arsB* is found in the *Firmicutes* and γ-*Proteobacteria*, whereas ACR3 is present in *Actinobacteria* and α-Proteobacteria (Achour et al. 2007). An ArsB system is composed of the 429 residues which are the integral proteins, including the 12 membrane-spanning segments (Fig. 18.5). The membrane protein is a uniporter that works by using the membrane potential. The positive exterior is used for extruding the arsenite. Even though the pKa of 9.2 favors $As(OH)_3$ over $As(OH)_2O^{-1}$, the equilibrium ensures that some anion is always available to ArsB. *E. coli* has a chromosomal *ars*RBC operon that confers moderate resistance to arsenite. However, when ArsA is synthesized in *E. coli* from the plasmid R773 *arsRDABC* operon, cells are more resistant to arsenite because the ArsAB ATPase is much more efficient at arsenite extrusion than ArsB alone. Thus, mechanisms to cope with As(V) have evolved to use existing arsenite extrusion systems, which are present in prokaryotes. Incidentally, there is little information about the efflux machinery for arsenate (Slyemi 2012).

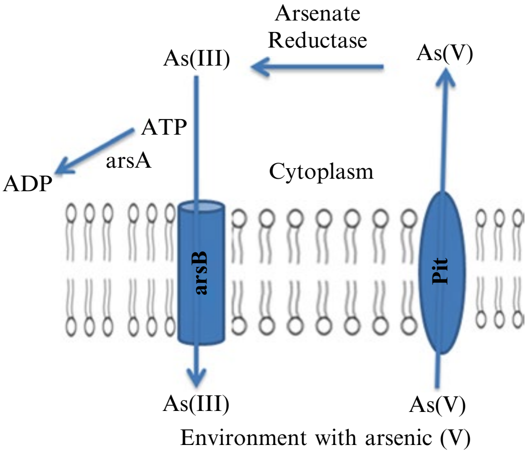


Fig. 18.5 Arsenate reduction and efflux mechanisms of a microbial cell

18.22 Summary

Understanding the fundamental mechanisms of As mobilization and immobilization will give a solution to the environmental problem arising due to As. Several reports indicate the involvement of microorganisms in the redox transformations of As on Earth. Reduction reaction will lead to environmental pollution problems, and oxidation reaction is considered as bioremediation process. In many parts of the world, human population is exposed to the natural As contamination in the drinking water. In these areas, As in the water should be within the permissible limits, because of the toxic effect on human health. Different types of adsorbents for removal of As from water have been reported. Isolating new As oxidizers from anoxygenic and reducer from oxygenic environment will expand the knowledge for constructing the functional bioremediation strategies using the microbes.

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Supriya Kore received her M.Sc. in Microbiology from the Government Institute of Science and Ph.D. degree from Dr. Babasaheb Ambedkar Marathwada University, Aurangabad. She was a recipient of senior research fellowship awarded by ICAR, New Delhi. She is working as an assistant professor in Dr. D.Y. Patil Biotechnology and Bioinformatics Institute at Pune since 2006. Her current interests are Natural Antimicrobials and Microbial Technologies.

Bio-Methane Production from Wastes: Focus on Feedstock Sources and Microbial Communities

19

Luigi Chiarini and Silvia Tabacchioni

Abstract

The anaerobic digestion process is a proven microbially mediated technology to achieve the reduction of organic wastes with simultaneous production of biogas. The number of biogas plants is continuously increasing worldwide. In Asia, millions of family produce biogas for domestic use by means of their own small-scale digesters. A number of new biowaste-based feedstocks are currently investigated as well as the efficacy of different substrate mixtures. During anaerobic digestion, biomass is degraded by microorganisms belonging to different functional groups performing their task through three sequential stages: hydrolysis and acetogenesis dominated by *Bacteria* and methanogenesis carried out by *Archaea*. A stable and efficient process relies heavily on the concerted and syntrophic activity of these microorganisms. During the last years, the application of culture-independent molecular techniques to samples from various anaerobic digesters has provided significant insights into these complex microbial communities revealing higher diversity at phylogenetic and functional level of bacterial communities than the archaeal ones. Greater efforts are needed to gain insights into the phylogeny, interspecies interactions, and function of key microorganisms involved in the first steps of anaerobic digestion as these details can provide the opportunity for enhancing methane yields through a more efficient production of substrates for methanogenesis.

19.1 Introduction

Anaerobic digestion (AD) is an anoxic biological treatment to stabilize organic matter while producing biogas, a mixture formed mainly of methane and carbon dioxide. Through this process, the amount of waste is significantly reduced while at the same time energy and solid or liquid fertiliz-

L. Chiarini • S. Tabacchioni (✉)
Sustainable Territorial and Production Systems Department, ENEA (Italian National Agency for New Technologies, Energy and Sustainable Development), via Anguillarese, 301-00123 Rome, Italy
e-mail: luigi.chiarini@enea.it; silvia.tabacchioni@enea.it

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ers are generated. The oldest and more widespread application of AD is the treatment of sewage sludge. After the first energy crisis in the1970s, AD experienced a remarkable growth, and now it can be considered a mature widespread technology all over the world. Indeed, millions of family-owned, small-scale digesters in Asia produce domestic biogas mainly for cooking and lighting (Al Seadi et al. 2008).

Hydrolysis, acetogenesis, and methanogenesis constitute the three main steps of AD. During hydrolysis, complex organic matter is degraded by hydrolytic enzymes secreted by microbes. Subsequently, the products of hydrolysis are converted to simple organic acids such as acetic acid and carbon dioxide (acetogenesis), and, finally, in the last stage (methanogenesis) methane is produced by methanogenic archaea in two ways: by cleavage of acetic acid or by reduction of CO_2 with hydrogen.

Substrates such as energy crops and biowastes have been extensively studied and some of them used in full-scale reactors for the production of biogas. However, the use of crops like maize, sugar beet, sunflower, or wheat for producing biofuels and bioenergy is not recommended as, being food and feed material, they lead to rise in food/feed prices. Nowadays, agricultural wastes such as straw and manure as well as the organic fraction of municipal solid wastes (OFMSW) and wastes from food processing industries are increasingly considered for biogas production, although not all are equally well-suited for AD, as demonstrated by frequent process instability after long-term reactor operation. Various parameters such as the concentration of slurry, pH, moisture, total solids, temperature, and carbon to nitrogen (C/N) ratio are among the main parameters affecting biogas production. In particular, the production of biogas mainly depends on the choice of feedstock and its C/N ratio (Wang et al. 2014). Optimum C/N ratio is rarely found in typical feed stocks. As reported in more detail below, a way to overcome this and other feedstock drawbacks is to digest simultaneously two substrates, the so-called co-digestion process.

The most applied reactor configuration for biogas production especially in agriculture is the single-phase continuously stirred tank reactor (CSTR). However, further research into reactor design and new avenues for the treatment of biowastes are necessary (Ganesh et al. 2014), and given the growing demand for energy recovery and efficient disposal of, in particular, solid wastes, such research is deeply needed. Indeed, single-phase anaerobic systems, in which all three reactions of hydrolysis, acetogenesis, and methanogenesis take place simultaneously in a single reactor, have been the preferred reactor design for the majority of wastes (Bouallagui et al. 2005) but present some drawbacks. For instance, the operation of such systems at a high organic loading rate (OLR) and for waste with large biodegradable organic content such as fruit and vegetable waste becomes difficult as this type of waste undergoes rapid acidification resulting in the inhibition of methanogenic activity (Bouallagui et al. 2009). Two-phase systems, in contrast, have the advantage of buffering the OLR in the first stage, which permits a relatively more constant feeding rate to the second stage, methanogenesis (Bouallagui et al. 2005), and, thus, achieving higher loading rates than those reported for single-phase systems (Rajeshwari et al. 2001; Bouallagui et al. 2004). Furthermore, the phase separation of hydrolysis/fermentation from methanogenesis in different reaction environments has been proposed as a strategy to increase overall process performances, in terms of stability and degradation efficiencies in both fermentation and methanogenesis phases and thereby in terms of overall energy recovery from biomass. A controlled acidogenic fermentation which allows efficient bio-hydrogen production has been considered the best pathway to pretreat raw biomass and enhance the methanogenic process. Efficient bio-hydrogen production and volatile fatty acids (VFA) liberation in the liquid during acidogenic phase would at the same time ensure energy recovery as H_2 and favor CH_4 production from VFAs in the methanogenic reactor.

Biogas production from biowaste is increasingly envisaged as integrated with a broader processes of producing fuels, power, heat, and value-added chemicals, the so-called biorefinery, from biomass. Indeed, the use of a wide range of biowaste feedstocks (e.g., agroindustrial wastes) coupled with the integration of biogas production into a multiproduct biorefinery, e.g., simultaneous production of bioethanol, biogas (methane), animal feed, and biofertilizer, would have distinct benefits, such as: (1) reduced production costs, (2) reduced usage of fossil fuels, and (3) reuse of by-products by integrating diverse technological processes (Bauer et al. 2008).

19.2 Feedstocks

AD, once almost exclusively applied to stabilize organic matter such as sewage sludge and manure, is successfully being used in the production of biogas from a steadily growing array of waste substrates (Table 19.1). A majority of waste feedstocks which have the potential for being used as substrates for AD can be categorized as: (a) OFMSW, (b) food industry waste, (c) agricultural residues, (d) manure, and (e) residual portions of waste water treatment plants (sewage sludge).

Notwithstanding the worldwide increasing diffusion of AD and beside the need to pretreat recalcitrant substrates such as lignocellulosic materials, some specific properties of the different waste substrates are linked to a few drawbacks that present AD of single substrates (mono-digestion) and that can cause instability and low yields of the overall process (Mata-Alvarez et al. 2014). For instance, (1) sewage sludge is characterized by low organic loads; (2)

Table 19.1 Characteristics of various feedstocks used for biogas production

Feedstock	Organic content	C/N ratio	Biogas yield (m^3 kg^{-1} VS)	References
Cattle slurry	Carbohydrates, proteins, lipids	6–20	0.20–0.30	Al Seadi (2001)
Pig slurry	Carbohydrates, proteins, lipids	3–10	0.25–0.50	Al Seadi (2001)
Poultry slurry	Carbohydrates, proteins, lipids	3–10	0.35–0.60	Al Seadi (2001)
Whey	75–80 % lactose		0.80–0.95	Al Seadi (2001)
	20–25 % proteins			
Corn stover	Carbohydrates, proteins	54	0.25	Li et al. (2013)
Rice straw	Carbohydrates, proteins	44	0.28	Li et al. (2013)
Straw (corn, wheat)	Carbohydrates, lipids	80–100	0.35–0.45	Al Seadi (2001)
Food waste	Carbohydrates (55–62%), Proteins (15 %), lipids (24 %)	13–24	NA	Zhang et al. (2014b)
Fruit wastes	NA	35	0.25–0.50	Al Seadi (2001)
Used animal oil	Lipids	NA	0.78[a]	Li et al. (2013)
Used vegetable oil	Lipids	NA	0.81[a]	Li et al. (2013)
Food processing bakery waste	Carbohydrates, proteins	19	0.53	Browne and Murphy (2014)
Food processing cheese waste	Carbohydrates, proteins	5	0.19	Browne and Murphy (2014)
Pig manure/potato waste (80:20 VS)	Carbohydrates, proteins, lipids	NA	0.30–0.33[a]	Kaparaju and Rintala (2005)
Cow manure/straw (70:30 VS)	Carbohydrates, proteins, lipids	NA	0.21[a]	Lehtomaki et al. (2007)
OFMSW/vegetable oil (83:17 DW)	Carbohydrates, proteins, lipids	NA	0.70[a]	Ponsà et al. (2011)

[a] Methane (CH_4) yield (m^3 kg^{-1} VS)

VS volatile solids, *DW* dry weight, *NA* not available animal manures have low organic loads and high nitrogen concentrations that may inhibit methanogens; in fact, the process of AD is quite unstable when the substrate has a low C/N ratio; (3) the OFMSW has improper materials as well as a relatively high concentration of heavy metals; (4) crops and agroindustrial wastes are seasonal substrates, which might lack N, and (5) food wastes include risks associated with the high concentration of N and/or long-chain fatty acids (LCFA), both potential inhibitors of the methanogenic activity. According to Li et al. (2010), the fat content of food waste is on average about 23 %, and lipid-rich waste can significantly contribute to the methane production (Wan et al. 2011); however, Appels et al. (2008) showed that long-chain fatty acids (LCFAs, 18-C), which are produced by decomposition of fat and lipids, can prove inhibitory at concentrations more than 1.0 g/L. Moreover, LCFAs can have a toxic effect on both syntrophic acetogens and methanogens (Hanaki et al. 1981) and affect negatively the transport of nutrients to cells because of their adsorption on the microbial surfaces (Pereira et al. 2005).

Most of these problems can be solved by the addition of a co-substrate in what has been recently called anaerobic co-digestion (AcoD). In fact, AcoD, the simultaneous AD of two or more substrates, is a feasible option to overcome the drawbacks of mono-digestion and to improve the economic viability of AD plants due to higher CH_4 production (Mata-Alvarez et al. 2014). The stability of the anaerobic process can be increased by AcoD of different organic substrates because of a better carbon to nitrogen (C/N) balance (Mshandete et al. 2004; El-Mashad and Zhang 2010). AcoD can also mitigate the inhibitory effect of high ammonia and sulfide concentrations (Hartmann et al. 2003) and show a more stable biogas production because of an increased buffer capacity (Nayono et al. 2010). Initially, because of the research perspective, AcoD focused on mixing substrates that favor macro- and micronutrient equilibrium, moisture balance, and/or dilute inhibitory or toxic compounds. Under these circumstances, co-digestion can produce more CH_4 than the addition of the CH_4 produced in both single digestions. However, as in full-scale industrial plants, the improvement of CH_4 production is achieved mainly by an increase of the OLR rather than synergism, almost all types of mixtures are used (Mata-Alvarez et al. 2014). Actually, the transport cost of the co-substrate from the generation point to the AD plant is the first selection criteria. Despite this fact, it is still important to choose the best co-substrate and blend ratio with the aim of favoring synergism, dilute harmful compounds, optimize CH_4 production, and not disrupt digestate quality.

In the following sections, a brief overview of the abovementioned feedstock categories will be given, with the exception of sewage sludge that has already been the subject of various reviews (Zhang et al. 2014a; Pilli et al. 2015).

19.2.1 Organic Fraction of Municipal Solid Waste (OFMSW)

It is generally assumed that OFMSW can be processed in an efficient and sustainable way through AD, providing a means of extracting some of the energy stored in this material. Therefore, AD is considered as a mature technology (Riggle 1998). On an industrial scale one-phase reactors for OFMSW digestion are predominant, the two-phase systems representing only 11 % of capacity at the end of the past century (De Baere 2000), probably because their maintenance and investment costs are lower. Overall, the treatment of OFMSW by AD shows a steadily increasing trend, although the quantity treated in the composting plants is still much higher.

Physical pretreatment, including milling and grinding, is essential in the case of solid wastes for improving the performance of AD because the particle size of these solid materials has a significant effect on the rate of hydrolysis. Kim et al. (2000) pointed out that the rate coefficient of the maximum substrate utilization doubled when the average particle size decreased from 2.14 to 1.02 mm. Izumi et al. (2010) also found that the particle size could significantly influence AD of food waste, which is a major component of

OFMSW. Approximately 40 % improvement on the total chemical oxygen demand was observed when the substrate was pretreated by a bead mill along with the mean particle size decreased from 0.843 to 0.391 mm, resulting in a higher CH_4 yield.

Although the number of production-scale plants for the treatment of OFMSW is increasing worldwide, research activity on OFMSW anaerobic digestion continues and focuses mainly on the biodegradation of the putrescent fraction of municipal solid wastes. For example, Pavan et al. (2000) studied the single-phase AD process, where OFMSW were segregated mechanically and at source, using a CSTR (continuously stirred tank reactor) type digester fed with different mixtures of both feedstocks. It was observed that in order to ensure the stability of the anaerobic digestion process, an OLR reduction was necessary when the contents of source-sorted OFMSW increased. Therefore, it may be advantageous to adopt a two-phase process, when using exclusively source-sorted OFMSW or fruit and vegetable wastes as substrate, because of the possibility of much higher loads in the reactor.

Biogas inhibition can often occur in the long-term operation due to nutrient imbalance, as already mentioned before. Indeed, trace elements (Zn, Fe, Mo, etc.) are rarely sufficient, whereas macronutrients (Na, K, etc.) can be in excess, and the C/N ratio can be outside of the optimum reported in literature (El-Mashad and Zhang 2010; Zhang et al. (2011a); Zhang et al. 2013). To counteract the inhibition and to overcome the disadvantages in single digestion, co-digestion of OFMSW with other organic substrates has been proposed. Most used co-substrate in industrial AD OFMSW plants is sewage sludge (SS) (Mata-Alvarez et al. 2014), because in many towns around the world, wastewater treatment plants are already equipped with aerobic digesters. Indeed, co-digestion between SS and OFMSW has been considered as a way to reduce the treatment costs of both wastes significantly. Although this particular mixture shows a high biogas potential, as shown in a review of benefits and constraints carried out by Iacovidou et al. (2012), AcoD of OFMSW: SS is not so easy in practice. Indeed, it is generally faced with (1) complex and unclear regulatory framework, (2) sorting pretreatment of the OFMSW prior its AD, (3) composition variability and seasonality, and (4) possible inhibitions caused by VFA accumulation, light metals, LCFA (adding biowaste rich in lipids), and/or NH_3 (adding biowaste rich in proteins).

19.2.2 Organic Waste from the Food Industry

The food industry generates large quantities of wastes worldwide. Treatment of food processing wastes prior to their disposal to the environment has been implemented through binding national legislation in many countries worldwide. As AD is often the primary technology in the treatment of food processing wastes and although the overall treatment process aims at a stabilization of the wastes for a safe environmental disposal, energy produced during the process in the form of biogas is increasingly utilized. After treatment, resulting anaerobic sludge can be applied as a fertilizer on agricultural land.

Protein and sugar containing whey from the dairy industry and citrus pulp from the juice manufacturing industry are among the agroindustrial wastes and by-products that can be anaerobically digested in mono- or co-digestion processes. Also various other crop and plant residues from industrial processing, usually treated via other routes or landfill, are amenable to anaerobic digestion. Indeed, industrial processing of citrus fruits, olives, and milk produces very high quantities of waste that could be extensively used as substrates for biogas production but have some drawbacks that have to be overcome, as shown in the following examples.

The production of orange juice and jam generates high volumes of orange peel waste, which are not permitted to be disposed in landfills and wastewater. Indeed, citrus wastes have high organic content, consisting of various soluble and insoluble carbohydrates, making these amenable and attractive for AD (Kaparaju et al. 2012). However, presence of D-limonene (a well-known antimicrobial agent) in peel press liquor, citrus wastewater, and peels can heavily limit the process of AD (Mizuki et al. 1990; Forgács et al. 2012; Kaparaju et al. 2012). Despite the known inhibitory effect of D-limonene, various processes have been developed to effectively biogasify orange peel waste. These methods typically involve size reduction (Kaparaju et al. 2012), coupled with pretreatment by steam distillation (Martín et al. 2010), solvent (hexane) extraction (Nguyen 2012), addition of enzymes (Srilatha et al. 1995), steam explosion, and combination of steam explosion and dilute acid hydrolysis (Forgács et al. 2012) to extract the D-limonene before the waste is digested in continuously fed, agitated, single-stage systems (Srilatha et al. 1995; Martín et al. 2010; Kaparaju et al. 2012). Another approach to overcome the inhibitory effects of D-limonene is to dilute its concentration by co-digestion with other feedstocks. Orange peel waste has been successfully co-digested with OFMSW in a continuously fed agitated single-stage system (Forgács et al. 2012). Also, glycerol is an excellent candidate for co-digestion with orange peel waste. Glycerol is a substance that is produced during the manufacturing of biodiesel, but the quantity generated exceeds the current demand for pure glycerol. A reduction of the inhibitory effect of some compounds as well as a correct nutrient balance could be obtained by co-digesting orange peel waste and residual glycerol (Martìn et al. 2013). Koppar and Pullammanappallil (2013) showed that in a reactor, operating under mesophilic temperature and semi-continuous conditions, OLR up to 2.10 g/L were compatible with stable anaerobic digestion of orange peel waste and glycerol. At higher OLR, process destabilization followed an accumulation of VFAs and a decrease in the pH.

Another attractive food waste susceptible to widespread use as a substrate for biogas production is cheese whey. Cheese whey is a by-product from cheese production. Between 115 and 160 million tons of whey are generated globally every year, half of which is transformed into food products or utilized for ethanol fermentation, while the rest is disposed of (Guimarães et al. 2010). Due to its worldwide availability and high carbohydrate content, whey is considered a suitable substrate to produce biogas via anaerobic degradation (Lin et al. 2014). Whey proteins have a relatively high value and are typically removed from the whey by ultrafiltration. Thus, it is mainly the whey permeate, i.e., a solution primarily composed of water, lactose, and salts, that is available for AD. Imbalanced C/N ratio and low alkalinity content (2.5 kg m^{-3} as $CaCO_3$) may affect the outcome of the fermentation process negatively. Indeed, the maintenance of stable process operating conditions is still a central issue in anaerobic digestion of whey. Therefore, co-digestion of whey permeate with cow manure or poultry waste has caught some interest because the latter feedstocks provide buffer capacity, nitrogen, and nutrients (Gelegenis et al. 2007). Kavacik and Topaloglu (2010) and Comino et al. (2012) showed that co-digestion of different waste types like manure and cheese whey makes the treatment of waste feasible, while the separate treatment of the same waste types would be highly problematic.

Olive mills represent a large proportion of the economy of the countries in the Mediterranean region (Anonymous 2003) and generate seasonal wastes such as olive mill wastewater (OMW) and olive cake, which are serious environmental hazards. OMW has a high content of polyphenolic compounds, suspended solids, volatile acids, polyalcohols, and nitrogenous compounds (Paraskeva et al. 2007; Zagklis et al. 2015), and, therefore, its mono-digestion is not easy to perform because of the inhibitory effect of the high concentration of phenols and, in particular, long-chain fatty acids (Beccari et al. 1998). However, co-digesting OMW with cow manure could result in a better performance of the anaerobic digestion process because of the dilution of phenolic compounds and long-chain fatty acids present in OMW. Moreover, the combination of these two substrates is likely to be more balanced in nitrogen and alkalinity, which are among the main parameters influencing the stability of the AD process. Fezzani and Ben Cheikh (2010) suggest that applying a two-phase reactor design to OMW fermentation can help overcome part of the abovementioned problems. Indeed, they showed that OMW degradation can be enhanced by co-digestion with olive cake in two-phase AD reactors without high dilution and added nitrogen substrate.

19.2.3 Agricultural Harvesting Residues and Manure

Manure and agricultural harvesting residues are often co-digested to enhance biogas production. Separate digestion of these two substrates gives relatively low yields of methane. Harvest residues comprise straw, stover, leaves, cobs, and also low-quality fruits and vegetables and can all be used as co-substrates for biogas generation; nevertheless, these residues must be employed in a sustainable way, with respect to ensuring that the rate of removal does not have a detrimental effect on soil fertility.

As it is economically unfeasible for biogas plants to run solely on manure (Angelidaki and Ellegaard 2003), co-substrates such as slaughterhouse waste and food industry wastes, which contribute to readily degradable organic matter, have been used to increase methane yields. However, these resources are nearly fully utilized so that biogas plants are forced to look for alternative co-substrates. The use of energy crops, which has increased dramatically in recent years, has been one option used in the biogas industry (Meyer-Aurich et al. 2012). In Europe, manure-digesting plants can be characterized as central and farm-scale plants (Wilkinson 2011). The "farm-scale" plants co-digest animal manure and, increasingly, bioenergy crops and agricultural wastes from one to three smaller neighboring farms. "Centralized" plants typically co-digest animal manure from multiple farms along with organic matter from industry and towns. The digestion capacities of these plants vary from a few hundred to several thousand cubic meters.

The use of energy crops is, however, undesirable as arable land is a scarce resource and energy crops compete for land with food production. Therefore, it is widely accepted that manure should be digested mainly with co-substrates that do not compromise food security and/or trigger land use changes. In this respect, the co-digestion of manure with harvest residues has been successfully implemented in recent years. Indeed, the combination of manure, with its buffering capacity and wide range of nutrients, and plant material, with its high carbon content, can result in an improved C/N ratio of the feedstock and, as a consequence, in a decrease of the risk of ammonia inhibition during the anaerobic digestion process. These positive synergistic effects should provide a potential for higher CH_4 yields than for the two types of substrates separately (Wu et al. 2010; Li et al. 2013).

19.3 Microbial Communities in Anaerobic Digesters

19.3.1 Seed Sources

The type of seed microorganisms and the need for more efficient inocula to be used at the start-up phase of the biomethanation process are among the most debated issues in methane production from AD of biomass. Microbial conversion of biomass to methane involves at least three metabolic groups of microorganism: primary fermenting bacteria, anaerobic oxidizing bacteria and methanogenic archaea. The first two groups degrade the biomass primarily to acetate, formate, and H_2, whereas the third group is responsible for the methanogenic degradation of acetate and the conversion of H_2 and CO_2 to $CH_4$by acetoclastic and hydrogenotrophic methanogens, respectively (Angelidaki et al. 2011). The balance within these distinct microbial groups is pivotal to the quality and yield of the methane produced and is directly connected to the overall stability of the process (De Francisci et al. 2015). Anaerobic sludge from wastewater treatment plants and cattle and swine manure are the primary sources of complex anaerobic consortia for the production of methane from biomass resources (Kampmann et al. 2012a; Merlino et al. 2012; Kim et al. 2013; Lim et al. 2013; Lu et al. 2013; Qiao et al. 2013; Guo et al. 2014; Town et al. 2014). However, studies on the efficacy of different inocula at the start-up of AD have been performed (Liu et al. 2009a; Pandey et al. 2011). Recently, syntrophic co-cultures of anaerobic fungi and methanogens have been isolated from the rumen of the herbivore Alpine ibex (*Capra ibex*) (Leis et al. 2014). The strict association between these two groups of microorganisms suggests their use in AD processes. Indeed, anaerobic fungi could improve hydrolysis in anaerobic digesters fed with agricultural residues and thus significantly increase their efficiency and yields (Gruninger et al. 2014).

A mixture of different microbial consortia has been used to improve methane production of a complex substrate. In a study, in which six different inocula were used for anaerobic thermophilic digestion of the OFMSW at dry conditions, the combination of swine excrement and digested sludge resulted to be one of the best inoculum (Forster-Carneiro et al. 2007). Furthermore, Quintero et al. (2012) tested different inocula composed by different combinations of rumen liquid with cow manure, pig waste sludge, and wastewater sludge to select the best microbial consortium to produce methane from fique's bagasse as substrate. They found that the inoculum composed by rumen liquid and pig waste sludge provided the best yields for bio-methane production because of the high hydrolytic activity of ruminal liquid and the high methanogenic activity of pig waste sludge.

To develop more efficient inocula at the start-up phase of the biomethanation process, several procedures have been carried out. For example, it has been proven that the inoculum that has previously been acclimatized to a given substrate gives the best methane yields (Browne and Murphy 2013; Guo et al. 2014). In both studies the seed sludge obtained either from a lab-scale CSTR which was previously fed with grass silage or from an anaerobic digester treating piggery wastewater was acclimated to low concentrations of food waste before inoculation into a digester fed with food waste. Similarly, Bertin et al. (2012) developed an efficient microbial consortium adapted to a mechanically sorted organic fraction of municipal solid waste (MS-OFMSW) substrate using a column recycled bioreactor that was initially filled with cattle manure and then continuously fed with only MS-OFMSW during a 3-month period. In another study, the sequential batch anaerobic composting (SEBAC) technology was used for the start-up of a laboratory scale CSTR reactor fed with synthetic OFMSW (Montero et al. 2010). In this study, two interconnected anaerobic digesters operating under thermophilic conditions with different types of wastes were used to create a flow of microorganisms and organic matter from the two reactors to obtain a more efficient inoculum.

19.3.2 Dynamics of Microbial Communities

The complex microbial communities existing in bioreactors play a crucial role in AD processes. Indeed, the success of the process depends on the actions and interactions of the different groups of microorganisms that make up the microbial consortium. Over the last two decades, there has been an increase in studies on the structure, function, and biological properties of the microbial communities involved in AD in order to fully understand the microbial ecology of the process and to link microbial community dynamics to anaerobic digester's performance. However, how the microbial component can affect the anaerobic digester performance is not entirely clear yet (Pervin et al. 2013). Several molecular techniques, such as cloning/sequencing, quantitative real-time PCR (qPCR), denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RLFP), single-strand conformation polymorphism (SSCP), fluorescence in situ hybridization (FISH), and catalyzed reporter deposition (CARD-FISH), have been used to provide qualitative and quantitative assessments of the microorganisms in anaerobic digestion systems (Cardinali-Rezende et al. 2012; Kampmann et al. 2012a; Merlino et al. 2012; Rademacher et al. 2012). In addition, high-throughput molecular techniques have been employed to get a more reliable picture of the structure and composition of the microbial communities in biogas reactors (Kröber et al. 2009;

Lu et al. 2013; Ziganshin et al. 2013; Lebuhn et al. 2014; Solli et al. 2014). Microbial community analysis of AD using clone libraries revealed the presence of only 69 operational taxonomic units (OTUs) (Rivière et al. 2009). In contrast, investigations of microbial communities from 9 full-scale ADs using HTP gene sequencing resulted in the discovery of thousands of OTUs (Werner et al. 2011).

Although phylogenetic profiles of microbial communities in anaerobic digesters have been investigated targeting mainly 16S rRNA gene (Pervin et al. 2013; Guo et al. 2014; Hagen et al. 2014), this molecular target provides limited information, because phylogenetically related microorganisms showing different functional capabilities cannot be easily recognized. More detailed information about microorganisms with specific functional capabilities can be achieved by tools targeting functional genes (e.g., genes involved in hydrolysis, acido-/acetogenesis, or methanogenesis). As an example, it is known that 16S rRNA primers/probes for *Euryarchaeaota* can detect microorganisms with unknown phylogeny or non-methanogens due to their scattered position within this phylum (Scully et al. 2005). The gene encoding for the subunit of key enzyme of methanogenesis, methyl-coenzyme M reductase (*mcrA*), has been employed to obtain a higher phylogenetic resolution of methanogens (Nettmann et al. 2008; Rastogi et al. 2008; Kröber et al. 2009; Song et al. 2010; Traversi et al. 2011; Lu et al. 2013; Munk and Lebuhn 2014). This enzyme catalyzes the final reaction step in methanogenesis and is only found in methanogens (Luton et al. 2002).

Studies focusing on the description of prokaryotic community at a particular operational time and on the community shift along with different operational conditions and periods have been performed (Schlüter et al. 2008; Bertin et al. 2012; Kampmann et al. 2012a; Hagen et al. 2014; Kampmann et al. 2014; Solli et al. 2014). Most studies on microbial communities in anaerobic digesters focused both on bacterial and archaeal populations; few studies have instead performed the analysis on the archaeal or bacterial communities separately (Bauer et al. 2008; Chen et al. 2012; Kampmann et al. 2012a; Merlino et al. 2012; Qiao et al. 2013; Hagen et al. 2014; Munk and Lebuhn 2014).

19.3.3 Lab-Scale Anaerobic Digestion Plants: Influence of Environmental and Operational Parameters

The structure and composition of the AD-associated prokaryotic community occurring in anaerobic digesters can be affected by temperature, pH, substrate type, and feed loading rate. Changes in bacterial communities have been correlated to pH fluctuations both in thermophilic and mesophilic reactors (Hori et al. 2006; Sun et al. 2014). Bauer et al. (2008) observed a prevalence of methanogens belonging to the order of Methanobacteriales and Methanomicrobiales in thermophilic and mesophilic reactors fed with maize silage as substrate, respectively. Changes in bacterial and archaeal populations during the increase of fermentation temperatures were observed in a two-phase biogas reactor supplied with rye silage and straw. At increasing temperatures over 65 °C, members of the Bacteroidales order became predominant in respect to the Clostridiales order in the hydrolytic phase whereas *Methanosarcina* sp. dominated among methanogenic *Archaea* resulting in the decrease of reactor's performance (Rademacher et al. 2012). Pervin et al. (2013a) observed that bacterial community in the pretreatment reactors of a temperature-phased anaerobic digestion (TPAD) system treating primarily sludge is strongly influenced by temperature. The diversity of the bacterial community dominated by populations affiliated to the *Firmicutes*, *Thermotogae*, *Proteobacteria*, and *Chloroflexi* decreased at increasing temperature with a progression from *Thermotogae* to *Lutispora* and *Coprothermobacter* resulting in a better hydrolysis performance than in the mesophilic pretreatment reactor. Similar results were obtained in another study of a TPAD pretreatment stage treating primary sludge, confirming the influence of temperature to select bacteria probably involved in hydrolytic processes (Pervin et al. 2013b). A study conducted using two parallel digesters operating at increasing OLR of food waste under mesophilic and thermophilic conditions revealed that richness and evenness of bacterial species in mesophilic reactors were greater than the thermophilic ones. Furthermore, with OLR elevation, the acetoclastic methanogens *Methanosaeta* gradually dominated the archaeal community. The high functional redundancy in bacterial community integrated with acetoclastic methanogenesis under mesophilic conditions resulted in a better anaerobic digester's performance, whereas the delicate interactions between hydrogen-producer and hydrogenotrophic methanogens under thermophilic conditions were more prone to disruptions (Guo et al. 2014).

Merlino et al. (2012) observed that bacterial communities from lab-scale batch digesters fed with different energetic crops and agroindustrial wastes varied in response to different substrates, whereas archaeal communities clustered together independently from the nature of the substrate treated. Recently, the impact of different pulses of proteins, lipids, and carbohydrates on the microbial composition of continuously anaerobic digesters fed with raw cattle manure was investigated by De Francisci et al. (2015). These authors show that the microbial composition of the three reactors, initially similar, diverged considerably after the substrate change. The greatest increase in diversity was observed in the reactor supplemented with carbohydrates where the microbial community became dominated by lactobacilli. On the other hand, the lowest level corresponded to the reactor overfed with proteins. Moreover, a common trend in all reactors resulting in a decrease of total methanogens abundance was observed. Similarly, Kampmann et al. (2012b) showed that *Bacteroidetes* and *Firmicutes* communities were severely affected by batch-feeding with casein, starch, and cream in 200-L biogas digesters.

The influence of substrate type on microbial community diversity was also demonstrated in a recent study on sequencing data from 78 anaerobic reactor samples representing 28 different studies. Indeed, this study revealed that bacterial sequences grouped by substrate type, independently from the study of origin, and that this grouping could be attributed to different bacterial lineages (Zhang et al. 2014c). In contrast to these findings, microbial community analysis of the end product digestate, obtained by co-digestion of ethanol by-products with different ratios of feedlot manure, revealed a numerically dominant core of 42 different bacterial OTUs and a roughly equal number of both hydrogenotrophic and acetoclastic methanogens in the reactors regardless of input material (Town et al. 2014). In another study, a stable archaeal community consisting of few hydrogenotrophic methanogens was observed in anaerobic digesters containing liquid manure that were consecutively fed with casein, starch, and cream; two most abundant species were found: one was closely related to *Methanospirillum hungatei*; the other one was only distantly related to other methanogens, with *Methanopyrus kandleri* being the closest cultivated relative (Kampmann et al. 2012a). The comparison of methanogens community between a pilot-scale and lab-scale reactors, both fed with swine manure, revealed the higher diversity in the former rather than in the latter. Only two hydrogenotrophic methanogenic species, *Methanoculleus receptaculii* and *Methanoculleus bourgensis*, were commonly detected in both reactors during the anaerobic digestion (Kim et al. 2013).

Four parallel CSTRs, operating under mesophilic conditions with maize silage as substrate, were used to investigate the effect of substrate feeding on the activity of methanogens. Different structures of methanogenic communities and functional variations were found in reactors demonstrating the importance of stochastic factors in affecting microbial community structure and functions (Lv et al. 2014). On the contrary, a recent paper (Luo et al. 2015) demonstrated a minor role of stochastic factors in shaping the profile of the microbial community composition and activity in three parallel biogas reactors supplied with cattle manure.

Population analyses of methanogens in three semicontinuously flow-through reactors under depletion of trace elements (mainly selenium, cobalt, and sodium) revealed that the whole (DNA-level) and the active (cDNA-level) fractions of methanogens can differ significantly. *Methanosarcina* spp. were most active in all reactors during the steady process state. This genus of methanogens was the most active when trace elements were sufficiently present. In another study, members of a hitherto undescribed genus of *Methanobacteria* (*Methanobacteriaceae* genus IV) were found to be the most active in the acidified control fermenter (Munk and Lebuhn 2014).

Methanosaeta were found to be the dominant methanogens in both pre- and post-overloading conditions in an anaerobic digester operating with dairy and poultry waste. Moreover, *Crenarchaeota*, a phylogenetic distinct group of non-methanogens, were identified as persistent constituents of the archaeal community during the AD process (Chen et al. 2012). *Crenarchaeota* were also found in other studies as major or minor components of archaeal populations in AD of various substrates (Collins et al. 2005; Zhang et al. (2011b); Qiao et al. 2013); however, their role in AD process is not clear.

19.3.4 Full-Scale Anaerobic Digestion Plants

A number of studies focusing on full-scale anaerobic digesters have been carried out in order to understand if differences in the microbial communities could account for the variability in reactor's performance (Table 19.2). Microbial community behavior at full-scale plants showed similar trend than those from lab-scale experiments. In seven full-scale mesophilic anaerobic digesters treating municipal wastewater sludge, Rivière et al. (2009), who analyzed the microbial communities using 16S rRNA gene cloning and sequencing, found that most bacterial sequences were affiliated with *Chloroflexi*, *Proteobacteria*, *Bacteroidetes*, and *Firmicutes*. In addition, they observed in each anaerobic digester a common core group of phylotypes composed of only six OTUs affiliated with *Chloroflexi*, *Betaproteobacteria*, *Bacteroidetes*, and *Synergistetes*. Instead, the *Archaea* community was represented by a restricted number of OTUs affiliated with *Methanosarcinales*, *Methanomicrobiales*, and Arc I phylogenetic groups among which the acetoclastic methanogens *Methanosarcinales* dominated in all digesters. Similarly, a meta-analysis of bacterial and archaeal 16S rRNA sequences, present in public databases, demonstrated a higher diversity of *Bacteria* than *Archaea* in anaerobic digesters. Dominant bacterial populations belonged to the phyla *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Chloroflexi*, whereas *Methanosaeta* and the uncharacterized WSA2 group were predominant among *Archaea* (Nelson et al. 2011). On the other hand, a study performed on six full-scale biogas plants fed with different liquid manures and agricultural crops revealed that each anaerobic digester shows a distinct methanogenic community structure with a prevalence of hydrogenotrophic methanogens belonging to the order *Methanomicrobiales*. In addition, three out of six biogas reactors revealed hitherto uncharacterized but potentially methanogenic species (Nettmann et al. 2010). Kröber et al. (2009) used an integrated approach, based on 16S rRNA gene cloning and sequencing and metagenome sequence data obtained by 454pyrosequencing, to gain insight into the prokaryotic diversity of a fermentation sample from a full-scale biogas plant supplied with maize silage, green rye, and liquid manure. Results revealed that the majority of the bacterial 16S-rDNA sequences were affiliated with the *Bacteroidetes* and *Firmicutes* phyla, with *Clostridia* as the most abundant class. Regarding the archaeal 16S-rDNA sequences, most of them were close to sequences of the hydrogenotrophic methanogen *Methanoculleus bourgensis*. These authors also found that most 16S rDNA metagenome reads could not be assigned to lower taxonomic ranks, revealing that a large fraction of microorganisms occurring in biogas plants are still unclassified or unknown. Similarly, a meta-analysis of 16S rRNA gene sequences present in public databases from anaerobic digester samples revealed that around 60 % of bacteria could not be assigned to any established genus (Nelson et al. 2011). Qiao et al. (2013) showed that the bacterial community of a

Table 19.2 Literature overview on microbial ecology studies in full-scale biogas reactors

Substrate	No. of reactors	T °C	Dominant microorganisms		Molecular techniques	Reference
			Bacteria	Archaea		
Cattle manure + maize silage	1	M		*Methanomicrobiales* (*Methanoculleus* sp.)	PCR-RLFP of clone libraries, qPCR (16S rDNA, *mcr*A)	Nettmann et al. (2008)
Maize silage + green rye + chicken manure	1	41 °C	*Firmicutes* (*Clostridiales*)	*Methanomicrobiales* (*Methanoculleus* sp.)	454 pyrosequencing	Krause et al. (2008)[a]
Maize silage + green rye + chicken manure	1	41 °C	*Firmicutes* (*Clostridium thermocellum*)	*Methanomicrobiales* (*Methanoculleus marisnigri*)	454 pyrosequencing	Schlüter et al. (2008)[a]
Maize silage + green rye + manure	1	41 °C	*Firmicutes* (*Clostridia, Bacteroidetes*)	*Methanomicrobiales* (*Methanoculleus*)	16S rDNA sequencing	Kröber et al. (2009)[a]
MWW+ industrial effluents	7	M	*Chloroflexi*, *Proteobacteria*, *Bacteroidetes*, *Firmicutes*	*Methanosarcinales*, Arc I, *Methanomicrobiales*, *Crenarchaeota*	16S rDNA sequencing	Rivière et al. (2009)
Pig manure	1	M	*Firmicutes*, *Bacteroides*, *Spirochaetes*	*Methanoculleus bourgensis*, *Methanosarcina barkeri* *Methanospirillum hungatei*	DGGE, 16S rDNA sequencing	Liu et al. (2009b)
Liquid manure + renewable raw materials	6	M		*Methanomicrobiales (Methanoculleus 5 reactors)* *Methanosaetaceae (1 reactor)*	FISH, qPCR, 16S rRNA cloning and subsequent ARDRA	Nettmann et al. (2010)
Maize silage + grains + manure	1	M		*Methanomicrobiaceae*	RLFP, RT-PCR	Bergman et al. (2010)
Brewery wastewater	9	M	*Bacteroidetes* *Syntrophobacterales* *Desulforomonadales* *Spirochaetes* *Synergystetes* *Chloroflexi*		454 pyrosequencing	Werner et al. (2011)[b]
OFMSW	1	35 °C	*Bacteroidetes*, *Firmicutes*	*Methanomicrobiales* (*Methanoculleous* sp.)	DGGE, ARDRA, qPCR, FISH, CARD-FISH	Cardinali-Rezende et al. (2012)[b]
Waste activated sludge	6	35–52 °C	*Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Chloroflexi*		454 pyrosequencing	Lee et al. (2012)[b]
Night soil	1					

Sewage sludge waste	1	30–44 °C	*Bacteroidetes*	*Methanomicrobiales*, *Methanosarcinales* (*Methanosaeta* sp.), Arc1	DGGE FISH	Regueiro et al. (2012)
Brewery wastewater	1		*Firmicutes*			
Dairy waste	1		*Proteobacteria*			
Sugar industry waste	1		*Syntrophomonadaceae*			
Yeast industry waste	1					
Dairy + fish waste	1					
Corn straw	1	M	*Firmicutes* (*Clostridiales*)	*Methanobacteriaceae*	16S rDNA sequencing	Qiao et al. (2013)
			Chloroflexi (*Caldilinea aerophila*)	*Methanospirillaceae* *Methanosaetaceae*		
			Actinobacteria (*Streptomyces hypolithicus*)	*Methanosarcinaceae*		
			Bacteroidetes (*Acetomicrobium faecale*)	*Methanoregulaceae unclassified*		
			Proteobacteria (*Smithella propionica*)	*Methanomicrobia*		
			Bacteria	Archaea		
Sewage sludge waste	7	M /T	*Firmicutes*, *Bacteroidetes*	*Methanosaeta* sp.	16S rDNA sequencing	Sundberg et al. (2013)
Co-digestion of various wastes: OFMSW, slaughterhouse, food, and industry wastes	14			*Methanoculleous* sp., *Methanobacterium* sp.		
OFMSW	1	29– 43 °C		*Methanosarcina*, *Methanosaeta*, *Methanocorpusculaceae*, *Mrc-2b* group	RT-qPCR (*mcr*A)	Traversi et al. (2014)[b]
Waste activated sludge	2	M	*Proteobacteria* (*beta-* and *deltaproteobacteria*), *Bacteroidetes Bacteroidia*, *Bacteroidetes* (class), Shingobacteriia, Flavobacteriia*)*, *Firmicutes* (*Bacilli, Clostridia*)	*Methanosaeta*, *Methanosarcina*	Illumina high-throughput sequencing	Yang et al. (2014)
Waste activated sludge	1	35 °C	*Proteobacteria* (*alphaproteobacteria*), *Firmicutes* (*Clostridia*), *Bacteroidetes* (*Bacteroidia*, Cytophagia, Flavobacteriia, Shingobacteriia), *Actinobacteria.*	*Methanosaeta, Methanosarcina*	Illumina high-throughput sequencing	Guo et al. (2015)

[a] These studies have been performed on the same sample from one reactor

[b] Overtime sampling

M mesophilic, *T* thermophilic, *M/T* mesophilic and thermophilic, *OFMSW* organic fraction municipal solid waste full-scale anaerobic digester treating corn straw presented higher diversity than the archaeal one. Sixty-nine and 11 phylotypes were identified among *Bacteria* and *Archaea*, respectively.

A study to assess the influence of temperature and substrate type on the composition of the microbial community was conducted using 454 pyrosequencing of 16S rRNA gene in samples from 21 full-scale biogas reactors operating at mesophilic and thermophilic temperatures. The substrate varied from sewage sludge or different combinations of slaughterhouses, restaurants, households wastes, and manures (Sundberg et al. 2013). Results revealed for all digesters a more diverse and abundant bacterial community compared to the archaeal one. *Firmicutes* and *Bacteroidetes* dominated in all reactors, although the relative occurrence of *Firmicutes* sequences was higher in reactors co-digesting various substrates than those digesting sewage sludge. Sequences from the acetoclastic *Methanosaeta* sp. dominated in sewage sludge reactors, whereas sequences from hydrogenotrophic *Methanoculleus* sp. and *Methanobacterium* sp. were predominant in reactors co-digesting various substrates. Moreover, microbial communities from all reactors clustered separately according to temperature. The influence of substrate type on the microbial community was also observed by Regueiro et al. (2012), who analyzed the microbial community structure in six full-scale anaerobic digesters supplied with various residues. They found a link between microbial activity and microbial community structure; in most anaerobic digesters, the higher hydrolytic and the methanogenic activity, the higher the *Bacteroidetes* and *Archaea* percentages, respectively, in the microbial communities. The understanding of the active populations present in anaerobic digesters can help to better select the inoculum source for the specific anaerobic digestion process and consequently avoid a poor start-up phase with a prolonged period of acclimation.

The above studies show a snapshot of the microbial community at a specific point in time. On the other hand, there are few studies investigating the microbial communities in production-scale reactors at different time points. Werner et al. (2011) characterized bacterial communities in nine full-scale granulated sludge reactors supplied with brewery wastewater by 454 pyrosequencing of 16Sr RNA gene over one year period. *Syntrophobacterales*, *Desulfuromonales*, *Bacteroidetes*, *Spirochetes*, *Clostridia*, *Chloroflexi*, and *Synergistia* resulted as prevalent bacterial communities. Moreover, syntrophic divisions *Syntrophobacterales* (an order of Deltaproteobacteria) and *Synergistia* were overrepresented throughout all locations, indicating that their populations were more stable and consistent than the average populations. Authors speculated that hydrolytic and fermentative populations rely on functional redundancy to maintain overall function and that resilience played an important role in maintaining syntrophic populations.

The prokaryotic communities of four full-scale anaerobic digesters fed with slaughterhouse and potato waste, WWTP sludge, and pig manure plus biowaste operating at different temperatures were analyzed using T-RLFP method during a time course of 45 days (Pycke et al. 2011). Results revealed that temperature greatly influences the composition of both *Archaea* and *Bacteria* as they group separately according to temperature. Richness varied during the time course for both domains; however, the archaeal community structure was more stable than the bacterial one under thermophilic conditions.

Traversi et al. (2014) developed bioindicators of efficiency for the anaerobic process through the quantification and characterization of the methanogens over time in a full-scale reactor fed mainly with OFMSW. Detection of methanogens by RT-qPCR revealed that *Methanosarcina* was the most abundant family, followed by *Methanocorpusculaceae* and *Methanosaeta*. Due to its high abundance, *Methanosarcina* was proposed as an indicator of stability, whereas *Methanosaeta* showing high sensitivity to temperature and OLR was proposed as a bioindicator of instability in anaerobic digesters supplied with OFMSW. The detection of microbial indicators coupled with physical-chemical monitoring can represent an advantage in applying corrective methods to maintain stable yields of methane production.

A study comparing bacterial communities of seven full-scale anaerobic digesters over time operating at a temperature range of 35–52 °C was carried out using 454 pyrosequencing of the V1, V2, and V3 regions of the 16S rRNA gene. Six out of seven anaerobic digesters were fed with waste-activated sludge (one of these in combination with smaller amounts of food waste) and one with night soil. It was found that *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Chloroflexi* were predominant, and the composition of microbial communities was mainly affected by temperature. Some of the phylotypes were weakly linked with some process performance parameters (Lee et al. 2012).

A 16S rDNA-targeted molecular approach including DGGE, ARDRA, qPCR, FISH, and CARD-FISH was used to investigate the bacterial and archaeal community dynamics of a full-scale OFMSW reactor from start-up to a steady-state condition (Cardinali-Rezende et al. 2012). By means of these molecular techniques, the authors observed shifts in the prokaryotic community of the OFMSW anaerobic reactor from start-up to steady-state conditions with an increase in the cell number of both *Bacteria* and *Archaea* over the time. *Bacteroidetes* and *Firmicutes* and the hydrogenotrophic *Methanomicrobiales* predominated among fermentative and methanogens microorganisms, respectively. Acetoclastic methanogens *Methanosarcina* and *Methanimicrococcus* were identified mainly with the reactor working in steady-state conditions, whereas *Methanosaeta* could be only detected by qPCR and FISH, revealing the sensitivity of these quantitative techniques (Cardinali-Rezende et al. 2012).

Significant shifts in the bacterial community structure were observed only after 99 days of the addition of brewery waste yeast in a production-scale wastewater reactor, whereas no significant shifts in the archaeal microbial community structure were observed neither during the first 99 days of anaerobic digestion nor during the period of 189 days (Zupančič et al. 2012).

19.4 Conclusions

An increasing number of studies are evaluating the possibility to use a wider range of biowastes for biogas production. Co-digestion of two or more feedstocks is a well-established technique that offers the possibility of increasing methane yield through synergistic effects of co-substrates and dilution of toxic compounds. Seed sources play an important role in the start-up phase of anaerobic digestion by ensuring a balance among the distinct microbial groups involved in hydrolytic and methanogenic activities. A mixture of different microbial consortia, as well as the use of adaptation and enrichment procedures, have been used to obtain more efficient inocula. However, there is a common tendency to separate the hydrolytic and methanogenic phases to improve digester performance, although, it is not known if a two-phase system does improve the digestion of all type of residues. Moreover, the application of anaerobic fungi into anaerobic digesters could be a possibility to improve hydrolysis in agriculturally used biogas plants due to their ability to degrade cellulose and hemicellulose. Major efforts should be directed to understand their phylogenetic and functional diversity and to isolate robust strains to be used in biogas plants. Molecular-based studies on the prokaryotic community inhabiting anaerobic digesters revealed a greater diversity of bacterial community than the archaeal one independently from the type of the study. Hydrogenotrophic methanogens seem to predominate among methanogenic microorganisms in both lab- and full-scale reactors fed with agricultural residues, whereas acetoclastic methanogen predominates in reactors supplied with municipal wastewater sludges.

19.5 Opinion

Biogas production from biowaste is a very fast growing market around the world. Several types of biowastes are under scrutiny for full-scale utilization. More investigations are needed to explore the potential of new biowaste-based feedstocks and the efficacy of different substrate mixtures. For an increased diffusion of biogas plants, improvements in the process efficiency and stability through a better reactor design (e.g., two-phase fermentation processes) coupled with a better understanding of factors governing efficient co-digestion of different substrates are necessary. Furthermore, a more in-depth comprehension of the microbial community structure and dynamics in biogas digesters and their influence on process stability and biogas yield would further boost biogas production. The development of microbial consortia specifically designed to maximize their hydrolytic and methanogenic activity on particular substrates would be highly recommended. Hence, greater efforts are needed to gain insights into the phylogeny, interspecies interactions, and function of key microorganisms involved in the different steps of AD.

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Luigi Chiarini received his degree in Biological Sciences, University of Rome "La Sapienza". He is presently senior scientist at ENEA-Sustainable Territorial and Production Systems Department. His research activity has focussed for many years on the microbial ecology of plant-growth promoting rhizobacteria and opportunistic human pathogenic bacteria. His current interests are the bioconversion of biomasses (in particular, the organic fraction of municipal solid wastes) into biofuels, i.e. hydrogen and methane, and the ecology of the bacteria involved.

Silvia Tabacchioni received her degree in Biological Sciences, University of Rome "La Sapienza". She is presently senior scientist at ENEA-Sustainable Territorial and Production Systems Department. She is a Fellow of Association of Microbiologists of Italy. Her research activity focussed on bacterial production of chemicals using wastes as substrate, microbial ecology of plant-growth promoting rhizobacteria and opportunistic human pathogenic bacteria. Her current interests are the bioconversion of biomasses into biofuels, i.e. hydrogen and methane and microbial ecology of bacteria involved in these biological processes.