Handbook of
Hydrocarbon and Lipid Microbiology
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Sang Yup Lee *Editor*

Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Production of Fuels and Chemicals

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Handbook of Hydrocarbon and Lipid Microbiology

This handbook is the unique and definitive resource of current knowledge on the diverse and multifaceted aspects of microbial interactions with hydrocarbons and lipids, the microbial players, the physiological mechanisms and adaptive strategies underlying microbial life and activities at hydrophobic material: aqueous liquid interfaces, and the multitude of health, environmental and biotechnological consequences of these activities.

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Editor

Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Production of Fuels and Chemicals

With 174 Figures and 33 Tables

Springer

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To Hye Jean Hwang and Gina Lee for their love, support and inspiration

Preface

Due to our increasing concerns on climate change, environmental problems, and also limited nature of fossil oil and gas, there has been much interest in developing bio-based processes for the production of fuels, chemicals, and materials from renewable resources. This book comprises four parts: bioproduction of fuels, bioproduction of chemicals, microbial facilitation of petroleum recovery, and global consequences of bioproduction of fuels and chemicals. Part 1 is about bioproduction of fuels. Following introductory chapter on bio-based production of fuels, the butanol-ethanol production is showcased. Four chapters are covering the state of the art of algal biofuels including a methodology for lipid screening. Also covered topics include lignocellulosics-based biofuels, synthetic biology for biofuels production by yeast, biomethane as an energy source, carbon dioxide to electofuels, and removal of sulfur from fuels. Part 2 is about bioproduction of chemicals. Following the introductory chapter overviewing the bio-based chemicals production, chapters covering enzyme mining and evolution are presented. Several chemicals including isoprenoids, flavors and fragrances, and fatty-acids and derivatives are covered in several chapters. As research works on alternative substrates are progressing, two chapters are allocated on the use of other substrates and also one carbon feedstocks. Chapters on the production of applications of biopolymers, rhamnolipids, and protein emulsifiers are also presented in this Part 2. As covered in the first edition of this book, Part 3 presents several chapters on microbial facilitation of petroleum recovery. In addition to microbially enhanced oil recovery, the use of biosurfactants in oil recovery and the use of microorganisms in processing and upgrading of crude oil are presented. Two interesting chapters are presented in Part 4 on global consequences of bioproduction of fuels and chemicals. This book providing up-to-date overview and global consequences on bio-based production of fuels, chemicals, and materials together with related tools and methodologies will be useful for those who are interested in environmentally friendly processes.

Finally, I would like to thank all the authors who wrote their chapters with state-of-the-art information and knowledge to make this book an important reference in the field. I also want to thank the series editor Dr. Ken Timmis for his guidance and colleagues at Springer for their excellent support.

KAIST, Republic of Korea Sang Yup Lee

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Kenneth N. Timmis
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Kenneth Timmis studied microbiology and obtained his Ph.D. at Bristol University. He undertook postdoctoral training at the Ruhr-University Bochum, Yale and Stanford, at the latter two as a Fellow of the Helen Hay Whitney Foundation. He was then appointed Head of an independent research group at the Max Planck Institute for Molecular Genetics in Berlin and subsequently Professor of Biochemistry in the University of Geneva, Faculty of Medicine. Thereafter, for almost 20 years, he was Director of the Division of Microbiology at the National Research Centre for Biotechnology (GBF)/now the Helmholtz Centre for Infection Research (HZI), and concomitantly Professor of Microbiology in the Institute of Microbiology of the Technical University Braunschweig. He is currently Emeritus Professor in this institute.

The Editor-in-Chief has worked for more than 30 years in the area of environmental microbiology and biotechnology, has published over 400 papers in international journals, and is an ISI Highly Cited Microbiology-100 researcher. His group has worked for many years, *inter alia*, on the biodegradation of oil hydrocarbons, especially the genetics and regulation of toluene degradation, and on the ecology of hydrocarbon-degrading microbial communities, discovered the new group of marine oil-degrading hydrocarbonoclastic bacteria, initiated genome sequencing projects on bacteria

that are paradigms of microbes that degrade organic compounds (*Pseudomonas putida* and *Alcanivorax borkumensis*), and pioneered the topic of experimental evolution of novel catabolic activities.

He is Fellow of the Royal Society, Member of the European Molecular Biology Organisation, Fellow of the American Academy of Microbiology, Member of the European Academy of Microbiology, and Recipient of the Erwin Schrödinger Prize. He is the founder and Editor-in-Chief of the journals *Environmental Microbiology*, *Environmental Microbiology Reports*, and *Microbial Biotechnology*.

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Dr. Sang Yup Lee is Distinguished Professor at the Department of Chemical and Biomolecular Engineering, Korea Advanced Institute of Science and Technology (KAIST). He is currently the Dean of KAIST Institutes, Director of BioProcess Engineering Research Center, and Director of Bioinformatics Research Center. He served as a Founding Dean of College of Life Science and Bioengineering. He has published more than 560 journal papers, 74 books/book chapters, and more than 630 patents, many of which licensed. He received numerous awards, including the National Order of Merit, National Science Medal, Ho-Am Prize, POSCO TJ Park Prize, the Best Science & Technology Award from the President of Korea, James Bailey Award, International Metabolic Engineering Award, Marvin Johnson Award, US Presidential Green Chemistry Challenge Award, Charles Thom Award, and Elmer Gaden Award. Professor Lee also delivered numerous named lectures around the world. He is currently Fellow of American Institute of Chemical Engineers, American Association for the Advancement of Sciences, American Academy of Microbiology, American Institute of Medical and Biological Engineering, Society for Industrial Microbiology and Biotechnology, the World Academy of Sciences, Korean Academy of Science and Technology, and National Academy of Engineering, Korea. He is also Foreign Associate of both National

Academy of Engineering, USA, and National Academy of Sciences, USA. He is honorary professor of University of Queensland, Shanghai Jiao Tong University, Wuhan University, Hubei University of Technology, and Beijing University of Chemical Technology. He is Editor-in-Chief of *Biotechnology Journal* and Associate Editor and board member of numerous journals. He has served as the Chairman of the Global Agenda Council on Emerging Technologies and also Biotechnology and is currently Chair of Global Future Council on Biotechnology at the World Economic Forum. He founded the World Council on Industrial Biotechnology in 2010 and served as a Founding Chair. He served as a member of the Presidential Advisory Council on Science and Technology of Korea and is currently a member of Government Performance Evaluation Committee. His research interests are metabolic engineering, industrial biotechnology, synthetic biology, systems biology, and nanobiotechnology.

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Part I

Bioproduction of Fuels

Bioproduction of Fuels: An Introduction

1

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Abstract

Production of biofuels by microbial fermentation is well established as illustrated by large-scale production of bioethanol. Recently, there has been focus on microbial production of advanced biofuels that can be used as drop-in fuels in both gasoline, diesel and jet fuels with the objective of providing an alternative to fuels derived from petroleum. Microorganisms have therefore been engineered to enable conversion of sugars into chemicals that can be used as biofuels, such as alcohols, fatty acid esters, and alkanes. Here we review recent progress on engineering microorganisms that can serve as cell factories for production of advanced biofuels.

1 Introduction

In the twentieth century, the global consumption of fossil fuels has continuously increased, while the planetary reservoirs are being depleted (Lee and Lee 2013). Increased fuel consumption causes increased greenhouse gas emissions that are associated with increased global warming. At the beginning of the twenty-first century, the problems of exhaustion of fossil fuels have encouraged the global community to search for alternative energy sources, including more environmentally-friendly liquid transportation fuels (Feofilova et al. 2010; Kurnia et al. 2016; Singh et al. 2016). Among many energy alternatives, biofuels, hydrogen, natural gas and syngas (synthesis gas) are likely to emerge as the four strategically important sustainable transportation fuel sources in the future. Within these four, biofuels are the most environmentally friendly energy source as they overall cause less greenhouse gas emissions (Stephenson et al. 2008). Biofuels are also favourable due to their renewability, biodegradability and an acceptable quality of the produced exhaust gases (Bhatti et al. 2008).

Biofuels such as alcohols, alkanes and biodiesel produced by microorganisms can replace liquid transportation fuels derived from petroleum. However, production of biodiesel derived from vegetable oils or animal fats and bioethanol derived from corn and crops can compete with food supply, thereby causing economic and ethical problems (Demirbas 2008; Zhang et al. 2011). Furthermore, biofuel production from food crops requires enormous amounts of water, large land mass and specific requirements for fertilizers. Current biofuels also suffer from various other problems, e.g., bioethanol has a lower energy density than petroleum fuels and is hygroscopic, preventing it from being transported through existing pipelines.

It is therefore necessary to progress beyond ethanol and towards the production of advanced biofuels from biomass, as this will overcome the above mentioned problems. Advanced techniques in synthetic biology and metabolic engineering offer a

platform to develop cell factories containing extensively engineered homologous and heterologous pathways that can be used for the production of advanced biofuels (Wackett 2011).

Biofuels from engineered microorganisms have great potential in enhancing processing steps such as fermentation, substrate separation and energy coupling. Metabolic engineering to produce biofuels from various microorganisms is one of the technologies which could be a promising alternative for creating truly sustainable, technically feasible and cost-competitive biofuels. The role of metabolic engineering for biofuel production is critical for the development of sustainable biofuels in the near future.

For the production of advanced biofuels by microbial fermentation, new insights into cellular metabolism are required. Advances in bioinformatics and mathematical modelling have enabled detailed and quantitative analysis of cellular metabolism. This kind of analysis has been the basis for more precise engineering of cell factories having an altered cellular metabolism where there is an increased flux directed towards the product of interest.

There are certain limitations like slow growth, high nutritional requirements, pathogenicity, stress sensitivity and low product yields in using natural microorganisms for biofuel production. Metabolic engineering has been exploited not only to improve traditional microbial fermentation processes, but also to produce chemicals that are currently used as fuels (Bailey 1991; Keasling 2010; Nielsen 2001; Nielsen and Keasling 2016) (Fig. 1).

In the most successful cases of industrial biofuel production, metabolic engineering has been applied to microorganisms such as *Corynebacterium glutamicum*, *Escherichia coli*, and *Saccharomyces cerevisiae*, resulting in the development of highly versatile cell factories as well as tailored producer strains that can be used for cost-competitive biofuel production.

2 Definition of Biofuels

Biofuels are broadly classified as primary and secondary biofuels. Primary biofuels are used in an unprocessed form such as chips and pellets from wood, and are directly used for heating, cooking or electricity production. Secondary biofuels are produced by processing crops or biomass to ethanol, biodiesel and dimethyl ether (DME). Depending on the raw material and the processing technology used for biofuel production, secondary biofuels are classified into first, second and third generation biofuels (Nigam and Singh 2011). First-generation biofuels are liquid type fuels that are generally produced from sugars (Diniz et al. 2014; Love et al. 1998), grains or seeds (Abraham et al. 2016; De Domenico et al. 2016; Eryilmaz et al. 2016; Pietrzak et al. 2016) in a relatively simple process. Ethanol is a well-known first-generation biofuel that is produced from fermented sugar extracted from plants and starch. However, as mentioned above first-generation biofuel production from different crops, plants and seeds is in potential conflict with our food supply. Furthermore, utilization of only a fraction of the total plant biomass reduces the soil

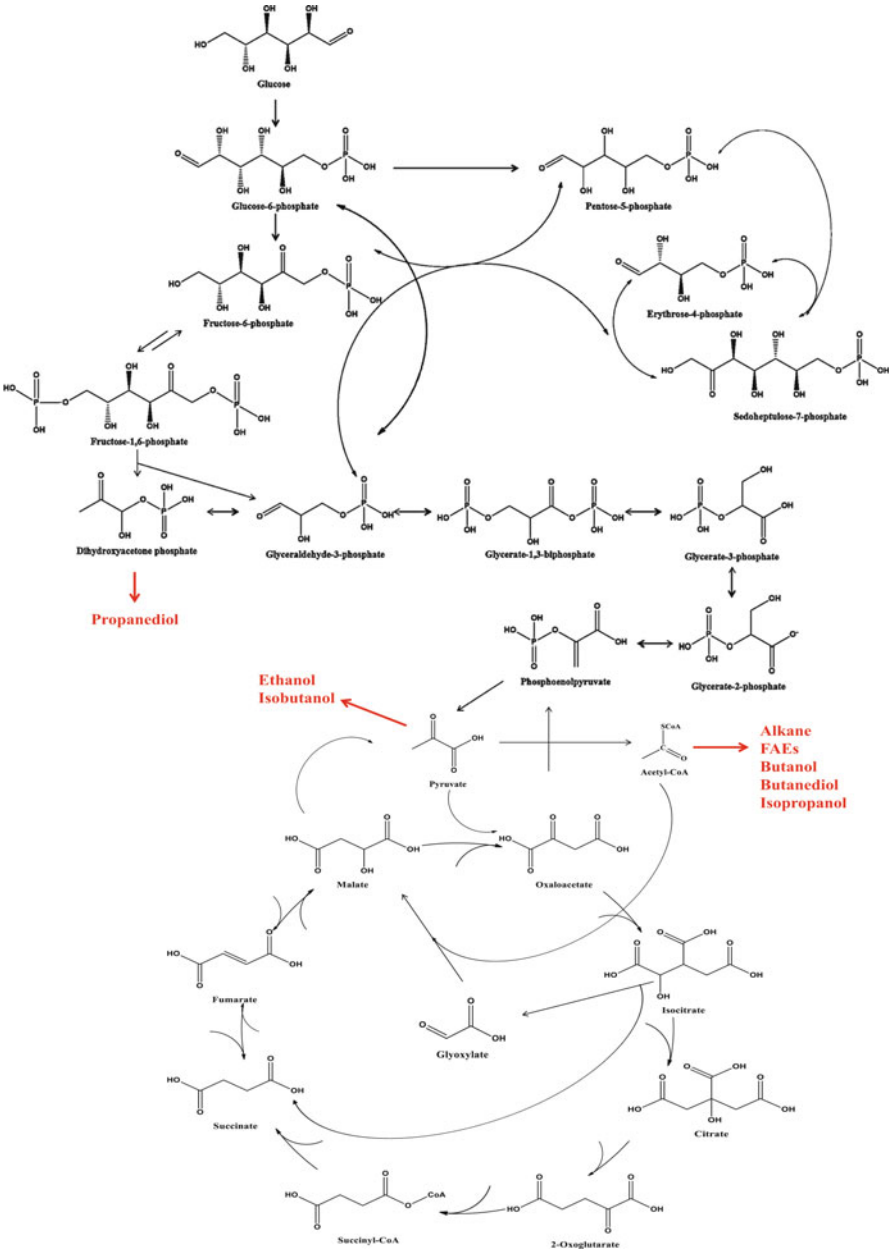

Fig. 1 Pathway for production of alkane, FAEs, butanol, butanediol, isopropanol, propanediol, ethanol and isobutanol

use efficiency (Nigam and Singh 2011). The use of non-edible biomass for the production of biofuels has therefore gained much interest, and when used for production of bioethanol this is normally referred to as second-generation biofuels. Second-generation biofuels are produced from non-edible sources such as residues of crop production or from whole plant biomass. The production of second-generation biofuels from non-edible biomass is advantageous in terms of land use efficiency, lower production costs and improved energy balance (Caspeta and Nielsen 2013).

Third-generation biofuels are hydrocarbons and other chemicals that integrate better with the current infrastructure as discussed above, and fare better than first and second-generation biofuels, as they have high energy density, better miscibility with existing fuels and compatibility with normal combustion engines. These are produced by microbes and microalgae and are considered to be a viable alternative energy source devoid of many drawbacks that are associated with first and second-generation biofuels.

3 Metabolic Engineering

Some microorganisms can naturally produce biofuels, but they often suffer from low growth rates, intolerance to toxic biofuel products, and incomplete carbon source usage (Lee et al. 2008; Li et al. 2010). To solve these problems, there is an increasing interest in using metabolic engineering to develop cell factories that can be used for the production of biofuels. Metabolic engineering has contributed to the improved performance of microorganisms in industrial production, including modification of terminal production pathways as well as flux redirection for elimination of undesired by-products or enhanced supply of building blocks, redox power or energy. Some microbial species such as yeast, fungi and microalgae can efficiently produce fatty acids that can be further converted to alkanes. These species can be used as potential sources for biofuel production, in particular hydrocarbons.

Synthetic biology and metabolic engineering has enabled the reconstruction of heterologous metabolic pathways in well-studied microbial hosts such as *E. coli* and *S. cerevisiae* for the production of biofuels. For many years these microorganisms have been used for industrial-scale production and can be engineered to tolerate toxic biofuels and to consume various carbon sources such as xylose and biomass (Alper et al. 2006; Buschke et al. 2013; Hahn-Hägerdal et al. 2001; Trinh et al. 2008). Extensive characterization of these hosts and the availability of different genetic manipulation tools enable the engineering of heterologous pathways not only to improve the production titer and yields but also to extend the range of biofuels. However, production yields of biofuels from engineered microorganisms are often too low to compete with and replace fossil fuels (Ezeji et al. 2007; Fischer et al. 2008), and it is therefore necessary to engineer the cell factory further for increasing titer, productivity and yield. Recently, a variety of engineered microorganisms, generated using metabolic engineering, integrated with genome engineering and synthetic biology, appear to be quite promising as they have increased the production

yields of biofuels such as biodiesel, butanol, terpenoids, syngas, and H_2 (Bokinsky et al. 2011; Lan and Liao 2011; Li et al. 2012; Srirangan et al. 2011; Westfall et al. 2012; Zhang et al. 2011).

4 Metabolic Design from Microorganisms

To design and engineer microorganisms for high yield production of biofuels we need to better understand how microbial cells can coordinate their intracellular metabolic pathways under different environmental conditions. This information will help to reconstruct efficient pathways for biofuel production and to optimize the balance between biomass production and biosynthesis in microorganisms (Johnson and Schmidt-Dannert 2008; Lan and Liao 2011, 2012; Portnoy et al. 2010; Trinh et al. 2008). Recently, engineered biosynthetic pathways for the production of biofuels in *E. coli* and *S. cerevisiae* have greatly expanded the candidate repertoire of biofuels, and in recent studies biofuels such as alkanes and alkenes have been produced from microbial cells (Rude et al. 2011; Schirmer et al. 2010; Zhou et al. 2016a). Engineering effort of a given target molecule in a given selected host comes with its own set of advantages and challenges and this knowledge can be used to guide future reconstruction of complete biosynthetic pathways. When building a pathway in a suitable host, it is advantageous to consider the use of native high flux pathways to provide key precursors. Heterologous enzymes, either found in nature or designed by protein engineering, can be expressed to catalyze the conversion of these precursors into the target compound. Preferably, the chosen heterologous pathway enzymes will be orthogonal to the host native pathways, meaning few intermediates will be used in the native cellular metabolism. Furthermore, limiting the use of precursors by competing pathways and creating irreversible steps in the engineered pathway can increase the flux towards the product. Finally, other key determinants of the overall yield and productivity, such as redox balance, ATP usage and cofactor requirements must be considered.

5 Industrial Microorganisms Used for Biofuel Production

It is important to choose the right cell factory for industrial production of biofuels (Fletcher et al. 2016), and this is often selected among *S. cerevisiae*, *E. coli*, *Aspergillus niger*, *Bacillus subtilis*, and *C. glutamicum*. These cell factories are generally robust and have already been used for large scale industrial production. Furthermore, the genetics and physiology of these cell factories are very well characterized and many metabolic engineering tools for genome editing and gene expression are available. It is therefore possible to develop accelerated and efficient biofuel producing cell factories using one of these platforms. In the following we will discuss the pros and cons of these different cell factory platforms.

5.1 *Saccharomyces cerevisiae*

S. cerevisiae has been the organism of choice in many industrial applications, not only because of its long history of application within the brewing and bread making industry, but also because it fits quite well with the characteristics mentioned above (Fletcher et al. 2016). It is the best-characterized eukaryote as it has wide biotechnological applications beyond foods and beverages with properties such as high robustness, a generally regarded as safe (GRAS) status and its capability to aerobically and anaerobically grow on diverse carbon sources. Companies such as Amyris and Sanofi have established large scale commercial production of the anti-malaria drug artemisinin from sugars using engineered yeasts. The production of microbially derived chemicals such as fragrances and food flavours has already been demonstrated at the laboratory scale (Carlquist et al. 2015).

5.2 *Escherichia coli*

The Gram-negative bacterium *E. coli* is probably the best-studied organism of all microbes. Metabolic engineering tools for *E. coli* are presumably the largest that exist for one particular organism. *E. coli* strains can consume various carbon sources including sugars and organic acids under aerobic or anaerobic conditions and can produce biofuels faster than other organisms (Fu et al. 2015). *E. coli* strains use amino acids as a precursor to produce biofuels (Blombach et al. 2011).

5.3 *Corynebacterium glutamicum*

The Gram-positive soil bacterium *C. glutamicum* was identified as a glutamic acid overproduction bacterium in 1956 (Uaka 1960). *C. glutamicum* is used for industrial production of L-amino acids such as leucine, lysine and glutamic acid. Besides these traditional products, advances in recombinant DNA technology has enabled the development of specialized cell factories for the production of various natural and un-natural compounds such as succinic acid, diamines, amino acids, alcohols, vitamins, pyrazines, diamines, L-ornithine, pyrimidines and polymers (Ahn et al. 2016; Hirasawa and Shimizu 2016; Lee and Kim 2015; Nguyen et al. 2015).

5.4 *Bacillus* spp.

The Gram-positive bacterium *Bacillus* spp. has an advantage (minimum genome for engineering) as a host for biochemical production of isobutanol Li et al. 2011), isoprene (Xue and Ahring 2011), ethanol (Romero et al. 2007) and 2,3-butanediol (Biswas et al. 2012). Development of genome editing techniques and specific gene expression as well as an expanding set of online tools has further enhanced the opportunity to use *Bacillus* species as cell factories in efficient and cost-effective

bioprocess. Advances in fundamental and applied research in *Bacillus* species further consolidate its position as one of the most dominant microbes used in industry (Liu et al. 2013b).

5.5 *Pseudomonas putida*

The Gram-negative soil bacterium *P. putida* is a well-established host for metabolic engineering. *P. putida* can degrade organic compounds and aromatic compounds such as toluene and xylene due to its high metabolic versatility (Kim and Park 2014) and has a potential to be used for the industrial production of biofuels and biochemicals (Poblete-Castro et al. 2012), rhamnolipids, terpenoids, non-ribosomal peptides and polyketides (Loeschcke and Thies 2015; Nikel et al. 2016). Also, *P putida* presents a high tolerance to oxidative stress and toxic compounds (Kim and Park 2014; Ramos et al. 2015), which is of interest for several technical applications, such as biofuel production.

5.6 Lactic Acid Bacteria (LAB)

Traditionally, LAB have been associated with food and feed fermentations, where they are widely used. In 1980s, the development of genetic tools such as cloning and gene expression enabled effective, deliberate and directed manipulation of LAB. A reasonably well understanding of cellular pathways combined with the availability of genetic tools enabled the deliberate and rational change of primary metabolism in LAB, which consequently allowed the generation of cell factories for the production of high-value biochemicals through metabolic engineering (de Vos and Hugenholtz 2004; Kleerebezem and Hugenholtz 2003; Neves et al. 2005). Recent studies demonstrated the engineering of LAB, genera *Lactococcus* and *Lactobacillus*, for the production of biochemicals such as L- and D-lactic acid, 1,3-propanediol, 2,3-butanediol, and biofuels.

6 Biofuels Produced by Microorganisms

6.1 Ethanol

Ethanol is the classical fermentation product of *S. cerevisiae,* both as an ingredient in alcoholic beverages and as a biofuel (Jang et al. 2012; Liu et al. 2013c). Thus, all current bioethanol is being produced using this yeast, and ethanol production with *S. cerevisiae* often comes close to the maximum theoretical yield of 0.51 g/g, with concentrations reaching more than 100 g/L industrially, and reported titers of about 50 g/L in academic studies (Liu et al. 2013c) (Fig. 2). These titers of ethanol could also be achieved by the expression of the ethanologenic pathway of *Z. mobilis* into *E. coli* (Ohta et al. 1991). Another study reported the construction of a heterologous

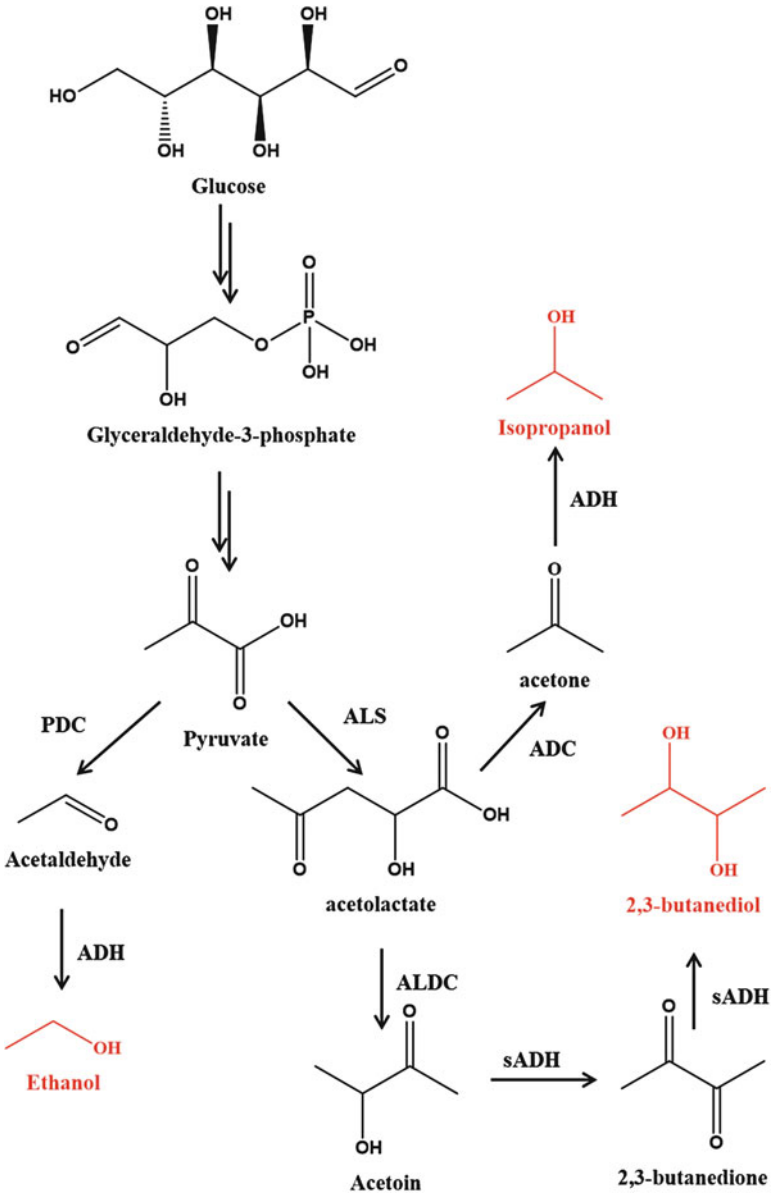

Fig. 2 Pathways for the production of ethanol, isopropanol and 2,3-butanediol. Enzymes: *PDC* pyruvate dehydrogenase, *ADH* alcohol dehydrogenase, *ALS* acetolactate synthase, *ALDC* acetolactate decarboxylase, *sADH* stereospecific secondary alcohol dehydrogenase, *ADC* acetoacetate decarboxylase

ethanol pathway accompanied by elimination of competing fermentative pathways through deletions of the genes encoding for fumarate reductase (*frdABCD*), lactate dehydrogenase (*ldhA*), acetate kinase (*ackA*) and pyruvate formatelyase (*pflB*) in *E. coli*. In this strain, pyruvate dehydrogenase was anaerobically expressed for redox balancing, allowing the overall production of ethanol with a 90% yield (Zhou et al. 2008). Co-fermentation of sugars was approached by the deletion of *mgsA* that is involved in control of sugar metabolism (Yomano et al. 2009). Ethanol fermentation by *C. glutamicum* strictly relies on heterologous genes, and an engineered strains expressing pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adhB*) from *Z. mobilis* that are additionally deficient in lactate dehydrogenase and PEP carboxylase produced 0.53 g/g ethanol under oxygen limited conditions (Smith et al. 2010).

6.2 Isobutanol and 1-butanol

The production of isobutanol and 1-butanol from engineered microorganisms is extensively studied. Butanols are attractive biofuels as they have a higher energy density than ethanol and are less hygroscopic, resulting in improved value for use in e.g., boat engines. The pathway of isobutanol biosynthesis is closely linked to the branched-chain amino acid pathway (Fig. 2). *C. glutamicum* engineered for valine over-production was successfully re-engineered for isobutanol production (Smith et al. 2010). Inactivation of lactate dehydrogenase and pyruvate carboxylase increased the isobutanol production to 4.9 g/L. *C. glutamicum* has also been engineered for the production of isobutanol from glucose by inactivation of L-lactate and malate dehydrogenases, expression of keto acid decarboxylase from *Lactococcus lactis*, alcohol dehydrogenase (*ADH2*) from *S. cerevisiae* and *pntAB* transhydrogenase from *E. coli*. Engineered *C. glutamicum* grown to high cell densities allowed the accumulation of 73 g/L isobutanol in the fermentation (Blombach et al. 2011). *E. coli* was engineered for isobutanol production through engineering the branched-chain amino acids pathway. To increase isobutanol production, the acetolactate synthase (*alsS* gene) from *Bacillus subtilis* was used instead of acetolactate synthase (*ilvIH* gene) from *E. coli*, resulting in the production of 22 g/L isobutanol (Atsumi et al. 2008).

The 1-butanol biosynthesis pathway from *Clostridium acetobutylicum* was implemented into *E. coli*, which then produced 600 mM 1-butanol (Atsumi et al. 2008) (Fig. 3). Another strategy for 1-butanol production used a synthetic pathway from threonine. This engineered *E. coli* produced titer of 2 g/L of both 1-butanol and 1-propanol in a ratio of 1:1 through de-regulation of amino acid biosynthesis and elimination of competing pathways (Shen and Liao 2008), though the acetyl-CoA derived biofuel production turned out to be more efficient. Reconstruction of this pathway by selecting genes from different organisms resulted in superior biofuel production compared to solely using *C. acetobutylicum* genes resulting in 30 g/L of 1-butanol production (Shen et al. 2011). The 1-butanol biosynthesis pathway has also been extended to1-hexanol production pathway in *E. coli* (Dekishima et al. 2011).

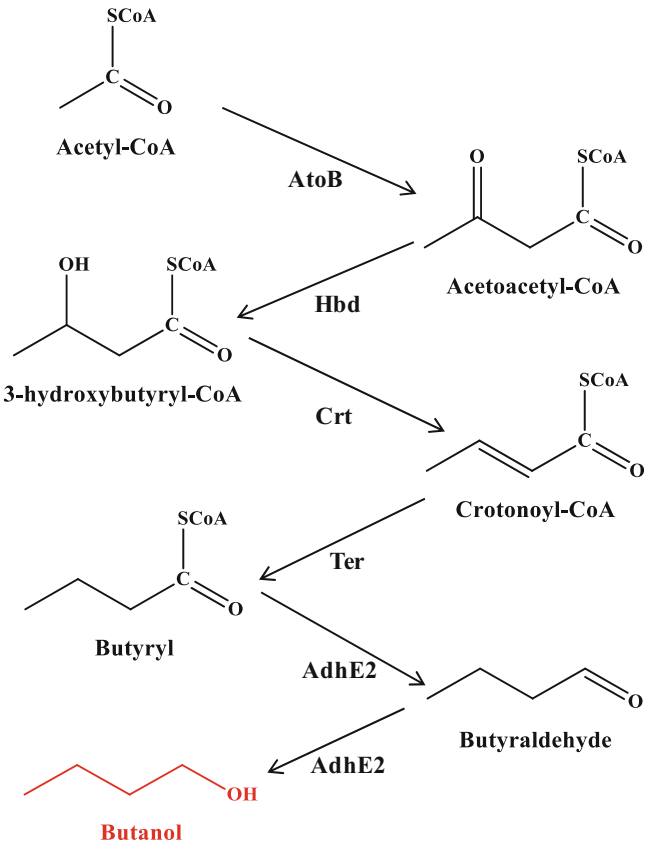

Fig. 3 Pathway for production of butanol. *AtoB* acetyl-CoA acetyltransferase, *Hbd* acetoacetyl-CoA thiolase, *Crt* crotonase, *Ter* trans-enoyl-coenzyme A reductase, *AdhE2* aldehyde/alcohol dehydrogenase

6.3 Propanediol

Dupont has developed a commercial process for production of 1,3-propanediol (1,3-PDO) from glucose using a recombinant *E. coli* strain. This process is highly efficient and the chemical is used for production of their polymer Sorona®. There have, however, also been many academic studies on production of 1,3-PDO, e.g., from glycerol by obligate and facultative anaerobic bacterial genera such as *Klebsiella*, *Clostridia*, *Lactobacillus* and *Enterobacter* (Fig. 4). Two sequential enzymatic reactions are needed for the conversion of 1,3-PDO from glycerol. In the first reaction, glycerol is converted to 3-hydroxypropionaldehyde (3-HPA) by glycerol dehydratase (GDH) and in the second reaction, 3-HPA is converted to 1,3-PDO by NADH-dependent 1,3-PDO dehydrogenase (Liu et al. 2016). Blocking the glycerol assimilation pathway improved 1,3-PDO production and reduced formation of by-products such as acetate, butyric acid, ethanol, lactic acid,

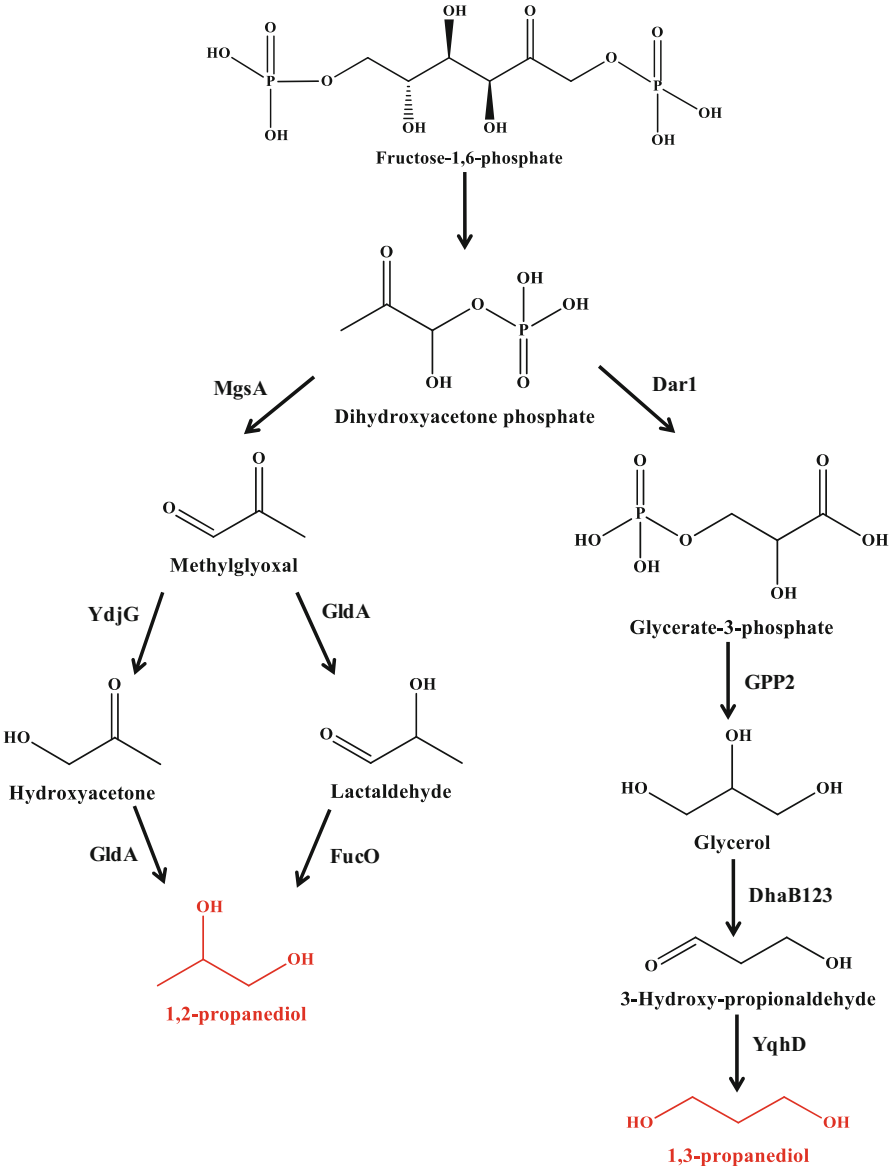

Fig. 4 Pathway for production of 1,2-propanediol and 1,3-propanediol production. *MgsA* methylglyoxal synthase, *DhaB123* glycerol dehydratase, *YdjG* methylglyoxal reductase, *GldA* glycerol dehydrogenase, *FucO* 1,2-PDO oxidoreductase, *YqhD* 1,3-PDO oxidoreductase, *Dar1* glycerol 3-phosphatase dehydrogenase, *Gpp2* glycerol 3-phosphate phosphatase

2,3-butanediol (2,3-BDO), and succinate. The 1,3-PDO production was increased by 45% compared with the wild type under anaerobic and aerobic conditions in a mutant strain containing a truncated lactate dehydrogenase (*ldhA* gene) where 104 g/L of 1,3-PDO has been reported using an engineered *E. coli* strain K-12 ER2925 (Tang et al. 2009). Engineered strains of *C. acetobutylicum* were created by introducing the 1,3-PDO operon of *C. butyricum* VPI 3266. 1,3-PDO production of 101.6 g/L from engineered strains is reported to be the highest in fed-batch culture under anaerobic conditions (González-Pajuelo et al. 2005).

The pathway for the production of 1,2-propanediol (1,2-PDO) from glucose was detected in *Clostridium* species and involves the generation of methylglyoxal from the glycolytic intermediate DHAP via the action of methylglyoxal synthase (Fig. 4). The production titer of 1,2-PDO from an engineered *E. coli* was 5.6 g/L where methylglyoxal synthase, glycerol dehydrogenase and aldehyde oxidoreductase were over-expressed (Clomburg and Gonzalez 2011) . *S. cerevisiae* was engineered to increase the glycerol uptake rate as a strategy to enhance 1,2-PDO production. The methylglyoxal synthase and glycerol dehydrogenase enzymes were over-expressed for the production of 1,2-PDO using a modified YEPD medium containing 1% glycerol and 0.1% galactose. The production titer of 1,2-PDO from engineered *S. cerevisiae* was 2.19 g/L (Jung and Lee 2011).

6.4 Isopropanol

Isopropanol and 1-butanol are relatively better alcohol fuels than ethanol because of their high energy density and low hygroscopicity. Isopropanol is produced by *Clostridium* species using the acetone pathway (Jones and Woods 1986) (Fig. 2). Three genes, *ctfAN* (acetoacetyl-CoA transferase), *thl* (acetyl-CoA acetyltransferase) and *adc* (acetoacetate decarboxylase) from *C. acetobutylicum* ATCC 824 were expressed for acetone and isopropanol production in *E. coli* (Bermejo et al. 1998).

6.5 Butanediol

The two butanediols (BDOs) 2,3-BDO and 1,4-butanediol (1,4-BDO) are broadly used as solvents, cosmetics, drugs, and polymers, but can also be used as biofuels. The company Genomatica has launched a commercial process for production of 1,4-BDO using a recombinant *E. coli* strain (Nielsen and Keasling 2016). Many microorganisms such as *Enterobacter*, *Klebsiella*, *Serratia* and *Bacillus* are capable of producing 2,3-BDO. In *E. coli*, 2,3-BDO production was realized by implementing acetolactate decarboxylase (*budA*), acetolactate synthase (*budB*) and butanediol dehydrogenase (*budC*) genes from *K. pneumoniae* (Liu et al. 2013c) (Fig. 2). A titer of 2,3-BDO of 17.7 g/L was achieved in engineered *E. coli* (Ui et al. 1997). An engineered *Enterobacter cloacae* strain produced a titer of 152.0 g/L (Li et al. 2015). Engineered *B. licheniformis* with deletions of *gdh* and *acoR* genes produced the highest titer of 2,3-butanediol, which is 98.0 g/L in fed-batch

fermentation (Qiu et al. 2016). In *S. cerevisiae*, the production was optimized by the elimination of competing pathways, overexpression of the biosynthetic route and accelerated glucose consumption (Kim et al. 2013).The highest titer of 96 g/L was yielded with glucose in engineered *S. cerevisiae*. *E. coli* was the first organism used for the bio-catalytic conversion of renewable feedstocks into 1,4-BDO with a reported production of 16.5 g/L (Tai et al. 2016) (Fig. 5).

6.6 Biodiesel

Biodiesel is mainly composed of fatty acid methyl esters (FAMEs), propyl esters (FAPEs), and ethyl esters (FAEEs) and is produced from animal fat waste or plant oils. However, as mentioned in the introduction there are certain limitations in the production of biodiesel from animal fats and plant oils, and metabolic engineering of microorganisms is therefore needed to make biodiesel production more desirable (Wackett 2008).

Microbial biodiesel production with *E. coli* was achieved through trans-esterification of ethanol with supplemented oleic acid. Endogenous production of fatty acids was increased by over-expression of fatty acid biosynthesis proteins and through inactivation of ß-oxidation. Fatty acyl-ACPs are intermediates in the fatty acid (FA) synthesis pathway and are precursors to FAEE synthesis (Fig. 6). Initially, fatty acyl-ACPs have to be converted to free fatty acids, which further need to be activated to fatty acyl-CoA and esterified with ethanol to produce FAEEs (Liu et al. 2013a). Biosynthesis of FAEEs was carried out by supplying FAs to the culture media. The titer of FAEE was observed to be 1.28 g/L with oleic acid in the medium. In another study with engineered *E. coli*, ethanol and FA overproduction pathways were manipulated resulting in FAEE production without supplying FAs to the medium (Steen et al. 2010b). Improving the intracellular fatty acyl-CoA pool with overexpression of *E. coli* genes encoding thioesterase I (*tesA*), acyl-CoA ligase (*fadD*), and acetyl-CoA carboxylase (*acc* genes) resulted in a final titer of 0.92 g/L FAEEs (Duan et al. 2011). Approximately 400 mg/L of FAEEs were produced from *E. coli* with expression of the genes of wax ester synthase (*atfA*), malonyl-CoA transacylase (*fabD*) and acyl-CoA thioesterase (*tesA*) (Steen et al. 2010a).

The production of biodiesel from *S. cerevisiae* was initiated by blocking the storage of lipids. Metabolic engineering of the fatty acid pathway with expression of a wax ester synthase enzyme from *Acinetobacter baylyi* enabled the production of a range of fatty acid esters, including ethyl, propyl, isobutyl, butyl and isoamyl esters (Lennen and Pfleger 2013). The combination of several metabolic engineering strategies for improved production of fatty acid ethyl esters in *S. cerevisiae* resulted in stable expression of genes (de Jong et al. 2015). Expression of heterologous wax ester synthase (WS2) from *Acinetobacter calcoaceticus* enabled the formation of different fatty acid esters. This engineered strain mostly produced FAEEs (18.2 mg/L), with a small proportion of FABEs (1.85 mg/L) and FAIEs (2.21 mg/L). A yeast strain with Maqu_0168 (wax ester synthase from *Marinobacter*

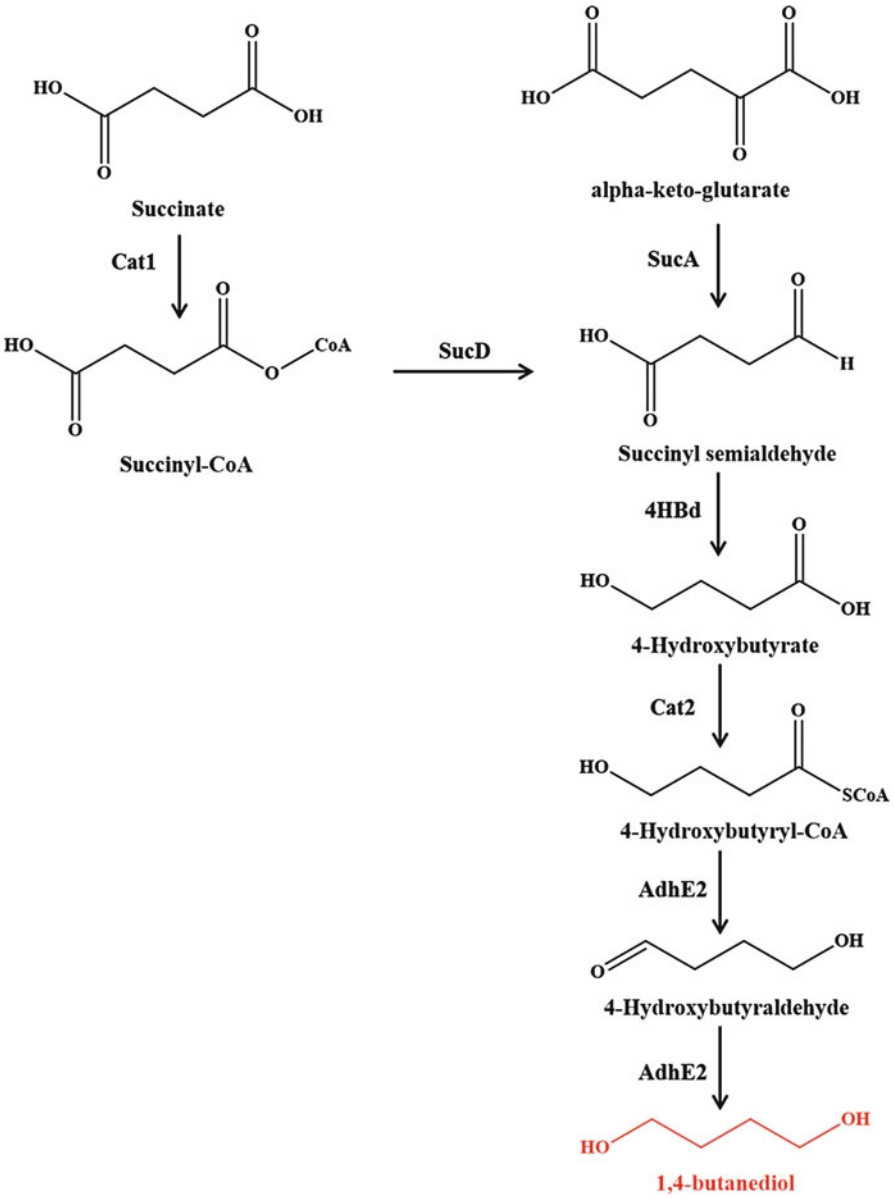

Fig. 5 Pathway for production of 1,4-butanediol. *Cat1* succinate-CoA transferase, *SucD* succinate semialdehyde dehydrogenase, *SucA* 2-oxoglutarate decarboxylase, *4HBd* 4-hydroxybutyrate dehydrogenase, *Cat2* 4-hydroxybutyryl-CoA transferase, *AdhE2* aldehyde/alcohol dehydrogenase

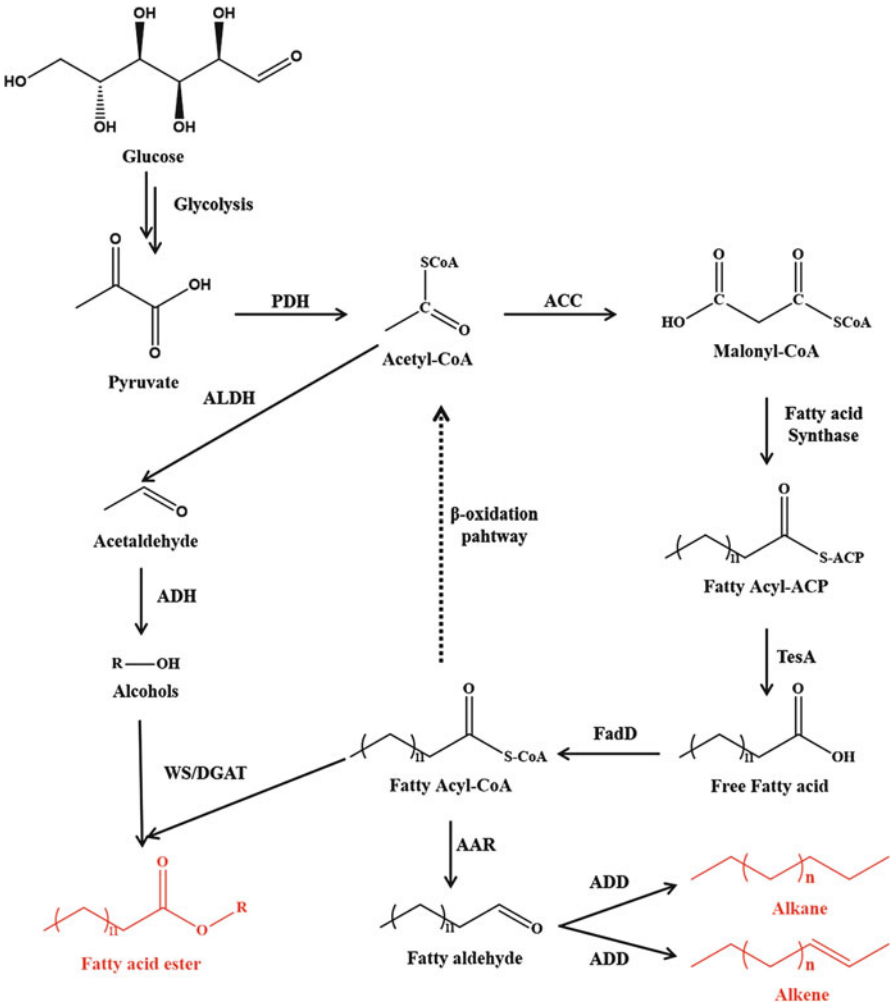

Fig. 6 Pathway for production of fatty acid esters, alkane and alkene. *PDH* pyruvate dehydrogenase, *ACC* acetyl-CoA carboxylase, *TesA* fatty acyl-ACP thioesterase, *FadD* malonyl-CoA-ACP transacylase, *AAR* fatty acyl-CoA reductase, *ADD* aldehyde decarbonylase, *WS/DGAT* wax ester synthase, *ALDH* acetaldehyde dehydrogenase, *ADH* alcohol dehydrogenase

hydrocarbonoclasticus) and acetyl-CoA carboxylase produced a large proportion of FAIEs (96.2 mg/L), less FAEEs (12.2 mg/L), and a small amount of FABEs (3.82 mg/L) (Barney et al. 2012; Teo et al. 2015). In another study, (over)expression of genes *WS2*, *ADH2*, *ALD6, ACC1*^{S1157A,S659A} and *acs*^{1641p} in *S. cerevisiae* led to production of 1,072 ± 160 μg of total FAEEs gCDW^{-1} (de Jong et al. 2015).

6.7 Alkanes

Alkane biosynthesis has been observed in cyanobacteria, and established in engineered *E. coli* and engineered yeasts. Alkanes are superior molecules compared to other biofuels in many aspects, having high energy density and hydrophobicity, and therefore being an ideal drop-in biofuel. Biosynthesis of alkanes from microorganisms is an environment-friendly solution to mitigate the heavy dependence of transport fuels on unsustainable fossil resources (Akhtar et al. 2013; Choi et al. 2014; Harger et al. 2013; Lennen and Pfleger 2013). Alkanes are synthesized intracellularly or extracellularly in small amounts in several microorganisms. Low capability of alkane biosynthesis in microbes is attributed to poor catalytic activity of the last enzyme of the pathway, fatty aldehyde decarbonylase (*ADO* gene) (Andre et al. 2013; Cao et al. 2016; Eser et al. 2011) and accumulation of intermediate fatty aldehydes that might inhibit the enzyme activity and cause toxicity to the cell (Jin et al. 2016; Kunjapur and Prather 2015; Rodriguez and Atsumi 2014). Biosynthesis of alkanes from microorganisms has gained enormous interest because of the above mentioned reasons.

Recently, it was observed that *Aureobasidium pullulans* var. *melanogenum*, isolated from a mangrove ecosystem was able to produce more 21.5 g/L long chain hydrocarbons and *Botryococcus braunii* cells produced 0.262 g/L of hydrocarbons (Xu et al. 2014).

Engineered *Synechocystis* strains, with over-expressed alkane biosynthesis genes (where the carbon was redirected to acyl-ACP), were found to produce 26 mg/L of heptadecane (Wang et al. 2013).

In *E. coli*, the alkane biosynthesis genes of acyl-ACP reductase (*AAR* gene) and ADO were expressed leading to a production of 101.7 mg/L of alkanes (Fig. 6). In another study, genes encoding acyl-coenzyme A dehydrogenase (*fadE*) and fatty acid metabolism regulator protein (*fadR*) were deleted, resulting in over-expression of fatty acyl-CoA reductase (*fabH*) and production of 600 mg/L of alkanes or alkenes (Choi and Lee 2013).

Saccharomyces oviformis, *Candida tropicalis*, and *Candida utilis* are yeasts that are naturally able to synthesize hydrocarbons (Ladygina et al. 2006). Recently, it was also observed that deletion of hexadecenal dehydrogenase (*HFD1* gene), expression of fatty acyl-ACP/CoA reductase (*FAR*) and fatty aldehyde deformylating oxygenase (*FADO*) from *S. elongatus* led to the production of tridecane, pentadecaneand heptadecane in *S. cerevisiae*. This recombinant yeast strain produced 22 μg/g of alkanes (cell dry weight) from glucose (Buijs et al. 2015). In another study, the biosynthetic pathway of alkanes was combined with engineering of the fatty acid biosynthesis resulting in production of 0.8 mg/L alkanes (Zhou et al. 2016a). Through further targeting of the alkane biosynthetic pathway into the peroxisomes alkane production could be further improved, resulting in titers exceeding 2 mg/L (Zhou et al. 2016b).

6.8 Sesquiterpenes

Sesquiterpenes are hydrocarbons derived from the farnesyl-pyrophosphate (FPP). These molecules can be branched and cyclic and therefore have good properties, in particular for jet fuels. The biotech company Amyris has together with the large energy company Total developed a commercial process for production of the sesquiterpene farnesane, which is a branched chain hydrocarbon with valuable properties as a biofuel (Tippmann et al. 2013; Meadows et al. 2016). This molecule can be produced simply by inserting a farnesane synthase into yeast, but in order to obtain recent production level it is, however, necessary to engineer the mevalonate pathway to ensure more efficient provision of FPP (Tippmann et al. 2015). Recently Amyris demonstrated that by complete rewiring of the central carbon metabolism it is possible to obtain a high yielding strain of farnesane (Meadows et al. 2016).

7 Concluding Remarks

In recent years many different biosynthetic pathways, from various organisms, have been engineered for microbial production of biofuels. Typically the reconstruction of a heterologous pathway for production of a novel biofuel results in very low titers, and it is therefore necessary to engineer the central metabolism and optimize the biosynthetic pathway in order to increase the productivity. When building heterologous and/or homologous pathways to engineer a host for biofuel production, it is advantageous to consider the use of native high flux pathways to provide key precursors and co-factors, e.g., to highjack the glycolytic pathway of *S. cerevisiae* as this is able to carry a very high flux during ethanol production. This is important as the efficiency of converting the carbon source into the desired biofuel have considerable impact on the economic feasibility of biofuel production. However, through several rounds of metabolic engineering it is generally possible to improve the yield and productivity of biofuel production as demonstrated in success stories on production of several chemicals.

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Biofuels (Butanol-Ethanol Production)

2

Lawrence P. Wackett

Contents

Abstract

Petroleum-based motor fuels generally consist of hydrocarbons or hydrocarbon fragments. This chemical functionality provides the fuel with a high energy density and a relatively low boiling point, viscosity, and vapor pressure. The premium petroleum fuels are isooctane and hexadecane for spark ignition and diesel engines, respectively. Conventional biofuels differ chemically, but new biofuels that are currently under development more resemble their petroleum counterparts. Current biofuels are principally ethanol and alkanoic acid methyl esters, or biodiesel. The next generation of biofuels will likely have a higher proportion of hydrocarbon fragments than ethanol. These include higher alcohols, ethers, alkanes, and alkenes.

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1 Introduction

Biofuel production is largely comprised of two major phases. Phase one consists of the degradation of biomass to release substrates, mainly sugars, for fermentation by microbes. The other phase consists of microbial fermentative conversion of sugars to one, or a mixture of, fuel molecules. Current commercial biofuels are largely ethanol and fatty acid methyl esters, or biodiesel. Ethanol is derived largely from plant sugars or starches. Biodiesel often derives from plant oils or animal fats. Effective biomass utilization will require more widespread use of cellulosic material. Efficient cellulose conversion to sugars will permit many different plants to be used and a more extensive utilization of waste materials. Cellulose is the most abundant polymer in nature. In nature, microbes annually hydrolyze 10^{11} tons of plant biomass, principally cellulose, and that could theoretically be converted into the energetic equivalent of 640 billion barrels of crude oil (Ragauskas et al. 2006). A major effort is underway to develop cellulose-derived biofuel fermentations. This research is the focus of the United States Department of Energy's Bioenergy Research Centers (Table 1).

Fuel fermentations must conserve a reasonable amount of the carbon of sugar substrate in the final product, deliver an adequate yield of product, and be reasonably robust with regard to process engineering. The current fuel of choice is ethanol because its biosynthesis is well understood biochemically and it, at least marginally, meets the requirements above. But other alcohols, fatty acid esters, and hydrocarbon fuels may prove superior. These different fuel choices are discussed below.

2 Alcohols

Ethanol production today does not differ much from what has been practiced for the past century. The organism used, *Saccharomyces cerevisiae*, is the same. The production facilities are not much different. One new approach for ethanol production,

Table 1 Internet resources for biofuels research

Biofuel resource	Description	URL or reference
BioFuels database	Chemical and biochemical pathways for biofuels	http://www.biofuelsdatabase.org
Joint BioEnergy Institute	Cellulosic ethanol; other renewable energy	http://jbei.lbl.gov
Great Lakes Bioenergy Research Center	Cellulosic ethanol; other renewable energy	http://www.greatlakesbioenergy.org
BioEnergy Science Center	Cellulosic ethanol; other renewable energy	http://www.bioenergycenter.org
American Oil Chemist's Society	Promoting science related to fats, oils, surfactants	http://www.aocs.org
Alternative Fuels Data Center	Alternative fuels: ethanol, hydrogen, methane, propane, biodiesel	http://www.eere.energy.gov/afdc/
Oil from algae	Links to info on algal oils	http://www.oilgae.com

developed in the laboratory, is to use a homoacetogenic bacterium to convert sugars to acetic acid (Eggeman and Verser 2006). The biologically-produced acetic acid is then chemically esterified with ethanol to make ethyl acetate. Ethyl acetate is largely immiscible with water and can thus be efficiently extracted. The ethyl acetate is then hydrogenated chemically. This overall process gives a stoichiometry of one mole of hexose sugar consumed to three moles of ethanol produced. This process could also effectively overcome one of the major limitations of the traditional ethanol fermentation, ethanol solvent toxicity to the producing cell.

The relatively low energy density of ethanol, and its problem of water miscibility, could largely be overcome by switching to a fermentation producing *n*-butanol, a compound that is more hydrocarbon-like than ethanol. An *n*-butanol fermentation by *Clostridium acetobutylicum* has been known for a century. However, the fermentation by this native organism suffers from a low titer of butanol and the co-production of ethanol and acetone. To overcome this, research is ongoing to produce recombinant bacteria that biosynthesize *n*-butanol. For example, *E. coli* strains have been engineered to synthesize acetoacetyl-CoA, 3-hydroxybutryl-CoA, crotonyl-CoA, butyryl-CoA, butyraldehyde, and ultimately *n*-butanol. Generally, low titers have been observed.

Some creative research by Atsumi et al. (2008) has demonstrated that pathways leading to "unnatural" alcohols can be engineered by co-opting amino acid biosynthetic pathways. The products, 2-methyl-1-butanol and 3-methyl-1-butanol, may be superior fuels to both ethanol and *n*-butanol.

3 Biodiesel or Fatty Acid Esters

Fatty acids consist mostly of hydrocarbon chains but the polar carboxylic acid moiety makes simple fatty acids a poor fuel. However, esterification of the acid, typically with methanol, makes a much more non-polar molecule which can be an excellent diesel fuel. As currently manufactured, the fatty acid fragment is derived from biological sources and the methanol typically derives from petroleum. Steinbuchel and his coworkers (Kalscheuer et al. 2006) have demonstrated that a completely biological ester can be prepared via an engineered bacterium that condenses a fatty acid with ethanol derived from the fermentation of glucose.

4 Ethers

Ethers can be excellent fuels. Dimethyl ether is currently used to power buses in Sweden. Dimethyl ether can be derived from biomass via syn-gas processes. A novel catalytic process was recently described to transform biomass-derived fructose into the cyclic aromatic ether, 2,5-dimethylfuran (Roman-Leshkov et al. 2007). 2,5-Dimethylfuran (DMF) has a high energy density of 30 kJ cm^{-3} and a research octane number of 119. Hydrogenation of DMF readily yields 2,5-dimethyltetrahydrofuran (DTHF). DTHF has a higher energy content than DMF

and may be more stable to long-term storage. The compatibility of these ethers with existing engines and their human and ecological toxicology still needs to be addressed.

5 Microbial Hydrocarbon Biosynthesis

Microbes biosynthesize hydrocarbons; alkanes, alkenes, arenes, and isoprenoid compounds. In many cases, the biosynthetic pathways have not been fully elucidated. Currently, there is a growing interest to use these metabolic pathways to generate molecules that can be used as fuels. The goal would be to engineer microorganisms that transform biomass sugars to hydrocarbons that could be used in place of petroleum-based gasoline or diesel fuel.

In this context, a great deal of excitement was generated by recent reports of high-level alkane production by *Vibrio furnissii* M1 (Park et al. 2001). Wild-type *V. furnissii* M1 was reported to biosynthesize diesel fuel alkanes from either pentose or hexose sugars. The yield was on the order of 35% of the sugars consumed. Accounting for energy and cell mass needs, this was near a theoretically optimum yield. Moreover, a novel biosynthetic pathway was proposed to proceed from fatty acids through the corresponding fatty acid alcohol to the respective alkane (Park 2005).

In the light of potential significance of these reports, the *V. furnissii* M1 strain was further investigated via genomic, whole cell metabolic, and cell-free enzyme studies (Wackett et al. 2007). In combination, these new observations all failed to detect alkane biosynthesis, alkane biosynthetic genes, or alkane catabolic genes. Moreover, other *Vibrio furnissii* strains reported by the same research group to produce alkanes were also negative in the follow-up study (Wackett et al. 2007). This points out the need for caution in reports of bacterial hydrocarbon formation. Hydrocarbon contamination is widespread in solvents, stopcock grease, glassware, and bacterial growth media. However, with so many reports of bacterial hydrocarbon biosynthesis (Ladygina et al. 2006), there are likely many good candidate organisms that can be productively investigated.

A separate series of observations were made with numerous *Micrococcus* species (formerly *Sarcina*) and their biosynthesis of a novel class of long-chain alkenes (Albro and Dittmer 1969). The alkenes were proposed to be derived from an unusual condensation of fatty acid molecules, or their thioesters, in a "head-to-head" manner. These alkenes are generally in the range of C_{25}–C_{31} with the double bond at the point of condensation in the middle of the chain. As such, these compounds are solid waxes. However, chemical cracking or biological chain length modification could be invoked to produce novel fuel molecules.

Plants are known to produce 1-alkenes. Mechanistic chemical studies have provided evidence that the compounds derive from a radical decarboxylation of fatty acid chains (Gorgen and Boland 1989).

6 University of Minnesota Biofuels Database

To facilitate research on hydrocarbon biosynthesis, and the generation of biofuels most broadly, the University of Minnesota BioFuels Database (UM-BFD) was created (Wackett 2008). In addition to hydrocarbons, the UM-BFD contains information on the chemical and biochemical synthesis of fuel alcohols, ethers, and esters (Table 1). The database will continue to develop with the assistance of the international biofuels research community.

7 Research Needs

There is still considerable need for new knowledge into the biosynthetic pathways for making desirable biofuel molecules. There is a need for testing new compounds for their compatibility with diesel and spark ignition engines. In parallel, biochemists and microbiologists will be required to identify the genes and enzymes underlying the production of natural products that resemble those fuel molecules. Then, the tools of biological engineering can be brought to bear for manipulating pathways for optimizing structures and product yields. The challenge of massive scale-up also looms prominently in the future. Current large industrial bioprocesses yield hundreds of millions of pounds of material; the market for fuels will require orders of magnitude higher output. This will require a massive investment in research and development.

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Chemicals and Fuels from Microalgae

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Seungjib Jeon, Byeong-ryool Jeong, and Yong Keun Chang

Contents

Abstract

Microalgae are emerging as excellent platforms for producing biofuels, chemicals, and other bioactive molecules. They are collection of distant photosynthetic organisms along the long evolutionary track from prokaryotes to multicellular eukaryotes. Quite different from familiar organisms such as bacteria,

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plants, and animals, they present challenges for research and industrial applications. On the other hand, their diverse characters offer unique opportunities for new products with higher efficiency. They are primary producers of glycerolipids, carotenoids, and other valuable chemicals, of which successful production necessitate understanding of their physiology and genetics. Fortunately, many of these have been found in microalgae, and biological research is following up to improve production of these materials in microalgae. Many microalgae have excellent carbon storage mechanisms for carbohydrates and/or lipids. Lipids in particular represent a wide variety of glycerolipids and carotenoids that can be converted to biofuels and nutraceutical ingredients. Their residues can also be used as feeds or processed to provide carbons for secondary production of value-added products such as fucose from other organisms. It should also be noted that microalgae can be an excellent host for production of recombinant proteins with pharmaceutical or therapeutic values. This review summarizes chemicals, biofuels, and other value-added products that can be produced from microalgae and present improvements and prospects on their successful production in large scale.

1 Introduction

Microalgae are diverse photosynthetic microbial organisms that can be excellent platforms for production of biofuels and other chemicals utilizing renewable and sustainable sources of energy and carbons. Their diverse characters also reflect matching metabolic pathways that can offer opportunities for obtaining unique chemicals (Wilkie et al. 2011; Gimpel et al. 2015; DOE 2016). Microalgae can also adapt to grow in a wide range of environmental conditions and do not compete with food resources. In addition, they are capable of photosynthetic productivity far exceeding that of plants (Hu et al. 2008; Roleda et al. 2012). Even though they lack in-depth understanding of biology and genetics, many techniques and knowledge learned from other model organisms can be applied to achieve significant improvements in producing useful chemicals including lipids and other value-added chemicals.

Lipids produced by microalgae are the most attractive products that can be converted to carbon-neutral biofuels. Glycerolipids including triacylglycerol (TAG) are one of the main groups of lipids from microalgae and can be converted to biofuels including biodiesel, green diesel, and biojet fuel, of which commercialization has already started. Their photosynthetic machinery can be hooked to the production of hydrogen instead of NADPH (Antal et al. 2011; DOE 2016). Microalgae are primary producers of polyunsaturated fatty acids (PUFAs) that can be accumulated in feeders such as fish and other aquatic animals. Nutraceutical values of PUFAs have long been recognized, and microalgae are now serving as feedstocks for PUFAs even at the commercial level for human consumption including baby formula (Hadley et al. 2010).

Carotenoids are another important group of lipids that can be produced by microalgae in large quantities. They are a group of terpenoids composed of eight units of the C5 isoprene (C40) and serve important biological functions not only in microalgal cells but also in organisms that feed on them. Some of them are involved in photosynthesis as photosynthetic pigments, as nonphotochemical quenching (NPQ) dissipating excess light energy into heat, and as antioxidants. Animals are not primary producers of carotenoids but accumulate and/or metabolize carotenoids through the food chain. Carotenoids still play crucial biological roles in animals including humans and thus offer important nutraceutical values. This has enabled commercialization of carotenoids as value-added products (Maoka 2011; Borowitzka 2013; Gimpel et al. 2015).

Furthermore, microalgae offer the potential for production of other exciting materials. They can be excellent hosts of production of therapeutic recombinant proteins with less concerns of toxicity that can be associated with bacterial endotoxins and human pathogens from animal cell culture (Chacón-Lee and González-Mariño 2010; Choi et al. 2010; Hadley et al. 2010). Microalgal extracts are already incorporated and commercialized as cosmetic products by NLP in Korea (Choi et al. 2015). They can also be used as substrates for secondary metabolism, where microalgal hydrolysates can be fermented by bacteria to produce ethanol together with value-added chemicals (Kim et al. 2015; Park et al. 2015). Even with all the beneficial characteristics, microalgae impose barriers to immediate commercialization mainly due to the production cost, which may be caused by paucity of biological and genetic understanding of these relatively unknown organisms compared to other model and industrial organisms. However, with the necessity to find carbon-neutral alternatives of petroleum-based fuels and chemicals, microalgae are considered as the next-generation feedstocks, and research efforts are following up to support this trends. For example, conserved metabolic pathways known in plants (and even in animals) have been used to build microalgal metabolic networks incorporating their unique features (Johnson and Alric 2012, 2013). Transcription factors are critical for regulating lipid metabolism in plants (Wang et al. 2007; Baud et al. 2009), and this concept has been applied to industrial microalgae to enhance lipid productivity (Kang et al. 2015). This review will focus on biofuels, chemicals, and other value-added products that can be produced by microalgae (summarized in Table 1) and formulate metabolic overviews (Figs. 1 and 3) for improved production of target molecules. Genetic engineering is in progress to improve production of these chemicals (briefly summarized in Fig. 2), and accompanying toolbox development is also presented (Table 2).

2 Metabolic Outlines for Biofuel Production in Microalgae

Microalgae, being photoautotrophic, can fix carbon dioxide into organic carbons by using carbon dioxide as a carbon source and sunlight as an energy source. The fixed carbons can be utilized for cell growth via the central carbon metabolic pathways including Calvin cycle, glycolysis, and tricarboxylic acid (TCA) cycle (Johnson and

Table 1 Chemicals and biofuels that can be produced from microalgae

Products	Host cells	Purpose	Production scheme and productivity	References
Lipids for biofuels				
Biodiesel	*Nannochloropsis oculata* NCTU-3	FAME as biofuels	Photo-bioreactor (800 mL) lipid productivity: 0.142 g L^{-1} d^{-1}	Chiu et al. (2009)
Green diesel	Microalgae oil (provided by Verfahrenstechnik Schwedt GmbH)	Upgraded hydrocarbons as biofuels	Increase of the C18 n-octadecane yield from 65% to 84%	Peng et al. (2012)
Biojet fuel	Algal triglycerides (provided by the Air Force Research Laboratory)	Upgraded hydrocarbons as biofuels	Conversion (TAGs to alkanes): 85% Cracking and isomerization: 43%, 59%, and 93%	Robota et al. (2013)
Lipids for food				
EPA	*Nannochloropsis oceanica* CY2	Health supplement	Semibatch operation (1 L) 14.4 mg L^{-1} d^{-1}	Chen et al. (2015a)
DHA	*Crypthecodinium cohnii*	Health supplement for reducing blood triglyceride in rats	Preclinical trial and commercial production (life's DHA™ by Martek)	Hadley et al. (2010)
DHA	*Aurantiochytrium limacinum* SR21	Health supplement	Fermentor (5 L) 122.62 mg L^{-1} h^{-1}	Huang et al. (2012)
Biohydrogen				
Biohydrogen	*Chlamydomonas reinhardtii* CC124 and D239–40	Hydrogen as biofuels	Erlenmeyer flask (150 mL): 140 mL L^{-1} d^{-1}	Oncel et al. (2014)
Biohydrogen	*Chlorella* sp. NIER-10003	Hydrogen as biofuels	Cylindrical glass reactor (1 L) 238 mL h^{-1} L^{-1}	Song et al. (2011)
Carotenoids				
Astaxanthin	*Haematococcus pluvialis* CCAP 34/8	Coloring substances	Continuous chemostat tubular (50 L): 8 mg L^{-1} d^{-1}	Garcia-Malea et al. (2009)
Beta-carotene	*Dunaliella salina* CCAP 19/18	Antioxidants as health supplement	Bubble-column bioreactor (450 mL): 2.45 mg m^{-2} d^{-1}	Hejazi et al. (2004)
Lutein	*Chlorella sorokiniana* 211–32 from UTEX	Food dye and nutraceutical agents	Batch culture (1 L) lutein: 4.2 mg L^{-1} d^{-1}	Cordero et al. (2011)

(*continued*)

Table 1 (continued)

Products	Host cells	Purpose	Production scheme and productivity	References
Recombinant proteins				
Antibody	*C. reinhardtii* 137c	Human antibody HSV8-lsc (pharmaceutical)	High levels of soluble protein accumulation	Mayfield et al. (2003)
Peptide	*C. Reinhardtii* 137c	Human VEGF isoform 121 (therapeutics)	Up to 2% of the total soluble protein	Rasala et al. (2010)
Peptide	*C. Reinhardtii* 137c	CSFV-E2 for vaccine	Up to 1.5–2% of the total soluble protein	He et al. (2007)
Hormone	*Chlorella ellipsoidea* KMCC C-20	fGH for aquaculture	Up to 400 μg/L of algal culture	Kim et al. (2002)
Biomass and other extracts				
AD substrate	*Nannochloropsis oceanica*	Hydrolysates for fermentation substrates	Production of alcohols (2,3-BDO and ethanol) by microorganisms	Kim et al. (2015); Park et al. (2015)
Cosmetics	*Chlorella ellipsoidea* and *Tetraselmis* sp.	Algal extract for cosmetics	Production of Yezena cosmetics	Choi et al. (2010)

AD anaerobic digestion, *2,3-BDO* 2,3-butanediol, *CSFV-E2* classical swine fever virus E2 viral protein, *DHA* docosahexaenoic acid, *fGH* flounder growth hormone, *PUFAs* polyunsaturated fatty acids, *VEGF* vascular endothelial growth factor

Alric 2013) as summarized in Fig. 1. Microalgae are flexible in metabolic modes and thus are capable of growth in the presence of organic carbons exclusively (heterotrophic) or together with carbon dioxide (mixotrophic) (Li et al. 2011b). Understanding these metabolic modes may be important for boosting biosynthesis of carbohydrates and lipids that are important components of biomass and biofuels, respectively. Under stress conditions including N starvation, microalgae switch metabolic pathways toward storage of carbons into starch and then into lipids (Li et al. 2011a). Organic carbon sources such as acetate and glucose are preferentially used for the synthesis of storage carbon molecules possibly due to reduced photosynthesis (Fan et al. 2012). These findings suggest that the mixotrophic mode of metabolism can lead to synergistic outcome of lipids production from inorganic carbon dioxide and organic carbons such as acetate and sugars. Both of the carbon sources are easy to obtain as industrial wastes, so this approach has multiple benefits of biofuel production. The metabolic outline presented in Fig. 1 is meant to provide a general direction; it thus lacks details of metabolic pathways that have already been reviewed in an excellent manner (Johnson and Alric 2012, 2013). Explanations of individual pathways are placed in the following section where appropriate.

When organic carbon sources are provided, there are a few genetic criteria that should be considered for successful production of biofuels. Firstly, genes required for the uptake of organic carbons should be present in the microalgae that are to be

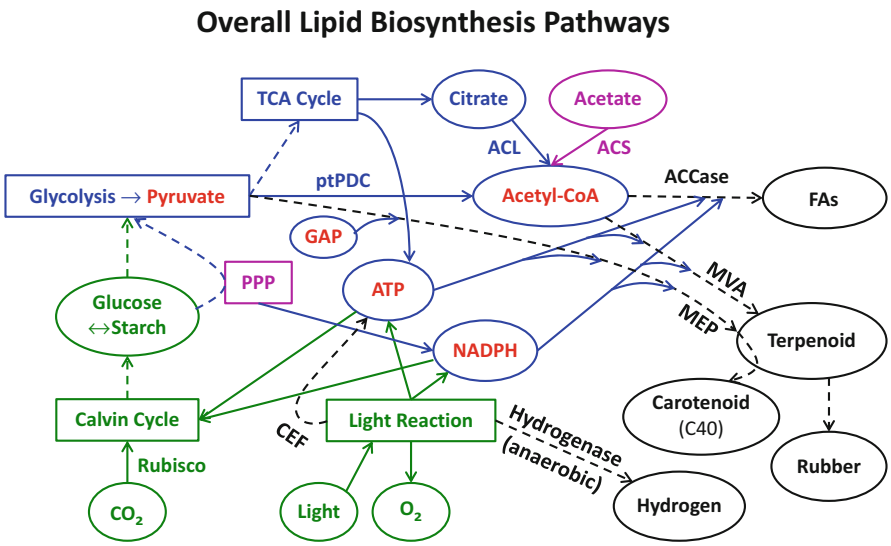

Fig. 1 Summary of metabolic pathways for production of lipids and hydrogen in microalgae. Central carbon pathways are colored *blue,* and five key metabolites are lettered in the *red* color. Photoautotrophic components are colored *green* and heterotrophic in *purple* with the exception that PPP can also be photoautotrophic. Pathways that may include multiple steps are indicated by *dotted lines*. Abbreviations: *ACCase* acetyl-CoA carboxylase, *ACL* ATP-citrate lyase, *ACS* acetyl-CoA synthetase, *CEF* cyclic electron flow, *GAP* d-glyceraldehyde 3-phosphate, *TCA* tricarboxylic acid cycle, *MEP* methyl-erythritol-4-phosphate pathway, *MVA* mevalonic acid pathway, *PPP* pentose phosphate pathway, *ptPDC* plastidic pyruvate dehydrogenase complex

used for biofuel production. Organic carbons are in general polar, and they cannot be diffused through the plasma membrane; they thus require specific transporters (Allen et al. 2011; Johnson and Alric 2012). Once inside cells, there should be proper metabolic enzymes and pathways for efficient use of the organic carbons. Acetate, for example, can readily be assimilated into acetyl CoA by acetyl-CoA synthetase (ACS), which has been known to be used for FA biosynthesis under N starvation in *Chlamydomonas* (Johnson and Alric 2013; Lauersen et al. 2016). ATP-citrate lyase (ACL) is considered as an "oleaginous gene" in yeast and can contribute to FA biosynthesis by transferring carbons from citrate to acetyl CoA (Vorapreeda et al. 2012). For FA biosynthesis, sources of energy (from ATP) and reducing power (from NADPH) are also important. Under normal conditions, ATP is produced by the normal photosynthetic process, called photophosphorylation, while NADPH by ferredoxin-NADP reductase (FNR) via linear electron flow. These photosynthetic products are required for carbon fixation via the Calvin cycle. However, under stress conditions, photosynthesis is inactivated, and ATP can be supplied by the cyclic electron flow (CEF) and NADPH by oxidative pentose phosphate pathway (OPPP), which are used for synthesis of FAs and other neutral lipids (Johnson and Alric 2012, 2013; Chen et al. 2015b).

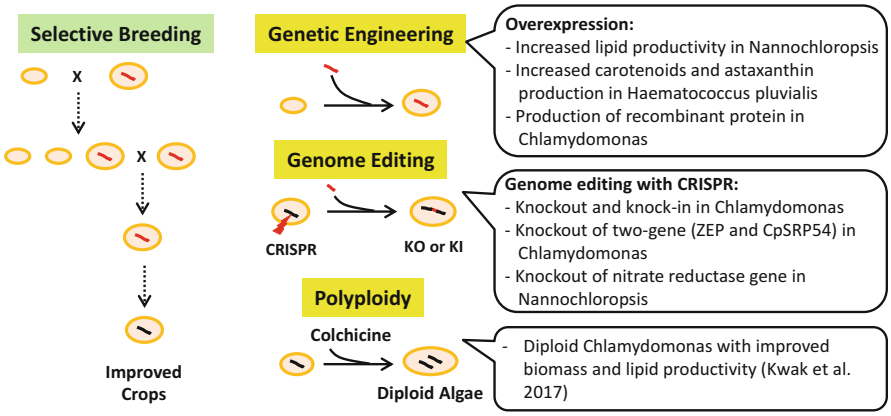

Fig. 2 Classic improvements of crops and possible strategies for genetic engineering of microalgae. Selective breeding has been employed by breeders as classic strategies of crop improvements (*left panel*). Modern genetic engineering technologies have enabled faster and more reliable improvements (*right panel*). These include direct introduction of beneficial gene(s), genome editing techniques using engineered DNases including CRISPR/Cas9, and generation of polyploidy using inhibitors of chromosome segregation such as colchicine. CRISPR/Cas9 is becoming very popular and can be used for many purposes including knockout (KO) and knockin (KI). These technologies are now being applied to algal biotechnology for the production of biofuels and biomaterials

3 Transformation Toolbox and Genetic Engineering in Microalgae

Genetic engineering of microalgae is a key step for improving production of biofuels and other biomaterials. Now that microalgal products are slated to appear commercially in a large scale, microalgae can be considered as a "crop," and we should reflect on the agricultural history of crop improvements. Humans have long been domesticating wild plants for staple crops, including rice, wheat, and many other plants (Doebley et al. 2006). The domestication process involved improvements in crop quantities as well as qualities, mostly by selective breeding of naturally occurring variants. This process inevitably included genetic selection and/or isolation of beneficial genes for the improvements. Modern science has devoted efforts to identify the beneficial genes and used these for improving crops by delivering and/or modifying the genes, which we may call genetic engineering of crop plants, as summarized in Fig. 2. Accompanying technologies have been developed to accomplish these improvements. These include direct introduction of genes into the plants by using transformation techniques, genome editing, and polyploidization. Among these, genome editing is now being performed by engineered DNases such as Cas9 in the clustered regularly interspaced short palindromic repeats (CRISPR) system (Kim and Kim 2014), which is now accomplished in microalgae (Shin et al. 2016). An interesting phenomenon accompanying crop improvements during the selective breeding is polyploidization that appears to improve biomass and stress

tolerance (Dubcovsky and Dvorak 2007), and this concept is being tested successfully in the model microalga *Chlamydomonas*.

Transformation techniques are essential for genetic engineering of an organism for improved production of biofuels and other biomaterials. Transformation of microalgae can be achieved by electroporation, particle bombardment, agitation with glass beads, and PEG-mediated delivery (Hawkins and Nakamura 1999; Kumar et al. 2004; Feng et al. 2009; Radakovits et al. 2010; Zhang et al. 2014; Kang et al. 2015) as summarized in Table 2. Glass bead agitation has been used for

Table 2 Summary of transformation toolbox and genetic engineering in microalgae

Algal species/ strain	Transformation technique	Markers/target genes	Outcome	References
C. Reinhardtii CC-124	Electroporation	MAA7, CpSRP45 and ChlM genes	Targeted gene knockout	Shin et al. (2016)
C. Reinhardtii 137 cc	Particle bombardment	aadA gene/CSFV-E2 for vaccine	Up to 1.5–2% of the total soluble protein	He et al. (2007)
C. Reinhardtii CC-503	Glass bead agitation	APHVIIIgene/shrimp yellow head virus	Improved 22% survival rate after YHV challenge	Somchai et al. (2016)
Chlorella ellipsoidea	Electroporation	Neomycin phosphotransferase II/soybean transcription factor GmDof4	Increase of lipid content by 46.4–52.9%	Zhang et al. (2014)
Chlorella sorokiniana ATCC-22521	PEG-mediated transformation	Neomycin phosphotransferase II/human growth hormone	Up to 200–600 ng/mL of algal culture	Hawkins and Nakamura (1999)
Chlorella vulgaris C-27	PEG-mediated transformation	Neomycin phosphotransferase II/human growth hormone	Up to 200–600 ng/mL of algal culture	Hawkins and Nakamura (1999)
Chlorella zofingiensis ATCC 30412	Particle bombardment	Modified phytoene desaturase gene	Resistance to norflurazon	Liu et al. (2014)
Dunaliella salina UTEX 1644	Glass bead agitation	GUS gene	Transgene expression	Feng et al. (2009)
Dunaliella salina CCAP 19/18	Particle bombardment	CAT gene/malic enzyme and ACCase genes	Increase of total lipid by 12%	Talebi et al. (2014)
Nannochloropsis sp. W2J3B	Electroporation	Shble gene/nitrate reductase and nitrite reductase	Targeted gene knockout	Kilian et al. (2011)
Nannochloropsis salina CCMP1776	Particle bombardment	Shble gene/bHLH transcription factor	Increased biomass productivity by 36%	Kang et al. (2015)
Nannochloropsis oceanica IMET1	Electroporation	HygR gene/nitrate reductase gene	Targeted gene knockout	Wang et al. (2016)

protoplasts of *Chlamydomonas* (Somchai et al. 2016); however, due to difficulties in removal of cell wall, other techniques are used for other microalgal transformation. Electroporation and particle bombardment are in general used for other microalgae due to the simplicity of the techniques (Feng et al. 2009; Maruyama et al. 1994; Kilian et al. 2011; Zhang et al. 2014; Shin et al. 2016).

Albeit at the infant stage of the true genetic engineering, compared to that of other industrially important dairy animals and crop plants, microalgae are now being subject to genetic engineering to improve production of biomaterials (Radakovits et al. 2010) as summarized in Table 1. Such examples can be found in *Dunaliella salina* for enhancing carbon flux to lipid biosynthesis by overexpressing metabolic enzymes (Talebi et al. 2014). In addition, overexpression of NsbHLH2, a bHLH transcription factor, has been shown to increase production of biomass and lipids in *Nannochloropsis salina* (Kang et al. 2015), opening an exciting possibility toward reliable genetic engineering of industrial strains of microalgae. *Haematococcus pluvialis* is the main producer of astaxanthin, and overexpression of beta-carotene ketolase in *H. pluvialis* has been shown to significantly increase total carotenoids and astaxanthin (Kathiresan et al. 2015). Recombinant proteins, including vaccines, antibodies, and hormones, are also important high-value products that can be produced in microalgae (Specht et al. 2010; Rasala and Mayfield 2015). In addition to the scheme of overexpression, genome editing techniques employing the most recent and popular CRISPR/Cas9 have been reliably enabled in microalgae (Baek et al. 2016; Shin et al. 2016; Wang et al. 2016). Development of these overexpression and knockout techniques is expected to accelerate production of biomaterials in microalgae to the commercial scale.

4 Production of Lipids for Biofuels from Microalgae

Microalgae are capable of accumulating lipids that can be converted into biofuels including biodiesel, biojet fuels, and green diesel (DOE 2016). Due to their significantly higher photosynthetic productivity compared to other biofuel feedstocks, they can offer higher lipid yield on a smaller area of land (Satyanarayana et al. 2011). Carbohydrates are also important biomass that can be converted to biofuels directly or indirectly, but direct conversion is less efficient due to their high oxygen content (Huber and Corma 2007). Indirect use of algal biomass containing carbohydrates via fermentation is also an attractive biofuel production scheme (Kim et al. 2015; Park et al. 2015). However, this review will focus on microalgal TAGs that are supposed to be the most efficient source of biofuels and infrastructures required for the conversion exist for TAGs originated from plants and animals (Huber and Corma 2007; MacDougall et al. 2011).

Biodiesel can be produced by catalytic transesterification of TAGs into fatty acid alkyl esters including fatty acid methyl esters (FAMEs) and offers advantages

over the conventional diesel such as renewability, carbon neutrality, environmental safety, and biodegradability (Kolesárová et al. 2011). Since the TAGs are the main component of biofuel feedstocks, it is critical to understand metabolic pathways for TAG biosynthesis, as summarized in Fig. 1. This summarizes overall metabolic flow of energy and carbon leading to biosynthesis of carbon-based molecules, as well as biohydrogen that will be described in the next section. Microalgae and plants, being at the bottom of the food chain, produce organic carbons from carbon dioxide, the waste products of the respiration of life and the combustion of man-made machines. The carbon fixation process accompanies production of oxygen from water by using the sunlight that truly represents renewable and sustainable production of essential materials for all life. Nevertheless, microalgae are better producers of certain materials than plants, because they have simpler life cycle and morphology leading to higher photosynthetic productivity. However, microalgae are different from plants, so are their metabolic pathways, which necessitate complete understanding of their metabolism in order to achieve successful production of materials. Their unique (as well as conserved) metabolic pathways including the central carbon metabolism are being elucidated in microalgae using the model organism *Chlamydomonas* (Johnson and Alric 2013). Oversimplified version of their central carbon metabolic pathways is presented in Fig. 1 with the emphasis of metabolic modes (autotrophic and heterotrophic) and sources of metabolic components (ATP for energy and NADPH for anabolic reducing power). The latter two are commonly required for most biosynthetic pathways including those for fatty acids (FAs) and terpenoids. Metabolic modes are also critical for biosynthetic processes, since the carbon sources (carbon dioxide for autotrophic and organic carbons for heterotrophic mode) may have preference for certain carbon partitioning into lipid (Fan et al. 2012; Johnson and Alric 2013; Polle et al. 2014; Jia et al. 2015). Understanding of these metabolic pathways is essential for metabolic engineering of microalgae for production of biofuel-related chemicals.

Other carbon-based biofuels, including the green diesel and biojet fuel, can also be obtained by alternative catalytic conversions of lipids. The prefixes “green” and “bio” signify fuel specifications that match the petroleum counterparts but should be originated from biological sources (DOE 2016). The main difference of these biofuels from the aforementioned biodiesel is that they lack oxygen in the final products. Production of these employs various catalytic conversions such as hydrocracking and hydroprocessing of lipids (Liu et al. 2015, 2016). There are technology and infrastructure that have been used to process petroleum-based fuels, but optimization for the biofuel remains, particularly removal of oxygen and other biological chemicals (Huber and Corma 2007; Kim et al. 2014; DOE 2016). Jet fuels are similar to diesel, but they require specifications for aviation purposes approved by the American Society for Testing and Materials (ASTM). These conversion technologies of biofuels started with plant lipids and now being applied to microalgal lipids (Robota et al. 2013; Liu et al. 2015, 2016; Yang et al. 2016b).

5 Biohydrogen from Microalgae

Hydrogen can be produced by microalgae under special anaerobic conditions if they contain hydrogenases and maturation proteins. Since it can also be produced chemically, microalgal hydrogen will be designated biohydrogen. Microalgae produce biohydrogen by using only water and sunlight, and, best of all, combustion of hydrogen does not produce the greenhouse gas carbon dioxide (Vieler et al. 2012; Gupta et al. 2013; Antal et al. 2015). Together with the fact that hydrogen is the most energy-dense fuel, it is considered as the most advanced and eco-friendly biofuel in the future (DOE 2016). Hydrogenases are the key enzymes required for the production of biohydrogen in microalgae; however, there are a few challenges in commercial production of biohydrogen, including low yield, high production cost, and anaerobic requirements of hydrogenases in the oxygen-evolving photosynthetic organisms (Gupta et al. 2013; DOE 2016). In addition to the use as biofuels, hydrogen can also be used for production of green diesel and biojet fuel via hydrocracking or hydroprocessing (Vardon et al. 2014; Gong and You 2015).

Biohydrogen production can be achieved through a few different routes: mainly photobiological and fermentative pathways. The photobiological pathway is more eco-friendly and requires light to initiate electron flow from splitting the water molecule and concomitant generation of protons, both of which are eventually converted to molecular hydrogen by hydrogenases (Melis et al. 2000; Gupta et al. 2013). Fermentation utilizes stored carbohydrate as electron donor for hydrogenase to produce biohydrogen (Kumar et al. 2016). However, fundamental problems exist in biohydrogen production, i.e., the extreme sensitivity of hydrogenases to oxygen. This may be solved by creating conditional anaerobic conditions in culture, such as sulfur starvation, or by genetic mutations including state transition mutant 6 (Stm6) that shows more efficient hydrogen production (Antal et al. 2011; Song et al. 2011; Oncel et al. 2014; Volgusheva et al. 2016). Under the anoxic conditions, microalgal cells can provide electrons through the alternative electron flow near the PSI, the so-called cyclic electron flow (CEF) as summarized in Fig. 1 (Antal et al. 2011, 2015). Genetic engineering can also be employed, since manipulation of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) can change carbon flux toward more efficient biohydrogen production (Kumaraswamy et al. 2013). Cyanobacterial hydrogenases are more oxygen tolerant, which can be beneficial for biohydrogen production if introduced into microalgae (Ghirardi et al. 2007).

6 Polyunsaturated Fatty Acids (PUFAs) from Microalgae

Fatty acids with the medium and long chain, particularly PUFAs with multiple double bonds, have excellent nutritional and pharmaceutical values. Microalgae are the primary producers of PUFAs, and they have enzymes required for biosynthesis of PUFAs, including desaturases and elongases. Some of these enzymes are

not present in animals including humans; therefore, we have to obtain PUFAs from food such as fish that are direct or indirect predators of microalgae. Microalgae are now excellent platforms for the production of PUFAs including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Adarme-Vega et al. 2012; Huang et al. 2012; Chen et al. 2015a), and a few products including life'sDHA™ have been commercialized by Martek (Hadley et al. 2010).

EPA (C20:5 n-3) and DHA (C22:6 n-3) are long-chain omega-3 fatty acids, and they have been used as food supplements for health benefits. They also have pharmaceutical values, entitling them as high-value coproducts of biofuel production from microalgae (Adarme-Vega et al. 2012). In general, heterokonts including diatoms, *Nannochloropsis*, and *Thraustochytrium* are good producers of PUFAs containing up to 30–40% of total fatty acids. Enhancement of biosynthesis of PUFAs can be achieved by biological and genetic engineering in microalgae. Biological engineering includes changes in cultivation conditions, where certain stress conditions induce biosynthesis of PUFAs (Sukenik 1991; Yongmanitchai and Ward 1991; Zhu et al. 2007; Chen et al. 2015a). Screening of mutations (Chaturvedi and Fujita 2006) and overexpression of one of the desaturases (Kaye et al. 2015) have been shown to increase the level of PUFAs.

7 Carotenoids from Microalgae

Biosynthesis of carotenoids is preceded by pathways to produce terpenoid precursors, which include methyl-erythritol-4-phosphate (MEP) and mevalonic acid (MVA) pathways (Fig. 1). The MEP pathway is present in cyanobacteria and in chloroplasts of plants and algae; hence, it is thought as the prokaryotic origin. Precursors for this pathway are pyruvate and glyceraldehyde-3-phosphate (GAP), which lead to the biosynthesis of carotenoids. The MVA pathway occurs in the cytoplasm and starts with acetyl CoA as the carbon donor for different sets of isoprenoids, and these two pathways can have crosstalk to share the precursors (Davies et al. 2015). The MEP pathway is activated by stress in microalgae, similar to the TAG biosynthesis pathway (Li et al. 2010). Carotenoids also accumulate as plastoglobuli in the chloroplast under stress, similar to TAG being accumulated in the lipid body (Davidi et al. 2015), which can be employed in increased production of carotenoids.

Carotenoids produced by microalgae include astaxanthin, beta-carotene, and lutein, to name a few. Astaxanthin is a red ketocarotenoid used in cosmetic, therapeutic, and food industries. *Haematococcus pluvialis* is the main producer of astaxanthin under phototrophic condition (Del Campo et al. 2007), even at industrial scale in outdoor photobioreactors (Garcia-Malea et al. 2009). Beta-carotene is used as provitamin A, antioxidant, and coloring agent and mostly produced by *Dunaliella salina*. Its accumulation is enhanced by stress conditions; the production involved two-step processes: firstly, cell growth followed by transfer of stationary culture to stress conditions including high-light stress (Hejazi et al. 2004). Lutein is used as

food dye, antioxidant, and nutraceutical agent against macular degeneration playing important functions in vision. Lutein can be produced in microalgae including *Chlorella zofingiensis* (Del Campo et al. 2004), *Coccomyxa acidophila* (Casal et al. 2011), *Scenedesmus almeriensis* (Sanchez et al. 2008), and *Chlorella protothecoides* (Shi et al. 2002).

Genetic engineering of carotenoid production is now being pursued in microalgae, thanks to further understanding of metabolic mechanisms of terpenoids including carotenoids in microalgae (Davies et al. 2015). Terpenoid biosynthesis in microalgae can be summarized as in Fig. 3, focused on the MEP pathway that is considered the only terpenoid pathway in microalgae including *Chlamydomonas*, *Botryococcus*, and *Nannochloropsis* (Molnár et al. 2012; Polle et al. 2014; Davies et al. 2015). The MEP pathway starts with the generation of carbon source molecules, glyceraldehyde-3-phosphate (GAP) and pyruvate from the Calvin cycle and glycolysis, respectively. The two molecules are combined to 1-deoxy-d-xylulose 5-phosphate (DXP), catalyzed by 1-deoxy-d-xylulose 5-phosphate synthase (DXS), the key enzyme for the terpenoid biosynthesis and has been used for genetic engineering (Davies et al. 2015). Next, the DXP is converted to methylerythritol-4-phosphate (MEP) by the enzyme coded by the 1-deoxy-d-xylulose 5-phosphate reductoisomerase (DXR). MEP is converted to precursors for terpenoids: isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), catalyzed by 4-diphosphocytidyl-2-C-methylD-erythritol synthase (IspD), 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (IspE), 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (IspF), 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase (IspG), and 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase (IspH). The interconversion of IPP and DMAPP is catalyzed by isopentenyl diphosphate isomerase (IDI) for balancing the ratio between IPP and DMAPP. The two precursors are combined to form terpenoids with different number of carbons. The shortest molecule is isoprene (C5), catalyzed by isoprene synthase (IspS). Isoprene production can be enhanced by genetic engineering of the *IspS* gene, and isoprene serves as an important monomer for the production of polymers including rubber (Razeghifard 2013; Lv et al. 2014). Further combination of IPP and DMAPP produces geranyl pyrophosphate (GPP) and geranylgeranyl pyrophosphate (GGPP). GPP is used for biosynthesis of monoterpenes (C10) and GGPP is used for biosynthesis of diterpenes (C20) and carotenoids (C40), catalyzed by phytoene desaturase (PDS) and phytoene synthase (PSY). The resulting β-carotene is further processed to astaxanthin, one of the most popular carotenoids from microalgae, and catalyzed by β-carotene ketolase (BKT). These enzymes have been overexpressed to improve the production of carotenoids (Gimpel et al. 2015). In addition to the metabolic engineering of carotenoid production, Fig. 3 is meant to propose another important aspect of engineering: supply of carbon and energy. The MEP pathway utilizes carbons, ATP, and NADPH from the photosynthetic machineries including photosystem I (PSI), photosystem II (PSII), and Calvin cycle. It should be noted that improvement of these photosynthetic machineries need to be preceded before improving the MEP pathway per se.

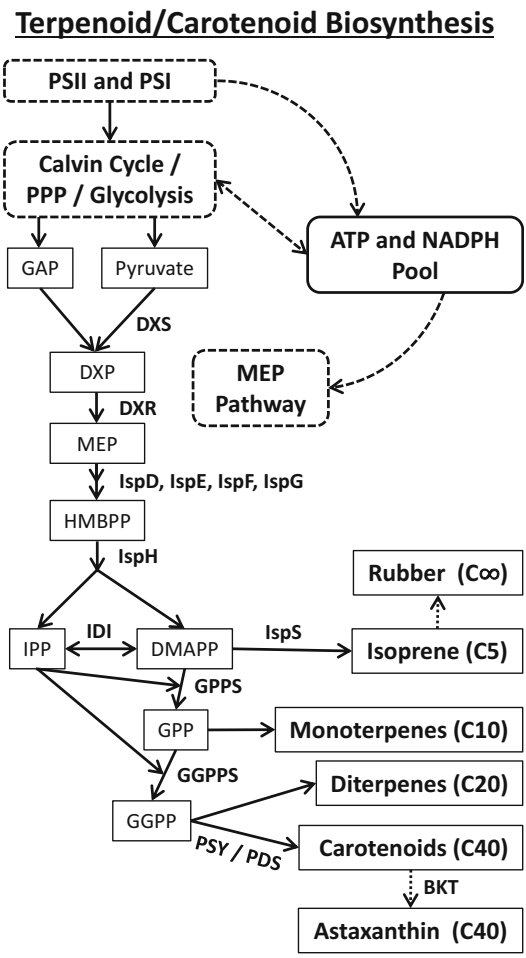

Fig. 3 Biosynthetic pathway and genetic engineering for the production of terpenoids and carotenoids in microalgae. Biosynthesis of terpenoids and carotenoids starts with the generation of metabolites for carbon sources including GAP and pyruvate, resulting in precursors of terpenoids including IPP and DMAPP. These precursors are combined into terpenoids with different carbon numbers including isoprene (C5) and carotenoids (C40). Abbreviations: *BKT* β-carotene ketolase, *DMAPP* dimethylallyl diphosphate, *DXP* 1-deoxy-d-xylulose 5-phosphate, *DXR* 1-deoxy-d-xylulose 5-phosphate reductoisomerase, *DXS* 1-deoxy-d-xylulose 5-phosphate synthase, *GAP* glyceraldehyde-3-phosphate, *GGPP* geranylgeranyl pyrophosphate, *GGPPS* geranylgeranyl diphosphate synthase, *GPP* geranyl pyrophosphate, *GPPS* geranyl diphosphate synthase, *HMBPP* 4-hydroxy 3-methyl-butenyl 1-phosphate, *IDI* isopentenyl diphosphate isomerase, *IPP* isopentenyl diphosphate, *IspD* 4-diphosphocytidyl-2-C-methylD-erythritol synthase, *IspE* 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase, *IspF* 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase, *IspG* 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase, *IspH* 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase, *IspS* isoprene synthase, *MEP* methylerythritol-4-phosphate, *PDS* phytoene desaturase, *PPP* pentose phosphate pathway, *PSI* photosystem I, *PSII* photosystem II, *PSY* phytoene synthase

8 Photosynthetic Production of Recombinant Proteins from Microalgae

In addition to the chemicals mentioned above, microalgae can be excellent platforms to produce recombinant proteins that can be highly valuable in the medical, pharmaceutical, and industrial applications. These include antibodies, vaccines, hormones, and bioactive enzymes, and microalgae emerge as excellent hosts that can compete with traditional hosts for recombinant proteins, including bacteria and other eukaryotic cells (Rasala and Mayfield 2015). Bacteria have been a good host for the production of recombinant proteins, particularly in large scale. However, they do not have proper posttranslational modifications that are necessary for correct functions in eukaryotes and are poor host for large and/or complex proteins where misfolded proteins end up in insoluble structures called inclusion bodies (Galloway et al. 2003; Demain and Vaishnav 2009). Eukaryotic systems including plant and animal cells may produce correct proteins, but they are expensive and hard to achieve large-scale production. Animal cells may also cause immunogenic or pathogenic responses when used in humans (Brooks 2004; Chebolu and Daniell 2009).

Microalgae can offer cost-effective and scalable production of heterologous proteins (Rasala and Mayfield 2015; Yang et al. 2016a). Many microalgae are in general safe for food and cosmetics, considered "generally regarded as safe" (GRAS). Actually microalgal products have been commercialized as nutritional supplements and cosmetics (Pulz and Gross 2004; Chacón-Lee and González-Mariño 2010; Choi et al. 2010; Hadley et al. 2010). They have less chance of immunogenic or pathogenic problems that have been associated with animal or bacterial cells (Brooks 2004; Chebolu and Daniell 2009; Rasala and Mayfield 2015). In addition, there have been interesting themes of microalgal feed technology. One such includes enhancement of growth of fish that feed on microalgae expressing cognate growth hormones, even though it has yet to be elucidated how hormonal peptides have biological effects after the digestive systems of fish (Kim et al. 2002). However, this opens an interesting opportunity to produce oral vaccines in microalgae for human use.

9 Research Needs

Biofuels and value-added products from microalgae are attractive renewable resources; however, it has limitations mainly in the cost of production. To reduce the cost, maximization and/or optimization are needed in many fields including genetic engineering, cultivation, downstream processing, and so on. Different from currently domesticated, and thus industrialized, animals and plants, microalgae are at the infant stage of such domestication, where most of the laboratory strains are the "wild" organisms that have evolved for their survival. We urgently need to develop customized improvements for microalgae to be employed in successful production of biomaterials. We are currently trying to solve the most critical bottleneck, i.e., extremely low transformation efficiency, even though this problem can be partially overcome by customized transformation strategies. Even after solutions

are established, we still need to optimize cultivation and downstream processing. It should be noted that genetic engineering can also improve these processes by repurposing microalgal cells for easier removal of cell walls, optimized oil composition, and secretion of oil contents. Such biological and biotechnological paradigms are already available and/or developed in animals and plants, and proper application of them will ensure successful industrialization of microalgal materials.

10 Concluding Remarks

Microalgae have emerged as excellent platforms for the production of sustainable and renewable biofuels that can replace traditional fossil fuels. They can also be used for value-added products such as carotenoids, PUFAs, and recombinant proteins. However, there are several issues associated with algal biofuels compared to other biofuels, including higher production/processing costs and technical problems associated with processing the whole biomass in contrast to the targeted biomass such as seeds of plants. These processing problems are due to the residual and/or contaminating cellular components including phosphorous from phospholipids, nitrogen from extracted proteins and metals (especially magnesium from chlorophyll), and chloride anions if cultivated in salty water (DOE 2016). These problems can be partly solved by making the production system more efficient. Nowadays, comprehensive system biology is employed to improve biofuel and other biomaterial productions (Jamers et al. 2009). Genetic engineering of microalgae is also being actively pursued for the same purpose. Many efforts have been focused on overexpression of metabolic genes (Yao et al. 2014; Xue et al. 2015); however, new trends of utilizing transcription factors emerge with good results in industrial strains of *Nannochloropsis* (Kang et al. 2015).

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Biodiesel from Microalgae

4

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Abstract

The development of industrial processes for large-scale production of biofuels, in particular biodiesel, is one of the most pursued purposes of research teams, companies, and governments all in the world, as consequence of a necessary reduction of CO_2 emissions and the need of renewable and affordable energy sources.

However, several constraints strongly limit biodiesel production, and its use, basically, is as additive blended with petrodiesel.

Microalgae are photosynthetic microorganisms which can convert CO_2 into triacylglycerols, and then, since decades, they have been considered as a potential innovative feedstock for biodiesel production, able to successfully replace oil crops.

Despite the considerable research and funding efforts, up to now biodiesel from microalgae is still an expensive process, because no significant reduction in cost of the downstream processing of biomass (biomass separation and drying and oil extraction) has been achieved.

Therefore, biodiesel production may be considered as part of a hypothetical process which produces several high-value added microalgae-based products, as pharmaceuticals or nutraceuticals.

However, research and capital investments in biodiesel production from microalgae show a positive trend up to date.

1 Introduction

In 1997, 37 industrialized countries and the European Community (at that time, formed by 15 member States) undersigned the Kyoto Protocol, a treaty in which the global warming and CO_2 emissions were recognized as the main cause of the climate changes. In this treaty, the countries committed to reduce greenhouse gas emissions produced by industry, in order to stabilize climate.

Not all the industrialized countries (e.g., the United States of America) ratified the Kyoto protocol, and several criticisms have been raised about the commitments and, later, the effective results obtained.

Recently, in December 2015, at the Paris Climate Conference, 195 countries accepted a new treaty, in which the governments agreed to aim to limit the global temperature increase to 1.5 °C, by reducing the greenhouse gas emissions.

To realistically achieve the expected results of Paris Climate Conference, two main guidelines should be followed, that is, assessment of large-scale systems for greenhouse gas sequestration and, overall, the progressive replacement of fossil fuels with other eco-friendly energy sources, such as biofuels.

Among biofuels, biodiesel is the most used for road transport in compression–ignition (diesel) engines.

Biodiesel is a renewable substitute for diesel fuel (Mittelbach and Remschmidt 2005), that is, CO_2 neutral, since the amount of CO_2 produced during its combustion

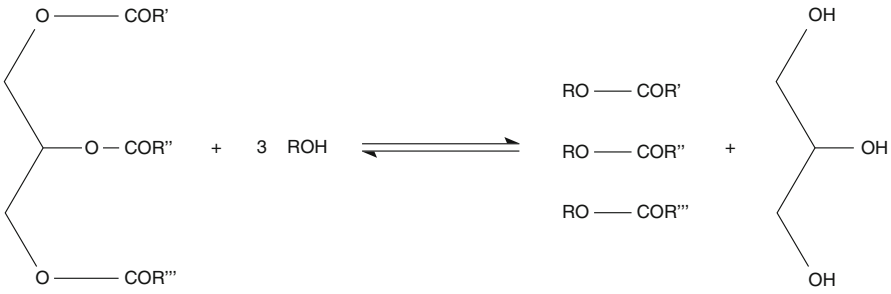

Fig. 1 Alcoholysis of a triacylglycerol for the production of fatty acid alkyl esters (FAAE) (Originally published in Salis et al. 2010a, published with kind permission of © Springer Science+Business Media New York, 2003. All rights reserved)

is the same that oleaginous plants absorbed during their growth. Traditional raw materials for worldwide biodiesel production are mainly coming from four oil crops: rapeseed oil, sunflower oil, soybean oil, and palm oil. In addition, some other oleaginous species such as *Jatropha curcas*, a plant that grows in harsh soils, and low value triacylglycerol feedstocks (restaurant grease, animal fat) are being used as cheap substrates for biodiesel production.

From the chemical point of view, biodiesel is a mixture of fatty acid alkyl esters obtained by alcoholysis of triacylglycerols in the presence of a catalyst (Knothe et al. 2005) (Fig. 1). Methanol is the most used alcohol, but in principle, ethanol and C_3–C_5 linear and branched alcohols might also be used (Salis et al. 2007).

Until today, biodiesel production and consumption are continuously growing. In the United States, thanks to the Energy Policy Act of 2005 that provided tax incentives and loan guarantees for renewable energy, biodiesel production increased 15 times up to 2014, and consumption had a parallel trend (U.S. Energy Information Administration EIA Monthly Energy Review 2016, Table 10.4).

The EU renewable energy directive of 2009 and the Federal Law 12,249/10 in Brazil have had a similar effect on the respective regional biofuel markets.

However, biodiesel production from vegetable oils has raised a contrast with food production. Over the past 10 years, many countries like Brazil, Kenya, Tanzania, and Indonesia have reduced arable land for food production and diverted it to fuel crops. According to a report written by the International Panel for Sustainable Resource Management (United Nations Environment Project 2009), 36 million ha of world cropland was used for biofuel production in 2008, and estimates of land requirements for future biofuels vary widely, ranging between 35 and 166 Mha in 2020, 53 Mha in 2030, and 1,668 Mha in 2050.

As alternative, microalgae have been considered as a no-food feedstock, because they are metabolically more relevant than plants for oil productivity and with no need of arable land. Also, innovative processes which contribute to a more sustainable biodiesel production, as those based on biocatalysis, have been described (Salis et al. 2007).

Despite the consistent investments in research by public institutions and private companies, biodiesel from microalgae is still quite far to be commercially available.

Many technical constraints on downstream processes, namely, biomass separation and drying and oil extraction, highly incide on the global cost of biodiesel production.

Nonetheless, several funding institutional programs and private investments for the next years indicate that the quest for an economically feasible solution is a challenge still open.

This chapter deals with the updated situation on microalgae technology, focusing on actual limits and future perspectives. In addition, updated results about biocatalytic process for biodiesel production are reported.

2 Microalgae: A New Feedstock for Biodiesel Production

2.1 Introduction to Algae

Algae are a very heterogeneous group of living organisms, which comprises both uni- (microalgae) and multicellular forms of life (macroalgae). They can be found in all aquatic environments, in freshwater, brackish water, and saltwater. Algae, as plants, are able to capture light energy thanks to pigments, such as chlorophylls, carotenoids, anthocyanins, phycobilins, phycoerythrins, and so on, through photosynthesis, thereby reducing CO_2 to organic compounds, such as sugars and then oil, and they are called *photoautotrophs*. However, several species are also able to use organic compounds for their life cycle, using a heterotrophic metabolism. Finally, other species can use both kinds of metabolism because they are mixotrophic organisms able to combine heterotrophy with photoautotrophy (Brennan and Owende 2010).

The wide variety of metabolic solutions has led to a great number of possible applications of microalgae.

Algae are very important from an ecological point of view, because they belong to the bottom of the food chain; moreover, they are the principal producers of oxygen on Earth.

They have also an economic relevance in human life, because they are used in different fields.

Their content of carbohydrates, proteins, fiber, vitamins, minerals, and polyunsaturated fatty acids (PUFAs, such as α-linolenic, eicosapentaenoic, and docosahexaenoic acids, which belong to the well-known ω-3 group) is exploited in human and animal nutrition. In Asian countries, microalgae, such as *Chlorella vulgaris*, *Haematococcus pluvialis*, *Dunaliella salina*, and *Spirulina maxima*, have been used as food for hundreds of years. Moreover, diatoms, which possess an exoskeleton of silicon, are used as feed supplement for livestock, in that silicon allows a better development of bones and cartilages. Also in aquaculture, microalgal species rich in PUFAs are used to feed fries, in order to increase the viability of population and the final number of adults.

The presence of pigments and antioxidants makes microalgae suitable in cosmetics and skin care products, for preparation of antiaging creams and regenerant products.

Microalgae can produce polymers, which can be employed as stabilizer, emulsifier, and gelifier in cosmetics, pharmaceuticals, and textile industry.

For their ability to synthesize and accumulate remarkable quantities of oil, algae have been considered as an alternative feedstock to oleaginous crops for the production of third-generation biodiesel.

2.2 Microalgae and Biodiesel

In 1970s, during the first energetic crisis caused by OPEC oil embargo in the autumn 1973, microalgae have been considered as a potential source of biofuels (Spolaore et al. 2006).

In the Aquatic Species Program, performed by US National Renewable Energy Laboratory (NREL), a specific R&D Program, from 1978 to 1996, was dedicated to alternative renewable fuels, including biodiesel from microalgae (Sheehan et al. 1998).

In particular, one of the main goals was to better understand the physiological and biochemical parameters underlying lipid synthesis in microalgae.

The study established that biodiesel from microalgae was technically possible, but it was also concluded that further R&D was needed for large-scale production.

Several advantages in using microalgae as feedstock are well known. First, cultivation of microalgae does not need the arable land required for oleaginous plants. Second, cultivation of microalgae can be performed on barred land, which cannot be considered for agriculture. Third, the oil yield (L/ha per year) of microalgae is 10–25 times higher than that produced by plants (Mata et al. 2010). For these reasons, cultivation of microalgae for oil production seems to be the most innovative tool for large-scale, cost-effective, clean energy production.

2.3 Composition of Microalgal Oil

In microalgae, fatty acid (FA) and triacylglycerol (TAG) biosynthetic pathways are still poorly characterized, even if the powerful tools offered by genetic manipulations have recently stimulated much research to understand the molecular basis which supports lipid biosynthesis in microalgae. One of the most intriguing findings is about the model microalga *Chlamydomonas reinhardtii*, which employs a distinct pathway that uses diacylglycerol derived almost exclusively from the chloroplast to produce TAG. The TAG formed in this pathway is stored in lipid droplets in both the chloroplast and the cytosol, differently from higher plants, in which TAG biosynthesis occurs in microsomal membranes with the TAG being deposited in ER-derived lipid droplets in the cytosol (Fan et al. 2011).

In general, it is assumed that what is already known for higher plants may be highly conserved also in microalgae lipid metabolism. FA biosynthesis is performed in the chloroplast and produces a C16 or C18 fatty acid or both, which are then used to synthesize all lipid-containing cell molecules, such as membranes and TAGs as storage lipids. The crucial step of FA biosynthesis is the carboxylation of acetyl CoA to malonyl CoA by acetyl CoA carboxylase.

TAG biosynthesis in algae is likely to occur via the sequential acylation of glycerol-3-phosphate in the chloroplast (Goncalves et al. 2016).

Oil production from microalgae can be performed by both photosynthesis and heterotrophy. FA composition may greatly vary in dependence of the species. An average oil from microalgae is mainly composed of a mixture of unsaturated FAs: palmitoleic acid (C16:1), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3). Saturated fatty acids, palmitic acid (C16:0) and stearic acid (C18:0), are also present although to a small extent.

It is already known that FA composition has direct influences on the overall quality of the final biodiesel that has to comply with the international standards (EN 14214 in UE and ASTM D 6751 in USA). For example, oxidative stability is a parameter critical for biodiesel time of storage as biodiesel oxidation results in the formation of gums, sedimentation, and engine deposits and increases the viscosity of the fuel through the formation of allylic hydroperoxides and several secondary oxidation products such as aldehydes, alcohols, and carboxylic acids (Hoekman et al. 2012). A high content of unsaturated FAs will correspond to a low oxidative stability. Indeed the oxidation of unsaturated compounds proceeds at different rates depending on the number and position of double bonds (Knothe et al. 2005). In microalgal oil unsaturated fatty acids are often highly represented, and then a microalgae-derived biodiesel may be quite susceptible to oxidation. As consequence, to increase oxidation stability, a hydrogenation step could be required, and this would contribute to increase the costs in a large-scale production.

On the other hand, oils and fats containing a high percentage of saturated fatty acids are responsible of poor low-temperature properties (i.e., high values of cloud point and pour point) of the biodiesel fuel that constitutes a problem in cold regions during winter.

It is known that microalgae oil composition and yield are influenced by nutritional stress. For example, nitrogen deficiency is known to greatly increase the amount of oil stored by several microalgal species, and same effect is observed in diatoms under deprivation of silicon, which is a fundamental compound present in cell wall. Moreover, in *Chlorella vulgaris* also iron or phosphorus limitations induce the same effect as nitrogen deficiency (Mallick et al. 2012) in comparison to balanced nutritional regimen, and a progressive nitrogen reduction has been used under heterotrophy, exploiting organic substrates contained in municipal wastewater (Robles-Heredia et al. 2015).

Also environmental factors, such as temperature (Fork et al. 1979) and light exposition (Holton et al. 1968), are also known to play a role in oil production.

In conclusion, an appropriate distribution of unsaturated and saturated FA in microalgal oil is required to meet acceptable levels of the international quality

standard criteria. For this reason, microalgal strain selection (bioprospecting) is of primary importance, to avoid the presence of useless FAs in oil mixture. Moreover, the knowledge of the role of nutritional and environmental parameters is strategical, because it represents an easy tool to modify, if needed, the distribution of saturated and unsaturated FAs.

On the other hand, several efforts have been made in order to apply metabolic engineering through genetic manipulations to improve microalgal fitness and increase oil production.

One of the most researched concerns has been the size reduction of light-harvesting antenna complexes to maximize photosynthetic light utilization efficiency, but it has been concluded that wild-type strains can outcompete their genetic variants, because large antenna complex is able to collect light also at low flux densities (Perrine et al. 2012).

The possibility to design and apply genetically modified microalgal strains with high oil productivity has introduced the concept of the fourth-generation biodiesel production (Lu et al. 2011).

Even if little is known about the regulation of lipid metabolism pathways in microalgae, it is encouraging that, in several microalgal genomes, genetic sequences are homologous with many lipid metabolism genes in higher plants (Radakovits et al. 2010).

Some interesting results have recently been obtained in *Chlorella ellipsoidea* (Zhang et al. 2014) and *Phaeodactylum tricornutum* (Ma et al. 2014; Xue et al. 2015), with remarkable increase in oil productivity.

Up to date, research has been performed on different strategies, and results demonstrated how microalgae are different for genome organization and regulation.

In the model microalga *C. reinhardtii*, overexpression of diacylglycerol acyltransferases, which are crucial enzymes involved in accumulation of TAGs in higher plants, did not significantly improve oil yield, even if mRNA levels were higher between 1.7 and 29.1 times than wild type (La Russa et al. 2012). Despite the successful increase in transcription, the data indicate the existence of unknown regulative factors which act at posttranscriptional level, playing a key role in oil yield.

On the contrary, the same strategy was successful in the diatom *P. tricornutum*, in which overexpression of diacylglycerol acyltransferase 2 resulted in 35% increase of neutral lipid accumulation without significantly affecting growth rate (Niu et al. 2013), indicating that in diatoms the global lipid biosynthetic pathway significantly differs from *C. reinhardtii*.

One very promising strategy for TAG accumulation increase is the funneling of solar energy toward lipid biosynthesis, by minimizing or abolishing energy-demanding ancillary pathways. In this perspective, Work et al. (2010) observed that mutants of *C. reinhardtii*, in which starch synthesis was abolished, could accumulate a higher quantity of lipids than wild type, so demonstrating that the elimination of metabolic pathways competing for the same precursor is a useful strategy to improve biotechnologically relevant features of microalgal strains. Similar mutants were also obtained from *Chlorella pyrenoidosa* (Ramazanov and

Ramazanov 2006) and *Scenedesmus obliquus* (de Jaeger et al. 2014); however, it has been observed that pilot studies are needed to verify the suitability of these mutants in productive processes (Hlavova et al. 2015).

Other studies on *C. reinhardtii* showed that overexpression of the endogenous thioesterase leads to variations in the fatty acid profile (Blatti et al. 2012), which then can be appropriately modified to comply the biodiesel quality standards. Further studies on *C. reinhardtii* endogenous thioesterase should verify whether this enzyme could be effective also in other strains and set up a feasible technique for efficient expression levels in other microalgal species.

The use of genetic manipulation is strategic to obtain biotechnologically robust microalgal strains with improved features in growth kinetics and oil accumulation. For some strains, genomic sequences are available, and then reverse genetics tools can be used to gain further information about regulative networks. For the most of microalgal species, however, little is known about genomics and metabolic pathways, and the possibility of genetic improvement is limited only to induction of random mutations (forward genetics).

Moreover, the most innovative vision in genetic engineering is synthetic biology, which allows to build genetic, regulatory and metabolic networks by chemical synthesis. In such a way, it is possible to chemically synthesize a mutated gene as well as an entire pathway for the production of a desired metabolite.

However, for a successful application of reverse genetics and synthetic biology, a deeper understanding about microalgal genomes and on regulative networks is necessary to obtain effective and stable genetically modified strains for a sustainable large-scale process.

3 Industrial Systems for Microalgae Production

Systems for microalgal biomass production have been designed according to two distinct parameters: economicity and productivity. Such parameters are the bases on which the most diffused cultivation systems *raceway ponds* and *photobioreactors* have been structured (Chisti 2007). Moreover, a number of cheaper systems are mentioned.

3.1 Raceway Ponds

A raceway pond is an economic open system for microalgae cultivation. It consists of a circuit of parallel tunnels placed at ground level, in which microalgae, suspended in culture medium, flow gently and are moved by a paddle wheel (Fig. 2).

The culture medium is usually prepared by commercial fertilizers (which contain sources of nitrogen, phosphorus, minerals, and trace elements), but mixtures of vegetable mass or agro-industrial wastes can also be employed. The circuit is built in concrete and may be lined with white plastic, in order to maximize sunlight into microalgal biomass. As open system, evaporation is significant as well as

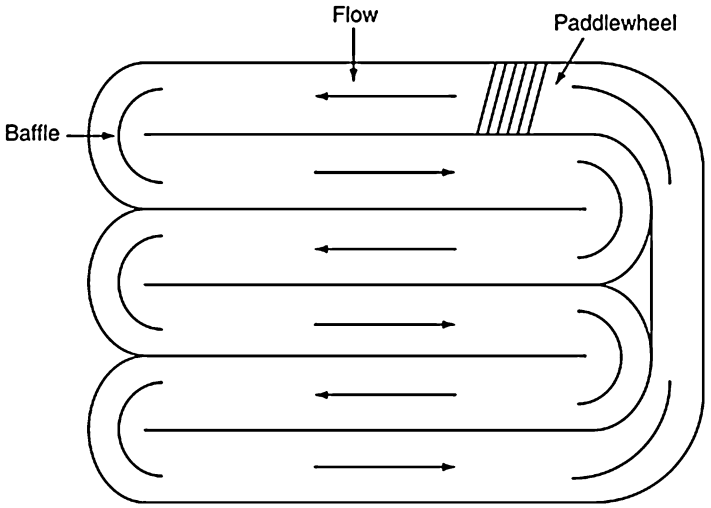

Fig. 2 Scheme of a raceway pond for microalgae production (Originally published in Salis et al. 2010a, published with kind permission of © Springer Science+Business Media New York, 2003. All rights reserved)

temperature fluctuations, and rainfalls can dilute the available nutrients, and hence, a greenhouse can be used to cover a raceway pond, in order to minimize such fluctuations (e.g., temperature shifts by heating) or, in some systems, they can be covered with a transparent removable top.

Further factors that negatively influence the biomass production are the contamination by microalgae-predating protozoa and other phototrophic competitors, and respiration, which can reduce biomass by as much as 25%. In such systems, biomass concentration is about 0.14 kg m^{-3}.

Actually, raceway ponds are used for large-scale production, because the plant and process costs allow the commercialization of microalgal biomass (mostly, *Chlorella* and *Spirulina*).

3.2 Photobioreactors (PBRs)

A photobioreactor is a system in which microalgae can be grown under controlled conditions. A common bioreactor (or fermenter) implemented with a light source can be considered as a PBR as, in general, it allows addition of nutrients, light exposition, pH, and temperature homeostasis, CO_2–O_2 exchange, biomass floatation, and light–dark cycles. In such a way, monospecies cultures can be performed, and continuous control of growth parameters allows a biomass concentration of about 4 kg m^{-3} (Fig. 3).

Actual PBRs consist of sets of clear plastic or glass parallel tubes, sometimes arranged vertically like a fence, called *solar collectors*, in which microalgal broth

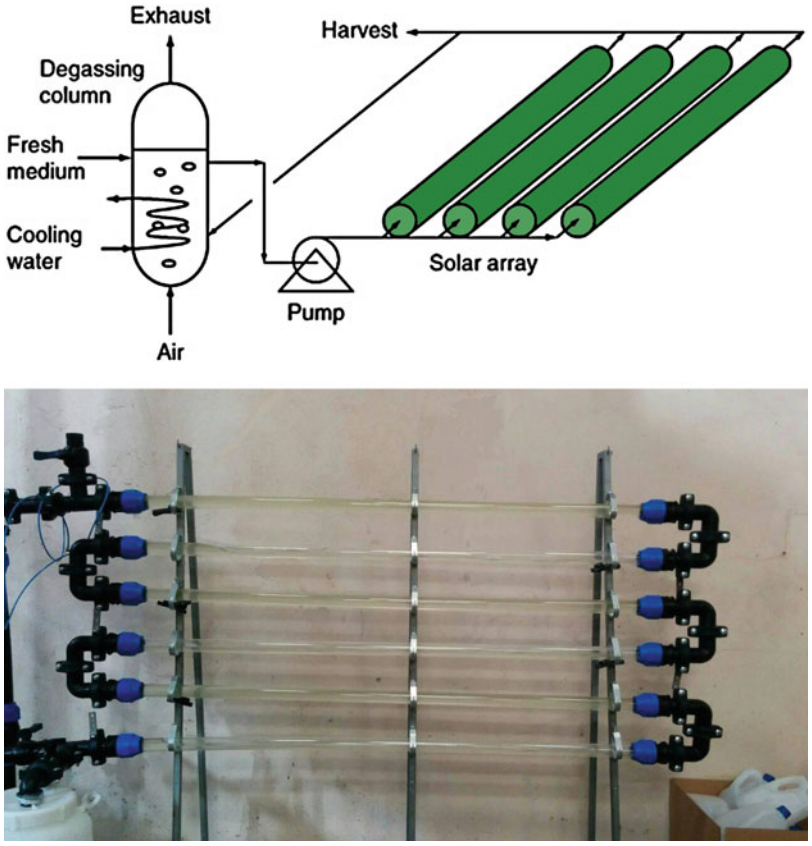

Fig. 3 *Top*: General scheme of tubular photobioreactor with parallel run horizontal tubes (Originally published in Salis et al. 2010a, published with kind permission of © Springer Science +Business Media New York, 2003. All rights reserved). *Down*: A 40 l tubular photobioreactor (produced by Plastica Alfa s.r.l, Caltagirone, Italy)

flows and develops. In order to minimize the reduction of sunlight penetration, the diameter of the tubes must not exceed 0.1 m. As a consequence, to manage consistent volumes of microalgal broth, the tubes require a considerable length, which can be as long as 80 m.

Such geometry gives a large surface area, with high light irradiation.

The dimensions of the tubes pose limitations to free diffusion of photosynthetic gases, and hence, oxygen accumulates, increasing the risk of photooxidative damage, whereas dissolved CO_2 is rapidly depleted and consequently medium pH increases. To skip such problems, a PBR has a degassing column, in which air is bubbled into microalgal broth, thus removing oxygen and enriching CO_2 concentration; however, air bubbling has to be calibrated to avoid shear stress. Sometimes, to have a good CO_2 availability, it is necessary to design and install several injection

points throughout the PBR circuit. Biofouling on the tubes' inner surface requires a periodic cleaning to avoid reduction of sunlight penetration into microalgal broth.

Linear tubular PBRs are the most diffused as pilot-scale tools and are subject to several scale-up studies, but actually their cost is prohibitive. Other than linear, tubes can be helical or coiled with an axial support equipped with a light source.

Flat panel PBRs are formed by a couple of transparent plastic or glass panels fitted together. The space between the panels is then filled with microalgal suspension, which is air bubbled from the bottom of the panel. Such system has lower oxygen accumulation than tubular PBRs and can also be automated for continuous orientation toward sunlight.

Bubble columns are vertical PBRs, usually made of transparent materials. Aeration is provided from below; mixing is excellent and is quite easy to clean.

3.3 Alternative PBRs

Other PBRs have been designed with cheap materials as bags or foils in polyethylene or polypropylene.

Soft plastic foils can be folded and sealed in a cylindric shape and used as bubble columns. Air or waste CO_2 is bubbled from the bottom, and cylinders are usually inclined with an angle of 45 °C on steel scaffolds to maximize sun exposition (Fig. 4).

The vertical growth reactor (VCR), produced by Valcent, is made of a sealed double sheet, in which a raceway circuit has been designed. The broth is withdrawn from the bottom and reinoculated at the top of the circuit by a pump.

Fig. 4 Bubble columns made of polypropylene foils molded in cylindric shape. Columns are supported by steel scaffolds and inclined toward sunlight

Sealed plastic sheets have also been used by Accordion to build up a cheap PBR. The double sheet may contain one or two chambers, in which microalgae are incubated. The double sheet is then weaved on parallel horizontal supports. When the double sheet is filled with microalgal suspension, it expands, but in correspondence of the horizontal supports, the section of the vessel is reduced, giving a series of bottlenecks. When air is bubbled from the bottom, the bottlenecks alterate the flow, increasing the turbulence and giving a good mixing effect.

Even if intriguing, it should be remarked that these systems have a limited sustainability as the foils have to be replaced from time to time and may have some problems of mechanical strength. However, if such foils could be easily cleaned and recycled more times before disposal, such system could be of remarkable interest.

4 Downstream Processing of Microalgae

After that biomass has been produced, it has to be harvested and then dried, and, finally, oil has to be extracted. Up to date, these three processes are performed with techniques that, at large-scale level, are too expensive and have strongly limited the widespread diffusion of microalgal oils or other products. In the following, the main technical procedures are described (Milledge and Heaven 2013).

4.1 Harvesting

It has been suggested that 20–30% of the costs of microalgal biomass is due to the costs of harvesting (Mata et al. 2010). Harvesting can be performed with several methods, that is, centrifugation, filtration, sedimentation, flocculation, flotation, or a combination of them. However, up to now, a universal method has not been developed.

4.1.1 Centrifugation

Centrifugation is the best method to recover almost the whole biomass rapidly, but the technique is quite expensive for energy requirement. Recently, the Dutch company Evodos has manufactured a very energy-efficient centrifuge, based on spiral plate technology, to separate microalgae from medium.

4.1.2 Filtration

Filtration is one of the most used tools for microbial mass recovery and does not require high amounts of energy, even if the operational time may be consistent. However, its efficiency is related to cell size, which has to be greater than filter pore diameter. On the other hand, especially with huge volumes of culture, clogging requires filter replacement or cleaning. Macrofiltration systems, such as presses, rotary vacuum filters, and belt filters work well with microalgal cells larger than 10 μm. However, an algae harvesting system that could dramatically reduce the

energy cost necessary to harvest, dewater, and dry algae by using a novel absorbent moving belt harvester (https://www.youtube.com/watch?v=PjHAROw31Sw) has been set up by Algaeventure Systems, which has also received funding from Advanced Research Projects Agency-Energy (ARPA-E, Department of Energy).

4.1.3 Sedimentation

Sedimentation exploits gravity and is based on the differences in density between particles to be separated and liquid, so the more dense the particles, the more rapidly they will sedimentate. Microalgal density is not very different from culture broth, so long operational times are required and low concentrations of cells can be recovered. However, some species of diatoms, a group of microalgae possessing a silicate shell, are quite heavy and usually do not need any additive to sedimentate.

4.1.4 Flocculation

Flocculation could overcome the limits of sedimentation and aims to increase the particle dimension by aggregation, induced by neutralization of cell-surface electric charges. Some microalgal species undergo autoflocculation, as consequence of environmental stress (nutrients, pH), but usually the addition of chemical or bio-derived flocculant is needed. The use of ferric and aluminum chloride and sulfate may alter the biomass quality and pose problems for disposal of exhausted cultural medium. In the last years, cationic polyelectrolytes are now considered as the most effective flocculants for the recovery of microalgae and showed to be more efficient than chemical salts. In their natural environments, bacteria and microalgae establish symbiosis, by which each partner produces and receives important organic compounds. As consequence, the cells aggregate in macroscopic groups (biofloc), which can easily be separated by gravity sedimentation. However, little is known about the relationships supporting symbiosis, and research should be addressed to understand whether such phenomenon could be realistically applied for large-scale biomass separation.

Flocculation can also be induced electrically, by electrocoagulation–flocculation and electrolytic flocculation. In such systems, negatively charged microalgae migrate toward the anode, losing their charge and generating aggregates. However, none of these methods has been assayed on large scale.

Recently, Maeda et al. (2016) presented an interesting system for biomass harvesting, mediated by cell-surface display. Silica-affinity peptide-displaying diatom cells linked silica particles in the medium and sedimented quickly and then could be easily harvested. If this system will be suitable for biotechnologically relevant microalgal strains, it will be very helpful in biomass harvesting.

4.1.5 Flotation

Flotation is promoted by bubbling air into a liquid containing microalgae, and bubbles drag cells to liquid–air interface. Often the addition of flocculants (see above) is needed to optimize the process. However, the use of flocculants raises the problem to dispose the exhaust medium properly. Up to now, flotation is not considered as a universal technique for microalgal biomass separation.

4.2 Drying

Drying the biomass is needed before oil extraction. The cheapest procedure is the exposition of biomass to sun. However, its application depends on the weather and may require a considerable time, enough for microbes to develop. In such a way, free fatty acids (FFAs), produced by oil biodegradation, may increase, thereby reducing transesterification efficiency, because of saponification if NaOH is used as alkaline catalyst. Roller drying, spray drying, and freeze-drying are largely used in food industry and can also be applied to microalgae, but their high operational cost is not compatible with biodiesel production. Convective drying, which is performed in ovens, do not alter the content of FFAs and actually can be considered as the most useful drying procedure.

4.3 Oil Extraction

Oil extraction is the final step of downstream processing, before biodiesel production. Many methods exist, divided in mechanical and nonmechanical (Show et al. 2015).

Mechanical methods are procedures which aim to disrupt microalgal cells to pour oil out and are usually employed to break down microbial cells. They include pressing, bead milling, ultrasound, autoclave, and homogenization. All these systems require high amounts of energy and work well at high cell concentrations; often, to improve the overall efficacy, they are combined to solvent extraction and can follow acidic or alkaline and enzymatic pretreatments which weaken cell surface.

Mechanical methods have the advantage to avoid chemical residues in the final product.

Nonmechanical methods are hexane solvent method, supercritical fluid extraction, enzymatic extraction, osmotic shock, and freeze–press. Hexane is one of the most efficient systems, but handling, storage, and distillation at large-scale level imply high costs; in the same manner, supercritical fluid extraction has high cost in capital investments, despite have much less risk than solvent extraction. Osmotic shock is too much time expensive, whereas enzyme extraction could be an interesting approach if the enzyme source has a limited cost. Freeze–press, as stated above about freeze-drying, has remarkable energetic requirements.

Recently, hydrodynamic cavitation (HDC) has been proposed as a biomass pretreatment to weaken cell surface, before cell disruption (Lee et al. 2014).

5 Critical Points and Limits of Industrial Production

Large-scale production and commercialization of microalgae, until today, is limited only to the whole biomass of some species, mostly *Spirulina* and C*hlorella*, as food supplement for human diet.

A wide range of microalgal products, other than biodiesel, could be commercially relevant; however, their widespread diffusion is severely limited by the actual procedures of cultivation and downstream processing. In the case of a high-value added product, it is likely that the operative costs would not be so limiting as they are when we talk about biodiesel, which ideally should get a price lower than petrodiesel.

Despite the high number of researchers involved and the conspicuous institutional funding and private investments, large-scale biodiesel from microalgae has still several bottlenecks from the economic point of view. In this way, several efforts have to be made to optimize the actual production systems and to design conceptually new systems, in which functionality and plant costs have to be considered simultaneously.

The global process consists of four stages: microalgae selection and cultivation, biomass separation and drying, oil extraction, and biodiesel production. In the following sections, the critical points of the stages are listed.

5.1 Microalgae Selection and Cultivation

A microalgal strain should be selected on the basis of well-defined criteria, such as stability of the strain, time of generation (i.e., yield in biomass in a considered time), nutritional requirements, yield in oil, and fatty acid profile. Such step has to be conducted at laboratory level, to elucidate the influence of growth parameters on biomass production and on oil yield and composition. Then the scale-up production, in which a key role is played by the chosen system, can be carried out.

Using a photobioreactor, its design is crucial to have a cost-effective optimal production. However, some aspects have an intrinsic limitation, such as the investment and maintaining costs and technician personnel for the process and plant. Another critical factor is the diffusion of CO_2 and O_2, which has to be monitored continuously in closed systems. Moreover, also temperature and biofouling on the inner surfaces of tubes have to be controlled.

Using raceway ponds, the principal problems to be considered are evaporation, rainfalls, temperature changes, contamination of microalgal broth by competing/predating organisms, and sedimentation of biomass.

5.2 Biomass Separation and Drying

After cultivation, a quick, efficient, and cheap system for separation of cells from the exhausted medium should be suitable to run huge volumes of liquid. Unfortunately, the best harvesting methods need high amounts of energy. However, flocculation (in particular, autoflocculation and bio-based flocculants) and belt filtration could be cost-effective solution systems, because they are potentially suitable for large volume cultures. On the other hand, whichever is the method used, it is very important that eventual chemical compounds have a negligible polluting impact, as the spent medium has to be disposed in a safe and economical way. Biomass drying is also very important, and the method is strictly dependent

on the molecule to be extracted. In the case of oil, convective drying is the most useful method, with no incidence on the content of FFAs, but requires energy to be performed.

5.3 Oil Extraction

Dried biomass, usually, is then pressed to extract a large percentage (70–75%) of the oils out of algae. The remaining pulp can be mixed with solvents to extract the remaining oil content. Nevertheless, the best oil-extracting agent – hexane – is neurotoxic, and therefore, its use and disposal need care and represent a cost. As an alternative process, supercritical extraction is a very efficient method, but still highly expensive.

Several pretreatments to weaken cell surface, such as enzymes and hydrodynamic cavitation, have been proposed, but large-scale studies are lacking.

After oil has been extracted, the exploited biomass has to be disposed, and as for exhausted growth medium, environmental impact has to be low. In general, it can be used as fertilizer, but several efforts are addressed to use it as feedstock for other coupled processes, such as production of bioethanol, by cellulose hydrolysis and subsequent fermentation of monosaccharides, or biogas.

5.4 Current Industrial Methods for Biodiesel Production

Most of biodiesel in the market comes from vegetable oil (rapeseed, sunflower, soybean, palm oil, etc.), animal fat, or waste cooking oil. Whatever the source of triglycerides, including microalgal oil, it needs to be transformed in biodiesel (fatty acid methyl ester: FAME) through a transesterification reaction. In this reaction three moles of methanol react with one mole of triglycerides to produce three moles of FAMEs and one mole of glycerol (Fig. 1). After purification, glycerol can be used for cosmetic and pharmaceutical purposes. Traditionally, biodiesel plants use homogeneous chemical catalysts to convert triglycerides in biodiesel. Typical alkaline catalysts are sodium, or potassium, hydroxide, or sodium methoxide. These catalysts require the use of expensive high-quality oil feedstock, because low-quality oil, with either high free fatty acid content or even other low-content contaminants (i.e., water, phospholipids, etc.), complicates the process. For this reason current processes require harsh and expensive refining operations, which often limit conventional biodiesel production (Christopher et al. 2014).

6 Enzymatic Biodiesel Production

Most of the drawbacks of the alkaline process for biodiesel production can be overcome through the enzymatic process. This process uses lipases (triacylglycerol hydrolases, E.C. 3.1.1.3) as biocatalysts for the transesterification of oil feedstock. The reaction is carried out at low temperature (i.e., 30 °C), atmospheric pressure, and in a solvent-free environment composed only by a mixture of the reagents (oil and

methanol). The process yields biodiesel and glycerol (without any additional by-product) which are easily separated due to their reciprocal immiscibility. In the previous version of this chapter (Salis et al. 2010a), we described the enzymatic biodiesel production mainly as an interesting research subject but still immature technology. Nowadays, the things have changed since pilot plants of enzymatic biodiesel are operating (Christopher et al. 2014). Moreover, very recently also some "full" plants are operating with enzymatic technology (Hobden 2014). The main issue that for years has hindered the development of the enzymatic process was the high price of the enzymes compared to that of the homogenous alkaline catalyst. Since the cost of the chemical catalyst was estimated to be about 25 USD/ton biodiesel (Nielsen et al. 2008), the enzyme biocatalyst should approximately have the same cost to be competitive. In fact, that estimation is rather rough since a change of catalyst means also very different plant and operation costs. Both of them would be lower for the enzymatic process respect to the alkaline one (Guldhe et al. 2015).

One of the used strategies to decrease biocatalyst's price is the immobilization of the enzyme on solid supports. This methodology allows both the reuse of the biocatalyst for several reaction cycles (Salis et al. 2010b) and the operation or continuous processes (Poppe et al. 2015). For the sake of truth, it should be said that the immobilization introduces an additional cost due to the use of the support (Cesarini et al. 2015). For this reason the use of a "liquid lipase" (an aqueous solution of lipase stabilized with glycerol or sorbitol and preservative against microbial growth) biocatalyst has been proposed (Cesarini et al. 2015). Indeed it has been estimated that the liquid lipase has a price about 30–50 times lower than the immobilized one (Cesarini et al. 2015). On the other hand, the liquid lipase is less recyclable than the immobilized one.

7 Research Needs

Biodiesel production from microalgae is a goal that still needs much research, despite the huge amount of resources invested. At present, although more than 60 companies are operating in microalgae sector, no large-scale processes are available. However, research on the several constraints is going on, and in the next years significant innovations are expected. Moreover, economic resources are allocated by public and private investors. In Washington, US Department of Energy recently announced up to $15 million for three projects aimed at reducing the production costs of algae-based biofuels and bioproducts through improvements in algal biomass yields.

To this aim, several aspects have to be approached.

First of all, bioprospecting of new strains has to be pursued, to find those that are more valuable for biodiesel production. Then, a systematic study on nutritional factors which allow maximization of biomass and oil yield and their role on FA composition is the basis to understand the variables for a hypothetical productive process. In parallel, a deeper knowledge on metabolic regulation, by functional genomics, proteomics, transcriptomics, and metabolomics, is strongly needed to

allow the use of genetic manipulation and obtain more efficient, productive, and stable strains. In such a way, it would be possible, for example, to reduce the presence of polyunsaturated fatty acids that have a negative effect on the stability of the final biodiesel mixture.

Other aspects are connected to the cultivation systems; therefore, though optimization of raceway ponds and photobioreactors is currently in progress, it is desirable that innovative and alternative cultivation systems and materials (e.g., with antifouling properties), more efficient and less expensive, would be designed.

Particularly relevant are the constraints deriving from downstream processing. Even if some new approaches have been observed in the recent years, much R&D is still required to propose technical solutions suitable for microalgae-based large-scale processes.

The disposal of exhausted growth medium and exploited biomass, the simultaneous production of other valuable compounds, the use of organic waste as substrates, and the CO_2 sequestration from industrial settlements, according to the biorefinery concept, are actually the most interesting perspective for economic sustainability of biodiesel production.

Finally, the development of new production methods, as those based on biocatalysis, will help to get a more economic and environmentally sustainable microalgal biodiesel fuel.

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Hydrocarbons from Algae

5

J. G. Qin

Contents

Abstract

Hydrocarbons are detected in species of all algal phyla, but their contents are generally below 2% of algal dry weight skewed toward odd-carbon number, typically at C_{15}, C_{17}, or C_{21}. *Botryococcus braunii*, a green colonial species (300–500 μm), contains exceptionally high hydrocarbons. Among the three races of *B. braunii*, race A contains C_{25}–C_{31} *n*-alkadienes/trienes up to 61% dry weight and race B contains C_{31}–C_{37} botryococcenes (triterpenes) up to 86% of dry weight. Race L contains lycopadienes (tetraterpene) $C_{40}H_{78}$ up to 8% dry weight. Cultures with 0.3% CO_2-enriched air could shorten mass doubling time by 3.6 times. Nitrogen deficiency favors lipid accumulation, but nitrogen required for growth should be above 0.2 mg L^{-1}. The optimal temperature for *B. braunii* is 20–25 °C with a light intensity of 60–100 Wm^{-2}. Slow growth is the major hurdle retarding the production of hydrocarbon at a large scale. The combined approach of molecular biology, genetic engineering and ecology is recommended

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to escalate the algal growth and hydrocarbon production to yield a commercially competitive alternative for renewable biofuels from algae.

1 Introduction

Hydrocarbons are considered the most stable group of naturally occurring compounds and are one of the Earth's most important energy resources (McMurry 2000). Due to oil crisis, a lot of research on hydrocarbon in algae was conducted in the 1970s, but this momentum did not last long. The majority of hydrocarbons found naturally occur in crude oil, but many oil shales may be originated from algae (Cane 1969). In the last century, the hydrocarbon content of coorongite was discovered to be the rubbery deposit derived from the alga *Botryococcus braunii* (Douglas et al. 1969). Recently, due to high petrol price, and more importantly due to the concern of climate change associated greenhouse gas emission to the atmosphere, the production of hydrocarbons from algae has received great attention (Ladygina et al. 2006; Qin 2005). One of the hydrocarbons of algal origin is botryococcane, an acyclic alkane derived from the freshwater green alga *Botryococcus braunii* (Maxwell et al. 1968). Botryococcane has been identified in all weathered bitumens that are regularly washed ashore along the coastline (McKirdy et al. 1986). Thus, *B. braunii*, the only synthesizer of botryococcene, is capable of contributing hydrocarbons that are potential sources of botryococcanes and long-chain *n*-alkanes. This chapter aims to discuss hydrocarbons from algae in attempt to shed a light on the possible exploration of biofuels from algae. This chapter first reviews the nature of hydrocarbons and their overall occurrence in algae, and then focuses on the description of *B. braunii*, a hydrocarbon-rich green alga, covering its morphology, taxonomy, and the hydrocarbon content. Finally, the culture methods and growing conditions of *B. braunii* are discussed before research needs are commented.

2 Hydrocarbon and Its Occurrence in Algae

Hydrocarbon is an organic compound consisting entirely of hydrogen and carbon, including aromatic hydrocarbons, alkanes, alkenes and alkyne-based compounds. Saturated hydrocarbons (alkanes) are the most simple hydrocarbon species and are composed entirely of single bonds between carbon atoms saturated with hydrogen. The general formula for saturated hydrocarbons is C_nH_{2n+2}. Unsaturated hydrocarbons have one or more double or triple bonds between carbon atoms. Those with one double bond are called alkenes with the formula C_nH_{2n}. Those containing triple bonds are called alkynes with a general formula C_nH_{2n-2}. Nevenzel (1989) reviewed biogenic hydrocarbons and found that the content of hydrocarbon in algae is generally low and most hydrocarbons are straight chain alkanes and alkenes. The characteristics of hydrocarbon of marine algae are (1) a distribution of chain lengths highly skewed toward odd-carbon number, typically centered at C_{15}, C_{17}, or C_{21},

frequently unsaturated, and (2) usually one or two components constitute over 80% of the total (Nevenzel 1989).

In blue-green algae (Cyanophyceae), the hydrocarbons of six species averaged 0.14% of dry weight ranging from 0.02% to 0.44% (Table 1). In *Spirulina platensis*, hydrocarbons account for 0.07% dry weight including 84.9% of the 17:1 compounds (carbon number: double bonds) (Rezanka et al. 1977). The most common hydrocarbon pattern in blue-green algae is 68–69% of 17:0 compounds, and C_{15}–C_{19} components accounting for 54–78% of the total hydrocarbons. The relative components of these hydrocarbons were shown to be strongly influenced by the age of the culture in *Anabaena variabilis* (Fehler and Light 1970).

Red algae (Rhodophyta) are eukaryotic, mostly multicellular marine species. Among 24 species analyzed, hydrocarbons account for 0.02% dry weight, ranging from 0.0003% to 0.073% (Table 1). The main hydrocarbons contain 17 carbons (17:0) which account for 58% of the total hydrocarbon (Nevenzel 1989). Nishimoto (1974) found that 17:0 was the largest component of the C_{13}–C_{39} hydrocarbons with 20% of the total alkanes in *Gloiopeltis furcata* (about 0.03% dry weight), 36–64% in *Grateloupia turuturu* (about 0.03%), and 12–15% in *Gracilaria textorii* (about 0.055%). In *Porphyra leucosticta*, however, the most abundant component is 19:5, possibly a derivative from the well-known 20:5($n-3$) fatty acid (Drew and Ross 1964). The longer chain hydrocarbons are also detected in various red algae (*Porphyra umbilicus*, *Plocamium coccineum* and *Ceramium rubrum*) (Nevenzel 1989). In contrast, various C_1 and C_3 hydrocarbons have been detected in *Asparagopsis taxiformis*, *A. armata* and *Bonnemaisonia hemifera* and these short chained, volatile hydrocarbons could be released to the water column by living algae (Gschwend et al. 1985).

In dinoflagellates (Cryptophyta and Pyrrhophyta), hydrocarbons averaged 1.34% dry weight with a range of 0.015–5.8% (Table 1). In Cryptophyta, Blumer et al. (1971) reported that 21:6 hydrocarbon was the most abundant in *Cryptomonas ovata* and *Rhodomonas lens*, but the absolute amount of 21:6 hydrocarbon was affected by the age of culture. The amount of 21:6 hydrocarbon biosynthesis is greatest during

Table 1 Hydrocarbon variation in various algae (Recalculated from Nevenzel 1989)

Algal groups	Mean and range of hydrocarbon (% dry weight)	Number of species included
Blue green algae	0.14 (0.02–0.44)	6
Red algae	0.02 (0.0003–0.073)	24
Dinoflagellates	1.34 (0.015–5.8)	9
Diatoms	0.56 (0.0041–0.66)	15
Brown algae	0.049 (0.00049–0.66)	45
Golden algae	0.76 (0.0034–0.0034)	4
Englenoids	0.51 (0.22–0.79)	3
Yellow algae	1.75 (1.4–2.1)	2
Green algae	0.68 (0.000044–17)	33

the log phase growth and falls off markedly in the stationary phase (Blumer et al. 1970). Lee and Loeblich (1971) found 21:6 to make up 20% of the total hydrocarbons (2.2% dry weight) in *C. ovata*, and 45% in *R. lens* (2.1% dry weight), but Antia et al. (1974) did not found 21:6 hydrocarbon in *Chroomonas salina*, but C_{17}–C_{33} hydrocarbons were dominant, accounting for 0.4% dry weight. In Pyrrhophyta, 21:6 hydrocarbon appears in free-living photosynthetic dinoflagellates, but is absent from colorless heterotrophic species. Similarly, culture age significantly impacts hydrocarbon compositions in Pyrrhophyta. The relative amount of 21:6 hydrocarbon in *Peridinium trochoideum* decreases from 80% of the total hydrocarbon in log phase cultures (12 days) to 32% in the stationary phase culture (17 days) (Blumer et al. 1971).

In diatoms (Bacillariophyta), hydrocarbons are averaged at 0.56% of dry weight, ranging from 0.00049% to 0.66% in 15 species (Table 1). In general, the 21:6 hydrocarbon is the major component, constituting more than 90% of the total hydrocarbons (Nevenzel 1989). Culture age has more effect on the amounts of total hydrocarbons than the chain length distribution in *Skeletonema costatum*. The total hydrocarbons increase four- to fivefold from the third to the eighth day with 21:6 hydrocarbon accounting for 80–90% in this species (Perry et al. 1978). For *Chaetoceros curvisetus*, the 21:6 hydrocarbon reaches 90% (Lee and Loeblich 1971).

In brown algae (Phaeophyta), hydrocarbons account for 0.049% dry weight with a range from 0.00049% to 0.66% in 45 species (Table 1). According to the hydrocarbon composition, Nevenzel (1989) divided brown algae into three categories. Category I includes the orders of Ectocarpales and Desmarestiales with 21:1 being the major component (73–98%). Category II includes Dictyotales and Chordariales with 15:0 as the major component (55–98%). In category III, 15:0 is a major component (23–47%) and 19:5, 21:5, and 21:6 vary from 19% to 66%, but the taxonomy groups in this category do not show a clear-cut distinction.

In golden algae (Chrysophyta), englenoids (Euglenophyta) and yellow algae (Xanthophyta), the mean hydrocarbons are 0.76%, 0.51%, and 1.75% dry weight, respectively (Table 1). Most marine chrysophytes contain 21:6 hydrocarbon, but this component was also detected in yellow algae (Blumer et al. 1970). In *Tribonema aequale* (Xanthophyta), C_{15} accounts for 86% of the total hydrocarbons, while in *Syracosphaera carterae* (Chrysophyta), the main component is C_{17} (46%). In contrast, in *Emiliania huxleyi* (Chrysophyta), the main components are straight chain hydrocarbons (31:2, 48% and 37:3, 32%) (Blumer et al. 1971). In euglenoids (*Euglena gracilis* and *E. viridis*), 21:6 is the main component with 17:0 being the next most abundant compound (Lee and Loeblich 1971).

In green algae (Chlorophyta), hydrocarbons accounts for 0.68% dry weight in 33 species (Table 1). The most common hydrocarbon for green algae shows that 17:0 is the main component, totaling more than 80% in 10 species and more than 25% in 24 species (Nevenzel 1989). *Botryococcus braunii* is unique both in the amount of hydrocarbon produced and the composition of C_{27}–C_{31} dienes and the methylated triterpenoids – botryococcenes (Cox et al. 1973). The potential use of this alga for renewable energy is discussed in the next section. Patterson (1967) reported that

Chlorella vulgaris grown heterotrophically on glucose in the dark synthesized a large amount of 25:1 and 21:1 hydrocarbons, whereas when grown autotrophically in the light only small amounts of saturated C_{17}–C_{36} alkanes were detected. Similarly, *Chlorella kessleri* synthesizes five times as much hydrocarbon heterotrophically as it does when grown autotrophically (0.01% vs. 0.002% dry weight) (Rezanka et al. 1977). Under autotrophic conditions, the hydrocarbon production is usually less than 1% of dry weight. For example, *Scenedesmus accuminatus* produced hydrocarbon of 0.31% dry weight (with 17:1 accounting for 32.5%; 26:1 for 22.3%; 17:0 for 15.8% and 27:1 for 12.2%) and *S. acutus* produced hydrocarbon of 0.28% dry weight (27:1 for 35.2% and17:0 for 12.2%) (Rezanka et al. 1977).

3 *Botryococcus braunii*: A Hydrocarbon Rich Alga

Botryococcus braunii is a unicellular colonial green alga and contains hydrocarbon up to 86% of the dry weight (Brown and Knights 1969). This alga is widespread in fresh and brackish waters of all continents (Chisti 2007; Metzger and Largeau 2005). A unique characteristic of this alga is its conspicuous ability to synthesize and accumulate a variety of lipids (Li and Qin 2005). These lipid substances include numerous hydrocarbons, i.e., highly reduced compounds comprising only carbon and hydrogen as elements, and a number of specific ether lipids (Metzger et al. 1991).

3.1 Morphology

Under a light microscope, the colonies *B. braunii* are yellow-green in color. Their shape is generally rounded to elliptical. The colonies are 300–500 μm across and each cell measures 5–9 μm wide and 8–13 μm long. The typical structure of this alga consists of various cylindrical and ramified tubes that branch off of the colony center. The colonies are of varied shapes (Fig. 1b, c), being composed of a number of spherical aggregates with radically arranged cells (Fig. 1a) embedded within a tough mucus envelope. Often, smaller colonies are united by interconnecting strands into larger ones (Fig. 1d). At times the cells can be squeezed out of their envelopes, which are so firm that they retain their shape. Each cell appears to be lodged within a funnel-shaped mucilage-cup (Fig. 1g, h) the base of which is prolonged into a thick stalk which extends to the center of the aggregate. The usually ellipsoid cells possess a thin membrane (Fig. 1e, h) which harbors abundant oil. This species can be identified as golden-brown lumps of mucilage containing oil with cells in the colony.

3.2 Taxonomy

Botryococcus braunii is a green alga – Phylum: Chlorophyta, Order: Tetrasporales, Family: Dictyosphaeriaceae (Smith 1950). Significant morphological heterogeneity

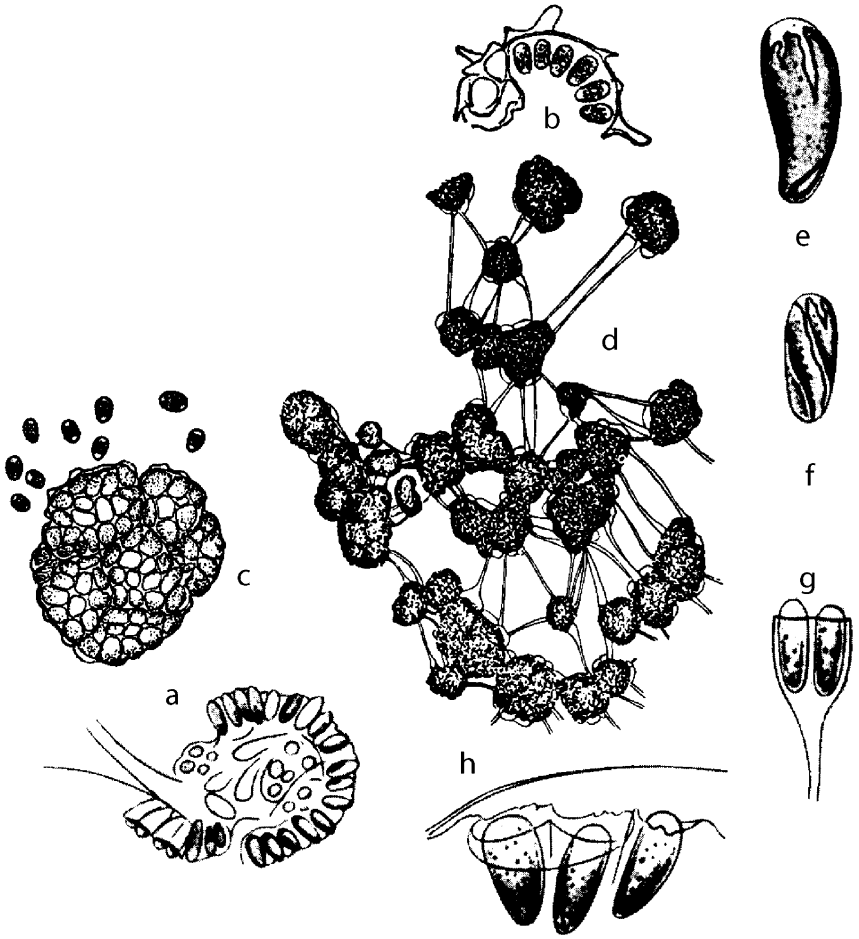

Fig. 1 Morphology and anatomy of *Botryococcus braunii* (From Smith 1950)

exists within this alga from lakes and cultivation in the laboratory. Based on morphological differences, Komárek and Marvan (1992) found that some of these features varied in relation to age and culture conditions. Recently, 18S rRNA sequences also reveal that *B. braunii* belongs to three chemical races A, B, and L (Senousy et al. 2004). Race B produces its major hydrocarbon botryococcene. Race A is named after its major product of alkadiene, and race L after its major hydrocarbon product of lycopadiene.

These races can be differentiated generally on the basis of the characteristic hydrocarbons and algal morphology (Table 2). Race A produces C_{25}–C_{31}, odd-numbered *n*-alkadienes and alkatrienes. These linear olefins can constitute up to 61% of the dry cell mass of the green active-state colonies (Gelpi et al. 1970). The B race produces polymethylated unsaturated triterpenes, called botryococcenes

Table 2 Distinctive features of three races of *Botryococcus braunii*

Botryococcus braunii			
	Race A	Race B	Race L
Hydrocarbon property	C_{25}–C_{31} odd numbered n-alkadienes/trienes	Botryococcenes (triterpenes) C_nH_{2n-10}, n = 30–37	Lycopadienes (tetraterpene) $C_{40}H_{78}$
Algal colour in stationary phase	Pale yellow or green	Orange reddish or orange brownish	Orange reddish or orange brownish
Long chain alkenyl phenols	Present	Absent	Absent
Cell size	13 × 7–9 μm	13 × 7–9 μm	8–9 × 5 μm

(C_nH_{2n-10}, n = 30–37). In natural populations, botryococcene can constitute from 27% to 86% of the dry cell mass (Brown and Knights 1969). The L race produces a single hydrocarbon $C_{40}H_{78}$, a tetraterpene, known as lycopadiene (Metzger et al. 1990). This hydrocarbon accounts for 2–8% of the dry biomass (Metzger and Casadevall 1987). The colony color in the stationary phase also differs among the races. The B and L race algae turn red-orange and orange brownish from green color, while the A race alga becomes pale yellow from green in the stationary phase. Long-chain alkenyl phenol is detected in the A race, but not in race B and race L. The cells of the L race (8–9 × 5 μm in size) are relatively smaller than the cells of races A and B (13 × 7–9 μm) (Metzger et al. 1988). Although biochemically different, these races coexist in natural environment. Mixed populations of race A and race L have been found in Australian lakes (Wake and Hillen 1981)

3.3 Hydrocarbon Content

The hydrocarbon contents of *B. braunii* have been previously reviewed by Banerjee et al. (2002) and Metzger and Largeau (2005). About 30 chemical structures have been determined from cultivated strains and wild samples of *B. braunii* race A (Metzger and Largeau 1999). All these compounds, ranging from monoenes to tetraenes and almost all odd-carbon-numbered, show a terminal unsaturation (Fig. 2). Generally, dienes predominate and comprise one mid-chain unsaturation (Fig. 2a). The most frequently found trienes exhibit two conjugated mid-chain unsaturations (Fig. 2b). Botryococcenes, specific hydrocarbons of *B. braunii* race B, are triterpenoids comprising acyclic and cyclic compounds. Among 50 botryococcenes identified, about 15 structures have been determined (Metzger and Largeau 1999). The C_{30} botryococcene from a cultured strain is the precursor of all higher homologous compounds (Fig. 2c); and botryococcene distribution is strain dependent (Metzger et al. 1985). In addition, algae of race B synthesise squalene ($C_{30}H_{50}$) which is a precursor of triterpenoids and C_{31}–C_{34} methylated squalenes (Fig. 2d) (Huang and Poulter 1989). The L race produces a single hydrocarbon $C_{40}H_{78}$, a tetraterpene, known as Trs-trs-lycopadiene (Fig. 2e) (Metzger et al. 1990).

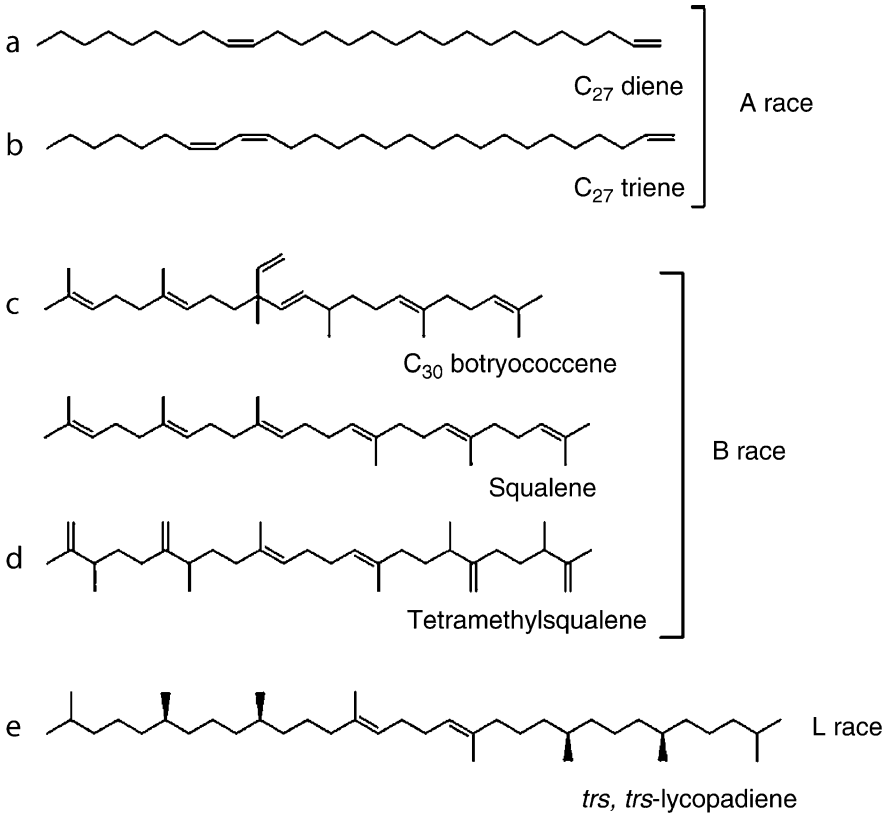

Fig. 2 Hydrocarbons produced by the three chemical races of *Botryococcus braunii* (Adapted from Metzger and Largeau 2005)

Race L contains the lowest hydrocarbon among other races, (Metzger and Casadevall 1987); therefore, with a generally higher hydrocarbon content, races A and B may be superior to race L for commercial extraction of hydrocarbon fuel.

4 Culture and Growth of *B. braunii*

Much work has been done on establishing the optimal nutritional requirements and culture conditions for producing *B. braunii* hydrocarbons (reviewed by Banerjee et al. 2002). However, the production of hydrocarbons in *B. braunii* appears to depend on growth, but the growth rate of the alga is slow. A modified Chu-13 medium is often used to culture *B. braunii* with satisfactory growth. This medium has the following composition (g L^{-1}): KNO_3 (0.2), K_2HPO_4 (0.04), $MgSO_4 \cdot 7H_2O$ (0.1), $CaCl_2 \cdot 6H_2O$ (0.08), ferric citrate (0.01), citric acid (0.1), boron (0.5 ppm),

manganese (0.5 ppm), copper (0.02 ppm), cobalt (0.02 ppm), and molybdenum (0.02 ppm) (Largeau et al. 1980).

Cultures with 0.3% CO_2-enriched air have a much shorter mass doubling time (40 h) compared with 6 days for cultures supplied with ambient air (Wolf et al. 1985). CO_2 enrichment favors the formation of lower botryococcenes (C_{30}–C_{32}), whereas cultures with ambient air accumulate higher botryococcenes (C_{33}–C_{34}). Nitrogen deficiency favors lipid accumulation, but nitrogen is required for growth (Ben-Amotz et al. 1985). Casadevall et al. (1983) reported that an initial NO_3-N concentration of >0.2 mg L^{-1} favors hydrocarbon production. When the cells are exposed to 5 mM NH_4^+ for 24 h, the nitrate reductase enzyme becomes inactive. The NH_4^+-N-related toxicity in the late exponential phase of growth could cause irreversible damage to algal cells (Lupi et al. 1994). Phosphorus is required for the growth of *B. braunii*, and it is usually supplied in the form of K_2HPO_4. Phosphate levels can decline to below 0.5 mg L^{-1} early in the exponential phase (Casadevall et al. 1983), but algae may rapidly absorb phosphate in amounts exceeding the requirement of the cell. In the later stages of culture, a large amount of phosphate can be released in the medium as the cells lyse. An increase in the initial amount of phosphate in the medium, beyond the level already present in the concentrated Chu-13 medium, does not affect the growth, the nature of hydrocarbons, and their relative abundance (Casadevall et al. 1983).

In addition to nutrients, recently, Li and Qin (2005; Qin and Li 2006) compared three strains of *B. braunii* from China (N-759), United Kingdom (CCAP 807/1), and Japan (N-836). The UK strain and the Japanese strain grew faster at 25 °C than at other temperatures; while the Chinese strain performed equally well at 20 °C and 25 °C. The UK strain contained the highest hydrocarbon content, but the Chinese strain had the lowest lipid content at most temperatures. The highest growth rate was found in the UK strain and the lowest growth rate was observed in the Japanese strain. The UK and the Japanese strains contained more hydrocarbons than the Chinese strain at 60 and 100 Wm^{-2}. Our results indicate that the Chinese strain and the UK strain grow faster than the Japanese strain, but the UK and the Japanese strains produce more hydrocarbon than the Chinese strain. The UK strain seems be a potential *B. braunii* strain for the exploitation of renewable energy.

5 Research Needs

Hydrocarbons from algae are a potential renewable source of biofuels that can be used as a replacement of liquid transport fuels. In the middle of the last century, most research focused on the screening of hydrocarbons from algal species. Toward the end of last century and the last decade, research has focused on the environmental requirements of hydrocarbon production and the physiological pathways of hydrocarbon synthesis in algae. Despite the research on the chemical and ecological requirements of algae, cultures of algae at a large scale for renewable hydrocarbons have not yet proved to be financially feasible. The slow growth rate of this alga is not a consequence of a limited supply of nutrients, but it seems that the high content of

hydrocarbon and fast algal growth rate are mutually exclusive. The accumulation of hydrocarbon may retard the growth rate in *B. braunii*. It is necessary to understand the physiological and biochemical pathways of hydrocarbon production in algae. Okada et al. (2000) cloned the squalene synthase gene from a strain of *B. braunii* race B in *Escherichia coli*, which offers promising prospects for the production of hydrocarbons by fast-growing micro-organisms. Therefore, the approach of molecular biology, genetic engineering and ecology should be combined toward the production of hydrocarbon from algae at a commercial scale.

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Screening for Lipids from Marine Microalgae Using Nile Red

6

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Abstract

The fluorescent stain Nile Red has been used extensively for the quantification of lipids in phytoplankton, including microalgae, because it preferentially stains neutral lipids and it is economical and sensitive to use for screening purposes. Although its basic application has not changed for several decades, recent improvements have been made to improve its utility across applications. Here we describe additional refinements in its application and interpretation as a high-throughput method for the rapid quantification of neutral lipids in liquid cultures of marine phytoplankton. Specifically we address (1) interspecies comparisons, (2) fluorescence excitation and emission wavelengths, and (3) the time course of the Nile Red signal in the context of using bulk or cell-specific fluorescence to quantify neutral lipids of live or preserved cells. We show that with proper caution in its interpretation across species and physiological states the quantity of lipid in hundreds of small volume samples can be reliably assessed daily using a refined Nile Red protocol.

Abbreviations

DAG	Diacylglycerol
DGCC	1,2-Diacylglyceryl-3-(*O*-carboxyhydroxymethylcholine)
DGDG	Digalactosyl diacylglycerol
DGTA	1,2-Diacylglyceryl-*O*-2″-(hydroxymethyl)-(N,N,N-trimethyl)-β-alanine
DGTS	Diacylgycerol-*N*-trimethylhomoserine
MGDG	Monogalactosyl diacylglycerol
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylgycerol
SQDG	Sulfoquinovosyl diacylglycerol
TAG	Triacylglycerol

1 Introduction

Along with proteins, carbohydrates, and nucleic acids, lipids represent one of the four major biochemical components of cells, and the ratio of these components varies widely among cell types, taxa, and physiological state. In particular, the lipid composition of phytoplankton, including microalgae, is strongly sensitive to nutritional status (Marchetti et al. 2010; Shifrin and Chisholm 1980) and has been shown to vary greatly among different species of phytoplankton including microalgae (Ben-Amotz et al. 1985; Shifrin and Chisholm 1981). In addition to such basic

research characterizing the quantity and classes of lipids, recently there is renewed interest in lipids from microalgae because of potential biotechnological uses; fast growth rates, lack of cellulosic cell wall in most species, and high lipid content make microalgae a top candidate for the production of biofuels and other bioproducts (DOE 2016; Gordon and Polle 2007; Greene et al. 2016; Huntley et al. 2015; Walsh et al. 2016; Williams and Laurens 2010). Towards this goal, rapid but accurate lipid quantification of microalgae is required (1) to identify which strains have the most desirable lipid profiles, (2) to identify the growth conditions that optimize the production of lipids, and (3) to develop microalgae strains through genetic modifications that increase lipid production (Greenwell et al. 2009; Miller et al. 2009).

Direct extraction of total lipids is most commonly done with either the Folch method (Folch et al. 1957) using an 8:4:3 $CHCl_3$:MeOH:H_2O solvent mixture or the refined Bligh and Dyer method (Bligh and Dyer 1959) using a 1:1:0.8 $CHCl_3$:MeOH:H_2O solvent mixture to extract lipids from cells (or tissue). Both of these classic methods, which require the harvesting of dilute cells from their growth liquid medium via filtration or centrifugation, rely on solvent phase separation of the lipids; the organic (lower) phase is removed and concentrated by evaporation, leaving behind lipids. Total lipids may then be determined by gravimetry. While this approach is generally robust and has been used for more than 50 years, it is not compatible with screening efforts involving large numbers of samples because of processing time involved with extraction. Further, relatively large volumes (i.e., mass) of samples are required for quantification by gravimetry because the basis of the measurements is mass difference between a sample and blank, and is therefore limited by the precision of the balance. If specific lipid classes are of interest, other expensive and time-consuming chromatography (gas, high pressure liquid, thin layer, etc.), nuclear magnetic resonance, or mass spectrometry-based methods are required (Choi et al. 1993; Gurr and James 1980; Harvey 1991; Holčapek et al. 2003).

Lipid-specific stains have been developed and offer an alternative to extraction-based protocols because they require neither large amounts of material nor solvent extractions. Nile Red (9-diethylamino-5*H*-benzo[α]phenoxazine-5-one) (Greenspan et al. 1985), LipidTox™ (Invitrogen™) and 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene-2,6-disulfonic acid, disodium salt (BODIPY® 492/515 disulfonate Invitrogen™) are common fluorescent stains used to characterize and quantify lipids in cells. Nile Red, which was originally developed as a modification of Nile Blue, has an intense fluorescence signal and has been used as an economical method to quantify the abundance and location of lipids in different cell types since its introduction 30 years ago (Greenspan et al. 1985). Because microalgae are naturally fluorescent, modifications by Cooksey (Cooksey et al. 1987) broadened its general applicability for microalgae. Specifically, fluorescence is quantitatively related to lipid content and highly correlated with both gravimetrically determined total- and neutral lipids. This method has been further refined for the purposes of screening microalgae in small volumes (Chen et al. 2009; Elsey et al. 2007); however, as its application has grown, additional uncertainties have been raised (Natunen et al. 2014; Rumin et al. 2015). Specifically, although the fluorescence

intensity has been reported to increase in bound versus free Nile Red (Cooksey et al. 1987; Greenspan et al. 1985; Pick and Rachutin-Zalogin 2012), there have been no reports documenting the relative excitation and emission spectra of bound and free Nile Red in aqueous phase even though this information is essential in selecting the proper wavelengths and optimizing fluorescence detection in the presence of a strong background fluorescence signal of phytoplankton. Also, recent advances in molecular level lipid class quantification allow a comprehensive comparison between Nile Red quantification and more laborious but detailed solvent-based extraction protocols across different phytoplankton strains (Chen et al. 2009). Finally, recent reports show the importance of temporal variability of the Nile Red signal for intact model microalgae and their plasma membrane or lipid globules, suggesting a role of physiology and potentially taxonomy in modulating the Nile Red signal (Pick and Rachutin-Zalogin 2012). These considerations lead to a reassessment of the application and interpretation of the Nile Red technique for the rapid quantification of lipids in low biomass samples of phytoplankton reported here.

2 Materials and Methods

2.1 Cultivation and Storage of Phytoplankton Samples

Marine phytoplankton cultures were grown in 40 mL Pyrex culture tubes or polystyrene tissue culture flasks using modified f/2 or H medium (Andersen 2005; Guillard and Ryther 1962) (100 μM NO_3^-, 36.3 μM PO_4^{3-}, 250 μM $SiOH_4$ (that was neutralized to pH = 7.0 prior to addition to medium (Mclachlan 1973)), f/20 metals, 0.2 g L^{-1} sodium bicarbonate). Cultures were grown at 30 °C under high light (photosynthetically active radiation ~550 μmol quanta m^{-2} s^{-1}) provided by cool white fluorescent bulbs with a 14:10 h light:dark cycle. Additional marine phytoplankton cultures for lipid class experiments were grown in standard f/2 medium in 2 L Pyrex bottles aseptically bubbled with air and stirred with a magnetic stir bar. These cultures were grown in the laboratory window using natural sunlight and at ambient laboratory temperatures of ~25 °C. Phytoplankton cultures did not exceed 3 mM C biomass. The strains used in this part of the study were obtained from the University of Hawaii culture collection and represent a range of taxa and species that include: *Opephora* sp. C003, *Opephora* sp. C010, *Nanofrustulum* sp. C015, *Desmochloris* sp. C046, *Bacillariophyta* sp. C077, *Skeletonema* sp. C129, *Nitzschia* sp. C315, *Staurosira* sp. C323, *Chlorella* sp. C596, *Chlorella* sp. 599, *Thalassiosira* sp. C-D, *Chaetoceros* sp. MT16, and *Chaetoceros* Ch60.

Cultures for flow cytometry were grown in 40 mL Pyrex tubes in a growth medium that consisted of Artificial Seawater (Goldman and Mccarthy 1978; Mclachlan 1973) with modified f/2 nutrients (100 μM NH_4, 36 μM PO_4, 106 μM $SiOH_4$, 1 mM TAPS, f/20 metals, f/2 vitamins). Cultures were grown at 25 ± 0.2 °C with ~350 μmol quanta m^{-2} s^{-1} provided by cool white fluorescent bulbs with a 14:10 h light:dark cycle. Three strains of marine phytoplankton including *Staurosira* sp. C323, *Chlorella* sp. C596 and one strain isolated from samples provided by the

Sahara Forest Project in Qatar and identified as *Navicula sp.* based on microscopy observation. For all experiments, phytoplankton strains were harvested at different stages of their growth phase (e.g., exponential, early stationary, late stationary) to obtain populations that were in different stages of nutrition stress, physiology, and carbon allocation and therefore different lipid content (Bittar et al. 2013).

"Live" samples for Nile Red lipids were measured within 1 h after subsampling from culture flasks. "Frozen" samples of 2 mL culture were immediately frozen at either −80 °C or −196 °C in cryovials (VWR # 16001–102) until later analysis, typically within 1 week. No difference was detected between frozen storage at different temperatures. Longer term storage (up to a month) also did not influence the results. "Preserved" samples were treated similarly to "Frozen," except the preservative glutaraldehyde (Tousimis #1057A) was added (0.125% final concentration) (Vaulot et al. 1989).

2.2 Nile Red Solutions

Nile Red (9-diethylamino-5*H*-benzo[α]phenoxazine-5-one) (Sigma # N2013) was dissolved (1 mg mL^{-1}) in either acetone or dimethyl sulfoxide (DMSO) (Greenspan and Fowler 1985; Greenspan et al. 1985; Rumin et al. 2015). Nile Red stock solutions were stored in air-tight Pyrex tubes at room temperature in the dark and made fresh weekly.

2.3 Nile Red Determined Lipids Using Microplate Reader

Nile Red solution (1 mg mL^{-1}) was added to phytoplankton cultures, final concentration 10 μg Nile Red mL^{-1}, and thoroughly vortexed. Higher concentrations (up to 30 μg Nile Red mL^{-1}) did not significantly modify the signal. In pilot studies, the choice of solvent (acetone or DMSO) did not affect the magnitude or variability of the signal. Treated samples were placed into a black 96-well microplate (VWR #82050-728) (300 μL each well) and fluorescence quantified using a Synergy 4 multimode microplate reader equipped with a Xenon flash lamp. Fluorescence was measured every 5 min for a total of 30 min. For each microplate well, the peak fluorescence signal over this 30 min time period was used as the reported signal. Fluorescence was excited at 540 ± 12.5 nm (Synergy part #7082249) and emission measured at 590 ± 10 nm (Synergy part #7082225). To account for nonspecific fluorescence, Nile Red signals were corrected by subtracting a medium blank with Nile Red and then subtracting the difference between the phytoplankton with solvent (with no Nile Red) and medium with solvent. Fluorescence excitation and emission spectra were measured similarly except that excitation or emission was provided and measured in dual monochromator mode with 5 nm resolution.

To account for potential machine and stain variability, a secondary solid standard plate (Synergy part # 7092092) was run weekly to ensure machine stability. Nile Red stain efficiency and stability was verified with each microplate analysis using a

standard solution made from chemically defined lipid concentrate (Invitrogen # 11905-031) preserved with 0.125% glutaraldehyde and stored in cryovials at −196 °C (in liquid nitrogen) until each standard vial was sacrificed.

2.4 Total Lipids

Samples for lipid analysis (50–100 mL) were collected by filtration onto combusted 47-mm glass fiber filters (nominal porosity of 0.7 μm) and gently rinsed thrice with 0.5 M ammonium formate to remove external salts (Zhu and Lee 1997). Samples were stored at −80 °C prior to express mail shipping on dry ice to the University of Hawaii. Upon arrival, samples were stored at −80 °C until further analysis. Prior to extraction, the frozen sample filters were freeze-dried overnight using a bench top Labconco lyophilization unit, set at −40 °C and a vacuum of at least 0.06 mbar for a minimum of 12 h. The samples were freeze-dried to eliminate the water barrier and allow organic extraction solvents to permeate the sample matrix during the following accelerated solvent extraction. A microscale analytical approach was developed for the determination of total lipid concentration as only small amounts of microalgae were available for analysis. The lipid extraction procedure is based on that described by Bligh and Dyer (Bligh and Dyer 1959). Total lipid concentrations are determined via gravimetric analysis following extraction using a DIONEX ASE 200 system and HPLC-grade solvents (VWR International). The ASE 200 flow-through solvent extraction system allows rapid extraction of up to 24 solid or semisolid organic matter samples using 11 mL stainless steel extraction cells with independent control of temperature and pressure using organic or aqueous solvents. The sample extract and solvent mixture of each cell were pumped into pre-cleaned 40 mL glass extraction vials. The total lipid content of each sample was calculated by weighing the glass extraction vials before and after the accelerated solvent extraction.

The following steps were carried out in preparation for extraction. The pre-cleaned and uncapped extraction vials were equilibrated for 30 min in a rack using a timer. Following the equilibration, the vials were weighed on a five-place analytical balance (Mettler Toledo, XS105DU). The weight value (g) was recorded as pre-weight to 0.01 mg. Internal and external calibration was carried out daily. The DIONEX extraction cells were cleaned using a programmed cleaning cycle (preheat =0 min, heat =5 min, N2 pressure = 1500 psi, temperature = 60 °C, static time = 0 min, flush % = 5, purge =20 s, cycle =1, and solvents =35% chloroform and 65% methanol). The 11 mL DIONEX extraction cells were lined with 19.88 mm DIONEX cellulose filters. The folded (in-half) sample filters were transferred individually in each cell with the crease facing the inside wall of the DIONEX cell. To ensure even contact of the sample material with the extracting solvents, the folded filters were carefully opened with forceps. Once the filter was positioned in the cell, Ottawa sand (20–30 mesh) was poured into the void volume up to 1–2 mm under the rim of the cell. Dispersing with an inert material allows increased permeation of the sample matrix, prevents sample adhesion and compaction, and hence maximizes extraction efficiency. For each set (24 cells) we ran three combusted 47 mm GFF

filters as blanks in nonsequential order. For the lipid extraction, DIONEX instrument settings were as follows: preheat =0 min, heat =5 min, N_2 pressure = 1500 psi, temperature = 60 °C, static time = 5 min, flush % = 20, purge =60 s, cycle =2, and solvents =35% chloroform and 65% methanol.

Following the accelerated solvent extraction, the glass vials containing the sample extract were loaded in a Genevac EZ-2 evaporator for 1.75 h. at 40 °C using the "low BP mixture" setting to evaporate the contents of the pre-weighed extraction cells under vacuum to dryness, and then placed in a rack uncapped for a 30 min equilibration time. For gravimetric "total lipid" analysis, the weight of the dry residue in the collection vials was used to calculate the amount of lipids in the original sample. The average blank value was subtracted from the final weight. For storage and further analysis, the dried lipid material was redissolved in 2 mL dichloromethane (HPLC-grade, Fisher Scientific) and transferred into 4 mL glass vials. One mL of dichloromethane was added twice to each sample and then sonicated for 30 s, for a final volume of 2 mL. Once all samples were transferred into the 4 mL storage vials, they were purged with nitrogen gas for 10 s and closed with Teflon caps. Each vial was sealed with Teflon tape and stored at −80 °C until further analysis.

The precision of the method was determined in triplicate on two different days by extracting a 47 mm GF/F filter plus 0 (blank), 10, 20, 30, 40, and 50 mg of dried Chlorella (SOLGAR #K-1250, broken cell-wall dietary supplement) in triplicate using the procedure described above. Blank values were subtracted and the two data sets were combined. The extraction efficiency was linear (R2 = 0.987) over this range of dry weights, and yielded a mean and SD of 17.4 ± 1.4 weight percent (WT%) total lipids (CV = 8.0%). This value is not statistically different ($P > 0.05$) from that (15.9 ± 0.4 WT% total lipids) determined for Chlorella samples run in parallel using a Folch et al. (1957) validated (Des Moines, IA; www.eurofins.com/) DIONEX macroscale protocol that requires an individual sample size of ~500 mg.

2.5 Neutral Lipids

Neutral lipids in the lipid extracts (see above) were determined by high-performance liquid chromatography (HPLC) coupled with evaporative light scattering detection (ELSD) using a modified version of the Silversand and Haux (1997) procedure (Silversand and Haux 1997). Lipid extracts (50 µL) were injected onto an Agilent Technologies 1200 HPLC system (left and right column temperatures: 30 °C, flow rate: 1 mL min-1) equipped with a Supelguard LC-DIOL guard column (4 x 20 mm, 5 µm particle size), a Supelguard LC-DIOL analytical column (4 x 250 mm, 5 µm particle size), and a Varian 385-LC ELSD (evaporator temperature: 35 °C, nebulizer temperature: 35 °C, N2 flow rate = 1.5 SLM). A binary solvent system was employed for the separation of neutral lipids: eluent A (hexane:acetic acid, 99:1, v:v) and eluent B (hexane:2-propanol:acetic acid, 84:15:1, v:v:v). The following solvent gradient program was used for analyte separation: t = 0 min (100% A), t = 6 min (30% A, 70%B), t = 7 min (100% B), t = 9 min (100% B), t = 11 min (100% A), and t = 14 min (100% A). Eluting peaks were identified by comparing their retention times

(Rt, min) with those of standards obtained from Sigma-Aldrich: monoolein, 1,2-diolein, 1,3-diolein, and triolein mix (1787-1AMP, certified reference material) and cholesterol (C8667). Neutral lipids were quantified by peak area (EZChrom Elite software) and expressed as percentage of total peak area. Triacylglycerols (TAGs) were quantified using C18:1 TAG (triolein, Sigma-Aldrich, T7140) as a reference standard over a concentration working range of 0.48–2.86 μg injection-1. The on-site precision for this method (2.1% RSD; $n = 30$, over >15 days) was established using a diatom reference material (*Staurosira* sp.) prepared in 1 g quantities by High-Purity Standards (Charleston, SC; www.highpuritystandards.com/).

2.6 Lipids Classes

Twelve samples were selected for molecular-level lipid analyses by HPLC mass spectrometry (HPLC/MS). Lipids were extracted from phytoplankton filtered onto glass fiber filters (VWR, Grade 691) which were extracted using a modified Bligh and Dyer solvent extraction protocol (Bligh and Dyer 1959). Briefly, filters were immersed in dichloromethane, methanol, and phosphate-buffered saline (PBS) in a ratio of 10:20:8, sonicated for 15 min, and let to stand overnight under nitrogen at −20 °C. The next day, dichloromethane and water were added in a ratio of 10:10; samples were centrifuged for 10 min, and the organic phase was removed and concentrated as the total lipid extract; this step was repeated twice more. Phosphatidylethanolamine-*N*-(2,4-dinitrophenyl) (DNP-PE; Avanti Polar Lipids) and triheptadecenoin (17:1/17:1/17:1, NuCheckPrep) were added as internal standards at the first step of the extraction.

Total lipid extracts were analyzed for polar-diacylglyceride lipids (polar-DAGs) by using HPLC/electrospray ionization mass spectrometry (HPLC/ESI-MS) as described by Sturt et al. (2004), Van Mooy et al. (2006, 2009), and Levitan et al. (2015) on an Agilent 1100 HPLC and Thermo LCQ Deca XP ion trap MS (Levitan et al. 2015; Sturt et al. 2004; Van Mooy et al. 2006, 2009). The molecular ion chromatograms were extracted for each individual IP-DAG species at their appropriate retention time, integrated, and applied to external standard curves. The standard curves were composed of triplicate measurements at four concentrations of the following IP-DAGs: the recovery standard, DNP-PE; PG, PE, and PC, which were obtained from Avanti Polar Lipids Inc.; MGDG and DGDG, which were obtained from Matreya, LLC; SQDG and DGTS, which were isolated from phosphorus-starved cultures of *Synechococcus* WH8102 (kindly provided by E.A. Webb, University of Southern California) and *Chaetoceros gracilis* (kindly provided by S. Dyhrman, WHOI), respectively, by using preparative HPLC (Van Mooy et al. 2009); DGTS was applied as the standard for DGTA and DGCC (Van Mooy et al. 2009), since none of the betaine lipids were commercially available.

Total extracts were analyzed for TAGs and DAGs by using reversed-phase nonaqueous HPLC/atmospheric pressure chemical ionization mass spectrometry (NARP-HPLC/APCI-MS) on an Agilent 1200 HPLC and Agilent 1200 single quadrupole MS. We identified 31 distinct TAGs by their [M + H] + and

[M + H-ROOCH] + mass spectra using previously described identification protocols (Holčapek et al. 2003, 2005). External standard curves were composed of triplicate measurements of the following TAGs at five concentrations: trihexadecenoin (16:1/16:1/16:1), triheptadecenoin (17:1/17:1/17:1; recovery standard), trioctadecenoin (18:1/18:1/18:1), trioctadecatrienoin (18:3/18:3/18:3), and trieicosapentaenoin (20:5/20:5/20:5). Response factors for the TAG standards were linearly regressed against both retention time ($r^2 = 0.94$) and equivalent carbon number (ECN = acyl carbon atoms-2(acyl double bonds); $r^2 = 0.91$). Response factors for unknown TAGs were calculated from the regression against retention time, and then used to determine the concentrations of the unknown TAGs. DAGs were observed in a few samples, and these were quantified as TAGs.

2.7 Microscopy

Phytoplankton cultures for lipid observation and localization were placed in flat bottom 24 place cell wells (Becton Dickinson # 351147). Nile Red solution (0.1 mg mL^{-1}) in acetone was added to cultures for a final concentration of 1 μg Nile Red mL^{-1}. Cultures were allowed to stain for ~5 min before observation. Cells were viewed with a 100 W epifluorescense Olympus IX71 inverted microscope, and pictures were acquired with a MacroFire digital camera and PictureFrame 2.3 software. Cells were initially viewed with a filter set typical for chlorophyll observation (excitation (ex) 460–490 nm, dichroic mirror =500 nm, emission (em) >520 nm) in which lipid bodies stained a bright yellowish-green in contrast to the red autofluorescence of the chlorophyll. Several other combinations of excitation and emission filters were examined, designed to target both neutral and polar Nile Red stained lipid bodies. For comparison with the microplate method, cultures were viewed with a similar filter combination (ex. 546 ± 13 nm, DM = 560 nm, em. 585 ± 20 nm) and yellow fluorescing neutral lipid bodies accounted for the majority of the signal in most strains.

2.8 Flow Cytometry

Cellular Nile Red fluorescence was quantified with a FACSCalibur flow cytometer using argon-ion laser excitation at 488 nm and an emission wavelength of 585 ± 42 nm (FL2 channel) as previously described (Bittar et al. 2013; Johnson et al. 2010). Both live and preserved samples were measured. Preserved samples were stored at −80 °C and measured within 2 days of the live samples. Samples for flow cytometry were stained with Nile Red to a final concentration of 6 μg L^{-1} and vortexed, then kept in the dark. Aliquots were taken from the sample and measured with the flow cytometer at 5 min intervals from 0 to 30 min, with the sample vortexed before each interval. Fluorescent microspheres (Fluoresbrite Microspheres, Polysciences, Inc.) were added as internal fluorescence standards. Measurement

duration lasted an average of 40 s. Unstained cells were also used to check that no background fluorescence occurred in the same range as Nile Red fluorescence. When comparing microplate reader and flow cytometer results directly, flow cytometrically determined cell concentrations and Nile Red fluorescence per cell were multiplied to calculate the total fluorescence of a given culture and compared to bulk estimates from the microplate reader.

3 Results

The Nile Red stain was initially examined using qualitative analyses to determine the localization of the staining within lipid-containing marine microalgal cells. Using a broad excitation and emission filter set for chlorophyll *a* fluorescence optimized for visual inspection, cells that are large and have defined organelles and cellular bodies show that the majority of Nile Red fluorescence occurs in defined regions of the cells (e.g., Fig. 1a). This specificity of staining is also apparent when using excitation and emission filters that have been optimized for neutral lipid (i.e., TAGs) bound Nile Red fluorescence detection (Fig. 1b). Cells that were too small to observe specific subcellular features using standard transmission microscopy did not show any specific regions of staining, but still stained and fluoresced similarly. When viewed with the appropriate filter sets, the subcellular patterns of staining of Nile Red were nearly identical to the patterns observed with other commercially available lipid stains that target neutral lipids such as LipidTox™ and BODIPY® and are consistent with specific staining of lipid vesicle within the cells.

To determine the influence of nonspecific fluorescence in high-salt aqueous phase (i.e., saltwater medium) across wavelengths, excitation and emission spectra were measured for a variety of microalgae cultures and medium controls in the presence and absence of the Nile Red stain (Fig. 2). Excitation spectra (with emission measured at 590 nm, the peak of triacylglyceride-bound fluorescence) of stained microalgae show a significant excitation peak at 530 nm, while unstained cells and

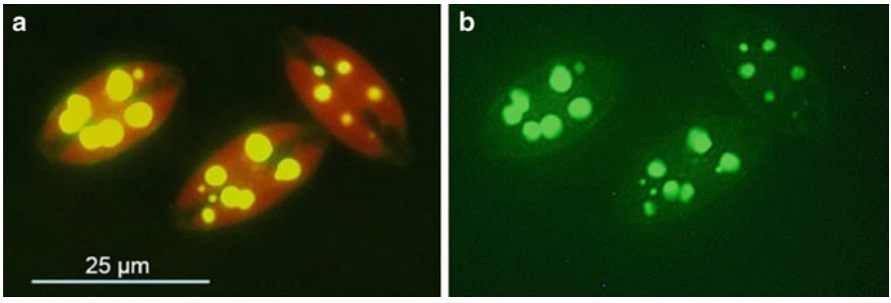

Fig. 1 Localization of Nile Red stain in microalgae. (**a**) Epifluorescence image using typical chlorophyll filter set. *Yellow* bodies are stained lipid droplets and red hue is chlorophyll (auto) fluorescence. (**b**) Epifluorescence image using narrow excitation and emission filters targeting bound neutral lipid fluorescence and optimized for microscopy. Note the localization of the stain in both images to the lipid droplets

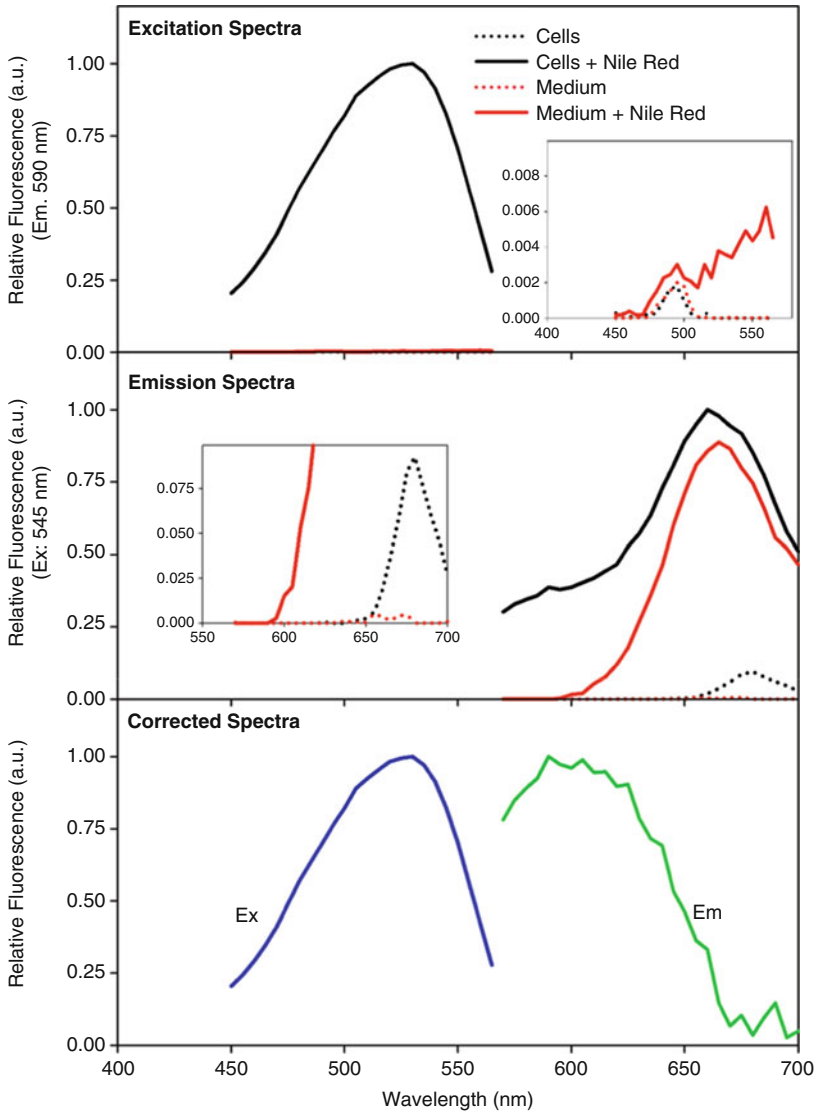

Fig. 2 Representative excitation and emission spectra of control and Nile Red stained medium and cells. *Top panel*: excitation spectra (emission 590 nm) with stained cells (*black solid*), unstained cells control (*black dotted*), stained medium control (*red solid*), and unstained medium control (*red dotted*). *Middle panel*: emission spectra (excitation 545 nm) (lines as *top panel*). *Bottom panel*: excitation (*blue line*) and emission (*green line*) spectra corrected for natural fluorescence of cells and medium and nonspecific binding

stained and unstained medium controls are all 3 orders of magnitude below this signal (Fig. 2 top panel) demonstrating that the contribution of nonspecific fluorescence is not dependent on the excitation wavelength. Emission spectra (with excitation provided at 545 nm) of stained microalgae have broad peaks at 660 nm (Fig. 2

middle panel) that are substantially longer than the 590 nm peak expected for pure Nile Red bound-triacylglyceride (Invitrogen). But unlike excitation spectra, emission spectra of the stained medium (i.e., no cells) control also have a significant broad peak at ~660 nm. Further, unstained microalgae have a minor fluorescence peak at ~680 nm that corresponds to the presence of chlorophyll *a* (Jeffrey et al. 1997), while unstained medium does not have any significant emission fluorescence. Taken together, the excitation spectra corrected for the presence of natural (i.e., unstained) fluorescence and nonspecific fluorescence (i.e., Nile Red stained medium) are essentially identical to the raw Nile Red-stained cell cultures (Fig. 2 bottom panel) and have one major excitation peak at 530 nm. However, correcting for Nile Red stained medium, and to a lesser degree natural fluorescence of microalgae, significantly changes the dominant peak of the emission wavelength spectra to ~590 nm. This wavelength is significantly lower than the raw Nile Red emission peak wavelength at ~660 nm, but has the least interference from nonspecific Nile Red fluorescence; it also more closely matches the emission peak of Nile Red bound to triacylglycerides. Thus, previous reports that show emission maxima at wavelengths >600 nm could be influenced by the presence of significant background fluorescence of Nile Red (i.e., unbound stain) in the aqueous phase (Cooksey et al. 1987) and potentially misinterpreted to indicate high levels of phospholipids, which has ~50–75 nm red-shifted excitation and emission peaks for Nile Red. This may be of particular importance when using marine microalgae because of the presence of high levels of salt in the medium. Based on the peak-corrected excitation and emission wavelengths, the availability of filters for microplate readers and the desire to target neutral lipids (i.e., triacylglycerides), an excitation of 540 ± 12.5 nm and emission of 590 ± 10 nm were used for further plate reader based assays.

Others have reported significant variability in the fluorescence signal of Nile Red as a function of time (Chen et al. 2009; Cooksey et al. 1987; Natunen et al. 2014), and therefore fluorescence is routinely measured over the course of 30 min or more (Chen et al. 2009; Elsey et al. 2007). The dynamics of these changes are variable and influenced by multiple factors including microalgae taxa, physiological conditions, and composition of extracted lipids that together result in fluorescence maxima from 0 to >30 min after the addition of Nile Red (e.g., Fig. 3). For the majority (78%) of more than 500 assays representing numerous strains, physiological conditions, and presumably cellular biochemical composition, there is a time delay between the addition of Nile Red and the fluorescence maximum (Fig. 3). The subsequent decreases over time were not due to the decay associated with the measurement process (i.e., photobleaching) as the decrease was independent of the number of times Nile Red fluorescence was quantified during the time course. To further investigate these temporal patterns, we compared standard time-course measurements from bulk estimates (i.e., microplate reader based, e.g., Fig. 3) to integrated cell-specific Nile Red fluorescence (i.e., flow cytometry) (Fig. 4) for both fresh and preserved samples. Although some differences exist in the relative fluorescence between methods as well as in the timing of the fluorescence maxima, perhaps due to differences in the temporal precision, overall bulk and cell-specific techniques show similar trends suggesting that the bulk signals

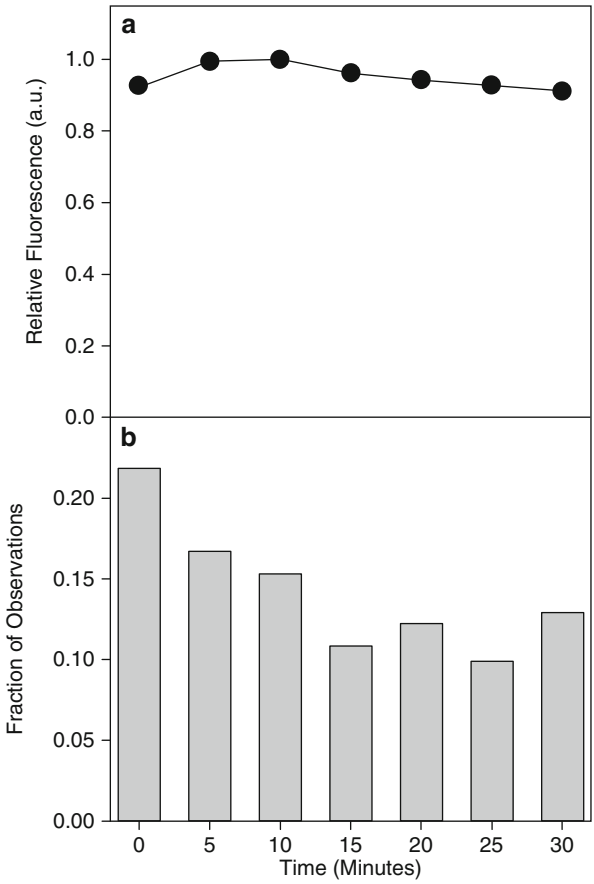

Fig. 3 (**a**) Representative temporal variability in the Nile Red fluorescence for microalgae. Error bars (standard error, $n = 24$) are smaller than the symbols. (**b**) Fraction of observations ($n = 515$, triplicate analyses) across different strains or physiological conditions with Nile Red fluorescence maxima at a given time point

are quantifying cellular properties (and not medium or other nonspecific extracellular binding artifacts).

While the bulk and cell-specific approaches show similar temporal trends, there are notable differences between some of the live and preserved samples, which can be different between the strains. For example, live *Chlorella* had fluorescence maxima near time zero, whereas preserved *Chlorella* had fluorescence maxima that were more stable in the first 15 min (Fig. 4). *Chlorella* also had the greatest temporal dynamics (specifically for fresh samples), which are driven in part by two populations of cells (stained and unstained) that begin to emerge after 5 min (Fig. 4). Preserving the cells prevented formation of two distinct populations, dramatically reducing these temporal dynamics. Conversely, *Staurosira* was relatively constant over the time course, regardless of treatment and did not have multiple populations. Finally, *Navicula* also had substantial temporal dynamics that were greatly reduced when the cells were preserved. However, unlike for *Chlorella*, the patterns for *Navicula* are not driven by heterogeneous cellular responses (i.e., multiple populations), but rather consistent increases and decreases among the cells

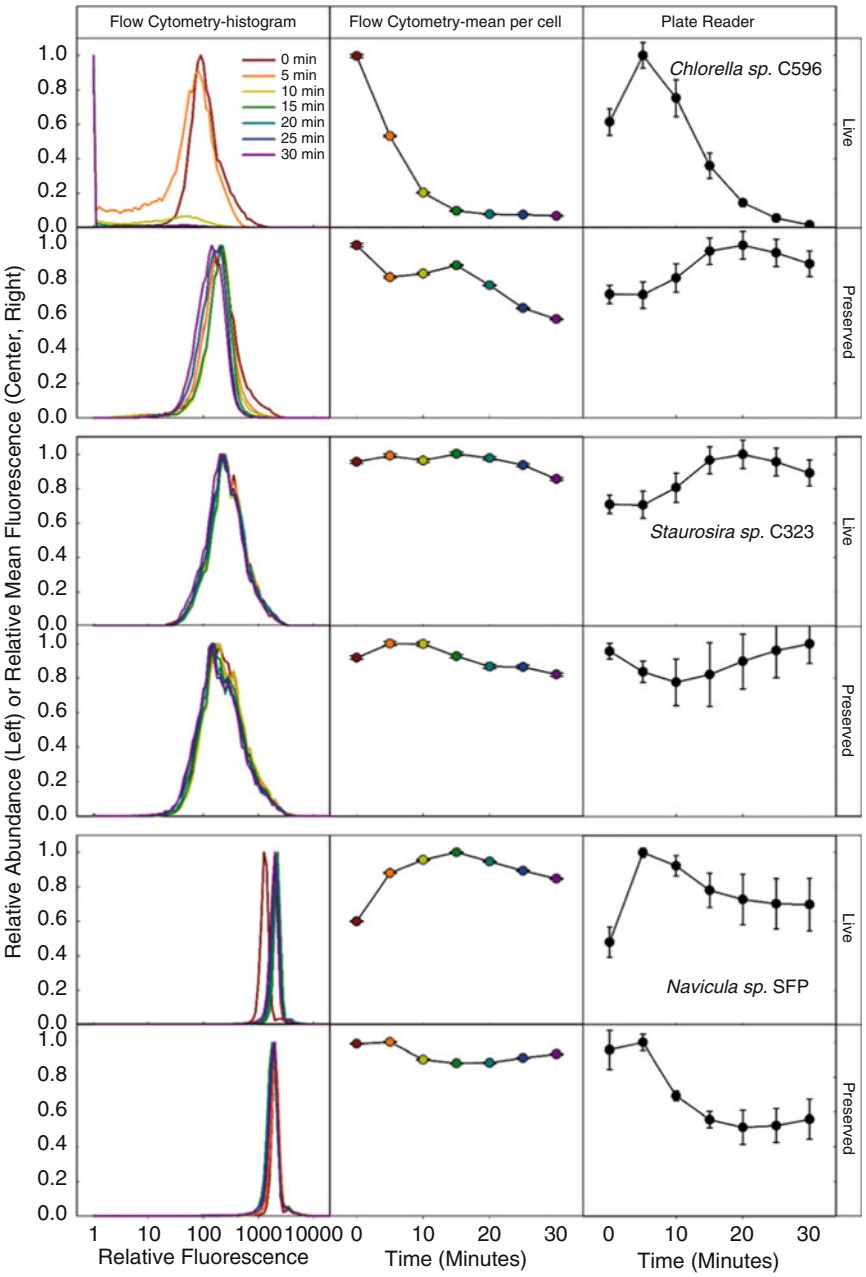

Fig. 4 Temporal variability of fluorescence per cell (*left column*), mean cellular fluorescence (*middle column*), or microplate reader (bulk) (*right column*) with live (*upper row*) or preserved (*lower row*) cells for *Chlorella* sp. C596 (*top box*), *Staurosira* sp. C323 (*middle box*), and *Navicula* sp. SFP (*lower box*). Each curve is normalized to its maximum value. Histograms represent composite population values. Means and standard error bars are for $n \geq 9$ technical replicates

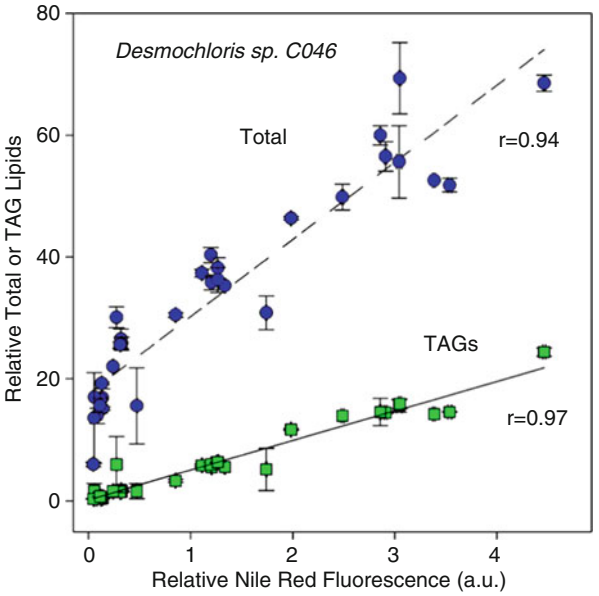

Fig. 5 Relationship between Nile Red fluorescence versus total lipids and TAGs for *Desmodesmus* sp. C046

(Fig. 5). Thus, temporal dynamics observed for bulk Nile Red signals can be manifest by both changes in population frequencies (i.e., either stained or unstained such as with *Chlorella*) or from changes in the relative efficiency of the stain such as with *Navicula*.

We compared our refined Nile Red protocol that monitors the temporal dynamics, which includes monitoring the temporal dynamics and selecting wavelengths optimized for detection, to a recently developed high precision technique for quantifying lipid classes. As expected, an individual strain grown under a variety of environmental conditions and sampled at different points in the growth curve to maximize the biochemical heterogeneity had substantial variability in its relative total and TAG lipid contents (Fig. 5). Moreover, Nile Red fluorescence was highly correlated with both total and TAG lipid content ($r = 0.94$, $n = 32$, $p < 0.01$; $r = 0.97$, $n = 32$, $p < 0.01$, respectively). This relationship was investigated across numerous strains and showed that Nile Red fluorescence is positively correlated with total cellular lipids quantified by gravimetry (Fig. 6), but with reduced fidelity ($r = 0.76$, $n = 57$, $p < 0.01$) compared to the relationship for an individual strain. Similarly, Nile Red fluorescence across strains are most strongly correlated with the TAG lipids ($r = 0.84$, $n = 57$, $p < 0.01$). However, Nile Red lipids were poorly correlated ($r = 0.45$, $n = 57$, $p < 0.01$) with the non-TAG lipids suggesting that the increased variability across species in the Nile Red – total lipids relationship was due, in part, to differences in these non-TAG lipids.

This relationship was investigated in more detail by quantifying the molecular lipid classes of selected strains (Table 1). As expected, the concentration of the various specific lipid classes varies widely but is mostly dominated by TAGs

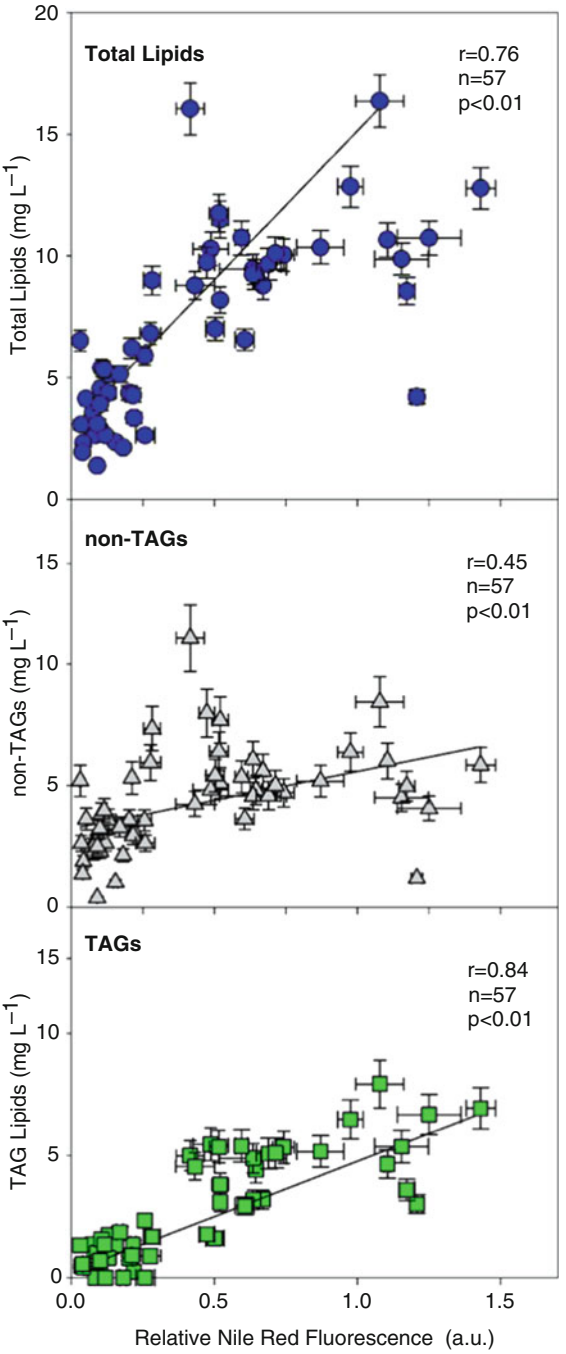

Fig. 6 Relationships between Nile Red fluorescence and total, non-TAGs, and TAGs lipids for a range of microalgae taxa poised at different physiological states

Table 1 Correlation between Nile Red fluorescence and molecular lipid classes. Statistically significant correlations ($n = 5, p < 0.05$) are in bold

Lipid class			% Total[a]	CV (%)	r-live	r-frozen	r-preserved
Polar DAGs							
	Glycolipids						
		MGDG	5.6	145	0.10	0.26	0.47
		SQDG	2.6	54	0.00	0.00	0.24
		DGDG	1.8	117	0.32	**0.96**	0.72
	Phospholipids						
		PC	2.0	137	0.46	**0.96**	0.71
		PG	1.4	91	0.36	0.74	0.36
		PE	0.5	88	0.22	0.39	0.00
	Betaine Lipids						
		DGCC	1.1	129	0.77	**0.97**	0.73
		DGTS	0.1	129	0.69	0.61	0.79
		DGTA	0.1	183	0.66	0.56	0.74
Nonpolar							
DAGs		20:0/20:0	0.6	224	0.14	n/a	n/a
		20:1/20:1	0.3	139	0.41	0.48	0.20
TAGs							
		16:1/16:0/16:1	9.7	79	**0.93**	**0.95**	**0.98**
		16:0/16:1/16:0	8.0	70	0.48	0.36	0.76
		16:1/16:1/14:0	7.4	132	**0.90**	**0.97**	0.75
		16:0/16:1/14:0	7.0	111	**0.89**	**0.98**	0.77
		16:1/16:0/20:5	4.3	77	**0.97**	**0.99**	**0.85**
		16:1/16:1/16:1	4.3	117	**0.87**	**0.99**	**0.82**
		16:0/18:2/20:4	4.2	136	**0.84**	**0.97**	0.74
		14:0/16:1/20:5	3.9	145	**0.92**	**0.96**	0.73
		16:1/16:1/20:5	3.8	130	**0.95**	**0.98**	0.77
		Unidentified TAGs	3.0	193	**0.86**	**0.97**	0.73
		16:1/16:1/20:4	2.1	121	**0.85**	**0.99**	0.80
		16:1/14:0/20:4	1.8	136	0.73	**0.97**	0.73
		16:0/16:0/20:5	1.3	63	0.45	0.57	0.76
		16:0/16:1/20:4	0.8	150	0.22	0.30	0.17
		16:0/16:0/20:4	0.6	94	0.35	0.30	0.71
		18:2/18:2/18:3	0.5	121	**0.92**	**0.97**	0.76
		14:0/14:0/18:2 + 14:0/14:0/17:1	0.5	147	**0.92**	**0.97**	0.74
		16:0/14:0/20:5	0.4	174	0.73	**0.91**	0.62
		18:3/16:0/16:1	0.4	176	**0.87**	**0.99**	**0.84**
		18:1/16:0/16:1	0.3	105	**0.98**	**0.96**	**0.98**
		20:4/16:0/20:4	0.2	120	0.00	0.39	0.00
		20:5/16:0/20:5	0.1	159	0.57	0.51	0.50

(*continued*)

Table 1 (continued)

Lipid class			% Total[a]	CV (%)	r-live	r-frozen	r-preserved
		17:1/17:0/14:0	0.1	154	0.14	0.10	0.39
		18:0/16:0/16:1	0.1	224	0.00	0.10	0.39
		16:0/16:0/16:0	0.1	224	0.00	0.10	0.39

[a]Percent of lipid class normalized to gravimetrically measured lipids, averaged across strains and replicates

Table 2 Correlation between Nile Red fluorescence and lipid class totals for five microalgae strains. Statistically significant correlations ($n = 5$, $p < 0.05$) are in bold

Lipid class	% Total	CV (%)	r-live	r-frozen	r-preserved
Polar DAGs	15.1	110	0.24	0.60	0.24
Nonpolar DAGs	0.3	139	0.41	0.48	0.20
TAGs	65.1	95	**0.97**	**0.99**	**0.88**
Gravimetric Lipids	100.0	58	**0.95**	**0.99**	**0.87**

(Table 2). In general, Nile Red fluorescence is most highly correlated with the most abundant TAG classes including 16:1/16:1/20:5, 14:0/16:1/20:5, 16:1/16:0/20:5, 16:1/16:1/16:1, 16:1/16:1/14:0, 16:0/18:0/20:4, 16:1/16:0/16:1, and 16:0/16:1/14:0. Another abundant TAG (16:0/16:1/16:0) is not strongly correlated with Nile Red (but this low correlation is driven by one point and would otherwise be >0.85). Other polar and nonpolar diacylglycerols (DAGs) are also not significantly correlated with Nile Red fluorescence. Nile Red fluorescence measured on live, frozen, or preserved samples generally showed the same correlations with the various lipid classes, and there were no statistically significant differences between the treatments as an ensemble, even though there are differences among some of the molecular lipid classes. Summing the specific lipid classes into major categories of polar-DAGs, nonpolar DAGs, TAGs or total gravimetric lipids and comparing to Nile Red fluorescence shows similar patterns to the larger data set using higher throughput approaches (Fig. 6). Overall there are no statistically significant differences between Nile Red fluorescence and major lipid classes for samples that were live, frozen, or preserved (Table 2). In summary, Nile Red fluorescence is most strongly correlated with TAGs and less strongly correlated with other lipid components and is in bulk unaffected by common sample preservation methods.

4 Discussion

Here we show that nonspecific binding of the Nile Red stain can lead to substantial fluorescence emission at longer wavelengths. In particular, in a high ionic strength water matrix (such as the saltwater used here or standard laboratory cell buffers like

phosphate buffer saline) the majority of the fluorescence emission signal above 600 nm is from unbound Nile Red and therefore not quantitative for lipids. Proper selection of wavelengths for lipid quantification (i.e., neutral vs. polar) or consideration of blanks and background corrections is essential.

Upon Nile Red addition to microalgae suspensions, the fluorescence intensity generally increased over time to a maximum and then decreased (Fig. 3b) – most (65%) of the temporal peaks in Nile Red fluorescence occurred between 5 and 25 min with an additional 22% at time zero. These temporal patterns occur with both live and dead (frozen or preserved) for bulk suspensions or cell-specific metrics (Fig. 4). However, for some strains these dynamics are modified (or attenuated) by preservation suggesting that the temporal patterns are related to a combination of active and passive cellular processes (Fig. 4). While the preservation of microalgae prior to dye addition does not dramatically affect the interpretation of bulk Nile Red fluorescence for major lipid class contents (e.g., Tables 1 and 2), cell-specific results can be impacted (e.g., Fig. 4 – *Chlorella* sp.) highlighting the importance of maintaining observations over a period of time and monitoring for population heterogeneity. (In microtiter plates used here, cell settling will not affect the fluorescence intensity since all contents of each well are equally quantified, regardless of their position within the cell well.) Others have observed this temporal variability (Pick and Rachutin-Zalogin 2012) including bi-modal peaks (Elsey et al. 2007) (which we did not observe) and shown that it can be driven by a combination of biotic (e.g., active dye pumping) and abiotic factors (e.g., diffusion; dye-dye; or dye-matrix interactions). Beyond the specific mechanisms responsible for this temporal variability, for practical purposes much of this variability can be reduced by preserving the cells and monitoring fluorescence intensity over time, without the need for lipid separation via density gradients or solvent extraction (Pick and Rachutin-Zalogin 2012).

Regardless of the wavelength pair used to visualize fluorescence, the majority of the Nile Red signal originates from specific subcellular locations (namely the lipid vesicles) of the microalgae (Fig. 1). These lipid vacuoles, which are essentially 100% lipid, are comprised of specific types of lipids, and in most strains Nile Red is targeting these lipids more strongly than other common cellular lipids such as membrane associated lipids (phospholipids, glycolipids, and sterols) or other structural features. Cells that have been subject to various disruptions including freeze/thaw or ultrasonic disruptions can show different staining patterns suggesting that this localization can be altered by compromising subcellular membranes or by altering the fluidity permeability and binding sites for Nile Red (Dempster and Sommerfeld 1998). This is evident in some differences among the live, frozen, and preserved samples in specific molecular classes of lipid (Table 1). Nevertheless, the overall bulk Nile Red – lipid class relationship holds regardless of treatment (Table 2).

Consistent with the localization of stain within the cell, the Nile Red signal is most highly correlated with neutral lipids and specifically TAGs within the cell (Tables 1 and 2). These lipid classes are the dominant energy storage lipids within

the cell. Other major structural lipids which are present such as the polar glycolipids are not well-quantified by Nile Red, making the stain a good candidate for targeting the neutral lipids without significant interference from other potentially abundant lipids. Using this approach there is a robust relationship between Nile Red fluorescence and neutral lipids for a given strain (Fig. 5). While this relationship is less strong across strains, due to differences in cell lipid composition (Table 1) and other biological factors (e.g., Fig. 4), Nile Red is still sufficiently robust to identify taxa with elevated TAGs without the need to optimize solvents or additives (Natunen et al. 2014). In turn, the conditions that optimize neutral lipids in these candidate strains can then be more precisely defined because of the robust intraspecific Nile Red-neutral lipid correlation. The Nile Red method to quantify neutral lipids in microalgae in solution provides a rapid screening tool for comparing the lipid quantity of hundreds of samples in a day. Because of the low volume (<300 μL per assay well) and biomass requirements (<3 mM C), numerous cell lines can be assayed in small volume microtiter plates. Moreover, the same cell lines can be grown under a factorial of environmental conditions (temperature, light, growth medium, etc.) that cause variation to identify conditions that optimize lipid profiles (Bittar et al. 2013). This screening approach may be of particular use in comparing or developing (through genetic modification by either random or targeted mutagenesis) microalgae strains for next generation biofuels where neutral lipids (i.e., TAGs) are of particular interest and where there is great potential for increasing lipid production through changes in environmental conditions or strain modification (Doe 2016; Georgianna and Mayfield 2012; Williams and Laurens 2010).

5 Research Needs

Nile Red has been used to quantify cellular lipids for ~30 years (Greenspan et al. 1985) and improvements over time have increased its utility for specific types of cell lines including microalgae (Chen et al. 2009; Cooksey et al. 1987; Elsey et al. 2007). Yet because of its increased use in the screening for lipids of collections of microalgae, additional refinements in protocols and interpretation may be necessary for broader application. In particular, the differences observed between the microalgae strains (e.g., Fig. 4) point to additional areas where protocols can be refined to overcome the likely fundamental differences in biology. Comparing the responses of other lipid binding stains (e.g., BODIPY, LipidTox) to Nile Red in the context of temporal, live/dead, and strain to strain variability may provide insight into the mechanisms of these varied responses. Finding solutions or work-arounds to these challenges will further enhance the utility of Nile Red as a tool for screening marine microalgae for lipids.

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Advanced Biodiesel and Biojet Fuels from Lignocellulosic Biomass

7

Tian Tian and Taek Soon Lee

Contents

Abstract

Global energy demands and environmental concerns have stimulated interest in renewable, carbon-neutral diesel and jet fuel from biomass. Lignocellulosic biomass is considered as a promising resource for the future bioindustry. The

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plant cell wall is a polymer network comprised largely of the sugar polymers such as cellulose and hemicellulose, and the polyphenolic lignin, and considerable efforts have been made toward the conversion of lignocellulose into fermentable sugars for their use in microbial fuel synthesis. Genetically engineered microbial hosts can utilize these sugars as a carbon source to biosynthesize a broad panel of bioproducts including fatty acid-, isoprenoid-, and alcohol-derived compounds, which can be used as precursors or directly as fungible alternatives to diesel and jet fuel. In this chapter, we review the principles of biofuel synthesis from biomass-derived sugar, summarize the promising technologies of biomass deconstruction and pathway engineering, and discuss the current applications of biodiesel and biojet fuels.

1 Introduction

Concerns over global petroleum supply, energy security, and environmental sustainability have generated tremendous interest in the search for alternative transportation fuels. Currently, ethanol produced from corn, sugar cane, or beets and biodiesel produced from vegetable oil and animal fats are predominantly used in many nations including USA, Brazil, European Union, and China (Antoni et al. 2007). Because of its low energy density and high hygroscopicity, ethanol is not an ideal replacement for liquid fuel (Fortman et al. 2008). Additional debate has surfaced regarding the ethics of using arable land for the production of ethanol from food crops, as well as using vegetable oil and animal fats for biodiesel production (Vasudevan and Briggs 2008). Lignocellulose offers an appealing solution to these concerns as it does not compete with food supplies and can be grown on land unfit for food crops. Over the past decades, microbial fermentation has prevailed as a promising alternative for fuel production with several advantages: (1) microbes grow fast; (2) microbial fermentation usually requires mild conditions; (3) microbes can utilize biomass directly or simple sugars from biomass hydrolysis as carbon sources; (4) recent advances in metabolic engineering and synthetic biology have enabled microbes to produce various candidate molecules for biofuels (Lee et al. 2008; Markham and Alper 2015).

In this chapter, we focus on advanced biodiesel and biojet fuels produced by genetically engineered hosts capable of utilizing lignocellulosic precursors (Fig. 1). First, we will briefly discuss physicochemical properties of biodiesel and biojet fuels, and describe the selection criteria of the molecules targeted for microbial production. The biofuel compounds covered in this chapter are mainly derived from central metabolites from glycolysis such as phosphoenolpyruvate (PEP), pyruvate, and acetyl-CoA (Fig. 2). We do not cover biofuels derived from lignin here. The biosynthesis of each fuel type will be discussed based on pathway construction, host selection, and strain optimization. We will also highlight some recently developed synthetic biology tools and their significance in pathway engineering and genomic

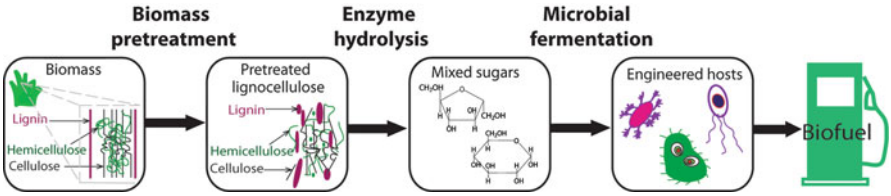

Fig. 1 Conversion of biomass to biofuels by microbial fermentation

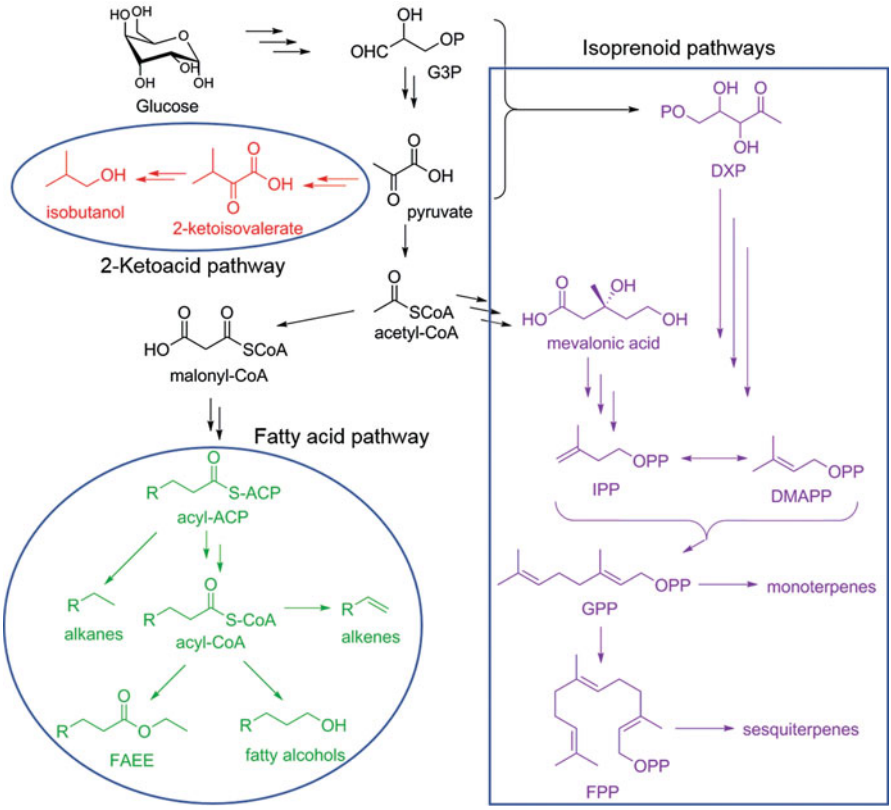

Fig. 2 Microbial conversions of glucose to diverse biofuel products. 2-keto acid pathway, isoprenoid-derived pathway, and fatty acid pathway are highlighted in *boxes* and *circle*. G3P, glyceraldehyde 3-phosphate; DXP, 2-C-methy-D-erythritol-4-phosphate; ACP, acyl carrier protein; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; FAEE, fatty acid ethyl ester

regulation. Finally, we will briefly describe and compare various lignocellulosic biomass deconstruction technologies and will report several commercialization examples of biodiesel and biojet fuel production from biomass.

2 Advanced Biodiesel Production from Microbial Hosts

2.1 Fuel Properties and Examples of Biodiesel

The carbon number of diesel fuel typically varies from 9 to 23, with an average of 16 of the mixed hydrocarbons (Table 1). Advanced biodiesel includes mixtures of biosynthetic alkanes, alkenes, long chain alcohols, and esters in this carbon number range. The properties of biodiesel are determined by what types of species are present in the fuel – each contributes to various properties including cetane number, heat of combustion, melting point, lubricity, viscosity, and toxicity (Rude and Schirmer 2009).

Cetane number is an indicator of the ignition properties for diesel fuel. A cetane number from 40 to 55 is generally sufficient for use in a diesel engine. The heat of combustion is also an important property that measures the combustion quality and that of cetane (or hexadecane) is 2,559.1 kcal/mol (at 20 °C). Other properties such as melting point (or freezing point), lubricity, and viscosity are also important factors since they affect the formation of engine deposits. Generally, low melting point, high lubricity, and low viscosity are desirable properties to avoid solidification of saturates in engines and facilitate fuel transportation in the existing distribution infrastructure.

The relationship between desired fuel properties and chain length/structure of hydrocarbons is complicated, and no simple rule is to follow when selecting the optimal candidate. In general, cetane number and energy content increase with increasing length and degree of saturation of the hydrocarbon chain. However, long chain hydrocarbons may lead to high melting point, and unsaturated compounds may exhibit better lubricity. For unsaturated compound, the configuration of double bounds in the chain can also influence viscosity of the fuel. For example, *cis*-double bond renders lower viscosity than *trans*-double bound. Additionally, branched chain and bulky substituents in the chain can lead to low freezing point which enables the use of diesel fuel in cold environment. Therefore, to select proper candidates for diesel fuel replacement, multiple factors need to be considered and balanced. By far, fatty acid alkyl ester (FAAE); long chain alcohols and alkanes; linear, branched, and cyclic isoprenoids have been proposed to meet the criteria as potential biodiesel candidates (Kang and Lee 2015; Vasudevan and Briggs 2008). In the next section, we discuss the strategies for making those biodiesel candidates

Table 1 Desired properties of diesel and jet fuels

Fuel type	Major components	Properties	Potential advanced biofuels
Diesel	C_9-C_{23} hydrocarbons	Cetane number 40–60	Alkenes, alkanes, linear or cyclic isoprenoids, fatty alcohols, FAEE, FAME
Jet fuel	C_8-C_{16} hydrocarbons	High energy density;very low freezing temperature	Linear, cyclic isoprenoids, branched alkanes, isobutanol-derived kerosene

based on biosynthetic routes selection, pathway construction, and genome modification.

2.2 Microbial Biodiesel Production from Sugars

2.2.1 Fatty Acids-Derived Biodiesel

The fatty acid biosynthesis pathway is the most prevailing route for biodiesel synthesis from microorganisms. As a component of cell membranes, fatty acids are synthesized in high flux and converted to phospholipids. Although fatty acids cannot be directly used as diesel fuel, its derivatives, including fatty alcohols, fatty alkanes/alkenes, and FAAEs are all desirable diesel substitutes (Liao et al. 2016; Rottig et al. 2010). The native fatty acids biosynthesis pathway starts from the ubiquitous precursor acetyl-CoA, which is converted to malonyl-CoA by acetyl-CoA carboxylase (ACC), and further converted to malonyl-ACP (acyl carrier protein) (Fig. 3). The condensation of malonyl-ACP and acetyl-CoA generates acetoacetyl-ACP (β-ketoacyl-ACP), which is subsequently transformed into acyl-ACP by β-keto reduction, dehydration, and enoyl reduction catalyzed by enzymes FabG, FabZ, and FabI, respectively. The elongation of fatty acyl chain initiates on acyl-ACP. The condensation of acyl-ACP and malonyl-CoA catalyzed by FabB results in the formation of β-ketoacyl-ACP with addition of two carbons to the initial hydrocarbon chain. The β-ketoacyl-ACP then can be further transformed to an elongated acyl-ACP compound through the same repeated cycle catalyzed by FabG, FabZ, and FabI (Lennen and Pfleger 2012).

The long acyl chain generated in the native fatty acids biosynthesis pathway is hydrophobic and energy rich, which makes it a preferred precursor for biodiesel. Acyl-ACP can be further transformed to fatty acids by endogenous thioesterase (TesA), and the excessive fatty acids can be activated to acyl-CoA by FadD for degradation through the β-oxidation pathway carried out by FadE, FadB, and FadA (Rottig et al. 2010). Thus, high yield of fatty acids biosynthesis can be achieved by deleting *fadE* in the chromosome to eliminate flux towards degradation of fatty acids and overexpressing a leaderless version of TesA (Kang and Lee 2015; Liu and Khosla 2010). This truncated TesA is secreted to cytosol rather than to periplasm, which disrupts the inhibition mechanism of membrane phospholipid synthesis and lead to fatty acids overproduction.

To produce fatty acid ethyl esters (FAEE), Steen and coworkers introduced heterologous genes encoding acyltransferase (*atfA*), pyruvate decarboxylase (*pdc*), and alcohol dehydrogenase (*adhB*) in *E. coli*. *AtfA* from *Acinetobacter baylyi* strain ADP1 can catalyze the formation of FAEE using ethanol and acyl-CoA as substrates. Since the endogenous ethanol production in *E. coli* is insufficient for de novo FAEE production, *pdc* and *adhB* from *Zymomonas mobilis* were also selected to carry out ethanol production. To further improve the flux toward making acyl-CoA, the genes encoding the truncated thioesterase TesA (‘TesA) and FadD were both overexpressed and *fadE* was deleted. The resulting FAEE production reached 0.67 g/L with 2% glucose in the presence of an overlay (Steen et al. 2010). Building on this

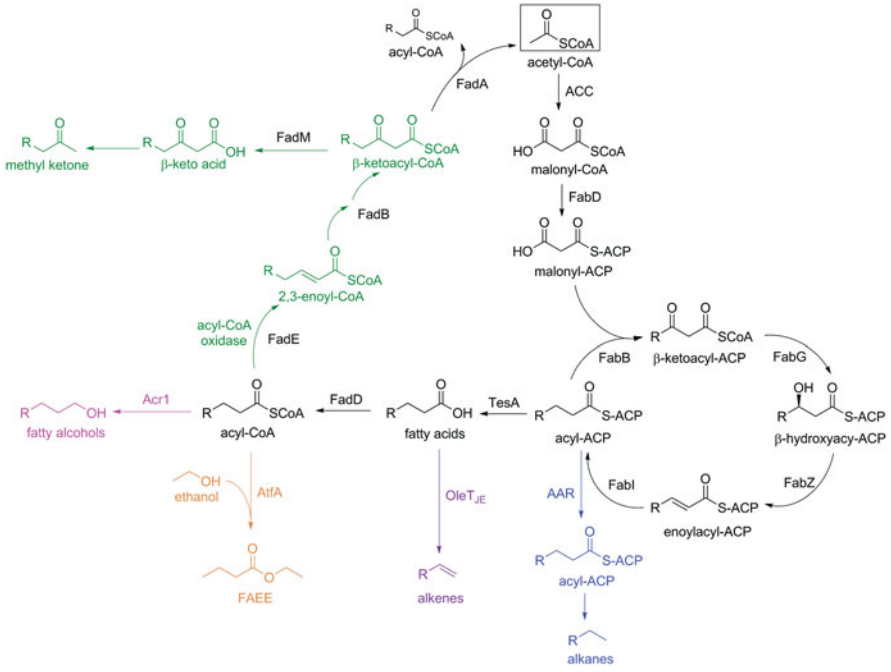

Fig. 3 Pathways for fatty acid-derived biodiesel and biojet fuel alternatives starting from acetyl-CoA (*boxed*). The pathways lead to various target products: fatty alcohols, FAEE, alkenes, and alkanes. The biosynthesis of methyl ketones utilizes an incomplete β-oxidation pathway where heterologous acyl-CoA reductase is expressed instead of endogenous FadE. Native enzymes FadB and FadM are also overexpressed and FadA is knocked out. ACC, acetyl-CoA carboxylase; FabD, malonyl-CoA:ACP transacylase; FabB, β-ketoacyl-ACP synthase I; FabG, β-ketoacyl-ACP reductase; FabZ, β-hydroxyacyl-ACP dehydratase; FabI, enoylacyl-ACP reductase; TesA, acyl-ACP thioesterase; FadD, acyl-CoA synthase; FadE, acyl-CoA dehydrogenase; FadB, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase; FadA, β-ketoacyl-CoA thiolase; FadM, thioesterase; Acr1, acyl-CoA reductase; AtfA, wax ester synthase; AAR, acyl-ACP reductase; ADO, aldehyde-deformylating oxygenase; OleTJE, a cytochrome P450 enzyme that reduces fatty acids to alkenes

study, Duan et al. overexpressed genes encoding acetyl-CoA carboxylase (ACC) to improve malonyl-CoA production, which further improved the production titer to 0.92 g/L with rich growth medium (Duan et al. 2011).

Like FAEE, fatty alcohols constitute much of biodiesel. To produce fatty alcohols, *acr1* encoding a fatty acyl-CoA reductase from *Acinetobacter calcoaceticus* BD213 was overexpressed in *E. coli*. To redirect flux toward acyl-CoA production, similar strategies including overexpressing truncated TesA and native *E. coli* FadD were also applied while knocking out *fadE* (Steen et al. 2010).

In addition to FAEE and fatty alcohols, medium chain methyl ketones are also suitable biodiesel candidates. In studies performed by Goh et al., methyl ketones (primarily C11-C15) were overproduced by taking advantage of an incomplete β-oxidation pathway and a subsequent conversion of β-ketoacyl-CoA to β-keto

acids (Goh et al. 2012). In *E. coli*, the native β-oxidation pathway initiates on acyl-CoA through dehydrogenation by FadE to produce a *trans*-2-enoyl-CoA. Then the hydration and oxidization of enoyl-CoA generates β-ketoacyl-CoA by FadB. The last step of β-oxidation pathway is catalyzed by FadA through thiolytic cleavage of β-ketoacyl-CoA, which creates acetyl-CoA and a shorter (n-2) acyl-CoA. To convert β-ketoacyl-CoA to β-keto acids, which can spontaneously be transformed to methyl ketones, an endogenous thioesterase FadM was overexpressed. To enhance the flux toward β-ketoacyl-CoA to make methyl ketones, *fadA* was also deleted from the chromosome, and after a series of pathway optimizations and genome modifications in fed-batch fermentations using glucose, a titer of 3.4 g/L methyl ketones production was achieved in *E. coli* (Goh et al. 2014).

Medium chain alkenes are also among those compounds suitable as biodiesel substitutes. Overexpression of heterologous cytochrome P450 enzyme $OleT_{JE}$ from *Jeotgalicoccus* sp. ATCC 8456, which can catalyze the conversion of fatty acids to terminal alkenes (α-olefins), has been reported for the production of 1-pentadecene and 1,10-heptadecadiene (Rude et al. 2011).

Among the potential biodiesel targets, alkane biosynthesis remains least explored in the past decades (Rude and Schirmer 2009). Recently, the discovery of two cyanobacteria enzymes enabled the overproduction of fatty acid derived alkanes in *E. coli*: an acyl-ACP reductase (AAR) and an aldehyde-deformylating oxygenase (ADO). The pathway consists of two steps: the reduction of acyl-ACP to fatty aldehydes, followed by a deformylation of fatty aldehydes to alkanes. By expressing these two heterologous genes from *Nostoc punctiforme* PCC73102 in *E. coli*, Schirmer and coworkers achieved more than 300 mg/L alkanes (C_{15} to C_{17}) (Schirmer et al. 2010). Later, an alternative acyl-CoA reductase from *Clostridium acetobutylicum* and a fatty aldehyde decarbonylase from *Arabidopsis thaliana* were also identified and introduced to *E. coli*, showing alkane (C_9-C_{14}) titers up to 580 mg/L (Choi and Lee 2013).

2.2.2 Isoprenoid-Derived Biodiesel

Isoprenoids are broadly involved in a variety of essential biological functions, including cell wall assembly, electron transport, respiration, and hormone regulation. Since methyl branches and the ring structure of isoprenoids contribute to low freezing point and high energy density, there is a growing interest in using isoprenoids for biodiesel (George et al. 2015). C_{15} isoprenoids, farnesene and bisabolene, have been extensively studied for microbial production (Beller et al. 2015). They can be chemically hydrogenated to farnesane and bisabolane with acceptable cetane ratings of 58 and 52, respectively. Farnesol (FOH) has also been evaluated as a biodiesel alternative, and some C_{10} isoprenoids such as myrcene and ocimene have also been found to decrease the cloud point of consumer grade diesel when hydrogenated and blended at 5–10% (Tracy et al. 2009).

Isoprenoid biosynthesis involves in the production of two universal C_5 precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). The chain elongation reactions are achieved via condensation of IPP to prenyl diphosphates of various lengths such as DMAPP (C_5), geranyl diphosphate (GPP, C_{10}), and

farnesyl diphosphate (FPP, C_{15}) to generate precursors of monoterpenes (C_{10}), sesquiterpenes (C_{15}), and diterpenes (C_{20}), respectively.

The C5 precursors IPP and DMAPP can be synthesized via two pathways as seen in Fig. 4: the 2-methyl-D-erythritol-4-phosphate (MEP) pathway (also called the 1-deoxyxylulose-5-phosphate (DXP) pathway) and the mevalonate (MVA) pathway. The MEP pathway is utilized in most bacteria and in plant plastid, whereas the MVA pathway is found in archaea, fungi, plant cytosol, and other eukaryotes as well as some bacteria. In the MEP pathway, pyruvate and glyceraldehyde 3-phosphate (G3P) substrates are utilized by DXP synthase (DXS) in conversion to DXP. DXP is reductively isomerized to MEP by DXP reductoisomerase (DXR), and MEP is then activated (by 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase (MCT)), phosphorylated (by 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol kinase (CMK)), and cyclized (by 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MDS)) to produce 2-C-methyl-D-erythritol-2,4-cyclodiphosphate (MEcPP). The final two steps are catalyzed by two iron-sulfur enzymes: hydroxymethylbutenyl diphosphate synthase (HDS) and hydroxymethylbutenyl diphosphate reductase (HDR) to yield both IPP and DMAPP (Zhao et al. 2013). The MVA pathway was discovered and engineered much earlier than the MEP pathway and begins with the condensation of two molecules of acetyl-CoA to acetoacetyl-CoA by a thiolase. Acetoacetyl-CoA is further condensed with an acetyl-CoA to generate 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by HMG-CoA synthase (HMGS). HMG-CoA is then reduced by HMG-CoA reductase (HMGR) to generate mevalonic acid (MVA). The resulting MVA is subject to two sequential phosphorylations by separate kinases, mevalonate kinase (MK) and phosphomevalonate kinase (PMK), to give mevalonate diphosphate (MVA-PP). The acid diphosphate is subsequently decarboxylated by mevalonate diphosphate decarboxylase (PMD) to yield IPP. Unlike the MEP pathway where both IPP and DMAPP are generated from a precursor via a single enzymatic step simultaneously, the MVA pathway relies on an isomerase (IDI) to convert IPP to DMAPP. The expression level of IDI controls the ratio between IPP and DMAPP production (Vranova et al. 2013).

The expression of the heterologous MVA pathway in *E. coli* led to high level production of isoprenoid precursors by bypassing the endogenous regulation which frequently limits the engineering of native pathway (Martin et al. 2003). This strategy has been applied to the biosynthesis of sesquiterpenes such as farnesene, and bisabolene. To increase metabolic flux towards terpene production, genes encoding the MVA pathway and the target sesquiterpene synthase are overexpressed. For farnesene, Wang et al. reported a titer of 380 mg/L using a de novo synthesized, codon-optimized farnesene synthase and exogenous MVA pathway in *E coli* (Wang et al. 2011). Later, Zhu et al. produced 1.1 g/L farnesene using the guided in vitro MVA pathway reconstitution approach (Zhu et al. 2014). For bisabolene, Alonso-Gutierrez et al. used a proteomics-aided approach to engineer the MVA pathway, boosting titers to 1.15 g/L in *E. coli* (Alonso-Gutierrez et al. 2015). Upon optimizing the native MVA pathway in yeast strains, several success examples have also been seen. Yang and coworkers used a fusion protein of codon-optimized FS and FPPS in *Yarrowia lipolytica* and produced 0.26 g/L farnesene with batch fermentation (Yang

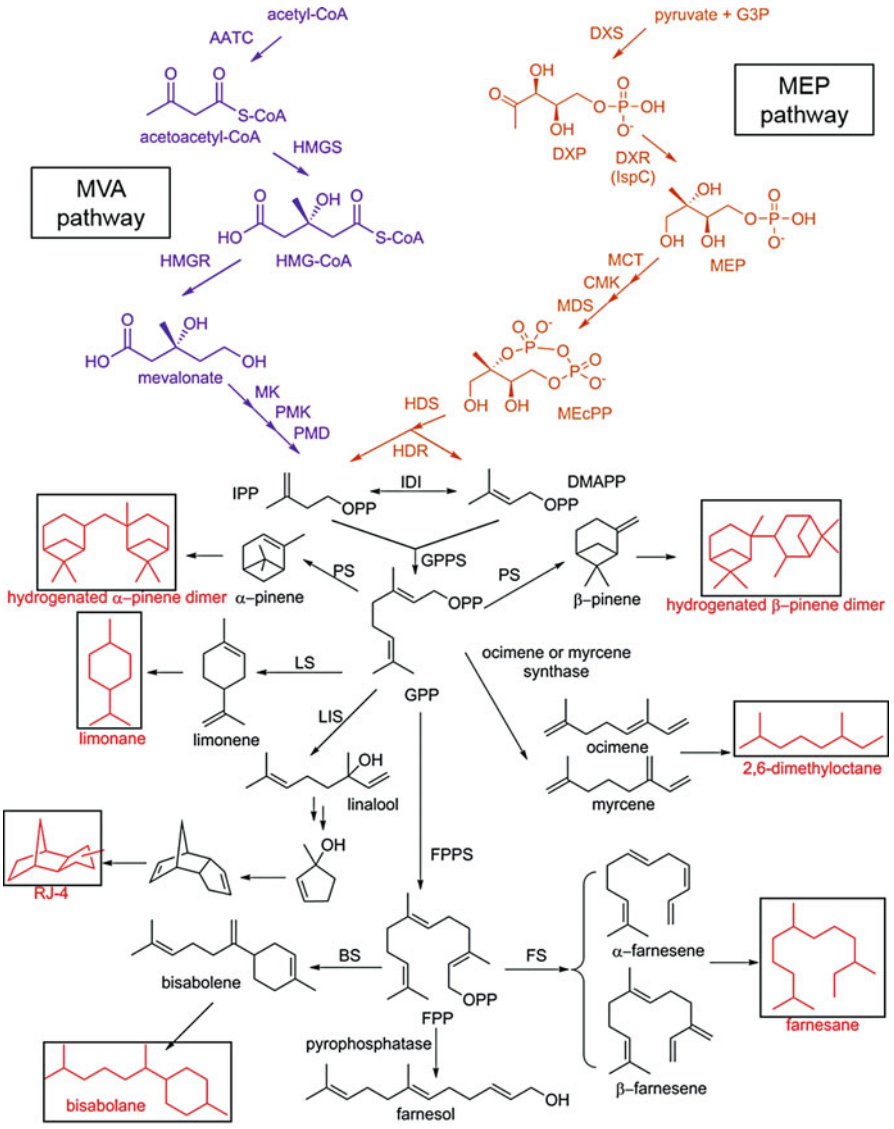

Fig. 4 Isoprenoid-derived biodiesel and biojet fuel products from MEP and MVA pathways. Examples of monoterpenes- and sesquiterpenes-derived biofuels are *boxed*. AACT, acetoacetyl-CoA thiolase; HMGS, HMG-CoA synthase; HMGR, HMG-CoA reductase; MK, mevalonate kinase; PMK, phosphomevalonate kinase; PMD, mevalonate diphosphate decarboxylase; IDI, isopentenyl diphosphate isomerase; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; MEP, 2-C-methyl-D-erythritol 4-phosphate; MCT, 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase; CMK, 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol kinase; MDS, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; MEcPP, 2-C-methyl-D-erythritol-2,4-cyclopyrophosphate; HDS, hydroxymethylbutenyl diphosphate synthase; HDR, hydroxymethylbutenyl diphosphate reductase; IPP, isopentenyl diphosphate;

et al. 2016). In *Saccharomyces cerevisiae* chromosomal deletions were performed and the strains were selected by a carotenoid-based phenotypic screening method, resulting in bisabolene titers of 0.8 g/L in batch and 5.2 g/L in fed-batch fermentation (Ozaydin et al. 2013).

The optimization of the native MEP pathway in many bacterial species has also been explored. The modulation of native MEP pathway and expression of farnesene synthase in cyanobacterium *Anabaena* sp. led to production of 0.3 mg/L farnesene (Halfmann et al. 2014), while the expression of the bisabolene synthase in cyanobacterium *Synechococcus* sp. generated 0.6 mg/L bisabolene (Davies et al. 2014). In *Streptomyces venezuelae*, over 10 mg/L bisabolene was obtained by deletion of other native sesquiterpene and monoterpene synthases downstream of the MEP pathway, with simultaneous overexpression of endogenous FPP synthase and exogenous bisabolene synthase (Phelan et al. 2015). In addition to farnesene and bisabolene, farnesol (FOH) is also certified as drop-in biodiesel. In *E. coli*, FOH has been generated by solely overexpressing FPPS either with the heterologous MVA pathway (135.5 mg/L) (Wang et al. 2010) or with the endogenous MEP pathway (389 μg/L) (Ohto et al. 2009b). Similarly, in *S. cerevisiae*, Ohto reported 145.7 mg/L FOH production along with other prenyl alcohols by overexpressing genes in the MVA pathway and prenyl diphosphate synthases (Ohto et al. 2009a).

Microbial production of monoterpenes (C_{10}) such as myrcene and ocimene are less explored as biodiesel fuels compared to sesquiterpenes (C_{15}) such as farnesene and bisabolene. Monoterpenes are synthesized from geranyl diphosphate (GPP) by GPP synthase (GPPS), mostly originated from plant. Unlike farnesyl diphosphate synthase (FPPS) whose activity appears to be ubiquitous in most living organisms, GPPS activity is not as prevalent in native microbial systems, and as a result, heterologous expression of GPPS is required to increase GPP pool (Chen and Liao 2016). Recently, synthesis of myrcene in *E. coli* has been reported by heterologous expression of the MVA pathway, IDI, GPPS from *Abies grandis*, and a myrcene synthase from holm oak at the titer of 58 mg/L with glycerol as a carbon source (Kim et al. 2015).

3 Biojet Fuel Production from Microbial Hosts

3.1 Fuel Properties and Examples of Biojet Fuel

Most jet fuels are a mixture of hydrocarbons with chain lengths ranging from 8 to 16 carbons. The physicochemical properties of jet fuel are similar to those of biodiesel but require higher energy density and lower freezing point (Table 1). To meet those criteria simultaneously, long linear alkanes are almost excluded due to

Fig. 4 (continued) DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GPPS, GPP synthase; FPPS, FPP synthase; PS, pinene synthase; LS, limonene synthase; LIS, linalool synthase; BS, bisabolene synthase; ADS, amorphadiene synthase

their poor cold temperature properties. The specifications of military jet fuels (JP-5, JP-10, JR-5, RJ-4) designed for rocket propulsion are even more constrained than commercial aviation fuels (Jet-A) (Meylemans et al. 2012). Medium chain terpenes with cyclic/ring structure and methyl branches make them ideal drop-ins or precursors for jet fuels; ring-strained fuels have a high heat of combustion while methyl-branched fuels have a low temperature viscosity (Harvey et al. 2015).

Biojet fuel precursors proposed to date include the isoprenoids limonene, linalool, pinenes, myrcene, ocimene, farnesene, farnesol, and bisabolene. These precursors can be converted to tactical fuel substitutes through a series of chemical modifications. In addition to isoprenoids, biodiesels and hydro-processed ester and fatty acids (HEFA) can also be used in the aircraft's engines. Lastly, branched short chain alcohols such as isobutanol are catalytically converted to a mixture of predominantly C_{12}-C_{16} hydrocarbons and have been used as kerosene jet blend stock (Taylor et al. 2010).

3.2 Microbial Biojet Fuel Production from Sugars

3.2.1 Fatty Acids-Derived Jet Fuel

As mentioned above, fatty acids and esters produced by microorganisms can be chemically converted into biojet fuel. HEFAs have been approved for blends into conventional aviation fuel and also have been reported to reduce green gas emission compared to petroleum-based fuels (Pearlson et al. 2013). Moreover, methyl-branched fatty acids produced by microorganisms exhibit low cloud and pour points, which is superior to straight chain fuels for aviation purposes (Knothe 2009). In the previous section, we have elaborated the fermentation of fatty acid-derived biodiesel targets, and here, we mainly focus on studies to produce branched fatty acids and esters as biojet fuel candidates.

The native *E. coli* is not capable of initiating fatty acids synthesis with branched acyl-CoA starter units, and a heterologous gene expression is required to produce branched fatty acids in *E. coli*. Haushalter and coworkers expressed genes from *Bacillus subtilis* to catabolize branched chain amino acids into branched acyl-CoAs (Haushalter et al. 2014). The branched acyl-CoAs are then condensed with malonyl-ACP via the native fatty acid biosynthesis pathway to generate iso- and anteiso-branched fatty acids. Later, this strategy was optimized by overexpressing *E. coli*'s native branched α-ketoacid synthesis genes and by introducing an exogenous lipoylation pathway (Bentley et al. 2016). The resultant branched chain fatty acids production reached 181 mg/L and 72% of the total free fatty acids. Recently, *Saccharomyces cerevisiae* has also been engineered for branched chain fatty acids and ester production. Teo et al. managed to produce fatty acid branched-chain alkyl esters using the endogenous fatty acids and alcohol synthesis pathways (Teo et al. 2015). A combinatorial approach was also employed to engineer the native Ehrlich pathway expression, which improved the production of short branched-chain fatty acids to 387.4 mg/L (Yu et al. 2016).

3.2.2 Isoprenoids-Derived Jet Fuel

In addition to the sesquiterpenes (i.e., farnesene and bisabolene), which also serve as biodiesel targets, monoterpenes including pinenes, limonene, and linalool are being explored as precursors of jet fuels (Beller et al. 2015; Fig. 4). Hydrogenated pinene dimers have been chemically synthesized and shown to have comparable properties to the aviation fuel JP-10 (Harvey et al. 2010). Hydrogenated limonene is a good candidate to enhance cold weather performance in jet fuel mixtures (Tracy et al. 2009), and linalool was reported as a precursor for the high-density fuel RJ-4 via chemical transformation (Meylemans et al. 2011). The biosynthesis of these monoterpenes requires expression of a GPP synthase, the monoterpene synthase corresponding to the target monoterpene, and the MVA or MEP pathway to produce the universal precursors, IPP and DMAPP. The highest limonene titer in a batch scale has been reported using proteomics-guided engineering of the MVA pathway at a titer of 605 mg/L in *E. coli* using a GPPS from *Abies grandis* and a limonene synthase (LS) from *Mentha spicata* (Alonso-Gutierrez et al. 2015). In more recent work, a library of diverse monoterpene synthases was employed in *E. coli* and produced over 30 different monoterpenoids, some of which have potential to be used as biojet fuels (Leferink et al. 2016). For example, earlier work on pinene production showed difficulty in high titer production due to the low activity of pinene synthase (PS). Low titers were addressed with a PS from *Pinus taeda* and a to achieved 550 mg/L pinene production (Leferink et al. 2016). The production of linalool remains low, with highest titers reported in shake flask at 132 μg/L in *Saccharomyces cerevisiae* with a *Clarkia breweri* linalool synthase (LIS) (Rico et al. 2010). More recent work employed a protein fusion of *Actinidia argute* LIS and native FPPS in *Saccharomyces cerevisiae* and achieved 240 μg/L in batch fermenter (Deng et al. 2016).

3.2.3 2-Keto Acids-Derived Isobutanol as a Jet Fuel Precursor

Amino acid biosynthesis is a high-flux pathway and universally conserved among microorganisms. Precursors for amino acids biosynthesis, 2-keto acids, are abundant in the cell and can be converted to diverse products including C_4-C_8 alcohols, aldehydes, C_4-C_5 carboxylic acids, and their acetate esters (Atsumi et al. 2008; Peralta-Yahya et al. 2012). Among those products, the biosynthesis of short chain alcohols, such as n-butanol, isobutanol, and propanol, have been well studied and developed as gasoline alternatives (Connor and Liao 2009). Recently, isobutanol has also been used as a precursor to produce high-density jet fuel. Biosynthesized isobutanol is dehydrated to isobutylene, then oligomerized and hydrogenated to form iso-paraffinic kerosene (IPK), which is a blend stock for jet and missile fuel (Taylor et al. 2010; Vasudevan and Briggs 2008).

Short chain alcohols can be produced from 2-keto acids via the Ehrlich pathway, where 2-keto acids are first converted to aldehydes by 2-keto acids decarboxylase (Fig. 5) and then reduced to alcohols by alcohol dehydrogenase (ADH) or aldehyde reductase (ALR) (Tashiro et al. 2015). Atsumi et al. heterologously expressed the genes *kivd* from *Lactococcus lactis* and *ADH2* from *Saccharomyces cerevisiae* to convert 2-ketoisovalerate to isobutanol in *E. coli* (Atsumi et al. 2008). The *ilvIHCD*

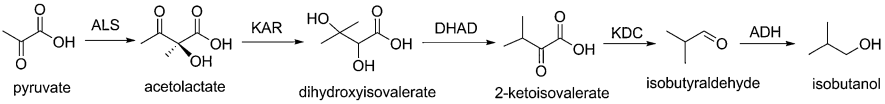

Fig. 5 Isobutanol biosynthesis via 2-keto acid pathway. ADH, alcohol dehydrogenase; ALS, acetolactate synthase; DHAD, dihydroxy-acid dehydratase; KAR, ketol-acid reductoisomerase; KDC, keto-acid decarboxylase

gene cluster was also overexpressed to direct more carbon flux from valine pathway toward isobutanol biosynthesis. To decrease the competition for pyruvate and further optimize isobutanol production, several genes (*adhE, ldhA, frdAB, fnr, pta,* and *pflB*) were deleted. In addition, *ilvIH* was replaced by the *alsS* gene from *Bacillus subtilis* due to its higher affinity for pyruvate. The combined genetic modifications led to a maximum titer of 22 g/L, the highest isobutanol production in shake flask to date (Atsumi et al. 2008). In situ product removal further improved isobutanol production to 50 g/L in laboratory scale fermentation (Baez et al. 2011). Isobutanol production has also been implemented in other microbial hosts such as *Saccharomyces cerevisiae* (0.14–0.18 g/L), *Corynebacterium glutamicum* (4.9–12.6 g/L), *Bascillus subtilis* (2.62 g/L), and *Shimwellia blattae* (6 g/L) using glucose as the substrate (Blombach et al. 2011; Felpeto-Santero et al. 2015; Lee et al. 2012; Li et al. 2011).

4 Biodiesel and Biojet Fuels Production from Biomass

Efficient biomass deconstruction is one of the fundamental goals of the biofuels industry. Lignocellulose pretreatment is a crucial step that significantly affects the cost of lignocellulosic biofuel production. The aim is to simplify the complex lignocellulose polymer matrix into its constituent sugar and nonsugar components. Over the past decades, various pretreatment approaches have been investigated on a wide variety of feedstocks. In this section, we review the features of the promising and commonly used biomass deconstruction technologies.

4.1 Lignocellulosic Biomass as Carbon Source

Lignocellulosic biomass, or plant cell walls, can generally be divided into three components: cellulose, hemicellulose, and lignin. Cellulose is a straight chain polymer consisting hundreds to thousands of glucose monomers condensed by β-glycosidic bonds. The multiple hydroxyl groups on the glucose form hydrogen bonds between polysaccharide chains, holding the chains firmly together and forming microfibrils with crystalline structure. Cellulose also contains amorphous regions which can be broken up into nanocrystalline cellulose. Chemical depolymerization of cellulose into its constituent glucose monomers requires the use of concentrated acids and high temperature. By using cellulase enzymes, however, the

cellulolysis process can be achieved under relatively mild conditions (Sindhu et al. 2016). Unlike cellulose, hemicellulose is a matrix polysaccharides of various sugar monomers, which has a random amorphous structure. The monosaccharides of hemicellulose include pentose (xylose, arabinose, rhamnose) and hexose (glucose, mannose, galactose). Hemicellulose can be hydrolyzed easily by diluted acid or base as well as myriad hemicelluloses (Agbor et al. 2011; Jonsson and Martin 2016).

Lignin is a cross-linked phenolic heteropolymer that "glues" the other lignocellulosic components. Because of the adhesive properties, lignin is detrimental to enzyme hydrolysis and fermentation processes since it physically sequesters the enzyme-accessible lignocellulose surface. Furthermore, the degradation of lignin generates phenolic compounds that deactivate cellulolytic enzymes and are toxic to microbial growth. Due to these negative impacts, the pretreatment process also aims to maximize delignification, limit the formation of inhibitory phenolic products, preserve the sugar from hemicellulose, and provide easy recovery of lignin-derived products (Alvira et al. 2010).

4.2 Features of Promising Pretreatment Technologies

Pretreatment technologies can be classified into four general categories: physical, physiochemical, chemical, and biological processes. Each type of pretreatment has its own advantages and drawbacks and can be selected based on the lignocellulosic composition of different feedstocks (Table 2).

4.2.1 Physical Pretreatment

Common physical pretreatment includes mechanical shearing, pyrolysis, and irradiation. These methods can increase porosity of lignocellulose and decrease the crystallinity of cellulose. However, some of these methods can be also very energy demanding and cost ineffective. Thus, physical pretreatment methods are often combined with chemical pretreatments. For example, the combination of irradiation and acid treatment has been proven to enhance enzymatic hydrolysis and lignin removal, while irradiation alone is insufficient for delignification (Taherzadeh and Karimi 2008). Similarly, pyrolysis at temperatures greater than 300 °C can decompose cellulose slowly, whereas pyrolysis at 97 °C with mild acid treatment can reach higher conversion of cellulose to glucose in shorter time (Kumar et al. 2009).

4.2.2 Physiochemical Pretreatment

Physiochemical pretreatment combines chemical and physical process simultaneously to more effectively breakdown lignocellulosic biomass. Recently, many physiochemical pretreatment methods have received substantial attention, including steam explosion, CO_2 explosion, and ammonia fiber explosion (AFEX).

In steam explosion, biomass is subjected to pressurized steam for a period of time and suddenly depressurized, which cause fibers to separate owing to the explosive decompression. In this process, autohydrolysis takes place since acetyl groups in hemicellulose can form acetic acid, meanwhile, water can act as acid at high

Table 2 Advantages and disadvantages of popular pretreatment technologies

Pretreatment method		Advantages	Disadvantages
Physical		Reduces cellulose crystallinity	High energy consumption and cost ineffective
Physiochemical	Steam explosion	Causes lignin transformation and hemicellulose solubilization	Forms inhibitory compounds
	AFEX	Increases digestibility	Not efficient for biomass with high lignin content
	CO_2 steam explosion	Disrupts the structure of cellulose and hemicellulose	Does not affect lignin
Chemical	Acid	Improves enzyme accessibility of cellulose, solubilizes hemicellulose and lignin	Forms inhibitory compounds
	Ozone	Removes lignin, improves susceptibility of cellulose under mild condition, no require for solvent removal	Cost-ineffective
	Alkaline	Increase cellulose digestibility	Requires long residence time
	Organic solvent	Causes lignin and hemicellulose hydrolysis	Solvents need to be recycled, high cost
	Ionic liquid	Disrupts structure of cellulose, hemicellulose, and lignin. Limited formation of inhibitors	Ionic liquid needs to be removed and recycled
Biological		Low capital cost, no hazardous byproduct, and no recovery requirement	Requires long residence time, high sugar loss

temperature (Jonsson and Martin 2016). To enhance the effect of such chemical hydrolysis, in some studies, external H_2SO_4 is added, so that cellulose can be partially hydrolyzed. As the most widely used physiochemical pretreatment, steam explosion has been demonstrated to effectively remove lignin with high sugar yield after enzyme hydrolysis, but inhibitory compounds can be generated from the acidic hydrolysis condition (Alvira et al. 2010).

In contrast to steam explosion, CO_2 steam explosion and AFEX form low levels of biological inhibitors. CO_2 steam explosion uses supercritical CO_2 to penetrate the lignocellulose. This method disrupts cellulose structure and increases enzyme digestibility. However, it does not affect lignin. The addition of other cosolvents can further improve delignification. It has been reported that utilization of supercritical CO_2 with an ethanol-water cosolvent at high pressure can achieve a delignification extent of 93% for sugar cane bagasse and 88% for *Pinus taeda* wood chips (Pasquini et al. 2005). AFEX share most common strengths and drawbacks with CO_2 steam explosion. In AFEX, biomass is treated with ammonia at high pressure and temperature between 60 °C and 140 °C. The pressure is then dropped resulting in expansion and disruption of biomass fibers (Wu et al. 2010). To take full

advantage of ammonia's potential to reduce cellulose recalcitrance and increase removal of lignin, recently, a new liquid ammonia pretreatment methodology, called extractive ammonia (EA), was developed (Sousa et al. 2016). Compared to AFEX, this method employs a high ammonia-to-biomass ratio at a low moisture level to enable the formation of a highly digestible cellulose allomorph and selectively extract lignin. It has been shown that EA-pretreated corn stover has higher fermentable sugar yield compared to the AFEX process using 60% lower enzyme loading.

4.2.3 Chemical Pretreatment

Chemical pretreatment is known for its effectiveness in delignification as well as decreasing the degree of polymerization and crystallinity of cellulosic components. In comparison with physical and physiochemical process, chemical pretreatment requires relatively lower energy and milder process condition and is thus more economically feasible for industrial purposes. Most prevalent chemical pretreatments use acid, alkali, organic solvent, ozone, and ionic liquids. Here, we compare the advantages and disadvantages of each approach and highlight the recent advances in chemical pretreatment processes.

Alkaline pretreatment, mostly using NaOH, $Ca(OH)_2$ (lime), or ammonia, can increase cellulose digestibility and partially remove hemicellulose and lignin. Alkaline pretreatment can be performed at room temperature with concentrated base and relatively long time. It is described to be more efficient in sugar preservation than acid pretreatment, and it is more suitable for agricultural residues than wood materials (Jonsson and Martin 2016; Kumar et al. 2009). The addition of oxidation agents such as hydrogen peroxide can improve the performance by enhancing lignin removal. Furthermore, no sugar degradation compounds such as furfural or hydroxymethylfurfural (HMF) were detected with alkaline peroxide pretreatment which favors the biological fermentation process (Alvira et al. 2010).

As a powerful oxidant, ozone has been employed for fractionation of lignocellulosic biomass. In ozonolytic process, lignin can be substantially degraded, and the susceptibility of cellulose is improved. Ozonolysis can be performed at room temperature and normal pressure, decreasing the chance of forming sugar degradation product or lignin-derived toxic compounds. However, owing to the large amount of ozone required for fractionation of lignocellulose, ozonolysis may not be cost-effective for industrial applications (Alvira et al. 2010).

Acid pretreatment can improve enzyme accessibility of cellulose, solubilize hemicellulose and lignin. The main advantage of acid pretreatment is saccharification of hemicelluloses with high sugar yield for a wide range of lignocellulosic materials. However, the disadvantage of this process is the generation of toxic inhibitors. Acid pretreatment can be either operated at high temperature with dilute acids or at low temperature with concentrated acids. Concentrated acid pretreatment, although less energy-demanding, is accompanied with equipment corrosion problems and high maintenance cost. On the other hand, dilute acid pretreatment appears more favorable for commercial scale-up. As the most well-studied pretreatment method, many types of dilute acids have been tested, including inorganic acids such as hydrochloric acid, sulfuric acid, nitric acid, and phosphate acid and organic

acids such as fumaric or maleic acids, though use of organic acid increases capital investment and further study is needed for optimizing solvent recycling processes (Alvira et al. 2010; Zhang et al. 2016).

Ionic liquid (IL) pretreatment is a promising approach owing to its low toxicity, limited inhibitor formation, and mild process conditions (Elgharbawy et al. 2016). ILs are mixtures of salts, typically containing organic cation and inorganic anions. ILs disrupt the noncovalent interactions between cellulose, hemicellulose, and lignin without leading to significant degradation. ILs pretreatment has been applied to a myriad of feedstocks such as wheat straw, wood chips, and flour (Alvira et al. 2010; Behera et al. 2014). In general, short processing time, high enzyme digestibility, and high sugar yield has been observed. Moreover, ILs combined with oxidative reagents, alkali or acid, can further improve cellulose conversion with lower enzyme:substrate loading (Heggset et al. 2016; Uju et al. 2016; Vasheghani Farahani et al. 2016). However, although formation of inhibitors is limited in this process, the remaining ILs are still detrimental to fermentative microorganisms and hydrolytic enzymes. Thus, future studies need to be carried out regarding the development of energy-efficient recycling approaches and screening of potentially IL-resistant microorganism (Elgharbawy et al. 2016).

4.2.4 Biological Pretreatment

Biological pretreatment is an eco-friendly strategy which utilizes microbes that are capable of naturally degrading lignocellulose materials in ecosystem such as brown-, white-, and soft-rot fungi. Brown fungi primarily hydrolyze cellulose, whereas white-rot and soft-rot fungi can attack cellulose, lignin, and hemicellulose via production of a series of enzymes (Keshk 2016; Sanchez 2009; Taherzadeh and Karimi 2008). For example, white-rot fungus has been studied as a model for its effective delignification due to the production of multiple lignolytic enzymes. These enzymes include lignin peroxidase, manganese peroxidase, and laccase (Limayem and Ricke 2012; Saritha et al. 2012). Soft-rot fungi such as *Aspergillus niger* and *Trichoderma reesei* secrete hemicellulase and cellulase mixtures, but their efficiency and mechanism of lignin depolymerization needs further investigation (Imran et al. 2016; Salihu et al. 2015; Shallom and Shoham 2003). Biological pretreatment presents advantages including low capital cost, no hazardous byproduct, and no recovery requirement. Drawbacks that require additional attention include low hydrolysis rates and saccharified sugar lost to fungal metabolism.

4.3 Enzymatic Hydrolysis of Cellulose

The process of pretreatment generally removes lignin and hemicellulose, significantly enhancing the digestibility and porosity of cellulose. However, further conversion of cellulose to glucose often requires the addition of cellulases for subsequent microbial growth and fermentation. Cellulases are a mixture of enzymes that act serially or synergistically to decompose cellulosic material, which primarily contains endocellulase (EC 3.2.1.4), exocellulase (EC 3.2.1.91), and β-glucosidase

(EC 3.2.1.21). Endocellulases randomly cleave internal β-1,4 linkages of amorphous cellulose to create free chain ends. Then, exocellulases cleave two to four units from the ends of the exposed chains, generating tetrasaccharides or disaccharides. Finally, β-glucosidase hydrolyze the oligosaccharides created by endocellulase and releases glucose (Chaturvedi and Verma 2013). Both bacteria and fungi have been exploited extensively for cellulase production. Many bacterial species belonging to Clostridium, Bacillus, Streptomyces, and *Bacteroides cellulosolvens* are all capable of producing cellulases. Fungi including *Trichoderma*, *Aspergillus*, and *Humicola* species are also widely employed in industry. Compared to bacterial cellulase production, fungal production generally achieves higher cellulase yields. For this reason, commercial cellulases are primarily expressed in fungi. Bacterial cellulases are argued as compelling alternatives, because bacteria are more genetically tractable and grow faster in harsh conditions (Imran et al. 2016; Keshk 2016; Sanchez 2009).

4.4 Studies on Conversion from Biomass to Diesel/Jet Fuel

Combining the biomass deconstruction technology and biofuel synthesis, a few studies have been pursued to demonstrate the production of biodiesel and biojet fuel from lignocellulosic materials. Bokinsky et al. introduced cellulase, xylanase, β-glucosidase, and xylobiosidase in *E. coli* which enabled the strains to grow on ionic liquid-treated switchgrass (Bokinsky et al. 2011). The authors further expressed pinene and FAEE biosynthesis genes into the previously engineered *E. coli* strains to achieve biofuel production from biomass. This study provided an approach that could be adapted by other microbes for use of generating advanced biofuels. However, titers produced using this strategy remained insufficient for industrial application and required future improvements in pathway engineering, enzyme screening, and pretreatment condition optimization.

Recently, synergistic microbial communities have inspired researchers to utilize synthetic consortia for fuel production. Minty et al. developed fungal-bacterial consortia which can directly convert AFEX-pretreated corn stover to isobutanol (Minty et al. 2013). A previously constructed model *E. coli* strain with high isobutanol productivity was selected, which contained *kivd* from *L. lactis*, *adh2* from *S. cerevisiae, alsS* from *B. subtilis*, along with native gene cluster *ilvCD* being overexpressed (Atsumi et al. 2008). Fungus *T. reesei* was then selected due to its prodigious cellulolytic activity and compatibility with *E. coli*. The consortia with optimal ratio achieved titers up to 1.88 g/L and yields up to 62% of theoretical maximum, which offered a model system for stable, tunable, and modular consortia for consolidated bioprocessing of lignocellulosic biomass to valuable products.

Another study of isobutanol production was conducted with *Corynebacterium crenatum* from diluted acid-pretreated duckweed. In this study, Su et al. modified *C. crenatum* strains by introducing exogenous genes converting pyruvate to 2-keto acids from *S. cerevisiae*, *kivd* gene from *L. lactis cremoris*, and *adh2* gene from *S. cerevisiae*. With the combined effort, the authors reported 1.15 g/L isobutanol production from liquefied duckweed hydrolysate (Su et al. 2015).

4.5 Applications and Commercialization

Driven by the goal substrate cost reduction and greenhouse gas mitigation, several synthetic biology-based biofuel and biochemical companies have initiated the manufacturing of advanced biodiesel and biojet fuel from abundant feedstocks. Two companies, LS9 (currently REG Life Science) and Synthetic Genomics have been engaged in fatty acids-derived fuel production. LS9 bred *E. coli* strains to convert cellulosic biomass to fatty acid-derived biodiesel. Their engineered strains express exogenous hemicellulases, ethanol biosynthesis, and wax-ester biosynthesis pathways to produce FAEE and fatty alcohols (Alibhai et al. 2016; Cardayre 2013; Schirmer et al. 2014). Synthetic Genomics employed a secretion protein from *Pseudomonas* or *Vibrio* species to enhance synthesis of fatty acids in *E. coli*, thus improving the production of fatty aldehydes and fatty alcohols – ideal biodiesel fuel targets (Brennan et al. 2012).

For isoprenoid-based biofuels, Amyris has commercialized farnesene production in modified yeast strains under the name "Biofene." In 2010, they reported 104.3 g/L farnesene production with a productivity of 16.9 g/L/day from sugarcane and sweet sorghum biomass as substrate. Later, they also patented amorphane as a jet fuel alternative. In 2014, Amyris collaborated with a French oil company TOTAL and a Brazilian airline GOL and flew the first commercial flight on Jet A fuel blended with bio-derived farnesane.

For isobutanol, three companies Butamax, Butalco (currently Lesaffre), and Gevo have commercialized isobutanol production in yeast strains. Butalco was a Swiss start-up company and was acquired by French company Lesaffre, a well-established yeast company in 2014. Butalco developed an isobutanol biosynthesis technology by adopting yeast's endogenous cytosolic pathway to convert pyruvate to isobutanol and expressed *Clostridium phytofermentans* xylose isomerase to digest hemicellulosic substrates. Butamax is a joint venture of British Petroleum (BP) and DuPon. They have retained the yeast mitochondrial pathway for isobutanol production and are still at their pre-revenue stage by far. In contrast, Gevo optimized their yeast cytosolic pathway and have produced over 1000-gallons cellulosic jet fuel derived from isobutanol. In 2016, Gevo also announced its completed production of the isobutanol-derived Alcohol-to-Jet fuel (ATJ) from wood waste, and they will partner with Alaska Airlines to fly their first commercial flight using this renewable ATJ produced from exclusively lignocellulosic residue.

5 Research Needs

Recent advances in synthetic biology and metabolic engineering have provided many new enabling technologies for efficient genomic modification, gene expression optimization, and dynamic pathway regulation to improve microbial biofuel production titer, rate, and yield. For example, the clustered regularly interspaced short palindromic repeats (CRISPR) system can be used as a universal scarless genome modification tool (Fineran and Dy 2014; Hsu et al. 2014; van der Oost

et al. 2014), and small RNA (sRNA) and antisense RNA can also be modulated for controlling gene expression in metabolic biosynthesis pathways (Yang et al. 2015). In addition, identification of biosensors that can detect intermediate metabolites have enabled real-time dynamic pathway regulation and cell population control to optimize biofuel fermentation (Dahl et al. 2013; Liu et al. 2015; Xiao et al. 2016; Xu et al. 2014; Zhang et al. 2012).

While synthetic biology tools can enlarge our capability for multi-dimensional pathway optimization, various omics technology will serve as enabling tools for characterization and analysis of the producing host variants. Omics technology can also enrich our understanding of the cellular metabolic network and the correlations among gene transcription/translation, enzyme expression, and metabolite production. There are increasing demands of high-throughput screening of high producing variants, and to meet these demands, efforts need to be made on optimizing the current analytical methods, constructing efficient screening platform, designing biosensors that can measure a given molecule in the cell, and establishing robotics-aided automation platform which will provide rapid, error-free sample preparation and analysis in a high-throughput manner.

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Synthetic Biology for Biofuels in *Saccharomyces cerevisiae*

8

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Abstract

The yeast *Saccharomyces cerevisiae* has crucial features to facilitate successful genetic engineering and industrial scale fermentations for producing drop-in biofuels. Short-chain alcohols, fatty acid derivatives, and isoprenoids are potential drop-in biofuels where their biosynthesis can help mitigate climate change while ensuring sustainability of energy supply. Here, we review the drop-in biofuel molecules that have been produced in engineered *S. cerevisiae*. Efforts to diversify and optimize biofuel production using synthetic biology and metabolic engineering approaches are discussed. Much improvement will be required to achieve commercial viability.

1 Introduction

The production of biofuels from microbes contributes to ensuring energy security and protecting the environment from destructive drilling for fossil fuels. However, the conventional biofuel, bioethanol is incompatible with current transportation infrastructure and can only be used as blends with gasoline. As a result, the development of drop-in biofuels that can be directly used in fuel distribution systems to power present-day engines holds great importance.

S. cerevisiae has important characteristics as a host organism for biofuel production. This includes the abundance of knowledge about its genetics, physiology, biochemistry, and methods for its genetic manipulation, which will facilitate its engineering. In addition, the ability of *S. cerevisiae* for robust growth in large-scale fermentations at low pH and its resistance to phage attack are crucial for scaling up the bioprocesses. Differing from bioethanol which is its native product, production of drop-in biofuels in *S. cerevisiae* is made possible with metabolic engineering strategies aided by synthetic biology tools.

1.1 Metabolic Engineering

Metabolic engineering emerged early in the 1990s and was first defined as *the directed modulation of metabolic pathways using methods of recombinant technology for the purpose of overproducing fuels and chemical and pharmaceutical products* (Bailey 1991). At its core, metabolic engineering aims to design, construct, and optimize metabolic pathways from a systemic point of view. Strategies such as overexpression of rate-limiting enzymes, deletion of competing pathways, cofactor balancing, and tolerance enhancement are central to metabolic engineering. However, initial implementation of such metabolic engineering strategies was often labor-intensive and restricted to the reconstruction of native metabolic pathways within the host organisms due to a lack of effective tools for engineering.

1.2 Synthetic Biology Advances Metabolic Engineering

While the goal of metabolic engineering to realize the commercially viable microbial production of biochemicals is clearly stated, the means for this goal to be achieved are less well defined, and this is exactly where synthetic biology comes into play. Synthetic biology is an emerging scientific discipline that aims to apply engineering principles of modularity, characterization and standardization to the design, and construction of novel biological systems for the investigation of fundamental biological questions as well as the generation of novel applications. Although still regarded as a relatively young discipline, this field is fast developing – evident by the expanding number of works published and the collection of synthetic biology toolsets currently available.

The realization of high-throughput DNA synthesis not only enabled the advancement of synthetic biology, it also improved how metabolic engineering is carried out. With the availability of genome databases of various organisms and enhanced sequence-to-function knowledge, novel synthetic metabolic pathways can now be constructed through integration of heterologous enzymes and pathways into host organisms to either improve existing functions or create novel functions such as non-native substrate utilization and product formation abilities. Such efforts will be challenging without the availability of a myriad of synthetic biology toolsets that assist at different levels of engineering. Firstly, novel biological part assembly and genome editing tools facilitate combinatorial assembly of DNA elements and genomic modification, speeding up pathway, strain, and library construction. Secondly, well-characterized modular genetic regulatory parts including promoters, terminators, riboswitches, and biosensors allow dynamic temporal regulation of pathway gene expression, permitting fine-tuning of metabolic fluxes to boost product formation. Thirdly, spatial engineering strategies such as pathway compartmentalization, synthetic scaffolding, and protein fusion force components of multistep enzymatic pathways into closer physical proximity, improving pathway efficiency through reduction of intermediate loss and transit time. And lastly, genetic circuit- or biosensor-guided directed evolution accelerates strain optimization, permitting the isolation of genetic variants with phenotypes of improved product formation or improved tolerance toward toxic intermediates or end products (Fig. 1). For the latest reviews on the application of synthetic biology tools for yeast metabolic engineering, readers are referred to (Jensen and Keasling 2014; Williams et al. 2016; Zhang et al. 2015).

Clearly, the emergence of synthetic biology has advanced metabolic engineering by transforming and accelerating the ways how modulation and optimization of metabolic pathways and production strains could be done. In the following parts of this review, the scope will be focused on synthetic biology and metabolic engineering efforts to engineer *S. cerevisiae* for production of drop-in biofuels. These drop-in biofuels include short-chain alcohols, fatty acid derivatives, and isoprenoids.

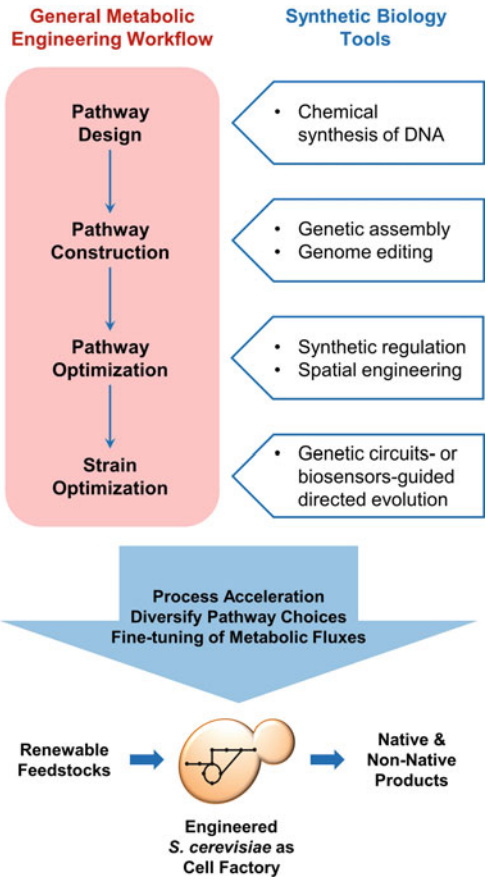

Fig. 1 Incorporation of synthetic biology tools into metabolic engineering workflow and their advantages

2 Short-Chain Alcohols

Ethanol is the most well-known and commonly used short-chain alcohol biofuel. New short-chain alcohol biofuel molecules, mainly butanol isomers, have been identified as viable petrol substitutes due to their high octane number and low hygroscopy (Generoso et al. 2015). The scope of this section will focus on drop-in short-chain alcohol biofuels produced using engineered *S. cerevisiae* (Fig. 2 and Table 1).

2.1 Isobutanol

S. cerevisiae naturally produces small amounts of isobutanol via the Ehrlich pathway from the catabolism of valine (Hazelwood et al. 2008). Isobutanol can also be produced from pyruvate in a biosynthetic pathway, where two molecules of pyruvate are

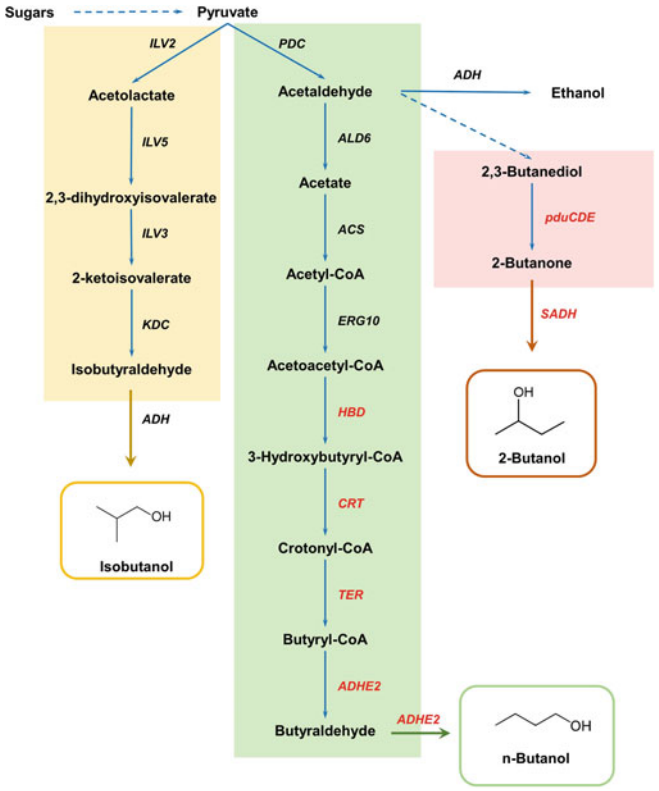

Fig. 2 Biosynthetic pathways of short-chain alcohol biofuels in engineered *S. cerevisiae*. Heterologous genes are colored in *red*. *Dotted lines* indicate multistep reactions. *ILV2* acetolactate synthase, *ILV5* acetohydroxyacid reductoisomerase, *ILV3* dihydroxyacid dehydratase, *KDC* 2-ketoacid decarboxylase, *ADH* alcohol dehydrogenase, *PDC* pyruvate decarboxylase, *ALD6* aldehyde dehydrogenase, *ACS* acetyl-CoA synthetase, *ERG10* acetyl-CoA acetyltransferase, *HBD* 3-hydroxybutyryl-CoA dehydrogenase, *CRT* crotonase, *TER* trans-2-enoyl-CoA reductase, *ADHE2* butyraldehyde dehydrogenase, *pduCDE* diol dehydratase, *SADH* secondary alcohol dehydrogenase

condensed into acetolactate (ALAC) by acetolactate synthase (ILV2). ALAC is then reduced to 2,3-dihydroxyisovalerate (DIV) by acetohydroxyacid reductoisomerase (ILV5). This is followed by conversion of DIV to 2-ketoisovalerate (KIV) by dihydroxyacid dehydratase (ILV3). KIV is decarboxylated by 2-ketoacid decarboxylase (KDC) to isobutyraldehyde, which is then reduced to isobutanol by alcohol dehydrogenase (ADH).

In the first report to engineer yeast for isobutanol production, the biosynthetic enzymes ILV2, ILV3, and ILV5 and the branched-chain amino-acid aminotransferase BAT2 were overexpressed (Chen et al. 2011). Observing that the isobutanol biosynthesis pathway occurred partly in the mitochondria and partly in the cytosol, researchers hypothesized that locating all the enzymes within the same compartment

Table 1 Short-chain alcohol biofuels produced by engineered *S. cerevisiae*

Biofuel	Titer (mg/L)	Carbon source (g/L)	References
Isobutanol	154	Glucose (40)	Chen et al. (2011)
	63	Glucose (20)	Matsuda et al. (2012)
	143	Glucose (20)	Kondo et al. (2012)
	151	Glucose (40)	Lee et al. (2012)
	630	Glucose (40)	Brat et al. (2012)
	58	Glucose (20), glycine (15)	Branduardi et al. (2013)
	635	Glucose (100)	Avalos et al. (2013)
	1620	Glucose (100)	Matsuda et al. (2013)
	377	Glucose (100)	Park et al. (2014)
	224	Glucose (20)	Ida et al. (2015)
	600	Galactose (38), glucose (2)	Yuan and Ching (2015)
	331	Glucose (20)	Park et al. (2016)
n-Butanol	2.5	Galactose (20)	Steen et al. (2008)
	16.3	Glucose (20)	Krivoruchko et al. (2013)
	92	Glucose (20), glycine (15)	Branduardi et al. (2013)
	120	Glucose (20)	Lian et al. (2014)
	243	Glucose (20)	Si et al. (2014)
	20	Glucose (20)	Lian and Zhao (2015)
	130	Glucose (20)	Schadeweg and Boles (2016)
	1050	Glucose (20)	Shi et al. (2016)
2-Butanol	4	Glucose (20)	Ghiaci et al. (2014)
3-Methyl-1-butanol	130	Glucose (100)	Avalos et al. (2013)
	766	Glucose (100)	Park et al. (2014)
2-Methyl-1-butanol	118	Glucose (100)	Avalos et al. (2013)

would increase the production of isobutanol. This proved true as relocating ILV2, ILV5, and ILV3 out of the mitochondrial matrix into the cytosol improved isobutanol production (Brat et al. 2012; Matsuda et al. 2012). Relocation of the metabolic pathway into the mitochondria by relocating KDC and ADH also increased isobutanol production (Avalos et al. 2013; Yuan and Ching 2015).

Efforts to further optimize isobutanol production through the elimination of competing pathways were carried out. These include (i) channeling pyruvate toward isobutanol production instead of acetyl-CoA biosynthesis through deletion of LPD1, a subunit of pyruvate dehydrogenase (Matsuda et al. 2013); (ii) reducing ethanol flux via pyruvate by deletion of PDC1, a major pyruvate decarboxylase (Kondo et al. 2012); (iii) blocking the isobutyrate, pantothenate, or isoleucine biosynthetic pathways through deletion of aldehyde dehydrogenase (ALD6), ketopantoate hydroxymethyltransferase (ECM31), or threonine deaminase ILV1, respectively (Ida et al. 2015); and (iv) deleting branched-chain amino acid aminotransaminase (BAT1), involved in valine biosynthesis (Park et al. 2014).

Furthermore, isobutanol production was improved through codon optimization and screening for highly active enzyme candidates (Brat et al. 2012; Lee et al. 2012), as well as expression of a constitutively active Leu3Δ601 transcriptional activator (Park et al. 2014) to increase transcription of endogenous genes in the valine and leucine biosynthetic pathways. As cofactor imbalance limits isobutanol production, overexpression of enzymes responsible for transhydrogenase-like shunts such as pyruvate carboxylase, malate dehydrogenase, and malic enzyme boosted NADPH cofactor availability and improved isobutanol production (Matsuda et al. 2013). Recently, the mitochondrial-localized pathway was optimized by increasing the mitochondrial pool of pyruvate through mitochondrial pyruvate carrier expression and removal of competing pathways by deletion of BAT1, ALD6, and LPD1 (Park et al. 2016).

Despite the aforementioned engineering approaches, isobutanol production in yeast is still unsatisfactory, indicating the existence of rate-limiting steps. Possible reasons for suboptimal yields were explored using a cytosolic, cofactor-balanced isobutanol pathway. Massive accumulation of pathway intermediates and ALAC degradation products was observed, which is consistent with the hypothesis that the enzyme ILV3, which possesses iron–sulfur clusters, could be a key bottleneck (Milne et al. 2016).

2.2 n-Butanol

The initial pathway used to produce n-butanol in yeast was first derived from *Clostridium spp.* (Steen et al. 2008). In this pathway, two molecules of acetyl-CoA are condensed to acetoacetyl-CoA, which is converted to 3-hydroxybutyryl-CoA and then into crotonyl-CoA. Crotonyl-CoA is reduced to butyryl-CoA, followed by butyraldehyde, and finally, n-butanol. In the first demonstrated n-butanol production in *S. cerevisiae*, enzyme homologs from different organisms were screened for highly active clostridial n-butanol biosynthetic pathway enzymes (Steen et al. 2008).

To boost n-butanol production, researchers sought to increase the availability of cytosolic acetyl-CoA. This was done by expressing alcohol dehydrogenase (ADH2), ALD6, acetyl-CoA synthetase (ACS), and acetyl-CoA acetyltransferase (ERG10), in strains lacking malate synthase (MIS1) or citrate synthase (CIT2) (Krivoruchko et al. 2013). n-Butanol production was further improved by redirecting glycolytic flux toward acetyl-CoA through inactivation of alcohol dehydrogenases (ADH1 and ADH4) and glycerol-3-phosphate dehydrogenases (GPD1 and GPD2) which are involved in ethanol formation and needed for glycerol production, respectively, and expression of pyruvate dehydrogenase (PDH), PDH-bypass, and ATP-dependent citrate lyase (ACL) (Lian et al. 2014). Availability of acetyl-CoA was also increased by overexpressing pantothenate kinase (CoaA), adding pantothenate to the medium and expressing an ATP-independent acetylating acetaldehyde dehydrogenase, ADHE (A267T/E568K) (Schadeweg and Boles 2016).

Besides the clostridial pathway, other n-butanol biosynthetic pathways have also been discovered. An endogenous n-butanol pathway dependent on the catabolism of threonine in *S. cerevisiae* was found (Si et al. 2014). The leucine biosynthetic pathway enzymes, KDCs, and ADHs were able to convert glucose via threonine into n-butanol. By overexpressing pathway enzymes and deleting competing pathways (ADH1 and ILV2), n-butanol production was improved. The researchers further showed that a mutant HOM3 allele encoding a feedback-insensitive aspartate kinase enabled deregulation and overproduction of threonine. The localization of the n-butanol pathway to the mitochondria further increased the production level. In addition, a citramalate synthase (CIMA), which enables an alternative route to α-ketobutyrate from pyruvate and acetyl-CoA, was able to act synergistically with the threonine degradation pathway. Overexpression of LEU1, LEU2, and LEU4 which catalyze reactions converting α-ketobutyrate to α-ketovalerate, LEU5, a mitochondrial carrier protein involved in the accumulation of CoA in the mitochondrial matrix, NFS1, a cysteine desulfurase to stabilize LEU1, CIMA, ADH, and KDC, further improved n-butanol production (Shi et al. 2016). Other notable approaches to n-butanol production were attempted including the reversal of the β-oxidation pathway in the cytosol (Lian and Zhao 2015) and the use of a putative glycine degradation pathway (Branduardi et al. 2013).

2.3 Other Short-Chain Alcohols

In order to produce 2-butanol, a heterologous pathway which converts 2,3-butanediol to 2-butanol via 2-butanone was engineered (Ghiaci et al. 2014). Using a TEV-cleavage-based system, all three subunits of a B12-dependent diol dehydratase from *Lactobacillus reuteri* and its two-subunit activating enzyme were expressed in equal amounts. The mature dehydratase enzyme, together with a secondary alcohol dehydrogenase from *Gordonia spp.*, enabled 2-butanol production. Production was boosted by increasing the availability of NADH, which was achieved by deleting GPD1 and GPD2 and growth under anaerobic conditions.

S. cerevisiae naturally produces small amounts of 3-methyl-1-butanol and 2-methyl-1-butanol via the Ehrlich pathway from the catabolism of leucine or isoleucine, respectively (Hazelwood et al. 2008). By engineering yeast to produce isobutanol through relocation of the isobutanol pathway into the mitochondria, the production of 3-methyl-1-butanol and 2-methyl-1-butanol was also increased. This could be attributed to the considerable overlap in the upstream ILV genes (ILV2, ILV3 and ILV5) and identical downstream, Ehrlich degradation pathways of KDC and ADH (Avalos et al. 2013). In another work, leucine biosynthetic genes LEU2 and $LEU4^{D578Y}$, a feedback inhibition-insensitive mutant of LEU4, were additionally overexpressed in a strain engineered for isobutanol production, resulting in increased 3-methyl-1-butanol production (Park et al. 2014).

3 Fatty Acid Derivatives

Fatty acid derivatives, such as fatty alcohols, alkanes, alkenes, and fatty acid esters, have a wide range of applications such as surfactants, lubricants, detergents, and fuels. With the introduction of novel pathways in yeast, endogenous fatty acids can be converted into useful fatty acid derivatives (Fig. 3 and Table 2).

3.1 Fatty Alcohols

To produce fatty alcohols in *S. cerevisiae*, NADPH-dependent fatty acyl-CoA reductase (FAR) from mouse (*Mus musculus*), mFAR1, which reduces fatty acyl-CoAs in the cell into fatty alcohols, was expressed. Overexpression of fatty acid biosynthetic genes, specifically acetyl-CoA carboxylase (ACC1), fatty acid synthase

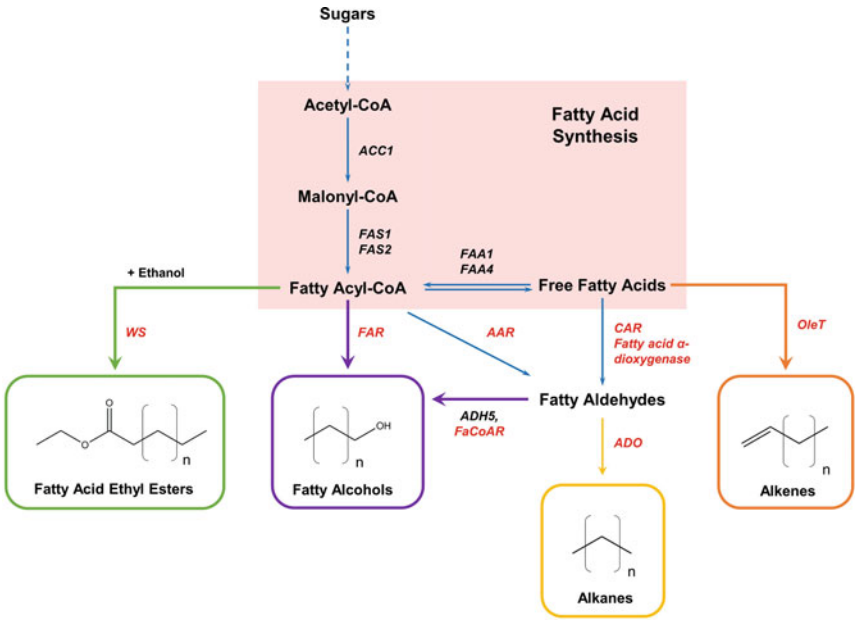

Fig. 3 Biosynthetic pathways of fatty-acid-derived biofuels in engineered *S. cerevisiae*. Heterologous genes are colored in *red*. *Dotted lines* indicate multistep reactions. *ACC1* acetyl-CoA carboxylase, *FAS1* fatty acid synthase 1, *FAS2* fatty acid synthase 2, *FAA1* fatty acyl-CoA synthetase 1, *FAA4* fatty acyl-CoA synthetase 4, *WS* wax ester synthase, *FAR* fatty acyl-CoA reductase, *AAR* fatty acyl-CoA/ACP reductase, *CAR* carboxylic acid reductase, *ADH5* alcohol dehydrogenase 5, *FaCoAR* bi-functional fatty acyl-CoA reductase, *ADO* fatty aldehyde deformylating oxygenase, *OleT* fatty acid decarboxylase

Table 2 Fatty acid derived biofuels produced by engineered *S. cerevisiae*

Biofuel	Titer (mg/L)	Carbon source (g/L)	References
Fatty alcohol	98	Galactose (18), glucose (2)	Runguphan and Keasling (2014)
	98	Galactose (60)	Tang and Chen (2015)
	1111	Glucose (100)	Feng et al. (2015)
	1300	Glucose (30)	Sheng et al. (2016)
	1500	Glucose (~320)	Zhou et al. (2016)
	20.3	Glucose (20)	Jin et al. (2016)
Alkane	0.022 mg/gDCW	Glucose (30)	Buijs et al. (2015)
	0.07	Galactose (18), glucose (2)	Foo et al. (2015)
	0.8	Glucose (30)	Zhou et al. (2016)
Alkene	3.7	Glucose (180)	Chen et al. (2015)
Fatty acid ethyl ester	n.i.	Galactose (20)	Kalscheuer et al. (2004)
	520	Glycerol (20), sodium oleate (1)	Yu et al. (2012)
	8.2	Glucose (20)	Shi et al. (2012)
	5.4	Galactose (18), glucose (2)	Runguphan and Keasling (2014)
	15.8	Glucose (20)	Shi et al. (2014a)
	17.2	Glucose (20)	Valle-Rodríguez et al. (2014)
	25.4	Glucose (114)	Thompson and Trinh (2014)
	48	Glucose (20)	Shi et al. (2014b)
	5100 mg/gCDW	Glucose (20)	de Jong et al. (2014)
	4.4	Glucose (20)	de Jong et al. (2015)
	10.5 mg/gCDW	Glucose (20)	Eriksen et al. (2015)
	n.i.	Glucose (20)	Lian and Zhao (2015)
Fatty acid short- and branched-chain alkyl esters	234	Glucose (20), galactose (20)	Teo et al. (2015)

n.i. not indicated in the publication, *gDCW* g dry cell weight

1 (FAS1), and fatty acid synthase 2 (FAS2), as well as the malic enzyme from the oleaginous fungus *Mortierella alpina* to increase cytosolic NADPH level, led to increased fatty alcohol production (Runguphan and Keasling 2014).

Fatty alcohols were also produced by expressing a FAR from barn owl (*Tyto alba*), TaFAR. Together with ACC1 overexpression, the production of fatty alcohols

was enhanced by expressing a heterologous ATP-dependent citrate lyase, which increases the cytosolic acetyl-CoA supply, and knocking out RPD3, which negatively regulates the INO1 gene involved in phospholipid metabolism (Feng et al. 2015). Fatty alcohol production titers could also be improved by using the same TaFAR enzyme and deleting the DGA1 gene to block the fatty acyl-CoAs-dependent pathway of TAG synthesis (Tang and Chen 2015).

Medium chain fatty alcohols were produced via targeted expression of TaFAR in the peroxisome of *S. cerevisiae*. Compartmentalizing TaFAR into the peroxisomal matrix enabled interception of medium chain fatty acyl-CoAs generated from the β-oxidation pathway and their conversion into medium chain fatty alcohols. Along with the previously mentioned ACC1, overexpression of PEX7 to enhance TaFAR targeting efficiency into the peroxisome further improved fatty alcohol production (Sheng et al. 2016).

Instead of using FARs to convert fatty acyl-CoAs into fatty alcohols, a pathway utilizing free fatty acids (FFAs) was engineered. The expression of *Mycobacterium marinum* carboxylic acid reductase (MmCAR) converts FFAs into fatty aldehydes, which are then reduced to fatty alcohols by overexpressed ADH5. To increase fatty alcohol production, FFA intermediates are accumulated through deletion of the main fatty acyl-CoA synthetase-encoding genes FAA1 and FAA4, which prevent reactivation of FFAs into fatty acyl-CoAs. Further, POX1 encoding the fatty acyl-CoA oxidase was also deleted to prevent FFAs degradation through β-oxidation. Deletion of the aldehyde dehydrogenase-encoding genes HFD1 and ADH6, coupled with expression of a bi-functional fatty acyl-CoA reductase from *Marinobacter aquaeolei VT8* (FaCoAR) which has high activity toward long-chain fatty aldehydes, further increased fatty alcohol production (Zhou et al. 2016). Introduction of a biosynthetic pathway involving cytosolic thioesterase, rice α-dioxygenase, and endogenous aldehyde reductases into a yeast strain lacking FAA1 and FAA4 genes enabled production of odd chain-length fatty alcohols (Jin et al. 2016).

3.2 Alkanes and Alkenes

To produce alkanes in yeast, *Synechococcus elongatus* fatty acyl-CoA/ACP reductase (SeAAR), which converts fatty acyl-CoAs into fatty aldehydes, and fatty aldehyde deformylating oxygenase (SeADO), which reduces fatty aldehydes into alkanes, were expressed. Elimination of HFD1 and expression of a redox system were found to be essential for alkane biosynthesis (Buijs et al. 2015). In a bid to avoid using fatty acyl-CoAs as substrates for alkane production, an alternative pathway was engineered to convert sugars to alkanes via FFAs, where a fatty acid α-dioxygenase from rice was expressed in *S. cerevisiae* to convert FFAs to fatty aldehydes. Co-expression of a SeADO converted the aldehydes into the desired alkanes. Alkane production was increased by overproducing FFAs through deletion of FAA1 and FAA4 (Foo et al. 2015). Production of alkanes from FFAs was also carried out using MmCAR to catalyze FFAs conversion into fatty aldehydes. HFD1

and POX1 were deleted to boost fatty acid supply, whereas ADH5 deletion led to an increased alkane production and decreased fatty alcohol accumulation. To further increase flux toward alkanes, the expression of SeADO was upregulated, one copy of MmCAR was integrated into the genome, and *Nostoc punctiforme* NpADO was expressed (Zhou et al. 2016).

By introducing a one-step fatty acid decarboxylation pathway into yeast, terminal alkenes were produced (Chen et al. 2015). In an attempt to boost production level, fatty acid decarboxylases (OleT) homologs were screened to select highly active enzymes and the best performing OleT was found to be from *Jeotgalicoccus sp. ATCC 8456* (OleTJE). The engineering of precursor and cofactor availability and OleTJE gene expression tuning further improved production.

3.3 Fatty Acid Esters

Fatty acid ethyl esters (FAEEs) are biodiesels converted industrially from chemical transesterification and esterification processes. Bioconversion of fatty acyl-CoAs into FAEEs requires a fatty acyl-CoA:alcohol transferase (wax ester synthase, WS) that can accept ethanol as the alcohol substrate. FAEEs were first detected in yeast expressing WS from *Acinetobacter calcoaceticus ADP1* devoid of four storage lipids biosynthetic genes, namely, diacylglycerol acyltransferase (DGA1), a phospholipid: diacylglycerol acyltransferase (LRO1), and two acyl-coenzyme A:sterol acyltransferases (ARE1 and ARE2) (Kalscheuer et al. 2004).

To improve production of FAEEs, researchers carried out various engineering strategies. Heterologous WS homologs were compared to determine the best performing FAEE-producing WS where WS from *Marinobacter hydrocarbonoclasticus DSM 8798* (ws2) showed the highest specificity for ethanol as the alcohol substrate (Shi et al. 2012). As ACC1 overexpression resulted in limited FAEE production improvement, ACC1 activity was improved by mutating the enzyme at Ser659 and Ser1157, abolishing posttranslational regulation by protein kinase SNF1 (Shi et al. 2014a). Elimination of nonessential and competing fatty acid utilization pathways through deletion of ARE1, ARE2, DGA1, LRO1, and POX1 genes enabled accumulation of more FAEEs (Valle-Rodríguez et al. 2014). Integration of six copies of ws2 expression constructs into chromosomal delta sites, and further overexpression of acyl-CoA binding protein (ACB1) involved in the transport of acyl-CoA esters and attenuation of the feedback inhibitory effect of acyl-CoAs on enzymes such as fatty acid synthetase, acetyl-CoA carboxylase, and long-chain acyl-CoA synthetase led to increased FAEEs production (Shi et al. 2014b). Another strategy used was the channeling of carbon flux toward acetyl-CoA by overexpressing ADH2, ALD6, and *Salmonella enterica* ACS^{L641P} (de Jong et al. 2014). Engineering the availability of redox cofactor NADPH through expression of bacterial NADP+-dependent glyceraldehyde-3-phosphate dehydrogenase (gapN) (Shi et al. 2014b) and phosphoketolase pathway (de Jong et al. 2014) increased FAEE production. Single copies of ws2, ADH2, ALD6, *S. enterica* ACS^{L641P}, $ACC1^{S1157A,\ S659A}$, and ACB1 were introduced into a *S. cerevisiae* strain lacking

ARE1, ARE2, DGA1, LRO1, and POX1. However, this led to lower FAEE titer than reported in earlier studies, where a single copy of ws2 was believed to be rate limiting (de Jong et al. 2015).

Production of FAEEs was also increased by overexpressing fatty acid biosynthetic genes, ACC1, FAS1, and FAS2 in a strain lacking POX1 (Runguphan and Keasling 2014). Alternatively, deletion of enzymes responsible for acyl-CoAs peroxisomal import, acyl-CoA transporter PXA2 and acyl-CoA synthetase FAA2, enabled blocking of β-oxidation pathway, as well as deletion of ACB1, which can increase transcription levels of fatty acid biosynthetic genes, resulted in enhanced cytosolic acyl-CoAs levels and FAEEs production (Thompson and Trinh 2014). Another work to produce FAEEs in yeast includes introducing an orthogonal route for fatty acid synthesis through heterologous expression of a type-I fatty acid synthase from *Brevibacterium ammoniagenes* coupled with ws2 (Eriksen et al. 2015), reversing the β-oxidation cycle in the yeast cytosol which enabled endogenous acyl-CoA/ethanol O-acyltransferases, EEB1, and EHT1 to catalyze the synthesis of medium-chain FAEEs (Lian and Zhao 2015) and using glycerol as well as sodium oleate as substrates (Yu et al. 2012).

Fatty acid esters with branched-chain alcohol moieties have superior fuel properties. This includes improved cold flow characteristics, as one of the major problems associated with biodiesel use is poor low-temperature flow properties (Knothe 2005). By expressing ws2 or Maqu_0168 from *Marinobacter sp.*, fatty acid short- and branched-chain alkyl esters, including ethyl, isobutyl, isoamyl, and active amyl esters, were produced using endogenously synthesized fatty acids and alcohols (Teo et al. 2015). Deletion of OPI1, a negative regulator of the INO1 gene in phospholipid metabolism, and expression of isobutanol pathway enzymes (ILV2, ILV5, ILV3, ARO10, and ADH7) targeted into the mitochondria enhanced fatty acid esters production.

4 Isoprenoids

Isoprenoids isoprenoidsare a diverse class of natural products derived from the sequential condensation of the basic C_5 isoprene units, isopentenyl pyrophosphate (IPP), and dimethylallyl pyrophosphate (DMAPP) via the mevalonate (MVA) pathway in *S. cerevisiae.* Due to their lower hygroscopy, higher energy content, and good fluidity at lower temperature (Gupta and Phulara 2015), recent years have seen much interest in the exploration of the fuel potential of the C_5 hemiterpenes, the C_{10} monoterpenes, and the C_{15} sesquiterpenes (Fig. 4 and Table 3). Specifically, the hemiterpene isoprene is a potential fuel additive for gasoline, diesel, or jet fuel (Bentley et al. 2014), while monoterpenes including limonene, cineole, linalool, sabinene, and geraniol are good candidates of biofuel precursor and aviation fuel alternative or additive (Meylemans et al. 2011; Ryder 2012). Moreover, sesquiterpenes such as farnesol, farnesene, and bisabolene are prospective biosynthetic biodiesel either on its own or in its hydrogenated form (Renninger and McPhee 2008, 2010; Peralta-Yahya et al. 2011).

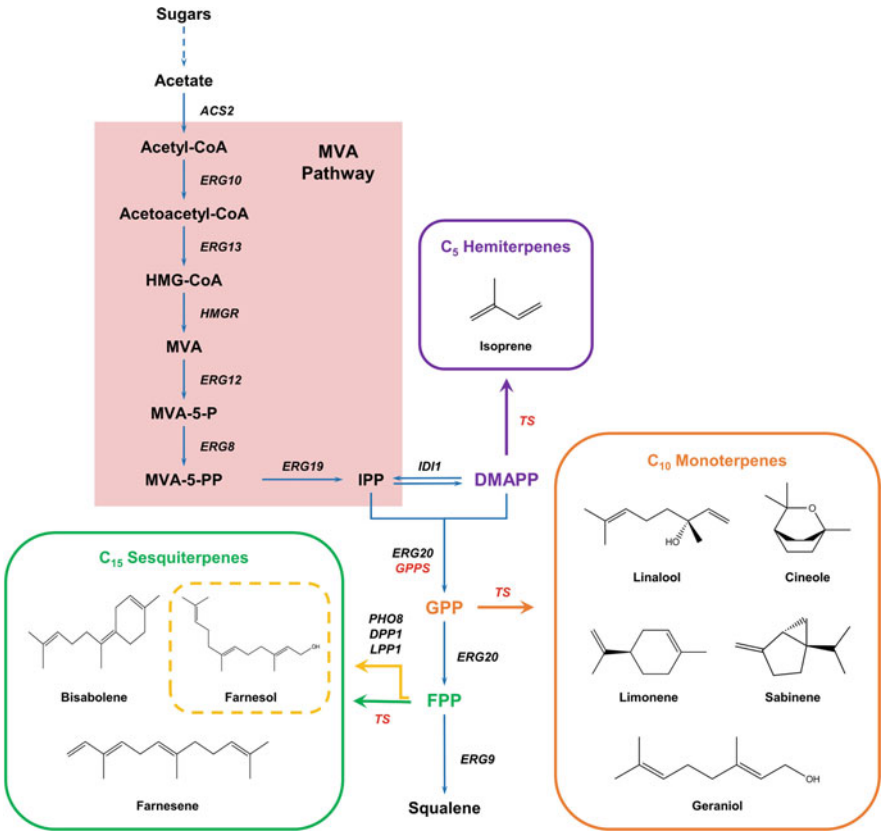

Fig. 4 Biosynthetic pathways of isoprenoid biofuels in engineered *S. cerevisiae*. Heterologous genes are colored in *red*. *Dotted lines* indicate multistep reactions. *MVA* mevalonate, *HMG* 3-hydroxy-3-methylglutaryl, *IPP* isopentenyl pyrophosphate, *DMAPP* dimethylallyl pyrophosphate, *GPP* geranyl pyrophosphate, *FPP* farnesyl pyrophosphate, *ACS2* acetyl-CoA synthetase, *ERG10* acetyl-CoA acetyltransferase, *ERG13* HMG-CoA synthase, *HMGR* HMG-CoA reductase, *ERG12* mevalonate kinase, *ERG8* phosphomevalonate kinase, *ERG19* mevalonate pyrophosphate decarboxylase, *IDI1* IPP isomerase, *ERG20* FPP synthase, *ERG9* squalene synthase, *TS* terpene synthase, *GPPS* GPP synthase, *PHO8* alkaline phosphatase, *DPP1* diacylglycerol pyrophosphate phosphatase, *LPP1* lipid phosphate phosphatase

Efficient microbial production of isoprenoids requires first accumulation of the corresponding branch-point intermediates, namely, IPP/DMAPP, geranyl pyrophosphate (GPP), and farnesyl pyrophosphate (FPP) for hemiterpenes, monoterpenes, and sesquiterpenes, respectively, and their subsequent conversion into desired isoprenoids by specific terpene synthase. As such, engineering strategies for isoprenoid production in *S. cerevisiae* are mostly devoted toward increasing these intermediate pools. These strategies include (i) upregulation of the upstream MVA pathway to improve precursor supply and (ii) downregulation of downstream competing metabolic branches to minimize metabolic flux loss. To improve precursor supply, the rate-

Table 3 Isoprenoid biofuels produced by engineered *S. cerevisiae*

Biofuel	Titer (mg/L)	Carbon source (g/L)	References
Isoprene	0.5	Galactose (20)	Hong et al. (2012)
	37	Sucrose (6)	Lv et al. (2014)
		Galactose (14)	
Limonene	1.48	Galactose (18)	Behrendorff et al. (2013)
		Glucose (2)	
	0.49	Galactose (20)	Jongedijk et al. (2015)
Cineole	~100 mg/gDCW	Galactose (20)	Ignea et al. (2011)
		Raffinose (10)	
Linalool	0.077	Glucose (20)	Herrero et al. (2008)
	0.132	Glucose (20)	Rico et al. (2010)
	0.127	n.i.	Sun et al. (2013)
	0.095	Galactose (20)	Amiri et al. (2016)
	0.241	n.i.	Deng et al. (2016)
Sabinene	17.5	n.i.	Ignea et al. (2014)
Geraniol	1	Glucose (10)	Oswald et al. (2007)
	5.4	Glucose 10	Fischer et al. (2011)
	36	Glucose (20)	Liu et al. (2013)
	8	Glucose (20)	Pardo et al. (2015)
	11.4	n.i.	Campbell et al. (2016)
	293	n.i.	Zhao et al. (2016a)
Farnesol	4950	n.i.	Millis and Maurina-Brunker (2004)
	~80	n.i.	Takahashi et al. (2007)
	22.6	Galactose (20)	Asadollahi et al. (2008)
	146	Glucose (50)	Ohto et al. (2009)
	79	Glucose (20)	Zhuang and Chappell (2015)
Farnesene	762	n.i.	Renninger and Mcphee (2010)
	170	n.i.	Tippmann et al. (2016)
Bisabolene	994	Galactose (18)	Peralta-Yahya et al. (2011)
		Glucose (2)	
	5200	n.i.	Ozaydin et al. (2013)

n.i. not indicated in the publication, *gDCW* g dry cell weight

limiting 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) (Ohto et al. 2009), its truncated, cytosolic variant, tHMGR engineered to bypass feedback inhibition (Ohto et al. 2009; Rico et al. 2010; Behrendorff et al. 2013; Sun et al. 2013; Lv et al. 2014; Amiri et al. 2016; Tippmann et al. 2016), or its stabilized variant, $HMG2^{K6R}$ (Ignea et al. 2011), was overexpressed. In addition, expression of the IPP isomerase, IDI1 (Ignea et al. 2011; Liu et al. 2013; Sun et al. 2013; Zhao et al. 2016) and the mutated global transcriptional regulator of the sterol biosynthetic pathway, UPC2-1 (Peralta-Yahya et al. 2011; Behrendorff et al. 2013; Zhao et al. 2016) were also upregulated to improve precursor supply. To divert flux away from the competing yet growth-essential ergosterol biosynthesis into the corresponding intermediate pools, the

expression of the FPP synthase, ERG20 (for DMAPP), or the squalene synthase, ERG9 (for GPP and FPP), was attenuated, either through promoter replacement (e.g., P_{MET3}, P_{HXT1}, or P_{CYC1}) (Asadollahi et al. 2008; Peralta-Yahya et al. 2011; Ozaydin et al. 2013; Lv et al. 2014; Amiri et al. 2016; Tippmann et al. 2016) or complete knockout in a sterol uptake enhancement background strain (Takahashi et al. 2007; Zhuang and Chappell 2015).

Apart from these, other genes constituting the acetyl-CoA formation and the MVA pathways are also prospective manipulation targets for improved intermediate pools. Overexpression of ACS2 and ERG10, along with deregulation of tHMGR and ERG20 improved isoprene production through enhanced acetyl-CoA precursor supply (Lv et al. 2014). In addition, upregulation of the whole MVA pathway through overexpression of 3-hydroxy-3-methylglutaryl-CoA synthase (ERG13), tHMGR, mevalonate kinase (ERG12), and phosphomevalonate kinase (ERG8) has also been undertaken to improve flux toward GPP accumulation for geraniol production (Campbell et al. 2016). Besides, ERG20 overexpression has been suggested for improved FPP accumulation (Peralta-Yahya et al. 2011; Ozaydin et al. 2013). To direct flux away from the undesirable tRNA biosynthesis into the DMAPP pool, overexpression of MAF1, the negative regulator of tRNA biosynthesis has also been attempted for improved geraniol production (Liu et al. 2013).

The metabolic engineering of *S. cerevisiae* for monoterpene production is largely hindered by the low cytosolic availability of GPP, given that GPP is a transitory intermediate of the two-step FPP biosynthesis catalyzed by ERG20 (Rico et al. 2010; Fischer et al. 2011; Ignea et al. 2014). To circumvent this, efforts have been directed toward the identification of ERG20 variants with improved GPP-synthesizing ability. Overexpression of the protein variants, $ERG20^{K197E}$ (Oswald et al. 2007; Campbell et al. 2016; Deng et al. 2016), $ERG20^{K197G}$ (Fischer et al. 2011; Liu et al. 2013; Jongedijk et al. 2015), or $ERG20^{F96W\text{-}N127W}$ (Ignea et al. 2014; Zhao et al. 2016) along with specific terpene synthase resulted in the production of a range of monoterpenes including limonene, linalool, sabinene, and geraniol. $ERG20^{F96W\text{-}N127W}$ was reported to yield slightly higher geraniol production in comparison to $ERG20^{K197G}$ in a recent study (Zhao et al. 2016). Furthermore, expression of a heterologous GPP synthase (GPPS) that exclusively produces GPP, such as that from *Abies grandis*, was also undertaken to enhance the GPP pool (Campbell et al. 2016). However, in another study, decreased geraniol yield was reported for heterologous expression of GPPS from *A. grandis*, *Picea abies*, or *Catharanthus roseus* (Zhao et al. 2016).

Apart from engineering the accumulation of intermediates, the identification of an efficient terpene synthase to catalyze the final step of isoprenoid formation from the corresponding branch-point intermediate is of equal significance and is regarded as one of the key limiting steps in the microbial production of isoprenoids (Kirby et al. 2014). Such enzymes are generally endogenously absent in *S. cerevisiae*, and thus, heterologous sources, especially of plant origin, are usually sought. The use of plant-derived enzymes necessitates additional protein modifications including codon optimization and removal of the plastid-targeting sequence, to improve heterologous protein expression and to avoid potential protein misfolding and inclusion body

formation, respectively (Behrendorff et al. 2013). The significance of these was well evident by improved limonene, geraniol, and bisabolene production utilizing modified terpene synthases in comparison to their wild-type counterparts (Peralta-Yahya et al. 2011; Liu et al. 2013; Jongedijk et al. 2015; Zhao et al. 2016). Besides, an additional strategy has been suggested to improve the capacity of a plant terpene synthase by co-expressing its plant-interacting proteins that are involved in its correct folding, stability, or localization. Using a two-hybrid screen, a luminal binding protein, a SEC14 homologous protein, and a heat shock protein 90 (HSP90) were isolated for their specific interaction with the *Salvia fruticosa* cineole synthase. Co-expression of the cineole synthase and HSP90 protein increased cineole production in *S. cerevisiae* (Ignea et al. 2011).

The choice of terpene synthase utilized is also of particular importance and would have a significant impact on the final isoprenoid production. Comparison of the product formation efficiency of homolog enzymes from various sources was attempted to identify the best performing variant. In a recent study, three plant geraniol synthases were evaluated for geraniol production, with the highest titer observed from *Valeriana officinalis* geraniol synthase, in comparison to those from *Lippia dulcis* and *Ocimum basilicum* (Zhao et al. 2016). Notably, the *O. basilicum* enzyme variant was the most commonly used geraniol synthase in earlier studies, of which unsatisfactory production titers were reported (Oswald et al. 2007; Fischer et al. 2011; Liu et al. 2013; Pardo et al. 2015; Campbell et al. 2016). Similar enzyme comparison approaches were also undertaken for limonene synthase (production level with enzyme from *Perilla frutescens* > *Citrus limon*), farnesene synthase (*Malus domestica* > *C. junos* > *Artemisia annua*), and bisabolene synthase (*A. grandis* > *Pseudotsuga menziesii* > *Arabidopsis thaliana*) (Peralta-Yahya et al. 2011; Jongedijk et al. 2015; Tippmann et al. 2016).

To enhance the efficiency of substrate's channeling between enzymes of two sequential reactions, a fusion protein strategy was employed. The fusion of ERG20 (native or mutated variants) with specific terpene synthase resulted in improved linalool, sabinene, geraniol, and bisabolene production (Ozaydin et al. 2013; Ignea et al. 2014; Deng et al. 2016; Zhao et al. 2016). In addition, a strategy to improve protein expression by modulating mRNA stability and translation was also demonstrated. Using a Tween-20 surfactant-based screening method, an alternative 3′ coding sequence of *A. grandis* bisabolene synthase that yielded higher bisabolene synthase protein level and elevated bisabolene production was isolated (Kirby et al. 2014).

Differing from other isoprenoids, farnesol production was achieved mainly via engineering strategies that elevate the FPP pool without the need to express any heterologous terpene synthase (Takahashi et al. 2007; Asadollahi et al. 2008; Ohto et al. 2009; Zhuang and Chappell 2015). Farnesol is a dephosphorylated product of FPP, and its formation was suggested to result from promiscuous hydrolysis by endogenous phosphatases including PHO8, LPP1, and DPP1 (Faulkner et al. 1999; Song 2006). Noteworthy, deletion of LPP1 and DPP1 was suggested to improve sesquiterpene production through enhanced FPP pool (Scalcinati et al. 2012).

However, negative results were seen when such an approach was applied for sabinene production (Ignea et al. 2014).

5 Research Needs

While drop-in biofuels can potentially replace conventional fuels in our daily lives, much work remains to be done to make the current production processes commercially viable. Achieving theoretical maximum yields while coupling the technology with cheap biomass feedstock fermentation is crucial for cost considerations. Bottlenecks that limit the productivity of biofuels will need to be solved in order to attain more efficient production pathways. Work that needs to be done include the discovery and design of new enzymes and pathways, engineering efficient use of cellular resources, and development of microbial host strains capable of withstanding high biofuel concentrations.

Here, we have reviewed the progress of engineering yeast to produce drop-in biofuels including short-chain alcohols, fatty acid derivatives, and isoprenoids. Efforts to produce fuels with properties most suitable for substituting conventional fuels will be centered on producing fuel molecules of a range of chain lengths, increasing the branch structure of the molecules as well as production of saturated hydrocarbons. Using synthetic biology tools and high-throughput iterative design-build-test-analyze cycles, yeast variants can be constructed and characterized to deliver synthetic yeast strains capable of powering the future.

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Biomethane as an Energy Source

9

J. Robson, A. Alessi, C. Bochiwal, C. O'Malley, and J. P. J. Chong

Contents

Abstract

Microbe-mediated anaerobic digestion (AD) occurs in a wide range of natural and man-made habitats and results in the production of biogas. In order to use biogas as a transport fuel, it must undergo a costly conversion to biomethane (>95% methane). Biomethane can replace natural gas as a clean fuel in vehicles as it produces fewer harmful emissions than petrol, diesel, or LPG in spite of the considerable improvements made to these fuels in recent years. In addition to producing fewer emissions, biomethane poses fewer environmental hazards than other fuels. In the event of an accident, biomethane dissipates into the atmosphere rather than spilling onto the ground – a major benefit for waterways and wildlife. The main barrier for the use of biomethane as a transport fuel is the requirement for additional infrastructure such as refueling stations. This restriction does not apply to the use of biogas or biomethane for static energy generation such as electricity and heat production. Currently, biomethane from large AD plants can

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be injected directly into natural gas distribution networks. While AD has been extensively utilized in wastewater treatment, industrial-scale application of this technology to solid organic wastes has only recently begun to be adopted, probably due to a number of not fully defined and poorly understood parameters that influence the efficiency and robustness of microbes in this process.

1 Introduction

Microbial processes in the global carbon cycle are responsible for the production of approximately 1 billion metric tonnes of methane per year (Thauer et al. 2008). Biomethane is an end product of the biological decomposition of organic matter in anaerobic environments such as peat bogs and marshes, lake sediments, flooded soils, landfill, and the guts of ruminants and humans (Pimentel et al. 2012). Anthropogenic activities such as farming and waste processing are also a significant source of biological methane production. At present, a large proportion of the biomethane from human activities escapes or is released to the atmosphere. As methane has a global warming potential some 23 times that of carbon dioxide, relatively small volumes of this gas make a significant contribution to global warming (Scheehle 2006; Thauer et al. 2008).

Biomethane is formed via a process known as anaerobic digestion (AD). Biogas from anaerobic digesters is usually a mixture of 55–70% methane (CH_4), 30–45% carbon dioxide (CO_2), and other impurities such as hydrogen sulfide (H_2S) and water vapor (Krich et al. 2005). Biogas that is less than 60% CH_4 cannot be used as a fuel directly due to high CO_2 concentrations and must be transformed into high-grade "biomethane" (typically $\geq$95% CH_4), which is equivalent to natural gas. Upgraded biogas can be used on-site for heat and electricity, injected directly into the natural gas grid or converted to vehicle fuel, hydrogen, or methanol. The technology required for the transmission and utilization of biomethane is essentially the same as that for natural gas, and therefore a good infrastructure exists for using biomethane.

2 The Anaerobic Digestion Process

The microbial conversion of biomass into methane is the most efficient way of transforming organic matter into an energetically useful product (Schink 2008). A mixed microbial community effects breakdown of organic material to its constituent components and ultimately results in the production of carbon dioxide and methane via methanogenic archaea (Schink 2008). A wide variety of organic waste can be used such as vegetable foliage, pig slurry, or corn husks, thus making AD an inexpensive solution for waste remediation (Holm-Nielsen et al. 2009). Anaerobic digestion can be divided into two major steps: (1) biological conversion of the organic components of biomass into simpler products like acetate, carbon dioxide,

and hydrogen by a mixed population of non-methanogenic bacteria and (2) utilization of these products by a mixed population of methanogenic archaea to produce CH_4 and CO_2 (Fig. 1). Usually in this context, the non-methanogenic, organic acid-producing bacteria are relatively fast growing compared to methanogens which are perceived as rate limiting in the process, although under laboratory conditions, methanogenic species in pure culture can display 2–3 h doubling times (Jones et al. 1983; Whitman et al. 1982).

Since two distinct types of microbial consortia are required for AD, industrial biomethane production is sometimes separated into two phases, somewhat mimicking the process as it occurs in ruminants (Weimer 1998). Methanogenesis is performed under strictly anaerobic conditions, which is halted by oxygen concentrations greater than 10 ppm; however these conditions are created effectively in natural ecosystems during the hydrolysis/acidogenesis/acetogenesis phases. Numerous microbial species have been isolated from anaerobic digesters receiving various biomass and waste feeds, by ensuring strict anaerobic conditions during isolation work (Mori et al. 2000; Savant et al. 2002; Imachi et al. 2008). However, syntrophic

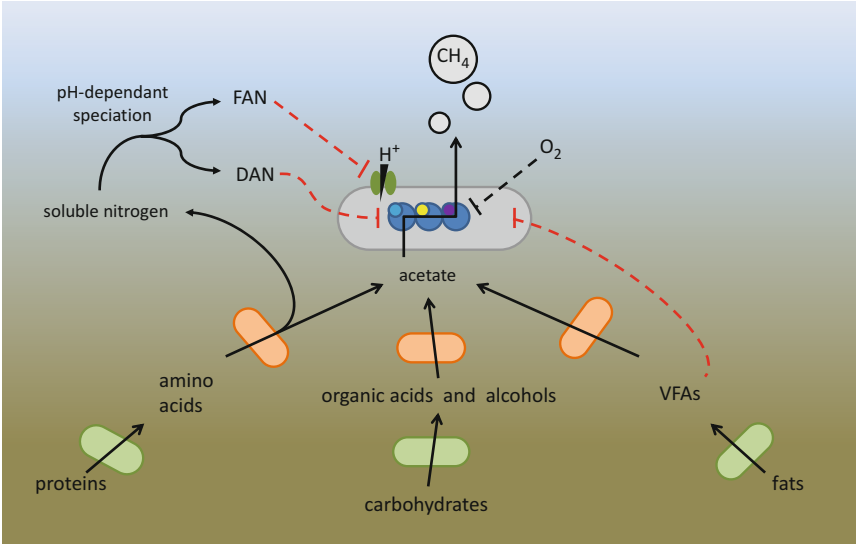

Fig. 1 The degradation and flow of molecules in an anaerobic digester. Hydrolytic microbes (*green rods*) such as *Clostridium* degrade biological polymers into oligomers and monomers using extracellular enzymes. Fermentative organisms (*orange*) such as *Actinomyces* further degrade monomers into simple carbon molecules such as acetate. Acetate molecules are cleaved to yield methane and carbon dioxide by methanogens (*grey*) such as *Methanosaeta*. Enzyme complexes (*blue circles*) used in methanogenesis require trace metals, e.g. cobalt, nickel and molybdenum (*light blue*, *yellow* and *purple dots*) and are strongly inhibited by oxygen (*dashed black line*). Mechanisms of other inhibitors vary (*red dashed lines*). VFAs interfere with the cell wall making it "leaky" and difficult to maintain homeostasis. Nitrogen species differentiate into either FAN, (NH_3) which interferes with homeostasis and causes cellular stress responses, or DAN (NH_4^+) which inhibits enzyme active sites

interactions between different species make discovery of novel microorganisms from AD samples difficult. This problem has been overcome by the application of novel molecular techniques such as metagenomics or metatranscriptomics, enabling the reconstruction of novel methanogenic pathways from uncultured AD microorganisms (Evans et al. 2015; Stolze et al. 2015). These approaches provide a better understanding of the community structure by providing a measure of microbial diversity and the means to track responses to environmental stimuli while bypassing culture-dependent steps (He et al. 2015). Since the complexity and diversity of the microbial population in AD communities can number thousands of species, culture-independent studies are gaining more importance in efforts to improve AD processes by creating substrate-tailored microbial communities (Campanaro et al. 2016).

All anaerobic digesters work on the same fundamental principle. Small-scale digesters for household and community use are commonplace in rural communities in Asia and Africa. These simple farm or household digesters consist of a simple (stirred) subterranean tank with a waste inlet and a gas outlet. Ambient soil temperature is the only source of heat. Such systems are fed with animal manure and household wastes, and the resulting biogas is used for cooking and lighting. Hydraulic retention times within this type of reactor tend to be higher than in those based on more sophisticated technology. In developed countries, AD is performed in digesters or reactors designed to produce high methane yields and high methane production rates at low cost. High methane yields are achieved through long solid retention times, high organic loading rates, and short hydraulic retention times. While in the recent past AD was dogged by a reputation for being unstable and difficult to control (Gerardi 2003), a better understanding of the physical and chemical parameters of AD processes has improved the robustness and productivity of anaerobic digesters, resulting in the operation of more than 17,000 biogas plants in Europe by 2014 ("The European Biogas Report" 2015). Our understanding of anaerobic digester microbiology is still relatively basic but offers great potential for further improving the effectiveness and efficiency of this process.

3 AD of Liquid Waste

In developed countries AD technology is most commonly used to reduce the organic content of sludges generated through sewage and wastewater treatment (Gerardi 2003). Very dilute (<1% solids) wastes tend to use fixed-film reactors where organisms are attached to inert media to reduce microbial washout and facilitate low (<1 day) retention times. Retention times can be low because wastes treated in this way are already highly soluble. Intermediate (5–10% solids) wastes are usually treated in digesters where the solids and microorganisms are recycled upon settling, either within the tank or in a secondary tank. While AD technology in the wastewater industry is clearly very advanced, most of this technology is not suitable for the solid waste arena.

One of the biggest technological advances in the treatment of wastewaters was the development of the upflow anaerobic sludge blanket reactor (UASBR) in the 1980s

(Lettinga 1995) (Fig. 2). This design was developed to treat water from the paper and sugar industries and now dominates high-strength wastewater treatment. Wastewater (influent) is dispersed into the base of the reactor and rises through a microbial sludge blanket considered "solid." The sludge comprises of small agglomerations of microorganisms that are captured by a specific arrangement of blades in the upper portion of the reactor. This allows microbial aggregates to grow to a size of 1–5 mm where the granules will sediment and therefore be retained in the reactor. Aggregated

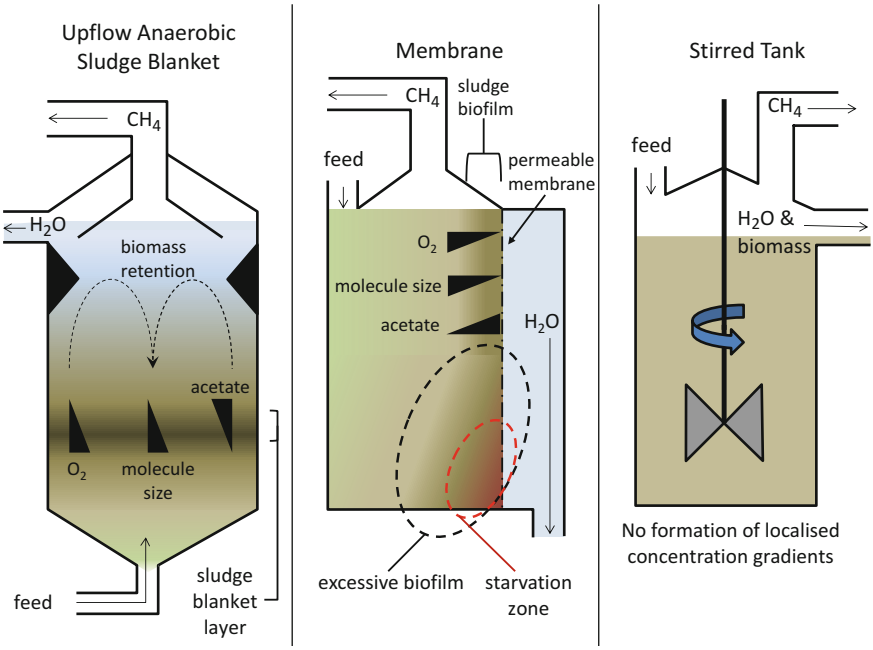

Fig. 2 Shared and distinct features of three common anaerobic digester reactor designs. The Upflow Anaerobic Sludge Blanket reactor operates by adding feedstock from the base of the reactor. The feedstock seeps through a microbial "sludge blanket" layer. Only one side of the sludge blanket is exposed to oxygen-containing feedstock thus creating a localised concentration gradient for both oxygen and incoming biopolymers. After diffusing through the sludge blanket, breakdown products such as acetate are taken up by methanogens. Methanogens, adhering to methane bubbles, rise to the top of the reactor and are captured by the inverted funnel before settling back to the sludge blanket. Membrane reactors utilise the same principle of a sludge layer to create localised concentration gradients. The permeable membrane allows active removal of soluble inhibitors such as ammonium and VFAs. Excessive biofilm formation (*dashed black circle*) on the membrane leads to areas of nutrient deprivation (*dashed red circle*) and death of part of the microbial population. Membranes need to be regularly cleaned to avoid excessive biofilm formation so more maintenance is needed compared to a UASB or a stirred reactor. Stirred reactors are the simplest design where feedstock is added and mixed with the community using motorised paddles. The feedstock and microbial community is fully homogenised which prevents formation of any localised concentration gradients. Compared to other systems, this potentially exposes the methanogenic population to increased levels of inhibition. The lack of an integrated method of biomass retention means biomass is lost alongside water during the addition of feedstock

biomass that settles back into the blanket area of the reactor allows a higher level of microbial retention compared to traditional reactors in which effluent, containing microorganisms, is discharged. The latter characteristics of UASBR enhance reactor stability by maintaining high densities of microbial consortia for long periods and overcoming problems associated with slow microbial growth and washout (Lettinga 1995; Schink 2008; Stams et al. 1997). Spatial organization and functional specialization of the microbial community within granules have been observed, with hydrolytic microorganisms located in the outermost granule layers and methanogenic archaea at the center. Separation of different functional groups of microorganisms, analogous to the approach taken with multi-tank systems, exposes them to different environmental stimuli leading to increased methane production (Show et al. 2004). UASBRs have also been reported to be effective in the treatment of semisolid wastes (Körner et al. 2003).

Elaboration of the phase separation principle behind the UASBR design resulted in membrane bioreactors (MBR) (Fig. 2) and fixed bed membrane reactors (FBMR) being developed. Both designs utilize a membrane to retain the majority of solids and microorganisms. MBRs filter bioreactor liquid and concentrate solids allowing for a smaller reactor footprint with a more concentrated microbial community. A concentrated community is also achieved in FBMRs by having liquid waste trickle over a microbial community grown on an inert surface. Specialized membranes can be included to treat difficult-to-digest feedstocks, for example, by removing toxins such as ammonium (a by-product of protein degradation in animal products), thus preventing inhibition of the ammonium-sensitive methanogenic community (Awobusuyi 2016).

4 AD of Solid Waste

Some 475 kg of municipal solid waste (MSW) are produced per capita per year in Europe (Mata-Alvarez et al. 2000). It is projected that by 2025 over 6 million tonnes of MSW will be produced globally per day (Hoornweg and Bhada-Tata 2012). Most MSW has traditionally been disposed of via landfill sites, which produce appreciable quantities of methane-rich biogas via uncontrolled and unoptimized AD processes. Where landfill sites have not been specifically designed for the collection of biogas, CH_4 enters the atmosphere (Mor et al. 2006). Methane released by landfill is estimated at 30–70 million tonnes per year and is a serious environmental concern (Bo-Feng et al. 2014). This biogas is often flared to comply with local regulations designed to reduce greenhouse gas emissions. Increasing numbers of landfill sites are harvesting, upgrading, and utilizing this biomethane (Powell et al. 2016). However, landfill gas tends to be of poor quality (~50% CH_4) (Rasi et al. 2007) and contains higher levels of impurities, because the organic fraction is not separated from inorganic waste, which is often toxic to the microorganisms required for AD.

MSW is better processed in specifically designed AD plants where conditions can be monitored and controlled. This approach allows the mass of waste to be reduced more quickly than via AD in landfill. The resulting solids can be used as a soil

conditioner, so that organic waste is almost entirely recycled. However, high solid (>10%) feedstocks normally require either stirred (Fig. 2) or leach bed batch systems. Longer retention times are needed to hydrolyze recalcitrant material such as lignin. On a municipal or agricultural scale, current solid waste AD technologies require large installations and significant investment in feedstock and product processing.

A major obstacle to the AD of solid waste is variability of feedstock, presenting process control issues that are compounded by unknown microbial populations being introduced with the waste into the process. These have potential for both positive and negative impacts on digestion efficiency. If the microbial ecosystem is skewed in favor of a particular group of organisms, quality and/or yield of biogas produced could be affected. Similarly, the successful seeding of digesters with anaerobic sludge is contingent on the microbial community contained within the sludge being able to successfully digest the feedstock provided. Any required change in the relative proportions of different microbes in the AD population in order to digest the feedstock can result in long lag times before solids are degraded and gases evolved, meaning increased retention times and less efficient CH_4 production. Approaches to overcoming community stability include pasteurization of feedstocks, thermal hydrolysis of incoming material, which has the additional benefit of breaking down material recalcitrant to digestion, and the mixing of ruminant slurry with MSW or lignocellulosic feedstocks for "co-digestion"(Ward et al. 2008). Agriculture produces sizable amounts of usable waste; livestock slurry in particular is both a rich source of organics and of microbial inoculant. A positive attribute of agricultural wastes on most modern farms is their relatively homogenous nature. These consistent feedstocks facilitate stabile operation of the AD process.

5 AD Process Optimization

Simple changes to the AD process can improve biomethane production efficiency. Solid feedstocks have increased particle sizes that restrict microbial and enzymatic access, hampering rapid breakdown. Feedstock pretreatments enhance digestibility of recalcitrant feedstocks, improving methane production. Pretreatments can use physical, chemical, thermal, or a combination of methods to achieve faster digestion rates. Mechanical pretreatments increase initial chemical oxygen demand, causing early onset of methane production and thus quicker digestion periods and increased (20–50%) biogas yields (Krich et al. 2005; Montgomery and Bochmann 2014).

Ultrasonic pretreatment utilizes cavitation produced via 15–20 kHz sound waves to create collapsing microbubbles with localized temperatures and pressures of up to 4,000 °C and 180 Mpa. This has been used to increase levels of reducing sugars from corn slurry almost threefold (Montalbo-Lomboy et al. 2010). Heated chemical treatments such as strong alkali, acid, and oxidative attack offer improved lignocellulose solubility and hydrolysis rates but tend to produce inhibitory cyclic by-products such as phenolics and furfurals. Physical and chemical treatments can be effectively combined in steam or ammonia explosion where a heated mixture is

subjected to high pressures (~200 bar) which is suddenly released to rupture cells and fibers. Explosive decompression treatments are effective at reducing particle size, solubilizing lignocellulose and improving enzymatic access to fibers. Gentler methods such as enzymatic pretreatments avoid the production of inhibitory molecules and can even act to remove toxic compounds. Aerobic degradation of cellulose by enzymes derived from white rot fungi is able to remove harmful cyclic compounds and toxins, but the introduction of oxygen by this method increases the volume of CO_2 produced. Addition of microbial stimulants like Aquasan and Teresan (saponified steroid products derived from plant extracts) can be used to activate microbes in the sludge. Addition of 10 ppm Teresan to mixed cattle manure produced ~35% more methane (Singh et al. 2001).

Other AD process parameters, such as operating temperature, pH, loading rate, etc., can be altered. Thermophilic (~50 °C) AD conditions increase methanogen growth rate and in turn increase the methane output. Temperature, pH (methanogenesis tends to be inhibited at non-neutral pHs, although methanogens adapted to acidic conditions exist), and agitation rates can all be used to better control the AD process. To prevent system failure and subsequent loss of revenue, system parameters, feeding regime and levels of potential inhibitors such as volatile fatty acids (VFA) and ammonia are typically monitored. In most cases, system failures arise from events such as increased organic loading rate (OLR) leading to a pH drop due to accumulation of VFA. Anaerobic bacteria produce VFA as a fermentation end product. Low VFA levels indicate a balanced microbial community, whereas VFA accumulation may indicate an overactive bacterial community which irreversibly affects the methanogenic population, causing the system to crash.

Methanogens are inhibited by high levels of dissolved ammonium nitrogen (DAN) and free ammonia nitrogen (FAN) derived from protein-rich substrates. DAN inhibits methane-producing enzymes, whereas FAN diffusion triggers intracellular stress responses. Acetoclastic methanogens are more sensitive to FAN than hydrogenotrophic species (Rajagopal et al. 2013), but the exact biological effects of ammonia excess are still unknown. Effective methods to control ammonia toxicity include pH reduction, addition of zeolites, microbial community acclimatization, and use of ammonium rejecting membranes (Rajagopal et al. 2013; Awobusuyi 2016). Processes such as struvite precipitation can limit or reverse ammonia accumulation. High protein wastes such as animal products and slaughterhouse waste can be co-digested with carbon-rich sources to maintain an optimal carbon/nitrogen ratio of 25:1 (Puyuelo et al. 2011).

Another consideration in AD process optimization is trace elements. These are essential for microbial growth, but an excess can be toxic. Various metals such as molybdenum, tungsten, cobalt, and nickel are part of enzymes' catalytic sites and used as electron carriers in the methanogenesis pathway. When metal concentrations are too high, anaerobic microorganisms use the metals as terminal electron acceptors, causing their precipitation and inhibiting methanogenesis. Understanding metal composition and concentration in a feedstock can provide a basis for tailored supplementation (Thanh et al. 2016).

Anaerobic digestion is a low-energy environment; microorganisms in this process use a wide variety of terminal electron acceptors such as CO_2, metals, and sulfur. Sulfur is present in certain amino acids and links peptide chains by a strong disulfide bridge. Sulfate-reducing bacteria use sulfate as a terminal electron acceptor, reducing it to corrosive sulfide. This process is coupled with methane depletion and its conversion into formate during anaerobic oxidation of methane (AOM). High sulfate levels in waste provide an energetically favorable environment for methane depletion. Excess metals and sulfate are both involved in diverting electrons from methanogenesis so removal of these compounds, or the identification of microbial species that lack these metabolic pathways, could increase methane production, especially in high protein digesters.

6 Gas Cleanup

Conversion of biogas to biomethane ("biogas upgrading" or "sweetening") can result in 95% CH_4 that can be used in exactly the same way as natural gas. Upgrading raw biogas requires removal of impurities such as CO_2, water vapor, H_2S, etc. Water scrubbing is most commonly employed to remove CO_2 and improve the quality of biogas. This typically results in the loss of less than 2% CH_4 due to the differences in solubility between CO_2 and CH_4 (Krich et al. 2005). H_2S is present in biogas derived from animal manure at 1,000–2,400 ppm. As well as being toxic, this gas is highly corrosive. Controlled injection of air into the biogas holder allows *Thiobacilli* present on the surface of the sludge to convert up to 95% of this gas into hydrogen and elemental sulfur (Krich et al. 2005). A range of chemical scrubbing methods can remove hydrogen sulfide. Water vapor can be introduced into the biomethane from both the AD process and gas scrubbing and is generally removed by condensation using cooling water or refrigeration.

7 Storage, Transport/Distribution, and Use

Biodigesters running on household waste have been in widespread use in the developing world for many years. In this case biogas is produced in small-scale digesters and used at source as a cooking and heating fuel in homes. Traditionally, biogas obtained directly from AD of landfill or agricultural waste is a low-grade fuel with low calorific value that is not economic to transport, and so it is usually burned on-site in biogas-capable combined heat and power plants to produce electricity that is either used locally or introduced into the grid. Upgraded biomethane can substitute for natural gas and be directly injected into a local distribution system (Krich et al. 2005) or be compressed or liquefied for use as a transport fuel. Both of these forms are lower-density fuels than traditional hydrocarbons, but biomethane can also be used as a feedstock for catalytic conversion to methanol or Fischer-Tropsch synthesis for hydrocarbon fuel production.

8 Conclusions

Currently anaerobic digestion and biomethane are underutilized in the developed world. This is probably due to a lack of innovation in the technology base and a lack of fundamental understanding of the dynamics of the microbial consortia involved in the AD process. Both of these deficits have been exacerbated by the availability of cheap fossil fuels. The AD process occurs readily in organic waste resulting in CH_4 emissions that increase with anthropogenic activities. As our understanding of climate change and the need for robust sources of renewable energy increases, so a better understanding of the AD process could be of benefit. Recent "green" tax incentives have driven an increase in the number of AD installations. However, these are based on exactly the same principles as those used in undeveloped regions and have not yet improved the efficiency of the AD process through a better understanding of the microbiology underlying the AD process. AD and biomethane have the potential to be part of the solution to an increasing need for renewable energy, but further research and development of these technologies is clearly required.

9 Research Needs

To facilitate greater uptake of anaerobic digestion as an "energy-from-waste" technology, the process must first become adept at tackling variable and polluted feedstocks. Understanding the roles and availability of trace metals and nutrients would prevent deficiencies occurring in enzymatic pathways. High-throughput sequencing techniques are likely to unveil novel functions and abilities of significant but thus far uncultured anaerobic microbes. With this knowledge, the ability to measure, model, and manipulate microbial populations in anaerobic digesters could facilitate the development of tailored consortia, resistant to excessive metals or toxins in previously indigestible material. An understanding of the molecular and genetic stress responses of AD microorganisms and how this effects output would provide defined procedures for rescuing or improving struggling digesters. Advancements in analytical equipment and real-time monitoring capabilities to detect fluctuations in the chemical environment of the AD process coupled to an understanding of how this affects the microbial consortia could deliver more effective process control and therefore more robust and efficient AD systems.

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Microbial Conversion of Carbon Dioxide to Electrofuels

10

Jongoh Shin, Yoseb Song, Sangrak Jin, Suhyung Cho, and Byung-Kwan Cho

Contents

Abstract

Electrofuel produced by microbes utilizing CO_2 and electricity as carbon and energy sources, respectively, has received much attention as an alternative to fossil fuels. Based on the inherent capabilities of microorganisms, extracellular electron transfer (EET) was demonstrated with various modes of cathodic electron transfer. With extensive studies on *Geobacter sulfurreducens* and

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Shewanella oneidensis, it was confirmed that cytochromes located in the outer membrane are essential for direct EET. Although a few electroactive bacteria are cytochrome independent, key compounds potentially involved in EET can be determined based on their redox functions, which were successfully demonstrated in electroactive acetogens and *Ralstonia eutropha*. Electroactive acetogens reduce CO_2 with electric power at the cathode and direct sunlight with a self-photosensitized nanoparticle for the production of organic compounds. Furthermore, a hybrid water splitting-biosynthetic system, which consists of advanced catalysts and genetically modified *R. eutropha*, exhibited production of diverse electrofuels with high CO_2 reduction efficiency. To improve the production of electrofuels, basic research and engineering of microorganisms and modification of electrodes is essential.

1 Introduction

In the last few decades, much of the concerns over the depletion of fossil fuel, importance of alternative energy, and increase in atmospheric CO_2 levels have promoted the development of several biological approaches for conversion of CO_2 into fuels or chemical products. Not surprisingly, many microorganisms can utilize various forms of reducing power to fix CO_2 into biomass (i.e., autotrophic growth) (Fig. 1) (Nybo et al. 2015). For example, photoautotrophic bacteria capture photon from light to obtain high-energy electrons, which are further used to reduce CO_2. Additionally, microorganisms can incorporate electrons extracted from reduced chemicals (such as NH_4^+, H_2S, or Fe^{2+}) into their metabolism. Several bacteria are of particular interest because of their potential for direct or indirect electron transfer from a cathode to obtain reducing power, which is referred to as microbial electrosynthesis (MES) (Rabaey and Rozendal 2010). MES is a highly attractive method to

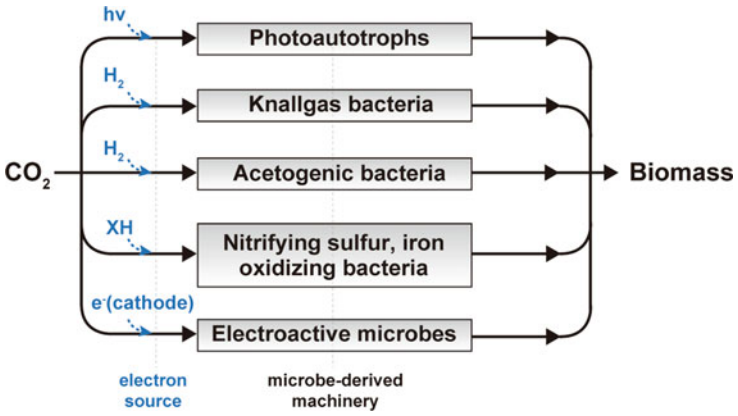

Fig. 1 Autotrophic microbial growth using various electron sources to reduce CO_2 to biomass

convert CO_2 into fuels, known as electrofuels, by utilizing only the electrons obtained from an electrode. Additionally, a unique system is needed to catalyze the biochemical reactions between an electrode (i.e., the cathode) and bacterial cells, which is called a bioelectrochemical system (BES). Although the conversion of chemical bond energy into electrical energy at the anode, called microbial fuel cell (MFC) technology, has been extensively studied (Nevin et al. 2010; Rabaey and Rozendal 2010), reversing the reaction at the cathode is thermodynamically unfavorable, where bacteria produce reduced products, usually methane, ethanol, or butanol.

Electrofuel production is an alternative avenue in biofuel production owing to several advantages (Rabaey and Rozendal 2010). Photon-to-fuel efficiency using a solar panel is higher than the efficiency of natural photosynthesis (Zhang 2015). Owing to the early stage of technology development, current CO_2 reduction rate and product diversity are limited (Tremblay and Zhang 2015). However, metabolic engineering and synthetic biology approaches will accelerate the efficiency of electrofuel production in various ways. In this chapter, we highlight the latest findings on the electrofuel production and describe how electricity and CO_2 facilitate electrofuel production in the most extensively studied microorganisms.

2 Bioelectrochemical System

Electron transfer reactions are essential metabolic processes in living organisms. The electrons are transferred from an electron donor with lower potential to an electron acceptor with higher potential in microorganisms, and this process allows the microbes to generate energy and drive their metabolism. Most microorganisms have different electron transport chains (ETC) (Hernandez and Newman 2001). Electrons are transported from an electron donor to a final electron acceptor via a series of redox reactions of membrane-associated electron carriers, which are involved in the establishment of an electrochemical gradient across the membrane. The net energy gain (Gibbs free energy, ΔG) in the ETC is governed by the difference in redox potential (ΔE) between electron donor and acceptor. Among microbes, some unique bacteria called exoelectrogens, which transfer electrons from the outer membrane to the external environment, or vice versa, have been studied (Potter 1911).

A decade ago, a special phenomenon was observed in some bacteria, wherein they were able to produce or receive electric currents because of the oxidation of organic compounds (Gregory et al. 2004). These unique microbes have been used to develop a novel electro-bioreactor system, which is also called as BES (Fig. 2a). BES contains two electrodes (anode and cathode), membrane between the electrodes, and extracellular electron transfer (EET) microbes. In MFC, microbes oxidize a supplied organic carbon or electron donor to generate a flow of electrons from the anode surface to the cathode surface (Rabaey and Rozendal 2010), where compounds like microbes or oxygen are reduced. Additionally, electricity can be

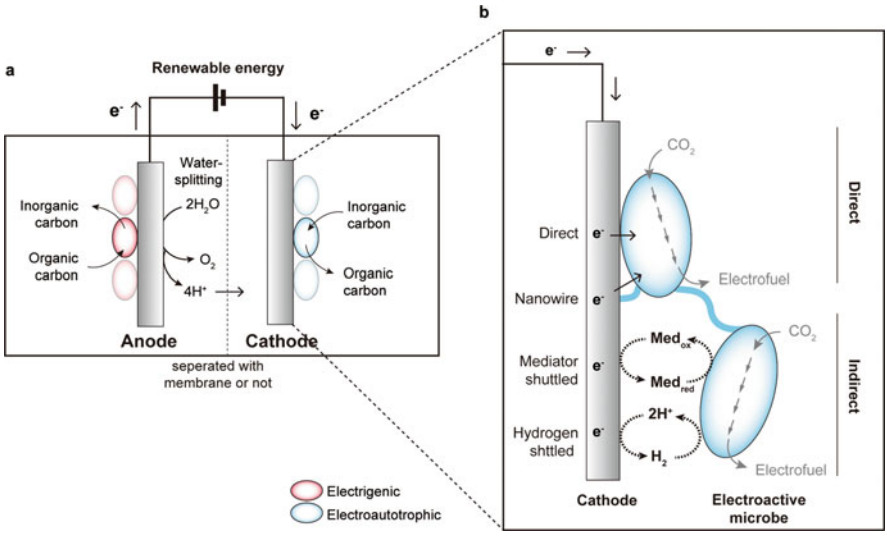

Fig. 2 (**a**) Schematic representation of the bioelectrochemical system. (**b**) Electrofuel production of cathodic electron transfer modes. Extracellular electron transfer proceeds by direct interaction (through direct contact or nanowire) or indirect interaction (through a mediator or a hydrogen shuttle)

supplied to the BES from renewable sources to permit the thermodynamically unfavorable metabolic process.

Based on the BES system, MES, potentially, is a carbon fixation process that produces organic molecules such as acetate (Nevin et al. 2010, 2011) or methane (Cheng et al. 2009) by using electricity as an energy source and electrochemically active microbes as biocatalysts to reduce CO_2, a carbon source (Rabaey and Rozendal 2010). Therefore, production of electrofuels largely depends on the interaction between microbes and surfaces of electrodes.

3 Electron Transfer from a Cathode to Microbes

To transfer the electrons received from cathode directly or indirectly to microorganisms, membrane-associated electron carriers are required. For this process, redox reactions are catalyzed spontaneously by different membrane-associated electron carriers. In general, based on the inherent capabilities of microorganisms, two methods of cathodic electron transfers are proposed (Rabaey and Rozendal 2010): (1) the microbe physically interacts with the cathode by pili or pilus-like appendages (called as nanowires) or (2) the microbe indirectly interacts through certain mediators or hydrogen shuttles (Fig. 2b).

3.1 Direct Electron Transfer

A physical interaction between bacteria and cathode, called direct electron transfer, is an attractive process without a requirement for the diffusion of an electron carrier. *Geobacter sulfurreducens* and *Shewanella oneidensis* MR-1 are metal-reducing bacteria that have been extensively studied. Several studies have reported that direct EET typically involves at least a series of outer membrane complexes, and outer-membrane c-type cytochromes are essential for the direct EET for both bacteria (Shi et al. 2009; Mehta et al. 2005)

G. sulfurreducens utilizes the branched OMCs system, which consists of several multiheme c-type cytochromes. The system enables the transfer of electrons between the extracellular metals and the cellular menaquinone (MQ) pool to establish an electrochemical gradient (Lovley et al. 2011). In addition, the electrons can be directly transferred to the electrode surface through conductive pili or pilus-like appendages (Reguera et al. 2005). Similar to *G. sulfurreducens*, *S. oneidensis* MR-1 uses the Mtr pathway, which consists of decaheme c-type cytochromes, to transfer electrons beyond the cell envelope (Bretschger et al. 2007). Additionally, both strains have membrane-bound NADH-hydrogenase, soluble electron carriers and H^+ ATPase. Additionally, it was reported that both bacteria can take up electrons from the cathode. For electron uptake by *S. oneidensis* MR-1, the reversed Mtr pathway is used (Ross et al. 2011), but a different pathway is used in *G. sulfurreducens* (Strycharz et al. 2011). In addition, the mechanisms of the cathodic reaction have not been studied in detail compared to those of the anodic reaction in BES.

3.2 Indirect Electron Transfer

Alternatively, electrons can be transported indirectly via the reduction of mediators (Fig. 2b). Previous studies reported that *G. sulfurreducens* secretes cytochrome (OmcZ) as a redox mediator within the biofilm matrix (Richter et al. 2009). For *S. oneidensis*, electrons can be transported indirectly via self-produced flavins as an electron shuttle (Marsili et al. 2008). Although, indirect electron transfer is estimated to be inefficient on a small scale, the carrier-mediated transfer can be sustainable with minimal cost on a large scale. Under these circumstances, hydrogen and formic acid can be used for this purpose (described below).

3.3 Electron Transfer Without Cytochrome

Several microbes have been reported to exchange electrons via the electrode (direct/indirect EET), but their EET mechanisms have not been well understood. Interestingly, a few organisms, which show the absence of the membrane-bound cytochrome (e.g., *Clostridium ljungdahlii*), have shown a possibility of being capable

of direct EET (Nevin et al. 2011; Köpke et al. 2010). According to their function, the possible mechanisms of electron transfer carriers have been postulated as follows:

1. NADH dehydrogenase: NADH dehydrogenase catalyzes the transfer of electrons from NADH to the quinone pool in the ETC.
2. Ferredoxin-dependent proteins: Iron-sulfur is the prosthetic group of ferredoxins, which mediate electron transfer in diverse metabolic reactions. The Rnf complex is a membrane-associated NADH:ferredoxin oxidoreductase contributing to proton (or sodium) motive force for ATP synthesis in acetogens such as *C. ljungdahlii* or *Acetobacterium woodii* (Tremblay et al. 2012). Additionally, the Ech complex (energy-conserving hydrogenase) catalyzes the reduction of ferredoxin with an H^+ gradient generated in acetogens such as *Moorella thermoacetica* (Pierce et al. 2008). Membrane-bound Rnf and Ecf complexes were hypothesized to be key components in electron transport.
3. Cytochromes: Heme is the prosthetic group of cytochromes and participates in electron transfer. Especially in metal-reducing bacteria, such as *Shewanella* spp., iron is used as the electron carrier and is oxidized or reduced during electron transport processes. Outer membrane cytochromes are key components for extracellular electron transfer, which was demonstrated in *Geobacter* and *Shewanella* spp. (Shi et al. 2009).
4. Flavoproteins: Flavin mononucleotide (FMN) is the prosthetic group of flavoproteins. There are many different flavoproteins that participate in either one- or two-electron transfers in the ETC.
5. Quinone: The compound contains lipophilic and catalytic cofactors with soluble electrons and protons, which are located inside the membrane. Quinone plays a crucial role in coupling electron flow to proton movement.
6. Hydrogenase: The protein catalyzes the reduction and oxidation of hydrogen. Recently, the hydrogenase of an electromethanogenic microorganism showed properties of direct electron transfer from a cathode (Deutzmann et al. 2015).

3.4 Electron Transfer via Hydrogen

Hydrogen and formic acid can be used as soluble electron carriers for lithoautotrophic bacteria. Several lithoautotrophic bacteria, including acetogenic bacteria, utilize hydrogen as the energy source; among these, *Ralstonia eutropha* has been extensively studied. Hydrogen can be produced from the cathode electrochemically in a hybrid microbial-water-splitting catalyst system, leading to the reduction of CO_2 to electrofuels (Fig. 2b) (Torella et al. 2015). Current electrolytic hydrogen generation is 50% more efficient and sustainable than direct electron transfer on a large scale (Torella et al. 2015). Because of this advantage, water splitting is a promising mechanism for large-scale conversion using solar energy or other renewable sources of energy (Reece et al. 2011; Hou et al. 2011). Alternatively, formic acid could also be used as a soluble electron carrier. In contrast to hydrogen,

formic acid is highly soluble in culture medium and a safe chemical compound, and is produced using electrons, water, and CO_2 (Ikeda et al. 1987).

4 Microorganisms for the Production of Electrofuels

For the electricity-driven synthesis of electrofuels, microbes should be able to convert CO_2 to intermediate chemicals or fuels by using electrical energy. Some methanogens, acetogens, and oxygen-reducing bacteria have been reported that have the ability for reductive processes at the cathode, resulting in the reduction of CO_2 to methane or organic compounds by electric power. In recent years, sunlight has been reported to be directly used by acetogens (*M. thermoacetica*) for the production of organic compounds, mimicking natural photosynthesis by using self-photosensitized nanoparticles (Sakimoto et al. 2015, 2016).

4.1 Acetogenic Bacteria

Acetogenic bacteria are a physiologically defined group of bacteria that can synthesize acetyl-CoA as central metabolic intermediate from CO_2 or CO via the Wood-Ljungdahl pathway (Drake 1994). The unique carbon respiration process of acetogens offers the possibility for the development of a novel approach for the conversion of CO_2 into substitute fuels or valuable chemical products. Previous reports indicate that a number of acetogens, including several *Clostridium* and *Sporomusa* spp. and *M. thermoacetica*, accepted electrons from the cathode, reducing CO_2 primarily to acetate (Nevin et al. 2010, 2011).

4.2 Energy Conservation and EET in Acetogens

The enzymatic reactions associated with the Wood-Ljungdahl pathway and coupled energy conservation system have been well characterized, especially in *M. thermoacetica* as a model organism (Huang et al. 2012; Mock et al. 2014; Schuchmann and Müller 2014). However, the mechanism of electron transfer to acetogens from a cathode is largely unknown. In the system, membrane-associated cytochrome enzymes and other electron transfer carriers can be suggested as key components involved in the EET (Schuchmann and Müller 2014; Kracke et al. 2015).

M. thermoacetica has b- and d-type cytochromes (Gottwald et al. 1975; Pierce et al. 2008), which are hypothesized to be involved in direct EET at high coulombic efficiency (Nevin et al. 2011) with mechanism similar to that determined in *Shewanella* and *Geobacter* species. Additionally, other membrane-associated electron carriers, which have integrated electron transport involved in the generation of a proton gradient across the membrane, such as NADH dehydrogenase, hydrogenase,

menaquinone, energy-converting hydrogenase (Ech complex), could be possible candidates for the electron transfer machinery of *M. thermoacetica* (Fig. 3a).

Furthermore, the soluble NfnAB and Hyd complexes of *M. thermoacetica* catalyze the reduction of two $NADP^+$ with one NADH and one Fd_{red}^{2-} (electron bifurcation) and the reduction of Fd and NAD^+ with hydrogen (Fig. 3a), respectively (Wang et al. 2013). Another acetogenic species, *Sporomusa ovata* is phylogenetically close to *M. thermoacetica* and contains membrane-associated cytochromes (b- and c-types) and quinones that can directly utilize electrons from a cathode. *S. ovata* also received electrons with the reduction of CO_2 to acetate and 2-oxobutyrate (Nevin et al. 2010).

However, *C. ljungdahlii* is able to transfer electrons directly from a cathode despite the absence of cytochromes and quinones. Alternatively, the membrane-bound Rnf complex (ferredoxin:NAD^+-oxidoreductase) was observed in *C. ljungdahlii*, which translocates protons across the membrane to synthesize ATP, during autotrophic and heterotrophic growth (Tremblay et al. 2012). Outer membrane redox components, as well as soluble intracellular complexes, are potential part of the electron transfer machinery.

With acetogenic bacteria, MES has been performed using diverse cathode materials ranging from a graphite stick (Nevin et al. 2010) to nickel nanowire (Nie et al. 2013), with cathode potentials from −0.4 V (vs. SHE) to −0.6 V (vs. Ag/AgCl). More recently, novel approaches (Sakimoto et al. 2015, 2016) have been reported. First, cadmium sulfide (CdS) nanoparticles were used to study the production of acetate by precipitation on the membrane of *M. thermoacetica* (Fig. 3a). Bacteria consumed the electron from the CdS nanoparticle and the photooxidative catalyst (TiO_2), which are used as light harvesters to eliminate the need for solid-state cathodes. This approach eliminates the need for hydrogen, which is difficult to store and transport, and solid cathodes, which are difficult to store, transport, and scale up.

4.3 Carbon Fixation and Extended Electrofuel Pathway in Acetogens

With the energy generated from the electron source, acetogens reduce CO_2 via the Wood-Ljungdahl pathway by using several electron carriers and enzymes (Ragsdale 2008; Drake et al. 2008). The pathway is coupled with energy conservation systems to permit the occurrence of the thermodynamically unfavorable reaction and is reported to be the most efficient CO_2-fixation pathway, among pathways including the Calvin-Benson-Bassham (CBB) cycle, 3-hydroxypropionate cycle, and reverse TCA cycle (Fast and Papoutsakis 2012).

The first reaction of the pathway is the reduction of CO_2 to formate by the action of formate dehydrogenase with a ferredoxin- or NADH-dependent reaction. A series of enzymatic reactions involving the methyl and carbonyl branch of the Wood-Ljungdahl pathway results in the conversion of formate to acetyl-CoA (Fig. 3b). Subsequently, the main product, acetate, is produced as a core feature through

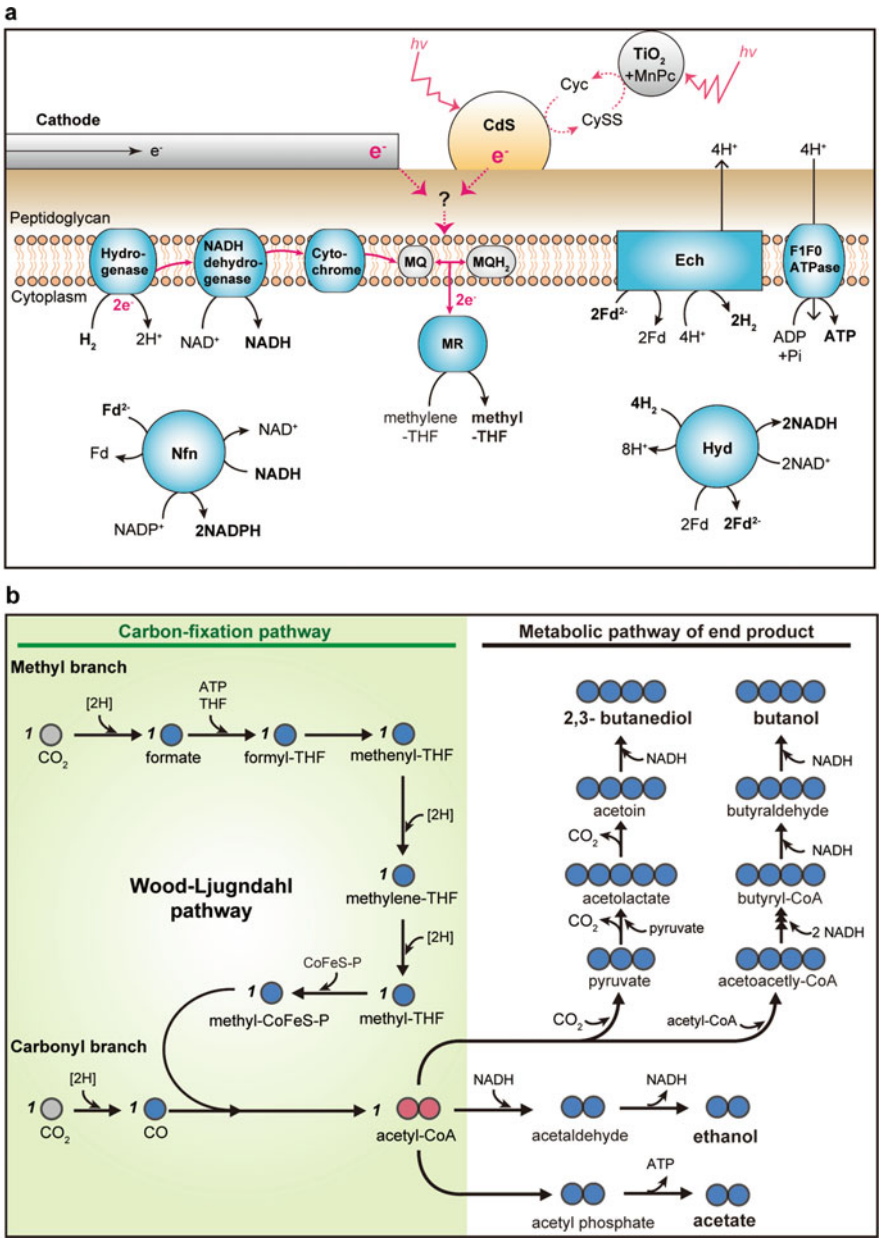

Fig. 3 (**a**) Schematic representation of the energy conservation system and applied bioelectrochemical system in *Moorella thermoacetica*. Abbreviations: *MQ* menaquinone, *Nfn* electron-bifurcating transhydrogenase (Nfn complex), *Hyd* electron-bifurcating hydrogenase, *THF* tetrahydrofolate, *MR* methylene-THF reductase, *Ech* energy-conserving hydrogenase (Ech complex), *Fd* ferredoxin, Fd^{2-} reduced ferredoxin, *Pi* inorganic phosphate. (**b**) The Wood-Ljungdahl pathway in acetogenic bacteria with the fuel production pathway. Abbreviations: *THF* tetrahydrofolate, *CoFeS-P* corrinoid [Fe-S] protein, *[H]* reducing equivalent

phosphotransacetylase and acetate kinase in most acetogens (Drake 1994; Ragsdale 2008; Schuchmann and Müller 2014). Additionally, synthesis of targeted products has been demonstrated by introducing biosynthetic pathways for ethanol, 2,3-butanediol, and butanol into *C. ljungdahlii*, which is known to be a genetically tractable strain for the production of value-added chemicals (Köpke et al. 2010, 2011; Leang et al. 2013). In the near future, under the right circumstances, a rationally designed and genetically modified strain will be able to produce a target product, increase the reaction rate, or tolerate various environmental conditions.

4.4 *Ralstonia eutropha*

R. eutropha is a gram-negative beta-proteobacterium that is isolated from soil and fresh water environments. *R. eutropha* proliferates with high cell densities (> 200 g/L) and takes up CO_2/H_2 autotrophically under aerobic conditions (Lu et al. 2016). The microorganism is considered to be a great platform for the production of electrofuels owing to its ability to route most reduced carbons generated from the CBB cycle to the accumulation of polyhydroxybutyrate (PHB) with CO_2/H_2 (Brigham et al. 2012). Using available genetic tools, numerous knockout studies involving PHB synthetic pathway have demonstrated that the carbon flux can be redirected from stored PHB into other compounds. Because of these advantages and its genetic tractability, *R. eutropha,* as a model industrial organism, has been extensively engineered to produce other value-added compounds like alcohols, fatty acids, or esters (Brigham et al. 2012).

4.5 Lithoautotrophic Metabolism of *R. eutropha*

The central carbon metabolism of *R. eutropha* completely relies on CO_2 fixation, which requires a series of high energy-intensive reactions. *R. eutropha* produces two [NiFe]-hydrogenases – membrane-bound hydrogenase (MBH) and a cytoplasmic soluble hydrogenase (SH) –to conserve the energy supply through the oxidation of H_2 (Cramm 2009; Bowien and Kusian 2002). H_2 oxidation is catalyzed by oxygen/carbon monoxide-tolerant hydrogenases. MBH is linked to ETC and SH, which is a bidirectional hydrogenase and produces energy by reducing NAD^+ with the oxidation of hydrogen (Fig. 4a).

Carbonic anhydrase (CA) and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) are the key enzymes in the CBB cycle (Bowien and Kusian 2002). CA can catalyze the interconversion between CO_2 and bicarbonate and can control intracellular pH homeostasis more efficiently by trapping CO_2 inside the cell (Fig. 4a). RuBisCo is the second key enzyme used in the carbon fixation step (Fig. 4b), and it (in Form 1C) is more suitable for O_2-containing environments (Badger and Bek 2008).

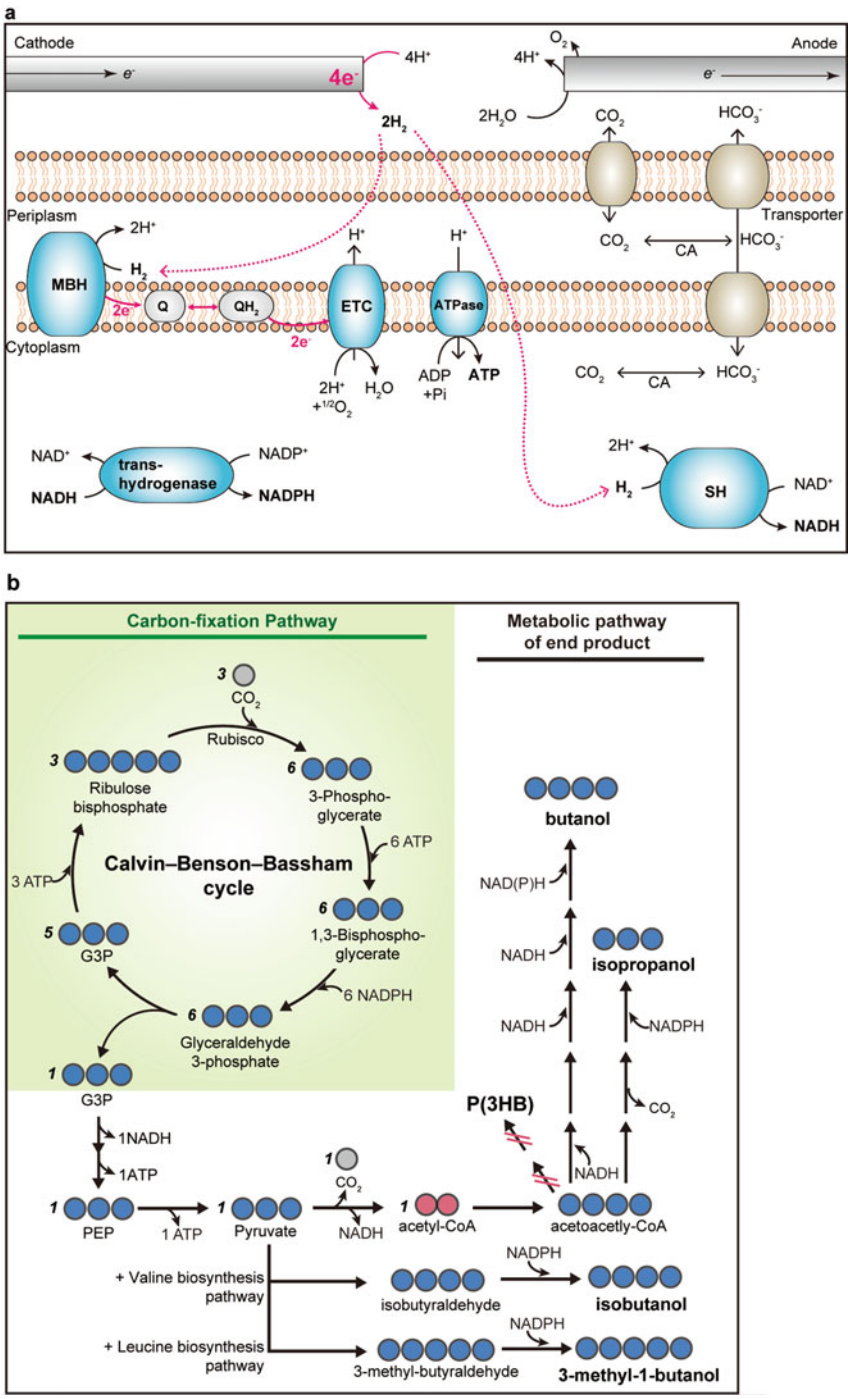

Fig. 4 (**a**) Schematic representation of lithoautotrophic metabolism of *Ralstonia eutropha*. Abbreviations: *CA* carbonic anhydrase, *MBH* membrane-bound hydrogenase, *ETC* electron transport

4.6 Hybrid Water Splitting-Biosynthetic System

For the production of electrofuels, a hybrid water splitting-biosynthetic system has been proposed, which consists of water-splitting catalysts and engineered *R. eutropha* (Torella et al. 2015; Liu et al. 2016). The system depends on the key catalysts (e.g., cobalt-phosphorus electrode) that split water and produce hydrogen to generate biomass and isopropyl alcohol. *R. eutropha*, the biocatalyst, thrives autotrophically on CO_2 and H_2 as the carbon and energy sources, respectively. In this system, *R. eutropha* converts CO_2 to multicarbon compounds via the CBB cycle (Fig. 4b).

When *R. eutropha* faces carbon abundance and nutrient deprivation, PHB was accumulated via the CBB cycle as a form of intracellular carbon storage (Pohlmann et al. 2006). Recently, the carbon flux was reported to be rerouted to prevent the accumulation of PHB, which causes the strain to secrete pyruvate and NADH abundantly and produce biofuel (Lu et al. 2012). By redirecting the rescued pyruvate and NADH into biosynthetic biofuel pathway, the production of isopropanol (Li et al. 2012; Torella et al. 2015; Liu et al. 2016), butanol, isobutanol (Liu et al. 2016), and 3-methyt-l-butanol (Kracke et al. 2015; Liu et al. 2016; Lu et al. 2012) was demonstrated (Fig. 4b). Although butanol production has not been reported yet in *R. eutropha*, it can be achieved by metabolically engineered *R. eutropha*.

To generate and transfer hydrogen efficiently from water and electricity to microbes via water splitting, several electrodes such as Pt, stainless steel (SS), cobalt phosphate (CoPi), and NiMoZn alloy have been used (Torella et al. 2015; Li et al. 2012; Schuster and Schlegel 1967). However, these systems are lethal to bacteria because of the generation of reactive oxygen species (ROS) or leaching of Ni particles. The results of a very recent study on catalyst design showed that the combination of an ROS-resistant cobalt-phosphorus alloy cathode and a cobalt phosphate (CoPi) anode increased the high CO_2 reduction efficiency (~10%) without ROS production and Ni leaching.

5 Conclusions and Future Research

In summary, electrofuel utilizes CO_2 and H_2 as carbon and energy sources, respectively. With extensive studies, several microorganisms were demonstrated and modified successfully for the conversion of CO_2 into various alternative fuels by

Fig. 4 (continued) chain, *SH* soluble hydrogenase, *Pi* inorganic phosphate, *Q* coenzyme Q. (**b**) A modified Calvin-Benson-Bassham pathway for the production of biofuels in *Ralstonia eutropha.* Abbreviations: *G3P* glyceraldehyde 3-phosphate, *PEP* phosphoenolpyruvate, *P(3HB)* poly (3-hydroxybutyrate)

using electricity. However, the underlying mechanism of extracellular electron transfer remains unclear. In addition, the performance of MES systems including final product titer and the CO_2 consumption rate is still limited. To improve the efficiency of the MES system, elucidation and modification of the EET and the involved metabolic networks are required to develop novel and efficient electrofuel production via systems and synthetic biology approaches. Furthermore, advanced cathode modifications will resolve the remaining technical challenges, such as ohmic voltage loss or the scale-up issue.

6 Research Needs

Electrofuel technology is an attractive and paid by great attentions in recent years, because carbon dioxide can be sequestered and stored as alternative fuels through the microbial conversion of carbon dioxide with renewable energy. Thus, the importance for the economic feasibility and environment problems will continue to grow. With extensive studies, several microorganisms were demonstrated and modified successfully for the conversion of CO2 into various alternative fuels by using electricity. However, the underlying mechanism of extracellular electron transfer remains unclear. In addition, the performance of MES systems including final product titer and the CO2 consumption rate is still limited. To improve the efficiency of the MES system, elucidation and modification of the EET and the involved metabolic networks are required to develop novel and efficient electrofuel production via systems and synthetic biology approaches. Furthermore, advanced cathode modifications will resolve the remaining technical challenges, such as ohmic voltage loss or the scale-up issue

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Genetic Engineering for Removal of Sulfur from Fuel Aromatic Heterocycles

11

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Contents

Abstract

Aromatic sulfur heterocyclic (ASH) compounds are among the most toxic and recalcitrant contaminants of fossil fuels and may cause serious environmental (e.g., acid precipitation), industrial (e.g., catalyst poisoning), and health problems (e.g., cardiopulmonary diseases). Different biochemical pathways for ASH degradation have been described in a wide variety of microorganisms. These pathways, which are encoded either on plasmids or on the host chromosome, usually are not essential for growth but rather allow to exploit a specific environmental niche or condition. Dibenzothiophene (DBT) is widely use as model ASH compound. A sulfur-specific pathway for DBT biodesulfurization (dsz pathway or 4S pathway) has been extensively studied at the physiological, biochemical, and genetic levels. The distribution and conservation of the *dsz* genes in a wide variety of bacteria strongly suggest that these genes are commonly subjected to horizontal gene transfer in nature. Despite the fact that an efficient ASH biodesulfurization depends on the expression, activity, feedback inhibition, and

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substrate range of the *dsz* gene products, host cell contributions also play an essential role in achieving higher activities, which are pivotal from a biotechnological point of view to develop a commercially viable biodesulfurization process. Factors, such as the cell-reducing power, cytoplasmic oxygen levels, transmembrane trafficking of substrates and products, solvent tolerance, and the ability of the cells to access and uptake the aromatic compounds, may influence strongly the biodesulfurization efficiency. A large number of recombinant bacteria have been engineered to overcome the major bottlenecks of the biodesulfurization process. The increased use of high-throughput *omic* techniques, as well as systems biology and synthetic biology approaches, is contributing significantly to unravel the intricate regulatory and metabolic networks that govern the degradation of ASHs. These studies will pave the way for further metabolic flux modeling and for the rational design of synthetic metabolic pathways or bacterial consortia for upgrading large volumes of fossil fuels, one of the greatest challenges addressed by current biotechnology. Moreover, existing desulfurization biocatalysts can also potentially be used in a variety of applications, e.g., synthesis of higher-value oil-based chemicals that have barely begun to be explored.

1 Introduction

Fossil fuels and, in particular, crude oil are heterogeneous mixtures of organic molecules including aliphatic and aromatic hydrocarbons. Within the aromatic hydrocarbons, some may also contain oxygen, sulfur, or nitrogen in their composition, and they are named as heteroaromatic/heterocyclic compounds. Aromatic sulfur heterocyclic (ASH) compounds are among the most potent environmental pollutants. Global society moves toward zero sulfur fuel, and hydrodesulfurization is the conventional industrial process that has been employed by refineries to remove organic sulfur from liquid fuels. However, deep desulfurization has become an important research subject due to the upcoming legislative regulations to reduce sulfur content. Hydrodesulfurization has several disadvantages to achieve such deep desulfurization, and, therefore, alternative solutions are needed. Microbial desulfurization or biodesulfurization (BDS) of organosulfur pollutants is an attracting alternative and complementary approach to hydrodesulfurization because of cost-effectiveness, environmental friendliness, and high selectivity toward compound recalcitrant to hydrodesulfurization (Nuhu 2013; Boniek et al. 2015; Pokorna and Zabranska 2015; Mohebali and Ball 2016).

Dibenzothiophene (DBT) is widely used as model compound for ASHs (Xu et al. 2006). Other sulfur-containing heterocycles have been scarcely studied (Schreinier et al. 1988; Kayser et al. 1993; Kirkwood et al. 2005, 2007; Ahmad et al. 2014). These compounds can be degraded by bacterial pathways, encoded either on plasmids or on the chromosome, that usually are not essential for growth but rather allow to exploit a specific environmental niche or condition. Studying and improving the methods for ASH biodegradation have attracted much attention both in basic

research and for bioremediation of polluted ecosystems and enhancing oil refining operations (Kilbane 2006; Xu et al. 2006). In this chapter, we will address the genetics of naturally occurring and engineered pathways for the removal of some ASHs, which are a major cause of environmental acid precipitation and industrial catalyst poisoning.

2 Genetics of Aromatic *S*-Heterocycles Biodegradation

Organosulfur compounds typically found in diesel fuel are mainly alkylated DBTs. In natural systems, some bacteria assimilate the sulfur from ASHs in very small amounts for their maintenance and growth. Desulfurizing bacteria are widespread in different environments and geographic locations, suggesting an important and common survival strategy for these bacterial species (Duarte et al. 2001; Mohebali and Ball 2016). Three different types of reactions have been described for bacterial DBT desulfurization: (1) sulfur oxidation, (2) carbon-carbon cleavage, and (3) sulfur-specific cleavage (Gupta et al. 2005; Xu et al. 2006; Soleimani et al. 2007; Mohebali and Ball 2016).

The sulfur oxidation of DBT is catalyzed by some fungal laccases and bacterial ring-hydroxylating dioxygenases that oxidize DBT to DBT-sulfone and other sulfur-containing hydroxylated derivatives as dead-end products (Gupta et al. 2005; Xu et al. 2006; Soleimani et al. 2007; Mohebali and Ball 2016).

The oxidative and carbon-carbon cleavage of DBT is known as the "Kodama pathway" (Kodama et al. 1973) and consists of three main steps including lateral dioxygenation of one of the homocyclic rings, ring cleavage, and hydrolysis yielding hydroxy-formyl-benzothiophene as the end product (Fig. 1) (Gupta et al. 2005). The Kodama pathway for DBT degradation is usually plasmid encoded in different *Pseudomonas* strains (Ohshiro and Izumi 1999). A 9.8-kb DNA fragment from a 75-kb plasmid of *Pseudomonas* sp. strain C18 was shown to contain nine genes, *doxABDEFGHIJ* (dox for DBT oxidation), responsible for the conversion of naphthalene to salicylate. DBT serves as an alternate substrate for these naphthalene-degrading enzymes (Fig. 1) (Denome et al. 1993). Although hydroxy-formyl-benzothiophene was reported as dead-end product, other possible products and even a complete mineralization by mixed bacterial cultures have been described (Gupta et al. 2005). Other organisms able to degrade DBT via the Kodama pathway are *Burkholderia fungorum* DBT1 (Andreolli et al. 2011), *Xanthobacter polyaromaticivorans* 127W (Hirano et al. 2004), *Beijerinckia* sp., and *Rhizobium meliloti* (Frassinetti et al. 1998; Mohebali and Ball 2016).

A different type of carbon-carbon cleavage pathway for DBT and DBT-sulfoxide degradation has been reported in *Brevibacterium* sp. DO and *Arthrobacter* sp. DBTS2 strains, respectively (van Afferden et al. 1990). These two organisms use these heterocycles as sole source of carbon, sulfur, and energy. Although their desulfurization pathway was reported to be partly similar to the sulfur-specific pathway with the formation of DBT-sulfoxide and DBT-sulfone (see below), it was still carbon destructive because the aromatic compounds were degraded finally

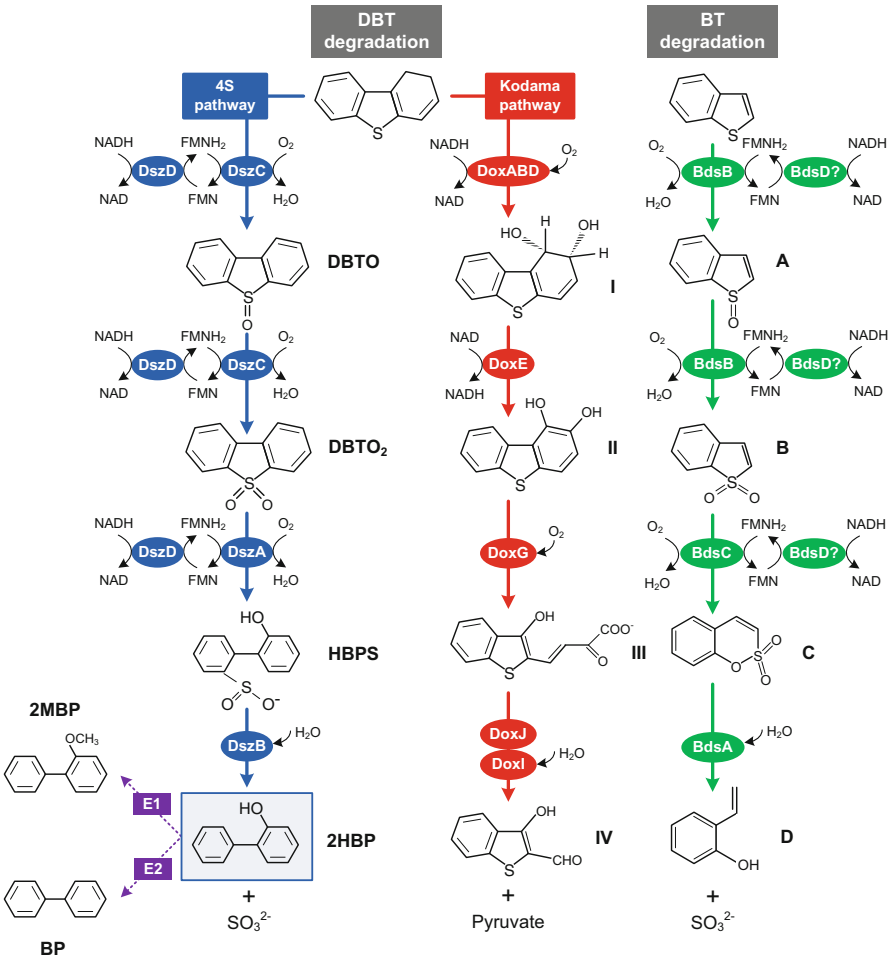


Fig. 1 Scheme of the main aerobic pathways for DBT and BT degradation. Four major pathways are shown: the 4S pathway (Adapted from Denome et al. 1993) (*blue arrows*) and two extended 4S pathways, E1 and E2 (Xu et al. 2006; Akhtar et al. 2009) (*purple arrows*), the Kodama pathway (Adapted from Kodama et al. 1973) (*red arrows*), and the BT degradation pathway proposed for *Gordonia terrae* strain C-6 (Adapted from Wang et al. 2013) (*green arrows*). The metabolites shown are *DBT* dibenzothiophene; *DBTO* dibenzothiophene sulfoxide; *$DBTO_2$* dibenzothiophene sulfone; *HBPS* 2-hydroxybiphenyl-2-sulfinate; *2HBP* 2-hydroxybiphenyl; *2MBP* 2-methoxybiphenyl; *BP* biphenyl; *I* *cis*-1,2-dihydroxy-1,2-dihydro DBT; *II* 1,2-dihydroxyDBT; *III* *cis*-4-[2-(3-hydroxy)-thionaphthenyl]-2-oxo-3-butenoate; *IV* 3-hydroxy-2-formyl-benzothiophene; *BT* benzothiophene; *A* benzothiophene *S*-oxide; *B* benzothiophene *S,S*-dioxide; *C* benzo[c][1,2]oxathiin *S,S*-dioxide; *D* *o*-hydroxystyrene. *?* indicates a proposed enzyme so far unknown

to CO_2, sulfite, and water (Ohshiro and Izumi 1999). The genes/enzymes of this pathway have not been yet characterized (Bressler and Fedorak 2000).

However, much of the impetus on microbiological desulfurization of fossil fuels comes from the discovery of a highly specific "4S pathway" (sulfur-specific

pathway) in the organism *Rhodococcus erythropolis* strain IGTS8 (ATCC 53968), which was isolated in 1990 by the Institute of Gas Technology (USA). Denome et al. (1993) isolated from IGTS8 cells a large linear plasmid, pSOX (120 kb), which contained a 4.0-kb DNA fragment responsible for the sulfur oxidation trait and capable of conferring this ability to non-desulfurizing strains. The *soxABC* genes (later renamed as *dszABC*) responsible for DBT desulfurization in strain IGTS8 are arranged in an operon-like structure next to an IS*1166* element (Fig. 1) (Denome et al. 1994; Piddington et al. 1995). Since then, several microorganisms have been described with DBT-degrading pathways similar to that of *R. erythropolis* IGTS8 (Nuhu 2013; Mohebali and Ball 2016).

The *dszC* gene encodes the DBT monooxygenase (DszC) that catalyzes the oxidations of DBT to DBT-sulfoxide and the latter to DBT-sulfone. The *dszA* gene encodes the DBT-sulfone monooxygenase that catalyzes the oxidation of DBT-sulfone to 2-hydroxybiphenyl-2-sulfinate (HBPS). Finally, the *dszB* gene encodes the HBPS sulfinolyase (desulfinase) that catalyzes the conversion of HBPS into 2-hydroxybiphenyl (2HBP) and sulfite (Fig. 1) (Gray et al. 1996). Nevertheless, the 4S pathway requires a chromosome-encoded additional gene, named *dszD*, which is responsible for the NADH-FMN oxidoreductase (DszD) that allows the regeneration of the $FMNH_2$ cofactor needed for the DszC- and DszA-catalyzed reactions (Fig. 1) (Gray et al. 2003). Genome sequence of the desulfurizing bacteria *R. erythropolis* XP also localized genes *dszABC* on a plasmid, while *dszD* is located on the chromosome (Tao et al. 2011a).

The biochemistry of the dsz pathway has been well studied using purified DszA, DszB, and DszC enzymes from several bacteria (Kilbane 2006). DszB desulfinase is present in the cytoplasm at concentrations severalfold lower than those of DszA and DszC and is the slowest of the three Dsz enzymes, becoming the rate-limiting step in the whole biodesulfurization process (Gray et al. 1996). Moreover, it was recently shown that HBPS, the substrate of DszB, can be secreted out of the cell to the medium and is unable to enter again into the cell, reinforcing the critical role of DszB in the BDS efficiency (Martínez et al. 2016). Purified Dsz enzymes from *Mycobacterium goodii* X7B showed that DszC can use both $FMNH_2$ and $FADH_2$ (Li et al. 2009) and DszD is a homodimer with each subunit binding one FMN or FAD (Li et al. 2012). The crystal structure of DszC from *Rhodococcus* strains revealed that two distinct conformations, named "open" and "closed," occur in the flexible lid loops adjacent to the active site and might show the status of the free and ligand-bound DszC enzyme. Site-directed mutagenesis study shows that mutations in the residues involved either in catalysis or in flavin or substrate binding result in a complete loss of enzyme activity, suggesting that the accurate positions of flavin and substrate are crucial for the enzyme activity. The DszC C-terminus, which is located in the interior of the protein, plays a crucial role in tetramerization and in the formation of the substrate-binding pocket (Duan et al. 2013; Liu et al. 2014; Zhang et al. 2014; Guan et al. 2015). DszA seems to proceed via a flavin-N5-oxide intermediate (Adak and Begley 2016). Different mutations at the active site of DszD were evaluated, explaining previous experimental results and enabling the design of DszD mutants with increased activity (Sousa et al. 2016). The three-dimensional structure of DszB revealed that this enzyme belongs to a new family

of desulfinases that share a catalytic cysteine residue and whose overall fold is similar to that of the periplasmic substrate-binding components of sulfur-regulated ABC-type transporters (Lee et al. 2006).

Despite the fact there is no indication that the *dsz* genes are inducible, they are strongly repressed by sulfate and sulfur-containing amino acids, which constitute an important bottleneck when designing efficient DBT desulfurizer biocatalysts (Li et al. 1996). The transcription start site of the *dszABC* operon mapped 46 nucleotides upstream from the ATG initiation codon of the *dszA* gene. At least three regions that affect *dsz* expression have been identified: the first one (−263 to −244) reduces *dsz* repression, but its deletion did not affect repression of gene expression; the second one (−146 to −121) could bind an activator; and the third region (−98 to −57) could be a repressor binding site (Li et al. 1996). The analysis of the *dsz* operon of *Gordonia alkanivorans* RIPI90A showed a high similarity with that of *Rhodococcus erythropolis* IGTS8, but there were differences in *dsz* promoter sequences. The promoter region from −156 to −50 is responsible for full promoter activity in strain RIPI90A (Shavandi et al. 2010).

By screening the available genomic databases, novel potential DBT-desulfurizing microorganisms belonging to different bacterial genera were identified (Bhatia and Sharma 2010a). The distribution and conservation of the *dsz* genes in a wide variety of mesophilic bacteria strongly suggest that these genes are commonly subjected to horizontal gene transfer in nature (Duarte et al. 2001; Gray et al. 2003; Kilbane 2006). Moreover, desulfurization has been shown to occur also in thermophilic bacteria. The first such microorganism to be identified was *Paenibacillus* sp. A11-2, and its desulfurization *tdsABC* genes are very similar to the *dszABC* genes. A *tdsD* gene not linked to the *tdsABC* genes encodes a flavin reductase that shows similarity to the Fre-like reductases rather than to the DszD-like reductases (Ishii et al. 2000). Other DszABC/TdsABC homologs have been found in the thermophilic *Paenibacillus naphtalenovorans* 32O-Y, but with low identity with Dsz and Tds proteins from *R. erythropolis* IGTS8 and *Paenibacillus* sp. A11-2 (26–48%, respectively) (Wang et al. 2015). Curiously, the desulfurizing capacity of *P. naphtalenovorans* 32O-Y is enhanced in a mixed culture with *Paenibacillus* sp. 32O-W, a non-desulfurizing bacterium (Butler et al. 2016). The thermophilic *Klebsiella* sp. 13T is also able to desulfurize DBT into 2HBP through the 4S route (Bhatia and Sharma 2012). Other desulfurization competent moderately thermophilic cultures have been isolated, and although the desulfurization genes of *Bacillus subtilis* WU-S2B (*bdsABC* genes) and *Mycobacterium phlei* WU-F1 are identical to each other, they exhibit only 61 % and 58 % identity with the *dszABC* and *tdsABC* genes, respectively (Kirimura et al. 2004). The flavin reductase from *M. phlei* WU-F1 (Frm) exhibited a high activity over a wide range of temperatures, and the overexpression of the *frm* gene encoding this protein with the *bdsABC* genes was critical to achieve a high desulfurization efficiency (Furuya et al. 2005). Interestingly, *M. phlei* WU-0103 is able to degrade a wide range of DBT, benzothiophene (BT), and naphthothiophene derivatives (Ishii et al. 2005). In contrast to what has been observed in strain IGTS8, the DszC ortholog (BdsC) appears to be the

rate-limiting enzyme in the desulfurization pathway of *B. subtilis* WU-S2B (Ohshiro et al. 2005). This BdsC monooxygenase is also able to oxidize indole to the blue pigment indigo in the presence of a heterologous flavin reductase in *E. coli*, and this feature was used to clone a gene (*frb*) encoding a flavin reductase from *B. subtilis* WU-S2B (Furuya et al. 2004). The flavin reductase Frb shows no appreciable similarity to DszD, but it is stable under high temperature, couples to monooxygenases, and also presents nitroreductase activity (Takahashi et al. 2009). Psychrophilic strains, e.g., *Sphingomonas subarctica*, that show a significant desulfurization activity have been also isolated (Gunam et al. 2013).

Despite the standard 4S pathway produces 2HBP as final product, an "extended 4S pathway" has also been reported in some thermophilic *Mycobacterium* strains. In this pathway, 2HBP becomes partially methoxylated to 2-methoxybiphenyl likely by the action of an *O*-methyltransferase (Fig. 1) (Xu et al. 2006). Compared to 2HBP, 2-methoxybiphenyl has a reduced inhibition effect on cell growth and desulfurization activity. This extended 4S pathway was also found in other *Mycobacterium* strains (Chen et al. 2009), and it was recently described in Gram-negative bacteria such as *Achromobacter* sp. and *Chelatococcus* sp. (Bordoloi et al. 2016). In a *Rhodococcus* strain, an extended 4S pathway that converts 2HBP to biphenyl has been also described, but the genes/enzymes involved have not yet been identified (Fig. 1) (Akhtar et al. 2009, 2015).

Bacterial cultures that have very similar *dsz* genes can differ significantly as regards their substrate range, desulfurization activity, optimal temperature, and concentration of metabolites, indicating the key role of the bacterial host contributions to the functioning of the whole desulfurization pathway. Factors such as the reducing power (NADH and $FMNH_2$ cellular levels), the cytoplasmic oxygen levels, the transmembrane trafficking of substrates and products, and the ability of the cells to access and uptake the aromatic compounds may influence strongly the biodesulfurization efficiency (Kilbane 2006). Thus, whereas *Pseudomonas* cells must access hydrophobic substances from the water (and therefore at low concentrations), *Rhodococcus* cells are able to access the very hydrophobic compounds directly from the oil, although no DBT transport genes have been identified so far in *Rhodococcus* or *Mycobacterium* strains (Kilbane 2006; Monticello 2000). Transcriptomic studies revealed the upregulation of several putative ABC transport systems when the desulfurizing *Gordonia terrae* C-6 strain was cultivated in the presence of BT, thus suggesting their participation in the transport of BT and its metabolites (Wang et al. 2013). On the other hand, ASHs may exert a stress response on the desulfurizing bacteria, including a change in the structural-functional properties of the host cell membrane that may result in overexpression of different stress proteins, antioxidants, and enzymes associated with the energetic metabolism. Thus, a proteomic study with *Chelatococcus* cells grown in DBT revealed the upregulation of putative transporters (e.g., enolase and MFS transporters) that may play an important role in transporting DBT and its metabolites across the cell membrane, as well as the upregulation of the ATP synthase enzyme to provide the required energy supply for the transporters. Several stress proteins and chaperons were also

upregulated, confirming their role in DBT desulfurization and adaptation of bacteria to this ASH (Bordoloi et al. 2016).

The inhibition caused by the intermediates of the 4S pathway is also an important bottleneck in BDS, and the two final products (HBPS and 2HBP) are responsible of the major inhibitory effects on the Dsz enzymes and cell growth (Chen et al. 2008; Alves and Paixão 2011; Abin-Fuentes et al. 2013). The ability of degrading DBT derivatives is limited not only by the metabolic capacities of the biocatalyst but also by the structure of the compounds. In this way, it has been reported that it is difficult to desulfurize long-chain alkylated DBT because an increase in the carbon number of the alkyl substituent group results in an increase in size and hydrophobicity (Bhatia and Sharma 2010b). Nevertheless, several bacterial isolates have been shown to desulfurize sterically hindered DBTs (Mohebali and Ball 2016). Thus, some monooxygenases, such as MdsC from *Mycobacterium* sp. G3, have a broad substrate range and oxidize different alkyl DBTs to each sulfone form (Nomura et al. 2005). Despite the chemical similarity between DBT and BT, the two desulfurizing pathways are mutually exclusive in most bacteria (Gilbert et al. 1998). Only a few bacteria harboring *dszABC* genes are capable to biodesulfurize both DBT and BT, although the participation of Dsz enzymes in benzothiophene desulfurization has not yet been confirmed (El-Said Mohamed et al. 2015a). Comparative transcriptomics in *G. terrae* C-6, a strain capable of desulfurizing BT and its derivatives but not DBT and its derivatives, revealed a three-gene operon, named *bdsABC*, responsible for BT degradation through a pathway similar to the 4S pathway (Fig. 1). Expression of *bdsABC* genes in *E. coli* allowed the conversion of BT into *o*-hydroxystyrene as final product (Wang et al. 2013).

The released sulfite from DBT desulfurization via the 4S pathway should be assimilated into bacterial biomass. A genome-scale in silico metabolic model of *R. erythropolis* has been developed (Aggarwal et al. 2011a) and shown to be useful in determining possible pathways of incorporation of sulfur from DBT into biomass. *R. erythropolis* was proposed to convert sulfite into sulfide that can be assimilated into biomass via sulfite reductase (reduces sulfite to sulfide that can be incorporated into sulfur-containing amino acids, CoA, and other metabolites) and/or sulfite oxidoreductase (oxidizes sulfite to sulfate) pathways (Aggarwal et al. 2012).

Sulfate-reducing bacteria have been also reported to anaerobically desulfurize model compounds and fossil fuels (Kim et al. 1995). *Desulfovibrio desulfuricans* M6 is a bacterial strain which anaerobically reduces DBT to biphenyl and H_2S (Kim et al. 1995). Other anaerobic microorganisms, such as *Desulfomicrobium escambium* and *Desulfovibrio longreachii*, have been reported to desulfurize DBT following a pathway in which biphenyl was not the end product. The desulfurization of oil under anaerobic conditions avoids costs associated with aeration and has the advantage of liberating sulfur as a gas. However, the low rate and extent of petroleum desulfurization by currently available anaerobic cultures, and the lack of knowledge on the biochemistry and genetics of such microorganisms, make the development of a commercial process unlikely in the near future (Gupta et al. 2005).

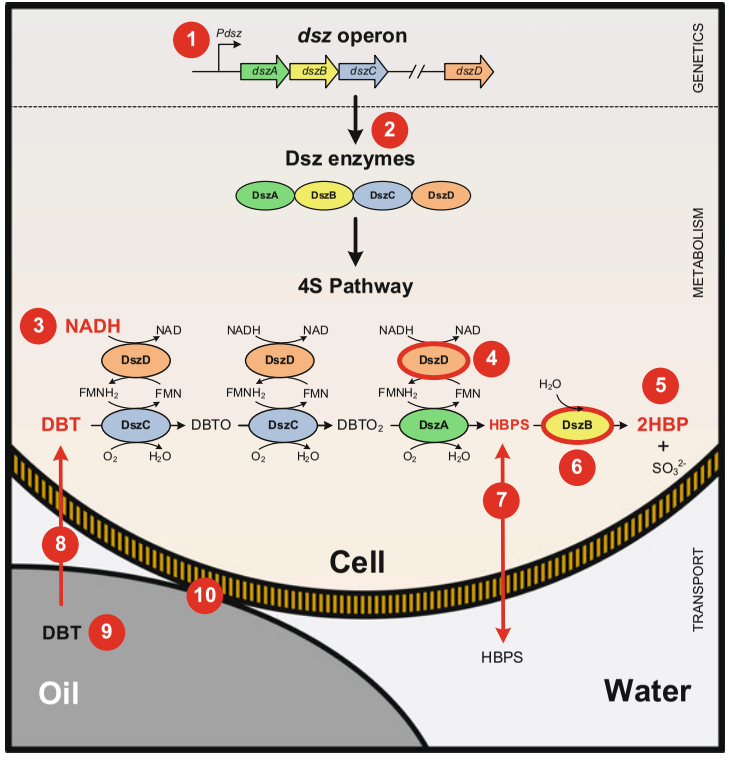
3 Engineered Recombinant Biocatalysts for Biodesulfurization

The 4S pathway is a complex enzyme system, and its cofactor requirement precludes the use of purified enzymes rather than whole cells for a practical BDS process. Resting cells are regarded as the best biocatalysts. However, the desulfurization activity of naturally occurring bacterial cultures is low in comparison to the requirements of a commercial process, and genetic manipulation has been used to address the major bottlenecks of the biocatalysts that preclude to reach higher desulfurization rates (Fig. 2). Nevertheless, it should be noted that for optimizing this industrial process, a lot of research on process development has to be accomplished (e.g., bioreactor design, separation of desulfurized oil fraction, efficient wastewater treatment, etc.) (Mohebali and Ball 2016), but these topics will not be reviewed in this article.

3.1 Engineering the 4S Pathway

The first attempts to create recombinant desulfurizers involved the cloning of the *dsz* genes from *R. erythropolis* IGTS8 in a plasmid that was able to confer a desulfurization phenotype to *R. erythropolis* UV1 (an IGTS8 mutant strain cured of the pSOX plasmid harboring the *dsz* genes) and to *Rhodococcus fascians*, but not to *E. coli* (Denome et al. 1993). Since then, a high number of recombinants were developed to try to overcome the major bottlenecks of the desulfurization process at the biocatalyst level (Fig. 2).

To alleviate the mechanism of sulfur repression, the *dszABC* operon from strain IGTS8 was engineered under the control of heterologous broad-host-range regulatory signals ($lacI^q/P_{trc}$) and stably inserted into the chromosomes of different *Pseudomonas* strains. The recombinant bacteria were able to desulfurize DBT more efficiently than the native host. Furthermore, these new biocatalysts combine relevant industrial and environmental traits, such as production of biosurfactants, with the enhanced biodesulfurization phenotype (Gallardo et al. 1997). Similar approaches that alleviated sulfur repression and increased desulfurization rate involving the expression of the *dsz* genes under the control of heterologous promoters have been described in *Rhodococcus* strains (Gupta et al. 2005). Two mutants of *R. erythropolis* KA2-5-1, which express about fivefold higher level of desulfurization activity than the parental strain in the presence of sulfate, were isolated using a transposome technique. Gene analysis of the mutants revealed that the transposon was inserted within the *cbs* gene encoding a cystathionine synthase (Tanaka et al. 2002). A putative promoter P_{kap1} which is not affected by sulfate was isolated from *R. erythropolis* KA2-5-1 and successfully used for expressing desulfurization genes (Noda et al. 2002). Recombinant cells of *G. alkanivorans* RIPI90A were constructed harboring its own *dszABC* operon in a plasmid under the control of the *Plac*



	BOTTLENECKS	GENETIC ENGINEERING APPROACHES
1	Sulfate-dependent repression of the *Pdsz* promoter	*dszABC* gene expression under heterologous promoters (*Ptrc, Phsp,…*)
		Disruption of the *dszB* gene for HBPS accumulation
		Transposome mutagenesis of a host-encoded *cbs* gene
		Mutagenesis of sufur metabolism-related genes (e.g. *cbs*)
2	Low DszABCD activity and thermostability	Cloning and expression of the *dsz* genes in multicopy plasmids under the control of strong heterologous promoters
		Cloning and expression of the *dsz* genes in thermophiles (e.g., *Thermus thermophilus*)
		Designing thermo-stable Dsz enzymes à la carte
3	Low levels of NADH	Optimizing metabolic fluxes using systems biology tools (e.g. Genome-Scale Metabolic Models)
4	Low levels of flavin reductase	Cloning and expression of heterologous flavin reductases in *dszABC*-harboring recombinant strains
5	2HBP-dependent retro-inhibition of Dsz enzymes	Further biotransformation of 2HBP by expressing heterologous enzymes (e.g., *carABC* genes)
		Disruption of the *dszB* gene
		Engineering synthetic microbial consortia that express individualized Dsz enzymes
6	DszB-dependent rate-limiting step	Optimizing *dszB* expression by replacing its translation signals
		Re-arranging the *dsz* operon (e.g., *dszBCA*)
7	Secretion of HBPS outside the cells	Increase of DszB activity/production
		Membrane permeabilization (e.g. expression of HBPS uptake systems)
8	Uptake of DBT from the oil phase	Expression of hydrophobic compounds transport systems (e.g., *hcuABC* genes)
		Heterologous expression of *dsz* genes in appropriate recipient cells (e.g., biosurfactant producers)
9	Narrow range of DBT-derived (e.g. alkyl-DBTs) substrates	Gain-of-function mutations of *dszA* and *dszC* genes
		Molecular protein evolution (e.g., RACHITT on DszC)
10	Low viability of the biocatalyst in petroleum	Expressing the *dsz* genes in solvent-resistant strains (e.g., *Pseudomonas putida* Idaho)

Fig. 2 Main bottlenecks in DBT biodesulfurization and genetic engineering approaches proposed to overcome them

promoter. This plasmid-based strategy increased the maximum desulfurization activity in a 2.6-fold and solved the sulfate repression limitation (Shavandi et al. 2009). Sulfate repression can be also overcome by deleting the *dszB* gene from the *dsz* operon. Deletion of this gene eliminates the slowest step of the Dsz pathway and allows the accumulation of HBPS, which is a more valuable product than sulfate because it can be used for surfactant biosynthesis. This strategy also helps to overcome the toxic effect derived from the production of 2HBP (Monticello 2000).

The bacterial toxicity and desulfurization inhibition caused by 2HBP could be diminished by engineering a biocatalyst that can convert this phenolic compound into a less toxic metabolite. In this sense, the carbazole-degrading enzymes have been proposed to transform 2HBP into benzoate, and a recombinant biodesulfurizer strain, *R. erythropolis* XPDN harboring the *carABC* genes from *Pseudomonas* sp. strain XLDN4-9, was shown to convert 2HBP into benzoate (Yu et al. 2006). The extended 4S pathway described in some desulfurizing bacteria (see above) is another mechanism to overcome the feedback inhibitory effect of 2HBP on the Dsz enzymes. Since the 4S pathway consists of three serial and irreversible modules, it was possible to develop a collection of synthetic *dsz* cassettes formed by different combinations of the *dsz* genes that were functional both alone and by combination in different *P. putida* biocatalysts (Martínez et al. 2016). Compartmentalization of the functional units of the 4S pathway into different hosts allows for their individual optimization and demonstrates that through division of labor, synthetic consortia can overcome process limitations, e.g., 2HBP feedback inhibitions, difficult to achieve when using monocultures. Thus, the generation of an artificial 4S pathway by combining the *dszC1-D1* and the *dszB1A1-D1* cassettes in an optimized synthetic bacterial consortium, and the *dszB1* cassette as a cell-free extract, is a promising alternative to the use of naturally existing or recombinant *dsz* pathways for enhanced conversion of DBT into 2HBP (Martínez et al. 2016).

Biodesulfurization can be enhanced by expressing multiple copies of the *dsz* genes, and, thus, when the *dszABC* and the *dszD* genes from *R. erythropolis* KA2-5-1 were reintroduced into the same strain cloned in a multicopy plasmid, the recombinant strain showed a higher desulfurization capacity (Hirasawa et al. 2001). Moreover, genetically modified biodesulfurizer strains that contain multiple copies of the *dsz* genes were reported to desulfurize alkylated derivatives of DBT, which are the main target molecules when extremely low sulfur levels are desired (Mohebali and Ball 2016).

To overcome the rate-limiting step caused by the low DszB activity, the *dszB* gene was overexpressed by increasing its copy number and optimizing its ribosome-binding site (Reichmuth et al. 2004). In a different approach to increase the DszB level within the host cell, the *dsz* operon was redesigned by uncoupling the translation of *dszB* from that of the *dszA* gene. A further improvement was done by modifying both transcription and translation levels, and a rearranged *dszBCA* operon was engineered to provide a higher desulfurization (12-fold increase) when expressed in *R. erythropolis* DS-3 (Li et al. 2008b).

Since the *dszABC* operon does not contain the reductase-encoding *dszD* gene, designing recombinant biocatalysts that produce the right amounts of NADH-FMN

reductase for the activity of the DszC and DszA enzymes is crucial to reach an optimum desulfurization potential. To this end, Galán et al. (2000) used the *hpaC* gene that encodes the NADH-FMN flavin reductase from the 4-hydroxyphenylacetate degradation pathway of *E. coli* W to construct a recombinant *P. putida* strain, *P. putida* KTH2 (pESOX3), which contained the *dszABC* genes from *R. erythropolis* IGTS8 cloned into a broad-host-range plasmid and the *hpaC* gene stably inserted into the chromosome. The resulting strain showed a significant increase in desulfurization activity with respect to the parental strain lacking the *hpaC* reductase gene. In a different study, a flavin reductase from *Vibrio harveyi* was overexpressed in *E. coli* cells that contained a compatible plasmid harboring the *dszABC* genes, and an increase in the rate of DBT removal, but a decrease in the rate of 2HBP production, was observed (Reichmuth et al. 2000). Different flavin reductases have different abilities to couple to the DBT monooxygenases and reduced flavin species, and in some cases, flavin reductases coming from non-desulfurizers are superior to those coming from desulfurizer strains (Gray et al. 2003). Site-directed mutagenesis was used to enhance up to sevenfold DszD activity of *R. erythropolis* IGTS8 revealing the critical role of residue 62 in enzyme activity (Kamali et al. 2010).

The broad substrate range of the Dsz enzymes is one of the driving forces for the development of BDS as a commercial process. Modifications of the Dsz enzymes have been shown to be an efficient way to improve biodesulfurization. Gain-of-function mutations in *dszA* and *dszC* genes generated new monooxygenases able to cope with BT and other non-DBT sulfur compounds present in crude oils (Arensdorf et al. 2002; Gray et al. 2003). Additional efforts to enhance biodesulfurization implied protein engineering of the Dsz enzymes. DszC enzymes with significantly improved rates of substrate turnover and specificity were developed by a molecular protein evolution technique (RACHITT) that allowed in vitro recombination of *dszC* genes from *Rhodococcus* and *Nocardia* strains (Coco et al. 2001). In a different approach, the utilization of mixed bacterial cultures with different substrate specificity was also used to enhance BDS efficiency toward a variety of sulfur compounds, e.g., mixtures of DBT and BT (Li et al. 2008a).

The interest on isolating thermophilic bacterial strains and enzymes is evident due to the fact that the petroleum refining process requires high temperatures. The possibility of the *dsz* genes from mesophilic bacteria being cloned and expressed in extreme thermophiles, such as *T. thermophilus*, has been explored. The *dszC* gene from *R. erythropolis* IGTS8 was successfully expressed in *T. thermophilus*, but the thermolability of the other Dsz enzymes prevented the functional expression of the full desulfurization pathway in this host (Kilbane 2006). The known three-dimensional structure of DszB and sequence comparison analyses with its thermostable orthologs (TdsB and BsdB) allowed the rational design of a double-mutant enzyme that showed higher thermostability without loss of catalytic activity or affinity for the substrate (Ohshiro et al. 2007). Since *T. thermophilus* grows at temperatures ranging from 55 ° to 85 °C, it might be possible to use directed evolution and selective pressure to gradually evolve desulfurization enzymes able of functioning at higher temperatures (Kilbane 2006).

3.2 Engineering the Host Cells

Contributions of host cells to the functioning of the 4S pathway have been shown to be another major key factor for efficient biodesulfurization. This concept has been well illustrated when the same *dszABCD* transposon was introduced into 22 *Rhodococcus* or *Mycobacterium* strains, and a wide range of desulfurization phenotypes, including differences in the substrate range susceptible of desulfurization, were found (Kilbane 2006). Some of the host functions that may influence oil desulfurization are those related to the uptake of substrates, tolerance to the organic solvents present in the crude oil, and temperature at which maximum desulfurization activity is detected. Biodesulfurization activity also depends on the culture conditions of the host cells. Thus, it was possible to optimize the biodesulfurization capacity of the recombinant *P. putida* CECT52479 cells by changing the media composition and culture conditions (Martín et al. 2004, 2005). Moreover, it was also found that the activity of each enzyme of the 4S pathway depends on the cellular age of the *P. putida* CECT5279 cells (Calzada et al. 2009) and the biodesulfurization capacity was enhanced using co-substrates, e.g., acetate, to increase the global reducing power of the cell (Martínez et al. 2015). The effect of Zn^{2+} on growth and desulfurization activity by *G. alkanivorans* 1B cells was also reported (Alves et al. 2008).

An important bottleneck in desulfurization is the acquisition of the low solubility substrates from the oil to the cytoplasm of the biocatalysts. The *hcuABC* gene cluster (*h*ydrophobic *c*ompound *u*ptake genes) of *P. aeruginosa* NCIMB9571 seems to encode a specific transport system of hydrophobic compounds, such as DBT, from the oil. When the *hcuABC* genes were cloned in a recombinant *P. putida* IFO13696 strain carrying the *dszABCD* genes, the resulting cells acquired the ability to desulfurize DBT in *n*-tetradecane, likely due to the increase in the uptake of DBT from the solvent (Noda et al. 2003). The *hcuABC* genes of *P. delafieldii* R-8 were introduced into the desulfurizing bacteria *R. erythropolis* LSSE8-1, and the desulfurization ratio of DBT using the resulting recombinant strain was increased by 19% (Wang et al. 2011). Transcriptomic studies revealed the upregulation of several putative ABC transport systems when the desulfurizing *G. terrae* C-6 strain was cultivated in the presence of BT, thus suggesting their participation in the transport of BT and its metabolites (Wang et al. 2013). On the other hand, it was shown that a transposon-induced mutation likely altered the D9-unsaturated fatty acid composition increasing the fluidity of cell membranes and enhancing the desulfurization activity in a *R. erythropolis* strain (Watanabe et al. 2003). The beneficial role of biosurfactants in the acquisition of hydrophobic compounds from the crude oil has been also explored in biodesulfurization (Gallardo et al. 1997; Ma et al. 2006; Raheb and Hajipour 2011). A significant oxygen tension is also necessary during BDS, and some approaches to improve the supply, transfer, and store of oxygen in vivo have been accomplished. Thus, the *vgb* gene that encodes the *Vitreoscilla* hemoglobin Vhb was introduced into *R. erythropolis* LSSE8-1, and the resulting strain showed increased DBT desulfurization (Xiong et al. 2007). The combination of a biocatalytic oxidation of organosulfides and thiophenes with hemoproteins, e.g., fungal

peroxidases, to form more soluble sulfoxides and sulfones, with a further bacterial metabolism to remove the sulfur from the latter, has been also proposed as an improvement of the BDS process (Ayala et al. 1998). Nanobiocatalytic approaches offer another alternative to improve the transfer rate of sulfur compounds from diesel oil to the microbial cells. Al_2O_3 nanoparticles were assembled onto *P. delafieldii* 8-8 biodesulfurizing cells to selectively adsorb DBT from the organic phase resulting in an increase in BDS rate (Guobin et al. 2005).

Several organic solvent tolerant strains of *Pseudomonas* can be ideal candidates for biodesulfurization in petroleum. In this sense, the *dszABC* operon from *R. erythropolis* XP was cloned into the solvent-resistant *P. putida* Idaho strain to generate a solvent-tolerant, desulfurizing *P. putida* A4 strain (Tao et al. 2006). The *Pseudomonas azelaica* HBP1 and related strains express a MexAB-OprM efflux system that confers tolerance to the toxic 2HBP, thus being suitable candidates for the expression of *dsz* genes (Czechowska et al. 2013; El-Said Mohamed et al. 2015b). The combination of a solvent-resistant bacterial host, e.g., the *P. putida* S12 strain, with an organic solvent-responsive expression system that drives the transcription of the *dsz* genes, resulted in a high efficiency of DBT removal in a biphasic reaction with *n*-hexane (Tao et al. 2011b). The recombinant organic solvent-tolerant bacterium *Rhodococcus opacus* ROD2-8 carries the *dszABC* genes from *R. erythropolis* IGTS8, and it is able to efficiently desulfurize DBT in a biphasic reaction mixture. The enhanced desulfurization activity was suggested to be due to a combined effect of attenuated feedback inhibition of 2HBP and reduced DBT uptake limitations due to 2HBP diffusion from cells and accumulation of both DBT and biocatalyst in the emulsion layer (Kawaguchi et al. 2012). At enzyme level, the tendency to oil of DszC has been enhanced performing in silico point mutations that increase the hydrophobicity of the enzyme (Torktaz et al. 2012).

Modern technologies of synthetic biology and metabolic engineering have been also applied to BDS. The use of a secreted sulfur-rich polypeptide (sulpeptide S1) combined with directed evolution provides an example of a promising approach for developing improved desulfurization biocatalysts. When an *R. opacus* strain was cultivated with DBT as the sole sulfur source, it was dependent upon the production of the 4S pathway enzymes expressed from a plasmid. Because the S1 peptide gene was inserted within the *dsz* operon, the increased production of desulfurization enzymes also resulted in the increased production of the S1 peptide, thereby increasing the nutritional demand for sulfur. Several rounds of selective pressure for rapid growth on DBT led to the appearance of improved desulfurization biocatalysts that showed a 20-fold increase in specific desulfurization ability (Pan et al. 2013). The identification of the mutation(s) responsible for the increased specific activity requires further research, but it may lead to a better understanding of the desulfurization pathway and to alternative ways to improve it.

A genome-scale flux-based model was used to study in silico the sulfur metabolism of *R. erythropolis* (Aggarwal et al. 2011b). This model predicts closely the growth rates reported in literature and suggests ethanol and lactate as the best carbon sources for cell growth and desulfurization. In addition, it was used to identify essential genes and reactions for desulfurization activity (Aggarwal et al. 2011a).

In silico studies using a genome-scale model of *G. alkanivorans* show that the sulfur-containing amino acid cysteine and methionine decrease desulfurization activity and also the preference of BT over DBT due to the lower NADH requirements for BT metabolism (Aggarwal et al. 2013). It has been proposed that in addition to efficiently express the *dsz* genes and the requirement of reducing cofactors, the sulfite reductase and sulfite oxidoreductase enzymes involved in sulfur metabolism play also a critical role in enhancing DBT desulfurization in *R. erythropolis* cells grown in the presence of this ASH compound. Decreasing the expression of the *cysJ* gene to achieve lower sulfite reductase activity would starve cells for sulfur and result in an increased rate of DBT utilization provided that elevated levels of sulfite oxidoreductase are maintained to avoid the toxic accumulation of sulfite (Aggarwal et al. 2012). A proteomic study in *Chelatococcus* cells grown in the presence of DBT revealed the upregulation of the cysteine synthase enzyme, suggesting that the synthesis of the sulfur-containing amino acid cysteine is a predominant mechanism of sulfur assimilation in this bacterium. Moreover, the upregulation of thioredoxin also suggests the involvement of this protein in sulfur assimilation and as a target for improving desulfurization (Bordoloi et al. 2016).

4 Use of the 4S Pathway for Alternative Applications

The 4S pathway represents a model system with unique advantages that can be used to improve our understanding of microbial physiology but also to develop novel bioprocesses. Existing desulfurization biocatalysts can potentially be used in a variety of applications including the detoxification of some chemical warfare agents, e.g., mustard gas, creating higher-value products from organosulfur compounds in petroleum, making novel antibiotics that contain carbon-sulfur bonds, or producing novel biodegradable plastics derived from polythioesters (Kilbane and Stark 2016).

Since the 4S pathway enzymes have a broad substrate range, they can metabolize sulfides, disulfides, mercaptans, sulfoxides, sulfones, sulfinates, sulfonates, thiophenes, and benzothiophenes, so the potential applications of biodesulfurization enzymes extend to a wide range of chemicals (Kilbane and Stark 2016). With the low price of petroleum, the production of higher-value products derived from the organosulfur material present in oil is an economically viable process. Recombinant biocatalysts that express the DszC and DszA monooxygenases but lack the DszB desulfinase have been constructed to efficiently convert DBT into sulfonate compounds that can be useful for the production of surfactants (Martínez et al. 2016). On the other hand, efficient biocatalysts containing the complete 4S desulfurization pathway convert DBT into 2HBP that can be used as precursor of other phenolic compounds for the synthesis of polymers or as fungicides, disinfectants, preservatives, dyes, or antioxidants and that can be reacted to add nitrate, halogen, alkyl, or acyl groups to create a wide range of chemical derivatives (Kilbane and Stark 2016). Similarly, the phenyl styrene compounds derived from BT-like precursors could be polymerized to yield plastic material (Kilbane and Stark 2016). Desulfurization enzymes could potentially also be used to modify organosulfur compounds to

make novel monomers that can be polymerized to produce polythioesters employed as thermoplastics and biodegradable polymer films (Khairy et al. 2015). Some DBT monooxygenases are also able to oxidize indole to the blue pigment indigo in the presence of a heterologous flavin reductase, and this feature can be used as a selective indication that may be widely applicable for the screening of novel genes encoding either a flavin reductase or a flavin-dependent monooxygenase from two-component monooxygenases (Furuya et al. 2004). Flavin reductases may be beneficial in many biotechnological applications as enzymes coupling to monooxygenases or, in some cases, as nitroreductases (Takahashi et al. 2009).

The development of desulfurization-competent hosts, such as those adapted to express a sulfur-rich polypeptide (Pan et al. 2013), represents a new tool for studying the expression, protein folding, disulfide bond formation, and protein secretion of high-sulfur-content proteins. Polypeptides that contain a high concentration of cysteine/methionine include some of the most potent venoms and some therapeutic compounds such as bacteriocins, defensins, and various synthetic peptides with antimicrobial and antitumoral properties (Kilbane and Stark 2016).

5 Research Needs

Despite significant research has been made during the last years, it is clear that a more detailed knowledge on the biochemistry and genetics of the ASH catabolic pathways is required. Rational protein engineering, in vitro evolution, and isolation of new Dsz enzymes with higher catalytic efficiency and not susceptible to the inhibition caused by 4S intermediates are future challenges. Issues regarding gene regulation and trafficking of substrates and intermediates need further study. The genes/enzymes involved in the degradation of highly substituted and complex ASH that are commonly present in fossil fuels are also unexplored. The characterization of the genes/enzymes involved in the extended 4S pathways is also needed. Genome mining and metagenomics will permit search for novel pathways/ genes for biodegradation/biotransformation of ASHs. This approach will benefit from the use of genetic traps to survey new enzymatic activities in metagenomic libraries. Engineering regulatory circuits that respond to the presence of toxic ASHs would allow designing novel biosensors to monitor the presence and fate of these compounds in the environment (Carmona et al. 2008).

The development of bioprocesses for upgrading large volumes of fossil fuels is one of the greatest challenges addressed by biotechnology. It is evident that the most efficient biocatalysts reported so far are far to meet the ultra-low sulfur levels ($<$15 ppm) required by new diesel fuel specifications. The currently available biocatalysts require an activity increase in biodesulfurization rate of about 500-fold. Host cell contributions play a pivotal role in achieving the higher activities needed for developing a commercially viable BDS process. Some key research needs for improving biocatalysts for an efficient BDS process are the design of engineered cells with higher specific desulfurization activity, broader substrate

range, higher thermal and solvent tolerance, higher substrate affinity, longer duration in bioreactors, etc. (Kilbane 2006; Mohebali and Ball 2016).

The rising number of available complete genomes, the increased use of high-throughput omic techniques as well as systems biology approaches for addressing biological complexity from a holistic perspective, and the application of network theory to biology will certainly contribute significantly to unraveling the intricate regulatory and metabolic networks that govern the degradation of ASHs. These studies will pave the way for further metabolic flux modeling and for the rational design of synthetic metabolic pathways that can perform and integrate efficiently within the metabolic/regulatory network of the particular host biocatalyst or bacterial consortia.

So far, biocatalysts used are planktonic cells, usually very sensitive to the stressful conditions prevalent in bioreactors that are dispersed in large volumes of reaction medium. However, biofilms of catalytic bacteria may be advantageous over suspended cells not only in that the physical proximity limits the unwanted diffusion of intermediates but also in that they exhibit a superior tolerance to physicochemical insults and harsh reaction conditions and they allow the operation of catalysts in packed column reactors. Recently, it was shown that the physical form and the morphology of the active biomass can be genetically programmed in vivo by implanting a synthetic device on an endogenous regulatory network that controls the planktonic versus biofilm lifestyle in *P. putida*, thus boosting biodegradation of the target substrate (Benedetti et al. 2016). Similar synthetic morphology approaches should be taken into consideration when designing the next generation of desulfurizing biocatalysts.

Finally, it should be taken into account that desulfurization biocatalysts can potentially be used in a variety of applications, e.g., synthesis of higher value oil-based chemicals (Kilbane and Stark 2016), that have barely begun to be explored.

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Part II

Bioproduction of Chemicals

Bioproduction of Chemicals: An Introduction

12

Yokimiko David, Mary Grace Baylon, Sang Yup Lee, and Si Jae Park

Contents

Abstract

The successful transition of a petroleum-based economy to a more sustainable economy is highly dependent on the development of technologies that will meet the demands for the production of fuel and industrially important chemicals. Establishment of microorganism-based biorefineries is a promising route in realizing this goal through the application of metabolically engineered microorganisms capable of converting renewable biomasses to value-added chemicals. This review encompasses the constructed synthetic pathways and microbial strain

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improvement strategies developed to date for the direct production of building-block chemicals from renewable biomass.

1 Introduction

Petroleum plays an integral role as the major resource material for the fuel and chemical industries because of its high carbon content. Although petroleum is considered as the "black gold" that led the industrial revolution, its processing causes severe damage to the environment due to its extensive carbon footprint, ultimately resulting in climate change. Moreover, petroleum is a nonrenewable resource, and its reserves are insufficient to meet the increasing demands of future generations. These concerns have led to an active search for alternative methods to develop sustainable technologies that will decrease the global dependence on petroleum. Utilization of renewable biomasses, which also have high carbon content, is an ideal replacement for petroleum as sustainable resources and/or raw materials for fuel and chemical production.

Increasing research and commercial interests in the utilization of renewable biomasses has led to the development of biorefinery technologies with the aim of converting renewable biomasses into fuels and valuable chemicals. Development of biorefineries based on microbial fermentation is one of the most promising technologies at present. Progress in industrial biotechnology has allowed researchers to alter, manipulate, and engineer microbes and enzymes to catalyze the conversion of renewable biomasses and generate industrially useful chemicals. Unlike other biorefinery technologies, application of engineered microorganisms has particular advantages with respect to biomass processing and chemical production with high enantiopurity, thereby increasing its potential value.

In microorganism-based biorefineries, biomass is first pretreated and hydrolyzed to release the organic compounds, especially the sugar monomers, to make them available for microbial conversion. There are several pretreatment methods possible, including physical, biological, and/or chemical treatments (Oh et al. 2015). Following the pretreatment, the biomass-derived sugars are converted by native or metabolically engineered microorganisms to produce valuable chemicals. After fermentative production by microorganism, the target chemical product is separated from the impurities such as cells, cell debris, organics, and nutrients, and purified for commercial application (Fig. 1).

Although microorganism-based biorefineries may address the environmental concerns raised by the use of petroleum, the feasibility and cost-effectiveness of this technology is not yet sufficient to surpass the current petroleum-based technologies. This gap is mainly attributed to the lack of methods available for the conversion of renewable biomass and to achieve the high production of valuable chemicals. This review provides an overview of the current developments in the construction of direct metabolic routes and generation of efficient microbial strains for the production of building-block chemicals.

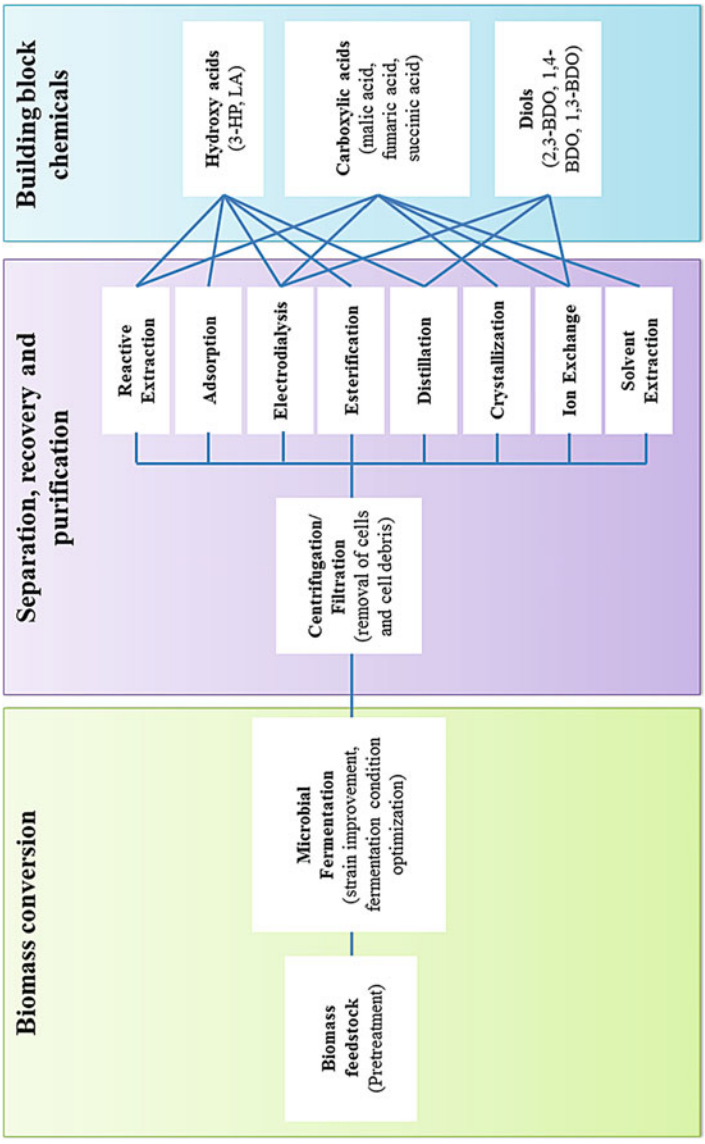

Fig. 1 Overview of a complete biorefinery process

2 Current Developments in the Production of Bio-Based Chemicals

2.1 Hydroxy Acids

Hydroxy acids are versatile feedstocks used in the synthesis of various industrially important chemicals. Among the known hydroxy acids, 3-hydroxypropionic acid (3HP) and lactic acid (LA) are considered two of the top value-added chemicals owing to their high market value and wide application.

2.1.1 3-Hydroxypropionic Acid

3HP is a non-chiral carboxylic compound. The occurrence of both a carboxyl and β-hydroxyl group in 3HP contributes to its versatility as a chemical feedstock for the synthesis of various commodities and specialty chemicals such as malonic acid, acrylate, and propiolactone (Valdehuesa et al. 2013) (Fig. 2). In a microbial system, 3HP can be produced through different routes, including 3-hydroxypropionate/4hydroxybutyrate cycle, acrylic acid and pyrimidine base (uracil and thymine) degradation, and the reductive glycerol utilization pathway (Kumar et al. 2013). Metabolic engineering of these pathways is necessary to efficiently produce 3-HP.

Among the native 3HP-producing pathways, the reductive glycerol utilization pathway has the advantage of utilizing glycerol, a cheap substrate, and requires a relatively low number of steps (2–4 steps) making it easier to manipulate (Valdehuesa et al. 2013). The synthesis of 3HP via the reductive glycerol utilization pathway proceeds via two routes after dehydration of glycerol to 3-hydroxypropionaldehyde (3-HPA): (a) direct oxidation of 3-HPA to 3HP and (b) conversion of 3-HPA to 3-hydroxypropionyl-CoA, 3-hydroxypropionyl-phosphate and 3HP (Fig. 3). Although this pathway is observed in several microorganisms from different genera, including *Klebsiella*, *Lactobacillus*, *Shimwellia*, *Clostridium*, *Enterobacter*, and *Citrobacter*, studies on the production of 3HP using the glycerol utilization pathway have mainly focused on *Klebsiella pneumoniae* and *Escherichia coli* strains (Valdehuesa et al. 2013). *K. pneumoniae* is a well-studied 3HP-producing strain, which can use both routes of the reductive glycerol utilization pathway. This bacterium is equipped with a stable glycerol dehydratase and has the ability to synthesize its cofactor, vitamin B_{12} (Valdehuesa et al. 2013; Kumar et al. 2013). Overexpression of aldehyde dehydrogenase encoded by the *PuuC* gene under the *tac* promoter, and deletion of acetate and lactate formation-related genes (*Δldh1*, *Δldh2*, and *Δpta*) coupled with optimization of fermentation conditions, has led to a high glycerol conversion ratio of 54% and a high titer of 3HP (83.8 g/L) (Li et al. 2016).

E. coli is a known workhorse microorganism that is widely applied in industrial biotechnology. Its genome and physiology have been extensively studied, and the genetic manipulation tools available for *E. coli* are well established. Thus, several studies have focused on optimizing 3HP production by conferring *E. coli* with the ability of reductive glycerol utilization through heterologous gene expression. A balanced expression of glycerol dehydratase and aldehyde dehydrogenase was achieved in

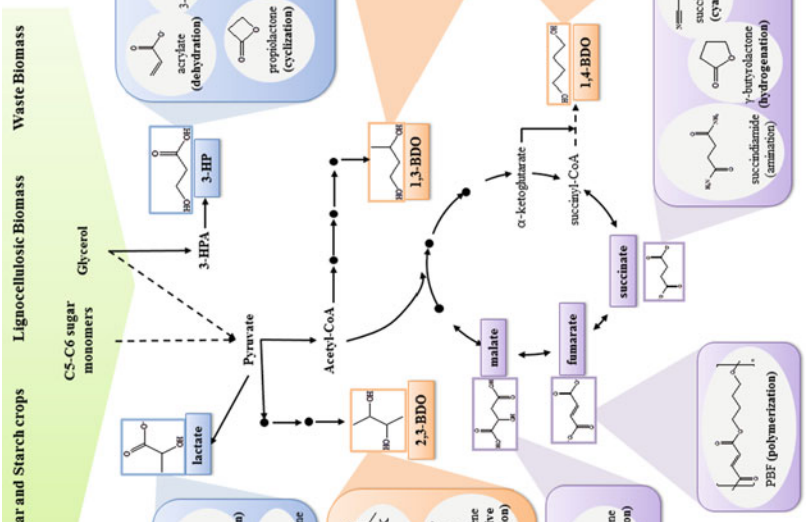

Fig. 2 Representative bio-based building block chemicals and their derivatives produced through microbial-based biorefineries. Biomass-derived sugars (green box) are converted to industrially important chemicals such as hydroxy acids (blue boxes), butanediols (orange boxes), and carboxylic acids (purple boxes) using metabolically engineered microbial cells

E. coli by fine-tuning the expression of *dhaB1*, which encodes the major subunit of glycerol dehydratase, using a specifically designed 5′ untranslated region. This prevented the accumulation of the cytotoxic compound 3-hydroxypropionaldehyde (3-HPA), thereby increasing cell growth and leading to the production of 40.51 g/L of 3HP with a yield of 0.97 g/g from glycerol (Lim et al. 2016). 3-HPA accumulation could also be attenuated by increasing the conversion rate of 3-HPA to 3HP. Aldehyde dehydrogenase with enhanced catalytic activity was developed through site-directed and saturation mutagenesis of *Cupriavidus necator* GabD4. Heterologous expression of mutant GabD4_E209Q/E269Q in *E. coli* resulted in a high titer (71.9 g/L) and productivity (1.8 $g{\cdot}L^{-1}{\cdot}h^{-1}$) of 3HP from glycerol (Chu et al. 2015).

Biosynthesis of 3HP from glucose does not occur in nature. Therefore, many researchers have attempted to design pathways for the production of 3HP from glucose. Several biotechnology companies such as Cargill, Genomatica, and OPX Biotechnologies have filed patents for novel pathways where glucose was used as the substrate. However, only a few of these patented pathways are thermodynamically favorable (Valdehuesa et al. 2013). Among these pathways, the malonyl-CoA, propionyl-CoA, and β-alanine pathways can be used for 3HP synthesis (Fig. 3).

The malonyl-CoA pathway involves malonyl-CoA reductase (MCR), acetyl-CoA carboxylase, and biotinilase for the conversion of acetyl-CoA to malonyl-CoA and 3-hydroxypropionic acid, respectively (Fig. 3). Heterologous expression of *mcr* (encoding malonyl-CoA reductase) from *Chloroflexus aurantiacus* DSM635 under the T5 promoter and overexpression of *accADBCb* (encoding acetyl-CoA carboxylase and biotinilase) and *pntAB* (encoding nicotinamide nucleotide transhydrogenase) in *E. coli* BL21 (DE3) resulted in the production of 0.19 g/L (2.14 mM) of 3HP (Rathnasingh et al. 2012). Moreover, heterologous expression of Acc from *Corynebacterium glutamicum* and codon-optimized MCR from *C. aurantiacus* in *E. coli* BL21 (DE3) resulted in the production of 10.08 g/L of 3HP with a productivity rate of 0.28 $g{\cdot}L^{-1}{\cdot}h^{-1}$ during fed-batch fermentation (Cheng et al. 2016). Through the propionyl-CoA pathway, 2.17 g/L (24.14 mM) of 3HP was produced in the recombinant *E. coli* mutant deficient in *ygfH* (encoding propionyl-CoA: succinate CoA-transferase) and *prpC* (encoding methylcitrate synthase) (Luo et al. 2016). This was achieved through the heterologous expression of propionyl-CoA dehydrogenase (from *Candida rugosa*), propionyl-CoA transferase (from *Megasphaera elsdenii*), and 3-hydroxypropionyl-CoA dehydratase (from *C. aurantiacus*) (Luo et al. 2016). 3HP production was also observed in a recombinant *E. coli* strain through the β-alanine route. In the constructed pathway, β-alanine was converted to malonic semi-aldehyde (MSA) and 3HP via β-alanine pyruvate transaminase (encoded by *P. aeruginosa pa0132*) and MSA reductase (encoded by *E. coli ydfG*), respectively (Fig. 3). This resulted in a 3HP titer, yield, and productivity rate of 31.1 g/L, 0.42 g/g, and 0.63 $g{\cdot}L^{-1}{\cdot}h^{-1}$ (Song et al. 2016). Since the above-mentioned pathways are initiated from either pyruvate or acetyl-CoA, these studies can also be used as a basis for developing novel pathways using other hexose and/or pentose sugars present in biomass.

Lastly, the glucose and glycerol pathways can be linked for the production of 3HP by the additional expression of GPD1 (encoding glycerol-3-phosphate

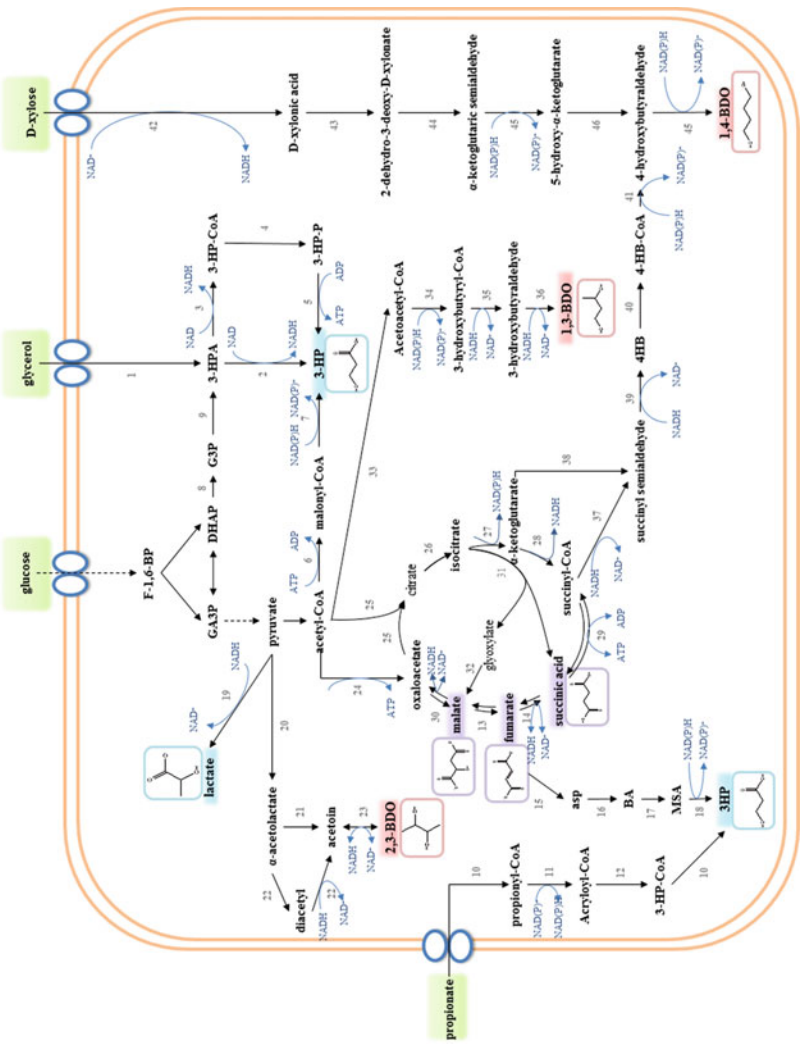

Fig. 3 Native and novel biosynthetic routes for the production of lactate, 2,3-butanediol (BDO), malate, fumarate, succinic acid, 3-hydroxypropionic acid (3HP), 1,3-BDO, and 1,4-BDO. Enzymes: (1) glycerol dehydratase, (2) aldehyde dehydrogenase, (3) propionaldehyde dehydrogenase, (4) phosphate

dehydrogenase) and GPP2 (encoding glycerol-3-phosphatase) from *Saccharomyces cerevisiae* along with *dhaB1B2B3*, *aldH*, and *xylR1R2* (encoding for the transcriptional activator of *xyl* operon) overexpression in the *E. coli ptsg* mutant. 29.7 g/L 3HP could be produced from mixed glucose and xylose with a 0.36 g/g yield and 0.54 $g \cdot L^{-1} \cdot h^{-1}$ productivity (Jung et al. 2015).

2.1.2 Lactic Acid

Lactic acid (LA) is the most abundant naturally occurring carboxylic acid, and has been extensively used in various applications such as food, pharmaceutical, and polymer industries. The petroleum-based chemical synthesis of LA yields a racemic mixture of D- and L-LA, while enantiopure LA can be obtained in a biological system (Biddy et al. 2016; Jung et al. 2010). The biological pathways involved in LA synthesis can be categorized as homofermentative and heterofermentative. The homofermentative pathways include the Embden-Meyerhoff pathway, pentose phosphate pathway, and glycolytic pathway, wherein an enantiopure LA is produced as the end product. By contrast, in the heterofermentative pathways such as the phosphoketolase pathway (PK), equimolar amounts of racemic LA, acetate, ethanol, succinate, and formate are produced as end products, thereby resulting into a low yield of LA (Wang et al. 2015).

Bacillus sp. WL-S250, a homofermentative isolate, can produce high concentrations of pure L-LA (225 g/L) with a yield of 0.99 g/g from glucose via multipulse fed-batch fermentation (Meng et al. 2012). *Enterococcus mundtii* QU 25, another homofermentative isolate, was found to have the ability to utilize xylose and could produce 129 g/L L-LA with a yield and productivity of 0.785 g/g and 0.768 $g \cdot L^{-1} \cdot h^{-1}$, respectively, from xylose (Abdel-Rahman et al. 2015).

Fig. 3 (continued) propanoyltransferase, (5) propionate kinase, (6) acetyl-CoA carboxylase, (7) malonyl-CoA reductase, (8) glycerol-3-phosphate dehydrogenase, (9) glycerol-3-phosphatase, (10) propionate-CoA transferase, (11) propionyl-CoA dehydrogenase, (12) 3-hydroxypropionyl-CoA dehydratase, (13) fumarase, (14) succinate dehydrogenase, (15) aspartase, (16) aspartate-α-decarboxylase, (17) β-alanine pyruvate transaminase, (18) malonic semi-aldehyde reductase, (19) lactate dehydrogenase, (20) α-acetolactate synthase, (21) α-acetolactate decarboxylase, (22) 2,3-butanediol dehydrogenase, (23) acetoin reductase, (24) PEP carboxykinase, (25) citrate synthase, (26) aconitase, (27) isocitrate dehydrogenase, (28) α-ketoglutarate dehydrogenase, (29) succinyl-CoA synthetase, (30) malate dehydrogenase, (31) isocitrate lyase, (32) malate synthase, (33) 3-ketothiolase, (34) acetoacetyl-CoA reductase, (35) butyraldehyde dehydrogenase, (36) alcohol/aldehyde dehydrogenase, (37) succinate semi-aldehyde dehydrogenase, (38) 2-oxoglutarate decarboxyalse, (39) 4-hydroxybutyrate dehydrogenase, (40) 4-hydroxybutyryl-CoA transferase, (41) 4-hydroxybutyryl-CoA reductase, (42) D-xylose dehydrogenase, (43) D-xylonic acid dehydratase, (44) 2-dehydro-3-deoxy-D-xylonate dehydratase, (45) alcohol dehydrogenase, (46) keto-acid decarboxylase. Abbreviations: F-1,6-BP, fructose-1,6-bisphosphate; GA3P, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone-phosphate; G3P, glycerol 3-phosphate; 3-HPA, 3-hydroxypropionaldehyde; 3-HP-CoA, 3-hydroxypropionyl-CoA; 3-HP-P, 3-hydroxypropionyl-phosphate; asp, aspartate; BA, β-alanine; MSA, malonic semi-aldehyde; 4HB, 4-hydroxybutyrate; 4HB-CoA, 4-HB-CoA

The relatively low yield and racemic LA production by heterofermentative microbes can be overcome by the deletion of genes responsible for by-product formation and selective expression of enantiospecific lactate dehydrogenase. For example, deletion of *ldhL1*, encoding L-lactate dehydrogenase, in the heterofermentative strain *Lactobacillus plantarum* NCIMB 8826 expressing *xylAB* genes, resulted in the formation of D-LA from agricultural residues with production ranging from 22 g/L to 27 g/L and an optical purity of 99–99.5% through simultaneous saccharification and cofermentation of corn stover and sorghum stalks (Zhang et al. 2016).

Although *E. coli* does not produce LA through the PK pathway, it nevertheless exhibits heterofermentative behavior because of the other organic acids produced aside from LA. Homolactic acid production (51 g/L) from glucose, with a high yield of 97%, was observed in recombinant *E. coli* SZ40 by knocking out the *pflB* (encoding pyruvate formate lyase) and *frdBC* (encoding fumarate reductase) genes to block the production of formate and succinate, respectively (Zhou et al. 2003). High production of D-LA was also observed in the *E. coli* TG114 strain following removal of the *pflB, frd, adhE* (encoding alcohol/aldehyde dehydrogenase), and *ackA* (encoding acetate kinase) genes. Methylglyocal is a precursor for the formation of both D- and L-LA. Additional deletion of *mgsA* (encoding methylglyoxal synthase) to block methylglyoxal synthesis in *E. coli* TG114 resulted in the production of 118 g/L of D-LA and increased the purity from 95% to 99.9% (Grabar et al. 2006).

2.2 Dicarboxylic Acids

Dicarboxylic acids are abundant in nature, given that they are intermediate products of central metabolism in most organisms. They are versatile chemicals, owing to their dual carboxylic groups, and are commonly applied for the production of solvents, polymers, and pharmaceuticals, as well as in the food industries. Among the various dicarboxylic acids, succinate, fumarate, and malate are some of the most commonly used building block chemicals. Since these chemicals are intermediates of the tricarboxylic acid (TCA) cycle, metabolic engineering strategies for these chemicals require critical modification to prevent growth retardation in order to achieve a high production yield.

2.2.1 Malic Acid

Malic acid is a building block for the synthesis of poly(β-malic acid) and its derivatives. Its application has also been extended to the pharmaceutical industry as carriers in drug delivery system (Cammas et al. 1996). Malic acid can be produced via the TCA cycle or through the sequential action of phosphoenol carboxylase and malate dehydrogenase to convert pyruvate to oxaloacetate and malate, respectively (Fig. 3). Heterologous expression of the phosphoenol pyruvate carboxylase gene (*pckA*) from *Mannheimia succiniciproducens* MBEL55E in *E. coli* resulted in the production of 9.25 g/L malic acid with a 0.56 g/g glucose yield and 0.74 $g \cdot L^{-1} \cdot h^{-1}$

productivity by allowing more ATP generation from the conversion of phosphoenolpyruvate (PEP) to oxaloacetate (Moon et al. 2008).

Inactivation of competing pathways has also been demonstrated to be an effective strategy to increase malic acid production. The *E. coli* mutant XZ658 (*ΔfrdBC*, *ΔscfA*, *ΔmaeB*, *ΔfumABC*, *ΔmgsA*, *ΔpoxB*, *ΔldhA*, *ΔackA*, *ΔadhE*, *ΔpflB*) could produce 34 g/L of malic acid in a two-stage cultivation (Zhang et al. 2011).

2.2.2 Fumaric Acid

Fumaric acid is commonly used in the food industry as an acidulant, and serves as a precursor of L-malic acid and L-aspartate. It is also used as a building block of several bio-based chemicals and polymers (Xu et al., 2012) (Fig. 2).

A fumaric acid-producing *E. coli* strain was developed by applying rational metabolic engineering and flux optimization. This aided in determining the appropriate deletion and overexpression strategies for *E. coli* W3110 in order to optimize the PEP carboxylase- and citrate synthase-catalyzed reaction fluxes (Song et al. 2013; Song and Lee 2015). The *E. coli* CWF4NSA (*ΔiclR, ΔfumCAB, ΔptsG, ΔlacIPsdh::Ptrc ΔaspA*) mutant overexpressing the *ppc* (encoding phosphoenolpyruvate carboxylase), *sdhCDAB* (encoding succinate dehydrogenase complex), and *gltA* (encoding citrate synthase) genes was able to produce 35.1 g/L fumaric acid with a yield of 0.49 g/g of glucose (Song and Lee 2015).

2.2.3 Succinic Acid

Succinic acid has become one of the most well-studied and valuable feedstock chemicals in the twenty-first century. It has been used in various applications, including the food and pharmaceutical industries (Ahn et al. 2016; Biddy et al. 2016). Moreover, it can be used as a precursor for the production of different commodity and specialty chemicals that are based on benzene and intermediate petrochemicals such as 1,4-butanediol (BDO), tetrahydrofuran (THF), linear aliphatic esters, and nylon monomers (adipic acid and n-methyl pyrrolidone/2-pyrrolidone) (Ahn et al. 2016) (Fig. 2). Due to its wide range of application and potential as feedstock for petroleum-based chemicals, succinic acid production has attracted much interest and has been recognized as one of the top-12 building-block chemicals (Biddy et al. 2016). Several biotechnology companies and production facilities such as Bioamber, Myriant, Reverdia, and Succinity have already started the industrial production of bio-based succinic acid. The large market and bio-based processing of succinic acid have nearly surpassed those of petroleum-based production (Biddy et al. 2016; Ahn et al. 2016).

The most common natural producers of succinic acid are ruminal bacteria, namely *Actinobacillus succinogenes, Anaerobiospirillum succiniciproducens, Mannheimia succiniciproducens,* and *Basfia succiniciproducens.* These organisms are anaerobic and capnophilic, thereby requiring high concentration of CO_2 for their growth. Other bacteria that can produce succinic acid as one of the main fermentation products include *L. plantarum*, *E. coli*, and *C. glutamicum* (Beauprez et al. 2010; Ahn et al. 2016).

The three naturally existing pathways for succinic acid production are the oxidative and reductive TCA cycles and the glyoxylate cycle (Beauprez et al. 2010; Cheng et al. 2012) (Fig. 3). Because succinic acid is a component of the central carbon metabolism, metabolic engineering should be carefully considered to prevent cell retardation and obtain a high production yield. In silico modeling and simulation employed with recombinant *M. succiniciproducens* has provided a good strategy to determine the genes that can be targeted for modification, overexpression, and deletion. Engineered *M. succiniciproducens* (*ΔldhA, Δpta, ΔackA*) was able to produce 90.68 g/L of succinic acid with an overall yield of 1.15 mol/mol glucose and low by-product formation. This enabled the high recovery of succinic acid (74.65%) with 99.997% purity (Choi et al. 2016).

2.3 Diols

Diols are organic compounds with two hydroxyl groups, which are commonly used as a feedstock for the production of polymers and specialty chemicals (Fig. 2). Among the BDOs, 2,3-Butanediol (2,3-BDO) is the only diol produced through natural microbial systems. Therefore, construction of novel pathways is required to develop strains capable of producing the nonnatural diols such as 1,4-BDO and 1,3-BDO from renewable biomass (Sabra et al. 2015).

2.3.1 2,3-Butanediol

During World War II, the colorless and odorless compound 2,3-BDO was employed for the synthesis of 1,3-butanediene in the production of rubber (Syu 2001). Since then, its application has been extended to the production of plasticizers, solvents, the fuel additive methyl-ethyl-ketone, and as an anti-freeze agent (Celińska and Grajek 2009).

Unlike other BDOs, 2,3-BDO exists naturally as a product of mixed-acid fermentation in species of the genera of *Klebsiella, Enterobacter, Serratia, Paenibacillus,* and *Bacillus* under microaerobic to anaerobic conditions (Ji et al. 2011). The metabolic function of 2,3-BDO is to serve as a stress response against acidic environments by producing neutral compounds that can be reutilized as a carbon and energy source (Ji et al. 2011).

2,3-BDO is naturally produced from pyruvate, which branches out into two possible routes for succinic acid production, as shown in Fig. 3. In brief, the first route involves the conversion of pyruvate to 2,3-BDO via three steps mediated by α-acetolactate synthase, α-acetolactate decarboxylase, and 2,3-BDO dehydrogenase. In the second route, α-acetolactate is converted into acetoin via diacetyl (Ji et al. 2011) (Fig. 3). Microbial fermentation of 2,3-BDO often yields a mixture of its isomeric forms L-(+)−/(2S,3S)-, D-(−)−/(2R,3R)-, and meso-2,3-BDO. Native 2,3-BDO producers can produce any of the isomeric forms depending on the stereospecificities of the BDO dehydrogenases present. The genes responsible for the stereospecific conversion of acetoin to 2,3-BDO were identified in *K. pneumoniae*. Overexpression of the *ardII* gene encoding L-2,3-BDO

dehydrogenase in *K. pneumoniae* (*ΔldhA, Δard*) resulted in the production of 67.5 g/L L-2,3-BDO dehydrogenase in a fed-batch fermentation (Park et al. 2014). *Bacillus licheniformis* MW3 was found to possess two BDO dehydrogenases, namely 2R,3R-BDO dehydrogenase (*gdh*) and meso-2,3-BDO dehydrogenase (*budC*). Deletion of the *budC* gene in *B. licheniformis* MW3 to block meso- and (2S,3S)-BDO resulted in the production of 123.7 g/L of (2R,3R)-BDO with 99.4% purity. Likewise, deletion of the *gdh* gene blocked (2R,3R)-BDO formation and resulted in the production of 90.1 g/L of meso-2,3-BDO with a yield, productivity, and purity of 0.49 g/g glucose, 2.82 $g \cdot L^{-1} \cdot h^{-1}$, and 99.6%, respectively (Ge et al. 2016).

Microorganisms capable of utilizing a wide range of substrates, such as *K. pneumoniae*, can be used for the cost-effective production of 2,3-BDO (Yu et al. 1982; Silveira et al. 1998). For instance, *K. pneumoniae* was found to convert up to 50% of the hydrolyzed wood hemicellulose toward 2,3-BDO production (Saddler et al. 1983). Aside from monomeric sugars, 2,3-BDO was also produced from whey permeate, agricultural wastes, and starch by *K. pneumoniae, Enterobacter aerogenes,* and *B. licheniformis* (Lee and Maddox 1986; Sun et al. 2009; Tsvetanova et al. 2014).

2.3.2 1,4-Butanediol

1,4-Butanediol (1,4-BDO) is an important chemical known for its application as a feedstock for the production of THF and polybutylene terephthalate, which are commonly used in the synthesis of a wide range of specialty chemicals, polymers, and plastics (Sabra et al. 2015; Biddy et al. 2016).

To date, the natural pathways for the in vivo synthesis of 1,4-BDO remain unknown. The highly reduced nature of 1,4-BDO is the main hurdle in developing a thermodynamically feasible pathway for the production of this compound. To overcome this hurdle, a novel biosynthetic pathway was constructed by applying a pathway-identification algorithm and genome-scale metabolic model of *E. coli* (Yim et al. 2011). Using these techniques, two routes were generated by expressing heterologous genes (Fig. 3). The first route involves the conversion of succinic acid to 1,4-BDO through the sequential action of *E. coli* succinyl-CoA synthetase (*sucCD*), *Porphyromonas gingivalis* semi-aldehyde dehydrogenase (*sucD*), *P. gingivalis* 4HB dehydrogenase (*suc4hbd*), *Clostridium beijerinckii* codon-optimized 4-hydroxybutyryl-CoA transferase (*cat2*), and *E. coli* alcohol dehydrogenase (Fig. 3). In the second pathway, instead of succinic acid, the reaction starts with α-ketoglutarate, which is converted to succinyl semi-aldehyde by *Mycobacterium bovis* 2-oxoglutarate decarboxylase (*sucA*). The second route has the advantage of being more thermodynamically favorable and has fewer steps compared to the first route (Fig. 3). Further optimization of 1,4-BDO production was achieved by using various plasmids having different copy number and blocking the competing pathways, which led to the formation of 18 g/L of 1,4-BDO from glucose using either of the two pathways (Yim et al. 2011).

Another 1,4-BDO pathway utilizing xylose was designed and developed in *E. coli*, wherein two modules were engineered: enzymatic and genetic controller modules. The enzymatic module pertains to the heterologous key enzymes xylose

dehydrogenase (*xdh*) and 2-dehydro-3-deoxy-D-xylonate dehydratase (*xylX*) from *Caulobacter crescentus*, and keto acid decarboxylase (*mdlC*) from *Lactococcus lactis*. These enzymes were overexpressed under the autoregulated genetic controller comprising *luxI* and the *Plux* promoter. The N-Acyl homoserine lactone (AHL) produced from the self-regulated catalysis of LuxI served as the inducer of the *Plux* promoter. The engineered *E. coli* strain was able to produce 0.44 g/L of 1,4-BDO with a yield of 0.07 mol/mol of D-xylose. The findings demonstrated that regulation through a quorum-sensing system is more promising than using isopropyl-β-D-thiogalactopyranoside (IPTG), which decreases cell growth and increases the production cost (Liu and Lu 2015).

2.3.3 1,3-Butanediol

1,3-Butanediol (1,3-BDO) is an optically active alcohol that exists in (*S*)- and (*R*)-forms. It is chemically synthesized from petroleum-based chemicals such as 4-hydroxy-2-butanone, resulting in a racemic mixture. (*R*)-1,3-BDO is known for its application as a precursor for polyester plasticizers and azetidone and its derivatives, which are used in the production of antibiotics (Matsuyama et al. 2001; Sabra et al. 2015).

Production of (*R*)-1,3-BDO with high enantiomeric excess (e.e.) from racemic 1,3-BDO was achieved using a recombinant *E. coli* JM109 strain expressing an (*S*)-specific secondary alcohol dehydrogenase (CpSADH) from *Candida parapsilosis* as a whole-cell biocatalyst (Matsuyama et al. 2001). Enantio-selective oxidation of (*S*)-1,3-BDO present in the racemate by the recombinant *E. coli* under optimized fermentation conditions resulted in the production of 72.6 g/L of (*R*)-1,3-BDO with a 95% e.e. and 48.4% mol/mol recovery yield (Yamamoto et al. 2002).

A novel synthetic pathway was designed for the biosynthesis of (*R*)-1,3-BDO from glucose. This pathway involves the conversion of acetyl-CoA through three sequential steps by 3-ketothiolase, NAD(P)H-dependent acetoacetyl-CoA reductase, and alcohol/aldehyde dehydrogenase (Kataoka et al. 2013) (Fig. 3). Heterologous expression of the *phaAB* (encoding 3-ketothiolase and acetoacetyl-CoA reductase) and *bld* (encoding butyraldehyde dehydrogenase) genes from *Ralstonia eutropha* and *Clostridium acetobutylicum,* respectively, in the recombinant *E. coli* led to the production of 9.05 g/L (100.4 mM) (*R*)-1,3-BDO with a 98.5% e.e. (Kataoka et al. 2013). The butyraldehyde dehydrogenase from *C. acetobutylicum* is oxygen-sensitive; thus, it would be expected that cultivation of the recombinant *E. coli* strain in an oxygen-limited environment would be favorable for (*R*)-1,3-BDO formation. In contrast to this expectation, an aerobic condition was found to be more suitable for fermentation with this strain. This is due to the fact that the key intermediate and cofactors acetyl-CoA and NAD(P)H/NADH required for the constructed pathway are mainly supplied by glucose metabolism. Therefore, the fermentation conditions, namely the oxygen supply and pH, were optimized. Under the optimal conditions, the recombinant *E. coli* BW-NK3 strain heterologously expressing the *R. eutropha phaAB* and *C. acetobutylicum bld* genes was able to produce 174.8 mM of (*R*)-1,3-BDO with a yield, productivity, and purity of 0.372 mol/mol glucose, 3.90 mM/h, and 98.6% e.e., respectively, by fed-batch fermentation (Kataoka et al. 2014).

3 Research Needs

For microorganism-based biorefineries to be successful, it is important to develop and design microbial strains that can efficiently convert fermentable sugars from non-food biomass into target chemical products with a high titer, yield, and productivity. Advances in synthetic biology and metabolic engineering strategies of microorganisms will aid in developing tools for the genomic manipulation and in identifying the appropriate strategies for optimizing cell condition and chemical production. It is also vital to develop computational approaches and models to predict the behavior of biological systems and for the reproducibility of microbial cell performance from lab scale to an industrial-scale setting.

4 Conclusion

Biorefineries are expected to grow with the advancement of industrial biotechnology that gives way to the development of efficient microbial cell factories. Development of biorefineries for the production of building-block chemicals from biomasses is a first step in transitioning from our dependence on petroleum to a more sustainable exploitation of renewable resources. Microbial systems developed for high production of building block chemicals can then be used for the production of its derivatives, which are normally produced from petroleum and other novel chemicals, which will create new markets and opportunities for bio-based chemicals.

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Biopetrochemicals via Biocatalysis by Hydrocarbons Microbes and their Enzymes

13

R. Karande, A. Schmid, and K. Buehler

Contents

Abstract

Hydrocarbon-degrading organisms are an important source for industrial relevant reactions. The respective degradation pathways harbor oxidoreductases, an enzyme class catalyzing highly interesting reactions for the production of high value added compounds and fine chemicals. Exploiting these reactions for biocatalysis requires the development of different reaction concepts, as hydrocarbons are often problematic substrates in terms of toxicity and solubility. This chapter will present the development of various reaction concepts for the technical utilization of hydrocarbon degrading organisms and their respective enzymes as biocatalysts.

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1 Introduction

Hydrocarbon (HC hereafter) degradation may be accomplished anaerobically (see Vol. 2, Part 3), as well as aerobically by various bacterial species (Fuchs 2008; Van Hamme et al. 2003; Widdel and Rabus 2001). However, solely oxygen-dependent reactions have been exploited industrially so far. As our possibilities to handle oxygen-sensitive strains and their respective enzymes are expanding, these organisms gain more and more importance and it may be expected that the first applications of such biocatalysts will be reported in the near future.

Microbes mineralizing hydrocarbons have been studied intensively since the 1940s with a focus on metabolic intermediates and their possible benefit for chemical synthesis. The first patent utilizing HC microbes for petrochemical production described the conversion of gaseous C1–C4 hydrocarbons into a mixture of unspecified oxygenated organic compounds by passing them through a trickling bed filter inoculated with a mixed culture of soil microbes originating from oil contaminated sites (Taggart 1946). Despite the recognition that microbes are able to produce petrochemicals from petroleum in this early patent, it took 20 years before the biochemistry of hydrocarbon degradation was actually understood. This set the stage to produce fine chemicals in a controlled manner utilizing hydrocarbon degrading microbes. The main enzymes active in hydrocarbon degradation pathways are oxidoreductases. Many of these enzymes can be applied for the enzymatic synthesis of fine chemicals from petroleum constituents. For bioremediation purposes, wildtype hydrocarbon degrading organisms can be applied converting their substrates to biomass and CO_2. However, in order to utilize such reactions for biocatalysis, it was necessary to develop solutions preventing the product of choice from being degraded. In the following, we will discuss exemplarily organisms and the responsible enzymes in the context of the development of different technologies and approaches in biotechnology, which opened promising opportunities for establishing efficient and competitive processes based on biopetrochemicals (Table 1). The chosen reactions represent, to the best of our knowledge, the first examples where these methods have been applied.

2 Utilizing Wildtype Organisms for Productive Biocatalysis

This chapter highlights examples of transforming liquid HC to value added compounds. Not included are examples from strains converting gaseous compounds. For such interested readers may refer to (Shennan 2006; Conrado and Gonzalez 2014). A pioneer strain for biopetrochemical-based catalysis is *Pseudomonas putida* GPo1 (former *P. oleovorans* TFL-1 L (van Beilen et al. 2001)). This organism metabolizes medium chain length linear alkanes (C_6–C_{12}) and 1-octene as sole source of carbon and energy and is able to epoxidize or hydroxylate a number of

Table 1 Reactions involving biopetrochemicals and hydrocarbon degrading organisms

#	Technologies	Catalyst		Biotransformation-substrate	Product	Final product ($g\ L^{-1}$)	Reaction time h (days)	References
		Enzyme(s)	Strain					
1	Cometabolism; 2-LP	AlkBGT	Growing *P. putida* GPo1	1,7-octadiene	7,8-epoxy-1-octene; 1,2–7,8-diepoxyoctane	7.46 g L^{-1}	72	Schwartz and McCoy (1977)
2	Enzyme kinetics; 2-LP	AlkBGT	Growing *P. putida* GPo1	1-octene	1,2-epoxy-octane	11 g L^{-1}	169	de Smet et al. (1981); (1983)
3	Cometabolism; enzyme kinetics; 2-LP	AmoAB	*Rhodococcus corallinus* B276 (resting & growing)	1-alkenes (C_3, C_4, C_{13} – C_{18})	Corresponding 1,2-epoxides	–	–	Furuhashi et al. (1981); Furuhashi (1986)
4	Cometabolism	XylMA BADH BZDH	Growing *P. putida* mt-2	2,5-dimethyl-pyrazine	5-methyl-pyrazin-2-carboxylicacid	24 g L^{-1}	54	Kiener (1992)
5	Recombinant system; engineered strain; 2-LP	AlkBGT	Growing *P. putida* pPS8141	Octane	1-octanol	5 g L_{org}^{-1}	24	Bosetti et al. (1992)
6	Recombinant system; 2-LP	AlkBGT AlkJ AlkH	Growing *E. coli* HB101 pGEc47	Octane	Octanoic acid	6.6 gL_{aq}^{-1a} 1.9 gL_{aq}^{-1c}	5[a,b]	Wubbolts et al. 1996; Rothen et al. (1998)
7	Recombinant system	AlkBGT AlkJ AlkL ω-TA AlaD	Resting cells *E. coli* JM101	Dodecanoic acid methyl ester (DAME)	12-aminododecanoic acid methyl ester (ADAME)	0.1 gL_{aq}^{-1}	1	Ladkau et al. (2016)
8	Engineered strain; 2-LP	todABC1C2 &. todD	Growing *P. putida* MC2	Toluene	3-methyl-catechol	6 g L_{org}^{-1}	55	Hüsken et al. (2001a); Hüsken et al. (2002)

(*continued*)

Table 1 (continued)

#	Technologies	Catalyst		Biotransformation-substrate	Product	Final product (g L^{-1})	Reaction time h (days)	References
		Enzyme(s)	Strain					
9	Recombinant; fed-batch biotransformation	CHMO	Growing *E. coli* TOP10 pQR239	Ketone[c]	Lactone[c]	3.8 gL^{-1}	6	Doig et al. (2002)
10	Biofilm catalysis; engineered strain; 2-LP	StyAB	Biofilm of *Pseudomonas* sp. strain VLB120ΔC	Styrene	Styrene oxide	60 g L_{org}^{-1}	(55)[d]	Gross et al. (2007)
11	Biofilm catalysis; engineered strain; 2-LP	CHX	Biofilm of *Pseudomonas* sp. strain VLB120	Cyclohexane	Cyclohexanol	0.4 g L_{aq}^{-1}[e]	(16)[d]	Karande et al. (2016)
12	Biofilm catalysis; engineered strain; 2-LP	CymA CymB CymC	*P. putida* GS1	(R)-(+)-limonene	(R)-(+)-perillic acid	0.87 g L_{aq}^{-1}[e]	(12)[d]	Willrodt et al. (2016)

AlkBGT alkane monooxygenase; *AmoAB* alkene monooxygenase; *XylMA* xylene monooxygenase; *BADH* benzyl alcohol dehydrogenase; *BZDH* benzyl aldehyde dehydrogenase; *AlkJ* alcohol dehydrogenase; *AlkH* aldehyde dehydrogenase; *todABC1C2* toluene deoxygenase; *todD* toluene dehydrogenase; *CHMO* cyclohexanone monooxygenase; *StyAB* styrene monooxygenase; *AlkJ* alkane uptake facilitator; *ω-TA* ω-transaminase; *AlaD* alanine dehydrogenase; *CymA p*-cumene monooxygenase; *CymB p*-cumic alcohol dehydrogenase; *CymC p*-cumic aldehyde dehydrogenase

[a]In fed-batch culture

[b]After induction

[c]Conversion of bicycle [3.2.0]oct-6-en-2-one to (-)-(1S, 5R)-2-oxabicyclo[3.3.0]oct-6-en-2- one and (-)-(1R, 5S)-3-oxabicyclo[3.3.0]oct-6-en-3-one

[d]Continuous process, actively terminated

[e]Average product concentration through out reaction time, measured at reactor outlet

other hydrocarbons, making it highly interesting for organic synthesis and selective C-H oxyfunctionalizations. Its economic potential and substrate range have been reviewed in Witholt et al. (1990) and Mathys et al. (1999). The biochemistry and genetics of the enzyme system responsible for hydrocarbon degradation have been investigated in detail and inspired many studies on hydrocarbon hydroxylation addressing the properties of the respective enzymes (Lode and Coon 1971; McKenna and Coon 1970; Peterson et al. 1967; Ruettinger et al. 1974), the bioconversion of aliphatic compounds (de Smet et al. 1981; Witholt et al. 1990), the organization of the respective genes (van Beilen et al. 1992b), and the expression of the genes in recombinant hosts (McKenna and Coon 1970; Staijen et al. 1997; van Beilen et al. 1992a, 1992b; Witholt et al. 1990). Conversion of hydrocarbons by *P. putida* GPo1 is initialized by the action of an integral cytoplasmic three component membrane alkane monooxygenase (AlkBGT), which incorporates one atom of molecular oxygen into the substrate yielding *n*-alkanol, while the second oxygen atom is reduced to water with electrons derived from NADH (Ruettinger et al. 1977). Electron transport is accomplished by two redox enzymes, namely an iron-sulfur rubredoxin (AlkG) (Peterson and Coon 1968) and a flavoprotein rubredoxin reductase (AlkT) (Ueda and Coon 1972). In course of the degradation pathway, *n*-alkanol is oxidized to the corresponding aldehyde and acid by an alcohol dehydrogenase (AlkJ) and an aldehyde dehydrogenase (AlkH), respectively, before it is coupled to acetylCoA and channeled into the β-oxidation cycle of the central carbon metabolism.

Schwartz and coworkers utilized wildtype *P. putida* GPo1 as a catalyst for the epoxidation of 1,7-octadiene to 7,8-epoxy-1-octene exploiting the cometabolic conversion of the biotransformation substrate 1,7-octadiene, while the organism was growing on *n*-octane as sole source of carbon and energy (Fig. 1; Table 1/#1) (Schwartz and McCoy 1977). A bulk phase of cyclohexane was added to prevent toxification of the biocatalyst.

This example combines two important concepts in biocatalysis, namely cometabolism and the two liquid phase concept, which are utilized in many reactions involving biopetrochemicals. Cometabolism refers to the conversion of a nonnatural substrate (e.g., 1,7-octadiene), which has a similar structure as the native one (e.g., octane) and thus will be attacked by the key enzymes of the respective degradation pathways. However, as the enzymes of central carbon metabolism are in general more specific (have a narrow substrate spectrum) and are often not able to metabolize substrate analogs, a so-called "dead end" product is produced, which will accumulate in the reaction broth and in the ideal case represents the product of choice. In order to

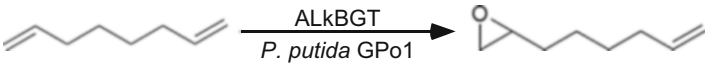

Fig. 1 Cometabolic conversion of 1,7-octadiene to 7,8-epoxy-1-octene using wildtype *Pseudomonas putida* GPo1 growing on 1-octane in a two phase system (Schwartz and McCoy 1977). *AlkBGT* Alkane Monooxygenase

keep the central carbon metabolism running and induce the respective genes needed for biotransformation, organisms concurrently need the native substrate as a source of carbon and energy in addition to the biotransformation substrate.

The two liquid phase concept is based on the addition of an organic solvent (here cyclohexane) to an aqueous system (in general growth media). It serves as a substrate reservoir and product sink depending on the partition coefficient of the solvent, if the educts are apolar. The two liquid phase concept is often applied in reactions involving hydrocarbon transformations, as these compounds are hardly soluble in water and/or rather toxic to the respective biocatalysts (see Vol. 2, Part 7, Cellular Ecophysiology Problems of Hydrophobicity and Bioavailability), which makes it necessary to keep their concentration in the aqueous phase as low as possible. On the other hand, the application of a toxic organic phase introduces significant stress on the organisms. Solvent stress is directly leading to an increase in the maintenance energy of the cell and thus impairs other energy depending reactions, like protein synthesis and biotransformations (Blank et al. 2008). Organic solvents tend to accumulate in the bacterial cytoplasmic membrane. At a log $P_{o/w}$ (partition coefficient between octanol and water) below 4.0, they will cause impairment of the cytoplasmic membrane function and its expansion resulting in leakage of cellular metabolic products (Sikkema et al. 1992). Microorganisms developed different ways to cope with solvent stress, which gave rise to a highly interesting field of research investigating solvent tolerance in microorganisms (Ramos et al. 2002).

Applying the AlkBGT system in *P. putida* GPo1 in a two liquid phase system, synthesis of 1,2-epoxyoctane from 1-octene was accomplished to a final product concentration of 11 g L^{-1} (Fig. 2; Table 1/#2). In this example, 1-octene served as biotransformation substrate as well as sole source of carbon and energy (de Smet et al. 1981; Schwartz 1973; Schwartz and McCoy 1973).

It was possible to utilize this reaction for preparative biocatalysis because the kinetics of the respective enzymes accounted for the accretion of 1,2-epoxyoctane. The initial oxygenation yielding the epoxide is not the rate limiting step in the degradation pathway and 1,2-epoxyoctane accumulates as long as 1-octene is present in excess in the culture broth. Exploiting enzyme kinetics for biotransformation is a straight forward and simple way to make such reactions usable for biocatalysis. No strain or protein engineering are required and the number of components present in the reaction broth is kept as low as possible, which is beneficial for the down-stream processing.

Successful examples for processes running at an industrial scale exploiting enzyme kinetics, cometabolism and/or the two liquid phase concept, respectively,

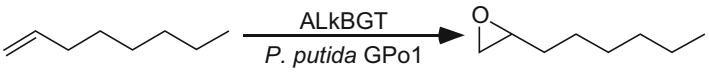

Fig. 2 Utilizing the kinetics of the hydrocarbon degradation enzymes of wildtype *P. putida* GPo1, 1,2-epoxyoctane is accumulated from 1-octene, which serves as biotransformation and growth substrate, in the presence of a second organic phase (de Smet et al. 1981; Schwartz 1973; Schwartz and McCoy 1973). *AlkBGT* alkane monooxygenase

are the following two reactions. The conversion of various alkenes into the respective epoxides (Fig. 3; Table 1/#3) is accomplished by utilizing *Rhodococcus corallinus* (formerly *Nocardia corallina*) B276 either in a two liquid phase or single aqueous phase set-up, depending on the educts. This process was developed at Nippon Mining Co. (now Nippon Mining and Metal Co.) and is running there at industrial scale (Furuhashi et al. 1981; Furuhashi 1986).

Various alkenes and some alkanes are converted by this strain by the action of alkene monooxygenase (AmoAB): short-chain gaseous compounds (C_3–C_5), short-chain liquid compounds (C_6–C_{12}), and long-chain olefins ($C_{>12}$). In contrast to the abovementioned examples, these processes employed glucose as the main carbon source for growth, which was possible as the respective genes encoding the epoxidation activity were constitutively expressed, while in *P. putida* GPo1 they are induced in the presence of the respective hydrocarbon. AmoAB is a two-component enzyme belonging to the class of nonheme diiron monooxygenases. Similar to AlkBGT, the enzyme system also comprises a redoxchain for electron transfer consisting out of an iron-sulfur flavoprotein and a coupling protein, which has a regulatory function, but does not contain any prosthetic group (Miura and Dalton 1995).

Cometabolism is exploited for the three step oxidation of 2,5-dimethylpyrazine to 5-methylpyrazine-2-carboxylic acid (MPCA) by the wildtype strain *Pseudomonas putida* mt-2 in a process running at Lonza (Fig. 4; Table 1/#4).

This strain is able to grow on toluene and *m*- and *p*-xylene as the sole source of carbon and energy by means of the xylene degradation pathway, which is well described in literature (Abril et al. 1989; Gibson and Subramanian 1984; Harayama et al. 1989). For the conversion of 2,5-dimethylpyrazine to 5-methylpyrazine-2-carboxylic acid, all three enzyme activities of the upper xylene degradation pathway are needed, namely xylene monooxygenase (XylMA) as the key enzyme in the pathway, benzyl alcohol dehydrogenase (BADH) and benzaldehyde dehydrogenase (BZDH) (Worsey and Williams 1975). XylMA is a two-component enzyme with XylM being the hydroxylase component, while XylA is functioning as an electron

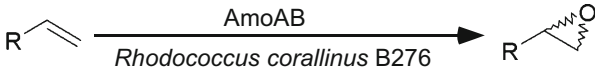

Fig. 3 Conversion of various 1-alkenes to the corresponding 1,2-epoxides using wildtype *Rhodococcus corallinus* B276 either in growing or in resting state (Furuhashi et al. 1981). *AmoAB* alkene monooxygenase

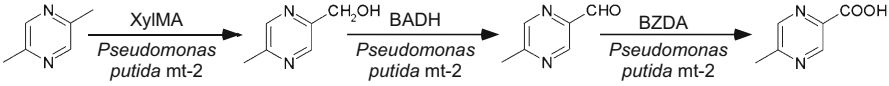

Fig. 4 Conversion of 2,5-methylpyrazine to 5-methylpyrazine-2-carboxylic acid using wildtype *Pseudomonas putida* mt-2 growing on *p*-xylene (Kiener 1992). *XylMA* Xylene monooxygenase, *BADH* benzyl alcohol dehydrogenase, *BZDH* benzyl aldehyde dehydrogenase

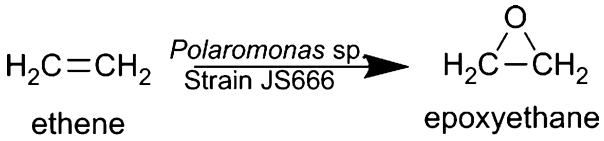

Fig. 5 Conversion of ethene to epoxyethane utilizing *Polaromonas* sp. Strain JS666 in a resting state. Specific activities of approximately 3 U (given in nmol/min/mg of protein) have been reached in a nonoptimized reaction set-up (Coleman et al. 2002)

shuttle transferring electrons from NADH to the hydroxylase (Harayama et al. 1989). 2,5-Dimethylpyrazine is converted cometabolically, while the strain is growing on *p*-xylene as carbon and energy source. The respective process utilizes growing cells, feeding a mixture of *p*-xylene 75% v/v (native substrate) and 2,5-dimethylpyrazine 25% v/v (substrate analogon). At a 1000 L scale a final product concentration of 24 g MPCA L^{-1} was achieved, corresponding to a yield of 95% (Kiener 1992). In addition, this strain is also capable of oxidizing various methylated heteroaromatic five- and six-member rings to the corresponding carboxylic acids.

A recent example for highly interesting HC degrading microbes in the context of productive biocatalysis is *Polaromonas* sp. Strain JS666. This strain was isolated from granular-activated sludge of a pump-and-treat plant (Dortmund, Germany) and is capable of growing on *cis*-1,2-dichloroethane (cDCE) as sole carbon and energy source under aerobic conditions (Coleman et al. 2002), as well as on heptane, octane, cyclohexanol, and cyclohexane carboxylate. Surprisingly, resting cells of cDCE grown JS666 were able to oxidize ethene stoichiometrically to epoxyethane although the compound could not be utilized as a carbon source (Fig. 5). Although this strain itself was so far not used for biocatalysis directly, it harbors highly interesting enzymes. Sequencing its genome unrevealed gene clusters encoding for enzymes involved in haloalkane, haloalkanoate, *n*-alkane, alicyclic acid, cyclic alcohol, and aromatic catabolism (Mattes et al. 2008). These have been in part utilized in recombinant host strains like *E. coli* as outlined in the section below.

Another fairly new representative in the field of HC degraders is *Acidovorax* sp. CHX100, a strain noted for its excellent growth rates on cyclic alkanes (C_5-C_8) (Salamanca and Engesser 2014). It was applied in a biotrickling filter for the removal of cyclohexane from gaseous emissions (Salamanca et al. 2017). The fairly high growth rates of 0.199 h^{-1}, 0.075 h^{-1}, and 0.201 h^{-1} on cyclohexane, cyclohexanol, and cyclohexanone, respectively, indicate highly active enzymes for these conversions present in this strain (Salamanca and Engesser 2014). Degradation of cyclohexane involves the interesting compound caprolactone, which is further metabolized in the natural host (Fig. 6). The key-enzyme catalyzing the hydroxylation of the alkane to the corresponding alcohol has been identified as a CypP450 monooxygenase. It was isolated and applied in a recombinant bacterial host system (see below). When growing on cyclohexane, *Acidovorax* sp. CHX100 is able to convert chlorocyclohexane to *cis*-and *trans*-4-chlorocyclohexanol, without further degradation of products (Fig. 7). Apart from this, the wildtype strain was so far not

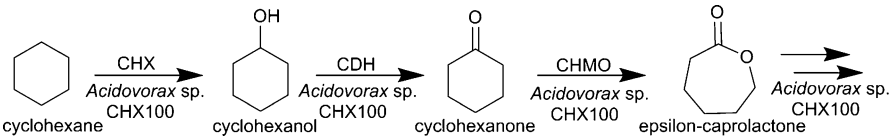

Fig. 6 Reaction pathway of *Acidovorax* sp. CHX100 starting from the cyclic alkane cyclohexane to the interesting intermediate caprolactone (Salamanca et al. 2017). *CHX* CypP450 monooxygenase, *CDH* cyclohexanol dehydrogenase, *CHMO* cyclohexanone monooxygenase

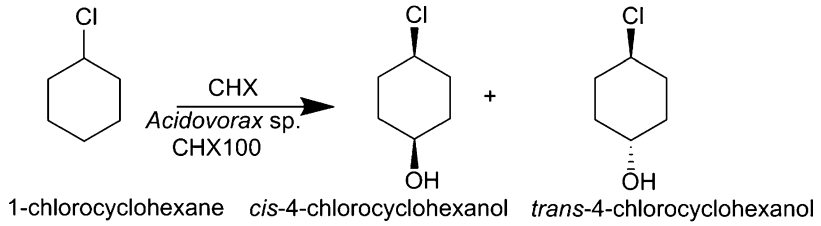

Fig. 7 Cometabolic conversion of chlorocyclohexane to *cis*- and *trans*-4-chlorocyclohexanol utilizing *Acidovorax* sp. CHX100 growing on cyclohexane (Salamanca and Engesser 2014). *CHX* cyclohexane monooxygenase

further developed as a production host, although it for sure holds a promising potential in this respect.

Other interesting strains with future perspectives for chemical synthesis are *Arhodomonas* sp. Strain Seminole (Dalvi et al. 2014) and *Rhodococcus* sp. OCT 10 DSM 45596^T (Dobslaw and Engesser 2012). *Rhodococcus* sp. OCT 10 DSM 45596^T is the first natural isolate described to be able to mineralize 2-chlorotoluene and 2-bromotoluene, while 2-fluorotoluene was transformed in a cometabolic way. *Arhodomonas* sp. Strain Seminole is a strain prospering in hypersaline environments (2–2.5 M NaCl). It was isolated from soil obtained from an oil production facility and is able to degrade various aromatic HC like benzene, toluene, phenol, and others as the sole sources of carbon and energy. Unlike their nonhalophilic counterparts, enzymes from such organisms possess unique features and maintain catalytic activity and stability at high salinity.

3 Utilizing Molecular Engineering Tools for Biocatalysis

Utilizing wildtype strains for biotransformations quickly reaches its limits in terms of productivity, especially when the respective biotransformation substrates at the same time are consumed as carbon and energy source by the microbe. With the expanding development of gene technologies, research in biocatalysis focused on applying recombinant systems or engineered strains using mostly *E. coli* or *Pseudomonas*, rather than wildtype organisms.

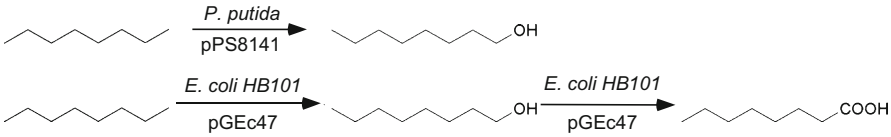

Fig. 8 Conversion of octane to octanol and octanoic acid, respectively, utilizing recombinant hosts in a two-liquid-phase system. *AlkBGT* alkane monooxygenase, *AlkJ* alkanol dehydrogenase, *AlkH* aldehyde dehydrogenase

Applying an engineered, recombinant host strain *P. putida* PpS8141 encoding the alkane hydroxylase genes (*alkBGT*) from *P. putida* GPo1 on a plasmid, production of alkanols from C_7–C_{11} alkanes was achieved (Fig. 8; Table 1/#5) (Bosetti et al. 1992).

P. putida PpS8141 is able to convert alkanes added in a bulk organic phase to the respective terminal alcohols. However, it turned out that this strain was only able to grow in the presence of the organic phase, if pyruvate was added as an additional carbon source, indicating a significant amount of stress introduced onto the organism by the apolar phase. Wildtype *P. putida* did not accumulate any of the respective alcohols, as the hydroxylation of the alkanes is the rate determining step in the degradation of such hydrocarbons. Therefore, the strain was engineered towards alcohol dehydrogenase deficiency, in order to accumulate the alcoholic metabolic intermediates in the organic phase.

E. coli strains as hosts for biotransformations are still fancied over *Pseudomonas* species because there is much more experience with this organism, regarding pathway engineering and general handling. However, if it comes to the conversion of apolar substances like hydrocarbons, where the introduction of a second liquid organic phase might be required, *E. coli* generally experiences more problems to adapt to the respective solvent as compared to the Pseudomonads. Although it was possible to produce octanoic acid from octane using recombinant *E. coli* HB101 pGEc47 in a two phase system (Fig. 8; Table 1/#6), growth depended on the supply of extra amino acids in rather high concentrations in addition to the glucose during the fed-batch to promote growth of the biocatalyst in the presence of the solvent (Wubbolts et al. 1996).

Recently, cascade reactions coupling the very same oxygenase (AlkBGT) from *P. putida* GPo1 and a transaminase from *Chromobacterium violaceum* in vivo have been successfully demonstrated in *E. coli* BL21(DE3) (Fig. 9; Table 1/#7). Through terminal alcohol and aldehyde transformation of the renewable dodecanoic acid methyl ester (DAME) from palm kernel oil, the aminododecanoic acid methyl ester (ADAME) has been produced (Ladkau et al. 2016). ADAME is a valuable monomer for the high-performance polymer Nylon 12. One of the key-limitations in biocatalyst performance was the insufficient transport of the substrate DAME across the cell membrane due to its hydrophobicity and bulkiness. Thus, a membrane spanning porin Alk L also originating from *P. putida* GPo1 was introduced into the outer membrane of *E. coli* host strains. This significantly facilitated substrate uptake and intracellular substrate availability and thus enhanced oxygenation and transamination activities by 8.3 and 7.6 fold, respectively. In addition the intracellular alanine

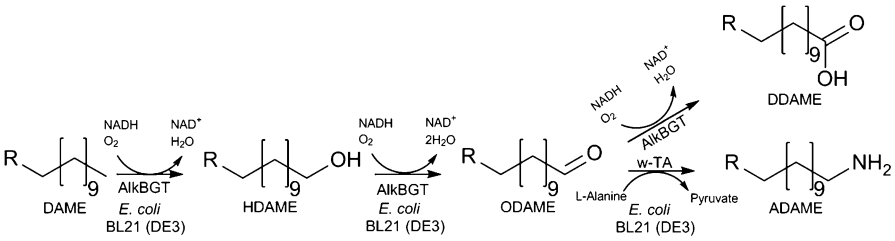

Fig. 9 Orthologous pathway in *E. coli* BL21 (DE3) for the production of ω-aminododecanoic acid methyl ester from renewable dodecanoic acid methyl ester utilizing enzymes from *P. putida* GPO1. Abbreviations: *AlkBGT* alkane monooxygenase, *AlkJ* alkanol dehydrogenase, *AlkL* alkane uptake facilitator, *ω-TA* ω-transaminase, *AlaD* alanine dehydrogenase (Ladkau et al. 2016)

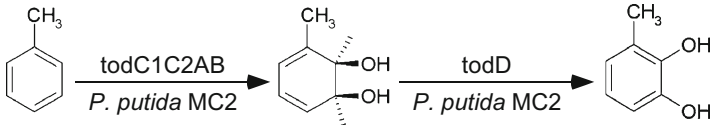

Fig. 10 Conversion of toluene to 3-methylcatechol utilizing the first two reactions of the toluene degradation pathway in *Pseudomonas putida* MC2. *todABC1C2* toluene dioxygenase, *todD* toluene dihydrodiol dehydrogenase

supply necessary for ensuring sufficient transaminase activity was optimized by coupling this reaction via a heterologous alanine dehydrogenase to the central metabolite pyruvate. Finally, a respiratory chain-linked alkanol dehydrogenase (AlkJ) was introduced to increase pathway flux and minimize overoxidation to the respective carboxylic acid. This process was evaluated on a pilot plant scale by Evonik Industries in Slovenska Lupca, as an alternative to petroleum-based laurin lactam production (Press release EVONIK, 2*013, www.corporate.evonik.de/en/media/search*).

An engineered variant of *Pseudomonas putida* F1 was used for the conversion of toluene to 3-methylcatechol (Fig. 10; Table 1/#8). *P. putida* F1 is able to utilize toluene as a sole source of carbon and energy. The initial reaction in the toluene degradation pathway is accomplished by a dioxygenase coupled to a redox chain, which is similar to the alkane hydroxylase system (Zylstra et al. 1988). The terminal dioxygenase is supplied with electrons from NADH via a flavoprotein reductase (Subramanian et al. 1981) and an iron-sulfur ferredoxin (Subramanian et al. 1985), thus catalyzing the incorporation of both atoms of molecular oxygen into toluene, forming a *cis*-toluene dihydrodiol (Yeh et al. 1977). This is subsequently oxidized to 3-methylcatechol by an NAD^+ dependent dihydrodiol-dehydrogenase (Rogers and Gibson 1977) and channeled via the *metha*-cleavage pathway into the central carbon metabolism (Zylstra and Gibson 1989).

The engineered variant *P. putida* MC2 displayed no ring cleaving dioxygenase activity anymore and thus the product 3-methylcatechol will accumulate. In order to enhance conversion rates, multiple copies of *todC1C2BAD*, encoding for the toluene dioxygenase and toluene dihydrodiol dehydrogenase, respectively, have been

integrated into the genome of the biocatalyst (Hüsken et al. 2001a). *P. putida* MC2 now displayed a fourfold increase in 3-methylcatechol production. However, in a single aqueous phase batch approach, only 10 mM of 3-methylcatechol could be achieved due to toxification of the biocatalyst by the product. Higher product yields of 25 mM were gained by introducing octanol as a second organic phase into the system. Remarkably, the biocatalyst was able to cope with the organic phase without being preadapted, although the conversion was rather slow, taking approximately 50 h, before the final product concentration was reached. In order to keep the lag phase rather short, LB medium was used for the 2-liquid phase set-up. Nevertheless it was reported that *P. putida* MC2 also grew on minimal medium in the presence of a second organic phase and produce comparable amounts of 3-methylcatechol (Hüsken et al. 2001b).

The second liquid phase concept is one possibility to circumvent product inhibition or toxification of the biocatalyst. However, if there is no appropriate solvent for the involved educts available or the host strain is not able to cope with the solvent stress, other solutions are necessary. A simple approach to prevent biocatalyst inhibition by the biotransformation substrate is the control of the feed, keeping the substrate concentration below the *ki*. This has been successfully applied to a reaction converting cyclic ketones to the respective lactones by the action of a Baeyer-Villiger monooxygenase (Fig. 11; Table 1/#9). This enzyme was originally derived from *Acinetobacter calcoaceticus*, a hydrocarbon degrading strain well known for its ability to excrete biosurfactants, and thus highly interesting in the field of bioremediation and microbial enhanced oil recovery (Banat et al. 2000; Rosenberg et al. 1988).

Acinetobacter calcoaceticus is able to convert cyclic ketones to the corresponding lactones by the action of cyclohexanone monooxygenase, a one component NAD(P)H dependent flavoenzyme. As it is considered an opportunistic pathogen (safety class 2), it is not very attractive as a biocatalyst for industrial applications. Cyclohexanone monooxygenase was therefore heterologously expressed in *E. coli* TOP10 [pQR239], which had the advantage of a 25-fold higher enzyme expression. In addition, no lactone hydrolase was present in this host and thus the product was not further degraded. *E. coli* TOP10 [pQR239] was strongly inhibited at substrate concentrations >0.4 g L^{-1} and maximal conversion rates occurred at a ketone concentration of 0.2 g L^{-1} (feed rate 0.6–1.1 g L^{-1} h^{-1}) (Doig et al. 2002, 2003).

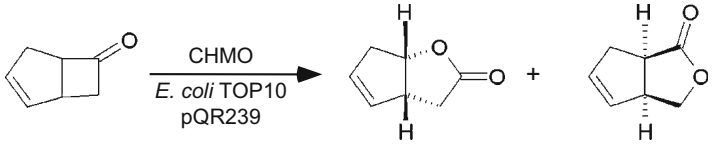

Fig. 11 Regiodivergent oxidation of bicyclo[3.2.0]hept-2-en-6-one to (−)-(1S,5R)-2-oxabicyclo [3.3.0]-oct-6-en-3-one and (−)-(1R,5S)-3-oxabicyclo[3.3.0]-oct-6-en-2-one utilizing cyclohexanone monooxygenase (*CHMO*) from *Acinetobacter calcoaceticus* recombinantly expressed in *E. coli* TOP10 [pQR239]

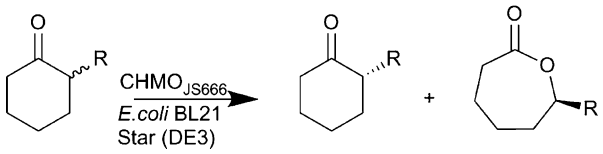

Fig. 12 Kinetic resolution of 2-substituted cyclic ketones transformed by cyclohexanone monooxygenase ($CHMO_{JS666}$) from *Polaromonas* sp. strain JS666 expressed in recombinant *E. coli*

A maximum product concentration of 4.5 g L^{-1} at a 200 L scale was achieved with this approach (Baldwin et al. 2008). Higher product yields were not possible due to product inhibition of CHMO (Doig et al. 2002, 2003). To overcome this problem, a couple of adsorbent resins have been investigated for in situ product removal (ISPR). This approach is analogous to the 2-liquid organic phase concept. The resin is added directly into the reaction broth, for substrate feed and product removal (in situ SFPR) (Hilker et al. 2004, 2005; Simpson et al. 2001). The product concentration was significantly enhanced to 20 g L^{-1} at a 50 L^{-1} scale using this technique.

As introduced above, *Polaromonas* sp. strain JS666 is so far the only known organism able to metabolize 1,2,-dichloroethane (cDCE). One of the key enzymes involved in the degradation of cDCE is a cyclohexanone monooxygenase (CHMO) which was recombinantly expressed in *E.coli* BL21 Star (DE3) (Alexander et al. 2012). It shares 62% amino acid identity with the CHMO from *Acinetobacter calcoaceticus* described above. Employing resting cell experiments, 32 ketone substrates have been evaluated as possible substrate for this enzyme. The performance of the biocatalyst was comparable to other CHMOs from other organisms, with an outstanding stereodiscrimination of 2-substituted cyclic ketones with an ee >99% ($E > 200$) (Fig. 12).

Subcloning of $CHMO_{JS666}$ also allowed investigating its role in cDCE degradation a bit more closely. So far it was hypothesized that it is responsible for the cometabolic cDCE degradation, resulting in accumulation of the toxic cDCE epoxide (2,3-dichlorooxirane). Surprisingly, no activity of $CHMO_{JS666}$ with sDCE was observed in the recombinant *E. coli* host strain, which contradicts former discussions (Alexander et al. 2012). Eventually, it could be shown that the initial step in the cDCE degradation is catalyzed by a P450 monooxygenase (Nishino et al. 2013). This enzyme belonging to the CYP153A family transforms cDCE to 2,2-dichloroacetaldehyde and an additional undefined metabolite. This work showed that cDCE monooxygenase transformed cDCE mainly to dichloroacetaldehyde rather than to epoxide (Fig. 13). In a comparative in vitro study based on CYP153A from *Polaromonas* sp., strain JS666 and CYP153A16 originating from *Mycobacterium marinum* have been evaluated. Both monooxygenases have been recombinant expressed in *E. coli,* purified, and reconstituted with putidaredoxin (CamA) and putidaredoxin reductase (CamB) to supply the necessary redox equivalents (Scheps et al. 2011). CYP153A exhibited higher terminal hydroxylase activity towards C_5 to C_7 alkanes and thus produced larger amounts of primary alcohols from alkanes then CYP153A16. In addition this enzyme showed diterminal hydroxylase

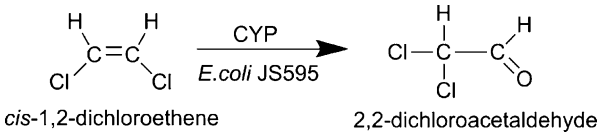

Fig. 13 Conversion of *cis*-1,2-dichloroethene to 2,2-dichloroacetaldehyde utilizing recombinant *E.coli* JS595 (Nishino et al. 2013)

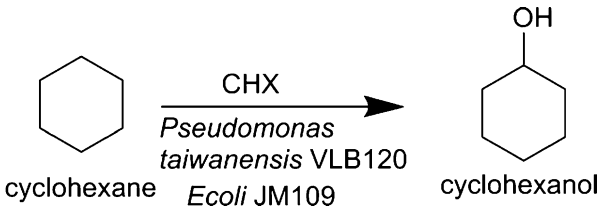

Fig. 14 Conversion of cyclohexane to cyclohexanol utilizing recombinant *Pseudomonas taiwanensis* VLB120 or *E.coli* JM109 (Karande et al. 2016; Salamanca et al. 2015). *CHX* cyclohexane monooxygenase

activity towards octane and nonane and formed 1,8-octanediol and 1,9-nonanediol, respectively. It was able to oxidize 1-octanol and 1-nonanol to the corresponding α,ω diol products; however, especially for the longer chained substrates further oxidation products emerged like aldehydes and fatty acids.

For *Acidovorax* sp. CHX100 not so much work on its enzymatic potential was conducted so far. As described above, it is able to grow on C_5-C_8 cycloalkanes as a sole source of carbon and energy with remarkably high growth rates (Salamanca and Engesser 2014). Also in this strain, a Cyp450 monooxygenase is responsible for initiating the alkane degradation by transforming it to the corresponding alcohol. To utilize this reaction for biotechnological purposes, the respective genes have been cloned into *E. coli*, together with ferredoxin reductase and ferredoxin completing the necessary redox chain (Salamanca et al. 2015). In comparison to other cycloalkanes, the highest amount of product was observed for the transformation of cyclohexane to cyclohexanol (985 μg mL^{-1}). In addition, cytochrome P450 genes were expressed in recombinant *Pseudomonas taiwanensis* VLB120 which allowed initial resting cell activities of 20 U g_{CDW}^{-1} (Karande et al. 2016). This initial activity corresponds to the turnover number (mol product (mol enzyme s)$^{-1}$) of 4 s^{-1} (Fig. 14).

4 Biofilms in Biocatalysis

A rather new approach in fine chemical production is the utilization of biofilms for biocatalysis. This concept exploits the robustness of biofilm organisms against various types of bactericides, including organic solvents and the self-immobilization

of the microbes for long-term processes (Branda et al. 2005; Li et al. 2006). Biofilms are microbial communities, which settle at the interphase of solid to liquid media and actively excrete extrapolymeric substances, which protect and strongly influence the shape of the biofilm. This example considers the production of (*S*)-styrene oxide by an engineered variant of *Pseudomonas* sp. Strain VLB120 growing as a biofilm in a tubular reactor (Fig. 15; Table 1/#10) (Gross et al. 2007). This strain consumes styrene as sole source of carbon and energy, via the enzymes of the styrene degradation pathway, well described in (Panke et al. 1998). The key enzyme is the highly enantiospecific two-component styrene monooxygenase (StyAB), StyA being the monooxygenase, while StyB is an NADH: FAD oxidoreductase necessary for delivering electrons for the epoxidation reaction (Otto et al. 2004).

The engineered strain *Pseudomonas* sp. strain VLB120ΔC (Park et al. 2007) lacks the styrene oxide isomerase activity and thus (*S*)-styrene oxide is accumulating in the presence of another C-source, which can be used for growth. This mutant was grown on glucose in the presence of a second phase of styrene and a productivity of 16 g L^{-1} d^{-1} could be achieved (Gross et al. 2007). Classical whole cell approaches utilizing the same strain report 46 g L_{aq}^{-1} d^{-1} (continuous) and 109 g L_{aq}^{-1} d^{-1} (fed-batch), respectively (Park et al. 2007).

In order to solve the severe mass transfer limitation regarding oxygen and substrate, and thus increase productivities, a novel reactor concept based on an aqueous-air segmented flow biofilm membrane reactor (Karande et al. 2014) was explored for several reactions (Fig.15b). Utilizing this reactor setup increased the productivity of the above-described styrene transformation to 46 g $L_{aq}^{-1}d^{-1}$ in the biofilm system. Employing a variant of *P. taiwanensis* VLB120 which was engineered for better biofilm attachment in this segmented flow system increased productivities even further to 140 g L_{aq}^{-1} d^{-1} (Schmutzler et al. 2016).

This reactor system was also used for the multistep oxyfunctionalization of (*R*)-(+)-limonene to (*R*)-(+)-perillic acid catalyzed via the *p*-cymene degradation pathway using the native host *P. putida* GS1 (Willrodt et al. 2016) as well as for the hydroxylation of cyclohexane to yield cyclohexanol employing P450 cyclohexane MO from *Acidovorax* sp. in recombinant *P. taiwanensis* VLB120 (Karande et al. 2016). Both reactions have in common that they comprise highly toxic and volatile reactants, which represents a challenge for the whole-cell biocatalyst. To overcome toxicity issues and to ensure continuous product formation, the aqueous-air segmented flow biofilm membrane reactor was evaluated. As shown in Fig. 15, the inner surface of the silicone membrane was used as a substratum to grow the cells, while the aqueous-air segmented flow pattern ensured a continuous supply of oxygen and consequently controlled biofilm growth. In addition the silicon membrane of the capillary prevented direct contact of the cells with the bulk phase of the toxic substrates reliving catalyst toxification. This biocatalytic reactor concept ensured continuous product formation for several days at an average volumetric productivity of 10 g L^{-1} day^{-1} for cyclohexanol (Fig. 15; Table 1/#11) and 34 g L^{-1} day^{-1} (*R*)-(+)-perillic acid (Fig. 15; Table 1/#12).

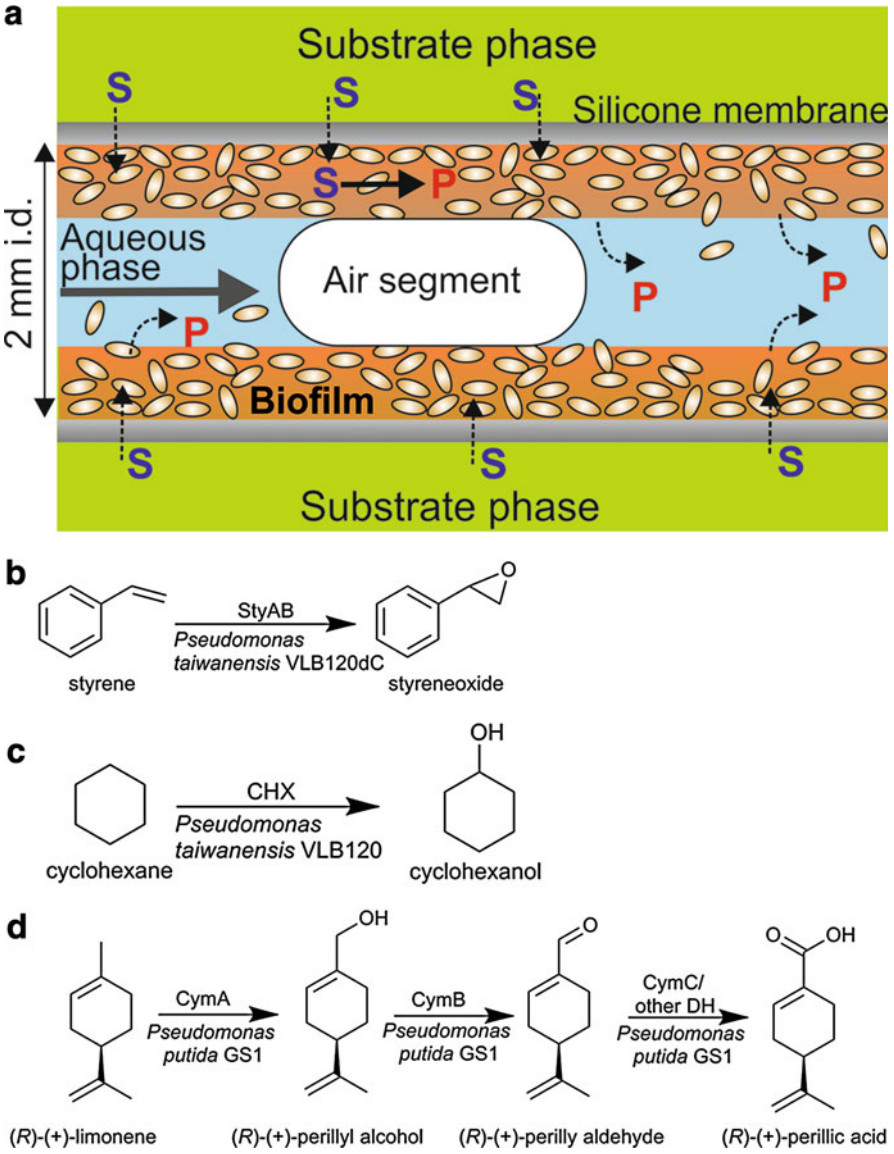

Fig. 15 Biotransformation of HC utilizing catalytic biofilms in segmented flow microreactors. (**a**): Overall concept of employing a microbial biofilm in a capillary microreactor with segmented flow. The biofilm is growing on the inside of the tubing and is being continuously flushed with aqueous growth medium. Oxygen is supplied via air segments, which are pumped through the tubing. The biotransformation substrate is supplied from the outside and enters the biofilm via diffusion. (**b**–**d**) show exemplarily reactions, which have been conducted in this set-up. (**b**): Enantiospecific epoxidation of styrene yielding (*S*)-styrene oxide utilizing styrene monooxygenase (*StyAB*) in *P. taiwanensis* sp. Strain VLB120ΔC. (**c**): Conversion of cyclohexane to cyclohexanol utilizing P450 monooxygenase from *Acidovorax* sp. recombinant expressed in *P. taiwanensis* VLB120. (**d**): Multistep oxyfunctionalization of (*R*)-(+)-limonene to (*R*)-(+)-perillic acid catalyzed via enzymes of the p-cymene degradation pathway in *P. putida* GS1

This reactor technology can be transferred to different biocatalytic reactions involving compounds that are toxic and volatile and have low water solubility to perform efficient product synthesis. However, although these promising examples show the proof of concept of this reaction set-up, this is a fairly young technology for fine chemical production and there is still a huge optimization potential to make this approach attractive for possible applications. Pending questions involve ongoing evolution in continuous living microbial systems as well as up-scaling and product work-up.

5 Research Needs

What will the future bring for hydrocarbon biocatalysis? It is likely, that new reactivities will be discovered and our toolbox for catalyst design will be challenged to come up with optimal catalyst features like selectivity, stability (total turnover number), and also economical and ecological prowess. One example for new reactivities might be anaerobic C-H oxyfunctionalization catalysts based on enzymes using water as oxidant for the conversion of, e.g., methane to methanol. Other challenges are the design of efficient biocatalysts for low prized bulk chemicals syntheses. These will preferentially be based on (living) whole microbial cells, whereas cell free, enzyme-based "off the shelf" catalysts will become available for syntheses of small amounts of products and of course for the design of biosensors.

The available technology to design and control biocatalysts, especially regarding whole cell and metabolic engineering, is enormous and its potential for biotechnology has by far not been exploited yet. We are only beginning to understand the complexity of cell metabolism, physiology, and physico-chemical reactions. Despite the fact that there are several successful examples for designing highly productive biocatalysts via metabolic engineering, these present the results of many years of research and are always tailored solutions for specific reactions only. We are still far away from being able to tune cell metabolism and physiology ad libitum and on a modular theoretical know how basis as we find it, e.g., in chemical engineering. Biocatalytic processes are often only economical for specialty and fine chemicals production. Technology platforms like systems biology and systems biotechnology will continue to develop into profiled research fields and certainly contribute to a comprehensive understanding of whole cell catalysis, also for bulk chemicals syntheses.

Will there be a need and interest for petrochemicals based biocatalysis or will the future be dominated by the use of regrowing material and biomass as carbon sources for the synthesis of value added chemicals and new materials? We are experiencing an advent of alternative energy sources but also a drastic increase of petroleum costs and the prize for glucose and other regrowing C-sources. This points to a future where regrowing C-sources will become much more important in the chemical industry, but also where fossil C-sources like petroleum, gas, and coal will continue to play a key role in the value chain of chemical industries.

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Metagenomic Mining of Enzyme Diversity

14

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Abstract

In the present there is a great necessity of suitable biocatalysts with high process performance, as a "greener" complementary alternative to the chemical synthesis. It is expected that in the coming decade, up to 40% of bulk chemical synthesis processes could be substituted by enzymatic catalysis. The identification and optimization of an appropriate enzyme represent important requirements to obtain a successful and efficient enzymatic process. In this context, the establishment of enzymatic processes in the industry is mainly a problem of finding and optimizing new enzymes. In this sense, nature is the richest reservoir from which enzymes can be isolated because they are continuously changing and evolving as a consequence of natural processes of selection. We are now taking advantages of sequencing and extensive screening technologies to develop enzyme discovery strategies and to identify microbial enzymes with improved and unusual activities and specificities. These approaches, in combination with modern protein engineering methods and distinct combinatorial and rational methods, will increase our chances to generate new stabilized biocatalysts that fit industrial requirements. Here, we review the methodologies, obstacles, and solving problems around metagenomics investigations to screen for enzymes with activities of interest.

1 Introduction

Microbes represent the most diverse and abundant group of organisms on Earth, making up to 60% of the total biomass of all living organisms. They dominate all ecosystems from soils and oceans to the habitats most hostile to life, such as polar environments or acidic streamers. They play a crucial role in biogeochemical cycling of all chemical elements and are responsible for a primary production and environmental cleanup (Phale et al. 2007). In human bodies, it has been estimated that there are ten times more microbial cells than human cells (Curtis and Sloan 2005; Turnbaugh et al. 2007), which collectively points at the necessity of understanding the microbial community structure and diversity for health and disease, microbe-host interactions, processes dynamics at small-to-global scales, and ultimately for understanding the evolution of life on Earth.

In the modern industrial era, microbes are exploited as a rich source of valuable industrial products with applications across all major areas. Microbial secondary metabolites are extremely important to our health and nutrition. For example, microbial products are used as antibiotics, antitumor agents, and immunosuppressants in the pharmaceutical industry and as biopesticides, antiparasite agents, and food-processing agents in the agricultural sector (Demain and Adrio 2008). Just as example, there are at least about 17,000 bioactive natural products with antibiotic properties found in bacteria, 8,700 natural antibiotics in *Actinomycetales*, and 4,900 in fungi (Bérdy 2005). Similarly, microbial products are widely used by the chemical industry for the production of amino acids, vitamins, organic acids, detergents, biocatalysts, and bioconversion agents and by environmental industries for bioremediation and the production of bioenergy.

The preference for the microbial production of a variety of compounds that can otherwise be isolated from plants or chemically synthesized is due to:

(1) The wide range of reactions that microorganisms are capable to perform
(2) Their ability to adapt to different environmental settings and thus the possibility to produce them in inexpensive media
(3) Easiness to genetically manipulate them for increased production
(4) The intrinsic great diversity, which leads different species to produce enzymes catalyzing the same reactions, but with a rich flexibility with respect to catalytic efficiency and stability to different physical and chemical conditions (Demain and Adrio 2008)

Enzymes of microbial origin are an exceptional natural resource of powerful biocatalysts capable of performing a wide range of reactions and accepting an extensive collection of complex molecules as substrates. In fact, from the wide range of sources of commercial enzymes used industrially, 88% are from microorganisms, and the remaining are divided between animal (8%) and plant (4%) sources (Gurung et al. 2013). Currently there is a great demand for suitable enzymatic biocatalysts with high process performances to use as a greener alternative to chemical synthesis (Davis et al. 2001; Koeller et al. 2001).

Due to the enormous natural diversity of the microbial community, which suggests a world of possibilities in terms of new catalytic activities, it is of a high interest to assess new natural environments for the isolation and characterization of novel enzymes. We have so far explored only a small fraction of this enormous diversity. Although prokaryotes cover the largest fraction of individual living organisms, with an estimated amount of 10^3–10^5 microbial species in just 1 g of soil (Schloss and Handelsman 2006), almost 99% of bacteria are recalcitrant to culturing. This technical limitation has over the years stimulated the development of new culture-independent tools to disclose and characterize microbial genomes. However, to date, only about 11,000 Bacteria and Archaea species have been classified, and each year, at least 600 new species are described (Kyrpides et al. 2014; Yarza et al. 2014). Metagenomics represents a powerful approach for accessing and examining the biological and molecular diversity existing in different natural environments. Metagenomics is currently thought to be one of the likely technologies to provide the candidate molecules required by the market (Ferrer et al. 2016).

In this chapter, we focus on the importance and the possible application of metagenomic studies, and we review the methodologies for the sequence homology and functional screening of libraries to discover and characterize new enzymes with activities of interest.

2 An Industrial Perspective

The market of enzymes is distributed over various application areas, comprising food (45%), detergents (34%), textile processing (11%), leather (3%), and pulp and paper (1.2%) (Demain and Adrio 2008).

Their versatility allows their use in many processes, such as the degradation of natural polymers including cellulose and proteins, as well as in fine chemical industry for the regioselective or enantioselective synthesis of pharmaceutical and agrochemical molecules (Gupta et al. 2002; Monsan and O'Donohue 2010; Nguyen et al. 2008). Enzymes can also offer an efficient strategy in the field of bioremediation by the degradation of polluting chemicals (Phale et al. 2007). For this reason, the annual demand for enzymes, which currently is growing almost 7% per year, is expected to be close to 10% in 2030.

At present, "white" and "red" biotechnological sectors are facing an important request for new enzymes and metabolites. The ideal biocatalyst for any industrial application needs to function sufficiently well according to several performance parameters (Table 1).

Food and beverage enzymes constitute the largest segment of industrial enzymes. Next in the list are lipases, valuable enzymes utilized in the digestion and processing of dietary lipids (e.g., triglycerides, fats, oils). Largely diversified in their enzymatic properties and substrate specificity, microbial lipases are more stable than animal or plant lipases. Moreover, lipases are widely used in a variety of other applications such as baking and detergents or in the conversion of vegetable oil into fuel (Guo and Xu 2005; Gupta et al. 2004).

A common industrial implementation of enzymes is their use as detergent additives. A suitable enzyme must be active under alkalophilic and thermophilic conditions. Proteases, along with lipases, amylases, oxidases, peroxidases, and cellulases, are commonly used in detergents, enhancing the ability to remove tough stains and to make detergents eco-friendly (Adrio and Demain 2014; Mitidieri et al. 2006).

In the starch industry, thermostable amylases are used for the enzymatic hydrolysis of starch into glucose, fructose, or maltose (Gomes et al. 2003).Other relevant applications of amylases comprise food industry (baking, brewing, fruit juices), paper industry (α-amylases for the modification of starch of coated paper), textiles, and fuel ethanol from starches (Gurung et al. 2013).

In the textile industry, enzymes play an important role in the development of cleaner processes and in reducing the use of raw material and production of waste, for example, cellulases for denim finishing and laccases for decolorization of textile effluents and bleaching (Araújo et al. 2008).

The replacement of existing chemical processes with enzymatic routes becomes a successful application when it contributes to an overall cost reduction, with an increased product titer, lower energy demand, and lower volumes of wastewater streams and amount of byproducts. A number of biocatalytic routes have been fine-tuned for pharmaceutical manufacturing, showing preeminence above the traditional chemical pathways (Bornscheuer et al. 2012). The market of antibiotics, e.g., semisynthetic penicillins and cephalosporins, is ruled by enzymatic processes. Another important application in biocatalysis is the generation of enantiomerically pure intermediates, especially when just one of the two isomers of a compound shows the desired activity; in that context, esterases, lipases, and proteases are widely applied in the preparation of chiral compounds (Demain and Adrio 2008).

Table 1 Set of criteria used to evaluate enzyme candidates from an industrial point of view. Most promising candidate enzymes can be selected for further process development (Lorenz and Eck 2005)

Activity
pH profile
Temperature profile
Specific activity (kat/kg, U/mg)
Turnover frequency (kcat)
Efficiency
Space-time yield
Product inhibition
By-product/ingredient inhibition
Producibility/expression yield
Stability
Temperature stability
pH stability
Ingredient/by-product stability
Solvent stability
Specificity
Substrate range
Substrate specificity (K_M, k_{cat}/K_M)
Substrate regioselectivity and enantioselectivity
Substrate conversion (%)

Most screening requests at the industrial scale for chemical synthesis address aldo-keto reductases, followed by transaminases and lipases (Ferrer et al. 2015).

Designing a process to fit a common/mediocre enzyme can be expensive and not profitable; on the contrary, it is reasonable to find a more efficient suitable natural enzyme, by the production of pre-characterized enzyme libraries using generic substrates. Once interesting enzymes or metabolites are found, they can be used as backbone for enzyme engineering to supply a biocatalyst able to optimally match process requirements. Scale-up and process optimization should lead to a viable industrial application.

2.1 Mining Biocatalysts from Extreme Environments

Extreme environments provide a great source of microbes (extremophiles) adapted to a wide range of extreme conditions, both physical such as temperature (−2 to 110 °C), pressure, or radiation and geochemical such as salinity (2–5 M NaCl) and extreme pH values (<2 and >9). Corresponding microbes have been isolated from samples collected in hot springs, near volcanoes, in the deep sea, in the Arctic and Antarctic ice, in deserts and arid areas, and in other extreme locations (Wilson et al. 2009).

The ability of these microorganisms to survive under extreme conditions strictly depend on peculiar enzymes that not only support microbial growth but also can constitute robust biocatalysts exhibiting enhanced features merging the industrial requirements for a variety of applications (Ferrer et al. 2005b; Herbert 1992; Van den Burg 2003).

Thermophilic and hyperthermophilic enzymes are stable and active at high temperatures (Hough and Danson 1999; Vieille and Zeikus 2001) and are therefore of biotechnological interest thanks to their adaptability to harsh industrial reaction conditions. The conveniences of conducting reactions at high temperature are well known and include an increased solubility of polymeric substrates, increased bioavailability, faster reaction rates, the decreased risk of contamination, and the cost saving due to the unnecessary cooling system. Extreme thermophiles, growing optimally at 60–80 °C, are widely distributed among the genera *Bacillus*, *Clostridium*, *Thermoanaerobacter*, *Thermus*, *Fervidobacterium*, *Rhodothermus*, *Thermotoga*, and *Aquifex.* Most of hyperthermophiles (with a growth optimum of 80–110 °C) belong to the archaea, for example, *Sulfolobus*, *Pyrolobus*, and *Thermofilum* for the Crenarchaeota phylum and *Thermococcus*, *Pyrococcus*, and *Thermoplasma* for the Euryarchaeota (Gomes and Steiner 2004).Thermophilic proteases, lipases, cellulases, and amylases are being identified by screening metagenomic libraries and used in many different industrial applications: detergent, food, starch, textile, and pulp and paper industries are the major users (Atomi et al. 2011; Cherry and Fidantsef 2003; de Carvalho 2011; Kumar et al. 2011). The thermostable DNA polymerase is the most successful application of a product extracted from an extremophile, *Thermus aquaticus*, which was found in the Lower Geyser Basin of Yellowstone National Park, USA (Saiki et al. 1988).

High temperatures and alkaline media usually denote processes in the paper and pulp industry, and the identification of thermostable and alkali-stable enzymes represents a major biotechnological goal in this field (Kumar et al. 2016). Cellulases and xylanases are used as bio-bleaching agents with economic and environmental advantages over chemical alternatives; moreover, they have biotechnological relevance in a number of other fields, including food, textile industries, as well as for saccharification of pretreated lignocellulosic biomass for the production of biofuels (Hess 2008).

Enzymes from halophiles are able to deal with high concentration of salts (up to 4 M KCl and over 5 M NaCl) (Hough and Danson 1999). Proteins from halophilic organisms, with an excess of negatively charged amino acid residues on their surface, remain stable and active at high ionic strength (Madern et al. 2000). The property of low solubility of halophilic enzymes has been exploited by applying them in organic solvents and nonaqueous media (Klibanov 2001). The production of halophilic enzymes, such as xylanases, amylases, proteases, and lipases, has been reported for some halophiles belonging to the genera *Acinetobacter*, *Haloferax*, *Halobacterium*, *Halorhabdus*, *Marinococcus*, *Micrococcus*, *Natronococcus*, *Bacillus*, *Halobacillus*, and *Halothermothrix* (Gomes and Steiner 2004).

Microorganisms that can survive under extreme pH values could be good sources of thermoalkaliphilic or acidophilic enzymes (Golyshina et al. 2016; Méndez-García et al. 2015). Proteases, lipases, cellulases, peroxidase, or oxidoreductases are used as

additives in detergents, in bio-bleaching of pulp and paper, or in the cleanup of effluent streams of the textile processing industry (Adrio and Demain 2014; Gomes and Steiner 2004).

The complexity in the cultivation of extremophiles, because of the longer generation times, lower biomass yields, and strict cultivation conditions than mesophilic microorganisms, requires different strategies to overcome the problems (Schiraldi and De Rosa 2002). Metagenomic approaches allow the screening of enzymes from environmental gene pools by cloning their sequences into suitable hosts such as *E. coli* or *P. pastoris* without the need to cultivate the original strains (Ferrer et al. 2005a).

3 Metagenomics and Its Approaches

Metagenomics is a powerful approach to unravel the biodiversity of microorganisms and activities, regardless of whether or not they can be cultured in laboratory. All metagenomic studies start with the isolation of genomic DNA from an environmental sample, bypassing the need for culturing the organisms that may serve as a source for enzymes. The following step is usually the construction of clone libraries, through the cloning of the environmental DNA into a suitable vector, transforming the cloned DNA into a host organism (typically *E. coli*) and subsequent analysis of the DNA using both bioinformatics and experimental methods (Handelsman 2004). Nowadays though, the environmental DNA is directly subjected to the de novo sequencing and data analysis, bypassing the intermediate (and obsolete) cloning step. The discovery of enzymes in metagenomes can be conducted using two different approaches: first, sequence-based metagenomic approaches that look for enzymes homologous to known biocatalysts in the (meta)genomic data and may include PCR-based methods that use primers designed according to conserved regions of known enzymes and, second, functional metagenomic approaches, where metagenomic libraries are established and clones screened with enzyme substrates (Lam et al. 2015; Sabree et al. 2009) (Fig. 1).

The latter approach allows the discovery of genuinely novel enzymes, since bioinformatics analyses are homology based and thus may only identify enzymes that are similar to characterized counterparts. Recently, the data on the discovery of new enzymes using metagenomic platforms has been reviewed for a period since 1998 (Ferrer et al. 2016). During that period, genetic materials from microbial communities from approximately 2,200 sites around the world, with an emphasis on microbes from extreme habitats, have been examined. Environments of every kind have been included such as terrestrial (soil, plant rhizosphere) and marine habitats (superficial and deep seawater, hydrothermal vents), alkaline lakes, acid mine drainage systems, and eukaryote-associated microbiomes (rumen, gut). However, only a small fraction of the sites (11.6%) have been subjected to enzyme discovery studies with only 7,000 enzymes or clones containing enzymatic activities of interest for the industry identified and characterized (Ferrer et al. 2015, 2016),

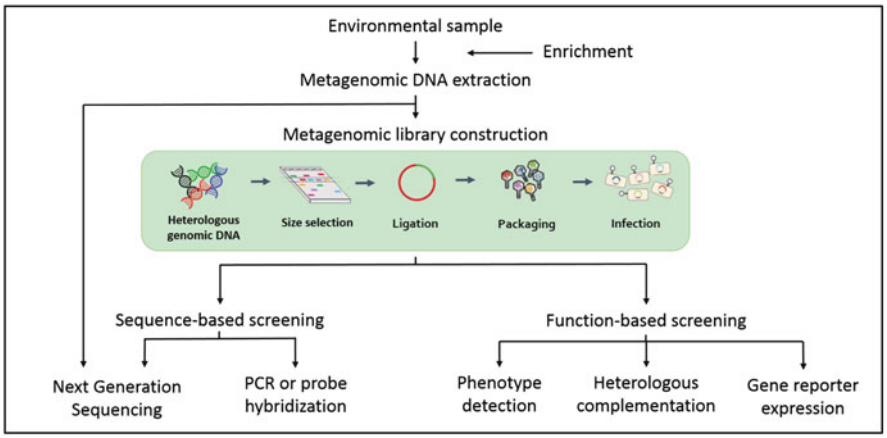

Fig. 1 A simplified scheme of metagenomic enzyme discovery pipeline, which highlights the difference in sequence- and function-based approaches

although in most cases rather superficially. This underlines that in spite of a number of success stories the biosphere is undersampled.

3.1 Screening Methods

3.1.1 Sequence-Based Enzyme Discovery

Sequence-based (homology-driven or "genome gazing") screening is based on the search for genes predicted to code for particular enzyme classes. This approach involves the design of PCR primers or hybridization of probes on conserved regions and motifs of known protein families and subsequent sequencing and comparison with homologous known enzymes (Ferrer et al. 2009). The approach is therefore only suitable to the discovery of members belonging to known gene families, since it completely relies on the available genome annotations. A large proportion of the open reading frames of newly sequenced genomes have little sequence homology with known enzymes, and this prevents the discovery of new protein classes with sequence elements that differ from the conserved sequences of the primers (Harrington et al. 2007).

The anchor used for the amplification or hybridization can be a 16S rRNA gene for a quick analysis providing a snapshot of the diversity within the community (Béjà et al. 2000) but can also be a gene encoding an enzyme of interest. Sequence-based approaches have led to the identification of genes encoding enzymes, such as lipases (Bell et al. 2002), glycerol dehydratases (Knietsch et al. 2003), chitinases (Hjort et al. 2010), nitrilases (Gong et al. 2013), and esterases (Ferrer et al. 2015).

Moreover, the advent of next-generation sequencing platforms had a great impact. Nowadays we have got the chance to analyze a huge set of sequence data,

and many screening projects bypass the cloning step in favor of direct sequencing of the extracted environmental DNA. The analysis of the resulting large data sets allows the exploration of the taxonomic and functional biodiversity and of the system biology of diverse ecosystems. On the other hand, as a consequence of the fast increase of sequences deposited in public databases, the majority of them have been poorly or mis-annotated, with most of the genes labeled as hypothetical or protein of unknown functions, since they have not been experimentally characterized (Fernández-Arrojo et al. 2010; Schnoes et al. 2009). A further disadvantage for the homology-based approach is that its efficiency is based on the quality and completeness of genome annotations in current databases (Hallin et al. 2008). Additionally, many of the identified sequences did not correspond to full-length proteins due to low sequence coverage.

Homology-based approaches mainly suffer from the long computation time required to search for homologs for each of the sequences within the typically massive metagenomic data sets. Recently, web-based metagenomic annotation platforms, such as the metagenomics RAST (mg-RAST) server, the IMG/M server, or JCVI Metagenomics Reports (METAREP), have been designed to analyze metagenomic data sets. The uploaded environmental data sets can be compared vs both protein and nucleotide databases. In this way, multiple metagenomic data sets derived from various environments can be compared at various functional and taxonomic levels (Goll et al. 2010; Meyer et al. 2008).

3.1.2 Function-Based Enzyme Discovery

Unfortunately, the generation of vast metagenome sequencing data is not followed, proportionately, by the discovery of new activities or functional characterization of proteins. Fewer than 2 out of 10,000 hits have led to the discovery of industrially relevant biocatalyst (Lorenz and Eck 2005). Activity-based metagenomics provides an opportunity to circumvent this limitation. The functional approach is not dependent on previous genomic knowledge and allows for the discovery of novel enzymes with unexpected peptide sequences with classical or new activities that would not be predicted based on DNA sequence alone. Thus, functional metagenomics complements sequence-based metagenomics, adding functional information or correcting incorrect functional assignments to nucleic acid and protein databases.

Function-driven screens usually begins with the construction of genomic expression libraries and the use of three different strategies for identifying clones of interest: direct phenotype detection using a specific colorimetric or fluorometric substrate (Handelsman 2004), heterologous complementation of host strains (Kazimierczak et al. 2009; Mirete et al. 2007), and detection following induction of biosensors or reporter gene expression (Uchiyama et al. 2005). The probability (hit rate) of identifying a certain gene depends on multiple factors that are intricately linked to each other: the DNA extraction method, the host-vector system, the size of the target gene, its abundance in the source metagenome, the assay method, and the efficiency of heterologous gene expression in a surrogate host.

3.1.3 Cloning Vectors/Vehicles and Library Construction

DNA is first harvested from environmental samples, then size-selected, end-repaired, and ligated to a vector, allowing packaging by lambda phage for subsequent infection of *E. coli*. According to the primary goal, small- or large-insert libraries can be preferred, each of them offering particular advantages and disadvantages.

To obtain a function encoded by a single gene, small-insert expression libraries can be built by cloning small-sized fragments (<10 kb, equivalent to four to eight genes) into standard cloning vectors (e.g., pUC derivatives, pTOPO-XL, pBluescriptSK$^+$, and pCF430) or lambda phage vectors (Sabree et al. 2009). Therefore, most genes are present in the appropriate orientation and under the influence of strong vector promoters, and thus they have a good chance of being expressed and detected by activity screens (Ferrer et al. 2009). Moreover, due to the lysis of *E. coli* cells at the end of the phage infection cycle, translated proteins are released to the extracellular matrix, which means an earlier detection of the screened activity and a negligible impact of the toxic effects associated with the expression of lethal genes. The clear disadvantage of above plasmid vectors is in their high (up to 700) copy numbers per cell, which may facilitate a leaky expression of some genes that are incompatible with the host well-being.

On the other hand, to obtain targets encoded by multiple genes, large fragments must be cloned into fosmids, cosmids, or bacterial artificial chromosomes (BACs), which can harbor fragments up to 300 kbp. The commercially available pCC1FOS vector has become a frequently used tool for cloning large DNA fragments (ca. 40 kbp) from various microbial communities. One of the advantages of using pCC1FOS vector, when transformed into the appropriate host (e.g., *E. coli* Epi300), is that there is a possibility to induce its copy number, which allows a higher gene dosage and eventually a better expression than from a single-copy uninduced vector (Wild et al. 2002). Multicloning sites also allow the Sanger sequencing of termini of cloned fragments and reveal important information about the native metabolic role of the enzyme in question or can help to identify the source organism of the DNA fragment (Popovic et al. 2015).

Enzyme activities are usually assayed on agar plates supplemented with enzyme substrates. By cultivating a metagenomic library on the plates, one can identify positive clones through visual screening for the appearance of a clear zone (halo) or color. Thousands of clones can be analyzed in a single screen, with the potential to identify novel classes of proteins with known or unknown functions. Agar plate-based screening has been successfully applied to screen different metagenomic gene libraries to mine industrially useful enzymes such as lipases, esterases, cellulases, proteases, laccases, and other activities (Ferrer et al. 2009, 2010; Placido et al. 2015; Tchigvintsev et al. 2015).

The hydrolysis of emulsified triacylglycerols, such as tributyrin, triolein, and olive oil, is generally applied to assess lipase activity, while soluble short-chain fatty acid esters are applied to study esterase activity (Placido et al. 2015). Seven lipase-producing thermophilic bacteria were identified from a Malaysian hot spring streaking the cultures on an agar plate containing olive oil, showing lipolytic activity up to 4.58 U/ml for the catalyst extracted from *Bacillus* (Sheikh Abdul Hamid et al. 2003).

The diversity of rumen hydrolytic enzymes was for the first time investigated by screening a metagenomic phage library of the rumen content of a dairy cow for hydrolase activity (Ferrer et al. 2005c). A total of 22 clones with hydrolytic activities were identified (12 esterases, 9 endo-β-1,4-glucanases, and one cyclodextrinase) and characterized, among them eight were entirely new, showing no sequence similarity to enzymes deposited in public databases.

If the target activities can be linked to the survivability of the host organism, which requires the addressed gene to grow under selective conditions, the screen becomes highly sensitive and high throughput. This method is applied frequently to screen for resistance genes to toxic compounds, such as antibiotics or heavy metals. The approach has been successfully applied in the detection of enzymes such as racemases (Chen et al. 2010), DNA polymerases (Simon et al. 2009), and β-lactamases. For example, 5.4 Gbp of soil DNA led to the identification of nine unique clones containing resistance to aminoglycoside antibiotics and one clone expressing resistance to tetracycline (Riesenfeld et al. 2004).

A functional study conducted on 12 marine environments that included extreme anoxic deep sea sites (deep hypersaline anoxic basins *Urania*, *Kryos*, and *Medee* in the Eastern Mediterranean Sea, and epibionts of the gut and gill of the deep sea shrimp *Rimicaris*), extremely low pH sites (Vulcano Island hydrotherms), and regions characterized by heavy industrialization and oil contamination (seawater of Messina harbor, Milazzo and Priolo refineries (Sicily), MT Haven shipwreck (near Genoa, Italy), superficial petroleum-polluted seawater near Kolguyev Island and Port of Murmansk (Russia)), has yielded to the identification of several putative enzymes (Popovic et al. 2015). The frequency of positive hits was of 1 per 9 Mbp of screened DNA for esterase/lipase and 1 hit per 28.4 Mbp for glycosyl hydrolases and per 23.9 Mbp for dehalogenase screens (Popovic et al. 2015). Among the identified biocatalysts, several esterases belonged to uncharacterized families or to proteins annotated to have alternate functions, proving the unique advantage of the functional screen over the sequence-based one.

4 Limitation in the Metagenomic Enzyme Discovery Process

4.1 Coverage, Representation, and Need for Enrichment

Some bottlenecks in the metagenomic enzyme discovery process lower the transition from the discovery stage of an enzyme to its application.

The library representativeness of the diversity present in the original environmental DNA is an important element that must be taken into consideration if we want to access the full potential of environmental metagenomes. Community coverage is possible only in simple or high specialized environments characterized by low species richness, such as acid mine drainage at Iron Mountain, California (Ram et al. 2005; Tyson et al. 2004). The relatively simple structure of its microbial community led to the assembly of five complete genomes. In contrast, the metagenome of a very complex community, such as that from the soil, cannot be

exhaustively sequenced at reasonable costs, and the reconstruction of genomes or metabolic pathways is only possible for few predominant organisms. Some sequences are less likely to be captured in libraries, and this inevitably represents a barrier to the screening.

A comparative study of shotgun sequencing of original sample to corresponding metagenomic libraries from human feces (Lam and Charles 2015) and cornfield soil (Cheng et al. 2014) highlighted how the relative abundance of phyla can differ in the library compared to its corresponding extract. Interestingly, the analysis indicated a bias in the relative abundance of each operation taxonomic units (OTU), with some being 1,000-fold overrepresented and others underrepresented in the library; AT-rich sequences appeared to be inadequately represented (Lam and Charles 2015). The sequencing of extremely large libraries is necessary for a complete coverage of species-rich samples. In addition, the enormous volume of data generated by environmental sequencing requires advances in bioinformatics and the development of new algorithms specifically designed for metagenomic data analysis (Wooley et al. 2010).

Due to the high diversity within the microbial communities, target genes encoding for novel enzymes represent a tiny fraction of the total nucleic acid sample extracted. However, microbial community composition can be actively manipulated prior to metagenomic library construction, in order to enrich for desired activities. For functional screening, pre-enrichment of environmental samples is usually obtained by amending with specific nutrients or substrates, favoring the growth of the microbes that express the desired biocatalyst. Selection pressure for enrichment can be obtained upon chemical or physical criteria. For example, a size-selective filtration was used for enrichment in the Sargasso Sea genome sequencing project (Venter et al. 2004). The main drawback of the artificial enrichment is the danger of enriching fast-growing microorganisms that do not utilize the supplied nutrients. A recent success example of the pre-enrichment method related to the research of new amidases for the enzymatic conversion of D-phenylglycine into intermediates for the synthesis of β-lactam antibiotics conducted on soil (Gabor et al. 2004). To increment the likelihood of finding useful enzymes, enrichment cultures were performed with D-phenylglycine amide as exclusive nitrogen source. 16S rRNA analysis showed a four times lower bacterial diversity in the enrichment cultures than the original sample. Clones exhibiting amidase activity were selected on a medium containing phenylacetyl-L-leucine or D-phenylglycine-L-leucine, allowing only the growth of recombinants capable of hydrolyzing the amide compounds. Amide-positive clones were identified, and after an extensive substrate profiling, a distinctive clone (pS2) was isolated, able to support a more than twofold higher maximal level of penicillin G accumulation than *E. coli* penicillin amidase (Gabor et al. 2004). In another study, the efficiency of screening metagenomic libraries for cellulose activities was improved more than three times by incubating the soil sample with crystalline cellulose before DNA extraction (Mori et al. 2014).

Similarly, microbial communities within contaminated ecosystems tend to be dominated by the organisms capable of consuming and/or tolerating toxic organic compounds. Phylogenetic analysis conducted on sediment from Milazzo bay

showed how the structure and composition of the bacterial community capable of growing in hydrocarbon-contaminated superficial sediments dramatically change in response to nutrient load and to addition of various hydrocarbons (Yakimov et al. 2005). Environmental samples can dynamically vary in phylogenetic and process equilibrium: the population of a closed niche may be modified until the system reach a new equilibrium where the key microorganisms have the working capacity to make the overall community work (Bargiela et al. 2015; Yakimov et al. 2005).

New PCR-independent amplification techniques that use multiple displacement amplification (MDA) with Φ29 DNA polymerase (Blanco et al. 1989) for whole genome amplification have allowed access to rare microbes, present in low numbers in bacterial communities or from single cells (Spits et al. 2006).

A method for enrichment is the so-called stable isotope probing (SIP), an approach that selectively enriches metabolically active microbes that consume a specific labeled substrate. The DNA that incorporates high amounts of heavy isotope (such as ^{13}C) belongs to metabolically active microorganisms, and it can be separated by density centrifugation. The labeled nucleic acid can be therefore used for the generation of a metagenomic enriched library (Neufeld et al. 2008; Radajewski et al. 2000). This methodology was successfully used to specifically enrich and label methylotrophic populations in sample of lake sediments able to use labeled C_1 compounds (Kalyuzhnaya et al. 2008). SIP was also useful for the isolation of various enzymes, such as novel biphenyl dioxygenase (Sul et al. 2009). Without preexisting knowledge on the identity of the microbes within the microbial community, it is thus possible to have a direct access to their genome. However, the stable isotope probing approach can result in a loss of novel catalysts that are only present in a small number of microbes, which can be superseded by fast-growing and more abundant bacteria.

Differential display (DD) is an alternate technique that can be used for the discovery of bacterial genes, and it can be applied to identify differentially expressed genes. DD is used to compare the mRNA pools: cells are grown under different physiological conditions; messenger RNA is then amplified at arbitrary sites by reverse transcription (RT), followed by RT-PCR (Galvão et al. 2005; Liang et al. 1993). Levels of gene expression are thus compared, and genes expressed only under a specific condition will give rise to RT-PCR bands. These are further analyzed by DNA sequencing to discover new ORFs (Brzostowicz et al. 2003). The application of this approach to the prokaryotic system requires the use of arbitrary oligonucleotide primers to initiate RT of the mRNA at random sites, while for eukaryotic mRNA, it is possible to take advantage of the poly(A) tails. Differential display has been successfully used to discover genes for cyclohexanone monooxygenase in mixed cultures from a wastewater bioreactor (Brzostowicz et al. 2003), to discover a putative operon for 2,4-dinitrophenol degradation in *Rhodococcus erythropolis* (Walters et al. 2001), and to explore the gene expression patterns in marine microbial communities using an adaptation of this technique called transcriptome fingerprinting analysis (TFA) (Coll-Lladó et al. 2011).

4.2 Need for Multiple Hosts

Currently, the bacterium *E. coli* is the most common host organism used for screening metagenomic libraries, although the heterologous expression remains a barrier for the extraction of the maximum information from functional analyses. The *E. coli* transcription-translation machinery is not completely compatible with the expression of genes harvested from environmental microbes: this can result in a low proportion of positive clones or in inactive enzymes after expression (Gabor et al. 2007; Loeschcke et al. 2013).

An alternative host is *Bacillus subtilis*, which is endotoxin-free and secretes proteins into the extracellular medium; unfortunately there is an insufficient number of expression vectors conceived for this bacterium that furthermore presents plasmid instability, misassembled proteins, and active proteases (de Carvalho 2016). Other alternative organisms for library construction and screening are being used, including strains of *Streptomyces* and *Pseudomonas* (Ferrer et al. 2007) or *Rhizobium leguminosarum* (Wexler et al. 2005). The adoption of a broader host range vectors capable of replication in several hosts will positively expand the range of detectable activities, although further optimization of the conditions for high transformation efficiency and for expression of, for instance, metagenomes derived from archaea-dominated communities, is still necessary. While expression systems in further bacteria (e.g., *Rhodobacter*, *Burkholderia*), archaea (*Haloferax*, *Sulfolobus*), and yeasts *(Pichia*, *Saccharomyces*, *Schizosaccharomyces)* (Craig et al. 2010; Liebl et al. 2014) are relatively well established for expression of individual proteins, none of these hosts have so far been utilized for metagenomic libraries construction and screening.

5 High-Throughput and Single-Cell Genomics

The impact of NGS technologies on metagenomics has been very profound; currently a typical metagenomic project is sequence-based and generates large amounts of sequence data. In fact, conventional function-based screening of metagenomic libraries faces some challenges such as a low hit rate of positive clones, labor intensiveness, low throughput, and taking an excessive time. In functional metagenomic screenings, the low frequency of positive clones is a significant limitation. As we already discussed, common screening methods are based on the conversion of easy-to-screen, general assays in solid or liquid media. The lack of relevant substrates and screening methods for rare enzymatic activities, as well as the poor performance of enzymes under nonnatural conditions, represents a major technological limitation (Fernández-Arrojo et al. 2010; Singh 2010). The development of multi-substrate approaches for high-throughput functional screening and the design of new chromogenic compounds that can mimic the real complex target substrates should be of high interest. High-throughput screening methods are inevitably necessary: new assays have been established in recent years, and they are in constant development along with the progress in robotics and functional assays.

5.1 Single-Cell Genomics

Single-cell genomic (SCG) is a novel technology that is paving the way to new methods of analyzing and understanding our environment. Specific organisms of interest can be targeted by processing a small number of cells that are relevant to a specific function in the environment. A complex sample can be enriched for a target cell population using fluorescence-activated cell sorting (FACS):it enables the physical separation of a user-defined subpopulation of cells of interest from a complicated mixture for further analysis, such as downstream genomics or proteomics (Bergquist et al. 2009; Kodzius 2016). Therefore, this approach allows the identification of biological activity within a single cell matching specific criteria, such as size, shape, or presence of specific nucleic acids or activities, using fluorescent dyes (Rinke et al. 2014a; Woyke et al. 2009). Among the many advantages of FACS are its high throughput, high sorting speed, and ability to sort live cells. In addition, FACS can detect the presence of cells in a droplet, rejecting the empty ones and directing the cells with desired properties into multiwell/microtiter plates (Kodzius 2016). Droplet microfluidic-based screening is recognized as an efficient tool for the assessment of cellular behavior at the single-cell level. FACS can be used to detect expression of certain types of genes by regulation of a fluorescent biosensor present in the same cell as the metagenomic DNA (Rinke et al. 2014b).The co-encapsulation of cells expressing target molecules (e.g., enzymes and antibodies) with a reporter molecule, such as a fluorescent substance, provides a rapid detection of droplets that contain cells producing molecules of interest (Agresti et al. 2010; Brouzes et al. 2009; Scanlon et al. 2014).

In a recent work, genes coding for lipolytic enzymes were retrieved from a soil metagenomic library through a gel microdroplet (GMD) technique combined with fluorescent-activated cell sorting (FACS) (Hosokawa et al. 2015). Directly after the library construction into the fosmid vector and the infection of EPI300-T1^{R} *E. coli* cells, the clones were encapsulated in agarose GMD with fluorogenic substrates (fluorescein dicaprylate) and IPTG via a microfluidic droplet generator. After incubation, the substrate diffused into the periplasm and was enzymatically hydrolyzed; microscopic observation enabled detection and counting of lipolytic-active clones from the library pools in a digital format, based on the presence or absence of green fluorescence products in the GMDs. A hit rate of 0.62% was estimated with this method compared to the 0.28% of the conventional agar-plate assay, thanks to a more clear identification of the fluorescent signal rather than the hydrolysis halo. Moreover, the GMD-based assay has the potential of reducing significantly the reagent volume required (approximately 100 pL/cell) and the time required to assess the activity of clones from 3 days to 12 h.

5.2 SIGEX

An efficient screening tool is the substrate-induced gene expression (SIGEX) which, in combination with FACS technology, allows the high-throughput screening of

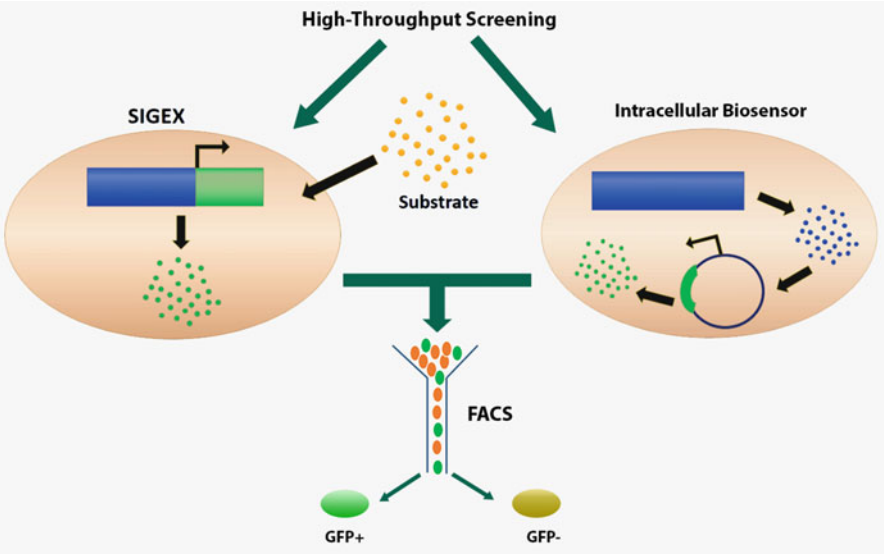

Fig. 2 Substrate-induced gene expression (SIGEX). Metagenomic DNA is cloned into a promoter-trap vector containing a *gfp*-reporter downstream of the cloning site. Promoters in the metagenomic DNA that respond to specific carabolites induce expression of GFP. An intracellular biosensor detects biologically active metagenomic gene products. GFP expression is dependent on the interaction of those small molecules with the biosensor proteins. Finally, FACS is used to sort the GFP+ and GFP− cells separately

30,000 clones/s of metagenomic libraries constructed in an operon-trap vector (Uchiyama and Miyazaki 2010). The operon-trap vector includes a co-expressed reporter gene, such as *gfp*. The GFP fluorescence is used to detect clones that contain metagenomic fragments containing genes that are expressed in response to induction substrates. This approach was successful applied to identify transcriptional activators responsible for aromatic compound degradation (Uchiyama and Miyazaki 2013; Uchiyama et al. 2005) using different aromatic inducing compounds, such as benzoate, naphthalene, salicylate, 3-methylcatechol and 4-chlorocatechol (Fig. 2).

5.3 Microarrays

The DNA microarray technology is an alternative tool with the potential to investigate simultaneously multiple genes. Functional gene arrays (FGAs) are microarrays that contain probes for key genes involved in microbial functional processes, such as biodegradation of environmental contaminants or biogeochemical cycling of C, N, S, P, and metals (He and Deng 2012).The development of a FGA implies a certain workflow: the selection of functional genes and the retrieval and verification of all the sequences from public databases; the design of oligonucleotide probes, usually with specific software tools; and finally the “in-house” or

commercially microarray construction spotting the probes onto glass slides (He and Deng 2012). Distinct FGAs were developed immobilizing PCR amplicons or oligonucleotides to target specific functional processes. For example, FGAs of 50-mer oligonucleotides were developed to monitor microbial communities in acidic environment and bioleaching systems (Yin et al. 2007). Yin et al. investigated acid mine drainage environments, and they designed the microarray containing 1,071 probes using 16S rRNA sequences and a diverse set of functional genes involved in carbon, nitrogen, sulfur, and iron metabolisms and metal resistance. Acidophilic microorganisms known to be in the acidic environments were used as keywords for identifying appropriate genes in the GenBank database to use as probe (*A. ferrooxidan*s, *Leptospirillum* sp., *Acidiphilum* spp.).The established microarray was used as a generic profiling tool that revealed differences among various microbial communities. A total of 80 16S rRNA genes probes and 150 functional genes probes were detected, proving that the 50-mer oligonucleotide array can be used as a specific and quantitative tool.

Park et al. (2008) developed another format of DNA microarray technology for rapidly identifying clones from metagenome libraries: fosmid clones (targets) were spotted on a slide, and specific gene probes were labeled and used for hybridization. This approach was used to investigate fosmid libraries obtained from marine sediments (Park et al. 2008). However, specificity, high hybridization efficiency, and detection sensitivity are the most critical parameters of this technique, and the target sequences extracted from the conserved regions of already known protein families radically reduce the chances of obtaining new proteins (Gabor et al. 2007).

5.4 The Way Forward: From Enzyme Discovery to the Preparation of Ready-to-Use Biocatalysts

It is clear that the establishment of enzymatic processes in the industry is mainly a problem of finding new enzymes. As mentioned above, one can take advantage of sequencing and extensive screening technologies to develop enzyme discovery strategies and to identify microbial enzymes with improved and unusual activities and specificities. However, novel and potentially more efficient enzymes obtained from metagenomics are likely to require further modification by protein or enzyme engineering to obtain increased enzymes properties and catalytic activities. In addition to that, enzymes are delicate materials that need to be stabilized to survive a range of challenges conditions typically used in industrial processes. Accordingly, new formulations are needed that can stabilize and protect enzymes from adverse conditions, including those originated from washing, leaching, and solvents attack. Here, distinct combinatorial and rational methods to generate such stabilized biocatalysts are required, regardless of the apparent relative improvements at the end of the process (Bommarius et al. 2013; Tran and Balkus 2011). The material may not be just an inert component that allows the reuse of the enzyme but rather may play a role in the enzyme activity and stability.

Nanotechnology has emerged as a promising tool to overcome problems of using enzymes for industrial purposes, providing improved stability, higher activity, and protection against protease attack and minimizing solubility-related issues (Kim et al. 2010). Various nanobiocatalytic approaches, using nanostructured materials, such as magnetic nanoparticles, polymer nanofibers, and nanoporous materials, have been successfully used and tested as nanoscale reactors (Ge et al. 2009; Lei et al. 2002).Uniform and well-controlled nanopores provide a large pore volume and high surface area, which can be used for improved enzyme and peptides loading. The covalent bonds between polymer and enzyme, in addition to the enzyme cross-linking via glutaraldehyde (GA) treatment, result in an aggregate coating with exceptional success in enzyme stabilization (Singh 2010). Moreover, this system is also resistant to protease activity as the nanoporous media prevent the enzyme aggregate from leaching out and from being attacked by proteases in the fermenter (Kim et al. 2010), which enable it to be reused. Other outstanding examples include the enzyme immobilization and stabilization in superparamagnetic silica/iron oxide nanocomposites with structured porosity (Valdés-Solís et al. 2009), maghemite (γ-Fe2O3) nanoparticles functionalized with a reactive malfunctional polymer (Shukoor et al. 2008), and dendronized nanoparticles (Gustafsson et al. 2015), to cite some.

Adsorption of enzymes on nanomaterials can improve enzyme activity and thermal stability. Magnetic nanoparticles, with an iron oxide core, covered by different types of polymers, allow an easy recovery of immobilized proteins. Xylanase from *Aspergillus niger* immobilized onto Fe_3O_4-coated chitosan magnetic nanoparticles showed increased thermal stability compared to the free enzyme and retained 87.5 % activity after seven successive reactions by magnetic separation (Liu et al. 2014).

6 Conclusions and Research Needs

The demand for industrial enzymes is on a continuous rise driven by a growing need for sustainable and "greener" economy. Microorganisms play part as the largest source of an extraordinary amount of biocatalysts with a potential wide range of applications across several industries. Although numerous microbes inhabit the biosphere, the majority of them are reluctant to be cultivated under standard laboratory condition. Recent developments in metagenomics, in combination with other tools such as proteomics and recombinant DNA techniques for gene synthesis, have facilitated the discovery of new microbial enzymes from nature and their evolution to variants with improved catalytic properties.

Functional metagenomics has proven to be highly successful for mining the enormous microbial diversity and thus providing biomolecules that merge industrial criteria. However, the refinement of methods in metagenomics will be crucial for further success in areas of industrial biocatalysis. The identification of obstacles and solving problems to cloning and screening will aid in the development of new tools and technologies for functional metagenomics. Improving *E. coli* (and other

bacteria, fungi, and archaea) as screening host and exploring the potential of different organisms will help overcoming limits at the level of transcription and translation and likely improve future hit rates. High-throughput techniques for the screening of millions of clones and for the further evaluation of enzyme performance will allow identifying highly active, efficient, and promiscuous biocatalysts.

Once a new or improved activity has been successfully identified by metagenomics, the process of integrating them at industrial scale may still be interfered if the expression of the pure proteins does not provide sufficient amounts of enzyme at reasonable costs. Further, it is recognized that the final catalytic properties of a biocatalyst are mainly the result of substrate arrangement in its active site driven by its natural or evolved polypeptide sequence and structure, and the influence that the immobilization process, needed to reuse and stabilize the enzymes, additionally exerts. For this reason, when starting a metagenomic investigation, all these factors need to be taken into consideration.

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Evolving Enzymes for Biocatalysis

15

Uwe T. Bornscheuer and Robert Kourist

Contents

Abstract

This chapter covers concepts developed for the directed evolution of enzymes. The principle strategy is given in comparison to rational protein design followed by a description of the most prominent methods for creation of mutant libraries. Screening and selection strategies to identify the best hits in these libraries are presented followed by several assays developed for a range of enzyme classes. Finally, selected examples for the successful application of evolutionary methods to optimize biocatalysts are given.

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1 Introduction

The application of enzymes in biocatalysis has become increasingly important in the last decades for several reasons such as: (i) enzyme processes are in general more environmentally friendly as they save energy, and organic solvents and transition metals can be avoided; (ii) enzymes show high chemo-, regio-, and stereoselectivity making the outcome of a given reaction selective and predictable; (iii) access to new enzymes has been greatly facilitated due to major progress in microbiology and especially molecular biology; and (iv) novel tools for protein design have been developed, which substantially facilitate the creation of tailor-designed biocatalysts for a given reaction or process (Bornscheuer et al. 2012).

For the latter approach, two different (but increasingly used in a complementary manner) strategies have been developed: rational protein design or directed (molecular) evolution. A general scheme of both methods is given in Fig. 1. Rather than being alternatives, both approaches are usually combined in order to make an optimal use of the available knowledge of an enzyme. Recent examples show that an efficient focusing of the randomization is often more successful than a comprehensive randomization. This chapter focuses on evolutionary methods to design biocatalysts, but also rational protein engineering tools are covered, and examples for their successful application are given. For more details, readers are referred to a number of books and reviews (Arnold and Georgiou 2003a, b; Bornscheuer et al. 2012; Lutz and Bornscheuer 2008; Packer and Liu 2015).

The question whether rational design or directed evolution is the better strategy has often been discussed and no simple conclusion can be drawn. In fact, rational design is hampered by the complexity of proteins and limited by our knowledge of sequence-function relationships. Also, it often does not provide the desired result and yields sometimes very surprising variants, which can be far away from the needed property. For example, thermostability is difficult to predict rationally, and therefore directed evolution appears to be the better choice here (Dombkowski et al. 2014). Nevertheless, in cases where sufficient structural knowledge is available, focused randomization (Koudelakova et al. 2013; Reetz et al. 2009) is highly successful.

2 Evolutionary Methods

Directed evolution (also named *in vitro* evolution or molecular evolution) is a technique of protein alteration and selection of the fittest individual. Within just a decade, this technology has emerged as an extremely powerful and widely used tool to improve biocatalysts. In general, a directed evolution strategy is comprised of a random mutagenesis followed by a high-throughput screening and/or selection step (Fig. 1). The process begins with the choice of a starting enzyme – which must be available in recombinant form and having a suitable expression system at hand – and in the identification of the property that needs to be optimized. A large mutant library, as unbiased as possible, is prepared by random mutagenesis of the protein-

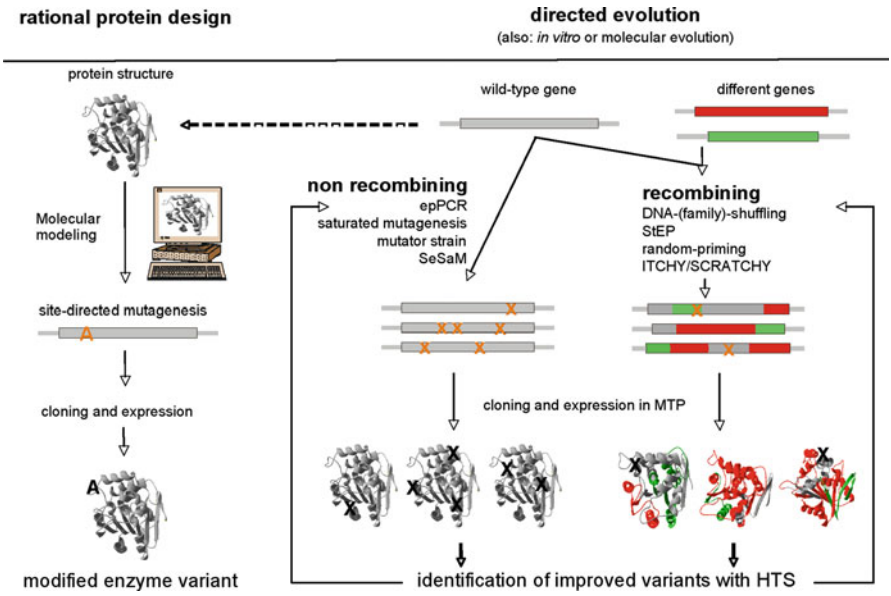


Fig. 1 A comparison of rational protein design and directed evolution. Rational design starts from a protein structure (or a homology model), from which key amino acid residues are identified and then modified by site-directed mutagenesis and finally verified for predicted properties. Directed evolution starts from one or several (homologous) genes, which are subjected to a range of random mutagenesis methods. From the resulting libraries of mutants or chimeras, desired variants are identified and verified by screening or selection

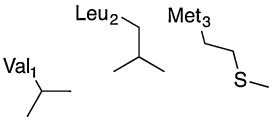
encoding gene, according to the techniques detailed below. After cloning and expression, a huge collection of enzyme variants typically in the range of 10^4–10^8 individuals is generated. This library is finally subjected to the identification of mutants with the evolved desired property using screening or selection methods. The best hits might subsequently serve as improved starting point for additional rounds of mutagenesis to accumulate beneficial mutations. Very often the information derived from mutant analysis can serve as a basis for additional rational protein design approaches.

Two different strategies for the generation of mutant libraries can be followed: (i) asexual (non-recombining) evolution, in which a parent gene is subjected to random mutagenesis to yield variants with point mutations, and (ii) sexual (recombining) evolution, in which several parental genes are randomly fragmented and then recombined to yield a pool of chimeras.

In the last 20 years, a plethora of methods (Bornscheuer et al. 2012; Lutz and Bornscheuer 2008; Packer and Liu 2015) have been developed, and some will be briefly described in the following paragraphs. As an aside, the term "directed evolution" was initially used because the method was inspired by natural evolution. However, both processes show considerable differences (Currin et al. 2015). Natural evolution is a highly complex mechanism in which the use of mutagenesis is

accurately controlled. Striking examples are fungal pathogens that use sexual recombination upon infection of their host mechanism, but are haploid during their saprophytic stage (Heitman et al. 2013). Similarly, highly accurate proofreading enzymes usually suppress spontaneous mutations during replication. Simultaneously, sophisticated and stringently controlled mechanisms generate a tremendous sequential and structural diversity, for which the antibodies from our immune system are a prominent case. A quick comparison to natural mutagenesis shows that the concepts covered in this chapter are far from the complexity of natural evolution. Nevertheless, random mutagenesis approaches coupled to high-throughput screens have proven their value as molecular optimization algorithms that are highly efficient for cases where a biocatalytic function is not fully understood. The most widely used asexual method is **e**rror-**p**rone **p**olymerase **c**hain **r**eaction (epPCR). Here, nonoptimal reaction conditions are used to create a mutant library (Caldwell and Joyce 1992; Leung et al. 1989). For example, increasing the Mg^{2+} concentration, adding Mn^{2+}, and usage of unbalanced dNTP concentrations can increase the error rate of the commonly used polymerase from *Thermus aquaticus* (*Taq*) from 0.001 to 1%. However, a homogeneous mutational spectrum (unbiased library) cannot be created using *Taq* DNA polymerase with Mn^{2+} and unbalanced nucleotides as the "polymerase or error bias" will result in a higher tendency to exchange the deoxynucleotides A and T compared to G and C. Improved DNA polymerases that exhibit a more homogeneous mutational spectrum are now commercially available, for example, Mutazyme™ I and II from Stratagene with a higher error rate, as well as Taq-Pol I614K (Patel et al. 2001) or Pfu-Pol (exo-) D473G (Biles and Connolly 2004). Another problem in epPCR is the so-called codon bias, resulting from the degenerated genetic code, which means that a specific amino acid change will be much less common than others. For example, a single nucleotide exchange in a valine codon results in only six alternative amino acid substitutions. Thus, the encoding of all 20 proteinogenic amino acids requires the exchange of all three (adjacent) codons, which is a rather unlikely event when an entire protein-encoding gene (i.e., 1,000 bp) is subjected to epPCR at a typical error rate of 1% (Neylon 2004). Despite these limitations, epPCR is still the method of choice for many researchers as it is rather easy to perform (Tee and Wong 2013) and is not protected by patents, which guarantees freedom to operate. Recently, combinations of directed evolution and rational design have been proposed, which have been named semi-rational design or rational evolution, but we prefer the term *focused directed evolution*. This includes methods such as **i**terative **s**aturation **m**utagenesis (ISM) (Brundiek et al. 2012; Miyauchi et al. 2011; Reetz et al. 2006) and CASTing (**c**ombinatorial **a**ctive-**s**ite saturation **t**est) (Reetz and Carballeira 2007; Reetz et al. 2006; Sandström et al. 2012). Both methods rely on the protein structure, but instead of distinct point mutations, several amino acids (usually near the catalytically active site in a radius of ~10 Å) are subjected to simultaneous random mutagenesis. This allows covering many more mutations and also includes cooperative effects of several neighboring point mutations. Table 1 shows the hypothetical diversification of a hydrophobic pocket as an example. A comprehensive simultaneous screening means a drastic effort for only three positions out of several hundred. Iterative

Table 1 Comparison of different mutagenesis approaches for the diversification of three adjacent hydrophobic amino acids forming a hydrophobic pocket. The close position of the three amino acids makes it likely that synergistic effects will have a strong influence on the function of this pocket



No	Approach	Library size for 95% coverage	Comment
1	Iterative saturation mutagenesis using NNK[a]-codon	3 rounds à 100 clones	300 is a best-case scenario; usually, several mutants need to be combined after identification of best substitutions
2	Simultaneous saturation mutagenesis using NNK-codon	98,304 clones	Useful when synergistic effects are expected; comprehensive for the chosen positions
3	Simultaneous saturation mutagenesis using NDT-codon[b]	5,184 clones	Only 12 representative amino acids in each position. The lower diversity can be justified by the reduced screening effort and thus the possibility to investigate a larger number of different position
4	Simultaneous saturation mutagenesis using VTK-codon	648 clones	Only Ile, Leu, Met, and Val covered

[a]K = A,C,G
[b]D = A,G,T

saturation mutagenesis is also comprehensive, but depends on decisions during the screening rounds (which variant is the best for the subsequent screening round?) and often fails to detect synergistic effects between neighboring mutations that would not be visible in iterative single-site mutagenesis (Gassmeyer et al. 2015). A promising strategy is to reduce the variety in the individual positions, either to a representative set or to a set with amino acids that are most likely to achieve an improvement (Sandström et al. 2012). If it is known that only hydrophobic amino acids will lead to active enzyme variants, the variety can be reduced to codons encoding for hydrophobic amino acids (Yoshida et al. 2015). Bioinformatics and structural considerations are highly useful tools to guide the mutagenesis. For instance, accurate structural alignments indicate which amino acids are usually present (and thus promising candidates) in the selected positions (Kourist et al. 2010). Consequently, the screening effort is substantially reduced compared to a classical directed evolution approach mutating the entire protein. Researchers at Codexis used protein structure-activity relationships (ProSAR), a similar approach. In comparison to CASTing, this approach is 3D-structure or homology model-independent, and this resulted in a substantial improvement in catalytic function (Fox et al. 2007).

When beginning from already selected variants or genes with sufficient homology, sexual recombination methods are preferred, which may combine the best features of

both parental donors. The first example was developed by Stemmer et al. (Stemmer 1994) and termed DNA- or gene shuffling. It consists of a DNAse I degradation and subsequent recombination of the fragments without primers (self-priming PCR) followed by a final PCR with primers. This method was further refined in the last decade and also named DNA family shuffling or molecular breeding. One alternative is the **st**aggered **e**xtension **p**rocess (StEP) developed by Zhao et al. (Zhao 1998). It consists of the amplification of short fragments of the parental genes, so that in subsequent cycles, the resulting short fragments can anneal on any other of the parental genes. Thus, the bias introduced by DNAse I digestion can be avoided. While shuffling of several variants of the same protein is straightforward (i.e., for the recombination of hits from other randomization methods), recombination of different genes bears the risk that essential amino acid interactions of the three-dimensional structure are destroyed. Bioinformatic approaches can guide the fragmentation of the proteins in order to minimize any disturbances of the structure (Meyer et al. 2006). In order to recombine several parental sequences without the need for high sequence homology, several methods have been developed. The first one was ITCHY (**i**ncremental **t**runcation for the **c**reation of **hy**brid enzymes) (Lutz et al. 2001; Ostermeier et al. 1999), which is based on the fusion of fragments of different sizes of two genes, which were generated by an exonuclease III and S1-nuclease digestion. Later, several improvements and variations were described (Lutz et al. 2001; Ostermeier and Lutz 2003; Udit et al. 2003).

3 Identification of Desired Variants

The key to a successful directed evolution experiment is a rapid and highly reliable assay system. Due to the huge number of possible variants, which are created in the random mutagenesis approach, desired hits can hardly be identified after individual expression of variants using classical analytical methods, such as gas chromatography or HPLC. Consequently, suitable high-throughput tools must be available to identify desired biocatalysts. The decisive criteria for such high-throughput screening systems are the number of variants to be screened, the signal-to-noise ratio, the reproducibility, and the practical effort needed to realize the screening (Acker and Auld 2014). All these technical parameters summarize our ability to break down a complex chemical reaction to a change of one single property that can be easily measured. The strategies can be subdivided into a selection or a screening approach, which are outlined below. Both approaches have their pros and cons, and a decision of which method is more appropriate has to be made case by case. A broader overview of recently described tools can be found in literature (Acker and Auld 2014; Reymond 2005).

3.1 Selection

Biological selections are based on complementation of auxotrophy or resistance markers that use tolerance toward cytotoxic agents such as antibiotics. If applicable,

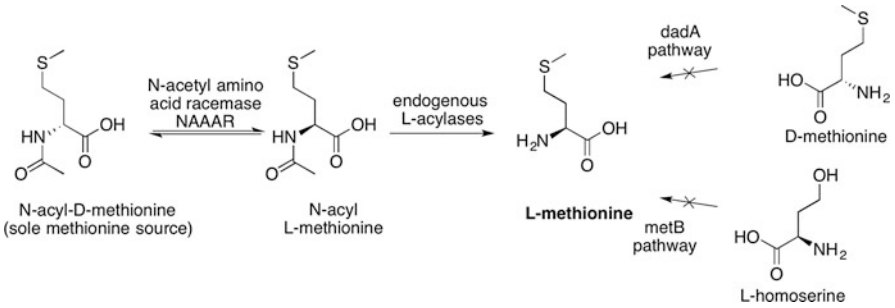


Fig. 2 Example for a complementation approach. Deletion of the dadA pathway and metB pathway resulted in an *E. coli* strain that cannot grow in media lacking L-methionine. Enzymes producing this metabolite confer a growth advantage to the cells. The selection assay was used for the optimization of an *N*-acetyl amino acid racemase (Baxter et al. 2012)

selection is a very powerful tool for screening large libraries and the discovery of protein mutants. However, the usage of phenotypic selection is usually limited to the isolation of catalysts for reactions that are of direct biological relevance or can be indirectly linked to a selectable phenotype. Such selections can be carried out *in vitro* or *in vivo* and either in solid phase or microtiter plates (MTPs). Mutagenesis techniques create libraries often ranging between 10^4 and 10^8 individuals. In order to screen a significant amount of sequence space, discarding inactive or uninteresting individuals can significantly reduce the size of the library and hence the efforts needed for identification of best hits.

Often, selection is performed as a complementation approach: only a mutated enzyme variant produces an essential metabolite (Fig. 2; Baxter et al. 2012). The main limitation of complementation assays is that the enzymatic reaction must be coupled to the cellular metabolism, which is usually only possible for reactions involving natural metabolites.

While selection assays often rely on simple experimental means such as agar plate assays, the use of a **f**luorescence-**a**ctivated **c**ell **s**orter (FACS) allows a vast throughput combined with an accurate characterization of the individual clones. Notably, FACS can be used both for screening and selection. By binding the (*R*)-enantiomer of a chiral carboxylic acid to a carbon source and the (*S*)-enantiomer to a toxic molecule, respectively, a selection assay was created that confers a growth advantage to cells bearing a highly (*R*)-selective esterase. Unselective variants produce the toxic by-product, whereas inactive variants do not release the carbon source (Fig. 3). Coupled to FACS selection, this assay proved to be successful to select esterase variants with higher enantioselectivity (Fernández Álvaro et al. 2011). Very recently, sophisticated ultrahigh-throughput systems were described for the discovery of desired enzyme variants (Bornscheuer 2016). One example uses a system containing up to 4 million individual *E. coli* cells per square inch, which are spatially separated in a microarray. A fluorescence readout allows to identify desired hits, which can then be retrieved through the use of a laser-based extraction system (Chen et al. 2016). In another example, metagenome-based libraries were analyzed using

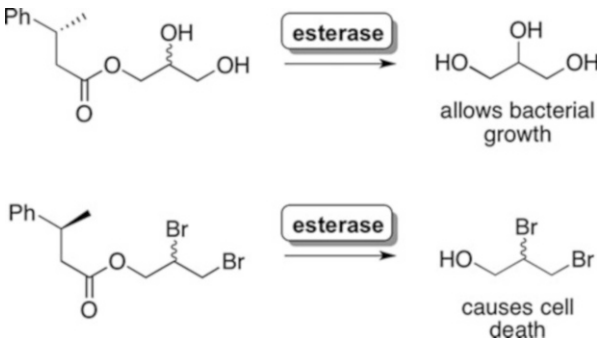


Fig. 3 Selection assay for the increase of the enantioselectivity of an esterase in the hydrolysis of chiral carboxylic acid esters

microfluidics using water-in-oil droplets of picoliter volume, which after addition of substrate and analysis of fluorescence could be sorted to identify promiscuous sulfate monoesterases and phosphotriesterases (Colin et al. 2015).

3.2 Screening

Many assays cannot be applied in a solid-phase format. Thus, individual clones must be grown and assayed in MTPs. These tests are significantly more time-consuming than solid-phase assays. However, by using robot automation and colony-picking technology, throughput can be substantially increased. Readers are referred to a recent example for a robotic platform used to identify improved enzymes from four different classes (Dörr et al. 2016). A major advantage is that screening provides significantly more information compared to a selection approach as the activity can be directly and quantitatively measured and even allow to determine the kinetics. Furthermore, screening enables the direct determination of the enantioselectivity of an enzyme, which is very often the key property, which needs to be improved. A large number of highly elegant assays were published, and some of them were successfully applied for directed evolution.

In all assays, it is advantageous to use the "true" compounds of interest compared to surrogates, i.e., nonnatural substrates designed to provide an intense, detectable signal when they are converted by the enzyme. In practice, however, colorimetric and fluorometric assays (i.e., releasing *p*-nitrophenol, fluorescein, resorufin, or coumarin after the enzymatic reaction) are undoubtedly the most widespread screens to determine enzyme activities in a high-throughput manner (Moore and Arnold 1996). For the determination of lipase/esterase activity and enantioselectivity, we developed an assay, in which acetates can be directly used as "true" substrate (Fig. 4). This method is based on a commercially available "acetic acid" test (R-Biopharm GmbH, Darmstadt, Germany), which couples the hydrolysis of acetates with an acetate-dependent enzymatic cascade leading to the stoichiometric formation of NADH (Baumann et al. 2001). If enantiomerically pure chiral (*R*)- and (*S*)-acetates are used in separate experiments – but using the same enzyme variant – the method allows the determination of the (apparent) enantioselectivity.

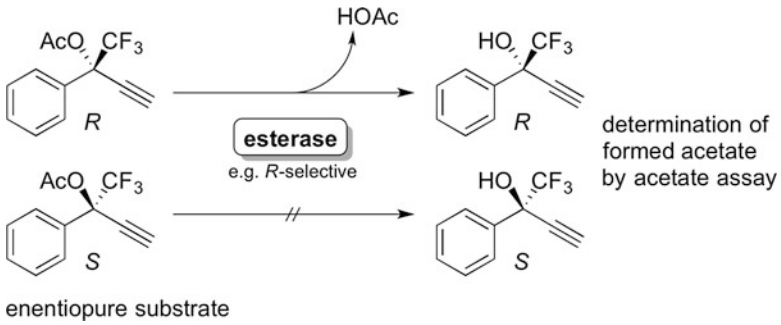


Fig. 4 Use of the enzyme-coupled acetate assay for the screening of esterase enantioselectivity in the hydrolysis of tertiary alcohol esters. Both enantiopure substrates are applied in separate wells

It was successfully used to identify an esterase variant with high enantioselectivity in the synthesis of 3-butyn-2-ol (Schmidt et al. 2006) and to completely invert the enantiopreference of an esterase in the hydrolysis of chiral tertiary alcohol esters (Bartsch et al. 2008).

Several elegant screens were developed by Reymond and coworkers, which are based on the release of resorufin or coumarin (Grognux and Reymond 2004; Leroy et al. 2003) or the formation of adrenochrome (Wahler et al. 2004; Wahler and Reymond 2002) and avoid the often observed instability of the enzyme substrate. Disadvantages are the need for synthesis of the specifically designed substrates and that only end-point measurements are possible rather than quantification of enzyme kinetics. A very simple and carefully optimized assay (Quick E) for hydrolases is based on the use of pH indicators and allows the rather accurate determination of enantioselectivity starting from the true substrate (Horsman et al. 2003; Janes and Kazlauskas 1997; Liu et al. 2001).For dehydrogenases, activity is commonly measured by cascade reactions to couple NAD(P)H generation with the formation of colored compounds. Examples are tetrazolium dyes like nitroblue tetrazolium (NBT) (Fibla and Gonzalezduarte 1993; Mayer and Arnold 2002). Oxidative activity can be monitored by coupling enzymatic H_2O_2 production to a horseradish peroxidase (HRP) reaction using ABTS as reagent (Yang and Shamsuddin 1996).

4 Examples

The majority of industrially used enzymes are hydrolases (65%), and thus many examples of evolved hydrolases can be found in literature. One of the first examples was an esterase variant from *Bacillus subtilis* (BsubpNBE) with 150 times higher activity in 15% DMF compared to the wild type, created by combining epPCR and shuffling. The enzyme is therefore applicable for the deprotection of a precursor in the production of the antibiotic loracarbef in the presence of DML as cosolvent (Moore and Arnold 1996). A related esterase (BS2), which differs only by 11 amino acids from BsubpNBE, was evolved by rational design in our group, and the enantioselectivity

toward the tertiary alcohol 2-phenyl-3-butin-2-yl acetate could be increased sixfold to E = 19 and toward linalyl acetate inverted from (*R*)- to (*S*)-preference with E = 6 (Henke et al. 2003). In a later study, this mutant (G105A) showed a good enantioselectivity toward 2-phenyl-3-butin-2-yl acetate (E = 54) in 20% v/v DMSO and an E-value of >100 toward the trifluoromethyl analogue (Heinze et al. 2007). Another mutant E188D gave similarly high enantioselectivity toward both substrates as well as a series of other tertiary alcohol acetates (Kourist et al. 2007). Using a focused random approach, we were also able to invert the enantioselectivity (Bartsch et al. 2008). While screening with the acetate assay described above, a double mutant (E188W/M193C) with an (*S*)-preference and an E-value of ~70 toward 1,1,1-trifluoro-2-phenylbut-1-yn-3-ol was identified. Notably, the single mutants E188W or M193C, which could have also been obtained by random mutagenesis, show only E = 16 for the (*S*)-enantiomer or low (*R*)-selectivity, respectively, and that only the combination, which would be rather unlikely to be obtained by "normal" random mutagenesis, results in the substantial inversion of enantioselectivity. This synergistic manner is thus an excellent argument for using focused directed evolution. Shortly after the first evolved esterase was described by the Arnold group, directed evolution of a lipase from *Pseudomonas aeruginosa* was reported by Reetz and coworkers. The initial enantioselectivity in the kinetic resolution of 2-methyl-decanoic acid *p*-nitrophenyl ester (MDA) was E = 1.1 (in favor of the (*S*)-acid), and after four rounds of epPCR, an E = 11 was obtained. Further improvements were achieved by combining mutations, using DNA shuffling and cassette random mutagenesis resulting in variants with high and practically useful enantioselectivities (Liebeton et al. 2000; Reetz and Carballeira 2007; Reetz et al. 2001).

The bacterial arylmalonate decarboxylase converts prochiral arylmalonic acids into optically pure arylaliphatic acids. While several (*S*)-arylpropionates possess pharmaceutical activity, the wild-type enzyme exclusively produces the non-desired (*R*)-enantiomers. The reason lies in the position of the catalytic residue C188 that catalyzes the stereospecific protonation of a planar intermediate (Fig. 5). On the basis of a homology model, Miyamoto et al. predicted that changing the position of the catalytic Cys to the opposite side of the active center would invert the enantiopreference. Indeed, the double mutant G74C/C188S produced the desired (*S*)-enantiomers in high optical purity. While the homology model correctly predicted the selectivity switch, it failed to anticipate that this variant had a drastically reduced activity (Ijima et al. 2005). Moreover, the model was not accurate enough to give clues for a rational strategy for activity recovery. In lieu of a crystal structure, epPCR was used to increase the activity of the (*S*)-selective variant tenfold (Terao et al. 2006). After the elucidation of the structure of the double mutant (Obata and Nakasako 2010), iterative saturation mutagenesis of a hydrophobic pocket recovered the activity 900-fold (Miyauchi et al. 2011). Simultaneous saturation mutagenesis using minimal codons such as VTK (*vide supra*) produced mutants with higher activity than the (*R*)-selective wild type in the synthesis of optically pure (*S*)-ibuprofen and (*S*)-flurbiprofen. Interestingly, the identified amino acid substitutions

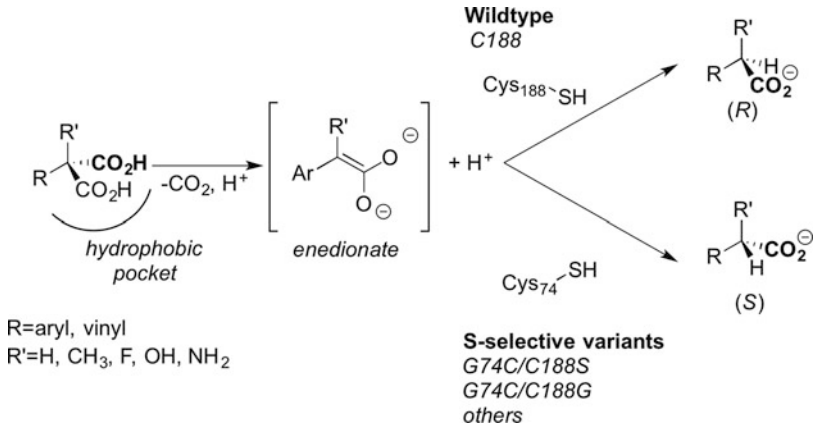


Fig. 5 Asymmetric synthesis of optically pure α-arylpropionates by arylmalonate decarboxylase and its variants

in the hydrophobic pocket resulted in a sixfold activity increase compared to the wild-type enzyme (Gassmeyer et al. 2016).

Directed evolution is a powerful technique to create new catalytic activities or to elevate activities not found in nature to synthetically relevant levels. A particular interesting example is the catalytic formation of carbon-silicon bonds (Kan et al. 2016). Despite the natural abundance of both elements, enzymes that catalyze this reaction have not been identified so far. Arnold and coworkers recently reported that heme proteins have the capacity to produce organosilicon compounds. The mechanism proceeds via a carbene insertion into silicon-hydrogen bonds. Interestingly, the substrate spectrum of the enzymatic reaction is broad, and chemoselectivity and stereoselectivity are very high in many cases. In a screening of several cytochrome c proteins, cyt c from *Rhodothermus marinus* showed outstanding stereoselectivity in the formation of C-Si bonds (Fig. 6). An analysis of the active site indicated that a methionine involved in the coordination of the heme group might be a promising target for saturation mutagenesis. Saturation mutagenesis of this residue resulted in a 12-fold increase of the total turnover number, which could be further increased by variation of two additional residues to a 36-fold improvement, resulting in total turnover numbers toward a series of substrates of $>1{,}500$, while the excellent stereoselectivity was conserved. While promiscuous or new enzyme activities can efficiently complement the catalytic toolbox, low activity of these catalysts is a serious limitation. The example of the cyt c from *R. marinus* highlights the tremendous potential of directed evolution techniques to create synthetically attractive enzyme variants.

In this light, it is a very interesting development that also the methodical scope of directed evolution was greatly increased. This is particularly true for enzymes that are poorly expressed in *E. coli*. Low expression levels simply prevented obtaining

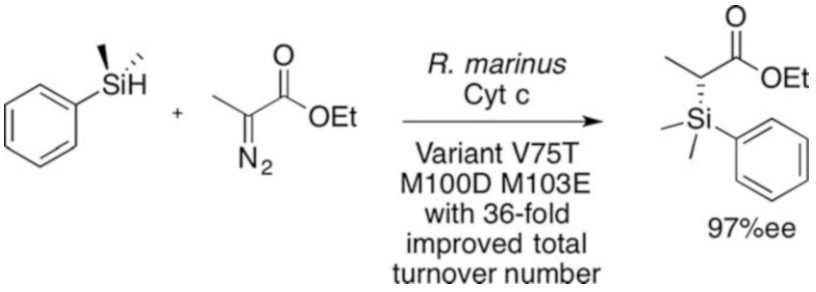


Fig. 6 Enzymatic formation of C-Si bonds catalyzed by engineered heme proteins

sufficient amounts in microtiter plate cultivation for high-throughput screening. Indeed, the lipase from *Pseudomonas aeruginosa* was chosen for the first directed evolution of enantioselectivity, simply because this enzyme is one of the few lipases that can be efficiently produced in bacteria (Liebeton et al. 2000; Reetz and Carballeira 2007; Reetz et al. 2001). The requirement for efficient expression in bacteria excludes a large number of highly interesting fungal exoenzymes such as laccases, lipases, and peroxygenases. Here, the presence of disulfide bonds and glycosylation lead to the formation of inclusion bodies in *E. coli*. Improved tools for the molecular biology of yeast expression systems efficiently removed this bottleneck. Use of an episomally replicating plasmid instead of genomic integration allowed a reliable expression of mutant libraries of lipase A from *C. antarctica* (CAL-A) in the yeast *Komagataella pastoris* (Engström et al. 2010; Sandström et al. 2009, 2012). This was used for the increase of the enantioselectivity toward several profens. In particular, a variant with enantioselectivity toward ibuprofen was achieved by simultaneous saturation mutagenesis of nine residues in the active site. Lipase CAL-A is a case where periplasmatic expression in *E. coli* (Brundiek et al. 2012) is also possible (which was used for an increase of its *trans*-selectivity by saturation mutagenesis), but where expression of the libraries in yeasts yields much higher enzyme concentrations. The expression of many fungal peroxygenases, however, has so far not been achieved. The capacity of these hydrogen peroxide-dependent enzymes to catalyze oxyfunctionalizations without need for nicotinamide cofactors and their outstanding stabilities make these fungal exoenzymes highly attractive biocatalysts. The development of a yeast expression system for a peroxygenase from *Agrocybe aegerita* by Miguel Alcalde and coworkers allowed for the first time saturation mutagenesis of this enzymes, resulting in a double mutant G241D/R257K with twofold increased peroxygenase activity, reduced peroxidative activity, and high regioselectivity for the formation of 1-naphtol (Fig. 7) (Molina-Espeja et al. 2014, 2016). Similarly, yeast expression systems made also laccases amenable for directed evolution (Mate and Alcalde 2015). These examples underline the importance of efficient expression systems for the directed evolution of enzymes that are difficult to express in *E. coli.*

The methodologies developed for directed evolution within just two decades for mutagenesis, screening, and selection together with the examples given clearly

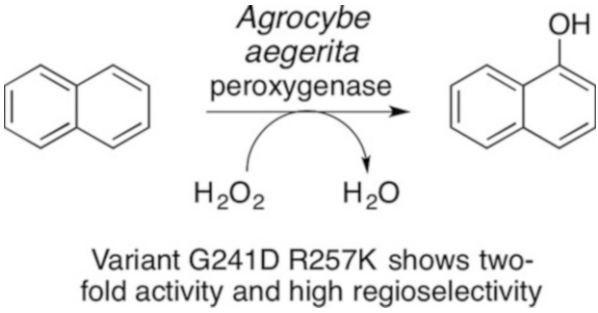


Fig. 7 Enzyme expression in yeast made the directed evolution of unspecific peroxygenase from *Agrocybe aegerita* possible

demonstrate that protein engineering is now substantially facilitated using evolutive methods. Furthermore, this tool already found its way into various industrial applications. The trend now clearly goes into the combination of directed evolution with rational protein design.

5 Research Needs

The challenges for protein engineering change follow the changing requirements for catalytic applications of enzymes. While biocatalysis until the early 2000s focused mostly on single-enzyme reactions, the assembly of several enzymes to enzyme cascade reactions greatly expands the catalytic possibilities (Enoki et al. 2016; Schmidt et al. 2015; Schrewe et al. 2013). Similarly, the progress in molecular biology has greatly facilitated whole-cell biotransformations (Köninger et al. 2016; Schmidt et al. 2015) and allows systematic and quantitative approaches in the so-called systems biotechnology. While the combination of enzymes from the same pathway is straightforward, cascades using enzymes from different organisms often have a limited scalability due to the different kinetic parameters and the occurrence of side reactions. The integration into artificial enzyme cascades and the suppression of side reactions pose very specific requirements to the kinetic parameters and the chemoselectivity of enzymes. Moreover, the engineering of the microenvironment of catalysts by scaffolding and compartmentalization extends the scope of protein engineering to multienzyme systems. From a methodological viewpoint, despite the availability of a broad set of mutagenesis methods, there is a further need for easy-to-use tools to create mutant libraries, which should contain all 20 proteinogenic amino acids in a highly balanced manner. Decreasing prices for synthetic genes and DNA libraries will fuel new mutagenesis strategies. The recombination of genes with low sequence homology and/or high G + C content is still challenging, and solutions are required to apply evolutionary methods more effectively. A constant need exists for screening and selection. Despite the progress made in the past decade, in most cases the development of suitable assays is the major bottleneck in directed evolution experiments especially as for each enzyme type to be studied, a tailor-designed method is usually needed.

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Screening for Enantioselective Enzymes

16

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Abstract

A wealth of novel enzyme genes is accessible from natural sources, from genome and metagenome sequencing projects, and from libraries created by directed evolution. However, the identification of novel enzyme activities with a high potential for biotechnological applications still represents a major bottleneck. Here, assays are needed allowing to screen for enzyme activities and enantioselectivities. Hence, we describe several assays carried out on agar plates or in solution which are based on photometry, fluorometry, chromatography, electrophoresis, spectrometry, and spectroscopy.

1 Introduction

The catalytic asymmetric synthesis of enriched as well as enantiomerically pure organic compounds is of rapidly growing academic and industrial interest (Drauz and Waldmann 2002; Liese et al. 2006). Two major groups of catalysts are commonly used for the asymmetric production of chiral compounds: (1) chiral synthetic catalysts (e.g., transition metal complexes) (Rothenberg 2008; Sheldon et al. 2007) and (2) biocatalysts, used as whole microbial cells or as isolated enzymes (Illanes 2008; Polaina and MacCabe 2008; Fessner and Anthonsen 2009). In addition to progress in biotechnological engineering (Liese et al. 2006), two important developments resulted in a significantly increased number of enzymes available for asymmetric catalysis: (1) the creation of enantioselective enzymes by directed evolution was demonstrated (Arnold and Georgiou 2003; Brakmann and Johnsson 2002; Brakmann and Schwienhorst 2004; Liebeton et al. 2000; Reetz et al. 1997; Reetz 2004) and (2) metagenomics allow to access a huge number of novel enzymes without the need to culture microorganisms (Beloqui et al. 2008; Ferrer et al. 2005; Steele et al. 2009). Both approaches generate a large number of enzyme genes, which usually need to be functionally expressed and the best candidates must be identified from 10^6 or even more samples within a reasonable time frame, ideally 1 or 2 days. To cope with this challenge, a set of different medium- and high-throughput methods was developed to evaluate enzymatic activity as well as enantioselectivity (Reymond 2006; Liebl et al. 2014; Classen et al. 2017). This chapter will give a short overview of well established assay systems which we have classified according to the analytical method used, namely visual inspection of agar plates, photometry, fluorometry, chromatography, electrophoresis, spectrometry, and spectroscopy, and enzyme fingerprinting approaches.

2 Photometric and Agar Plate Assays

Photometric and agar plate assays are inexpensive to operate, require minimal equipment, are easy to handle, and capable to analyze thousands of samples per day. Hence, these methods are preferably used in directed evolution and

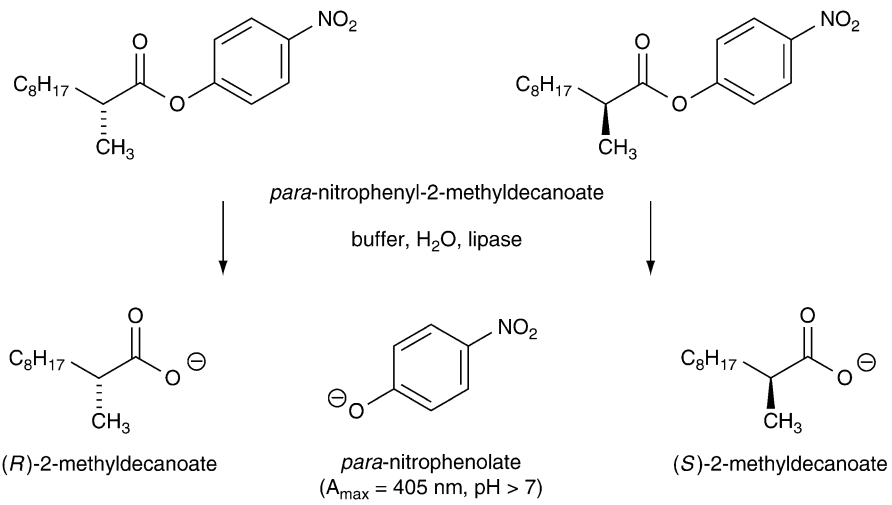

Fig. 1 *p*NP-ester-based assay (Reetz et al. 1997)

metagenomic projects, especially for prescreenings to identify active enzymes (Reetz 2006; Reymond 2006)

2.1 *p*NP-Ester-Based Assays

Photometric assays based on the hydrolysis of *para*-nitrophenyl esters (*p*NP-esters) represent simple and practical methods to determine lipolytic activities, but also enantioselectivities. All *p*NP ester-based assays have in common, that upon hydrolysis of the substrate in buffered solutions (pH > 7) *para*-nitrophenolate is released which shows a strong UV/Vis absorption at 405 nm (Fig. 1). Thus, activities and enantioselectivities can be calculated by measuring absorption as a function of time (typically during the first 8–10 min). Hydrolytic activities are determined with achiral *p*NP-esters (e.g., *p*NP-acetate or *p*NP-palmitate) (Winkler and Stuckmann 1979) whereas enantioselectivity determinations require chiral *p*NP-esters such as *rac*- *p*NP-2-methyl decanoate (Reetz et al. 1997). However, kinetic resolution of the racemate only allows to determine the overall reaction rate. This problem was solved by using enantiomerically pure (*R*)- and (*S*)-esters separately which enables the calculation of an apparent selectivity factor E_{app}. Usually, 48 enzyme variants can be tested simultaneously in a 96-well microtiter plate within a few minutes (Reetz et al. 1997). This assay was the first high-throughput assay (HTS-assay) suitable to determine enantioselectivities, however, it suffers from two major drawbacks: (1) the enzymes identified are those optimized to hydrolyze *p*NP-esters, which are normally not used in industrial applications; and (2) the (*R*)- and (*S*)-substrates are used in separate vessels, i.e., the enzymes do not compete for the two substrates, which may distort the results and requires to confirm the enantioselectivities determined for the best enzyme variants using other methods like GC or HPLC (Reetz 2006).

Therefore, a spectrophotometric assay named Quik *E* test was developed to measure enantioselectivities of hydrolases more accurately (Janes and Kazlauskas 1997). Competitive conditions were simulated by using a mixture of one enantiomer of a chiral *p*NP-ester and a resorufin ester taking the "role" of the second enantiomer. Both hydrolysis reactions are monitored by determination of the UV/Vis absorption of the two products, namely *para*-nitrophenolate and resorufin, at 405 nm and 570 nm. The same reaction is performed with the other enantiomer of the chiral *p*NP-ester. Although this method enables a more precise *E*-value determination, it still identifies enzyme variants evolved toward optimized hydrolysis of *p*NP-esters.

Recently, a modified *p*NP-assay was used to determine the synthetic activity of lipases in organic solvents (Teng and Xu 2007). Lipase-catalyzed transesterification reactions between *p*NP and ethanol in *n*-heptane were performed yielding in the formation of ethyl palmitate and *para*-nitrophenolate. Subsequently, the synthetic lipase activity was detected by extraction of *para*-nitrophenolate with 0.1 M NaOH and spectrophotometric determination at 410 nm (Teng and Xu 2007). Another spectrophotometric approach to measure the synthetic activity of lipases in organic solvents has been developed by Goujard and coworkers (2009). As model reactions the transesterification between vinyl stearate and pentanol in hexane at 30 °C or in decane at 50 °C has been performed. The conversion of vinyl stearate (A_{max} = 200 nm) to pentyl stearate was monitored through decreasing UV absorbance at 200 nm.

2.2 Enzyme-Coupled Assays

The anticipated results of any directed enzyme evolution experiment follow the general rule: "you get what you screen for" (Schmidt-Dannert and Arnold 1999). Thus, enzymes identified by assays using *p*NP-esters as well as other substrates bearing fluorogenic moieties or UV/Vis chromophores are not necessarily optimized toward a biotechnologically important or even the same, but chromophore-free substrate. To overcome this problem an enzyme-coupled assay can be used, in which one product of the enzymatic conversion of interest can be transformed by another enzyme into a secondary product which gives rise to a spectroscopic signal (Reetz 2006). An assay allowing to quantify *ee*-values was developed for the hydrolase-catalyzed kinetic resolution of chiral acetates (Baumann et al. 2001). Here the acetic acid formed in the hydrolysis of a chiral acetate is converted by acetyl-CoA synthetase to acetyl-CoA in the presence of ATP and coenzyme A. Subsequently, citrate synthetase catalyzes the reaction between acetyl-CoA and oxaloacetate to give citrate. The required oxaloacetate is formed from l-malate and NAD^+ in the presence of l-malate dehydrogenase, also forming as a side product $NADH + H^+$. Hence, the enzymatic reaction cascade stoichiometrically transforms chiral acetates into $NADH + H^+$; initial rates of acetic acid formation can be determined by the increase in adsorption at 340 nm. Furthermore, the apparent enantioselectivity E_{app} can be determined by using optically pure (*R*)- or (*S*)-acetates.

Nevertheless, the kinetic resolution of (*S*, *R*)-1-methoxy-2-propylacetate revealed that precise *E*-values could only be obtained for low enantioselectivities ($E = 1.4–13$), whereas at higher *E*-values ($E = 80$), the uncertainty was ±20% (Baumann et al. 2001).

The concentration of an enantiomerically pure product must be determined to calculate *ee*-values. Usually, HPLC or GC measurements are employed at conversion rates less than 100%. As an alternative, an enzyme-coupled assay was developed that allows *ee* determinations independent of the product concentration for reactions yielding chiral alcohol products (Li et al. 2004). Here, two highly enantioselective alcohol dehydrogenases were used each oxidizing one enantiomer of the product alcohol. The respective reaction rates were determined by measuring the increase in absorption at 340 nm caused by formation of $NAD(P)H + H^+$. Separate oxidation reactions with each chiral alcohol as well as with mixtures of both enantiomers in known ratios finally yield kinetic constants which can be used to calculate *ee*-values (Li et al. 2004).

2.3 pH-Indicator Assays

The hydrolysis of an ester by a lipase or an esterase results in the formation of an acid causing a decrease in pH. Thus, hydrolysis of two enantiomeric ester substrates can be followed by using appropriate pH-indicators. However, it should be noted that the pK-values of the pH-indicator (pK_i) and of the buffer (pK_b) used should be in the same range. Suitable indicators include *p*-nitrophenol (Janes et al. 1998), bromothymol blue (Morís-Varas et al. 1999; Wang et al. 2009), or tetra sulfonatophenyl porphyrin (Vinod Kumar et al. 2006); the respective color changes upon protonation can be monitored photometrically and *ee*-values calculated.

2.4 Agar Plate Assays

Agar plate assays represent the method of choice for easy and inexpensive activity screening in large libraries of enzymes (Turner 2006). Examples are the tributyrin (Lawrence et al. 1967) and rhodamine B plate assays (Kouker and Jaeger 1987) which are well established to determine hydrolytic activities of esterases and lipases constituting the most important group of biocatalysts for biotechnological applications (Jaeger and Eggert 2002). Tributyrin, a triglyceride substrate accepted by many lipolytic enzymes, is added to agar plates resulting in a turbid emulsion. Microorganisms which produce a lipolytic enzyme hydrolyze this substrate leading to the formation of clear halos around the colonies. Rhodamine B is a fluorescent dye which is added to agar medium additionally containing the lipase substrate triolein. Lipolytic activity is detected by UV-illumination (350 nm), because free fatty acids released from the triglyceride substrate form orange fluorescent adducts with the dye rhodamine B (Birner-Grünberger et al. 2006).

3 Fluorimetric Assays

Methods using fluorogenic substrates form a cornerstone within the portfolio of enzyme assays currently available (Reymond et al. 2009). They are usually very sensitive, less susceptible to compounds generating background signals, and the produced signal is directly related to the enzyme-catalyzed reaction. Nevertheless, fluorogenic substrates are usually not identical with the substrate of interest in a biotransformation, which can lead to erroneous results. Furthermore, those substrate esters are often unstable at high or low pH values and/or elevated temperatures and only work in aqueous solutions (Reymond 2009; Schmidt and Bornscheuer 2005).

3.1 Umbelliferone Assay

An elegant variation of the classical umbelliferone test (de Laborde de Monpezat et al. 1990) was developed by Reymond and coworkers to broaden its usability toward enantioselectivity determinations (Klein and Reymond 1999). Non-fluorescent chiral ethers of umbelliferone were used in a sequence of three coupled biocatalytic steps involving the hydrolytic enzyme of interest, a horse-liver alcohol dehydrogenase for the oxidation step, and bovine serum albumin accelerating the liberation of the fluorescent product by β-elimination (Fig. 2a). In the first step, the (*R*)- and (*S*)-substrate esters can be subjected separately to a lipase- or esterase-catalyzed hydrolysis. Subsequently, the fluorescence of umbelliferone released from both enantiomers can be determined giving the relative amounts of (*R*)- and (*S*)-alcohols. The determination of enantioselectivities of 20 different lipases and esterases with this assay revealed that the predicted and observed *E*- and *ee*-values matched within a range of $\pm$20% (Badalassi et al. 2000).

3.2 Resorufin Assay

Another simple and practical fluorescence assay using chiral resorufin esters was described to identify *P. fluorescens* esterase variants with enhanced enantioselectivities (Henke and Bornscheuer 1999). Optically pure resorufin-labeled (*R*)- and (*S*)-phenylbutyrate was subjected separately to esterase-catalyzed hydrolysis releasing (*R*)- and (*S*)-3-phenylbutyric acid as well as the fluorescent product resorufin (Fig. 2b). This method allows the determination of apparent enantioselectivities (E_{app}) as the hydrolysis of the two enantiomers has to be performed separately (Schmidt and Bornscheuer 2005). However, these E_{app}-values were in good agreement with the true enantioselectivities (E_{true}) (Henke and Bornscheuer 1999).

In this context, it should be noted that many fluorimetric assays suffer from the fact that the substrates are often insoluble in aqueous solutions and also show a high level of nonspecific hydrolysis with noncatalytic proteins (Beisson et al. 2000). To circumvent these general drawbacks monoalkylated as well as monoacylated

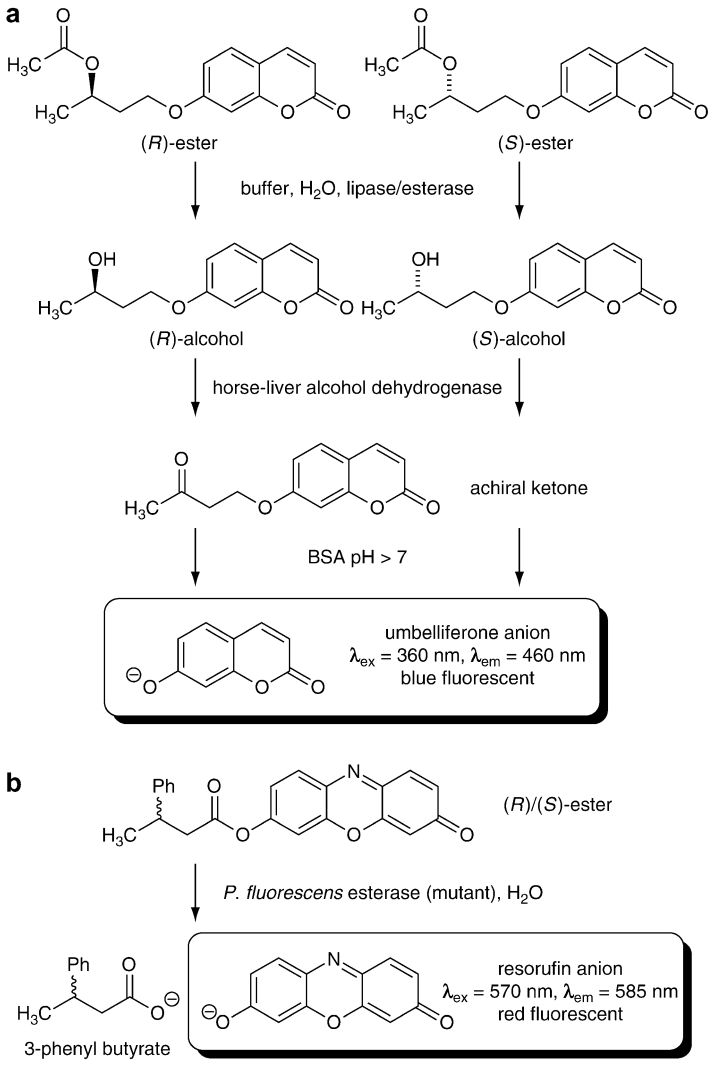

Fig. 2 Fluorimetric assays: (**a**) umbelliferone assay (Klein and Reymond 1999); (**b**) resorufin assay (Henke and Bornscheuer 1999)

fluorescein-derived substrates were synthesized which are highly reactive and stable in lipase and esterase assays performed in aqueous solutions (Yang et al. 2006).

As a variation of conventional fluorescence assays, the first high-throughput assay for hydrolase synthetic activity based on fluorescence detection was developed by Bornscheuer and coworkers (Konarzycka-Bessler and Bornscheuer 2003). The so-called NBD-H assay is based on the transesterification between an alcohol and a vinyl ester of a carboxylic acid. Acetaldehyde generated from the vinyl alcohol by keto-enol

tautomerization is reacted with a hydrazine (NBD-H: 4-hydrazino-7-nitro-2,1,3-benzoxadiazole) to the corresponding hydrazone ($\lambda_{Ex} = 485$ nm, $\lambda_{Em} = 520$ nm) which can be detected by measuring its fluorescence.

4 Chromatographic Assays

Chromatographic methods enable enantioselectivity determinations of enzyme-catalyzed reactions using true substrates which do not contain chromophores or fluorophores. Unfortunately, conventional gas (GC) or liquid chromatography (HPLC) based on chiral stationary phases can only handle a few dozen samples per day (Reetz 2006). A medium-throughput GC-assay enabling the fairly exact determination of 700–800 *ee*- and *E*-values per day was developed to screen for improved *P. aeruginosa* lipase variants catalyzing the kinetic resolution of racemic 2-phenyl-1-propanol to yield the corresponding acylated alcohols (Reetz et al. 2001). A setup consisting of two simultaneously operating GC-units (stationary phase of the columns based on the *β*-cyclodextrin derivative 2,3-di-*O*-ethyl-6-*O*-*tert*-butyldimethylsilyl-*β*-cyclodextrin), one prep-and-load manager, and one PC were used (Reetz et al. 2001). Later, this system was extended to three simultaneously operating GC-units to analyze enantioselectivities of cyclohexanone monooxygenases in Baeyer-Villiger reactions (Reetz et al. 2004a).

HPLC can also be exploited for medium-throughput *ee*-analyses, but the traditional setups had to be adapted to achieve the evaluation of several hundred samples per day. A successful HPLC-based *ee*-assay was established to analyze the enantioselectivities of cyclohexanone monooxygenase variants used for the asymmetric O_2-mediated oxidation of prochiral thio-ethers. The device consisted of a commercially available HPLC-unit equipped with a sample manager and an appropriate software to handle 96- or 384-well microtiter plates (Reetz et al. 2004b). An efficient system for the rapid analysis of the above mentioned reaction turned out to be benzoylated cellulose as a stationary phase with *n*-heptane and ethanol as mobile phases in combination with a short column (50 mm in length, 4.5 mm in diameter). This setup allowed the rough determination of at least 800 *ee*-values per day (Reetz et al. 2004b). For a more precise *ee*-determination of the identified hits, a conventional HPLC with a longer column (250 mm) was used subsequently (Reetz et al. 2004b).

5 Electrophoretic Assays

The major disadvantage of chromatography-based assays pertains to the fact that they cannot be used in a high-throughput manner analyzing thousands of samples per day (Reetz 2006). Instead, capillary array electrophoresis (CAE) turned out to be a reasonable alternative for high-throughput analysis of enzyme enantioselectivities (Reetz et al. 2000). Traditional capillary electrophoresis (CE) can serve to separate enantiomers by using an electrolyte that contains chiral selectors (e.g., *β*-cyclodextrin, chiral crown ethers, chiral surfactants, linear polysaccharides) (Blanco and

Valverde 2003). While this technique allows only a few dozen *ee*-determinations per day, the instrumental miniaturization nowadays available made the high-throughput application of CE possible.

For CAE-based assays, commercially available instruments have been developed, which contain a high number of parallel arranged capillaries (Reetz 2006). Miniaturization of the CAE instruments resulted in a rapid scale-up in complexity and density from 12, 48, 96, to finally 384 lanes within only a 5-year period (Paegel et al. 2005), enabling super-high-throughput *ee*-determinations. In this context, Reetz and coworkers used a 96-capillary unit (MegaBACE®, GE Amersham) to study chiral amines that are potentially accessible by enzymatic reductive amination of ketones, addition of ammonia to activated olefins or hydrolysis of acetamides (Reetz et al. 2000; Reetz 2006). Derivatization of the chiral amines of interest with fluorescein, separation by using several β-cyclodextrin derivatives as chiral selectors, and finally detection by laser-induced fluorescence (LIF) (Reetz et al. 2000) is needed. The agreement between *ee*-values determined by CAE and those of the corresponding (*R*)/(*S*)-mixtures measured by GC turned out to be excellent (Reetz et al. 2000). Appropriate optimization resulting in shorter analysis times for enantiomer separation may result in a daily throughput of 15,000–30,000 *ee*-determinations (Reetz 2006). An additional cheap variation is provided by CAE on microchips (Reetz et al. 2000).

Recently, an elegant Lab-on-a-Chip technique was developed combining a microfluidic reactor and a CE-based analysis unit on a single device which allowed the determination of *ee*- as well as *E*-values (Belder et al. 2006).

6 Spectroscopic and Spectrometric Assays Using *Pseudo*-enantiomers

In an achiral environment, enantiomers obviously have identical spectroscopic property and thus cannot be distinguished by conventional techniques. However, labeling or tagging of substrates prior to kinetic resolution of racemates or desymmetrization of prochiral compounds bearing enantiotopic groups enables their subsequent spectroscopic detection (Fig. 3). Here, isotope labels are most commonly used starting from *pseudo*-enantiomers or *pseudo-meso*-compounds, respectively.

While the enantiomerically pure substrates with and without the label must be provided first, the overall screening process is convenient since no additional derivatization is necessary. Mass spectrometry (MS), nuclear magnetic resonance (NMR), as well as Fourier transform infrared (FTIR) spectroscopy was applied in assays allowing 1,000–10,000 samples to be measured per day.

MS-based approaches are especially practical since the *pseudo*-enantiomeric compounds are directly distinguishable *via* their different masses, a method that was utilized for the determination of the configuration of secondary alcohols (Horeau and Nouaille 1990). High-throughput applications of isotope labeling for *ee*-assays were first reported in 1999: Hydrolase-catalyzed hydrolysis and esterification of *pseudo*-enantiomers and *pseudo-meso*-compounds (Fig. 4) were accessed

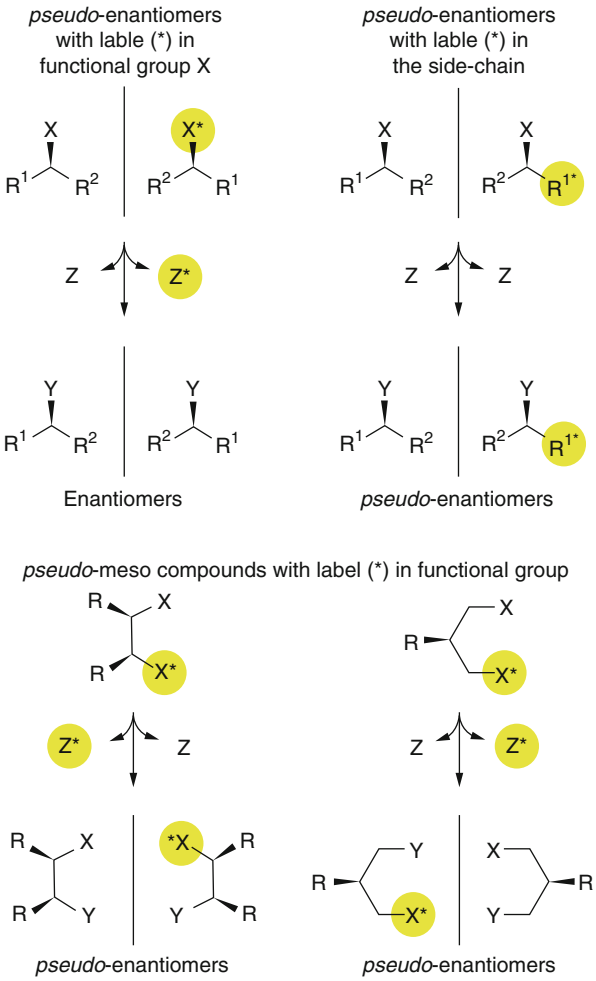

Fig. 3 *Pseudo*-enantiomers lead to enantiomers (label in functional group) or again to *pseudo*-enantiomers (label in side chain); *pseudo-meso*-compounds give *pseudo*-enantiomers that can be spectroscopically or spectrometrically distinguished

comparing the *ee*-values as determined by integrating the respective MS peaks with more accurate GC data (Reetz et al. 1999). The uncertainty in the *ee*-value was approximately ±5%. While any type of ionization can be employed, electrospray ionization (ESI) is commonly the method of choice.

The concept was extended, e.g., to nitrilase (DeSantis et al. 2003) and epoxide hydrolase (Cedrone et al. 2003; Reetz et al. 2004d) assays (Fig. 5). Not only the proof of concept was demonstrated, but applications in directed evolution projects were reported (Funke et al. 2003; Zha et al. 2003). Screening of up to 10,000 samples per day was possible when using a parallel eight-channel multiplex ESI-MS system (Schrader et al. 2002).

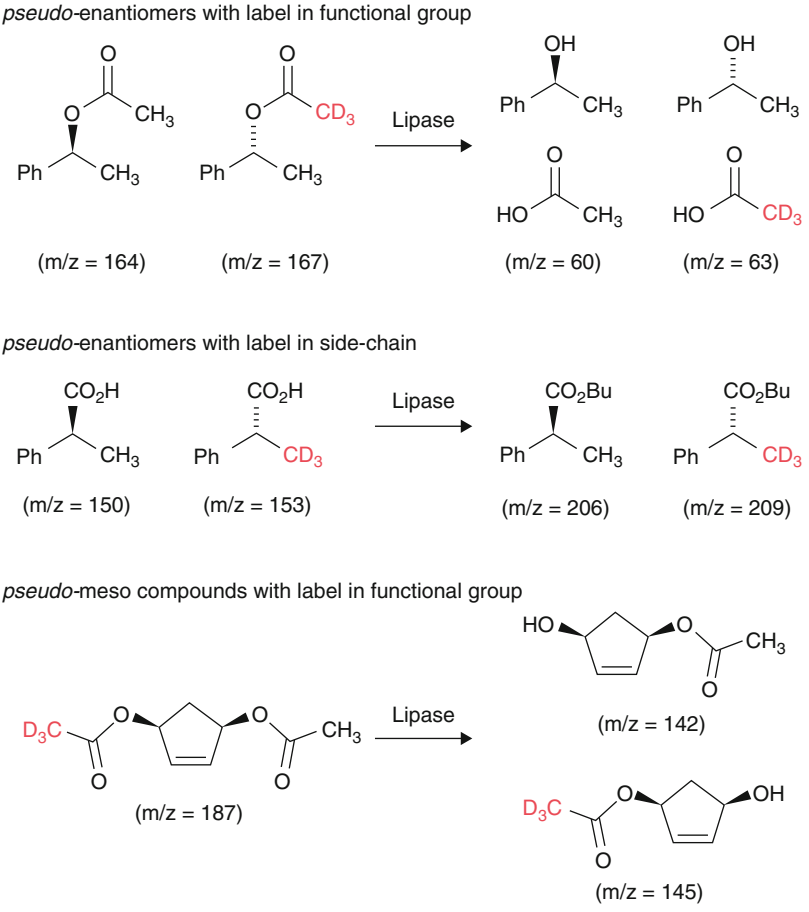

Fig. 4 MS-based assay for lipases (Reetz et al. 1999)

Technological improvements in recent years allowed speeding up NMR measurements: Designed flow-through NMR cells applied in combinatorial chemistry were utilized for distinguishing *pseudo*-enantiomers (Reetz et al. 2002). ^{13}C-labeling was shown to be practical for a ^{1}H NMR spectroscopic assay. While any carbon could be used for the label of either the (*S*)- or the (*R*)-enantiomer, an isolated methyl-group is most convenient; the CH_3-group does not show a ^{1}J coupling, hence giving a singlet, while the $^{13}CH_3$-group does lead to a doublet. The syntheses of the enantiomerically pure references – eventually simulating the racemate – is straight forward in the depicted cases (Fig. 6) since labeled acetyl chloride as well as methyl iodide needed for the derivatization are readily available. Transformations of

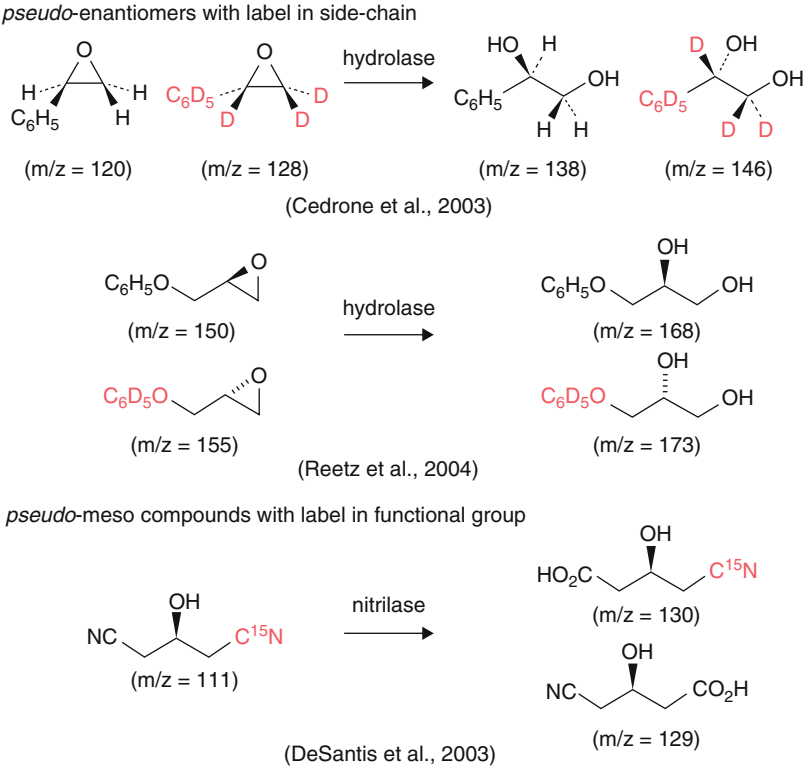

Fig. 5 Enantioselectivity assay for hydrolases based on MS spectrometry

prochiral compounds not involving enantiotopic groups can either be analyzed by traditional derivatization methods, e.g., using Mosher's reagent (Dale and Mosher 1973), or by adequately labeling the side chains as demonstrated in an assay screening for enantioselective reduction catalyst (Evans and Morken 2002). With the first generation set up, approximately 1,400 samples could be evaluated per day (accuracy ±5%); however, adopting the system to chemical shift imaging could increase the number by a factor of at least 4 (Reetz et al. 2004c).

A relatively low price alternative is Fourier transform infrared spectroscopy: While the accuracy is lower (approximately ±7%), a relatively high number of *ee*-values could be determined per day (up to 10,000). Again, a carbonyl group (as the functional group) is the ideal position for the label; the corresponding stretching vibration is intense and in a spectral region which is typically not interfering with other groups. The higher mass of the ^{13}C-labeled carbonyl compound – readily prepared from 1- ^{13}C-acetyl chloride – leads to a shift of the corresponding stretching band of 40–50 cm^{-1} to lower wave numbers (Fig. 6). Different kind of samples can already be analyzed on multiple 96- or 384-well microtiter plates automatically (Tielmann et al. 2003).

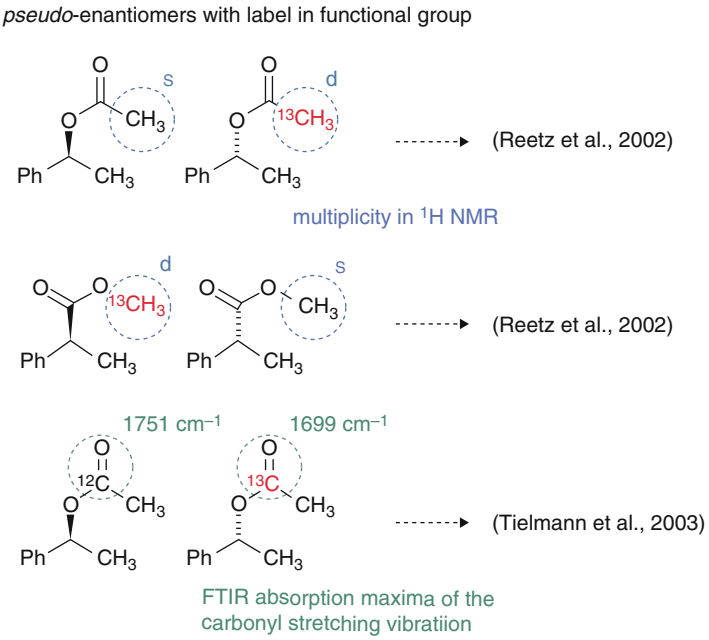

Fig. 6 Enantioselectivity assays based on ^{1}H NMR and FTIR spectroscopy

7 Enzyme Fingerprinting

The information content of conventional enzyme assays can be increased by simultaneous analysis of multiple substrates (Reymond 2009). A high-throughput determination of activity as well as enantioselectivity of a single enzyme under various experimental conditions or with a series of different substrates results in a reactivity profile that can be defined as an enzyme fingerprint (Reymond 2006; Wahler et al. 2001). Such a fingerprinting approach can be used to: (1) identify a specific enzyme of interest; (2) distinguish it from other closely related enzymes; and (3) as a tool for diagnostic and quality control of enzyme-containing samples (Reymond and Wahler 2002). The first multi-substrate analysis method named the APIZYM system was developed as a tool for classification and identification of microorganisms (Buissière et al. 1967). This assay, previously named Auxotab, uses a series of different chromogenic enzyme substrates to identify microorganisms on the basis of their expressed enzyme activities (Humble et al. 1977; Reymond 2009). Meanwhile, several enzyme fingerprinting assays have been developed focusing on the analysis of single enzymes (Reymond 2009; Reymond et al. 2009), namely parallel assays in microtiter plates, cocktail fingerprinting, and substrate microarrays.

7.1 Parallel Assays in Microtiter Plates

Microtiter plate assays using multiple chromogenic or fluorogenic substrates have been mostly used for hydrolase profiling (Liu et al. 2001; Wahler et al. 2001, 2002, 2004). Microtiter plates offer a sufficient number of small volume reactors to screen thousands of samples (Reymond and Babiak 2007). Using this approach, fingerprinting analysis of lipases as well as esterases with both enantiomeric forms of 16 different periodate-activated fatty acid ester substrates revealed that lipases were more active on long-chain substrates whereas esterases preferably hydrolyzed short-chain substrates. Moreover, a selectivity for intermediate chain length substrates was apparent as the second principal component of the observed diversity, an information not otherwise accessible (Grognux and Reymond 2004; Reymond 2009). Another study using a series of fluorescein-derived water-soluble esters revealed that lipases showed highest activity in a buffer containing 20% (v/v) dimethyl sulfoxide, whereas esterases were more active in pure aqueous buffer (Yang et al. 2006).

7.2 Cocktail Fingerprinting

In cocktail fingerprinting assays the ability of chromatographic devices to separate several different compounds simultaneously is used. Therefore, a single experiment is performed treating the enzyme-containing sample of interest with a mixture of different, selected substrates. Subsequently, the enzyme fingerprint can be extracted from the chromatogram of a single chromatography-based separation and quantification of the reaction products, e.g., by GC or HPLC (Reymond 2006). Recently, proof of principle was demonstrated for lipases as well as esterases by using a substrate mixture of 20 different monoacylglycerol analogs (Fig. 7) (Elend et al. 2006; Goddard and Reymond 2004; Yongzheng and Reymond 2005) and for proteases using five hexapeptides (Sicard et al. 2005). It should be noted here that substrate cocktails have also been analyzed by MS to profile activities and enantioselectivities of various enzymes such as nitrilases (DeSantis et al. 2003), epoxide hydrolases (Reetz et al. 2004d), proteases (Basile et al. 2002), and glycosyl transferases (Nagahori and Nishimura 2006).

7.3 Substrate Microarrays

Liquid handling at lower volumes as present in microtiter plates becomes problematic due to rapid evaporation and inefficient mixing of the test solutions (Reymond and Babiak 2007). In this context, solid-supported assays provide a good alternative to perform colorimetric and fluorescence screenings. This can be established either by pre-impregnation of silica gel plates with a fluorogenic substrate as the reaction medium (Babiak and Reymond 2005), by homogenous nanodroplet distribution of a solution containing fluorogenic substrates on a glass slide on which spots of enzyme had been previously printed (Gosalia and Diamond 2003), and by covalent

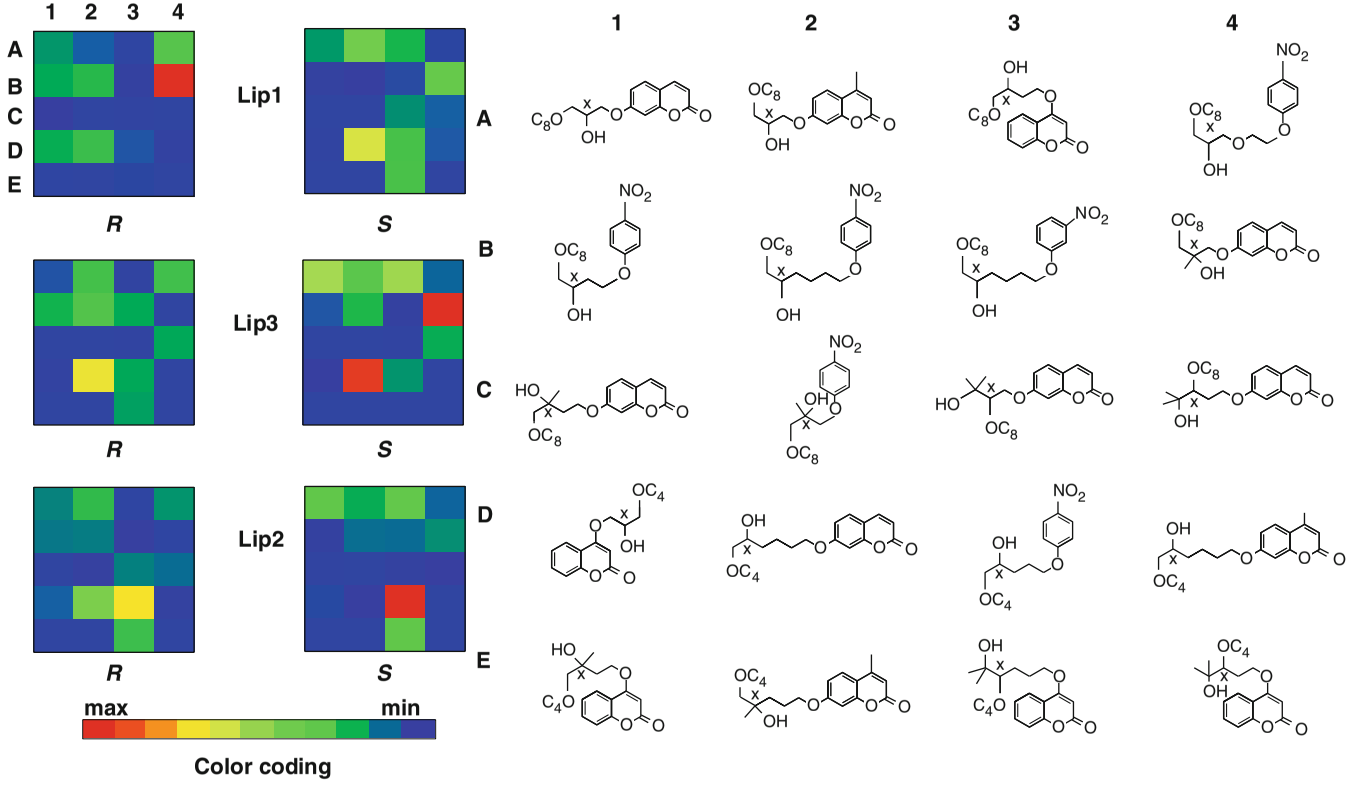

Fig. 7 Fingerprint analysis of three lipases isolated from metagenomic libraries towards activity against (*R*)- and (*S*)-enantiomers of 20 different ester substrates

attachment of fluorogenic substrates to the surface of a glass slide (Grognux and Reymond 2006). Using these different approaches, activity as well as enantioselectivity profiles could be generated for various types of enzymes (e.g., lipases, proteases, phosphatases) (Reymond 2009; Reymond et al. 2009).

8 Conclusions and Research Needs

Many different methods have been developed offering a broad diversity of options for activity, specificity, and enantioselectivity measurements for almost any enzyme of interest (Reetz 2006; Reymond 2009). On the other hand, each assay as well as analytical technique show its individual advantages and disadvantages, thus, the choice of an appropriate system depends on the experimental requirements and available equipment. An impressive number of medium- and high-throughput methods are already available, but most recent developments suggest that at least some of them, namely based on HPLC and GC, may further be extended in throughput. An example is the combination of on-column reaction chromatography with newly developed software tools used to directly determine reaction rate constants during chromatography allowing for high-throughput screening of activities and enantioselectivities (Trapp 2008).

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17 Functionalization and Modification of Hydrocarbon-Like Molecules Guided by Metagenomics: Enzymes Most Requested at the Industrial Scale for Chemical Synthesis as Study Cases

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Contents

Abstract

The end of the twentieth century has experienced a revolution in the life sciences and, specifically, in enzymology. In this sense, the relation between chemistry and biology is currently under a deep transformation influenced by the

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implementation of OMICS tools. We can now access uncultured bacteria, whose genomic material can further be a resource of enzymes for novel enzymology. Among enzymes of interest are esterases and lipases from the α/β-hydrolase fold superfamily, transaminases, and oxidoreductases such as aldo-keto reductases. These enzymes are the first-choice items from the toolbox for functionalization and modification of low-reactive hydrocarbon-like blocks, oils, and fats. Through a series of hydrolytic or synthetic reactions, they can be used for the economic and sustainable production of highly valuable customized and functionalized hydrocarbon-based materials. Through metagenomic studies, about 250 novel esterases and lipases, one β-transaminase, and about 60 alcohol dehydrogenases and aldo-keto reductases have been discovered and their properties described. Research on esterases, lipases, β-transaminases, and oxidoreductases from uncultivated bacteria has revealed unprecedented transformations. The study of their potential in the modification of about 200 different hydrocarbon-like molecules is analyzed herein. This chapter also highlights the catalog of the representative molecules whose functionalization and modification has been successfully achieved using esterases, lipases, β-transaminases, alcohol dehydrogenases, and aldo-keto reductases from uncultivated bacteria discovered through metagenomic approaches. Notably, these groups of enzymes are, at the industrial scale, the most desired means and tools for chemical synthesis.

1 Introduction

Our planet, with a surface area of about 510,072,000 km^2 and a longitudinal difference of close to 20,000 m (from the approx. 6,400 m above sea level of the Chimborazo mountain to approx. 11,000 m deep of the Mariana Trench), contains an extraordinary biodiversity, particularly of microorganisms (Kyrpides et al. 2014; Yarza et al. 2014). That extremely complex pool of organisms can be regarded as a superorganism, which is thought to be involved in the enzymatic conversion of a high proportion of the ever-expanding set of over some 10 million chemically distinct compounds known to date (Beloqui et al. 2008). It is continuously changing and evolving as a consequence of natural selection, which in many cases is forced by external factors, in which anthropogenic factors play a central role. Certain synthetic (unnatural) molecules occurred in the environment not only because these activities provoke the extinction of species and reduction in biodiversity but also because the introduction of new molecules to the environments forces microorganisms to develop a battery of enzymes that were not needed before. There are two recent investigations that exemplify this process, which are described below.

As an example, a new bacterium, *Ideonella sakaiensis,* able to degrade polyethyleneterephthalate (PET), one of the most common plastic used in the last 70 years, was recently discovered (Yoshida et al. 2016). This organism contains two key enzymes to facilitate the PET degradation. A PETase that is excreted to degrade the insoluble

polymer to an intermediate named MHET (mono(2-hidroxyethyl) terephthalate) that can be then transported inside the cells where a second enzyme is produced, a MHET hydrolase. This enzyme degrades this intermediate to the monomers with which industry produced PET: ethylene glycol, and terephthalic acid. By using these two enzymes, this bacterium is able to fully degrade the polymer in 6 weeks. The two enzymes of *I. sakaiensis* have a notable specificity for PET, which is surprising as they have had only 70 years to evolve, a quite short time frame for the evolutionary standards. In other recent study (Alcaide et al. 2013), the exposure of the environment to recalcitrant organic pollutants such as mono- and bi-cyclic aromatics was a cause for the evolution of α/β-hydrolase fold superfamily proteins with common carboxylesterase activity (carbon-oxygen cleavage) into a protein with *meta*-cleavage product hydrolase activity (carbon-carbon cleavage). These elegant studies exemplified the ability of α/β-hydrolase fold superfamily of enzymes to act over quite unreactive hydrocarbon-like molecules, such as PET and aromatic hydrocarbons. They also exemplify the potential of microbial enzymes in supporting such conversions.

Enzymes, whose diversity is practically unlimited in terms of amino acid combinations, are known to catalyze more than 5000 biochemical reaction types that are essential for supporting life (Beloqui et al. 2008). Enzymes are also powerful "biochemical catalysts" which are widely used in the Industry (Bommarius 2015). The increase in the annual demand for new enzymes is growing almost 7% per year and is expected to be close to 10% in 2030 (Ferrer et al. 2015). Initially, the processes for the enzymatic synthesis of molecules of interest were based on the exploration of the known commercial enzymes from microorganisms previously grown in the laboratory for the resolution (with more or less success) of reactions of interest. However, the number of commercial enzymes and the microorganisms containing them was, and continue being, reduced (Ferrer et al. 2016). For this reason, in the last 25 years the research focused on the identification of new enzymes isolated directly from environmental samples without the need to previously isolate and cultivate the microorganisms that contain them. Their application to chemical reactions of interest became a possibility. Enzymology is thus currently under a deep transformation as the search for new enzymes can be performed via either massive searches through direct sequencing of environmental genomic material or through selection of interesting activities in clone libraries created from environmental DNA (Niehaus et al. 2011; Mende et al. 2012; Wilson and Piel 2013; Chistoserdova 2014; Alcaide et al. 2015a, b). In the last 25 years, about 3000 different locations worldwide have been explored using these techniques (Ferrer et al. 2016), from which about 7000 clones containing activities of interest were identified and selected for characterization. Main targets are enzymes that are predominantly used in biocatalysis and industrial sectors (i.e., food, laundry, biofuels), such as nitrilases, transaminases, ketoreductases, glycosyl hydrolases, and lipases/esterases (Fernández-Arrojo et al. 2010; Bayer et al. 2011; Gong et al. 2013; Vergne-Vaxelaire et al. 2013), due to their remarkable market potential. Among them, esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3) from the α/β-hydrolase family have received a considerable attention as they belong to one of the most important groups of

biocatalysts for chemical synthesis with multiple commercial preparations available for industrial use (Nagarajan 2012; Turner and Truppo 2013). Also, transaminases or aminotransferases (EC 2.6.1) are versatile enzymes that catalyze asymmetric amine transfer reactions between an amine and a ketone (Perret et al. 2011). Finally, oxidoreductases such as alcohol dehydrogenases and aldo-keto reductases (AKRs) are capable of modifying a wide range of substrates such as aldehydes, monosaccharides, steroids, prostaglandins, polycyclic hydrocarbons, and isoflavonoids with NADP molecule as a cofactor (Penning 2015).

Indeed, most screening requests at industrial scale for chemical synthesis address aldo-keto reductases followed by transaminases and then lipases (Ferrer et al. 2015), which are the focus of the present chapter. Important parameters defining the relevance of esterases and lipases in biocatalysis are the ample substrate profile, high stereoselectivity, and high turnover rate (Singh 2010). They are also able (i) to hydrolyze large substrates, (ii) to cleave only one chiral ester when offered a racemic mixture of two esters, (iii) to perform a reverse reaction, e.g., synthesis of ester and amide bonds, (iv) to be produced in large quantities in a fermenter, and (v) to be amenable to enzyme evolution and improvement. Esterases and lipases have been extensively used for the functionalization and modification of low reactive hydrocarbon-like chemicals, oils, and fats. Ester compounds with small and large hydrocarbon chains of different length and chemical nature are accepted as substrates. They include open-chain compounds (whether straight or branched) containing saturated and unsaturated aliphatic hydrocarbon blocks with double or triple bonds, alicyclic (both aliphatic and cyclic) and aromatic (containing benzene rings) hydrocarbon-like derivatives, and esters containing complex hydrocarbon blocks. The other two groups of enzymes covered in the chapter are important for the introduction of chiral centers in hydrocarbon-like molecules. Chiral alcohols, for example, are important building blocks, widely applied for the introduction of chiral center into pharmaceuticals, agricultural chemicals, foods, and special materials, and some alcohol dehydrogenases and AKRs have been applied in the reversible oxidation of primary and secondary alcohols into aldehydes and ketones (Breuer et al. 2004) and in the asymmetric synthesis of chiral alcohols (Penning 2015), respectively.

In this chapter, we updated the diversity of hydrocarbon-like blocks that have been reported as being accepted by esterases, lipases, β-transaminases, alcohol dehydrogenases, and aldo-keto reductases, particularly, of those isolated and experimentally characterized by metagenomic approaches. We reviewed the state of the art as it stands in June 2016 and covered a total of circa 250 esterases and lipases, one β-transaminase, and about 60 alcohol dehydrogenases and aldo-keto reductases whose activity and capacity to modify and functionalize such molecules has been experimentally confirmed and validated. The facts presented here suggest that enzymes from metagenomes are of a great interest for promoting the functionalization and modification of hydrocarbon-like molecules of interest in the chemical industry. Examples of enzymes involved in the transformation of specific molecules are specifically given through the text.

2 Diversity of Hydrocarbon-Like Blocks as Potential Substrates

To date a total of about 250 esterases and lipases from metagenomes have been identified by using different high throughput methods (Peña-García et al. 2016) and characterized in terms of substrate specificity and their ability to act over different molecules (Ferrer et al. 2015). Their exhaustive analysis revealed that they can modify a total of at least 146 different types of esters containing complex hydrocarbon-like blocks. Modification through hydrolysis was the most common method, while in few cases modification through the reverse action (synthesis of ester bonds) was achieved. The most common substrates included tri-acyl-glycerol and *p*-nitro-phenyl esters, with acyl chains ranging from acetate to palmitate (as alkyl blocks) or oleate (as alkenyl block). The majority of the enzymes showed preference for shorter ($<C_{10}$) esters. Other hydrocarbon blocks included alkyl, alkenyl, aromatic, and aryl esters. In some cases, the enzymes did show a preference for the functionalization of only one chiral ester when offered a racemic mixture of two esters. Many halogenated compounds of low molecular weight, including hydrocarbon-like organochlorine insecticides, were also accepted as substrates. Other esters of different chemical nature were also modified that included polymer-like molecules, lactones, and sugar esters, to cite some. Below, details about the chemical nature of esters with different hydrocarbon-like blocks are given. For simplicity, they were grouped in six categories: (i) triglycerides, alkyl esters, and alkenyl esters, (ii) aromatic and aryl esters, (iii) racemic esters, (iv) halogenated esters, (v) polymers-like esters, and (vi) other esters.

Concerning the two other groups of enzymes covered in the chapter, the conversions of four aliphatic and aromatic amines catalyzed by β-transaminases, and about 60 chemically distinct alkyl, alkenyl, aryl, and aromatic alcohols, aldehydes, and ketones by alcohol dehydrogenases and aldo-keto reductases, have been reported.

3 Triglycerides, Alkyl Esters, and Alkenyl Esters

All metagenomic ester-hydrolases from the α/β-hydrolase family hydrolyzed different triacylglycerols or alkyl or alkenyl esters, albeit to different extents (Fig. 1). Among alkyl straight-chain derivatives, we found esters where the fatty acid chain varied from acetate to tetradecanoate and the alcohol chain from methyl to tetradecyl. Examples of hydrolases able to act towards shorter alkyl esters such as methyl acetate and methyl butyrate were reported by Meilleur et al. (2009) and Martínez-Martínez et al. (2013). Other examples of hydrolases capable of modifying larger alkyl esters (e.g., tetradecyl myristate) were published earlier by Chow et al. (2012). Unsaturated derivatives included methyl oleate and triolein (Lee et al. 2006), as well as vinyl esters such as vinyl acetate, vinyl butyrate, isopropenyl acetate, vinyl crotonate, and vinyl methacrylate (Martínez-Martínez et al. 2013, 94; Tchigvintsev et al. 2015; Gao et al. 2016).

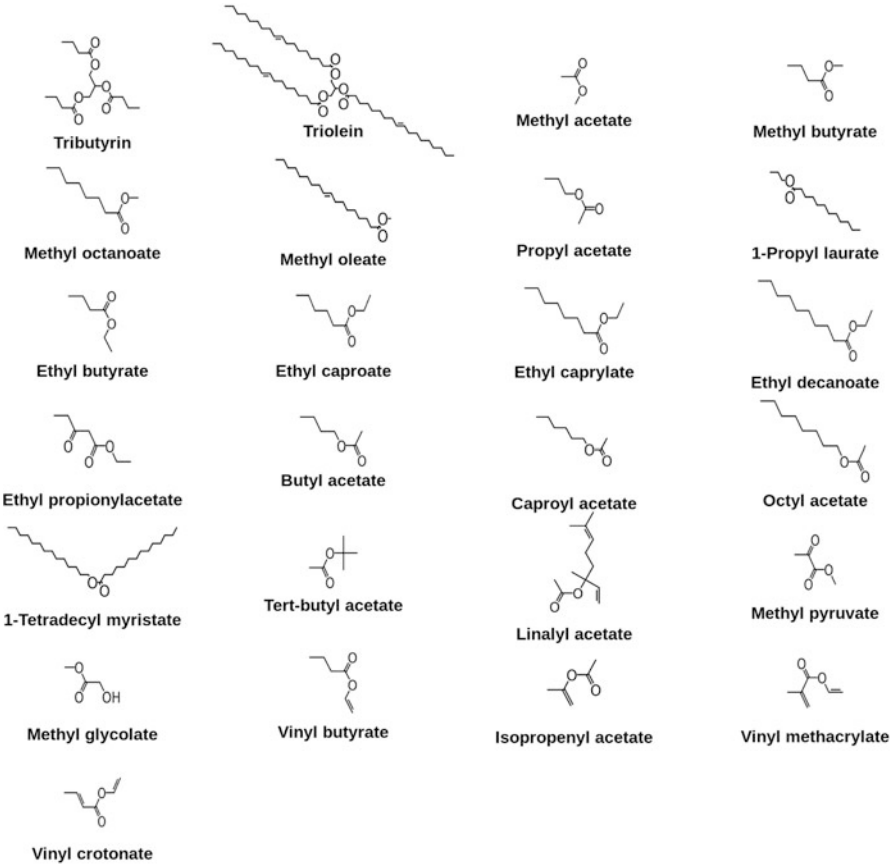

Fig. 1 Representative chemical structures of triglycerides, alkyl esters, and alkenyl esters whose functionalization and modification has been successfully achieved by esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3) from the α/β-hydrolase family discovered by metagenomic approaches. For simplicity only tributyrin and triolein are shown as representatives of triglycerides, although molecules from triacetin to triolein have been found to be transformed by metagenomic enzymes

The utilization of vinyl esters is highly appreciated as during transesterification reactions mediated by lipases and esterases, a vinyl alcohol, i.e., the enol form of acetaldehyde, is produced; this product is volatile, thus promoting the synthesis of the ester. The lipase-mediated polymerization of vinyl methacrylate allows the synthesis of polymers with pendant double bonds (Yhaya et al. 2012). Vinyl crotonate has been used for the lipase-catalyzed preferential acylation of the secondary hydroxy group of floxuridine (Zhao et al. 2009). The isomerization of the double bond of crotonate, which occurs in conventional organic synthesis, could be effectively avoided during the enzymatic acylation. Branched derivatives included isopropenyl acetate (Martínez-Martínez et al. 2013), *t*-butyl acetate, and trimethylsilylbutinol acetate (Elend et al. 2006). Methyl glycolate was also hydrolyzed by some enzymes (Alcaide et al. 2015a, b). Acyl

glycolates (GA) such as ethyl glycolate have been used for lipase-catalyzed copolymerization of ω-pentadecalactone (PDL) and the synthesis of poly(PDL-*co*-GA) copolymers (Jiang and Liu 2010). Finally, methyl pyruvate was found to be hydrolyzed (Tchigvintsev et al. 2015).

4 Aromatic and Aryl Esters

A number of metagenomic ester hydrolases from the α/β-hydrolase family hydrolyzed esters constituted by aromatic and aryl blocks (Fig. 2). Among the most common aromatic esters are those containing *p*-nitrophenyl and α-naphthyl blocks, with acyl chains ranging from acetate to palmitate. The ibuprofen derivative,

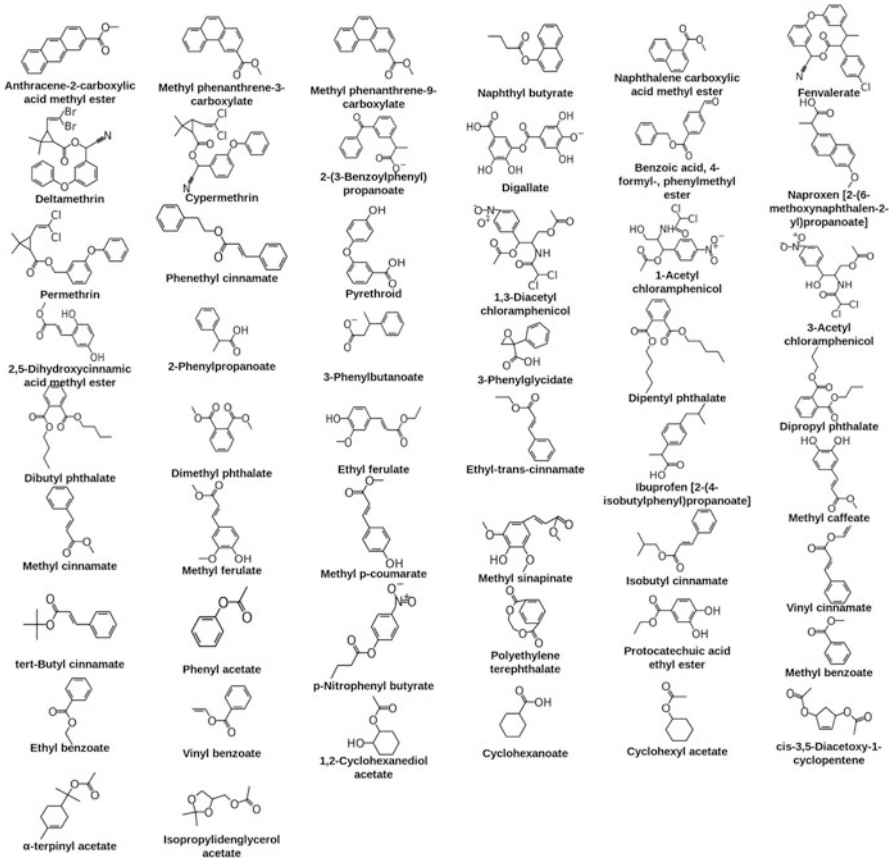

Fig. 2 Representative chemical structures of aromatic and aryl hydrocarbon-like ester blocks. For simplicity only α-naphthyl butyrate and *p*-nitrophenyl butyrate are shown as representatives of α-naphthyl and *p*-nitrophenyl esters, although esters with alkyl chains from acetate to palmitate have been found to be transformed by metagenomics enzymes

ibuprofen-*p*-nitrophenyl ester, was also used as a substrate (Elend et al. 2007). Hydrolysis of hydroxycinnamic acids that include methyl sinnapinate, methyl ferulate, ethyl ferulate, methyl *p*-coumarate, and methyl caffeate was also reported (Ferrer et al. 2005b; Chandrasekhaiah et al. 2012; Jiao et al. 2013; GeneBank acc. nr. KF709432, KF705200, HQ147564). Few enzymes additionally release ferulic acid and diferulic acid from untreated crude plant cell wall materials (Chandrasekhaiah et al. 2012) and one releases ferulic acid from rice bran, wheat bran, wheat-insoluble arabinoxylan, corn fiber, switchgrass, and corn bran (Wong et al. 2013). Phthalate esters such as dipropyl phthalate, dibutyl phthalate, and dipentyl phthalate were found to be hydrolyzed to their corresponding monoalkyl phthalate esters by one esterase (Jiao et al. 2013). Modification was also demonstrated for other similar molecules that included 2,5-dihydroxycinnamic acid methyl ester, ethyl-trans-cinnamate, isobutyl cinnamate, methyl caffeate, tert-butyl cinnamate, vinyl cinnamate, and the most complex block phenethyl cinnamate (Martínez-Martínez et al. 2013).

An esterase most active for hydrolyzing polyaromatic hydrocarbon (phenanthrene, anthracene, naphthalene, benzoyl, protocatechuate, and phthalate) esters was reported. The list of substrates included anthracene-2-carboxylic acid methyl ester, benzoic acid-4-formyl-phenylmethyl ester, methyl phenanthrene-3-carboxylate, methyl phenanthrene-9-carboxylate, naphthalene carboxylic acid methyl ester and protocatechuic acid ethyl ester, to cite some (Martínez-Martínez et al. 2014). Stereo-functionalization and modification of such hydrocarbon blocks with benzene rings are useful for synthesizing new materials.

Other molecules of interest included pyrethroid pesticides and rho-nitrophenyl esters of medium-to-short-chain fatty acids, which were hydrolyzed by the esterase ACJ07038.1 (Li et al. 2008). Additionally, an esterase (Elend et al. 2006) shows a high level of activity against a wide range of substrates including one secondary ester, 7-[3-octylcarboxy-(3-hydroxy-3-methyl-butyloxy)]-coumarin, which is normally unreactive. A lipase supports the hydrolysis of the triglyceride derivative 1,2-di-O-lauryl-rac-glycero-3-glutaric acid 6′-methylresorufin ester (Hardeman and Sjoling 2007). The esterase AEL88620.1 catalyzed the deacetylation of 1- and 3-acetyl and 1,3-diacetyl esters; the enzyme reactivated chloramphenicol from its acetyl derivative by counteracting the chloramphenicol acetyltransferase activity in *Escherichia coli* (Tao et al. 2011). Phenyl and benzoyl derivatives such as phenyl acetate, methyl phenyl-acetate, 3-phenylbutanoate, 3-phenylglycidate, 2-phenylpropanoate, 2-(3-benzoylphenyl)propanoate, methyl benzoate, ethyl benzoate,vinyl benzoate and ibuprofen-phenyl ester were also found as substrates (Elend et al. 2006, 2007; Chow et al. 2012; Martínez-Martínez et al. 2013; Ouyang et al. 2013; Alcaide et al. 2015a; Placido et al. 2015; GeneBank acc. nr. KM042178). Esters with naphthalene block such as naproxen (Elend et al. 2006), a nonsteroidal anti-inflammatory drug, permethrin, pyrethroid, deltamethrin, cypermethrin, and fenvalerate (Li et al. 2008) with a phenoxybenzyl block were also substrates. Finally, other aryl esters such as cis/trans-1,2-cyclohexanediol acetate, cyclohexanoate, cyclohexyl acetate, and isopropylidenglycerol acetate were also converted (Elend et al. 2006, 2007).

5 Racemic Esters

An ample set of enantioselective modifications have also been reported (Fig. 3). Chiral blocks included alkyl, alkenyl, aryl, and aromatic esters and halogenated chiral esters. For example, the stereoselective conversion (>91% enantiomeric excess [*ee*]) of esters of ibuprofen has been achieved using lipases/esterases from oil-contaminated soil (Elend et al. 2007; Chow et al. 2012). Stereoselective conversion for solketal esters (98% *ee*) has been achieved using esterases from deep-sea hypersaline anoxic basins (Ferrer et al. 2005a). While a novel (*S*)-ketoprofen-specific esterase has been identified using pools of metagenomes from the Microbank of Microbial Genomics and Application Center (Taejon, South Korea) (Yoon et al. 2007; Ngo et al. 2014), a (*R*)-ketoprofen-specific esterase (Kim et al. 2006) was found in soil metagenomes. Twenty one metagenome-derived esterases have also been effective for the enantioselective kinetic resolution of esters of phenylalkyl carboxylic acids (56 to >99% *ee*) and the

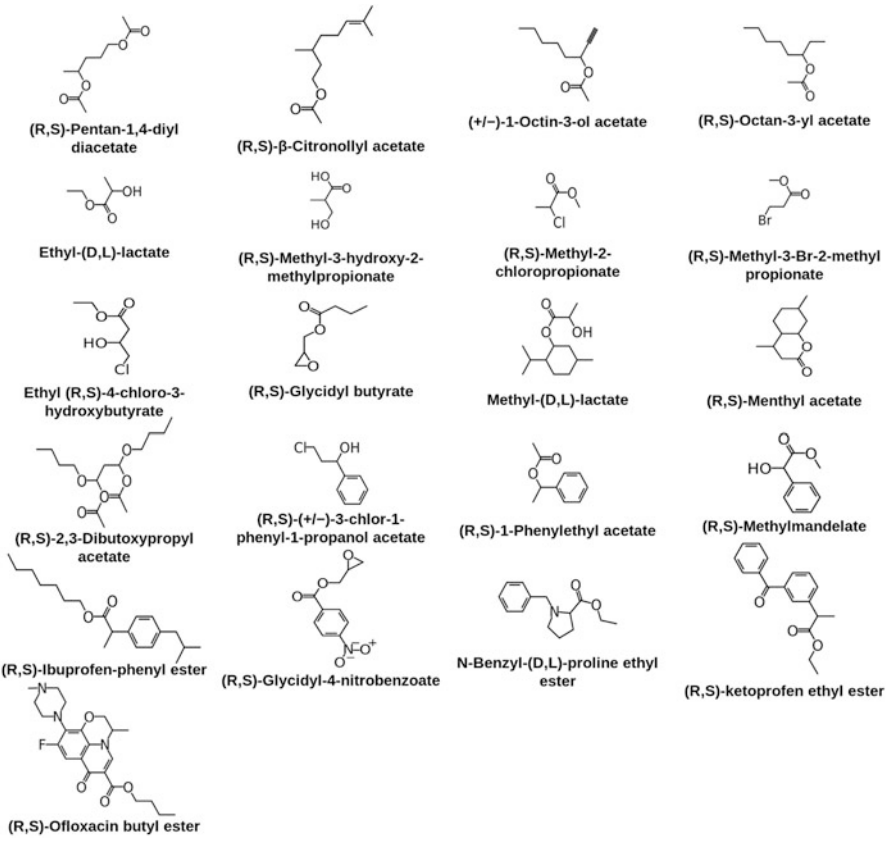

Fig. 3 Representative chemical structures of racemic hydrocarbon-like ester blocks. Only racemic mixtures (without showing preferred enantiomers) are shown

kinetic resolution of 1,1,1-trifluoro-2-phenylbut-3-yn-2-yl acetate and 3,7-dimethyl-1,6-octadien-3-yl acetate (enantiomeric ratio [*E*] > 100); the kinetic resolutions were performed in preparative scales (Kourist et al. 2007; Fernández-Álvaro et al. 2010). Two enzymes hydrolyzed methyl 3-phenylglycidate, a chiral synthon for the synthesis of the Taxol® side chain (Ouyang et al. 2013). Using (*R*,*S*)-1-phenylethyl acetate, Im-LipG9 (Martini et al. 2014) showed excellent enantioselectivity for the *R*-isomer (E > 200), giving an *ee* of higher than 95% for the products at 49% conversion. Using a racemic mixture, the capacity to hydrolyze preferentially (*S*)-racemic ofloxacin butyl ester with an *ee* of 70.3% was found using an esterase from arctic sediment (Jeon et al. 2009). Two novel esterases (Elend et al. 2006) isolated from drinking water also showed high enantioselectivity for (+/−)-1-octin-3-ol, *R*-(+)-3-chlor-1-phenyl-1-propanol, trimethylsilylbutinol, cis/trans-1,2-cyclohexanediol, and isopropylidenglycerol acetate. Finally, an esterase (Elend et al. 2006) was highly enantioselective for (+)-menthylacetate. Three esterases from Lake Arreo (Martínez-Martínez et al. 2013) were selective for (*R*,*S*)-methyl mandelate, whereas one lipase showed enantioselectivity (Martínez-Martínez et al. 2013) for (*R*,*S*)-glycidyl butyrate. Three esterases (Alcaide et al. 2015a) of gill chamber-associated microbiota in the deep-sea shrimp *Rimicaris exoculata* were also found to be enantioselective to different degrees and preferences (E values from 300 to 16) for 5 chiral esters that included methyl-(*R,S*)-mandelate, (*R,S*)-glycidyl-4-nitrobenzoate, (*R,S*)-methyl-3-bromo-2-methyl propionate, methyl-(*R,S*)- lactate, and menthyl-(*R,S*)-acetate. LIPESV12_9, LIPESV12_24, and LIPESV12_26 from hydrothermal vent sediments of the Levante Bay (Placido et al. 2015) were also found to be enantioselective, to different degrees and preferences, for at least seven chiral esters, including methyl-(*R,S*)-mandelate, methyl-(*R,S*)-lactate, (*R,S*)-menthylacetate, (*R,S*)-neomenthyl acetate, (*R,S*)-glycidyl 4-nitrobenzoate, (*R,S*)-pantolactone, and (*R,S*)-methyl-3-hydroxybutyrate. Using Gas Chromatography analysis (Elend et al. 2006), it was shown that EstCE1 was highly enantioselective for (+)-menthylacetate, with only the (+) enantiomer being converted (*ee*: 100%). EstCE1 and EstA3 showed different enantioselectivity when tested against the substrate *cis*-3,5-diacetoxy-1-cyclopentene, but due to a lack of pure reference compounds, the enantiomers could not be identified. Both EstCE1 and EstA3 were able to hydrolyze (+/−)-1-octin-3-ol, *R*-(+)-3-chlor-1-phenyl-1-propanol, and trimethylsilylbutinol (Elend et al. 2006).

6 Halogenated Esters

The selective hydrolysis of hindered and challenging esters such as the hydrolysis of esters on a number of halogenated ring structures is of interest in biocatalysis. As example, enzymes acting against these substrates can be used for the resolution of optically active secondary or tertiary alcohols (Kourist et al. 2007). As summarized in Fig. 4, the ability of metagenome-derived esterases-lipases to hydrolyze halogenated (including those containing bromo, chloro, fluoro, and iodo) alkyl, alkenyl,

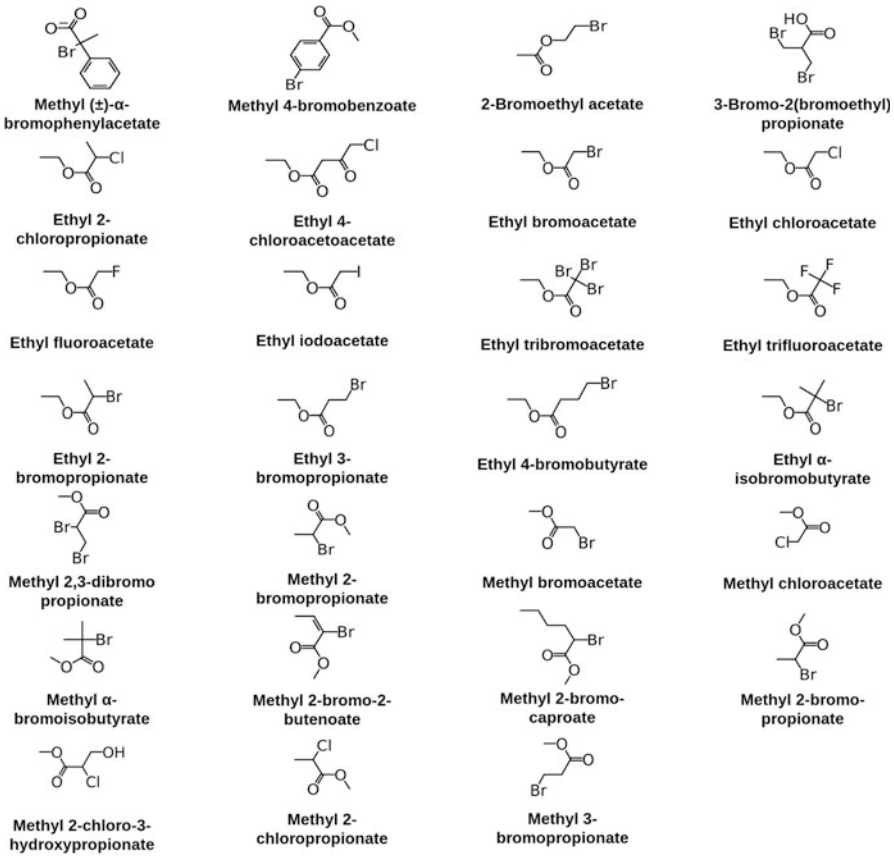

Fig. 4 Representative chemical structures of halogenated hydrocarbon-like ester blocks

and aryl esters has been demonstrated (Elend et al. 2006; Kourist et al. 2007; Fernández-Álvaro et al. 2010; Martínez-Martínez et al. 2013; Alcaide et al. 2015a, b; Tchigvintsev et al. 2015). A number of metagenomics-derived esterases-lipases did show outstanding properties. Particularly, one esterase showed excellent enantioselectivity ($E > 100$) in the kinetic resolution of 1,1,1-trifluoro-2-phenylbut-3-yn-2-yl acetate as confirmed by a preparative scale reaction, with preference for the (+)-enantiomer. Also, EstA3 derived from a drinking water metagenome and EstCE1 from a soil metagenome were able to hydrolyze R-(+)-3-chlor-1-phenyl-1-propanol (Elend et al. 2006). The hydrolysis and transformation of halogenated aromatic esters, particularly those containing a benzene-like ring, is broadly appreciated for the synthesis of food additives, drugs, cosmetics, preservative solvents, perfumes, pharmaceuticals, plasticizers, lubricants, solvents, detergents, soaps, and reaction intermediates. Few esterases-lipases from a karstic lake, particularly, the enzyme LaE6, have potential for such kind of transformations as they are able to transform

aryl/aromatic esters that included methyl (±)-α-bromophenylacetate and methyl 4-bromobenzoate (Martínez-Martínez et al. 2013). Finally, it is also noticeably the ability of some of these esterases-lipases to transform halogenated short and unsaturated carboxylic acids. As example, alkenyl esters that included methyl-2-bromo-2-butenoate (which is used in pharmacological research) have been reported as substrates for karstic lake-derived enzymes (Martínez-Martínez et al. 2013). Only two dihalogenated compounds, that included 3-bromo-2(bromoethyl)propionate and methyl 2,3-dibromo propionate, were reported to be hydrolyzed by esterases from microorganisms inhabiting marine environments (Tchigvintsev et al. 2015; Alcaide et al. 2015a, b). The presence of multiple halogen atoms and different alkyl and aryl esters is important for the functionalization of molecules with multiple chiral centers.

7 Polymers-Like Molecules

The example of the PET-degrading enzyme found in the bacterium *Ideonella sakaiensis,* mentioned in the introduction section, exemplified the capacity of microbial hydrolases to help degrading synthetic polymers, in weeks-time and at 30 °C, that have been recently introduced in our society and into the environment. Such enzymes are produced by microbes to degrade the polymer and to use it as carbon source. They can be also used in the industry for this purpose as more than 300 million of tons of plastics are produced every year worldwide (Yoshida et al. 2016). They can also be used in a reserve reaction in which one can use an intermediate that is functionalized by an esterase-lipase to start a polymerization reaction to produce new biodegradable polymers.

Some metagenomic esterases exhibit hydrolytic activity against polyester substrates making them attractive candidates for the development of enzyme-based depolymerization of polyester plastics. As example, polymers such as poly(dl-lactic acid) (PLA), poly(ε-caprolactone) (PCL), poly(ethylene succinate) (PES), poly (3-hydroxybutyric acid) (PHA), and/or polyethylene terephthalate (PET) with different weight-average molecular weights (Fig. 5) have been also found to be extensively modified by 2 metagenomics cutinases found in compost (Mayumi et al. 2008; Sulaiman et al. 2012), and 4 α/β-hydrolases from microorganisms inhabiting marine environments (Tchigvintsev et al. 2015; Hajighasemi et al. 2016). Thus they may have potential applicability for surface modification and degradation of polymers. However, although composting with such polymers is most effective at high temperatures (50–60 °C) when using such enzymes, the absence of transformation rate data limits the real potential of these enzymes in an industrial context. Following on from this, depolymerization activity of purified hydrolases against polymers is commonly performed either in solid phase using 1.5% w/v of emulsified substrates or in solution using commonly 20 mg of polymer in 1 ml of a buffer. Such low amount of substrates in combination with low degradation rates limits the application of known enzymes in a presumptive industrial process and exemplifies the need of novel screen programs for such enzymes. In

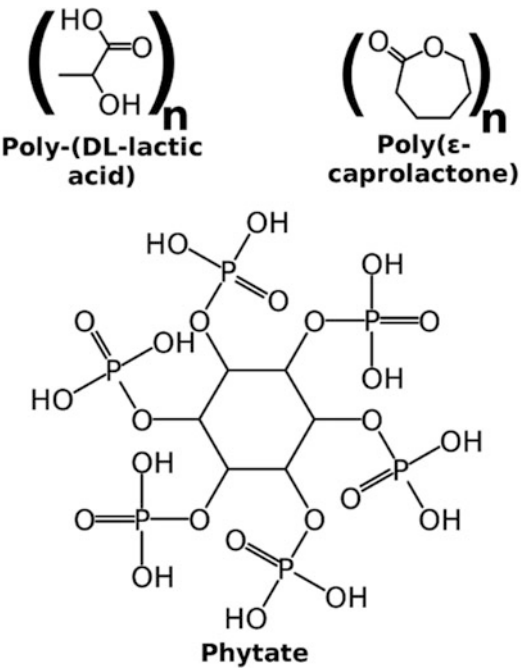

Fig. 5 Representative chemical structures of polymers-like molecules

addition to that, it is important to know that a limiting step is not the degradation rate itself, but rather the rate of access and absorption of the enzyme onto the surface of the substrate. By performing absorption experiments, it was found that the two compost-derived cutinases showed more absorption capacity for low molecular weight polymers compared to larger hydrophobic polymers (Mayumi et al. 2008).

Two enzymes had the capacity to hydrolyze phytate and can be thus considered as phytate esterases (GenBank acc. nr. KF709432, KF705200). These two enzymes were isolated from uncultured bacteria inhabiting wheat farm soil with long-term omission of phosphorus fertilization. Phytate esterase cleaves and frees the bound phosphates from the phytic acid molecule providing essential phosphorus needed for healthy (animal) nutrition. Phytase of interest must be optimally active in the pH range prevalent in the digestive tract (4.0–4.5) and must have a strong proteolysis resistance, and this is why actual screen programs are focused in screening metagenomics-derived enzymes fitting these requirements.

8 Other Esters

Figure 6 summarizes other hydrocarbon-like esters capable of being transformed by metagenomics esterases and lipases from the α/β-hydrolase family. Two were reported to be phospholipases with the ability to hydrolyze phosphatidylcholine

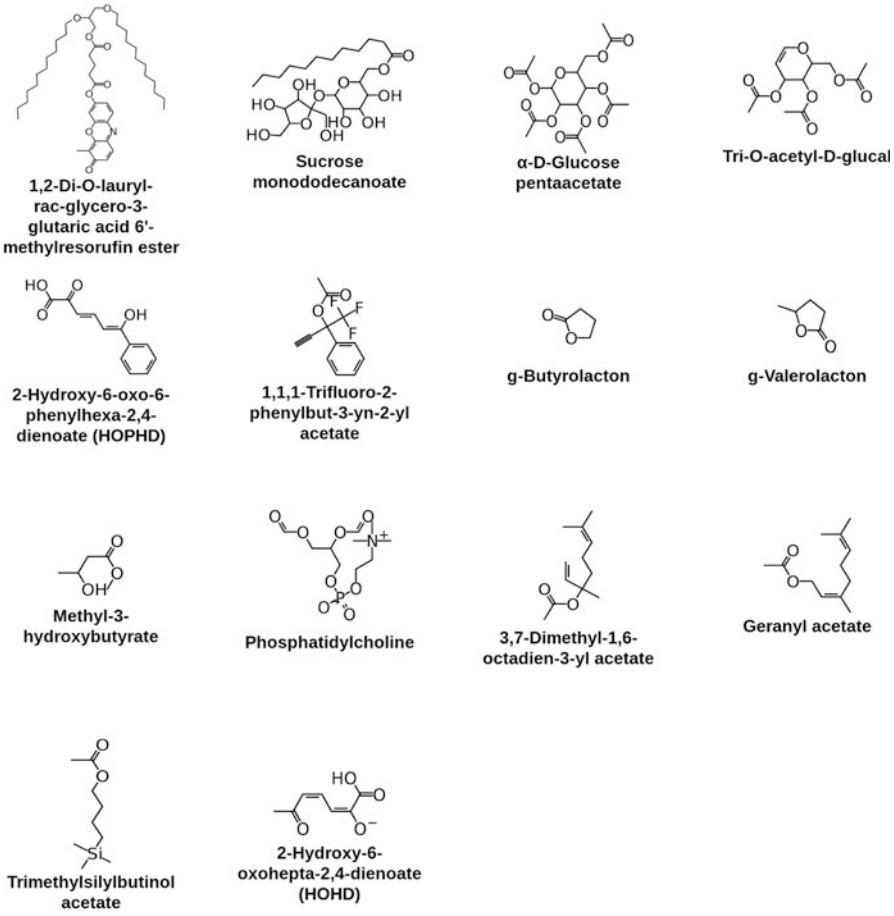

Fig. 6 Representative chemical structures of other hydrocarbon-like ester blocks

(Tirawongsroj et al. 2008; GenBank acc. nr. EU285670.1). Additionally, the first example of a metagenome-derived lipase (Martínez-Martínez et al. 2013) capable of producing sucrose esters that have broad applications (Plou et al. 2002) has been reported; the enzyme, isolated from the metagenome of an evaporite karstic lake, allowed transesterification reactions to produce sucrose esters by using long-chain vinyl esters and sucrose as substrates (Martínez-Martínez et al. 2013). Lactones such as γ-valerolactone and gamma-butyrolactone were also modified by an esterase from this lake (Martínez-Martínez et al. 2013). A number of esterases were also able to hydrolyze 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate and 2-hydroxy-6-oxohepta-2,4-dienoate by carbon-carbon bond cleavage (Alcaide et al. 2013). Finally, some

enzymes were able to accept tri-O-acetyl-glucal and the carbohydrate ester α-D-glucose pentaacetate (Martínez-Martínez et al. 2013; Alcaide et al. 2015a, b).

9 Amines, Alcohols, Aldehydes, and Ketones

To date, only one novel β-transaminase has been identified and kinetically characterized from a metagenome from the anaerobic digester of a wastewater treatment plant (Perret et al. 2011). This enzyme is involved in an alternative lysine fermentation pathway by converting 3-aminobutyryl-CoA into acetoacetyl-CoA with 2-ketoglutarate as amine acceptor. This reaction is biologically relevant. This enzyme catalyzed the transamination of other aliphatic and aromatic substrates. Thus, in the presence of α-ketoglutarate, this enzyme catalyzed the β-transamination of different *N*-acetylcysteamine (NAC) thioesters that included NAC thioesters of 3-aminobutyrate, β-homoleucine, and β-phenylalanine. They were converted to their corresponding oxo-compounds, namely, acetoacetyl-S-NAC, 5-methyl-3-oxohexanoate-NAC, and 3-oxo-3-phenylpropanoate-NAC.

Redox reactions that involve alcohols, aldehydes, and ketones catalyzed by oxidoreductases, including alcohol dehydrogenases and aldo-keto reductases (AKRs) that bind nicotinamide cofactor without a Rossmann-fold motif, are also important transformations. A number of alcohol dehydrogenases and AKRs from metagenomic origin have been described to date. An example is the Knietsch work where distinct environmental samples were enriched with glycerol and 1,2-propanediol as selective agents and a set of 16 such enzymes were obtained (Knietsch et al. 2003a). Using a similar approach, 15 alcohol oxidoreductases were isolated from metagenomic DNA libraries from three different soil samples (meadow, sugar beet field, cropland) (Knietsch et al. 2003b). These enzymes support the functionalization of 1,2-ethanediol, 2,3-butanediol, glycerol and 1,2-propanediol as well as the synthesis of dihydroxyacetone, used as a skin colorant, and the preparation of tritium-labeled compounds. Also, 23 alcohol dehydrogenase directly isolated from 17 samples of DNA extracted from soil have been described and used for the synthesis of optically pure alcohols from various ketones (Itoh et al. 2014). They include products obtained using acetone, chloroacetone, 2-butanone, 2-pentanone, 2-hexanone, 2-heptanone, 2-octanone, 3-octanone, 5-chloro-2-pentanone, 2′-chloroacetophenone, ethyl 3-oxobutanoate, methyl 4-chloro-3-oxobutanoate, ethyl 4-chloro-3-oxobutanoate, methyl benzoylformate, acetophenone, 2′-chloroacetophenone, 2-acetylpyridine, 1-phenyl-1,2-propanedione, methyl benzoylformate, and 1-phenyl-3-butanone. Finally, an alcohol/aldehyde dehydrogenase from a waste water treatment plant was also reported (Wexler et al. 2005). The enzyme was able to support ethanol and butanol modification.

Other recent example is the aldo-keto reductase MGS-M4 isolated from microbial communities from Lake *Medee*, the largest hypersaline deep-sea anoxic lake of the Eastern Mediterranean Sea (Alcaide et al. 2015a). The enzyme exhibited

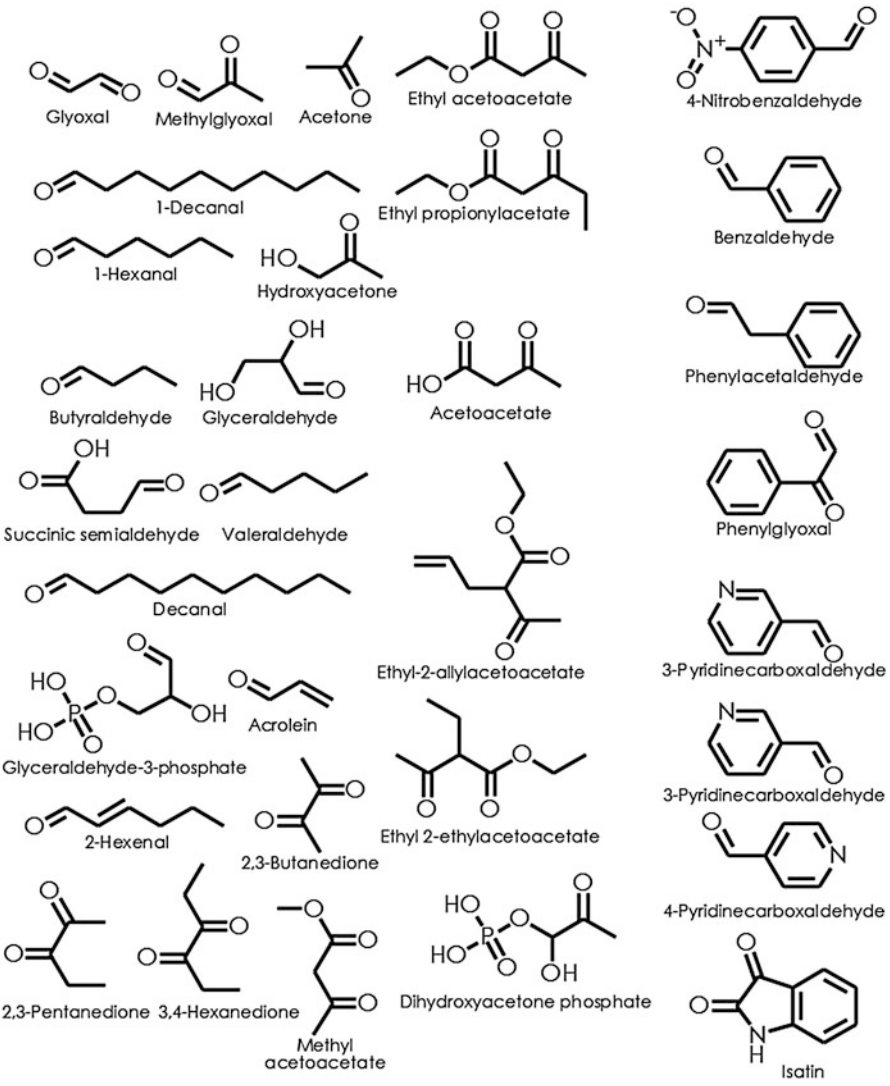

Fig. 7 Representative chemical structures of aldehydes and ketones successfully transformed by an aldo-keto reductase from microbial communities from Lake *Medee* (Alcaide et al. 2015a)

activity towards 41 aldehydes and ketones (Fig. 7). Methyl-glyoxal was the best substrate, while reduced activity was observed when ethyl-3-oxohexanoate was used as the substrate. The enzyme efficiently reduced aromatic derivatives such as 4-nitrobenzaldehyde, benzaldehyde, phenylacetaldehyde, phenylglycol, 3-pyridinecarboxaldehyde, 4-pyridinecarboxaldehyde, and isatin; it was also active towards alkyl and alkenyl (i.e., acrolein, 2-hexenal, and ethyl-2-allylacetoacetate) substrates.

These examples revealed the broad substrate spectra of oxidoreductases from yet uncultured microorganisms and their potential to be used as toolbox for the functionalization and modification of hydrocarbon blocks containing alcohol, aldehyde, and ketone groups.

10 Concluding Remarks and Research Needs

Industrial microbiology has more to offer than just providing a solution when the chemical synthesis does not work. The multiple examples in this chapter illustrate the enormous potential of enzymatic systems for the functionalization of unreactive hydrocarbon-like molecules. Microbial biodiversity hotspots to produce metagenomic resources for their further exploration and microorganisms with recently sequenced genomes will cover the whole diversity of microbial life, which is of a great interest to explore the new mechanisms for these conversions of relevance to the industrial implementation. This encompasses a potentially inexpensive and sustainable way of providing the chemical industry with high-value, customized, and functionalized molecules. The discovery that the limited set of metagenomics enzymes of industrial relevance (particularly, esterases, lipases, transaminases, alcohol dehydrogenases, and aldo-keto reductases) available are able to efficiently transform circa 200 different hydrocarbon-like molecules illustrates the role of metagenomics for future developments around the functionalization and modification of such molecules. For that, additional effort is needed to focus on characterizing and testing enzymes from uncultured microorganisms with as high chemical diversity of substrates as possible, to guarantee their industrial usefulness. This will require also the need for standardization of suitable high throughput screening methods, particularly for industrially relevant enzymes for which such methods are not yet standardized. Thus, while such methods are widely available to perform naïve screens for esterases and lipases in metagenomic clone libraries, they are not equally applicable for the screening of transaminases and oxidoreductases. Recently, a new assay for the high throughput screening of transaminases in liquid or solid-phase has been described (Baud et al. 2015), which uses an inexpensive amine donor, which could facilitate increasing the number of transaminases discovered by metagenomic approach in the future.

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Novel Sensors for Engineering Microbiology

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Maximilian Ole Bahls, Tsvetan Kardashliev, and Sven Panke

Contents

Abstract

The development of sustainable, biocatalytic routes to compounds otherwise derived from petrochemical processes is one of the major objectives in the field of biotechnology. Obtaining suitable microbial strains for this task still depends on the generation of strain variants and the subsequent screening or selection process. While the technical advances in DNA manipulation and synthesis allow rapid generation of millions to billions of metabolic pathway variants for a given product, the knowledge of which variants to generate and how to assess them in a

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high-throughput manner is lacking behind. The latter problem is increasingly tackled through the use of biosensors, by which product titers are coupled to easily detectable in vivo reporters such as fluorescent proteins. This in turn requires an interface where the presence of the desired product can trigger the formation of the reporter. Therefore, this chapter discusses how such an interface can be implemented and reviews the types of genetically encoded sensors available, their construction and applications, and how the specificities of future biosensors could be developed.

1 Introduction

Today's economy relies primarily on fossil resources and organic synthesis for its manufacturing needs. This industrial model is intrinsically unsustainable and causes significant burden on human health and the environment, alike. This has led to the concept of "Green Chemistry," which promotes the use of renewable resources and chemical technologies aiming at minimal environmental impact (Abraham and Nguyen 2003; Ribeiro et al. 2015). More often than not, such "green" manufacturing routes would employ biocatalysts in order to meet the 12 principles of sustainability (Anastas and Warner 1998). These principles include the transition to an economy that relies on renewables as a source of energy and building blocks, and bioconversions as the chassis for industrial chemical production. However, the rate of development of enzyme-driven processes is largely dependent on the pace at which new biocatalysts can be developed. This is in turn to a large extent a function of the speed and accuracy at which individual genetic designs of biocatalysts are assessed. Even though the advances in systems biology of microorganisms (which represent the largest fraction of biocatalysts for industrial biotechnology) increasingly facilitate a rational design of biocatalysts (Dai and Nielsen 2015) or semirational designs allow biasing diversification to meaningful outcomes (Jeschek et al. 2017), much of the biocatalyst improvement still rests on the assessment of large numbers of variants generated without exact knowledge of the underlying catalytic mechanisms and screening for the top-performing fraction (Tee and Wong 2013). Such variant libraries often originate from random or semirational diversity generation, such as random chemical mutagenesis or error-prone gene replication of a known, but inefficiently performing enzyme. The overwhelming fraction of the generated variants is thus comparable or inferior to the original catalyst. Therefore, one factor that limits the acceleration of development of novel biocatalysis schemes is the throughput of existing assay platforms for selecting the top-performing variants (Sundberg 2000; Xiao et al. 2015). For example, common screens on solid media or in microtiter plates allow the sampling of 10^4–10^5 events per laboratory evolution round, i.e., several orders below the average size of a library generated using state-of-the-art genetic diversity methods (Wong et al. 2006; Ruff et al. 2013). The advent of microfluidic and single cell analysis techniques has enabled sampling of $>10^7$ events per hour, likely representing the highest currently available throughput for single cell analysis. As a result, such single-cell analysis tools increase the maximum

throughput several orders of magnitude and narrow the gap to the library size that can be practically obtained via transformation (10^9–10^{10} cfu μg^{-1} DNA (Dower et al. 1988)).

A key requirement for harnessing the throughput of single cell-based screens is the availability of a fluorescence signal indicative of the performance of individual catalyst variants or genetic designs, as the majority of established laser-based detection methods used in microfluidic technologies rely on the measurement of fluorescence per cell. To this end, genetically encoded biosensors (GEBs) have attracted significant attention for application in fluorescence-activated cell sorting (FACS), as they provide the crucial link between catalyst variant performance and the fluorescence signal (Dietrich et al. 2010; Liu et al. 2015; Zhang et al. 2015; Williams et al. 2016).

Biosensors can be defined as two-component analytical "devices" comprising a module for recognition of an analyte of interest, the "receptor," and a module for signal output or transduction, the "reporter." At least one of these modules is derived from a biological system. Antibodies, regulatory proteins or protein binding domains, enzymes, nucleic acids, and even organelles and whole cells have all been used as modules of biosensors (Monošík et al. 2012; He et al. 2015). In the context of metabolic and protein engineering, in vivo screening protocols are advantageous as they generally enable higher analytic throughput, particularly in comparison to multistep protocols that require cell lysis and subsequent in vitro testing of library members. Therefore, the focus of this chapter is on biosensors that are encoded in their entirety on a plasmid or an organism's genome and render a functional sensing device upon transcription or translation of the genetic construct. Such biosensors provide a way to link the expression of a fluorescent protein to an intracellular target metabolite concentration while maintaining genotype-phenotype linkage as the cells remain intact throughout the screening process. Note that in principle also sensing of extracellular concentrations is possible as long as the genotype-phenotype linkage is retained, e.g., by suitable forms of compartmentalization (Meyer et al. 2015).

Noncoding functional RNA sequences, regulatory proteins and their cognate DNA sequences, or binding proteins and binding domains can all serve to develop sensing devices, making categorization of biosensors nontrivial. Based on the molecular architecture, RNA-based biosensors can be categorized as riboswitch biosensors and RNA-biosensors (composed of RNA mimics of fluorescent proteins, e.g., RmFP (You and Jaffrey 2015)) and protein-based biosensors can be grouped into transcription factor-based and fluorescence resonance energy transfer (FRET)-based biosensors. Another way to categorize GEBs is according to their mode of action. Here, riboswitch biosensors and transcription factor biosensors fall into the same category as they require the transcription/translation machinery of the host organism to generate the reporter molecules. FRET and RmFP biosensors comprise a second category for which sensing and signal generations take place independent of the host's transcription/translation machinery. An overview of receptor and reporter modules, modes of action, key features, and characteristics of GEBs from each subcategory is given in Fig. 1, and the recent examples of GEB design and

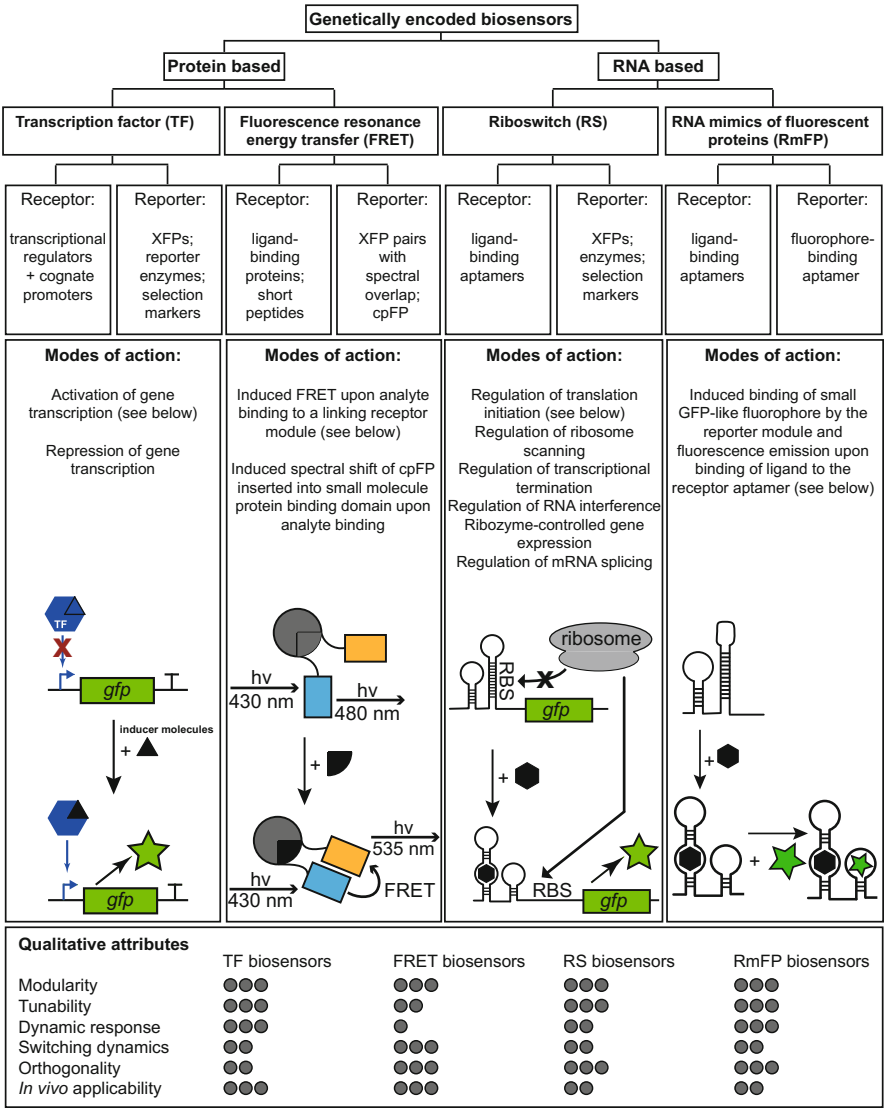

Fig. 1 Top: Classification and overview of available genetically encoded biosensor types. Middle: Modes of action and schemes for the most common sensor types. Bottom: Ranking of the biosensor classes. cpFP, circularly permuted fluorescent protein; GFP, green fluorescent protein; RBS, ribosome binding site; XFP, fluorescent protein

application in metabolic and protein engineering are discussed in more detail in the sections below. At the end of the chapter, we present brief guidelines for construction and troubleshooting of the most versatile, amenable, and robust category to date – that of transcription factor biosensors.

2 Genetically Encoded RNA-Based Biosensors

Noncoding RNAs (ncRNAs) are abundant and functionally versatile ribonucleic acids that become increasingly important in synthetic biology and applied biology in general (Morris and Mattick 2014). In particular, RNAs which are capable of specifically binding small molecules, i.e., aptamers, or can serve to regulate gene expression, i.e., riboswitches, prove useful for the development of genetically encoded sensing devices (Williams et al. 2016; Hallberg et al. 2017). Such ncRNAs do not only provide functionalities similar to those of regulatory and binding proteins but also have the advantage of comparative ease of RNA modeling, design, manipulation, and evolution. In addition, RNA-based regulation of gene expression is rapid as the transcription process is completed prior to the sensing event and enables spatial and also provides temporal and dosage control over gene expression (Berens et al. 2015; Krishnamurthy et al. 2015). In the following sections, the recent progress in the field of RNA-based biosensing is summarized including existing approaches for generating and applying such biosensing devices in the monitoring of biocatalytic reactions.

2.1 Riboswitch Biosensors

Riboswitches are short sequences found in the 5′-untranslated region (5′-UTR) of mRNAs that form secondary structures and thus modulate the expression of downstream sequences. Riboswitches consist of an aptamer, i.e., an RNA molecule that can specifically bind a given small molecule, and an additional regulatory component (e.g., a ribozyme, a terminator sequence, or a sequence complementary to a ribosome binding site) which can be conditionally (de)activated upon ligand binding to the appertaining aptamer. The binding of a small molecule to the aptamer leads to conformational changes in the 5′-UTR and consequently changes the state of the regulatory component.

Several mechanisms for ligand-dependent gene regulation by riboswitches exist in bacteria and eukaryotes, some of which exclusively pertain to one domain of life. One main mode of action of riboswitches in prokaryotes is cotranscriptional regulation as a consequence of the ligand-dependent formation of a terminator hairpin leading to dissociation of the RNA polymerase (Winkler et al. 2002; Hallberg et al. 2017). The inverse mechanism, i.e., ligand-mediated destabilization of terminators and subsequent upregulation of gene expression, is also possible (Hollands et al. 2012). Riboswitches in bacteria can also function by imposing control over translational regulation. In this case ligand binding to an aptamer upstream of the ribosome binding site (RBS) can either render the regulatory sequence inaccessible to ribosomes or, alternatively, expose the RBS, thereby upregulating gene expression (Winkler et al. 2004). A less common mechanism in prokaryotes is the ligand-mediated ribozyme activation in which binding of a small molecule to an aptamer component induces the self-cleavage of the catalytic RNA component and results in

rapid mRNA degradation (Davidson et al. 2013). More diverse modes of action are known in higher organisms (Wieland and Hartig 2008).

Indeed, significant effort has been invested to not only understand the molecular mechanism of action of RNA-based regulators but to improve their performance and robustness and to widen their in vivo applicability (Berens and Suess 2015; Hallberg et al. 2017). The latter has proven to be challenging, particularly for riboswitches that use humanmade aptamers, and to date, only a handful of those have been adapted to in vivo applications (see below). This is counterintuitive given the availability of an in vitro aptamer selection technology (Tuerk and Gold 1990; Robertson and Joyce 1990; Ellington and Szostak 1992) and computational methods for modeling and redesign of aptamer specificity (Penchovsky 2013; Clote 2015). The difficult transferability of artificially designed riboswitches to in vivo setting has been attributed to thermal instability of mRNA structures and RNA misfolding under physiological conditions, which have not been sufficiently closely mimicked during in vitro aptamer development (Berens et al. 2015).

The most successful strategy to identify artificial aptamers with acceptable in vivo functionality has been achieved by means of mutagenesis and recombineering of in vitro evolved riboswitches and activity screening within a host organism. In this way, an in vivo riboswitch biosensor for neomycin has been developed (Weigand et al. 2008). A green fluorescent protein (GFP)-based in vivo screen for regulated aptamers starting from an in vitro selected aptamer pool that bound the aminoglycoside antibiotic identified an aptamer that confers neomycin-depended control of translation initiation in yeast. It is noteworthy that the in vivo identified riboswitch was underrepresented in the original pool of aptamer variants with robust in vitro functionality.

Similarly, a riboswitch biosensor that activates protein translation in *Escherichia coli* cells in response to 2,4-dinitrotoluene (DNT) has been engineered (Davidson et al. 2013). This was achieved by incorporating degenerate bases between an in vitro selected trinitrotoluene (TNT) aptamer and the switching component (in this case, an RBS), and carrying out in vivo screening that relied on inducible expression of TEV protease and a FRET-substrate to detect riboswitch-upregulated protease expression in the presence of inducer. The isolated riboswitch exhibited a 10-fold relative increase in fluorescence in the presence of DNT. The aforementioned examples strongly suggest that in vivo screening is probably an indispensable step when developing riboswitch biosensors for application inside living cells.

At present, most engineered riboswitches still require high effector concentrations for switching and exhibit low dynamic range and high background activity in the absence of ligand (Berens and Suess 2015). Nevertheless, steady progress in riboswitch design and innovative uses of naturally occurring aptamers have resulted in the first successful in vivo screening applications of riboswitches. An early example describes the use of an RNA switch based on a naturally occurring aptamer for theophylline, a molecule of the xanthine family structurally similar to caffeine, to link theophylline concentrations and GFP expression levels in yeast (Michener and Smolke 2012). Quantitative high-throughput screening of large enzyme libraries, either in clonal cultures or in single cells by FACS, resulted in the identification of a

caffeine demethylase mutant with a 33-fold relative increase of activity and 22-fold improvement of selectivity. Similarly, a screening platform that employs a microfluidic static droplet array and an L-tryptophan riboswitch to analyze intracellular metabolite concentration from single microbial cells was used to isolate microbial strains with up to 145% increased productivity compared to its parental strain (Jang et al. 2016). In another study, a riboswitch based on a naturally occurring L-lysine aptamer and a selection module instead of a fluorescent reporter was used to identify aspartate kinase variants with 1.6-fold higher in vitro activity relative to the wildtype enzyme (Wang et al. 2015). The application of L-lysine riboswitches has been extended to the isolation of optimized lysine producer strains (Zhou and Zeng 2015a, b). Another report of riboswitch biosensor application in the context of metabolic engineering describes the use of the theophylline riboswitch biosensor to select strains with higher productivity of the drug methylxanthine (Michener and Smolke 2012).

Riboswitch biosensors based on ribozyme-type regulation have also been used in screening applications. For example, the natural *glmS* ribozyme was used in yeast to select for N-acetylglucosamine producing strains (Lünse and Mayer 2014). Another recent example describes the development of an elaborate strategy to identify *Bacillus subtilis* strains with improved vitamin B2 productivity (Meyer et al. 2015). *B. subtilis* strains that converted cellobiose to vitamin B2 were co-confined with *E. coli* sensor cells inside nL-size alginate beads. Product formation triggered a sequence of reactions in the sensor cells: (1) conversion of B2 into flavin mononucleotide (FMN), (2) binding of FMN by a natural FMN-sensitive RNA riboswitch, and (3) self-cleavage of RNA resulting in (4) the synthesis of GFP. The fluorescence intensity was then used to isolate more efficient vitamin B2 producers, while the co-confinement allowed retaining the link between genotype and phenotype.

Taken together, these examples demonstrate the potential and versatility of riboswitches as devices for in vivo screening. Given that riboswitch biosensors are still in their infancy, we anticipate that their importance to high-throughput screening will only increase in the years to come.

2.2 RNA Mimics of Fluorescent Protein Biosensors

An emerging category of genetically encoded sensors makes use of RNA aptamers that bind freely diffusible fluorophores and switch them to a highly fluorescent state (Paige et al. 2011). These RmFP aptamers were originally used for tagging and imaging specific RNAs in living cells (Juskowiak 2010; Dolgosheina and Unrau 2016). An emerging application of RmFPs is the sensing of intracellular metabolite concentrations. The fluorescent RNA aptamer-fluorophore complex can be converted into a sensor that emits a specific signal only in the presence of a small molecule inducer (Strack and Jaffrey 2013). This is achieved by fusing together a fluorophore-binding aptamer to a ligand-binding one in a way that only in the presence of a ligand the secondary structure of the fluorophore binding aptamer is correctly assembled, the small fluorophore can be bound and switched to a highly

fluorescent state. Sensors that employ the fluorophore binding aptamer "Spinach" (or derivatives thereof) as a reporter module and a different small molecule binding aptamer as receptor module have been built by inserting the latter into a structurally critical stem of the Spinach RNA (You et al. 2015; Strack et al. 2014). The target-binding aptamer is unstructured in the absence of the target molecule as a critical stem is disrupted thus preventing Spinach from folding and binding the fluorophore. However, when the aptamer binds its target, the correct folding of the critical stem in Spinach leads to fluorescence that can be detected both in vitro and in living cells. Sensors that bind *S*-adenosyl-methionine (SAM), ADP, and other metabolites have been created using this approach (Song et al. 2013). Moreover, these sensors have enabled imaging of the dynamics and turnover of SAM and ADP, cyclic di-GMP, and various proteins in living cells (Strack et al. 2014). A recent report demonstrates for the first time that in vivo detection of enzyme activity is also possible with RmFP biosensors. To this end, the authors used an optimized RmFP sensor for *S*-adenosyl-L-homocysteine (SAH) to measure methylthioadenosine nucleosidase (MTAN) activity in live *E. coli*, more precisely the increase of SAH levels upon chemical inhibition of MTAN (Su et al. 2016).

While we are yet to witness a "true" screening application, the potential for high-throughput enzymatic assays of these innovative genetically encoded sensors has been implied by the recent developments in the field. Nevertheless, it is noteworthy to mention that technical difficulties similar to those experienced with artificial riboswitch biosensors and FRET sensors (discussed below) can be anticipated along the way to robust in vivo RmFP biosensors capable of sensing arrays of chemically diverse compounds. In addition, due to their mode of action, RmFP biosensors appear to be more suited for sensing metabolite dynamics and their use in quantitative screening and identification of microbial producers will likely remain limited.

3 Genetically Encoded Protein-Based Biosensors

3.1 FRET Biosensors

FRET-based sensors typically involve a pair of donor and acceptor fluorophores linked by a ligand-binding protein domain such that upon ligand binding a conformational rearrangement is induced causing an alteration in the proximity of the donor and acceptor fluorophores and consequently a measurable FRET change (Hochreiter et al. 2015). In the FRET category, we also included sensors that are based on single, circularly permuted fluorescent protein (cpFP) fused to binding proteins in a way that ligand binding induces detectable changes in either fluorescence intensity or excitation and emission profiles (Baird et al. 1999). A characteristic feature of both of these protein sensor designs is that no transcription or translation event is required after sensing and that the exerted signal is generally reversible on shorter timescales. Despite advantages such as orthogonality to

existing metabolic processes of the host organism, high temporal resolution, and relative ease of construction, FRET sensors often suffer from low dynamic ranges and are less suited for monitoring of metabolite accumulation and predominantly are applied to monitoring of intracellular metabolite dynamics, rather than screening for producer strains (Schallmey et al. 2014; Zhang et al. 2015).

There is one screening application of a cpFP-based biosensor for hydrogen peroxide, HyPer, available in a novel screen to engineer enzymes for the enhanced production of H_2O_2. Cytochrome P450 BM3 variants were expressed in a biosensor strain and, using HyPer's ratiometric signal, variants that generated greater amounts of H_2O_2 than the wildtype enzyme via uncoupling were reported (Lim and Sikes 2015).

3.2 Transcription Factor Biosensors

In vivo genetic circuits are broadly reliant on transcription factors as regulatory proteins for controlled protein production (Polisky et al. 1976) that allow the on-demand activation or repression of gene transcription. However, in contrast to the RNA-based sensors, the sensing process requires the transcription/translation machinery of the cell for reporter activation. For the design of biosensor circuits, the transcription factor of interest is usually constitutively expressed by the cell and upon binding of an inducer molecule, the activated regulator is recruited to its operator in the cognate promoter region, thus activating gene expression (e.g., MalT and maltose (Hatfield et al. 1969)), for example of a reporter. Alternatively, the regulator can be a repressing (as opposed to activating) transcription factor. Here, the transcription factor blocks expression from its cognate promoter, until this repression is relieved by the addition of an inducer molecule (e.g., LacI and allolactose (Jacob and Monod 1961)). In addition, the repression can also be activated upon binding of a specific "inducer" (e.g., MetJ and *S*-adenosylmethionine (Shoeman et al. 1985)). However, if such a system should be used for sensing of an improved product titer due to increased reporter expression, it requires the inversion of the signal from repression to activation, thus making the biosensor circuit more complex to design and implement.

Many regulator-promoter pairs are known from previous studies which facilitate the a priori design of a circuit with specificity for the inducer molecule of interest (Fig. 2a). Even when not fully annotated, regulators can be found in metagenomic libraries for novel but natural target inducers, but also for xenobiotics only recently introduced to nature (de Lorenzo et al. 2010). Still, this set of pairs is markedly limited in terms of known compounds for which we can find suitable parts. However, the toolbox of synthetic biology and molecular biology workflows, in general, give increasingly access to engineered, bespoke regulator specificities as shown in Fig. 2 (Libis et al. 2016b; Rogers et al. 2016). The methods applied for the generation of tailormade regulators include directed evolution (comprising rounds of in vitro mutagenic regulator gene replication followed by in vivo selection of

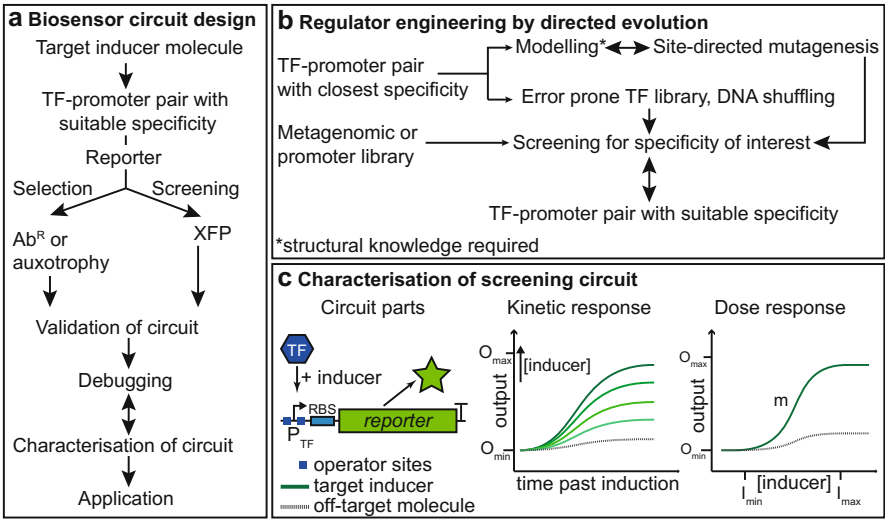

Fig. 2 (**a**) Biosensor design from target molecule definition to application. (**b**) Regulator engineering in order to obtain suitable target specificity. (**c**) Characterization of the biosensor circuits in terms of parts, kinetic-, and dose-response. Ab^R, antibiotic resistance; dynamic range of reporter output, O_{max}–O_{min}; I_{min}, detection threshold; I_{max}, maximum detection; m, sensitivity; TF, transcription factor XFP, fluorescent protein

improved variants (Galvao and de Lorenzo 2006)) but also extensive in silico modelling in order to predict regulator sites that correspond to a broadened or novel specificity (Jha et al. 2015). Here, a broadened specificity means that the original inducers are still working as inputs, while a novel specificity would require a switch to the novel inducer including a concomitant loss of function for the original inducer. So far, the model-based strategies are still more an envisioned scenario than an everyday reality in routine lab work but have a huge potential for simplifying the design of novel biosensors as discussed below.

Common to all circuits, independent of the input source, is an easily accessible, genetically encoded reporter. In general, two options are available: selection and screening strategies. In the case of selection, common reporters driven by the sensor circuit are antibiotic resistance proteins, proteins that complement auxotrophies, or proteins that implement a conditional phenotype based on the conversion of a substrate into a toxic product. All these reporters readily allow the selection of cells in the on-state, i.e., the sensor activated state, as resistant or cured cells in cultures outgrow other cells lacking the induced gene expression, and in the optimal case, exclusively survive under restrictive media conditions. We also include reporter proteins that convert a chromogenic substrate into this category, as identifying activated cells is still straightforward, e.g., by selecting blue colonies due to the expression of β-galactosidase (lacZ, e.g. (Tang and Cirino 2011; Cebolla et al. 1997)) on media including the corresponding colorless chromogenic substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), or by selecting colored

colonies producing pigments that do not require any particular precursor molecule or substrate (Santos and Stephanopoulos 2008). However, with the colorimetric assays, the enrichment of on-state cells is lost.

While the described selection systems are generally helpful if a rather digital on-or-off, switchlike information is sufficient or preferred, in metabolic engineering it is often of more interest to gradually increase a product titer over multiple rounds of improvement. In other words, there is no zero concentration off-state. Here, screening for improved variants has its strength, as it allows a distinct biosensor response depending on the accumulation of the target compound. Traditionally, transcription factor-based systems utilized various reporters including luciferase genes (*luxAB*) or chromogenic substrate conversion via *lacZ* expression but were almost exclusively replaced by fluorescent proteins (XFP) as also apparent from the previous RNA and FRET-based sensor examples. XFPs have the advantage of being almost self-sufficient reporters, meaning no substrate has to be added to the culture, as only oxygen is required for successful maturation of the chromophore. Crucially, fluorescent proteins have the essential feature of allowing high-throughput screens via FACS with a detection limit principally similar to the one observed for (catalytic) luciferase-based assays, which are not routinely implemented at the single cell level (Kohlmeier et al. 2007). Besides, transcription factor–based circuits using XFP variants with reasonably short half-lives allow the in vivo assessment of molecular fluxes over time (Rogers and Church 2016a). In particular for engineering of sensor specificities, schemes combining both selection and screening are highly useful as unwanted specificities and constitutive variants can be excluded by a round of negative preselection, followed by screening for increasing fluorescence with increasing product concentrations.

For more details and explicit tables of the known transcription factors and their cognate promoters, please refer to one of the numerous excellent reviews in the field (amongst others: (Daunert et al. 2000; Galvao and de Lorenzo 2006; van der Meer and Belkin 2010; Dietrich et al. 2010; Gredell et al. 2012; Eggeling et al. 2015; Mahr and Frunzke 2015; Rogers and Church 2016b; Rogers et al. 2016; Libis et al. 2016b). Here, we highlight the origin and construction of various transcription factor–based circuits available today and focus on how they were designed, including the engineering of novel transcription factor specificities and applications for screening in metabolic engineering. We conclude with a short section about circuit design and sensor debugging in order to facilitate the development and optimization of future biosensor circuits.

3.3 Circuits Based on Natural Transcription Factor Specificities

For many operons, often belonging to well-studied catabolic and stress-response pathways, the regulatory elements are known. They evolved mainly for the recognition and utilization of alternative nutrient sources under harsh environmental conditions lacking common nutrients; for example, for switching to hydrocarbons as energy and carbon source when sugars such as glucose are absent. Biosensor-

relevant regulatory elements are also often involved in the sensing of cellular damage, e.g., due to solvent exposure, or of the intracellular redox state. For several decades, such regulators were coupled to reporters including β-galactosidase and luciferases in order to build sensor circuits for the detection of environmental pollutants (de Lorenzo et al. 1993).

For multiple biomonitoring tasks, regulatory genes with their cognate promoters, in many instances originating from catabolic plasmids of *Pseudomonas putida*, were utilized. An exemplary set of such biosensors was built and characterized in *E. coli* and *P. putida* KT2442 by de Lorenzo and coworkers, including regulators for alkyl- and halobenzoates (based on XylS), alkyl- and halotoluenes (XylR), and salicylates (NahR), coupled with the expression of *luxAB* and *lacZ* (de Lorenzo et al. 1993). For the circuit utilizing the transcription factor XylS and its cognate promoter P_m fused to *luxAB*, the minimum detection level of extracellular *m*-toluate was found to be as low as 1 ppm (about 5–10 μM). The same set of regulators was also used as a basis for studies creating novel inducer spectra by engineering the regulator residues, which is discussed in the next section.

NahR was also utilized as a gas phase sensor for naphthalene vapor, by activating luciferase expression from its cognate promoter P_{sal} in *P. putida* (Werlen et al. 2004). Here the detection limit was found to be in the lower nM range of naphthalene. This study additionally highlights a potential source of error when exploiting circuit parts originating from biodegradative pathways. Usually, such pathways are not highly expressed as long as the preferred carbon sources of the cell are available, hence the activities of the corresponding regulators are downregulated (catabolite repression). In this study, the addition of 10 μM succinate to the culture medium leads to a 20% decrease in signal output, while at concentrations above 100 μM almost no significant sensor output was detectable.

Similar biosensors were developed for various molecules that are characteristic for hydrocarbon spills and contaminated groundwater samples by van der Meer and others (Sevilla et al. 2015; Jaspers et al. 2001). For instance, various whole-cell biosensors were built on the basis of AlkS, a regulator of an alkane-responsive system in *P. putida* GPo1. The reporters included *gfp* and *luxAB*, with the latter system allowing octane sensing in the nM range (Sticher et al. 1997). Additional sensor circuits for the detection of external toxic compounds, such as antibiotics and halogenated aromatics, were for instance based on the regulators TtgR (Espinosa-Urgel et al. 2015) and TodS-TodT (Lacal et al. 2006) of *P. putida*, respectively.

Intriguingly, many of the sensor specificities described above were published for the detection of spilled xenobiotics, but now might be of high value in future metabolic engineering approaches for the sustainable bioproduction of the very same compound classes. Nevertheless, the availability of sensor types for hydrocarbons is far from complete and in particular imperfect for recognizing particular molecular substitution patterns. Additionally, the sensor circuits described were mainly applied for sensing of extracellular molecules, which require the molecule to be able to translocate across the cell membrane. However, this uptake issue exists in the case of externally added target compounds, but not if the compound is

produced intracellularly, for instance in case of the production of medium chain length alkanes (Call et al. 2016; Wu et al. 2015).

More recently, the interest in biosensors extended toward applications in metabolic engineering workflows. Here, the sensors are used for the high-throughput in vivo screening of enzyme libraries, i.e., the assessment of millions of similar but slightly different microbe variants producing a target compound. This is in particular valuable for relatively inconspicuous small molecules, including amino acids, sugars, and various hydrocarbon products such as alkanes or alcohols, which are difficult to detect in a high-throughput fashion with standard chemical analytics.

A primary example for the development of such novel biosensors for FACS-based high-throughput screening of metabolites is the development of a LysG-based circuit for amino acids in *Corynebacterium glutamicum*. Here, Eggeling and coworkers fused the cognate promoter region of the transcription factor LysG to the gene for a yellow fluorescent protein (*eyfp*) in order to successfully screen for L-lysine overproducers with cytosolic concentrations in the lower mM range (Binder et al. 2012). Similar systems were applied for other amino acids, including L-arginine and L-histidine (Binder et al. 2013; Schendzielorz et al. 2014).

For the high-throughput assessment of medium chain length alcohols, a biosensor was built in *E. coli* by utilizing BmoR of *Thauera butanivorans* controlling the expression of a *gfp* gene from its cognate promoter. This circuit allowed a linear detection range for butanol from 0.01 to 40 mM and was applied to a proof-of-principle screen for improved producer variants (Dietrich et al. 2013). Another principle was used by Cheng et al., relying on the competition between a repressor (ArgR), which is activated by arginine and thus repressing GFP expression from its cognate promoter, and the enzyme arginine deaminase (ADI), which converts arginine into citrulline. Here, a more active ADI variant would lead to an increase in fluorescent reporter signal. This was utilized in a FACS-based screen for almost 10^7 ADI variants created by error-prone PCR (epPCR), yielding an improved ADI variant with both higher activity and a lower K_M (Cheng et al. 2015). More general sensing approaches include the development of a sensor for NADPH consumption, utilizing a [2Fe-2S]-cluster containing regulator, SoxR, and eYFP under the control of the SoxR-cognate promoter in *E. coli* (Siedler et al. 2014). In this study, NADPH-dependent alcohol dehydrogenase variant libraries were screened for improved activity, generally pointing to the feasibility of high-throughput screens for various other NADPH-dependent enzymes.

Besides, much data about regulator specificities and pathways that are regulated by molecules of interest is available in online databases. For instance, RegPrecise offers a manually curated database of prokaryotic regulons (Novichkov et al. 2013), Bionemo offers a search function for biodegradative pathways by molecules of interest, explicitly stating the involved transcription factors when available (Carbajosa et al. 2009), and more general pathway databases, e.g., the EAWAG Biocatalysis/Biodegradation Database (http://eawag-bbd.ethz.ch, including the former University of Minnesota Biodegradation/Biocatalysis Database and Pathway Prediction System (Gao et al. 2010)), allow to search for substrates and the related enzymes, which may indicate where to look for novel regulatory regions of interest.

When no suitable sensor protein is available, metagenomic and promoter libraries were successfully screened for suitable regulator specificities. For example, the Alon promoter library, including several thousand promoter-*gfp* fusions (Zaslaver et al. 2006), was mined for a phenylalanine responsive regulator system, which was then applied to a phenylalanine-overproduction screen in *E. coli* (Mahr et al. 2016). Additionally, substrate-induced gene-expression screening (SIGREX) allows the mining of genes from any environmental metagenome by cloning the fragmented DNA upstream of *gfp*, followed by screening for input molecule specific fluorescence (Uchiyama et al. 2005; Uchiyama and Watanabe 2008).

While many useful constructs were built with known or mined natural transcription factors, the development of novel sensor systems is still markedly limited by the availability of suitable regulator specificities. One workaround is the in vivo conversion of analytes after their formation into compounds for which sensors exist (Libis et al. 2016b), facilitated by software tools predicting the necessary enzymes for a given analyte (Delepine et al. 2016). For instance, cocaine was sensed after conversion into benzoic acid (BenR regulator (Libis et al. 2016a)) and 3-hydroxy propionate was sensed after conversion to acrylate (AcuR (Rogers and Church 2016a)). Concluding, the design of novel biosensor circuits based on transcription factors is a highly promising but ongoing task that has the potential of improving the in vivo screening for a great number of novel whole-cell catalysts, if the required specificities are found or engineered.

3.4 Circuits Based on Engineered Transcription Factor Specificities

In order to change the specificities of known regulators, the DNA sequence coding for the regulator can be varied and the resulting variants sampled for an altered inducer spectrum (inducers "a la carte" (Galvao and de Lorenzo 2006)). This strategy could either follow a random approach, in particular if no information about potentially valuable protein sites, such as the binding pocket, is available or a (semi)rational approach targeting particularly interesting residues as determined from crystal structures and homology models.

Studies following the random approach are mainly based on the error-prone in vitro regulator-gene replication via PCR, followed by rounds of in vivo screening for the novel specificities of interest. This approach frequently yielded regulator variants with the desired novel inducer spectra and altered affinities, in spite of the tremendous amino acid residue space that one can search through. Besides, such experiments provided valuable information about the location of potential protein sites involved in the inducer and DNA binding.

Classical examples for this strategy include work by Ramos et al., analyzing mutants of the XylS regulator originating from the TOL catabolic plasmid of *P. putida* mt-2. Mutants were selected by coupling the regulators to a gene for tetracycline resistance via its cognate P_m promoter, followed by culturing on media containing tetracycline and benzoate analogs which are not natural XylS

effectors. The spontaneous mutation rate was increased by adding ethyl methanesulphonate, a mutagenic compound, to the media. As a result, clones were obtained with either constitutive or novel and inducer specific XylS functionality, which were then further analyzed for their inducer spectrum with *lacZ* as the reporter (Ramos et al. 1986; Ramos et al. 1990). This enabled the discovery of multiple novel regulator specificities for various benzoate analogs in the low mM range.

Several mutant versions of NahR and XylR were generated by epPCR by de Lorenzo and coworkers. In the first case, new-to-nature specificities were found by fusing P_{sal}, the cognate promoter of NahR, to *lacZ*, followed by selection depending on the blue color of cells cultivated on media containing X-Gal and benzoate, a non-natural inducer of NahR. Upon specificity verification, about two-thirds of the variants were indeed responsive to externally added benzoate in the low mM range, while the other fraction of regulators consisted of constitutively active variants. Interestingly, and as seen in various studies before, none of the novel NahR regulator variants lost its responsiveness to the natural inducer salicylate (Cebolla et al. 1997). Similar observations were made when variants of XylR for the xenobiotic compound 2,4-dinitrotoluene were generated by epPCR. These variants were selected by fusing the cognate promoter P_U, regulating the upper TOL operon in the presence of *m*-xylene, to (1) a kanamycin resistance gene, (2) *pyrF* in an uracil auxotroph *P. putida* strain (both for selection), (3) *gfp* (for screening), and finally (4) *lacZ* for verification of the induction behavior. Remarkably, none of the altered amino acid residues were found to form the binding pocket itself. It was reasoned that the residues exchanged are involved in conformational changes upon inducer binding and thus change the signal transmission between different domains of the regulator protein. Moreover, the appealing concept of a multipotent stem form (Jensen 1976) was introduced to transcription factor engineering, according to which novel specificities arise as a result of the regulator adopting a more promiscuous form with a widened inducer spectrum. This stem form allows the sensing of various novel inducer molecules, but still includes the natural one(s). Building on such promiscuous regulator variants, ripening toward new specificities could take place via precise, stringent residue exchanges (Galvao et al. 2007).

Another strategy for the creation of new-to-nature regulator variants is the shuffling of DNA fragments generated by digestion of the coding sequences of closely related regulators, which nevertheless differ in their inducer spectrum. For instance, the N-terminal sequence of XylR, being responsible for effector binding, was shuffled with homologous sequences of the regulators DmpR and TbuT. The resulting variants were selected for various novel inducers including bulkier molecules like biphenyls or chemically altered side chains, e.g., nitrotoluenes. The novel regulators were selected by coupling DmpR's cognate promoter P_o to a kanamycin resistance gene, allowing cells cultured with a given inducer and kanamycin to survive only if they harbor a corresponding XylR variant. In order to exclude false positive, constitutive regulator variants, P_o was additionally fused to *sacB*. The expression of *sacB* resulted in a nonviable phenotypes for constitutive XylR variants if the whole-cell biosensors are cultivated with sucrose but without inducer (Garmendia et al. 2001).

The combination of epPCR with subsequent DNA shuffling of the variant hits showed intriguing results as well. Leadbetter and coworkers created novel biosensors based on LuxR, originating from the quorum sensing system of *Vibrio fischeri*, coupled to *gfp* expression via its cognate promoter P_{luxI}. The natural inducer spectrum of LuxR does not include butanyl homoserine lactone (C4HSL) having a shorter acyl-side chain length than the natural inducers. By rounds of directed evolution via error-prone replication of the regulator, LuxR variants were found that responded with half-maximal activity at a C4HSL concentration of 2.3 μM, while the wildtype regulator showed no response. The variants created were then subjected to another round of evolution by DNA shuffling, yielding further improved LuxR variants that allowed a half-maximal activity already at 150 nM of added C4HSL (Hawkins et al. 2007).

More recently, regulators for biosensors were engineered at specific amino acid sites, which were preselected due to their proximity to the inducer binding sites as judged from crystal structures. Here, the regulator variants are usually created via PCR with synthetic, mutagenic DNA oligomers corresponding to the chosen amino acid residues. Cirino and coworkers applied this strategy successfully to the AraC regulator protein, originating from the arabinose operon in *E. coli*. Initially, AraC mutants were created by both epPCR and targeted mutagenesis at four residues of the effector-binding pocket in order to find a molecular reporter specific for D-arabinose, as opposed to the natural inducer L-arabinose. As a reporter of the biosensor circuit, *gfp* was installed under the control of the cognate promoter P_{BAD}, allowing FACS-based screening of the AraC mutant libraries. In order to find suitable novel regulators, a dual screening workflow was used. Every round of positive screening with D-arabinose as the target inducer was followed by a round of deselecting cells that were either constitutively fluorescent or fluorescent in the presence of added L-arabinose. This allowed to create AraC mutants responsive to D-arabinose in the range of 10 nM to 1 mM while L-arabinose concentrations of around 10 mM or higher were required for a recognizable fluorescent output. Interestingly, all characterized AraC variants were found by the targeted approach, while the random, error-prone libraries yielded no highly improved variants (Tang et al. 2008). With a similar dual screening strategy, an AraC library, created by simultaneously mutagenizing five residues of the binding pocket, was used to generate a biosensor for mevalonate with a concentration range of 10–200 mM of mevalonate added to the culture. Mevalonate is a valuable input molecule for isoprenoid synthesis in *E. coli*, and its production was significantly improved by selecting producer variants with optimized expression levels of a reductase of the corresponding mevalonate pathway with the help of the biosensor system (Tang and Cirino 2011). Moreover, AraC-based sensors were further developed for triacetic acid lactone (TAL) at low mM concentrations, here with *lacZ* as the reporter. This system was applied to the selection of an improved 2-pyrone synthase variants converting malonyl-CoA more efficiently into TAL, resulting in variant with about 19-fold improvement in k_{cat}/K_M and a 20-fold improvement of the TAL titer (Tang et al. 2013).

A more rational engineering strategy is represented by the application of computational design to generate novel specificities, as exemplified by engineering a novel specificity for explosives, including TNT amongst others, into ribose-binding protein (Looger et al. 2003). However, additional experiments indicated that the specific concept applied in that work is not reliably working (Reimer et al. 2014). More recently, Baker et al. computationally designed and successfully expressed proteins in order to specifically bind ligands, namely the steroid digoxigenin (Tinberg et al. 2013), with high affinity and selectivity, pointing toward an increasing practicability of in silico design of novel specificities for biosensors so far not accessible. The Rosetta modeling protocol (Das and Baker 2008) was utilized for the creation of model-based, focused variant libraries of PobR, a transcription factor of *Acinetobacter* lacking an experimentally determined structure, for the novel inducer 3,4-dihydroxy benzoate (Jha et al. 2015). After FACS-based screening with a sensor circuit consisting of *gfp* under control of the cognate promoter of PobR, variants with activity for 3,4-dihydroxy benzoate in the μM range were isolated. The PobR variants also showed an increased response with the natural inducer 4-hydroxy benzoate, but no sensitivity for other structurally similar molecules.

In a comprehensive study of novel LacI variants, a repressor from the *lac* operon of *E. coli* (Jacob and Monod 1961), multiple of the aforementioned engineering strategies were utilized (Taylor et al. 2016). This included computational design, targeted residue saturation mutagenesis almost spanning the full protein length, and random epPCR mutagenesis. The LacI variants were screened for induction with four novel inducers, all being saccharides with varying degrees of structural similarity to the natural inducer allolactose. In the first round, nonrepressed designs were removed by coupling LacI to a fusion promoter P_{LlacO} driving the expression of a porin (TolC). In the presence of colin E1, a toxin, the TolC expression is toxic and thus eliminated undesired LacI variants. Next, FACS-based screens were employed in order to isolate variants induced by the target compounds, facilitated by fusing P_{LlacO} to *gfp*. For all compounds, novel LacI variants were found, showing sensor outputs similar to that of the wildtype LacI circuit. Noteworthy, for one target compound, sucralose, the random approach failed to deliver a novel LacI variant, while the successfully computationally designed variant contained four mutations. It was argued that the combinatorial space might have been too large for the epPCR strategy. The specificity of sensor variants with broadened specificity toward gentiobiose and sucralose was further improved by shuffling and combining beneficial mutations followed by additional rounds of FACS screening. This allowed increasing the maximum output significantly while the induction by isopropyl β-D-1-thiogalactopyranoside (a non-metabolisable mimic of allolactose) was drastically reduced and in the case of the gentiobiose-responsive variant almost completely eliminated. Intriguingly, this activity maturation of the intermediate LacI variants for gentiobiose and sucralose is in agreement with the above-discussed stem protein intermediate (Galvao et al. 2007), which likely might be required in order to find switched specificities, as opposed to broadened specificities.

4 Optimization of the Biosensor's Characteristics

Besides engineering the structure of the transcription factor and its physical interaction with the inducer molecule of interest, almost all other parts of the biosensor circuit are amenable to optimization for improved specificity and overall circuit behavior. As a result, debugging of unfavorable sensor features is possible and often necessary. The main characteristics of a whole-cell biosensor are depicted by a dose-response curve (also see Fig. 2c), indicating how the circuit's output changes with varied input concentrations – ultimately determining whether a sensor is useful for finding improved microbial producer strains. The dose-response curve shows the fold change between basal output (subject to undesired activity in the off-state known as "leakage") and the maximum obtainable output which is also known as the dynamic range, the steepness or slope of the response indicating the sensor sensitivity, and the minimum to maximum inducer concentrations between which meaningful changes in output are observable. In order to evaluate the suitability of the sensor circuit for high-throughput screening assays, in terms of reliability and false-positive rates, a dimensionless Z-factor can be calculated by relating the dynamic range to the data variation (Zhang et al. 1999). Also the response kinetics of the system provides valuable information in order to find the optimal time point for FACS-based screening. In addition, the output kinetics determines whether a given response is short-lived enough to be a useful measure for in vivo flux-analysis, which could be tweaked with mRNA- or protein tags for increased degradation rates. This could be necessary, for instance, when the formation and degradation of intermediates of a production pathway should be detected. If the cellular half-life of the sensor output is significantly longer than that of the transient formation of the input of interest, the signal output would be merely static instead of showing the actual metabolic flux.

The theoretical framework for sensor circuit design and its fine-tuning is significantly expanding with the progress of synthetic biology (for example, see Tabor et al. 2009; Ang et al. 2013), but most of the experimental implementations still require substantial design-build-test-learn cycles. Nevertheless, recent studies highlight how the debugging of imperfect sensor systems could be readily achieved with straightforward methods, as soon as the regulator of interest and the reporter are defined.

For an ArsR-based heavy metal biosensor, it was shown that the addition of a second ArsR binding site significantly decreased the leakage of the circuit, as it most likely increased the probability of interaction of the repressor with the RNA polymerase or simply sterically blocked it (Merulla and van der Meer 2016). Adding a binding site downstream of the promoter sequence (serving as a "roadblock") further decreased the basal expression in this study. Overall, this strategy allowed lowering the basal output while maintaining the inducible control and crucially a high maximum output, thus increasing the fold-change in output upon induction by a factor of approximately five. For a sensor system based on XylR, naturally responding to *m*-xylene and to a lesser extent also to 3-methylbenzyl alcohol, it was shown that rewiring of the circuit parts has an effect on the specificity without

changing the transcription factor itself (de las Heras et al. 2012). By introducing a positive feedback loop utilizing the P_S promoter for XylR expression and thus replacing a P_R promoter (negative feedback loop), the sensor became more specific for *m*-xylene. However, a similar positive feedback system with the stronger P_U promoter replacing P_R showed increased output for both *m*-xylene and 3-methylbenzoate but without improving the accuracy of discrimination. This suggests that the combination of the expression of an attenuated regulator with a positive feedback loop can adjust the activation threshold in a way that allows discriminating against the weaker inducer molecule 3-methylbenzyl alcohol. In addition, both the level of regulator expression and the physical localization of expression were shown to influence the biosensor features (Goni-Moreno et al. 2017): by utilizing a sensor system consisting of the activator XylS and the cognate promoter P_m driving *gfp* expression, it was shown that both high activator concentration and physical proximity of regulator and target promoter reduce the noise level and thus increase the obtainable dynamic range of the system in *P. putida*. The authors reasoned, supported by modelling, that this observation is caused by little to no expression of the reporter *gfp* as soon as the local concentration of inducer bound to XylS randomly becomes too low to allow binding to the promoter region, thus leading to a large spread of varied GFP concentrations in an induced population of genetically identical cells (Goni-Moreno et al. 2017).

Several studies underline how the exchange of standardized circuit parts facilitates the fine-tuning of sensor systems. For instance, a sensor based on DmpR was built for the screening of enzyme libraries for the production of phenol-derived compounds (Choi et al. 2014). Here the dynamic range was drastically improved by first replacing the existing RBS with an optimized RBS sequence, followed by the strict employment of standard transcriptional terminator sequences. In a similar manner, a set of biosensors for aromatic compounds was optimized (Xue et al. 2014). While several set-ups worked without further requirement for optimization, the unintentional transcription in the off-state of two sensors based on the transcriptional activators XylS and HbpR (the latter originating from the 2-hydroxybiphenyl pathway of *Pseudomonas azelaica*) prohibited a useful dynamic range. Thus it was reasoned that the activators were expressed at too high a concentration, or the RBS strength upstream of the reporter (*gfp*) was too high. In order to debug the system, first four weaker promoters were deployed for the expression of the regulators. This already led to a more suitable dose-response curve for the XylS-based circuit. The HbpR system was further improved by testing several weaker RBS upstream of the reporter gene, which also led to a significant improvement of the fold-change between the on- and off-state. Besides, this is a good showcase for how the availability of tested, standardized parts are facilitating the optimization of sensor circuits (e.g., Registry of Standard Biological Parts, http://parts.igem.org/Catalog). The suitability of RBS engineering for output optimization was further highlighted by the construction of a *p*-coumaric acid biosensor in *E. coli*, based on the repressor PadR of *B. subtilis* and its cognate promoter P_{PadC} driving *yfp* expression (Siedler et al. 2017). Here, two initially chosen RBSs upstream of PadR were either too weak or too strong in order to allow a useful dynamic range. While the very weak RBS

yielded a sensor with high basal output fluorescence (little repressor available), the second RBS tested was so strong that with the addition of 1 mM *p*-coumaric acid the fluorescence did not increase significantly (too much repressor available). However, by utilizing random RBS mutagenesis, several working sensor circuits were built with RBSs of intermediate strength, allowing up to a 130-fold change in output fluorescence upon addition of *p*-coumaric acid in the low mM concentration range.

Another common issue preventing the application of sensor systems is activation of the sensor circuit by off-target inducers. This can become a severe hindering factor, for example, when precursor molecules in the metabolic pathways of interest are similar enough to the product to unwittingly activate the sensor. In order to optimize the inducer spectrum, negative rounds of screening in the presence of the unwanted inducer (if it passes the membrane) can be applied. Here, all variants showing high basal fluorescence, either due to constitutive activation or induction with off-target molecules, are discarded. As mentioned before, a convenient strategy is to apply stringent negative selection first, in order to remove all variants showing off-target effects, due to a toxic phenotype. Then the workflow is continued with screens for the novel specificity of interest. This usually includes iterative rounds of screening for the cells exhibiting the highest fluorescence, as enrichment rounds are subject to cellular noise and thus include many false positive fluorescent cells if on- and off-state are not clear-cut (Siedler et al. 2017). An exemplary system for this dual selection/screening strategy was constructed for the engineering of a choline-inducible and -repressible transcription system, based on the transcriptional repressor BetI of *E. coli* (Saeki et al. 2016). The BetI variants were assessed by regulating the expression of a *sfgfp* gene but also of the genes for a herpes simplex virus thymidine kinase (*hsvtk*) and an aminoglycoside-(3′)-phosphotransferase (*aph*). While sfGFP served as a gradual indicator of the reporter-output, the latter reporters allowed negative and positive selection, respectively. In the presence of an artificial nucleoside, the expression of hsvTK is toxic, while the expression of APH is required for resistance against kanamycin. Using this sensor circuit under culture condition with and without externally added choline yielded BetI variants that were used for both choline-inducible and choline-repressive promoter systems. The discussed options and exemplary references for further details on biosensor engineering and debugging are briefly summarized in Table 1.

5 Research Needs

As highlighted above, in vivo biosensors can play an important role in high-throughput screening for improved biocatalysts. However, their application is dependent on finding the right sensor specificity and performance characteristics. The required engineering activities are well known, yet still laborious, opening a wide field for the development of rational, computer-based workflows. Such workflows have to take into account the actual in vivo conditions in order to avoid implementation problems as discussed for the RNA-based sensors, where in vitro selected aptamers often fail to operate satisfactorily in vivo. Until this is achieved, directed

Table 1 Optimization and debugging of common issues of transcription factor based genetic biosensor-circuits in microbial hosts for in vivo high-throughput screening

Issue	Possible solutions	References
Sensor specificity		
	Iterative rounds of positive screening of variants	Saeki et al. 2016
	Negative selection against unwanted inducers or side products of metabolic pathway	Tang et al. 2008
	DNA shuffling of hit variants	Hawkins et al. 2007
	DNA shuffling of homologous transcription factors	Garmendia et al. 2001
	Indirect detection via sensing enabling metabolic pathways	Libis et al. 2016a
Sensor output and dynamic range		
	Initial negative selection against high basal expression without inducer molecule present	Taylor et al. 2016
	Strength of promoter and RBS upstream of the transcription factor or reporter gene	de las Heras et al. 2012; Xue et al. 2014
	Operator multiplication upstream of reporter gene or utilization of the transcription factor as a "roadblock" for RNA polymerase	Merulla and van der Meer 2016
	Alternative carbon source in culture medium in order to avoid catabolite repression	Werlen et al. 2004
	Expression of importers for the target molecules (if not intracellularly made)	Call et al. 2016

evolution seems to be a good starting point for finding novel specificities. Finally, a standardized precise and quantitative description of sensor features would facilitate a more rapid exchange of genetic parts and thus the engineering of novel sensor systems.

6 Concluding Remarks

In this chapter, we reviewed the types and features of genetically encoded sensors available today. While RNA- and FRET-based sensors allow rapid detection of target compounds, the engineering of RNA tools which work under in vivo conditions with realistic cytoplasmic conditions, including high metabolite concentrations, physiological pH, temperature, and so on, is still difficult. However, the accuracy of in silico predictions for RNA binding motifs is constantly improving and will hopefully lead to more applications in the years to come. Biosensors based on transcription factors are in a more mature state. However, due to the reliance on the cellular transcription/translation machinery for the production of the reporter protein, their response is somewhat slower. In addition, the predictions for protein folding and protein binding to small molecules are at least as challenging as for RNAs. Fortunately, many interesting regulator specificities are already known from literature and databases today. Intriguingly, many of the systems initially developed for environmental monitoring of hydrocarbon spills might be of considerable interest for

metabolic engineering and high-throughput screening for products replacing certain petrochemicals.

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Microbial Production of Isoprenoids

19

J. Wong, L. Rios-Solis, and J. D. Keasling

Contents

Abstract

Isoprenoids are among the most diverse groups of compounds synthesized by biological systems; it has been estimated that there are approximately 30,000–50,000 known isoprenoids, which include the terpenoids and carotenoids.

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Isoprenoids are important in maintaining membrane fluidity, electron transport, protein prenylation, and cellular and organismal development and in controlling pests. Many isoprenoids have found application as fragrances and essential oils, pharmaceuticals, specialty and commodity chemicals, and most recently biofuels. To make all of these applications of isoprenoids possible, their production in microbial hosts is essential. Recently, there has been much progress in producing these complex hydrocarbons in both *Escherichia coli* and *Saccharomyces cerevisiae*. In this chapter, we review recent progress in this area.

1 Introduction

Isoprenoids are among the most diverse groups of compounds synthesized by biological systems; it has been estimated that there are approximately 40,000–70,000 known isoprenoids, which include sterols, carotenoids, and quinines (Beller et al. 2015; McCaskill and Croteau 1997). Isoprenoids are important in maintaining membrane fluidity, electron transport, protein prenylation, and cellular and organismal development, as fragrances and essential oils, antibacterial and antifungal agents, as well as high-value pharmaceuticals and fuel alternatives (McCaskill and Croteau 1997). Terpenes are classified by the number of isoprene (C5) units that they contain. Hemiterpenoids (C5) such as isopentenols; monoterpenes (C10), such as menthol and camphor; and sesquiterpenes (C15), such as zingiberene (ginger), are the major constituents of herbs and spices. Other sesquiterpenes and diterpenes (C20) are pheromones, defensive agents, and signal transduction agents (Fraga 2005; McGarvey and Croteau 1995; Wang et al. 2013). Higher molecular weight isoprenoids stabilize membranes (cholesterol and other C30 compounds) and serve as photoreceptive agents (carotenoids and other C40 compounds).

1.1 Isoprenoids as Pharmaceuticals

Many terpenoids have been found to exhibit potent biological activity, with several of them in development or in use therapeutically. The antimalarial drug artemisinin and the anticancer agent Taxol serve to illustrate the clinical importance of terpenoids, respectively (Kirby and Keasling 2009). Artemisinin, a sesquiterpenoid extracted from sweet wormwood (*Artemisia annua*), is a frontline treatment for malaria (Cui and Su 2009). Taxol, a diterpene extracted from the Pacific yew, is extremely effective in the treatment of certain cancers (ovarian, breast, lung and neck, bladder and cervix, melanoma, and Kaposi's sarcoma) (Jennewein and Croteau 2001; Skeel and Khleif 2011).Unfortunately, the complexity of the molecule precludes commercial total chemical synthesis (Engels et al. 2008). Taxol is currently produced either by semi-synthesis from 10-deacetylbaccatin III extracted from the needles of *Taxus* species or by extraction from plant cell suspension cultures grown with elicitors to improve production (Cusidó et al. 1999).

A range of medicinal diterpenoid compounds (i.e., phorbol esters and the related casbanes, lathyranes, jatrophanes, and ingenanes) are solely produced in Euphorbiaceae and Thymelaeaceae species (Vasas and Hohmann 2014) from casbene and neocembrene diterpene backbones (Kirby et al. 2010). These diterpenoids have gained interest due to unique anticancer, anti-HIV, vascular-relaxing, neuroprotective, anti-inflammatory, or immunomodulatory activities (Blumberg 1988; Halaweish et al. 2002; Jiao et al. 2009; Srivalli and Lakshmi 2012; Vasas and Hohmann 2014). Recently, the US FDA approved the use of a related diterpenoid, ingenol mebutate, for the treatment of the premalignant skin condition actinic keratosis (Vasas and Hohmann 2014). Prostratin, a phorbol ester found in *Homalanthus nutans* (Euphorbiaceae), is being developed as an adjuvant therapy to clear latent viral reservoirs, the primary obstacle to eradication of HIV (Wang et al. 2015). A related diterpene, resiniferatoxin, has potential utility in the treatment of chronic pain such as that resulting from osteosarcoma and osteoarthritis (Marwick 2005).

The monoterpene limonene and related derivatives are believed to inhibit farnesylation of the growth-promoting protein RAS, inhibiting malignant cell proliferation (Gelb et al. 1995; Gould 1997; Hohl 1996). Additionally, monoterpene indole alkaloids such as vinblastine, vincristine, and camptothecin are commonly used in chemotherapeutic treatments, and the pathways of these drugs are currently being researched for utilization in microbial production (Góngora-Castillo et al. 2012; O'Connor and Maresh 2006). The ability to produce terpenoid drugs in microbes could significantly reduce their production costs, reduce pressure on unsustainable plant-derived sources, and increase their chances of reaching clinical trials and the market.

1.2 Isoprenoids as Biofuels

There has also been a great deal of interest in terpenes as potential biofuels. The chemical structure of isoprenoids provides many beneficial aspects to act as a fuel alternative. This includes the common methyl branching, which lowers the freezing point significantly, as well as its usual cyclic nature which increases energy density (Beller et al. 2015). This has led to the recent production and testing of several isoprenoids as potential alternative fuels (Harvey et al. 2010; Mack et al. 2014; Yang et al. 2010). Esters of isoprenoid alcohols (C5, C10, and C15) have potential to be used as replacements for petroleum-based diesels (Fortman et al. 2008; Horton et al. 2003; Singh et al. 2008). Isoprenoid biosynthesis pathways provide additional routes to C5 hemiterpene alcohols (namely, isopentenol and isopentanol), which have higher energy contents than ethanol and high octane numbers (Yang et al. 2010), which supports their use as gasoline replacements and as antiknock additives (Cann and Liao 2010; Hull et al. 2006). Recently, due to their favorable energy content, the alcohols 3-methyl-3- and 3-methyl-2-butenol were shown to function as ideal antiknock additives in spark ignition engines (Mack et al. 2014).

In the case of monoterpenes, limonene and its fully hydrogenated form, limonane, are considered promising jet fuel replacements which have been evaluated as jet fuel additives (Chuck and Donnelly 2014; Tracy et al. 2009). Pinene dimers have also been shown to have a volumetric heating value similar to that of the tactical fuel JP-10 (Harvey et al. 2010).

Hydroxylated acyclic monoterpenoids such as linalool and geraniol (or its oxidized form geranial) are also potential biofuels (Hellier et al. 2013). Using linalool as substrate, a ruthenium (Ru)-based olefin metathesis reaction has been developed for the synthesis of 1-methylcyclopent-2-enol, a promising precursor for the synthesis of methylcyclopentadiene dimer, which can be converted to the high-density missile fuel RJ-4 (Meylemans et al. 2011).

The hydrogenated products of linear monoterpenes such as ocimene and myrcene are also considered biofuel replacements (Tracy et al. 2009). High-density renewable fuels have also been successfully synthesized through selective dimerization of α-pinene, camphene, limonene, and crude turpentine (Meylemans et al. 2012).

Diesel is composed of linear, branched, and cyclic alkanes with an average carbon length of 16, similar to the 15 carbon length of sesquiterpenes. The higher branching degree of sesquiterpenes gives them the benefit of more stability under high pressure, lower freezing point, reduced premature ignition, and increased octane number, although it also lowers the cetane number (Peralta-Yahya and Keasling 2010). Sesquiterpenes farnesane, farnesol, and bisabolene and its hydrogenated form bisabolane have been recognized to have great potential as second-generation biofuels (Rude and Schirmer 2009). Farnesane and bisabolane have cetane numbers of 58 and 52 which are within the range for diesel fuels (40–60) (Peralta-Yahya and Keasling 2010; Wang et al. 2011b). The carbon ring of bisabolane confers more energy density per volume of fuel than farnesane, while the latter has a better cetane number and it is the most promising for commercialization (Renninger and McPhee 2008).

Amyris, in collaboration with the French oil company TOTAL and the Brazilian airline GOL, has reached industry approval to use farnesane as a jet fuel replacement for commercial flights (George et al. 2015b). In addition, Amyris has also patented the sesquiterpene amorphane to be used as a jet fuel replacement (Ryder 2009; Tsuruta et al. 2009). In the next coming years, a wider range of biofuels can be expected to be commercialized.

2 Synthesis of Terpene Olefins

The primary building block (C5 unit) for the synthesis of isoprenoids is isopentenyl pyrophosphate (IPP) which is synthesized via two different pathways: mevalonate pathway and non-mevalonate or 1-deoxyxylulose-5-phosphate (DXP) pathway (Fig. 1). The mevalonate pathway is found primarily in eukaryotes and archaea, while the non-mevalonate pathway is found primarily in prokaryotes and in the plastids of photosynthetic eukaryotes (Eisenreich et al. 1998; Lange et al. 2000). The mevalonate pathway produces IPP from three molecules of acetyl-CoA (Hampton

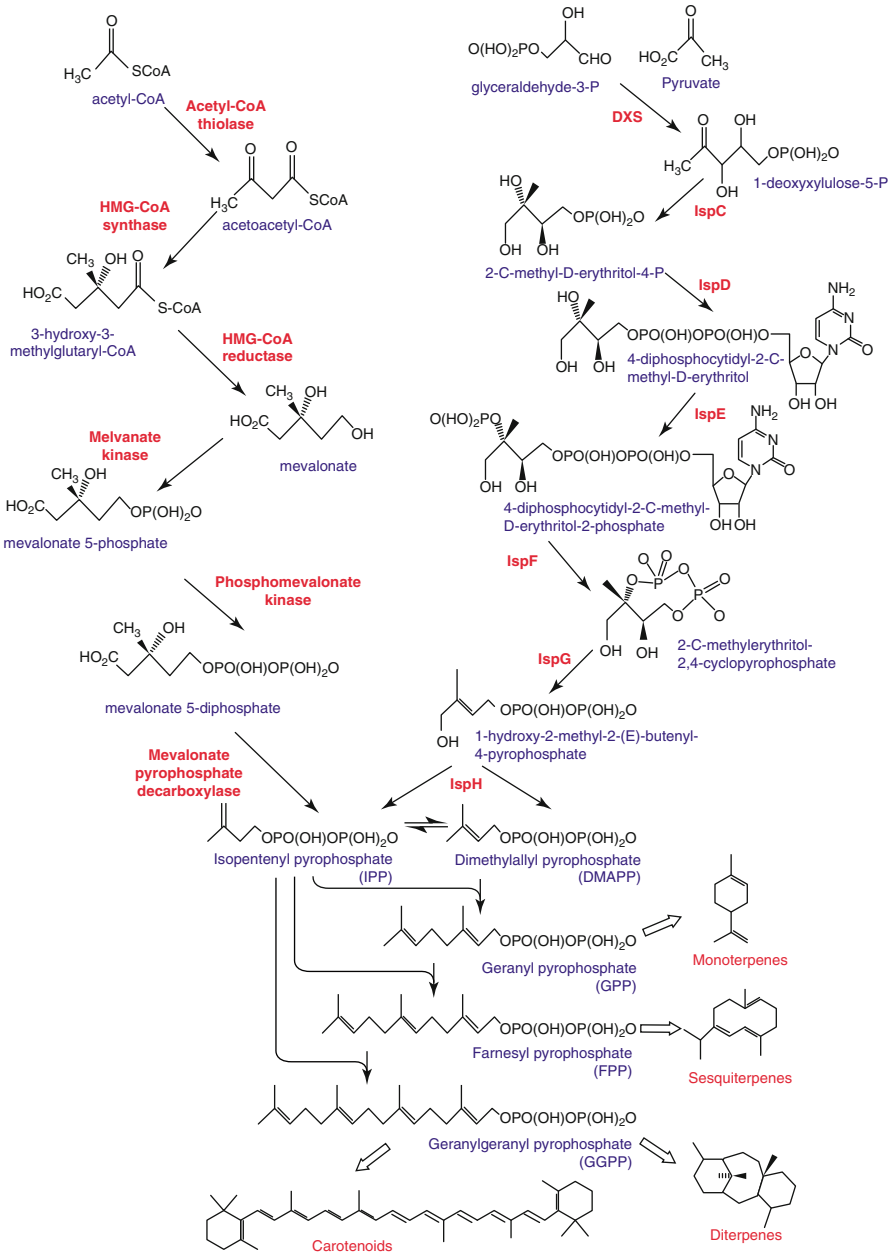

Fig. 1 Terpene biosynthetic pathways. *Left*: DXP pathway. *Right*: Mevalonate pathway

et al. 1996; Liao et al. 2016; Meigs et al. 1996; Szkopińska et al. 2000). The genes encoding the mevalonate-dependent pathway have been cloned from a number of organisms (Hahn and Poulter 1995; Phulara et al. 2016; Potter and Miziorko 1997; Takahashi et al. 1999; Weaver et al. 2015).

The more recently elucidated DXP (or MEP) pathway produces IPP and DMAPP from pyruvate and glyceraldehyde-3-phosphate (Campos et al. 2001; Lois et al. 1998; Rohmer et al. 1993; Schwender et al. 1996).

Synergy between both pathways has been observed in an *E. coli* engineered strain with both the DXP and the MVA pathway, where it was shown that overexpression of the dual pathway resulted in a 4.8-fold and 1.5-fold increase in the flux through the MEP and MVA pathway, respectively (Yang et al. 2016).

DMAPP acts as a primer for the sequential additions of IPP by isoprenyl pyrophosphate synthases to form C10 geranyl pyrophosphate (GPP), C15 farnesyl pyrophosphate (FPP), C20 geranylgeranyl pyrophosphate (GGPP), and larger isoprenyl pyrophosphates (Fig. 1). Several prenyltransferases have been characterized, and their genes cloned. These prenyltransferases are fairly selective for the chain condensation lengths and stereochemistries of their substrates and products (Leipoldt et al. 2015; Tarshis et al. 1994; Wang 2000).

Terpene olefins are synthesized from the linear terpene pyrophosphate esters GPP, FPP, and GGPP. Cyclization of GPP by terpene cyclases or synthases forms the monoterpenes, and cyclization of FPP creates the sesquiterpenes, while cyclization of GGPP forms the diterpenes (Dickschat 2016).

The terpene cyclases are similar to the prenyltransferases in structure and reaction mechanism. However, they differ from prenyltransferases in two respects: (1) terpene cyclases catalyze intramolecular reactions, whereas prenyltransferases catalyze intermolecular reactions; and (2) different terpene cyclases can take the same substrate and catalyze the formation of very different products (Chappell 1995). While some of the terpene cyclases form one or a few products, there are some cyclases that produce a variety of products from a single substrate type; for example, the δ-selinene and γ-humulene synthases of *Abies grandis* (Grand Fir) produce 34 and 52 total sesquiterpenes, respectively (Steele et al. 1998).

In general, the condensation and cyclization steps are independently catalyzed. Nevertheless, exceptions exist; some enzymes contain both a C-terminal isoprenyl transferase domain and an N-terminal cyclase domain which can catalyze both condensation and cyclization reactions (Chiba et al. 2013; Ozaki et al. 2014).

3 Metabolic Engineering of Isoprenoid Production

Due to the ease of genetic manipulation and industrial fermentation of *E. coli* and *S. cerevisiae*, most isoprenoid metabolic engineering efforts have focused on these hosts, although metabolic engineering in other host organisms for isoprenoid production has been promising (Melillo et al. 2013; Wriessnegger et al. 2014; Zhan et al. 2014). *E. coli* and *S. cerevisiae* have been engineered to produce high levels of mono-, sesqui-, and diterpenes for both biofuel and medicinal applications (Alonso-

Gutierrez et al. 2013; George et al. 2015a; Meadows et al. 2016; Paddon et al. 2013; Trikka et al. 2015).

3.1 Production of Terpenes in *E. coli*

E. coli was used to functionally express several native and codon-optimized plant terpene synthases and to confirm the production of the corresponding terpenes: myrcene and carene synthases (monoterpene synthases); δ-cadinene, epi-aristolochene, vetispiradiene, germacrene C, γ-humulene, selinene, amorphadiene, and epi-cedrol synthases (sesquiterpene synthases); and casbene, ent-kaurene, and abietadiene synthases (diterpene synthases) (Kim et al. 2015; Martin et al. 2001; Reiling et al. 2004). Originally, most of these terpenes were produced at levels of less than 1 μg L^{-1} (when using the native DXP pathway) in *E. coli*. This was approximately 500-fold lower than lycopene production in a similar system.

To address the difficulties expressing plant terpene synthases in *E. coli*, the two sesquiterpenes of *E. coli* codon-optimized genes of amorphadiene synthase (ADS) and epi-cedrol synthase (EPC) were synthesized. The cDNA clones for both genes had been previously obtained from *Artemisia annua* (Mercke et al. 1999, 2000) and were cloned into the high-copy expression vector pTrc99A. Expression of the codon-optimized synthetic genes increased both enzyme production and product formation. The production of amorphadiene was 13-fold higher than that of δ-cadinene when using endogenous FPP. An SDS-PAGE analysis of cells expressing the ADS gene showed a visible band near the predicted gene size of 64 KDa; the protein produced from the native plant gene was not evident on a gel, even when the rare codon tRNA plasmids were included. The synthetic genes were co-expressed with the genes for the latter steps of the mevalonate pathway (MBIS) with 20 mM mevalonate in the culture medium, *E. coli* produced greater than 1.7 mg L^{-1} amorphadiene. When the synthetic amorphadiene synthase gene was co-expressed with the full mevalonate pathway (Fig. 2), *E. coli* produced 10 mg L^{-1} amorphadiene (Martin et al. 2003). To prevent the loss of the volatile amorphadiene, dodecane was added to the culture medium. In this two-phase cultivation, we were able to produce nearly 1 g L^{-1} amorphadiene, with the amorphadiene accumulating in the organic phase (dodecane) (Newman et al. 2006). This system (engineered host plus production process) represented a production level approximately 1,000,000-fold higher than the previous best native plant gene system or the system based on the native *E. coli* isoprenoid biosynthetic (DXP) pathway.

The system was further improved by the identification of the two rate-limiting enzymes (mevalonate kinase (MK) and amorphadiene synthase (ADS)). By optimizing promoter strength to balance expression of the encoding genes, the pathway bottlenecks were alleviated and improved production fivefold (Anthony et al. 2009). Using gene variants of HMGS and HMGR derived from *Staphylococcus aureus* (mvaS and mvaA, respectively) as well as developing a high-density fermentation

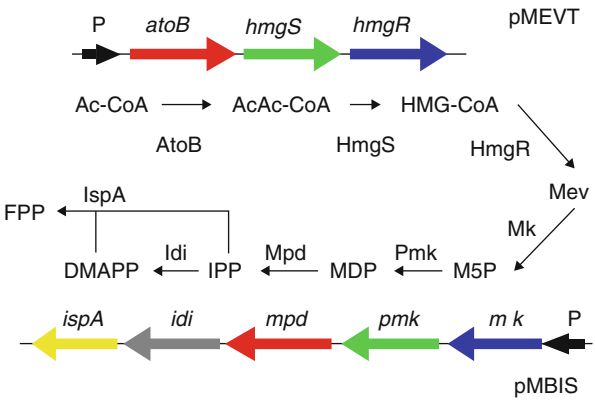

Fig. 2 Constructs used to express the mevalonate pathway in *E. coli*. The gene names are shown above each ORF. *P* promoter, *Ac-CoA* acetyl-CoA, *AcAc-CoA* acetoacetyl-CoA, *HMG-CoA* hydroxymethylglutaryl-CoA, *Mev* mevalonate, *M5P* mevalonate-5-phosphate, *MDP* mevalonate-5-pyrophosphate, *IPP* isopentenyl pyrophosphate, *FPP* farnesyl pyrophosphate

process, amorphadiene production was further improved to yield up 27.4 g L^{-1} in a 2L bioreactor (Tsuruta et al. 2009).

High-level production of mono- and diterpene synthases in *E. coli* requires the introduction of either GPP or GGPP production capabilities along with monoterpene or diterpene synthases. In addition to the genes involved in production of IPP and DMAPP, polyprenyltransferases have also been cloned and successfully expressed. To produce monoterpenes and diterpenes, *E. coli* FPP synthase gene (*ispA*) has been mutated at the fourth and fifth positions N-terminal to the first aspartate-rich domain using site-directed mutagenesis to convert the enzyme to either a GPP synthase or a GGPP synthase, respectively, similar to the research done on the FPP synthase of *B. stearothermophilus* (Narita et al. 1999; Ohnuma et al. 1996). The products of both mutant enzymes were verified by in vitro and in vivo production of monoterpenes and diterpenes in engineered *E. coli* (Reiling et al. 2004). The addition of these genes to the encoded IPP pathway created a host organism capable of overproducing precursors to monoterpenes, sesquiterpenes, diterpenes, and carotenoids. The engineered GGPP precursor generating system (GGPP synthase formed by the native *E. coli* FPP synthase) was evaluated for the production of the diterpenes casbene and ent-kaurene. Coupling the high-flux mevalonate pathway described previously for amorphadiene production to the GPP or GGPP synthase system allowed for the production of various isoprenoids like bisabolene, farnesene, limonene, pinene, casbene, or sabinene among others (Alonso-Gutierrez et al. 2013; Sarria et al. 2014; Wang et al. 2011a; Zhang et al. 2014).

Further engineering of the MVA *E. coli* platform guided by proteomics and metabolomics using a GPP synthase from *Arabidopsis thaliana* and a limonene synthase from *Mentha spicata* allowed the production of up to 650 mg L^{-1} of limonene (Alonso-Gutierrez et al. 2015). Using the same optimization strategy, a titer of 1.15 g L^{-1} of bisabolene was achieved using shake flasks by using a codon-optimized bisabolene synthase from *Abies grandis* (Alonso-Gutierrez et al. 2015; Peralta-Yahya et al. 2011).

The synthesis of monoterpenes pinene and myrcene in *E. coli* using the MVA pathway has also recently been achieved up to titers of 32 and 58 mg L^{-1},

respectively (Kim et al. 2015; Sarria et al. 2014; Yang et al. 2013). The lower titer of pinene in comparison to the titer of limonene suggested that pinene synthase is likely the bottleneck, considering that both pathways shared the same MVA pathway and precursors (Beller et al. 2015).

E. coli was used as a microbial system for the heterologous synthesis of β-phellandrene. Heterologous co-expression of the MVA pathway was required to confer β-phellandrene production up to 3 mg per g dcw (Formighieri and Melis 2014).

In the case of diterpenes, the major advances in heterologous production of the terpene olefin in *E. coli* have been achieved for taxadiene, which is the precursor of the anticancer drug Taxol (paclitaxel). In this case, the native DXP pathway was selected instead of the MVA pathway (Ajikumar et al. 2010), and using a multivariate-modular approach allowed the production of taxadiene up to 1 g L^{-1} in a 2L bioreactor, by using a previously identified truncated version of *Taxus brevifolia* taxadiene synthase (Huang et al. 1998). In this work, in order to optimize the production, the pathway was divided into an upstream module comprising the DXP pathway leading to IPP synthesis and a downstream module integrated by the *Taxus* geranylgeranyl pyrophosphate and taxadiene synthase. Both modules were combined with different promoters and gene copy numbers in order to identify the optimum combination for high diterpene production.

3.2 Production of Terpenes in *S. cerevisiae*

Yeast is a particularly attractive host because of its robustness in industrial fermentation, genetic stability, and greater capacity for P450 expression (relative to *E. coli*) (Chang et al. 2007; Ro et al. 2006). Using yeast as a heterologous host is now simplified due to recent advancements in markerless genome engineering using Cas9, which have significantly reduced the time to produce the necessary modifications for an engineered yeast strain (Horwitz et al. 2015; Lee et al. 2015). The development of yeast strains for artemisinin production has shown the potential of using yeast as a heterologous host for terpene production, even surpassing terpene titers in *E. coli* cell culture (Paddon et al. 2013). Terpenoid pathway engineering in *S. cerevisiae* has largely focused on the production of the C15-based sesquiterpenes whose titers have reached >10 g L^{-1} (in fed-batch bioreactors, >1 g L^{-1} in laboratory shake flasks) (Westfall et al. 2012). Development of monoterpenoid and diterpenoid production in yeast has lagged behind, with fewer studies and lower titers (<20 mg L^{-1} and <1 g L^{-1}, respectively) (Ignea et al. 2014; Trikka et al. 2015). For the Taxol pathway, the first enzyme, taxadiene synthase, has been functionally expressed in *E. coli* to produce >300 mg L^{-1} taxadiene in shake flasks (Ajikumar et al. 2010). However, only three of the subsequent enzymes could be functionally expressed and, until recently, only in a co-cultured *S. cerevisiae* strain since P450 expression in *E. coli* has proven problematic as these enzymes are typically membrane bound and require a P450 reductase to provide electrons for the oxygenation reactions

(Biggs et al. 2016; Zhou et al. 2015). Functional expression of taxadiene synthase in yeast has also been problematic; titers reported in the literature remain low (<10 mg L^{-1}) hampering further pathway identification and heterologous reconstitution (Dejong et al. 2006; Engels et al. 2008) (approximately 70 mg L^{-1} taxadiene has been reported (Ding et al. 2014); however, the authors' NMR and mass spectra do not match those previously published, leading us to discount this claim). For prostratin and other phorbols, functional expression of casbene synthase and subsequent enzymes in yeast has also proved challenging (Kirby et al. 2010; Luo et al. 2016).

The most successful instances of terpene production in *S. cerevisiae* rely on several approaches. Many groups have independently shown that HMGR is the rate-limiting enzyme of the isoprenoid pathway; this is due to negative feedback of the HMGR-encoded enzyme by pathway products, resulting in degradation of the enzyme (Hampton and Garza 2009). A truncated version of HMGR (tHMGR) was found to improve sesquiterpene titers, as this modification removes the endogenous regulation of this enzyme in yeast (Ro et al. 2006). Additionally, upregulation of every enzyme in the mevalonate pathway has been shown to improve titer by diverting flux of acetyl-CoA to isoprenoid production (Paddon et al. 2013). Recently, Amyris showed that modifying yeast central metabolism can produce significantly higher titers (Meadows et al. 2016). Removal of the PDH-bypass enzymes and replacement with heterologous enzymes for non-oxidative glycolysis to reduce carbon lost as CO_2 conserve ATP and reducing equivalents resulted in production of farnesene >15% by volume. Expressing multiple copies of a terpene synthase has been shown to improve final terpene titer. Fusions of terpene synthases with the preceding enzyme in the pathway have been used with varying levels of success in sesqui- and diterpene pathways (Albertsen et al. 2011; Ignea et al. 2014, 2015). An N-terminal fusion of yeast native Erg20pFPP synthase with patchoulol synthase resulted in a strain with a doubled patchoulol titer compared to strains with an unfused patchoulol synthase (Albertsen et al. 2011). Fusion proteins have been used with great success in yeast diterpene production. Ignea and colleagues fused a mutant GGPP-producing Erg20p, Erg20F96Cp, to the C-terminus of the type II 8OH-CPP synthase from *Cistus creticus*, resulting in a titer of 400 mg L^{-1} of sclareol (Ignea et al. 2015). They co-expressed this fusion enzyme with other type I terpene synthases, producing increased titers of these terpene products, cis-abienol and abietadiene, as well. Ignea and colleagues also developed a mutant Erg20p-producing GPP, Erg20F96W-N127Wp (Ignea et al. 2014). When fused to the N-terminus of sabinene synthase and combined with Erg20 and Erg9 down-regulation, they reported a final titer of 17 mg L^{-1} or 340-fold increase in sabinene relative to the starting strain. Trikka and colleagues reported the highest titer of any diterpenoid in yeast, 750 mg L^{-1}, of sclareol in shake flasks (Trikka et al. 2015). This titer was achieved by using a carotenogenic screen to identify gene deletion mutants with improved diterpene yield. Codon optimization has also provided mixed results for the production of terpenes in yeast (Andersen-Ranberg et al. 2016; Westfall et al. 2012); however, some of the highest diterpene titers have resulted from a combinatorial expression screen of codon-optimized type I and II

diterpene synthases, producing nearly 400 mg L^{-1} of various diterpenes (Andersen-Ranberg et al. 2016).

3.3 Production of Terpenes in Other Hosts

The past two decades have witnessed a significant effort to develop and optimize isoprenoid production in common hosts such as *Escherichia coli* or *S. cerevisiae*. Recently, many studies have focused on genetically modifying alternate microorganisms in order to develop novel hosts for isoprenoid production platforms.

Corynebacterium glutamicum has been engineered to produce pinene by co-expressing a geranyl diphosphate synthase and pinene synthase from *Pinus taeda* and *Abies grandis* in combination with an overexpressed native 1-deoxy-d-xylulose-5-phosphate synthase and isopentenyl diphosphate isomerase (Kang et al. 2014). This resulted in a pinene production of 27 μg g^{-1} dry cell weight.

The DXP pathway of *Streptomyces venezuelae* was engineered and combined with a codon-optimized bisabolene synthase from *A. grandis*, resulting in the production of up to 10 mg L^{-1} of bisabolene (Phelan et al. 2015).These titers still represent an approximate order of magnitude less of bisabolene production compared to *S. cerevisiae* (Peralta-Yahya et al. 2011), but titers are similar to pinene production in *E. coli* (Sarria et al. 2014).

Genetic engineering of the cyanobacterium *Synechocystis* allowed the production of isoprene from CO_2 and H_2O (Lindberg et al. 2010). This was achieved by heterologous expression of the codon-optimized *Pueraria montana* isoprene synthase in *Synechocystis*, enabling photosynthetic isoprene production with titers of up to 50 μg per g dry cell weight per day. *Synechocystis* sp. was also used to express a β-caryophyllene synthase gene from *Artemisia annua*, resulting in the synthesis of β-caryophyllene up to 46 mg L^{-1} (Reinsvold et al. 2011). *Synechocystis* sp. has also been engineered to produce limonene by overexpressing three native enzymes of the DXP pathway (*dxs*, *crtE*, and *ipi*) in combination with a codon-optimized *Schizonepeta tenuifolia* limonene synthase (Kiyota et al. 2014). The engineered strain allowed the photosynthetic production of limonene during 300 h reaching an overall production of 1 mg L^{-1}. Cyanobacterium *Synechococcus* sp. has been engineered to produce titers of 4 mg L^{-1} limonene and 0.6 mg L^{-1} α-bisabolene through heterologous expression of the *Mentha spicata* limonene synthase or the *Abies grandis* α-bisabolene synthase genes, respectively (Davies et al. 2014). This highlighted *Synechococcus* sp. as a promising platform for terpenoid biosynthesis.

Aspergillus nidulans has also been shown to be capable of heterologous terpene production like amorphadiene by encoding an *Artemisia annua* amorpha-4,11-diene synthase (Lubertozzi and Keasling 2008). Using the same host, Bromann et al. (2016) overexpressed the *Fusarium fujikuroi ent*-kaurene synthase and *Citrus unshiu* γ-terpinene synthase and demonstrated the production of *ent*-kaurene and γ-terpinene, respectively.

Although in the majority of the cases the production titers of terpenes in non-conventional hosts were still relatively low, these examples highlight the potential

for future applications and engineering endeavors to further improve traits for high-titer biofuels or high-value chemical biosynthesis.

4 Functionalization of Terpenes

The majority of terpenes of medical relevance are functionalized in at least one position on the hydrocarbon backbone. Functionalization, which may include glycosylation, acetylation, hydroxylation, benzoylation, and additional ring closures of the terpene backbone, expands further the diversity of possible terpenes. Relatively few of the enzymes responsible for terpene functionalization have been purified and/or characterized, and only a fraction of the genes encoding these proteins have been cloned. To date, the most extensive characterization has been done for the biosynthesis of the diterpenes Taxol (Ajikumar et al. 2010) and gibberellin. Most functionally characterized terpene-modifying enzymes to date have been P450s. These are the largest class of modifying enzymes by far, representing 1% of all encoded genes in plants (Hamberger and Bak 2013).

4.1 Monoterpenes

The monoterpenes menthol and carvone (from *Mentha x piperita* and *M. spicata*, respectively) are both synthetized from limonene but differ in the oxygenation position. The cytochrome P450 hydroxylases (limonene-3-hydroxylase and limonene-6-hydroxylase) that form these two products from limonene were cloned from mint trichome cDNA libraries, expressed in *E. coli* and *S. cerevisiae*, and characterized in vitro (Haudenschild et al. 2000; Lupien et al. 1995; Schalk and Croteau 2000; Wüst and Croteau 2002). Co-expression of a NADPH-cytochrome P450 reductase gene in *S. cerevisiae* or addition of the purified NADPH-cytochrome P450 reductase to *E. coli* lysate reconstituted the enzyme activity in vitro.

Croteau and colleagues introduced the carvone biosynthetic pathway into *E. coli* (Carter et al. 2003). They demonstrated that the region-specific cytochrome P450 limonene hydroxylase (Haudenschild et al. 2000) and menthofuran synthase (a cytochrome P450 monooxygenase) (Bertea et al. 2001) from mint could be functionally expressed in *E. coli* and *S. cerevisiae*. By introducing into *E. coli* a GPP synthase, limonene synthase, cytochrome P450 limonene-6-hydroxylase, and a carveol dehydrogenase, they were able to produce 5 mg L^{-1} of limonene. Unfortunately, most of limonene was excreted into the medium so that the final product was not detectable. Only when limonene was fed to cells was carvone detectable. Again, understanding the factors limiting P450 function could significantly impact the amount of final product formed.

In addition to limonene hydroxylases, a geraniol 10-hydroxylase has been cloned from *Catharanthus roseus* and expressed in *S. cerevisiae* for in vitro analysis (Collu et al. 2001). It was shown that a single amino acid substitution could convert a

C6-hydroxylase to a C3-hydroxylase (Schalk and Croteau 2000), indicating that plant P450s have great potential for protein engineering.

In the case of functionalization of limonene, a second cassette containing a cytochrome P450 from *Mycobacterium* sp. and a ferredoxin reductase was added to the *E. coli* strain encoding the MVA pathway, in combination with a geranyl diphosphate synthase from *Abies grandis* and a limonene synthase from *Mentha spicata*, resulting in the production of up to 100 mg L^{-1} of perillyl alcohol (Alonso-Gutierrez et al. 2013).

Recently three new cytochrome P450 monooxygenases were isolated and expressed in *E. coli* demonstrating the hydroxylation of 1,8-cineole (eucalyptol) to yield the products putatively identified as (1*S*)-2α-hydroxy-1,8-cineole or (1*R*)-6α-hydroxy-1,8-cineole (Unterweger et al. 2016). The hydroxylation of 1,8-cineole to (1R)-6β-hydroxy-1,8-cineole using *Pseudomonas putida* as a host in combination with a cytochrome P450 monooxygenase and its native redox partner cindoxin from *Citrobacter braakii* allowed a production of 13 g L^{-1} in a bioreactor (substrate 1,8-cineole was added exogenously) (Mi et al. 2016). This highlighted the ability of *P. putida* to resist various toxic compounds, including several monoterpenes which have been found toxic in *E. coli* (Chubukov et al. 2015).

The expansion of the enzyme toolbox for modification of monoterpenes will be critical to create starting materials to discover new functionalizing enzymes and has been used as hydroxylated derivatives in a range of industrial applications.

4.2 Sesquiterpenes

Many specific sesquiterpene hydroxylases have been identified. 5-epi-Aristolochene-1,3-dihydroxylase (CYP71D20), one of the first sesquiterpene-modifying P450s discovered, was found responsible for oxidizing 5-epi-aristolochene twice forming capsidiol in *Nicotiana tabacum* by co-expression with an *Arabidopsis thaliana* NADPH-cytochrome P450 reductase in *S. cerevisiae* (Ralston et al. 2001). Perhaps the most well-known sesquiterpene-modifying P450, amorphadiene oxidase (CYP71AV1), catalyzes three successive oxidations, using an amorphadiene substrate and forming artemisinic acid (Ro et al. 2006). More recently identified, CYP71BA1 forms 8-hydroxy-α-humulene from α-humulene in the zerumbone biosynthetic pathway in *Zingiber zerumbet* (Yu et al. 2011). In grape, VvSTO2 of the CYP71BE family forms (−)-rotundone via the oxidation of α-guaiene (Takase et al. 2016).

Many promiscuous sesquiterpene hydroxylases have also been identified. Cankar and coworkers found a P450 (CYP71AV8) from chicory that converts (+)-valencene to nootkatone, germacrene A to germacra-1(10),4,11(13)-trien-12-oic acid, and amorphadiene to artemisinic acid (Cankar et al. 2011). Premnaspirodiene oxygenase (CYP71D55) from *Hyoscyamus muticus* oxidizes premnaspirodiene, 5-epi-aristolochene, valencene, and 5-epi-eremophilene (Takahashi et al. 2007). (+)-δ-Cadinene-8-hydroxylase (CYP706B1), involved in the hydroxylation of (+)-δ-cadinene in the gossypol biosynthetic pathway, was found to oxidize

(−)-α-cubebene, (−)-α-muurolene, α-humulene, and (−)-δ-cadinene and has been expressed in *S. cerevisiae* (Luo et al. 2001; Wang and Essenberg 2010).

Other sesquiterpene-modifying enzymes have been found, though they are few. One example is a short-chain alcohol dehydrogenase that was found to form zerumbone from α-humulene (Okamoto et al. 2011).

A pathway in *E. coli* and *S. cerevisiae* was developed for the production of artemisinic acid, which can be converted directly into the antimalarial drug artemisinin in two chemical steps (Fig. 3) (Roth and Acton 1989). Addition of an amorphadiene oxidase (AMO) and associated redox partners from *A. annua* enabled production of artemisinic acid (Chang et al. 2007; Ro et al. 2006). The native gene (nAMO) presented no detectable in vivo or in vitro activity; nevertheless, a codon-optimized gene coupled with N-terminal transmembrane domain engineering gave two constructs highly competent to perform the first oxidation step in vivo to produce the alcohol congener of artemisinic acid at low levels (0.18–0.45 mg L^{-1}). The use of the redox partners with those from *A. annua* increased productivity 12-fold to 5.6 mg L^{-1} of alcohol. Finally, the use of the most appropriate promoters and expression vector allowed much higher in vivo productivity of fully oxidized artemisinic acid. Further co-expression of an alcohol dehydrogenase and aldehyde dehydrogenase in *S. cerevisiae* improved production of artemisinic acid from amorphadiene (Westfall et al. 2012).

4.3 Diterpenes

To date, most diterpene-modifying enzymes have been discovered in pathways related to labdane-type diterpenoids and taxane-type diterpenoids (Hamberger et al. 2011; Ignea et al. 2016; Zerbe et al. 2013). Hamberger and colleagues found P450s in the CYP720B family that oxidize diterpene resin acids producing anti-herbivory diterpenes in Sitka spruce trees (Hamberger et al. 2011). Additional steps of the carnosic acid biosynthesis pathway have been discovered; Ignea and coworkers identified four P450s – CYP76AH24, CYP71BE52, CYP76AK6, and CYP76AK8 – that oxidize intermediates in carnosic acid pathway by screening using heterologous expression in yeast (Ignea et al. 2016). In ingenane and phorbol ester biosynthesis, JcCYP71D495 and ElCYP71D445 oxidize casbene to 9-hydroxycasbene, while JcCYP726A35, JcCYP726A20, and ElCYP726A27 form 5-hydroxycasbene. When an enzyme from each group is co-expressed, they produce 9-hydroxy-5,6-hydroxycasbene. Co-expression with an ADH1 resulted in the unusual ring closure seen in the intermediate jolkinol using heterologous expression in *N. benthamiana* and in yeast microsomes (King et al. 2016; Luo et al. 2016).

The biosynthetic pathway to Taxol comprises approximately 20 steps, starting with the cyclization of the prenyl diphosphate geranylgeranyl pyrophosphate (GGPP) to the diterpene taxa-4(5),11(12)-diene, followed by multiple hydroxylations, two acylations, two benzoylations, oxetane ring formation, and side chain addition to generate the final product. Three taxadiene hydroxylating cytochrome P450s (taxadiene 5α-hydroxylase and taxane 10β-hydroxylase and

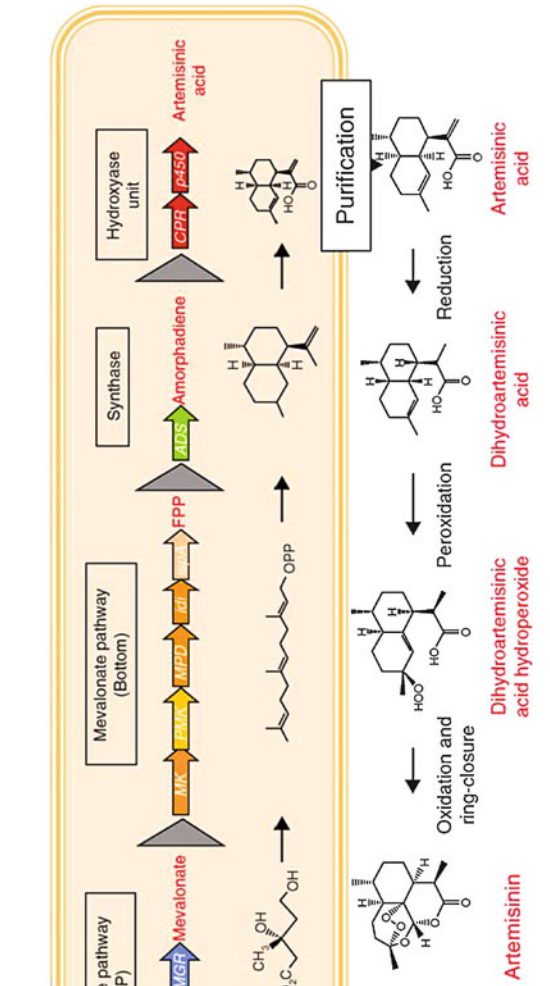

Fig. 3 Semi-synthesis strategy for producing artemisinin. The genes encoding the mevalonate-based FPP biosynthetic pathway were introduced from *Saccharomyces cerevisiae* and *Escherichia coli* into *E. coli*. The genes encoding the enzymes in the pathway were organized into two operons to allow for easy optimization. The MevT operon contains genes responsible for transforming three acetyl-CoAs into mevalonate, and the MBIS operon contains genes responsible for transforming mevalonate to FPP. In addition, the genes encoding the amorphadiene synthase, oxidase, and redox partners were also introduced into the host strain. The microbial strain produces artemisinic acid, which can be transformed to artemisinin using established chemistry

13α-hydroxylase) had been characterized by the early 2000s (Hefner et al. 1996; Jennewein and Croteau 2001; Schoendorf et al. 2001; Walker and Croteau 2001). Two of these hydroxylases, taxadiene 5α-hydroxylase and taxane 10*β*-hydroxylase, have been functionally expressed in *Saccharomyces cerevisiae* as a microbial consortium with *E. coli* providing the taxadiene precursor (Zhou et al. 2015). More recently, six additional taxoid hydroxylases were characterized, with taxane 2α-hydroxylase and 7*β*-hydroxylase directly contributing to Taxol biosynthesis (Kaspera and Croteau 2006). In addition, Walker and colleagues identified two acyltransferases and a benzoyltransferase in the Taxol biosynthetic pathway, with taxadien-5α-ol acetyl-transferase expressed in yeast (Walker and Croteau 2001; Zhou et al. 2015). While many enzyme candidates have been found, the specific order in which the enzymes act is still unknown. Biochemical characterization of many enzymes has proved difficult because of the lack of intermediates with which to test them. Functional co-expression of such isolated enzymes presents the possibility of fermentation-based production of complex and highly substituted natural products. However, significant challenges remain in determining all of the factors that must be co-expressed with the hydroxylases to produce the decorated terpene in sufficient quantities to examine subsequent steps in the pathway and eventually to produce the desired end product, in this case Taxol. Based on the hydroxylation pattern of previously related taxoid compounds, it has been proposed that the hydroxylation reaction order could be C5, C10, C2, C9, and C13 followed by C7 and C1 (Croteau et al. 2006). The hydroxyl groups of C2, C5, and C10 are then acetylated, and C2 further benzoylated. Late stage Taxol intermediates such as 1-deacetylbaccatin III require the oxidation of the hydroxyl group at C9 and the addition of the side chain β-phenylalanoyl at C13 (Croteau et al. 2006).

5 Production of Terpene-Based Biofuels

It has been shown that the isoprenoid pathway could be applied for the production of the proposed gasoline additives isopentanol and isoamyl acetate (Hull et al. 2006). For example, a *Bacillus subtilis* pyrophosphatase has been shown to dephosphorylate IPP to form isopentenol (Withers et al. 2007). The acetylation of isopentanol by an engineered *E. coli* has also been demonstrated (Horton et al. 2003; Singh et al. 2008). Additionally, saturated or lightly (mono- or di-)unsaturated mono- and sesquiterpenes may find use as diesel and jet fuels. The ability to engineer terpene cyclases for production of novel terpenes will drastically expand the number of potential fuels that can be synthesized microbially (Martin et al. 2001; Yoshikuni et al. 2006).

Biological platforms for the production of bisabolene and farnesene have been successfully developed (Peralta-Yahya et al. 2011, 2012; Wang et al. 2011a); nevertheless, the hydrogenation step of these sesquiterpenes to produce the corresponding biofuels bisabolane and farnesane is still limited in terms of titers (Kung et al. 2014). Therefore, at the current state, an option is to produce isoprenoid

biofuels using a hybrid process where the sesquiterpene is produced in a microbial host followed by a chemical step to produce the reduced fuel (George et al. 2015b).

In the case of bisabolene, after optimizing the MVA pathway to improve flux toward FPP and using a codon-optimized bisabolene synthase from *Abies grandis,* titers of bisabolene up to 900 mg L^{-1} in both *E. coli* and *S. cerevisiae* were achieved (Peralta-Yahya et al. 2011). After identifying gene deletions that improved isoprenoid production in shake flasks, bisabolene was produced at 5.2 g L^{-1}in bioreactors (Özaydın et al. 2013).

Farnesene synthase from different sources such as *A. annua* (Picaud et al. 2005), *Picea abies* (Martin et al. 2004), and *Zea mays* (Köllner et al. 2009) among others has been cloned and characterized in *E. coli*. Farnesene has been produced in *E. coli* from FPP via heterologous expression of a *Malus x domestica* farnesene synthase, reaching 320 mg L^{-1}(Wang et al. 2011a). Using the previously described *E. coli* and *S. cerevisiae* production platforms (Martin et al. 2003; Peralta-Yahya et al. 2011; Pitera et al. 2007) with the optimized MVA pathway and by recruiting the farnesene synthase from *A. annua* and *P. abies*, the production of farnesene in *E. coli* and *S. cerevisiae* reached up to 1.1 g L^{-1} and 0.72 g L^{-1}, respectively (Renninger and McPhee 2008). Farnesene is currently produced by Amyris from sugarcane by using an evolved *S. cerevisiae* PE-2 strain where mass yields higher than >50% have been achieved (Chandran et al. 2011; George et al. 2015b).

Novel process configurations integrating fermentation and product recovery, cell reuse, and low-cost technologies for product separation (Cuellar et al. 2013), in combination with novel synthetic biology tools applied for further strain engineering, will likely have a positive impact on biofuel commercialization.

6 Conclusions and Research Needs

Because of the sheer number of isoprenoids, they hold potential in both the fields of medicine and sustainable biofuels. Microbial production of isoprenoids offers a potentially more sustainable, higher-yielding alternative to chemical synthesis or extraction from the natural host. The ability to decorate the terpene backbone with oxygen in the correct position through the functional expression of terpene oxidases opens up the possibility of producing complex drugs and other important molecules. While remarkable progress has been made in improving titer of terpenes in microbial production, there is still a need for pathway discovery, particularly in the area of P450s and other modifying enzymes, to broaden the varieties of useful terpenoids produced in microbes.

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Efforts Toward Industrial Biosynthesis of Isoprene

20

Lidan Ye and Hongwei Yu

Contents

Abstract

With wide applications, isoprene faces a growing global market. The increasing concerns about the environmental problems brought by petrochemical industry propel the research on bioproduction of isoprene. To date, numerous efforts have

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been made in the characterization and engineering of the isoprene biosynthesis pathway, leading to successful production of isoprene by bacteria and yeast. This chapter summarizes the research progresses in isoprene biosynthesis and introduces the isoprene biosynthesis in nature, the key enzyme isoprene synthase, and the development of isoprene-producing microorganisms. In particular, the efforts made toward industrial biosynthesis of isoprene using bacteria and yeast are discussed in detail, with special attention given to the metabolic engineering strategies adopted to achieve isoprene-producing strains with improved performance indices. The persisting issues and future research needs are presented as well.

1 Introduction

Isoprene (C_5H_8), also known as 2-methyl-1,3-butadiene, is the simplest member of isoprenoids. In its pure form, it is a colorless volatile liquid with a boiling point of 34 °C and a low solubility in water. The conjugated double bond in its structure makes it chemically active and easy to polymerize. Its main industrial application is the production of synthetic rubber for tires and coatings. Other applications include use in adhesives and specialty elastomers, as well as synthesis of vitamins, pyrethroid pesticides, and fragrances such as linalool (Takabe et al. 1975). In addition, it can also be used for development of fuel additive for gasoline, diesel, or jet fuel (Bentley et al. 2014).

At the moment, the production of isoprene almost entirely relies on the refinery-based conversion of petrochemical feedstocks, including extractive distillation from C5 cracking fractions, dehydrogenation of C5 isoalkanes and isoalkenes, or chemical synthesis using isobutylene and formaldehyde as the starting materials (Weissermel and Arpe 1992) (Fig. 1). The heavy dependence on the C5 supply from petrochemical industry renders isoprene production largely influenced by the fluctuation of the petroleum market. Moreover, the energy-intensive and environment-unfriendly nature of the chemical manufacturing process raises increasing social concerns. Therefore, isoprene biosynthesis has attracted increasing interest in both industry and research institutions. Recently, researchers have comparatively analyzed the chemical and biological processes of isoprene production using green metrics (Morais et al. 2015) and pointed out that the chemical process could be substituted by the fermentative isoprene production with material and energy efficiency when the target was set to 50,000 tons per year.

In this chapter we will describe the efforts made so far toward industrial biosynthesis of isoprene. The isoprene biosynthesis pathway, the key enzyme isoprene synthase, and in particular the development of isoprene-producing microorganisms would be introduced.

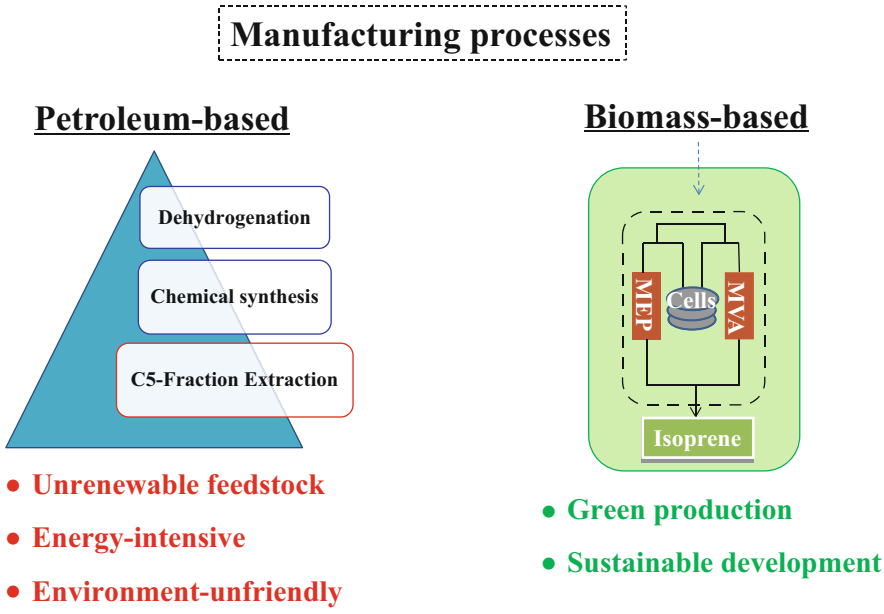

Fig. 1 Manufacturing routes of isoprene

2 Isoprene Biosynthesis in Nature

2.1 Natural Isoprene Producers

Isoprene emission is ubiquitous in nature, and its natural producers cover various eukaryotes and prokaryotes, with plants (especially tropical broadleaf trees) as the top contributors (Kuzuyama 2002). The yearly production of isoprene by vegetation reaches around 600 million metric tons, which accounts for around 40% of all biogenic isoprenoid emissions (Guenther et al. 2006). Marine is another important source of isoprene. The annual isoprene production from oceanic sources was estimated to be 1.9 million metric tons, with phytoplankton communities as the major contributors (Arnold et al. 2009). Various phytoplankton species have been reported to produce isoprene, with light temperature and organism size as the influential factors (Shaw et al. 2003). The global contribution of microbial isoprene production is relatively small, with *Bacillus subtilis* as the best natural bacterial producer, which produced isoprene at a yield of 12.78 nmol g^{-1} h^{-1} when cultivated in LB broth (Kuzma et al. 1995).

2.2 Isoprene Biosynthesis Pathway

Regardless of the emitting organisms, the universal precursors for biosynthesis of all isoprenoids including isoprene are isopentenyldiphosphate (IPP) and dimethylallyl diphosphate (DMAPP), the formation of which can be accomplished by two distinct and independent pathways, the methylerythritol-phosphate (MEP) pathway [also known as the 1-deoxy-D-xylulose-5-phosphate (DXP) pathway] and the mevalonate (MVA) pathway (Fig. 2). The MEP pathway initiates with condensation of glyceraldehyde-3-phosphate (G-3-P) and pyruvate to form DXP, which is then converted into MEP and further modified to yield hydroxy-2-methyl-2-butenyl-4-diphosphate (HMBPP), the substrate for IPP formation. The MEP pathway is present in most bacteria, cyanobacteria, green microalgae, and plant plastids (Xue and Ahring 2011). On the other hand, the MVA pathway is responsible for isoprenoid synthesis in eukaryotes, archaea, and cytosol of higher plants (Miziorko 2011). Acetyl-CoA is the primary feedstock for the MVA pathway, three molecules of which are condensed to form 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA). The subsequent reduction product mevalonate is often used as a node to divide the MVA pathway into an "upper section" and a "lower section." Mevalonate is then converted to IPP via phosphorylation and decarboxylation.

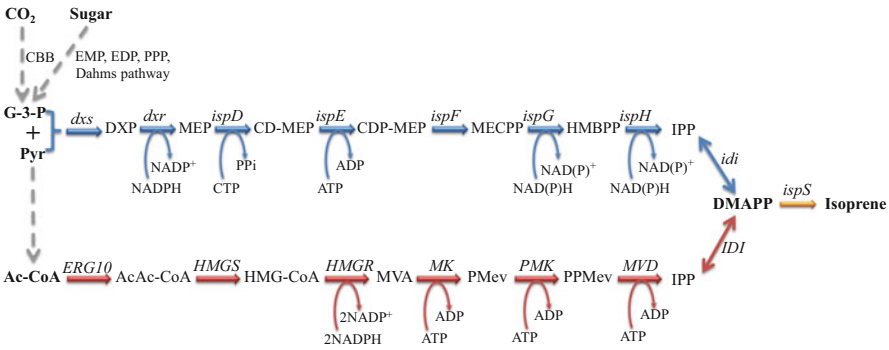

Fig. 2 Isoprene biosynthesis via MEP pathway (*blue arrows*) or MVA pathway (*red arrows*). Gene symbols and the enzymes encoded: *dxs* DXP synthase, *dxr* DXP reduction isomerase, *ispD* DXP-ME synthase, *ispE* CDP-ME kinase, *ispF* MECPP synthase, *ispG* HMBPP synthase, *ispH* HMBPP reductase, *idi* or *IDI* IPP isomerase, *ERG10* acetyl-coA acetyl transferase, *HMGS* HMG-CoA synthase, *HMGR* HMG-CoA reductase, *MK* mevalonate kinase, *PMK* mevalonate-5-phosphate kinase, *MVD* mevalonate-5-diphosphate decarboxylase, *ispS* isoprene synthase. Pathways: *CBB* Calvin–Benson–Bassham cycle, *EMP* Embden–Meyerhof pathway, *EDP* Entner–Doudoroff pathway, *PPP* pentose phosphate pathway. Pathway intermediates: *G-3-P* glyceraldehyde-3-phosphate, *DXP* 1-deoxy-D-xylulose 5-phosphate, *MEP* 2-C-methyl-D-erythritol 4-phosphate, *CDP-ME* 4-diphosphocytidyl-2-C-methyl-D-erythritol, *CDP-MEP* 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate, *MECPP* 2-C-methyl-D-erythritol 2,4-cyclopyrophosphate, *HMBPP* 1-hydroxy-2-methyl-2-(E)-butenyl 4-pyrophosphate, *IPP* isopentenyl pyrophosphate, *DMAPP* dimethylallyl pyrophosphate, *Ac-CoA* acetyl-CoA, *AcAc-CoA* acetoacetyl-CoA, *HMG-CoA* 3-hydroxy-3-methylglutaryl coenzyme A, *MVA* mevalonate, *PMev* mevalonate 5-phosphate, *PPMev* mevalonate pyrophosphate (Modified after Ye et al. 2016)

As shown in Fig. 2, there are a number of differences between these two pathways. Using glucose as the carbon source, isoprene synthesis through the MVA pathway generates a net of four NAD(P)H taking the glycolysis process into account, whereas the MEP pathway requires two NAD(P)H and three ATP for the synthesis of one isoprene molecule (Yang et al. 2016). In addition, initiation of the MEP pathway requires the heterogeneous condensation of two different precursors (pyruvate and G-3-P), which was believed to constrain the efficiency of the MEP pathway due to the doubled number of competition reactions and the imbalance in intracellular concentrations of these precursors (Liu et al. 2013; Korman et al. 2014). In contrast, the MVA pathway starts with homologous condensation of acetyl-coA, which largely simplifies the situation. Nevertheless, the MEP pathway was found to be more energetically balanced and have higher theoretical yield than the MVA pathway in converting sugars (30.2% vs. 25.2% mass yield on glucose) or glycerol to isoprenoids based on the stoichiometry and redox balance analysis (Anthony et al. 2009; Ajikumar et al. 2010).

In addition, when the MEP pathway was compared with the MVA pathway for conversion of CO_2 to IPP and DMAPP in cyanobacteria, the amount of CO_2 molecules needed to be fixed by ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) in the Calvin–Benson–Bassham (CBB) cycle for each IPP or DMAPP synthesized through the MEP pathway is lower than that through MVA pathway (6 vs. 9), suggesting higher carbon efficiency of the MEP pathway allowing faster photosynthesis of terpenoids (Gao et al. 2016).

No matter which isoprenoid precursor pathway is used, the interconversion of IPP and DMAPP is catalyzed by IPP isomerase (IDI). Experiments have shown that IDI could shift the balance of DMAPP-IPP pool toward DMAPP both in vitro and in vivo (Zhou et al. 2013). The final formation of isoprene from DMAPP is catalyzed by isoprene synthase.

3 Characterization, Engineering, and Discovery of Isoprene Synthase

3.1 Characterization of Isoprene Synthase

Isoprene synthase was first isolated and characterized in the 1990s (Silver and Fall 1991), and those from poplar and kudzu are the best characterized (Wildermuth and Fall 1996; Sasaki et al. 2005; Schnitzler et al. 2005). These plant isoprene synthases are all located in chloroplasts and show best activity at 40–50 °C and pH 7.0–8.5. The apparent Michaelis constants (K_m) are usually very high (2.45–8.7 mM) as compared to other terpene synthases (in μM range), which might be attributed to its presumable physiological role in stress fighting (Sharkey and Aspland 2013). Sequence analysis revealed conservation of metal binding motifs, in accordance with the dependence of DMAPP-to-isoprene conversion on metal ions such as Mg^{2+} or Mn^{2+} (10–15 mM) (Silver and Fall 1995).

The X-ray crystal structure of the isoprene synthase from gray poplar hybrid *Populus* × *canescens* (PcISPS) (Köksal et al. 2010) and *P. tremuloides* ISPS (Bott et al. 2012) showed an isologous dimer formed through significant interactions between C-terminal catalytic domains. Based on the crystal structure of PcISPS-DMASPP (dimethylallyl-S-thiolodiphosphate, an unreactive analogue of DMAPP), the formation of van der Waals interactions between the isoprenoid moiety of DMASPP and F338, V341, and F485 in the active site pocket of PcISPS was indicated (Köksal et al. 2010). Further structural analysis and kinetic measurements suggested that isoprene is generated from DMAPP via a syn-periplanar elimination mechanism in which the diphosphate-leaving group serves as a general base and 2-methylbut-3-enyl 2-diphosphate might be an intermediate during the catalytic process of ISPS (Faraldos et al. 2012).

On the other hand, the existence and characteristics of isoprene synthase in the natural isoprene-producing bacterium *B. subtilis* were explored. Although isoprene formation in *B. subtilis* was confirmed to be catalyzed through the endogenous MEP pathway and DMAPP was verified as the precursor (Wagner et al. 2000), the attempt to isolate the bacterial isoprene synthase failed (Julsing et al. 2007). Nevertheless, study using permeabilized cells and partially purified cell extracts of *B. subtilis* showed that the isoprene synthase activity had a pH optimum of 6.2 and required relatively low concentration of divalent cation (100 μM) (Sivy et al. 2002), which are more similar to the culture conditions of microorganisms as compared to those of plant ISPS.

3.2 Engineering of Known ISPS

For biotechnological production of isoprene, an ISPS capable of delivering high isoprene production rates in culture conditions is desirable. As microbial ISPS is yet to be identified, plant-derived ISPS is introduced for microbial synthesis of isoprene. However, the high K_m and low catalytic activity of the well-characterized kudzu and poplar ISPS becomes an obstacle to achieving high rates of isoprene synthesis. Furthermore, the environmental conditions in microbial fermentation are often suboptimal for enzymes from plants.

Protein engineering might provide a solution to the above problems. Truncation and L70R site mutation elevated the specific activity of *P. alba* ISPS (Bott et al. 2012), whereas introduction of S288C mutation significantly increased the solubility of ISPS from *P. alba* and willow species, leading to improved growth of the host strain (Rife and Wells 2014). *P. alba* ISPS mutants with increased isoprene forming activity were also found in site mutation libraries constructed based on the hydrophobicity, charge, conservativity, and location of amino acids (Beck et al. 2015). By means of single amino acid substitution or pi electron-based amino acid mutation, not only the existing ISPS could be modified but also related enzymes that do not naturally produce isoprene could be converted into isoprene-forming enzymes (Sharkey and Aspland 2013). Alternatively, a *P. alba* ISPS double mutant (F340L/A570T) with 3.8-fold enhancement of catalytic activity was obtained by directed

evolution using a high-throughput screening method based on DMAPP toxicity relief exhibited as improved cell growth (Wang et al. 2017).

3.3 Discovery of New ISPS

Discovery of new isoprene synthases provides another approach to enzymes with satisfactory catalytic performance. *Arachis hypogaea* ISPS has been used for isoprene production in *E. coli*, and its coexpression with a heterologous MVA pathway led to isoprene production of 35 mg/L/h/OD (Beatty et al. 2014). By using a homology-based cloning approach and database searching, nine new isoprene synthases within the rosid angiosperms were identified, among which, the ISPS from *Eucalyptus globulus* had the lowest K_m value (0.03 mM) and produced more isoprene than the *P. alba* ISPS (Sharkey and Aspland 2013; Sharkey et al. 2013). Similarly, sequence-based homology searches in combination with screening for "isoprene score" amino acids (F338, S445, F485, and N505 based on *P. alba* sequence) led to identification of three novel isoprene synthases, respectively, from *Ipomoea batatas*, *Elaeocarpus photiniifolius*, and *Mangifera indica.* Finally, based on the summarization and alignment of ISPS sequences, F338 and F485, which are present in all functional ISPS known to date, have been identified as essential for isoprene synthase activity (Ilmén et al. 2015).

4 Engineering Bacteria for Isoprene Synthesis

Although isoprene synthesis by plants is well documented and the isoprene yield is high, it is not possible to harvest this volatile product from the extended canopy of leafy plants for commercial applications. Therefore, production of isoprene via a microbial fermentation process becomes an attractive alternative and has been made possible by metabolic engineering in a number of chassis organisms, including the prokaryotic hosts *B. subtilis*, *E. coli*, and cyanobacteria and the eukaryotic host *S. cerevisiae*.

Compared to other bio-based chemicals, the low boiling point and water solubility of isoprene enable its recovery from the off-gas outside the bioreactor, leading to a number of potential benefits: (1) reduction of feedback inhibition by the product, (2) efficient recovery and purification of the product, and (3) possibility to use crude, low-cost feedstocks containing solid or liquid impurities without affecting the product purity (Whited et al. 2010).

Regardless of the species of the chassis organism, construction of isoprene-producing microorganisms involves expression of a plant-derived ISPS and engineering of the heterologous or endogenous isoprenoid precursor pathway. Aside from the efforts in protein engineering and discovery of ISPS, the upstream pathways responsible for precursor supply have been extensively explored to achieve a productive isoprene biosynthetic pathway (Fig. 3).

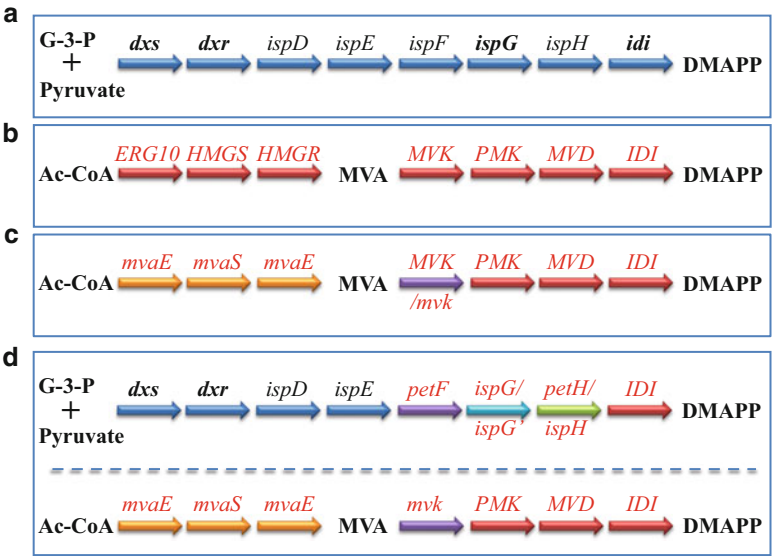

Fig. 3 Representative strategies in engineering the isoprenoid precursor pathways for isoprene synthesis in bacteria. (**a**) Overexpression of key enzymes in endogenous MEP pathway; (**b**) introduction of a heterologous MVA pathway, exemplified by genes from *S. cerevisiae*; (**c**) introduction of a hybrid MVA pathway; (**d**) introduction of both hybrid MEP pathway and hybrid MVA pathway. Genes in *bold* are overexpressed endogenous genes, while genes in *red* are introduced from heterologous sources. Different *colors of arrows* indicate different sources. In the hybrid pathways, genes from two or more different sources are used, and sometimes multiple copies of enzymes catalyzing the same step may be introduced from various sources (indicated by "/" between two genes)

The typical engineering strategies employed for development of isoprene-producing bacterial strains can be divided in to the following types: (1) engineering the endogenous MEP pathway, (2) introducing the heterologous MVA or MEP pathway, (3) engineering the feeding module for the isoprenoid precursor pathway, and (4) regulation of ISPS expression.

4.1 Engineering the Endogenous MEP Pathway

Engineering of the endogenous MEP pathway is a commonly adopted strategy in isoprenoids production, and the efforts include optimizing and balancing the expression of pathway enzymes by means of gene overexpression, use of different promoters, and replacement with genes from other sources (Ajikumar et al. 2010; Zou et al. 2013).

The supply of IPP and DMAPP as the precursors for isoprene synthesis in bacteria is determined by the contribution of MEP pathway enzymes to the flux control of the metabolic intermediates. DXS and DXR are the most extensively

studied enzymes in the MEP pathway. DXS has been shown as a general rate-limiting pathway enzyme by a number of studies on isoprenoid biosynthesis, whereas the importance of DXR regulation differs among different species. Overexpression of DXS but not DXR obviously enhanced the isoprene yield in *B. subtilis* (Xue and Ahring 2011). Overexpression of both endogenous and *B. subtilis*-derived DXS and DXR in *E. coli* significantly improved the isoprene production, and the exogenous enzymes showed higher efficiency than the native ones, probably due to the different protein structures of DXS enzymes (Zhao et al. 2011). However, for the cyanobacterium *Synechococcus elongatus*, the overexpression of DXS only had a modest effect on the photosynthetic production of isoprene, accompanied with impaired cell growth (Gao et al. 2016). Metabolite quantification showed 100-fold lower DMAPP/IPP ratio as compared to the reported value for isoprene-emitting kudzu leaves (Zhou et al. 2013), indicating the accelerated formation of IPP upon strengthening of the MEP pathway and the inefficient conversion of IPP to DMAPP. As a result, the activity of isoprene synthase was severely inhibited by the excessive IPP. In order to relieve the IPP toxicity, IDI was overexpressed to convert the accumulated IPP to DMAPP, leading to an obvious increase in isoprene production and relief of growth impairment. When expressed in the form of IDI-ISPS fusion, the isoprene production was further improved, which could be probably attributed to the substrate channeling effect (Gao et al. 2016). The function and significance of IDI were also demonstrated in another cynobacterium *Synechocystis*, where heterologous expression of the isopentenyl diphosphate isomerase (FNI) from *Streptococcus pneumonia* enhanced the DMAPP/IPP ratio, resulting in 250% increased rate and yield of isoprene production (Chaves et al. 2016). In *E. coli*, *idi* has also been reported as a key regulation target in addition to *dxs* and *dxr* for isoprene synthesis, overexpression of which resulted in obvious improvement of isoprene synthesis (Lv et al. 2013; Liu et al. 2014).

The contributions of other enzymes in the MEP pathway to the regulation of flux control in isoprene synthesis are less well characterized. Overexpression of *ispG* (encoding HMBPP synthase) in addition to *dxs* and *idi* was found to significantly improve isoprene production as compared to the parental strain overexpressing solely *dxs* and *idi* (Liu et al. 2013), indicating IspG as another bottleneck enzyme in the MEP pathway. A later patent supported this conclusion by showing enhanced isoprene yield in *E. coli* upon overexpression of native and exogenous *ispG* (Muir and Weyler 2014). Similarly, IspG was also identified as rate-limiting in the cyanobacterium *S. elongatus* by kinetic flux profiling, together with IspD, which was found to play a role in flux control after alleviation of the flux limitation by IspS, IDI, and IspG (Gao et al. 2016). Aside from strengthening the MEP pathway, the precursor supply can also be enhanced by reducing the competitive loss of IPP/DMAPP. Downregulation of *ispA* (encoding farnesyl diphosphate synthase) in the engineered *E. coli* strain (overexpressing *P. alba ispS*, *S. pneumoniae idi*, and endogenous *dxs*/*dxr* genes) resulted in a slight improvement of isoprene production (Liu et al. 2014).

Although production improvement was achieved by engineering of the native MEP pathway, the highly regulated nature and relatively poorly studied regulatory

mechanism of the MEP pathway cause difficulty for further enhancement of its efficiency. Meanwhile, the limited enzyme activities, in particular, the two iron-sulfur-containing enzymes IspG and IspH, may limit the kinetic capacity of the MEP pathway and cause internal balance among the pathway enzymes (Zhao et al. 2013). In addition, the accumulated indole resulted from the imbalanced pathway expression has also been reported as an inhibitory factor for the MEP pathway activity (Ajikumar et al. 2010).

4.2 Introducing the Heterologous MVA or MEP Pathway

As observed in biosynthesis of other isoprenoids (Li and Wang 2016), the introduction of a heterologous MVA pathway into bacteria obviously enhanced the isoprene production to a much greater extent than overexpression of the native MEP pathway. Zurbriggen et al. optimized the native MEP pathway and the bacterial hybrid MVA pathway (composed of endogenous *atoB*; *hmgS* and *hmgR* from *Enterococcus faecalis*; and *fni*, *mk*, *pmk*, and *pmd* from *Streptococcus pneumoniae*), respectively, by selection of translation initiation regions and adjustment of gene order in the superoperon structure and made a direct comparison on the efficiency of cellular organic carbon flux through these two pathways in an *E. coli* strain overexpressing *P. montana ispS*. The over 60-fold higher isoprene yield in the MVA pathway-expressing strain together with the isoprene-to-biomass carbon partitioning ratio of 0.78:1 suggested that the introduction of MVA pathway overcame the flux limitations imposed upon cellular regulation of the native MEP pathway (Zurbriggen et al. 2012), which was also validated in cyanobacteria. Introduction of the same hybrid MVA pathway in *Synechocystis* altered the photosynthetic carbon partitioning between isoprene and biomass, leading to an approximately 2.5-fold improvement in photosynthetic isoprene yield (Bentley et al. 2014). By providing an alternative route for DMAPP synthesis, the carbon partitioning of photosynthate toward the terpenoid biosynthetic pathway was increased by at least twofold upon heterologous expression of the MVA pathway. Since the host bacteria do not have the native regulatory elements to influence the flux through the MVA pathway, the introduction of an exogenous MVA pathway circumvented the strong regulatory checkpoints of the native MEP pathway by introducing a bypass in the flux of endogenous cellular substrate to IPP and DMAPP. Meanwhile, these results implied the insufficient precursor supply from the native MEP pathway for isoprene synthase.

To further boost the carbon flow to isoprene production, besides augmentation of the bacterial hybrid MVA pathway, the *E. coli* genome was engineered by deleting genes (*ackA-pta*, *poxB*, *ldhA*, *dld*, *adhE*, *pps*, and *atoDA*) related to accumulation of byproducts including acetate, lactate, and ethanol to reduce waste of carbon source (Kim et al. 2016). In this way, the acetyl-CoA flux directed to the MVA pathway was increased, finally leading to improved precursor supply for isoprene synthesis.

The source of MVA pathway enzymes also affects the final isoprene production. Introduction of the *S. cerevisiae* MVA pathway in *E. coli* resulted in higher isoprene titer as compared to the abovementioned strain harboring the bacterial MVA

pathway (532 vs. 320 mg/L) (Yang et al. 2012b). When a hybrid MVA pathway composed of the *E. faecalis* "upper MVA pathway section" and the *S. cerevisiae* "lower MVA pathway section" was introduced to *E. coli*, the isoprene synthesis was further enhanced. Subsequent coexpression of a site-mutated HMG-CoA synthase (A110G) in this strain led to production of up to 6.3 g/L isoprene (Yang et al. 2012a). Similarly, a bacterial/yeast hybrid MVA pathway consisting of the *E. faecalis* "upper MVA pathway section" and a more complicated "lower MVA pathway section" with enzymes from *S. cerevisiae* and *Methanosarcina mazei* (MVK, PMK, MVD, and IDI from *S. cerevisiae* and two additional copies of MVK from *M. mazei*) was introduced to *E. coli*. Overexpression of this hybrid MVA pathway together with the endogenous *pgl* gene (*ybhE*, encoding phosphogluconolactonase) and *P. alba* ISPS enabled production of more than 60 g/L isoprene at volumetric productivity of 2 g/L/h., and the cell productivity was calculated to be 0.85 g isoprene/g dry cell weight (Whited et al. 2010; Cervin et al. 2014). However, the yield was only 0.11 g/g glucose.

A recent study simultaneously utilized the MVA pathway and the MEP pathway for isoprene synthesis in *E. coli* (Yang et al. 2016). The bacterial/yeast hybrid MVA pathway was composed of genes from *E. faecalis*, *M. mazei*, *S. cerevisiae*, and *Thermosynechococcus elongatus*, whereas the hybrid MEP pathway contained *ispG*, *petF*, and *petH* from *T. elongatus*, *ispH* from *Anabaena sp.*, and endogenous *dxs*, *dxr*, and *ispG*. Overexpression of the dual pathway resulted in 4.8- and 1.5-fold increased fluxes of the MEP and MVA pathways and improved the isoprene yield by about 20-fold and threefold, respectively, as compared to overexpression of the MEP pathway or the MVA pathway alone. Meanwhile, ^{13}C metabolic flux analysis indicated negligible contribution of the native MEP pathway to isoprene production. Upon disruption of the MEP pathway by fosmidomycin addition, the flux through the MVA pathway in the strain harboring dual pathway was also reduced. Similar results were obtained in the mevalonate supplementation experiment. These results indicated the synergy of the dual pathway, which might be partly attributed to the complementary nature of the two pathways regarding the reducing equivalent demand and ATP requirement. The high NAD(P)H demand of isoprene synthesis through MEP pathway could be fulfilled by the coexisting MVA pathway which generates NAD(P)H, whereas the feedback inhibition of the lower MVA pathway enzymes such as mevalonate kinase might be alleviated by the MEP pathway through draining these downstream metabolites. Finally, the simultaneous utilization of the carbon-efficient MEP pathway and the energy-efficient MVA pathway resulted in the highest ever reported isoprene yield of 0.267 g/g glucose, reaching 86.4% of the maximum theoretical yield.

4.3 Engineering the Feeding Module for the Isoprenoid Precursor Pathway

In isoprene-producing strains, the isoprene biosynthesis pathway can be partitioned into two modules: (1) the carbon feeding module generating pyruvate and G-3-P or acetyl-CoA from substrates and (2) the MEP or MVA module producing isoprene as

the final product. Efficiency of the isoprenoid precursor pathways is not only controlled by the enzyme activities involved in the pathway itself but also influenced by availability of the starting materials (Chang and Keasling 2006). Therefore, engineering of the carbon feeding module can also lead to improved isoprene production.

In *E. coli*, Embden–Meyerhof pathway (EMP) is used as the carbon feeding module for the synthesis of naturally occurring isoprenoids. The imbalanced distribution of pyruvate and G-3-P generated by EMP leads to consistently higher intracellular concentration of pyruvate than G-3-P (Liu et al. 2013). Therefore, G-3-P becomes the limiting factor for the carbon flux toward the MEP pathway (Farmer and Liao 2001; Ramos et al. 2014). To balance the distribution between pyruvate and G-3-P so as to provide a more efficient carbon flux toward isoprene production, redirection of the sugar metabolism toward an alternative pathway simultaneously generating equimolar concentrations of the precursors might be a solution.

Among EMP, Entner–Doudoroff pathway (EDP), pentose phosphate pathway (PPP), and Dahms pathway, EDP is the only glycolytic pathway that simultaneously produces G-3-P and pyruvate within one reaction, whereas all other pathways generate one prior to the other. When they were tested respectively or in combination as the feeding modules for MEP-mediated isoprene biosynthesis in *E. coli*, the EDP-containing modules gave the highest isoprene production, demonstrating the importance of balanced supply of MEP pathway precursors. Taking also the energy and reducing equivalent requirement into account, EDP+PPP was found to be the best carbon feeding module of the MEP pathway (Liu et al. 2013).

Feeding module engineering can be also used to expand the feedstock spectrum for isoprene production. By introducing a heterologous dehydrogenase (*gld*) catalyzing the conversion of D-galactose to D-galactonate into an *E. coli* mutant strain with blocked Leloir pathway, D-galactose was exclusively directed toward the De Ley–Doudoroff (DD) pathway (Ramos et al. 2014). In combination with precursor supply enhancement by overexpression of *dxs* and *idi*, an isoprene production of 264 mg/L was achieved from galactose.

4.4 Regulation of ISPS Expression

ISPS has been clearly identified as a bottleneck enzyme in isoprene synthesis (Gao et al. 2016). Aside from ISPS engineering and discovery, efforts have also been made to improve its expression in bacterial hosts. For example, removal of the chloroplast targeting peptide and codon optimization of the *ispS* gene (Miller et al. 2001; Bott et al. 2012) have been frequently used to increase isoprene production. Codon optimization and expression regulation of the *ispS* gene in *E. coli* via adjustment of the RBS strength and inducer concentration increased isoprene production by 2.5–4.2-folds (Kim et al. 2016). In addition, regulation of ISPS expression by changing the driving promoter has been shown as an effective strategy to improve isoprene synthesis in cyanobacteria. When the expression of ISPS was

driven by a light-sensitive promoter (P*psbA2*), the isoprene production in *Synechocystis* was obviously enhanced in response to the increase of light intensity (Melis and Lindberg 2014). When the strong *rbcL* promoter was adopted to control ISPS expression in *Synechocystis*, an isoprene production of 336 μg/g dcw was achieved in open-cultivation system (Pade et al. 2016).

5 Engineering Yeast for Isoprene Biosynthesis

The heterologous MVA pathway introduced into *E. coli* and cyanobacteria has shown superior isoprene production capacity over the native MEP pathway, which triggers studies using yeast as an alternative host for isoprene biosynthesis. The eukaryotic model organism *S. cerevisiae* naturally employs the MVA pathway to synthesize physiologically important isoprenoids. Its high inherent capacity in isoprenoids production has been recently demonstrated by producing over 130 g/L of farnesene after rewiring the yeast central metabolism (Meadows et al. 2016). Isoprene synthesis has also been made possible in *S. cerevisiae* by introduction of a plant isoprene synthase. Codon optimization and multiple integration of the kudzu vine *ispS* gene in the *S. cerevisiae* genome resulted in constitutive isoprene production of 500 μg/L (Hong et al. 2012).

Practices in yeast metabolic engineering for enhanced isoprenoid synthesis have proven the carbon flow of MVA pathway and the supply of acetyl-CoA as important contributors to the accumulation of isoprenoids (Krivoruchko and Nielsen 2015). In order to improve isoprene synthesis in *S. cerevisiae*, the acetyl-coA supply and the MVA flux were simultaneously elevated using a comprehensive "push-pull-restrain" strategy (Lv et al. 2014). The precursor supply in the acetyl-CoA synthesis pathway and the MVA pathway was enhanced by overexpression of acetyl-CoA synthase, acetoacetyl-CoA thiolase, and HMG-CoA reductase (push-strategy), the isoprene branch was strengthened by increasing the copy number of *ispS* (pull-strategy), and the competing squalene synthesis pathway was downregulated by replacing the native promoter of farnesylpyrophosphate synthetase gene with a weaker promoter P_{HXT1} (restrain strategy). As compared to the parental strain harboring a single copy of *ispS*, the isoprene production was improved by 782-folds in the final engineered strain, reaching 37 mg/L.

The contribution of enhanced acetyl-coA supply to isoprene synthesis inspired us to approach to the rich acetyl-coA stock in mitochondria. Assembly of the mitochondria localization signal (MLS)-tagged isoprene synthetic pathway enabled compartmentalization of the complete pathway into mitochondria, leading to 1.7-fold improvement of isoprene production and 80% reduction of squalene accumulation as compared to its cytoplasmic engineering counterpart (Lv et al. 2016), suggesting reduction of precursor loss to competing pathways as another important advantage of mitochondria engineering. Other advantages of metabolic engineering in mitochondria may include higher local concentrations of the pathway enzymes, increased availability of intermediates, as well as relatively high reducing redox potential and rich ATP in the mitochondrial matrix (Hu et al. 2008).

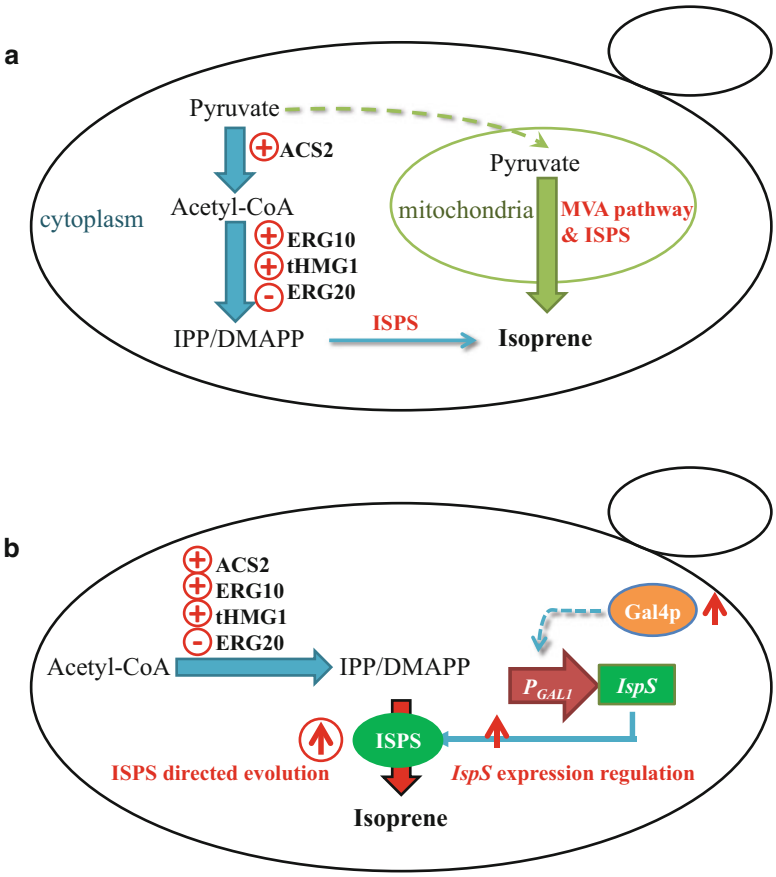

Fig. 4 Strategies for engineering isoprene synthesis in *S. cerevisiae*. (**a**) Dual regulation of precursor supply in cytoplasm and mitochondria by simultaneous augmentation of the cytoplasmic MVA pathway and introduction of the MVA pathway into mitochondria; (**b**) Gal4p-mediated expression enhancement and directed evolution of ISPS in the background of augmented MVA pathway; ⊕ represents overexpression, ⊖ represents downregulation, ⇧ indicates activity improvement, ↑ and indicates expression enhancement. Gal4p, transcriptional regulator

In order to make full use of intracellular acetyl-CoA pool so as to enhance the precursor supply for isoprene formation, dual metabolic engineering of cytoplasmic and mitochondrial acetyl-CoA utilization was conducted to further boost isoprene synthesis in *S. cerevisiae* (Fig. 4a). Mating of the mitochondrial engineered strain and the cytoplasmic push-pull-restrain engineered strain (Lv et al. 2014) generated the dual engineered diploid strain, which produced 2.5 g/L isoprene in fed-batch fermentation (Lv et al. 2016). Based on a set of control experiments, the advantages of the dual engineered diploid strain can be summarized as follows:

1. The rich acetyl-CoA pool in the mitochondria was made available to the isoprene biosynthetic pathway, which in combination with cytoplasmic engineering ensured sufficient precursor supply.
2. The loss of precursor DMAPP to competing pathways was reduced, and the flux directed into the target pathway was maximized.
3. Separation of the IPP/DMAPP pool into multiple subcellular compartments possibly relieved the cellular burden caused by accumulated cytotoxic intermediates.
4. The diploid strain possessed higher stress tolerance and improved cell health over haploid strains.

However, for the above strain, growth defects were still observed during fermentation, implying the existence of unsolved metabolic bottleneck in the biosynthetic pathway. After strengthening the acetyl-coA supply and the MVA pathway, the conversion of DMAPP to isoprene catalyzed by isoprene synthase becomes the rate-limiting step in the whole isoprene biosynthesis pathway. The low expression level and insufficient activity of plant isoprene synthases under microbial fermentation conditions does not only restrict the isoprene biosynthesis capacity but also led to accumulation of the cytotoxic DMAPP.

To enhance the relatively weak flux downstream of DMAPP, a recent study strengthened the isoprene formation step by a combinatorial engineering strategy integrating protein engineering and metabolic regulation of ISPS (Fig. 4b). In order to enhance the catalytic activity of ISPS, directed evolution was conducted using a novel high-throughput screening method developed based on the positive correlation between cell growth and ISPS activity in a parent strain with excessive DMAPP accumulation. Upon improvement of ISPS activity, the conversion of DMAPP to isoprene was accelerated, and the toxicity was relieved, leading to improved cell growth. Finally, a mutant with 3.8-fold higher activity was obtained. Meanwhile, overexpression of the transcriptional activator Gal4p together with deletion of the chromosomal *GAL* promoters was used to enhance the ISPS expression under control of P_{GAL1}. Combination of the above strategies obviously intensified the isoprene-forming pathway in a previously constructed isoprene-producing *S. cerevisiae* strain YXM08-ISPS with enhanced precursor supply (Lv et al. 2014) and led to an isoprene production of 3.7 g/L, which is the highest ever reported isoprene production by engineered eukaryotic cells (Wang et al. 2017).

6 Isoprene Biosynthesis via Nonnaturally Occurring Metabolic Pathways

Aside from the engineering efforts made in improving the natural isoprenoid precursor pathways, nonnaturally occurring metabolic pathways were also explored for isoprene production. Aiming to reduce the ATP consumption of the lower MVA pathway, the need for terminal alcohol phosphorylation was eliminated by

introducing two vinyl groups to synthesize isoprene from MVA, 2-methyl-2-oxopentanoate, 4-methyl-2-oxpentanoate, or isobutyryl-CoA (Botes and Conradie 2015). Isoprene production was made possible using whole cells or enzymes such as *R*-specific enoyl-CoA hydratase, MVD, mevalonate-3-kinase, and linalool dehydratase. In order to elevate the theoretical yield of isoprene and meanwhile enable anaerobic fermentation of isoprene from glucose, researchers designed four nonnaturally occurring metabolic pathways and comparatively discussed the redox balance, ATP balance, number of reaction steps, and unknown enzymes of these pathways. Using 2,3-dihydroxyisovalerate as the key intermediate, a theoretical yield (0.324 g/g) higher than those of the naturally occurring pathways was calculated (Coelho et al. 2014). The feasibility of methanol as an alternative carbon source for isoprene production has also been examined (Furutani et al. 2015). Methylotrophs such as *Pichia pastoris*, *Methylophilus methylotrophus*, and *Methylobacterium extorquens* were engineered by introduction of *P. nigra ispS* and the *idi* gene from *E. coli* or the *S. griseolosporeus* MVA pathway gene cluster, resulting in a conversion efficiency of 19–27% from assimilated methanol to isoprene. Isoprene production from methanol by non-methylotrophs exemplified by *B. subtilis* and *E. coli* was also made possible by introduction of methanol dehydrogenase from *B. methanolicus* as well as 3-hexulose 6-phosphate synthase and 6-phospho-3-hexuloisomerase from *Methylomonas aminofaciens*, in addition to the *P. nigra ispS* gene. However, all the abovementioned unnatural pathways remain to be further optimized (if possible) to give isoprene production comparable to those achieved using the natural pathways.

7 Research Needs

Despite the great progresses achieved in biosynthesis of isoprene using microbes, industrialization of bio-isoprene production is not yet realized. Future research efforts are required to solve the persisting problems. The potential of isoprene biosynthesis pathway remains to be fully explored, considering the gap between the theoretical yield and the actual production. Moreover, for large-scale production of bio-isoprene, cheap and sustainable feedstocks such as lignocellulosic hydrolysates should be used, which points to the necessity of engineering the sugar metabolism of isoprene-producing strains toward enhanced pentose uptake and utilization.

8 Concluding Remarks

Biotechnological production of isoprene has been made possible in prokaryotic and eukaryotic microbial hosts. The efforts toward industrial biosynthesis of isoprene include discovery, molecular engineering and expression regulation of isoprene synthase, engineering of the endogenous or exogenous isoprenoid precursor pathways and related carbon feeding modules, as well as design of nonnaturally

occurring metabolic pathways. Strengthening and balancing the metabolic flux through the isoprene biosynthesis pathway has been generally recognized as an efficient strategy. Moreover, comprehensive engineering of the key pathway enzymes through both protein engineering and metabolic regulation has been demonstrated to be powerful in providing simultaneous solutions to the insufficient endogenous supply of precursor and the limited inherent capacity of imported pathway to accommodate high precursor supply in the engineered microbes. With all these achievements and future efforts, we could expect industrial biosynthesis of isoprene in the near future.

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Microbial Production of Flavors and Fragrances

21

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Abstract

Synthetic biology opens a new door for sustainable and effective production of flavors and fragrances. It is achieved through the engineering of biosynthetic pathways for valuable compounds of interests in the microbial hosts such as *Saccharomyces cerevisiae* or *Escherichia coli.* This chapter focuses on the current state-of-art studies in pathway engineering for the production of functional isoprenoids, including monoterpenes and sesquiterpenes, as well as apocarotenoids. The relevant genetic manipulations on biosynthetic genes and enzymes performed in the last decade have been summarized. Various approaches, techniques to increase production titers of flavor compounds, and critical challenges have been highlighted and discussed in this chapter.

1 Introduction

The flavor and fragrance market is an expanding industry of an estimated value of USD 27 billion (Carroll et al. 2016). Chemically, these volatile or nonvolatile flavor and fragrance compounds comprise diverse molecular structures, which allow a wide variety of aroma and odor notes. Besides in food and beverages, they are widely applied in perfumes, cosmetics, detergents, cleaning, and hygiene products (Zebec et al. 2016).

Historically, flavors have been generated in situ during fermentation, by harnessing secondary metabolism of microorganisms to produce food and beverages (e.g., wine, beer, vinegar, or cheese), which determined their organoleptic properties. Today, extraction of flavor compounds from plant and animal material has become the major approach of their manufacture as additives. However, in many cases, low yield, process cost, and compound availability turned out to be the limiting factors (Longo and Sanromán 2006). Traditional breeding of plants to increase the content of flavor compounds showed limited success. On the other hand, the analysis and identification of the individual compounds opened the doorway to their chemical or synthetic production. Although chemical synthesis followed by purification was successful for some monoterpenes and carotenoids, it has been hampered due to the high process cost for production of more complex metabolites (Misawa 2011). Advances in biotechnology, as well as consumer preferences for "organically" or "naturally" produced flavors, led to the application of microorganisms as biocatalysts in biochemical synthesis of these high-value compounds. The phenotypic features related to productivity of producer strains (also called microbial cell factories (MCFs)) determine the economical parameters of the flavor production. Originally, traditional random mutagenesis and screening were applied to obtain MCF phenotypes relevant for industrial applications (Dai and Nielsen 2015). Nowadays, the combinations of novel and classical methods like genetic/metabolic engineering, adaptive laboratory evolution (ALE), directed evolution (DE), site-directed mutagenesis, and microorganisms' breeding supported with high-throughput screening (HTS) enable the construction and selection of successful MCF with the desired characteristics (Debabov 2015). Theoretically, it is possible

to produce every naturally occurring aroma or flavor molecule. This process requires the knowledge of a complete genome sequence, identification of suitable biosynthetic pathways, enzymes and genes with bioinformatics tools, and proper microbial host with efficient transformation and expression systems (Carroll et al. 2016). The present level of science enables the construction of metabolic pathways de novo in the MCFs for the production of chemicals, and there is a strong trend of generation of natural flavor compounds in a more sustainable manner (Berger 2008). Even though a considerable amount of current studies focusses on the production of flavors and aromas, only a few are obtained biotechnologically in industrial scale in mass quantities.

The aim of this chapter is to describe the current state of the art on bioprocesses for the production of flavors and fragrances to highlight the most relevant research achievements in this field. Since there hardly exist natural MCFs that produce high titers of flavors and fragrances, pathway and metabolic engineering of MCFs for the production of industrially relevant flavors and fragrances are the focus of the present chapter. This includes examples of selected hydrocarbons, oils and lipids, and, in particular, functional terpenoids, whose biosynthesis has been intensively studied in the last decade, including monoterpenes and sesquiterpenes, as well as apocarotenoids.

2 Terpenoids as Flavor Compounds

Terpenoids (or isoprenoids) comprise a very large family with over 70,000 known molecules. They are classified based on the number of carbons in their skeletal structure: hemi- (C_5), mono- (C_{10}), sesqui- (C_{15}), di- (C_{20}), and tri- (C_{30}) terpenoids as well as carotenoids (C_{40}). Primarily, mono- and sesquiterpenoids are considered as flavor compounds (Vickers et al. 2014, 2015). In addition, the C_9 and C_{13} apocarotenoids are also important flavor compounds.

All organisms possess at least one biosynthetic route toward terpenoid production, either a mevalonate-dependent (MVA) or a methylerythritol 4-phosphate (MEP, also called 1-deoxy-xylulose 5-phosphate (DXP) pathway (Fig. 1), both of which start at central carbon intermediates (Vickers et al. 2014). Usually, yeasts, animals, and archaea employ the MVA pathway, whereas bacteria use mainly the MEP route, with exceptions of some bacterial species, which use MVA route or both. The biosynthesis of all terpenes is dependent on two isoprene (C_5) precursors: isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). IPP and DMAPP are condensed head to tail to generate geranyl diphosphate (GPP, C_{10}), which is converted into farnesyl diphosphate (FPP, C_{15}) with IPP and prenyl transferase, e.g., FPP synthase. Further, FPP is condensed with IPP to form geranylgeranyl diphosphate (GGPP, C_{20}) with GGPP synthase. The GPP, FPP, and GGPP are the precursors of mono-, sesqui-, and diterpenes, respectively. Subsequently, the chemically diverse library of terpenoids can be created from GPP with terpene synthases/cyclases (TPS) family, including MTS, STS, and DTS for mono-, sesqui-, and diterpene synthases, correspondingly (Reiling et al. 2004). These

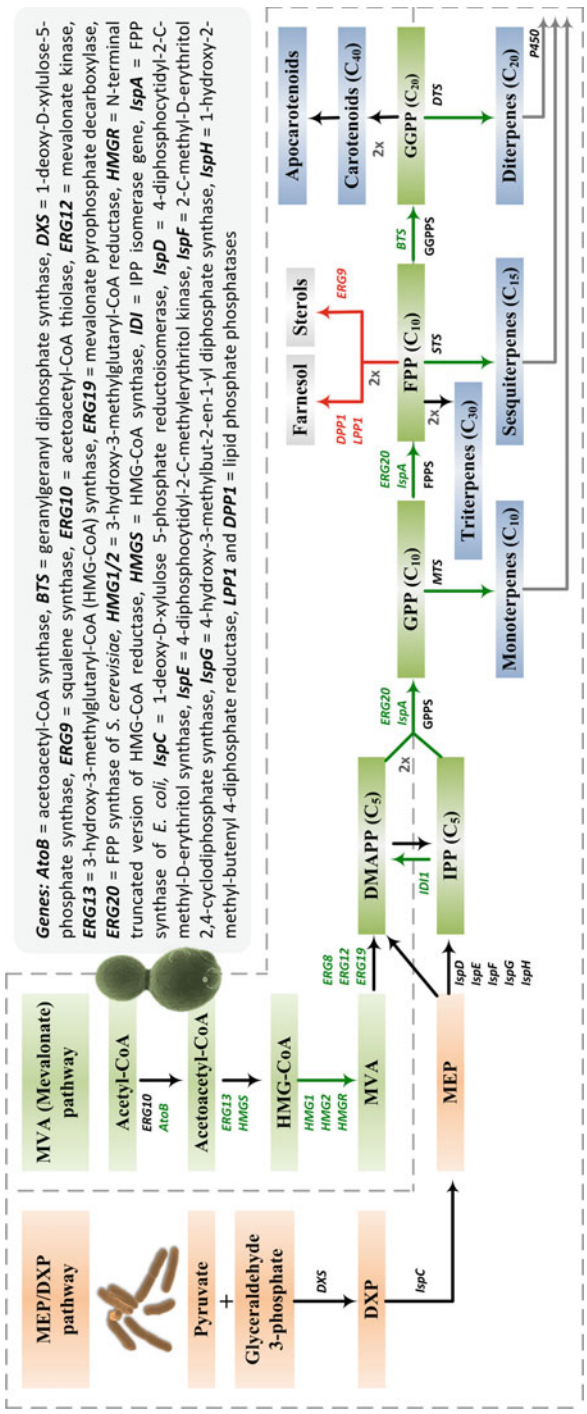

Fig. 1 The pathways of terpenoid biosynthesis indicating the genes involved and metabolic engineering interventions applied. Up- and downregulated genes are indicated in green and red, respectively

enzymes are commonly found in plants, but more recent studies demonstrated their presence also in bacteria (Misawa 2011; Yamada et al. 2015). Subsequent terpene-modifying enzymes, particularly cytochrome P450-dependent mono-oxidases (P450s), dehydrogenases, or various transferases, are responsible for structural diversity of these chemicals (Kampranis and Makris 2012).

3 Monoterpenes

3.1 General Overview

Monoterpenes are ten-carbon (C_{10}) isoprenoids consisting of two isoprene units (C_5). Limonene (orange peel), linalool (floral, citrus), geraniol (floral hops, citrus), and citronellol (floral) are the key flavor and fragrance constituents of increasing industrial interest (Fig. 2). Monoterpenes can be produced microbiologically, for example, by selected yeast in very small amounts (Carrau et al. 2005). Traditionally, for large-scale production, monoterpenes have been extracted from plants or synthetized chemically (Longo and Sanromán 2006). However, due to low yield of extraction from plants and the dependence on raw material availability as well as the high costs of their synthesis due to structural complexity, metabolic engineering has been suggested to create MCFs as sustainable resources for functional monoterpene

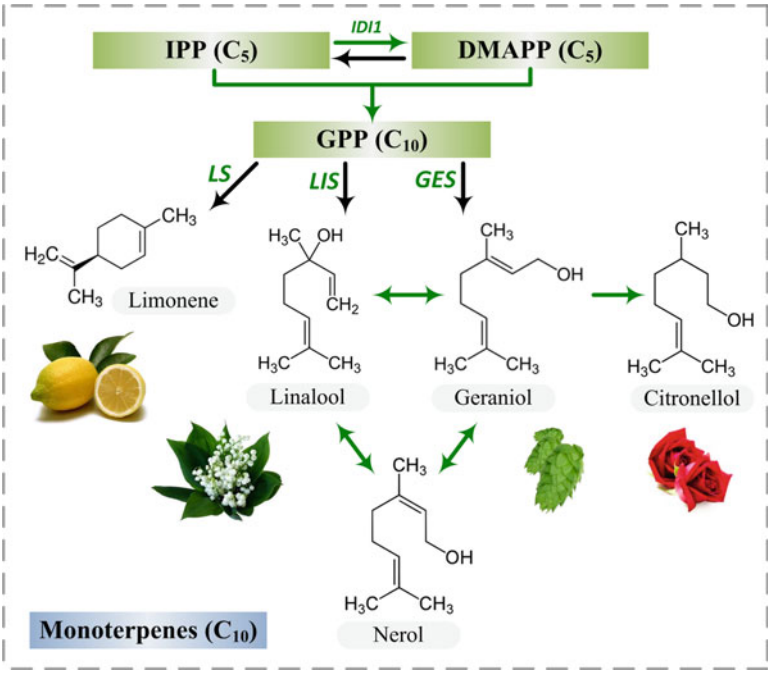

Fig. 2 The major industrially important monoterpenes for flavor and fragrance sector

synthesis (Zebec et al. 2016). In this regard, *Saccharomyces cerevisiae* and *Escherichia coli* are often employed as the microbial hosts for genetic manipulations (Vickers et al. 2014, 2015).

Over the last decade, numerous metabolic engineering strategies have been applied for monoterpene synthesis or improvement of their titers (Table 1). First engineering efforts of the bacterial native MEP pathway were focused on overexpression of the C_{10} precursors GPP from IPP and DMAPP (C_5). However, the obtained yield was very low in mg/L range (Carter et al. 2003; Reiling et al. 2004). Subsequently, appropriate plant-originated synthase has been introduced into the MCF to achieve increased yields of the desired monoterpenes. Nonetheless, to obtain reasonable titers, engineering of the upstream isoprenoid pathway flux (MVA and MEP), its optimization, and relevant gene expression are usually required (Vickers et al. 2014).

3.2 Research Efforts

Currently, limonene is one of the most demanded monocyclic monoterpenes in the flavor and fragrance sector. It is an olefin hydrocarbon ($C_{10}H_{16}$), which occurs in two optical forms. Most limonene is manufactured as a side product from citrus juice (Jongedijk et al. 2016). Limonene synthesis can be achieved in bacteria by expressing a plant limonene synthase (LS) using their innate MEP pathway. However, obtained yields were unsatisfactory, due to low availability of the limonene precursor GPP. Engineered *E. coli*, which expressed the GPP synthase gene from grand fir (*Abies grandis*) and the (−)-LS gene from spearmint (*Mentha spicata*), produced merely 5 mg/L of limonene (Carter et al. 2003). Using *E. coli*, Du et al. (2014) achieved a limonene titer of 35.8 mg/L by overexpression of genes encoding 1-deoxy-xylulose 5-phosphate synthase (DXS) and isopentenyl diphosphate isomerase (IDI), two rate-limiting enzymes in the endogenous MEP pathway, and establishing an optimized two-phase system. An alternative strategy for limonene synthesis is by engineering the MVA pathway into bacterial host. Alonso-Gutierrez et al. (2013) engineered *E. coli* with a nine-enzyme MVA pathway (from *Staphylococcus aureus* and *S. cerevisiae*) and LS (from *A. grandis*) for production of limonene, followed by coupling with cytochrome P450 hydroxylase (*Mycobacterium* spp.), which specifically hydroxylates limonene to generate perillyl alcohol. This strategy resulted in titer of 435 mg/L of limonene from glucose. Finally, the same group used multivariate, principal component analysis of proteomic data (PCAP) to optimize gene regulation and growth conditions of redesigned *E. coli* to improve limonene titers. Three clusters (CLs) of genes were introduced into *E. coli*, coding for the conversion of acetyl-CoA to mevalonate (CL1), further to isoprenoids precursors (CL2), and finally CL3 included GPP synthase and LS. It was found that the balanced pathway in terms of expression of intermediate steps and overexpression of LS led to an increased titer of 605 mg/L (Alonso-Gutierrez et al. 2015). The highest gram-scale limonene titers of 2.7 and 0.7 g/L were obtained from glycerol and glucose as carbon sources, respectively, using *E. coli* BL21 (DE3)

Table 1 Most relevant mono- and sesquiterpenoids for flavor and fragrance industry

Terpene	Natural synthesis	Engineered host	Pathway	Precursor source, genes up- or downregulation, genes heterologous expression, gene fusion	Terpene synthase	Terpene synthase source	Max yield (mg/L)	Reference
Monoterpene								
Linalool	*S. cerevisiae*	–	MVA	–			<0.1	Carrau et al. (2005)
Geraniol	*H. uvarum*	–	MVA	–			<0.1	Carrau et al. (2005)
Citronellol	*S. cerevisiae*	–	MVA	–			<0.1	Carrau et al. (2005)
Limonene	–	*E.coli* BLR (DE3)	MEP, GPPS	*Abies grandis*	LS	*Mentha spicata*	5.0	Carter et al. (2003)
Limonene	–	*E.coli* BL21 (DE3)	MEP, GPPS	*Abies grandis*	LS	*Mentha spicata*	35.8	Du et al. (2014)
Limonene	–	*E.coli* DH1	MVA, tGPPS	*AtoB*↑, *HMGR*↑, *HMGS*↑	LS	*Mentha spicata*	435.0	Alonso-Gutierrez et al. (2013)
Limonene	–	*E.coli* DH5α	MVA, tGPPS	*AtoB*↑, *HMGR*↑, *HMGS*↑, *ERG8*↑, *ERG12*↑, *ERG19*↑, *IDI1*↑	LS	*Mentha spicata*	605.0	Alonso-Gutierrez et al. (2015)
(+) Limonene	–	*E.coli* BL21 (DE3)	MVA, AGPPS2	*ERG8*↑, *ERG12*↑, *ERG19*↑, *IDI1*↑	LS	*Mentha spicata*	1,350, 2,700	Willrodt et al. (2014)
(+) Limonene	–	*S. cerevisiae*	MVA, GPP	*ERG20*↑	LS	*Citrus limon*	0.1	Jongedijk et al. (2015)
(−) Limonene	–	*S. cerevisiae*	MVA, GPP	*ERG20*↑	LS	*Perilla frutescens*	0.5	Jongedijk et al. (2015)
Geraniol	–	*E.coli* DH5α	MVA, GPPs	*ERG8*↑, *ERG12*↑, *ERG13*↑, *ERG19*↑, *HMG1*↑, *ΔyjgB*	tObGES	*Ocimum basilicum*	182.5	Zhou et al. (2014)

(*continued*)

Table 1 (continued)

Terpene	Natural synthesis	Engineered host	Pathway	Precursor source, genes up- or downregulation, genes heterologous expression, gene fusion	Terpene synthase	Terpene synthase source	Max yield (mg/L)	Reference
Geraniol	–	*S. cerevisiae*	MVA, GPPs	*HMG1*↑	GES	*Ocimum basilicum*	0.7	Pardo et al. (2015)
Geraniol	–	*S. cerevisiae*	MVA, FPPs	*ERG20*↑	GES	*Ocimum basilicum*	5.4	Fischer et al. (2011)
Geraniol	–	*S. cerevisiae*	MVA, GPPs	*ERG20*↑, *IDI1*↑, *tHMG1*↑, *UPC2–1*↑	tVoGES	*Valeriana officinalis*	293.0	Zhao et al. (2016)
Geraniol	–	*S. cerevisiae*	MVA, GPPs	*ERG20*↑, *IDI1*↑, *tHMG1*↑, *MAF1*↑	GES1	*Ocimum basilicum*	36.0	Liu et al. (2013)
Linalool	–	*S. cerevisiae*	MVA, GPPs	*tHMG1*↑, *ERG9*↓	LIS	*Lavandula angustifolia*	78.0	Amiri et al. (2016)
Sesquiterpene								
Valencene	–	*S. cerevisiae*	MVA, FPP	*ERG9*↓	GFTpsD	Grapefruit	0.6–3.0	Asadollahi et al. (2008)
Valencene	–	*S. commune*	MVA, FPP	*thn*↓	OptCnVS	*Chamaecyparis nootkatensis*	16.0	Scholtmeijer et al. (2014)
Valencene	–	*C. glutamicum*	MEP, FPP	Δ*crtE*, Δ*idsA*, *ispA(h)*	CnVS	*Callitropsis nootkatensis*	2.4	Frohwitter et al. (2014)
Nootkatone	–	*P. pastoris*	MVA, FPP	*HPO (h)*, *CPR (h)*, *tHMG1*↑, *ADH*↑	OptValS	*Callitropsis nootkatensis*	208.0	Wriessnegger et al. (2014)
Patchoulol	–	*S. cerevisiae*	MVA, FPP	*ERG9*↓	PatTps177	Patchouli	16.9	Asadollahi et al. (2008)

Patchoulol	–	*S. cerevisiae*	MVA, FPP	*ERG9*↓, *FPPS-PTS(h)*	PTS	*Pogostemon cablin*	23.0	Albertsen et al. (2011)
α-Farnesol	*S. cerevisiae*	–	MVA	–			<0.1	Carrau et al. (2005)
α-Farnesene	–	*Y. lipolytica*	MVA, FPP	*tHMG1*↑, *OptFS (h)-ERG20, IDI*↑	OptFS	Artificially synthesized	260.0	Yang et al. (2016)
α-Farnesene	–	*E. coli*	MVA, FPP	*ispA*↑, *ispA-aFS, IDI*↑	OptaFS	Artificially synthesized	380.0	Wang et al. (2011)
α-Farnesene	–	*Anabaena* sp.	MEP, FPP	-	OptFaS	Norway spruce	<0.1	Halfmann et al. (2014)
Cubebol	–	*S. cerevisiae*	MVA, FPP	*ERG9*↓	GFTpsC	Grapefruit	1.6	Asadollahi et al. (2008)
α-Santalene	–	*S. cerevisiae*	MVA, FPP	*tHMG1*↑,*ERG20*↑,*GDH2*↑, *ΔGDH1, ΔLPP1, ΔDPP1, ERG9*↓	OptSanSyn	*Clausena lansium*	25.0	Scalcinati et al. (2012b)
α-Santalene	–	*S. cerevisiae*	MVA, FPP	*ERG9*↓, *tHMG1*↑	SanSyn	*Clausena lansium*	92.0	Scalcinati et al. (2012a)

ADH alcohol dehydrogenase gene, *AtoB* acetoacetyl-CoA synthase gene, *CPR* cytochrome P450 reductase gene of *Arabidopsis thaliana, CrtE* and *IdsA* prenyltransferase genes, *ERG8* phosphomevalonate kinase, *ERG9* squalene synthase gene, *ERG12* mevalonate kinase gene, *ERG13* 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase, *ERG19* mevalonate pyrophosphate decarboxylase gene, *ERG20* FPP synthase gene of *S. cerevisiae, FPPS-PTS* fusion of farnesyl diphosphate synthase (FPPS) of *S. cerevisiae* and patchoulol synthase (PTS), *GDH1/GDH2* NADP-/NAD-dependent glutamate dehydrogenase gene, *HMG1/2* 3-hydroxy-3-methylglutaryl-CoA reductase, *HMGR* gene encoding N-terminal truncated version of HMG-CoA reductase, *HMGS* HMG-CoA synthase gene, *HPO* premnaspirodiene oxygenase gene of *Hyoscyamus muticus, IDI* IPP isomerase gene, *ispA* FPP synthase gene of *E. coli, LPP1* and *DPP1* lipid phosphate phosphatases genes, *OptFS* α-farnesene codon-optimized α-farnesene synthase gene, *tHMG1* truncated HMG-CoA reductase gene, *UPC2-1* gene encoding transcription factor regulating expression of genes involved in biosynthesis of sterols, *MAF1 thn* RGS regulatory protein gene, *yjgB* gene encoding geraniol dehydrogenase-like enzyme

equipped with the truncated and codon-optimized genes for LS (*M. spicata*) and GPP synthase 2 (AGPPS2) from *A. grandis*, in a two-liquid phase fed-batch fermentation system (Willrodt et al. 2014).

Yeasts cannot naturally produce monoterpenes due to lack of its precursor GPP and the deficiency of TPSs with some exception of wine yeast strains (Carrau et al. 2005). Natural synthesis of monoterpenes by yeast was described by Carrau et al. (2005) who reported 5 μg/L linalool production in wine *S. cerevisiae* and 3 μg/L geraniol synthesis by non-*Saccharomyces* yeast *Hanseniaspora uvarum*. Monoterpene production in yeasts as the hosts by expressing plant TPSs has hardly been explored. The strains of *S. cerevisiae*, which expressed (−)-LS from *Perilla frutescens* and (+)-LS from *Citrus limon*, produced titers of 0.48 mg/L and 0.12 mg/L, respectively, in the culture headspace (Jongedijk et al. 2015). Even though IPP and DMAPP overexpression was achieved and heterologous metabolic pathway or enzymes can be imported to the host, the molecular toxicity and high volatility of most monoterpenes are limiting factors for their biosynthesis in biotechnological conditions. The minimal inhibitory concentration (MIC) of limonene for *S. cerevisiae* is only 0.44 mM in solvent-free system (Brennan et al. 2012). Next to using two-phase fermentation systems, thus, adaptive laboratory evolution (ALE) can be used to alleviate limonene toxicity in *S. cerevisiae* cells (in the range 38–222 mg/L) to increase monoterpene production above their inhibitory limits (Brennan et al. 2015).

Other examples of bacterial monoterpene synthesis include the work by Zhou et al. (2014), who engineered *E. coli* with a foreign MVA pathway and geraniol synthase (GES) from sweet basil (*Ocimum basilicum*), which resulted in production of 105.2 mg/L of geraniol. However, loss of geraniol was observed due to its dehydrogenization and isomerization into other geranoids (nerol, neral, and geranial) which is catalyzed by geraniol dehydrogenase. Therefore, in order to eliminate this by-product formation, the geraniol dehydrogenase gene (*YjgB*) was deleted in *E. coli*, which increased synthesis of geraniol to 182.5 mg/L.

Recombinant wine yeast *S. cerevisiae*, which expressed the (S)-linalool synthase (LIS) gene from the higher plant, *Clarkia breweri*, excreted the linalool level (26 μg/L) exceeding the odor threshold under microvinification conditions (Herrero et al. 2008). Higher titers of 78 mg/L of linalool were achieved in *S. cerevisiae* equipped with LIS (*Lavandula angustifolia*) and induced *ERG9* repression and *tHMG1* overexpression (Amiri et al. 2016). Under microvinification conditions, wine yeast *S. cerevisiae* engineered with GES (*O. basilicum*) excreted 0.75 mg/L of geraniol, which was further metabolized to other monoterpenes citronellol, nerol, and linalool (Pardo et al. 2015). *S. cerevisiae* strains generated by site-directed mutagenesis of FPP synthase (at position K197 L in *ER20* gene) and transformed with GES (*O. basilicum*) produced up to 5.4 mg/L of geraniol and 0.3 mg/L of linalool and citronellol, which was 10- to 30-folds higher compared to using *S. cerevisiae* strains not harboring GES (Fischer et al. 2011). In the experiment of Zhao et al. (2016), the best *S. cerevisiae* strain produced 293 mg/L of geraniol in a fed-batch cultivation. This strain was engineered with a truncated GES (*Valeriana officinalis*) and co-overexpressing of

ERG20^{WW}, gene encoding transcription factor *UPC2–1*, and the rate-limiting enzymes for MVA pathway: truncated *HMG1* and *IDI1*.

4 Sesquiterpenes

4.1 General Overview

Sesquiterpenes are the most diverse group of isoprenoids with three five-carbon (C_5) isoprene units (Scholtmeijer et al. 2014). Sesquiterpenes have broad application in the flavor and fragrance industry. Examples of sesquiterpenes of high industrial importance include (Fig. 3a) valencene (an orange flavor), nootkatone (citrus flavor and fragrance, main grapefruit flavor constituent), β-elemene (ginger flavor), patchoulol (earthy aroma), nerolidol (floral and fruity flavor), α-farnesene (herb and organic wood-like flavor), and α-santalene (woody aroma).

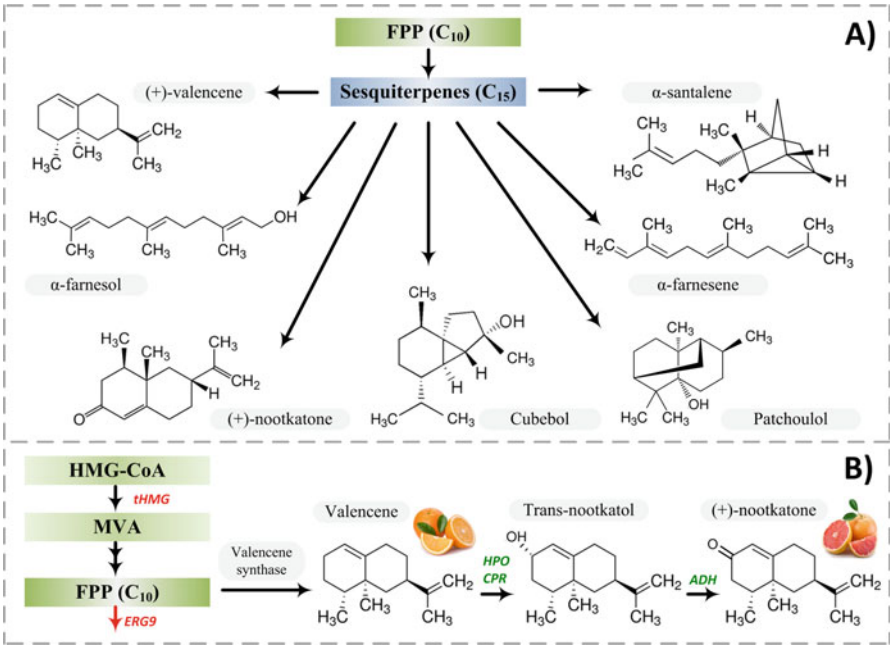

Fig. 3 The outline of the most industrially relevant sesquiterpenes (**a**), including an example of metabolically engineered pathway for the production of valencene and (+)-nootkatone (**b**). Up- and downregulated genes are shown in *green* and *red*, respectively. *tHMG* truncated HMG-CoA reductase gene, *ERG9* squalene synthase gene, *HPO* premnaspirodiene oxygenase gene, *CPR* cytochrome P450 reductase gene, *ADH* alcohol dehydrogenase gene

4.2 Research Efforts

Strategies of engineering yeast for sesquiterpene production have recently been broadly reviewed (Wriessnegger and Pichler 2013; Krivoruchko and Nielsen 2015). They involve (a) the expression of plant sesquiterpene synthases (codon optimized when necessary), (b) engineering of MVA pathway to increase the pool of sesquiterpene precursors (*tHMG1*, *ERG20, IDI*), (c) engineering of ergosterol synthesis (*ERG9*, *LPP1*, *DPP1*, *SUE*), and (d) fusion of the heterologous and/or host proteins of the sesquiterpene biosynthetic pathway (*FPPS-PTS*, *FS-ERG20*). Recent approaches include engineering of acetyl coenzyme A (acetyl-CoA) formation and protein engineering of cytochrome P450 enzymes (Renault et al. 2014; van Rossum et al. 2016). Lian and Zhao (2016) introduced the acetyl-CoA biosynthetic pathway from *E. coli*, namely, a pyruvate dehydrogenase and lipoylation machinery into *S. cerevisiae.* By increasing the cytosolic availability of acetyl-CoA, which is an important precursor in the pathways for terpenes, polyketides, and sterols synthesis, the levels of sesquiterpenes might be increased in metabolically engineered yeasts. Cytochrome P450s, both hydroxylases and reductases, are necessary for heterologous production of certain compounds originating from plants, such as nootkatone (Fig. 3b). However, they are poorly expressed in *S. cerevisiae* and *E. coli*. Therefore, one of the foci of metabolic engineering strategies is to increase the expression level of such enzymes in heterologous hosts (Renault et al. 2014). The co-expression of oxidoreductases, for example, alcohol dehydrogenase together with cytochrome P450 enzymes, might be necessary to further increase product yields (Renault et al. 2014; Wriessnegger et al. 2014).The engineering of transcription factors could be another valuable tool for modulation of multiple components of the metabolic pathway simultaneously. The gene *UPC2* is encoding a transcription factor that together with another transcription factor, *Ecm22*, is responsible for activation of several MVA and ergosterol pathway genes (Vik and Rine 2001). Although the overexpression of mutated *UPC2-1* alone had little effect on sesquiterpene production (Ro et al. 2006), the combined modulation of *UPC2-1*and other genes resulted in increased total production of sesquiterpenes (α-santalene and FPP-derived farnesol) in *S. cerevisiae* (Scalcinati et al. 2012a). In the same study, the upregulation of *GDH1* gene, encoding NADP-dependent glutamate dehydrogenase and deletion of *GDH2* gene encoding NAD-dependent glutamate dehydrogenase, in *S. cerevisiae* together with other modifications of the MVA and ergosterol pathways, increased α-santalene production, confirming that increased availability of reductive cofactor NADPH has a positive effect on sesquiterpene production (Scalcinati et al. 2012b).

Although *E. coli* is mostly used for monoterpene and carotenoid synthesis (Alonso-Gutierrez et al. 2013; Carter et al. 2003; Emmerstorfer-Augustin et al. 2016; Liu et al. 2016; Toogood et al. 2015), there exist a few examples of metabolically engineered *E.coli* for sesquiterpene production. For example, α-farnesene was heterologously produced using a codon-optimized synthase gene and an exogenous MVA pathway, resulting in 380 mg/l of the final product (Wang et al. 2011). High

titer was also reported for α-farnesene produced in *Yarrowia lipolytica* (~260 mg/l) (Yang et al. 2016).

In addition to engineering of MCFs to become efficient hosts for production of sesquiterpenes, it is possible to further increase titers and productivities by fermentation optimization and improved or improvement of recovery protocols (Wriessnegger and Pichler 2013). For example, a continuous fermentation process using a two-phase cultivation mode with in situ product removal was developed for production of compounds such as patchoulol, valencene, cubebol, and α-santalene in *S. cerevisiae* (Asadollahi et al. 2008; Scalcinati et al. 2012a, b).

5 Apocarotenoids

5.1 General Overview

Apocarotenoids are natural compounds derived from the oxidative cleavage of carotenoids (C_{40}, tetraterpenoids). In plants, the reaction is catalyzed by carotenoid cleavage dioxygenases (CCDs) (Harrison and Bugg 2014), and hence apocarotenoids have less than 40 carbon atoms. In particular, from C_9 to C_{13}, molecules are presently of industrial interest (Fig. 4). The latter, C_{13}-apocarotenoids (also known as norisoprenoids), including ionones (α, β, and γ isomers), dihydroionones (α and β isomers), damascones (α and β isomers), and β-damascenone, show a pleasant fruity

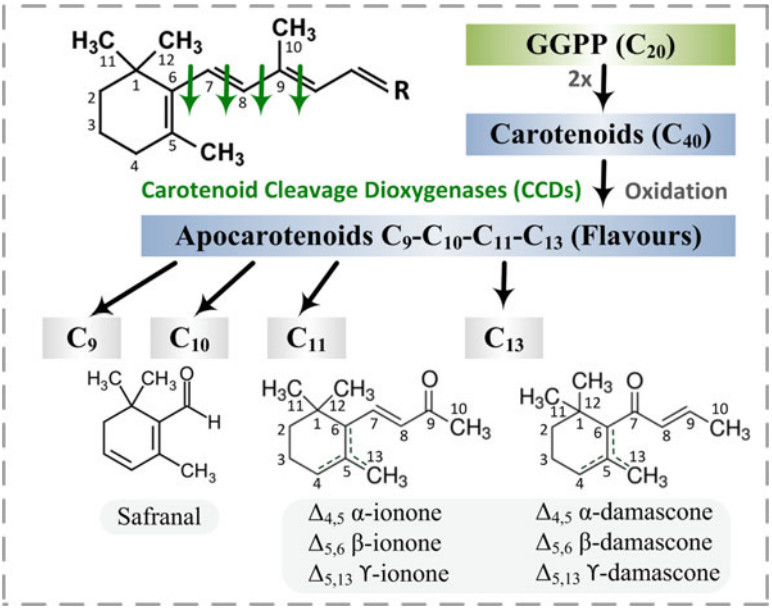

Fig. 4 The most relevant apocarotenoids for flavor and fragrance industry

Table 2 The most relevant apocarotenoids for flavor and fragrance industry

Apocarotenoid	Natural synthesis	Engineered host	Genes up- or downregulation, genes heterologous expression, gene fusion	Max yield (mg/L)	Reference
β-Ionone		*E. coli*	*CCD7, CCD4*	–	Huang et al. (2009)
β-Ionone		*S. cerevisiae*	*crtYB, crtI, crtE*	0.2	Beekwilder et al. (2014)
β-Ionone		*S. cerevisiae*	*BTS1, crtYB, crtI, PhCCD1*	5.0	López et al. (2015)
β-Ionone	*St. pasteuri*			13.2	Zhu et al. (2016)
Safranal	*S. cerevisiae*			4.0	Del Toro-Sánchez et al. (2006)
Safranal		*S. cerevisiae*		–	Raghavan et al. (2014)

St. pasteuri Staphylococcus pasteuri, *CCD* carotenoid cleavage dioxygenases, *crt* carotenogenic genes, *BTS1* geranylgeranyl diphosphate synthase gene, *PhCCD1* carotenoid cleavage dioxygenase gene from *Petunia hybrida*

and flowery aromas found in tea, grapes, roses, tobacco, and wine (Rodríguez-Bustamante and Sánchez 2007) (Table 2).

5.2 Research Efforts

Plants, specifically CCD1, catalyzes the oxidative cleavage of β-carotene producing the C_{13}-apocarotenoid, β-ionone. Different hosts have been studied for the production of carotenoids (Heider et al. 2012). For example, the overproduction of β-carotene in *E. coli* (Kim et al. 2006) and *S. cerevisiae* (Verwaal et al. 2007) has been achieved by introducing three carotenogenic genes (*crtI*, *crtE*, and *crtYB*) from the carotenoid-producing yeast *Xanthophyllomyces dendrorhous*. In addition, plant CCD enzymes have been cloned and expressed in *E. coli* and *S. cerevisiae* strains. Two most recent studies have reported de novo synthesis of β-ionone in *S. cerevisiae*. In the first one, the co-expression of the raspberry *CCD1* gene, together with the *X. dendrorhous* carotenoid genes (*crtI*, *crtE*, and *crtYB*), facilitated the β-ionone production in yeast from glucose. However, the low translational efficiency of this system limited β-ionone production to a final titer of 0.22 mg/L (Beekwilder et al. 2014). An alternative platform for β-ionone production was described by López et al. (2015), using differential expression of the carotenogenic genes *crtI* and *crtYB* and the plant gene *PhCCD1*in a farnesyl diphosphate (FPP)-overproducing *S. cerevisiae* strain

(Scalcinati et al. 2012a). In this study, a β-ionone titer of 5 mg/L was achieved after a 50 h fermentation using glucose as a carbon source (López et al. 2015).

Adaptive laboratory evolution (ALE) using oxidative stress as selection pressure has been successfully applied in engineered *S. cerevisiae* strains (Verwaal et al. 2007) to further increase the production of carotenoids (Reyes et al. 2014). The carotenoid production reached 18 mg/g dry cell weight (DCW) which was a threefold increase in carotenoid production as compared to the parental strain (Reyes et al. 2014). Nonadapted *S. cerevisiae* strains were unable to produce high levels of these compounds due to the membrane stress occurring as the result of carotenoid production (Verwaal et al. 2010). Recently, Olson et al. (2016) optimized the conditions for high production of carotenoids in bioreactors using evolved strains of *S. cerevisiae*. They achieved a yield of approximately 26 mg β-carotene/g DCW, increasing the carbon-to-nitrogen (C/N) ratio from 8.8 to 50 in standard yeast nitrogen base (YNB) medium, varying agitation speed to 800 rpm, and maintaining the pH at 4 (Olson et al. 2016). Nevertheless, the application of carotenoid hyper-producing *S. cerevisiae* strains was not enough for obtaining high industrial titers of β-ionone and other apocarotenoids. One of the main reasons is that apocarotenoids, similarly to other terpenoids, show strong biostatic and biocidal effects. For instance, 10 mg/L of β-ionone reduces the growth rate of *S. cerevisiae* by 20 %, while 50 mg/L completely stops yeasts growth (Cataldo et al. 2016). Different strategies have been reported to minimize this inhibitory effect of β-ionone. One of the approaches is the development of more resistant strains by using evolutionary engineering (Winkler and Kao 2014). Another strategy is to increase the solubility and consequently reduce the toxicity of some C_{13}-apocarotenoids. This could be achieved by cloning of glycosyltransferase genes as illustrated by the synthesis of vanillin in *S. cerevisiae* (Brochado et al. 2010). Other C_{13}-apocarotenoids that could be potentially targeted by this approach involve β-ionol or β-damascenone precursors (Cataldo et al. 2016).

Apocarotenoids are one of the main components responsible for color, taste, and aroma of saffron that is the world's most expensive spice present in *Crocus sativus* stigmas. In saffron, one can find the natural dyes, crocetin and crocins (Bathaie et al. 2014), bitter but odorless picrocrocin (Serra 2015), and C_9 and C_{10} apocarotenoids, such as keto derivatives and safranal, respectively, are responsible for the unique aroma of saffron (D'Auria et al. 2004). The production of the latter is thus of special interest for the flavor industry. Remarkably, other plants, such as *Ditaxis heterantha* seeds, that have similar properties to *C. sativus*, could be used for more economic production of safranal. Del Toro-Sánchez et al. (2006, 2015) described for the first time the ability of *S. cerevisiae*, isolated from the flower *Tagetes erecta*, to degrade the apocarotenoid heteranthin of *D. heterantha* seeds, which ultimately results – among other aromas – in safranal production. Hence, a search for microorganisms with the ability to degrade β-carotene and other carotenoids is very promising. There is also evidence that *Staphylococcus pasteuri*, isolated from sea buckthorn juice, has an ability to convert β-carotene to β-ionone and β-cyclocitral. The highest β-ionone production obtained in cell-free culture supernatant was 13.16 mg/L (Zhu et al. 2016).

6 Concluding Remarks and Research Needs

There is a strong move away from extraction of plants to microbial production of flavors and fragrances. A number of innovative biotech companies have extended their product portfolio to include apocarotenoids, sesquiterpenes, and monoterpenes such as nootkatone, α-farnesene, saffron, valencene, etc. This includes Swiss Evolva, Dutch Isobionics, and Amyris and Ginkgo BioWorks in the USA. They focus on the development of efficient MCFs or novel fermentation methods, and they have just or about to introduce their products into the market. Industrial production of terpenes remains a challenge as reported titers and productivities – at least from university research – are typically low. There is therefore a need to facilitate the development of more efficient MCFs and sustainable fermentation processes to compete with plant extraction and chemical synthesis methods. Hence, the future research should focus on:

1. Designing novel enzyme and protein engineering methods to enable a balanced expression of the biosynthetic pathways to terpenes and allow close to 100 % turnover rates
2. Developing novel methods for efficient expression of cytochrome P450 enzymes in microbial MCFs
3. Process engineering of efficient fermentation methods including more research on two-phase systems for simultaneous product recovery
4. Reduction of toxicity of intermediate and target terpenes

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Biotechnological Production and Significance of Triacylglycerols and Wax Esters

22

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Contents

Abstract

Global fat, oil, and wax ester demand is forecasted to increase in the next years. Steadily growing biodiesel requirements will lead to an increased demand for vegetable oils, in combination with a constant rise in the consumption of vegetable oil as food and feed. This situation will prompt the use of alternative sources for the production of oils and wax esters during the next years. In this context, microorganisms (yeasts, fungi, microalgae, and bacteria) are receiving increasing attention as alternative oils and wax esters sources. The knowledge acquired during the last decade about the production of bacterial triacylglycerols (TAG) and wax esters (WE) and their fundamental aspects could provide a new production platform for oils. The applied potential of bacterial TAG and WE may be

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similar to that of vegetable sources, such as additives for feed, cosmetics, oleochemicals, lubricants, and other manufactured products. In addition, bacterial oils could be used for biofuel production. The development of an industrial and commercially significant process may depend on the optimization of engineered cells and the technological procedures. This chapter will focus on the potential biotechnological application of TAG and WE produced by bacteria.

1 Introduction

Fats, oils, and WE are nowadays used for multiple commercial purposes. Global demand for fats and oils during the next years is forecast to rise and probably to exceed world production. World consumption of oils and fats has grown progressively during the last 25 years. Since 1995, the global per capita consumption of oils and fats has risen from 15.6 to 23.4 kg per year. In 2015, the world production of fats and oils was approximately of 205 million tons with 75% of vegetal origin. A large proportion of the vegetable oils are comprised of coconut, palm, and palm kernel oil from countries with tropical climates and soybean, rapeseed, and sunflower oils from moderate climates. Animal fat is obtained from the meat industry, and fish oil comes from the fishing industry.

Vegetable oils and WE are used as an ingredient or component in many manufactured products (Table 1). Over 80% of oils are used for edible purposes, including the production of cooking oils, margarines, and processed food. Half the remaining part is used in the animal feed industry, and approximately 14% goes to the chemical industry.

In recent years, biodiesel has also been gaining worldwide interest as an alternative energy source. This biofuel is basically a mixture of methyl esters produced by transesterification of vegetable oil. Increasing global appetite for biodiesel and other biofuels is driving the demand for vegetable oils to historic levels in world markets. Furthermore, steadily growing biodiesel requirements led to increased demand for vegetable oils, which is expected to coincide with progressive expansion in global demand for food and feed use. Utilization of vegetable oils as feedstock for biodiesel, as well as for generating electricity and for heating purposes, is expected to grow during the next years. This situation generates a new conflict between the use of oil

Table 1 Applications of vegetable oils and wax esters

Fats and oils	Wax esters
Culinary uses	
Components in manufactured products: soaps, candles, perfumes, cosmetic products, paints and other wood treatment products, lubricants, cooling agents, and oleochemicals	Components in manufactured products: cosmetics and pharmaceutical products, polishes, surface coatings, inks, and oleochemicals
Pet food additives	
Fuels	Biolubricants

for industrial purposes and food production. For all these reasons, the appearance of alternative sources for oils and WE is nowadays of great interest. In this context, microorganisms like cyanobacteria, fungi, yeasts, and bacteria are receiving increasing attention for their potential applications to the oils and WE industry.

2 Biosynthesis and Accumulation of Bacterial TAG and WE

TAG are nonpolar, water-insoluble fatty acid tri-esters of glycerol, which occur in most eukaryotic organisms, including animals, plants, yeast, and fungi. They are the most important storage material for energy and fatty acids required for lipid biosynthesis in cells. For several years, it has been considered that the formation of TAG as storage lipids was restricted to eukaryotic cells and that prokaryotes were unable to synthesize and accumulate them. Only recently, it has become obvious that TAG also occur frequently in certain groups of prokaryotes as reserve material, such as *Mycobacterium*, *Rhodococcus*, *Nocardia*, and *Streptomyces*, among others (Olukoshi and Packter 1994; Alvarez and Steinbüchel 2002; Bredemeier et al. 2003) (Table 2). Members of these genera produce variable amounts of TAG during cultivation on different carbon sources, and some species are able to accumulate very high levels of TAG in their cells (Fig. 1). The TAG content in *Streptomyces* species reached in some cases up to 50% of the cellular dry weight (Röttig et al. 2016), whereas fatty acids in acylglycerols in cells of *Rhodococcus opacus* PD630 accounted for up 76–87% of the cellular dry weight in gluconate- or olive oil-grown cells, respectively (Alvarez and Steinbüchel 2002). Such microorganisms may be considered as oleaginous bacteria since they accumulate more than 20% of their biomass as lipids. Gram-negative bacteria are also able to accumulate neutral lipids (Table 2). However, TAG represent only a minor component of the stored lipids, whereas WE are the main lipid compounds accumulated by these bacteria. WE are esters of long-chain fatty alcohols with long-chain fatty acids.

3 Potential Uses of Bacterial TAG and WE

In general, the applied potential of bacterial TAG may be similar to that of agricultural sources. Bacterial oils have still not been considered as an alternative source because such lipids were mostly unknown in prokaryotes until recently. However, bacteria should now also be considered as a source of lipids with potential application in the oil industry. The use of microorganisms for lipid production provides some advantage over agricultural sources with regard to the enormous variability of fatty acid composition depending on the carbon source used for cultivation of cells and the better accessibility of microorganisms to genetic and metabolic engineering (Alvarez and Steinbüchel 2002). In addition, our studies demonstrated that it is possible to direct the flow of metabolites to different storage compounds in oleaginous bacteria in order to obtain higher lipid contents or altered composition in storage lipids, which could have biotechnological implications (Alvarez et al. 1997).

Table 2 Occurrence of TAG and WE in bacteria. The order indicates the lipid predominance for each microorganism

Bacteria	Type of stored lipids
Gram-positive	
Rhodococcus opacus	TAG/WE
R. erythropolis	TAG
R. fascians	TAG
R. ruber	TAG
R. jostii	TAG/WE
Nocardia asteroides	TAG
N. corallina	TAG
N. globerula	TAG
N. restricta	TAG
Mycobacterium tuberculosis	TAG
M. smegmatis	TAG
M. ratisbonense	TAG/WE
M. avium	TAG
Dietzia maris	TAG
Gordonia amarae	TAG
Streptomyces coelicolor	TAG
S. albus	TAG
S. griseus	TAG
S. lividans	TAG
Gram-negative	
Acinetobacter baylyi	WE/TAG
A. lwoffi	WE/TAG
A. indicus	WE
Alcanivorax borkumensis	WE/TAG
A. jadensis	WE
Marinobacter hydrocarbonoclasticus	WE
Nitratireductor sp.	TAG

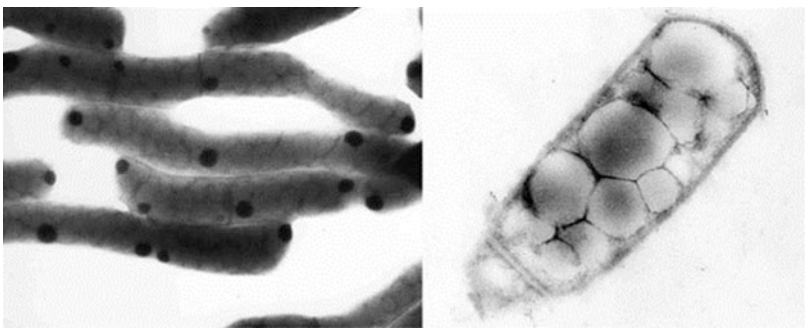

Fig. 1 Micrographs showing cells of *Rhodococcus opacus* with several lipid inclusion bodies (Pictures: F. Mayer (Georg- August- Universität Göttingen, Germany))

When the de novo fatty acid biosynthesis pathway was inhibited in *R. ruber,* which accumulates TAG and polyesters (PHA), by addition of cerulenin to the medium, more acetyl-CoA and propionyl-CoA became available for the synthesis of poly (3HB- *co*-3HV) resulting in an increase of the polyester content and in an alteration of the copolyester composition in cells (Alvarez et al. 1997). On the other hand, disruption of polyester biosynthesis in *R. ruber* by a mutation caused an increase in the cellular TAG content (Alvarez et al. 1997). The application of genetic engineering to oleaginous bacteria could extend and optimize the use of these microorganisms for the production of valuable oils. A good example of this is the use of lignocellulosic raw materials, which are important agro-industrial wastes, for this purpose. These materials possess recalcitrant components for bacterial cultivation. Thus, genetic engineering and evolutionary adaptation strategies were applied to some members of Actinobacteria for improving catabolism of xylose, cellobiose, and other waste components and, at the same time, the production of TAG (Hetzler and Steinbüchel 2013; Kurosawa et al. 2015). The successful implementation of these molecular strategies suggested the possibility that lignocellulosic biomass can be completely utilized by oleaginous bacteria to produce valuable oils.

Some oleaginous bacteria have already been used for high cell density cultivation to obtain high concentrations of TAG in bioreactors. Voss and Steinbüchel (2001) investigated *R. opacus* strain PD630 with respect to the fermentative production of TAG at the 30 L scale in a stirred tank bioreactor, which contained sugar beet molasses and sucrose as sole carbon sources. In addition, this fermentation process was successfully applied to a 500 L pilot plant scale. In addition, Kurosawa et al. (2010) reported the high cell density cultivation of *R. opacus* PD630 using high glucose concentrations for TAG production; thus, they highlighted the potential of this strain as a source of industrial biodiesel derived from starchy cellulosic feedstock that consists primarily of glucose polymers. According to these results, these oleaginous microorganisms could be applied to the biotechnological production of interesting single cell oils and probably other lipid-derived products as well, such as carotenoids, using a cheap residual C source from agricultural products. This is another interesting aspect of the bacterial lipid production in comparison to the agricultural sources. Inexpensive feedstock, such as organic wastes or residual materials from industry or municipal sources, can be used for lipid production. In this context, Gouda et al. (2008) reported the oil accumulation by *Rhodococcus* and *Gordonia* members from agro-industrial wastes, such as carob and orange wastes and sugar cane molasses. In other studies, whey was efficiently used as substrate for the production of cellular biomass and TAG by *R. opacus* strains (Herrero and Alvarez 2016). This procedure possesses not only an industrial but also an ecological importance. In addition, the availability of bacterial products does not depend on natural factors that normally affect the agricultural products, such as weather, climate, and possible catastrophes, although the feedstock used for lipid production can be influenced by these factors. Moreover, the bacterial lipid production does not compete for land. For these reasons, biotechnological production of valuable bacterial lipids may be advantageous. The diverse potential uses of bacterial oils and WE will be discussed below.

Bacterial TAG as edible oils: Most of vegetable oils are used for edible purposes. The use of bacterial oils for nutritional purposes is still uncertain due to economic reasons, and the lack of information on their digestion and absorption, considering that bacterial TAG exhibit a fatty acid distribution at the glycerol backbone and fatty acid composition, different from TAG obtained from plants and animals (Alvarez and Steinbüchel 2002). Beyond this, the use of bacterial oils for edible purposes would be not well socially accepted. However, oils from bacterial origin may be eventually used for feeding pets or commercially raised fish.

Bacterial TAG as components for manufactured products: Like vegetable oils, bacterial TAG could be produced in large amounts for use in soaps and detergents, plastics, personal care products, resins and lubricants, and oleochemicals, as ingredients for pharmaceutical products, or as protective coating for probiotic systems. Similarly, microorganisms are also a source of WE. Microbial WE could serve as components of lubricants, inks, surface coating, lacquers, cosmetics, and additives to biofuels. Currently, available WE are derived from plants and animals or from synthetic chemical processes. Long-chain WE are principally obtained from jojoba plant, which grows in the semiarid regions of Mexico and the USA. One disadvantage of jojoba WE is that the plant needs up to 5 years between the times of planting and first harvesting of the product. For these reasons, microorganisms could be considered as an alternative source of oils and WE, which can be produced in large amounts from inexpensive organic residues or wastes.

Bacterial lipids for specialized industrial applications: Oil-producing bacteria or their enzymes could be used for biotechnological production of unusual lipids with new technological applications. An interesting feature of microbial systems is that the chemical structure of oils or WE can be controlled by the composition of the feedstock used. In this context, it is worth mentioning the biosynthesis and accumulation of novel TAG and WE containing unusual components by members of *Rhodococcus* and related genera. We reported the accumulation of TAG and WE containing aromatic fatty acids, such as phenyldecanoic acid residues or isoprenoid fatty acids and fatty alcohols, such as 4,8,12-trimethyl tridecanoic acid or 2,6,10,14-tetramethylhexadecan-1-ol, after cultivation of Actinobacteria on phenyldecane, pristane, and phytane, respectively (Alvarez and Steinbüchel 2002; Silva et al. 2007). Another interesting aspect of lipid-accumulating bacteria is the potential use for biotechnology of the enzymes involved in the storage lipid metabolism. The identification and features of the wax synthase/diacylglycerol acyltransferase (WS/DGAT) enzyme from *Acinetobacter baylyi* ADP1 recently opened this new field. WS/DGAT catalyzes the last step of either TAG or WE biosynthesis by bacteria (Kalscheuer and Steinbüchel 2003). The importance of this enzyme is found in its highly unspecific acyltransferase activity, which confers a broad biocatalytic potential to this enzyme for industrial production of a large variety of lipids or biofuels in vitro or in vivo in prokaryotic as well as eukaryotic expression hosts. WS/DGAT is capable for utilizing a broad range of alcohols with different chain length as substrates, such as hexadecanol, ethanol, and isoamyl alcohol, as well as saturated and unsaturated fatty acids (Stöveken and Steinbüchel 2008). Monoacylglycerols could also be used as an alternative acyl

acceptor by the enzyme in vitro, in addition to diacylglycerols (Stöveken et al. 2005). WS/DGAT from strain ADP1 was also used for in vivo and in vitro biosynthesis of unusual storage lipids, like wax diesters consisting of 1,16-hexadecanediol esterified with long-chain fatty acids, in addition to thio and dithio WE (Stöveken and Steinbüchel 2008). On the other hand, some approaches to redesign the protein pocket of WS enzymes for extending binding to a higher diversity of ligands are in course (Barney et al. 2015). This strategy may improve binding of smaller or larger substrates to the enzyme in order to produce targeted oils. Such protein alterations could be beneficial for specific biotechnological applications. These results permit to predict further advances for establishing in vivo or in vitro production of bacterial oleochemical derivatives using whole cells or WS/DGAT enzymes.

Bacterial TAG or WE as source for biofuel production: The present forecasts a steady expansion in global demand for energy use. In this context, steadily growing oil requirements for bioenergy combined with a constant rise in the consumption of vegetable oil as food and feed may drive the use of alternative oil sources for the production of biofuels with similar efficiency as petroleum diesel in powering unmodified diesel engine. The use of nonfood grade microbial oils for biofuel production is now under consideration, and this topic became a very active field of research. As mentioned above, some microorganisms are able to produce large amounts of TAG or WE from organic compounds, which could be used for producing fatty acid esters (biodiesel) by a chemical process of transesterification. For these reasons, the main research efforts are currently focused on the biochemistry and genetics of oil-accumulating bacteria and also of bacterial strains which do not synthesize these compounds but could be biotechnologically important for designing a scalable and commercially viable oil-producing system. The main metabolic engineering strategies to enhance bacterial oil production involves gene overexpression, heterologous expression, deletion of genes from competitive processes, and combined strategies in order to achieve TAG/WE production. Genes belonging to central metabolism, fatty acids biosynthesis, TAG/WE metabolism, and uptake and catabolism of carbon sources that were engineered are depicted in Fig. 2. Moreover, some approaches to modify oil composition were performed to produce customized biofuels.

Another interesting approach is the in vivo production of fatty acid methyl or ethyl esters using engineered bacterial cells that condense methanol or ethanol with fatty acids. In this context, Eberly et al. (2013) demonstrated the in vivo ability of *Gordonia* sp. KTR9 to catalyze ester formation from exogenous short-chain alcohol sources for the production of biodiesel (fatty acid esters). On the other hand, Kalscheuer et al. (2006) reported the biosynthesis of fatty acid ethyl esters (FAEE) (referred as microdiesel) by metabolically engineered *Escherichia coli*. They combined the ethanol formation with its subsequent esterification with the acyl moieties of coenzyme A thioesters of fatty acids after heterologous expression in *E. coli* of the *Zymomonas mobilis* pyruvate decarboxylase and alcohol dehydrogenase and the WS/DGAT from *A. baylyi* ADP1. Recombinant cells produced fatty acid ethyl esters up to 26% of cellular dry mass after cultivation on glucose and oleic acid. This study demonstrated that the production of FAEE by bacteria is empirically feasible;

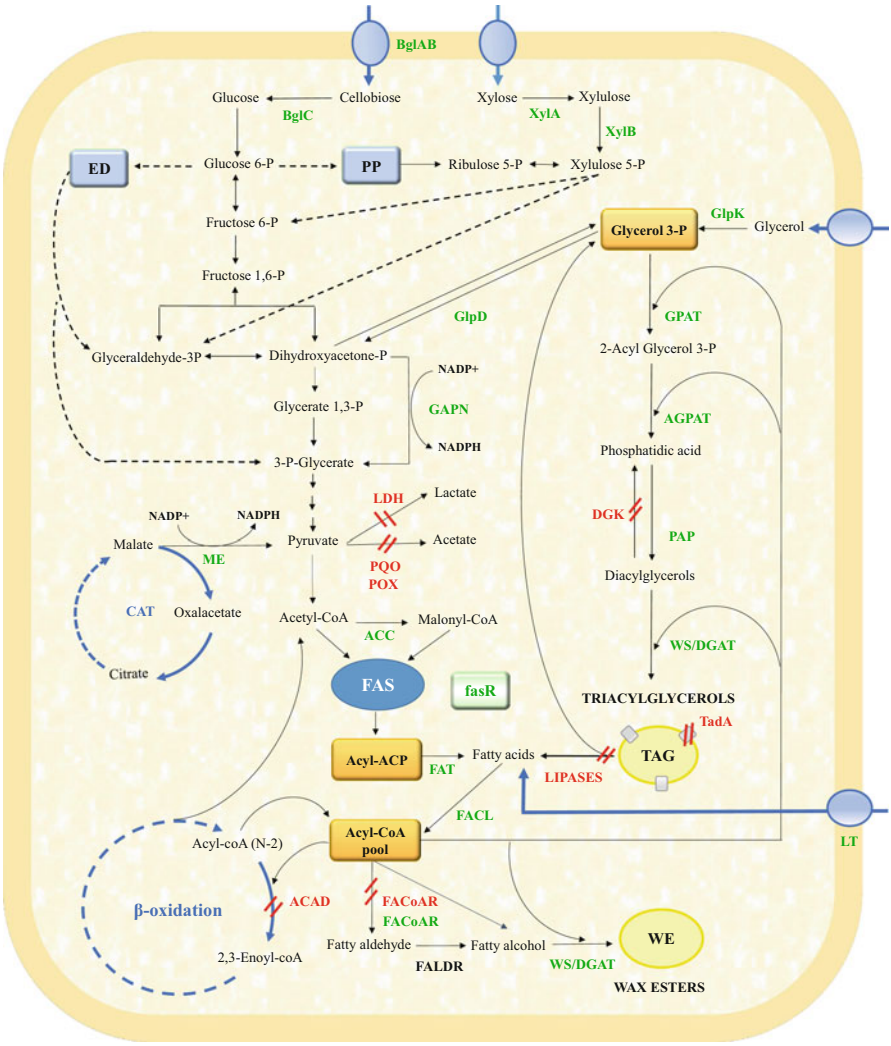

Fig. 2 Schematic representation of genes/proteins engineered to improve WE/TAG synthesis and accumulation in prokaryotes. Protein names in *red* indicate knockout reactions. Overexpression and/or heterologous expression of corresponding genes are depicted in *green*. Abbreviations: *ED* Entner-Doudoroff pathway, *PPP* pentose phosphate pathway, *FAS* fatty acid synthase, *TCA* tricarboxylic acid cycle, *TAG* triacylglycerols, *WE* wax esters, *XylA* xylose isomerase, *XylB* xylulokinase, *BglA and BglB* ABC cellobiose transport proteins components, *BglC* cytoplasmic β-glucosidase, *GlpK* glycerol kinase, *GlpD* glycerol-3-phosphate dehydrogenase, *GAPN* non-phosphorylative glyceraldehyde dehydrogenase enzyme, *ME* malic enzyme, *LDH* lactate dehydrogenase, *PQO* pyruvate/quinone oxidoreductase, *POX* pyruvate dehydrogenase, *GPAT* glycerol-3-phosphate O-acyltransferase, *AGPAT* acylglycerol-3-phosphate acyltransferase, *PAP* phosphatidic acid phosphatase, *DGK* diacylglycerol kinase, *WS* wax ester synthase, *DGAT* diacylglycerol acyltransferase, *ACC* acetyl-CoA carboxylase, *ACP* acyl carrier protein, *FAT* acyl-ACP thioesterase, *FACL* fatty acyl-CoA synthetase, *ACAD* Acyl-CoA dehydrogenase, *FadR* global lipid regulator, *TadA* structural protein of lipid bodies, *LT* lipid transporter protein, *FACoAR* fatty acyl-CoA reductase

however, further metabolic and genetic engineering is required for making this biotechnological process economically competitive. Some of the key points to consider for reducing production costs using engineered microorganisms would be (1) the use of WS/DGATs with higher specificity for ethanol, (2) the use of a recombinant system with the ability to de novo synthesize large amounts of fatty acids from sugars, and (3) the use of a recombinant system able to excrete FAEE outside the cell.

Moreover, the chemical structure of the produced biodiesel could be modified to obtain more stable biofuels. In this context, Tao et al. (2015) used engineered *E. coli* strains for the de novo biosynthesis of fatty acid branched-chain esters and branched fatty acid branched-chain esters through combination of the (branched) fatty acid biosynthetic pathway and the branched-chain amino acid biosynthetic pathway. These results highlight the flexibility of bacterial systems for their application in the biofuel industry.

4 Research Needs

The application of microbial oils and WE in the industry is currently a promising avenue (Fig. 3). However, a current problem to be solved is the reduction of the cost of microbial lipid production. The combination of fundamental knowledge of the biology of bacterial TAG accumulation, with genetic, metabolic, and process engineering will contribute to the economic feasibility of the bacterial oil production on an industrial scale. The available knowledge on the biochemistry and genetics of TAG and WE biosynthesis by bacteria is still fragmentary. For this reason, it is necessary to concentrate research on basic aspects of bacterial TAG and WE biosynthesis, such as the study of structural and regulatory genes involved in this process, the occurrence and characterization of additional lipid biosynthesis pathways in bacteria, and the metabolic interactions between pathways in oleaginous microorganisms. These studies will provide new insights, which will enable genetic and metabolic manipulation of selected microorganisms for lipid production at large scale. Moreover, it is of interest in this field to isolate new lipid-accumulating bacteria from diverse natural ecosystems and to characterize new WS/DGAT enzymes with different specificities for acyl- and alcohol substrates or different physical properties. The application of directed protein evolution methods to known WS/DGAT is other interesting possibility to be explored in more detail. On the other hand, some technological aspects need to be solved for making the bacterial lipid production economically feasible at industrial scale. Among the topics to be addressed are the optimal utilization of inexpensive organic residues for lipid production, the design and optimization of adequate bioreactors for high cell density cultivation of oleaginous microorganisms and the development of adequate methods for cell harvest, cell rupture, and lipid extraction. The intensive engineering of bacterial models, such as *E. coli*, for the direct production of diverse biofuels is another interesting avenue in the field.

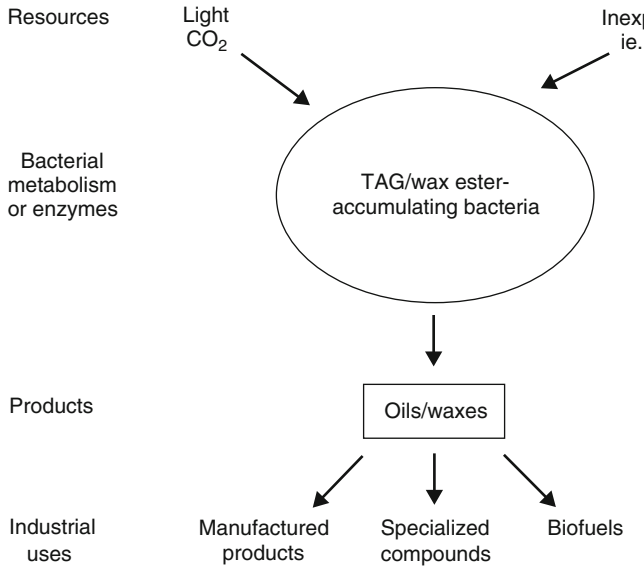

Fig. 3 Scheme of the potential use of microbial lipids for industrial purposes

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Production of Fatty Acids and Derivatives by Metabolic Engineering of Bacteria

23

Christopher R. Mehrer, Nestor J. Hernández Lozada, Rung-Yi Lai, and Brian F. Pfleger

Contents

Abstract

Metabolic engineering provides a powerful set of tools to engineer organisms such as bacteria, fungi, and plants to produce chemicals of interest. Fatty acid metabolism

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enables the sustainable production of many oleochemicals used as fuels, materials, and consumer products. In this chapter, we describe the biochemical pathways and metabolic engineering strategies that have been employed in bacteria to produce fatty acids and their related oleochemical products, such as alkanes, olefins, ketones, esters, alcohols, and polyesters. While microbial oleochemical production is promising, significant work remains to address the metabolic, physiological, and process engineering barriers that obstruct economic commercial deployment.

1 Introduction

Fatty acids are ubiquitous, energy-rich organic acids that make up the hydrophobic portions of biological lipids and oils. Fatty acids are also the precursors to a wide class of oleochemicals including alkanes, olefins, ketones, esters, alcohols, and polyhydroxyalkanoates (PHAs) that are used as fuels, surfactants, plasticizers, solvents, and materials. The vast majority of these molecules are made from plant oils and other lipid feedstocks that are in limited supply. Growing demand for biodiesel and other oleochemicals has motivated tropical deforestation in order to develop land for growing additional oil crops. Metabolic engineering offers an alternative strategy in which microbial biocatalysts are developed for converting renewable resources such as plant biomass to fatty acids and oleochemical derivatives (Woolston et al. 2013). Bacteria are common hosts for metabolic engineering because they naturally produce large amounts of fatty acids, are readily genetically manipulated, are used widely in industrial biotechnology, and have flexible metabolism capable of providing essential substrates and cofactors required for oleochemical synthesis. In this chapter, we describe the biochemical pathways and metabolic engineering strategies that are employed to produce fatty acids and oleochemicals in bacteria.

2 Overview and Paradigms of Fatty Acid Production

Fatty acids, lipids, and oleochemicals are synthesized from central metabolites by an iterative set of elongation and reduction reactions in which two carbons are added to the growing chain per cycle. For this reason, fatty acid biosynthesis (FAB) is categorized as a "+2" pathway in contrast to other iterative anabolic routes of building C-C bonds: "+5" pathway for building terpenoids from isoprene building blocks (Kirby and Keasling 2008), "+1" pathway for elongating intermediates in aliphatic amino acid biosynthesis (Marcheschi et al. 2012). The "+2" pathways act on acyl-thioester (either Coenzyme A – CoA – or acyl carrier protein – ACP) intermediates and can be subdivided into four phases, analogous to a polymerization reaction: (1) initiation, (2) iterative elongation and reduction, (3) termination, and (4) modification. The initiation reaction (Fig. 1a) generates a short β-ketoacyl thioester (either CoA or ACP) via a Claisen condensation reaction between two acyl-thioesters. The initiation reaction is the first of several chain elongation

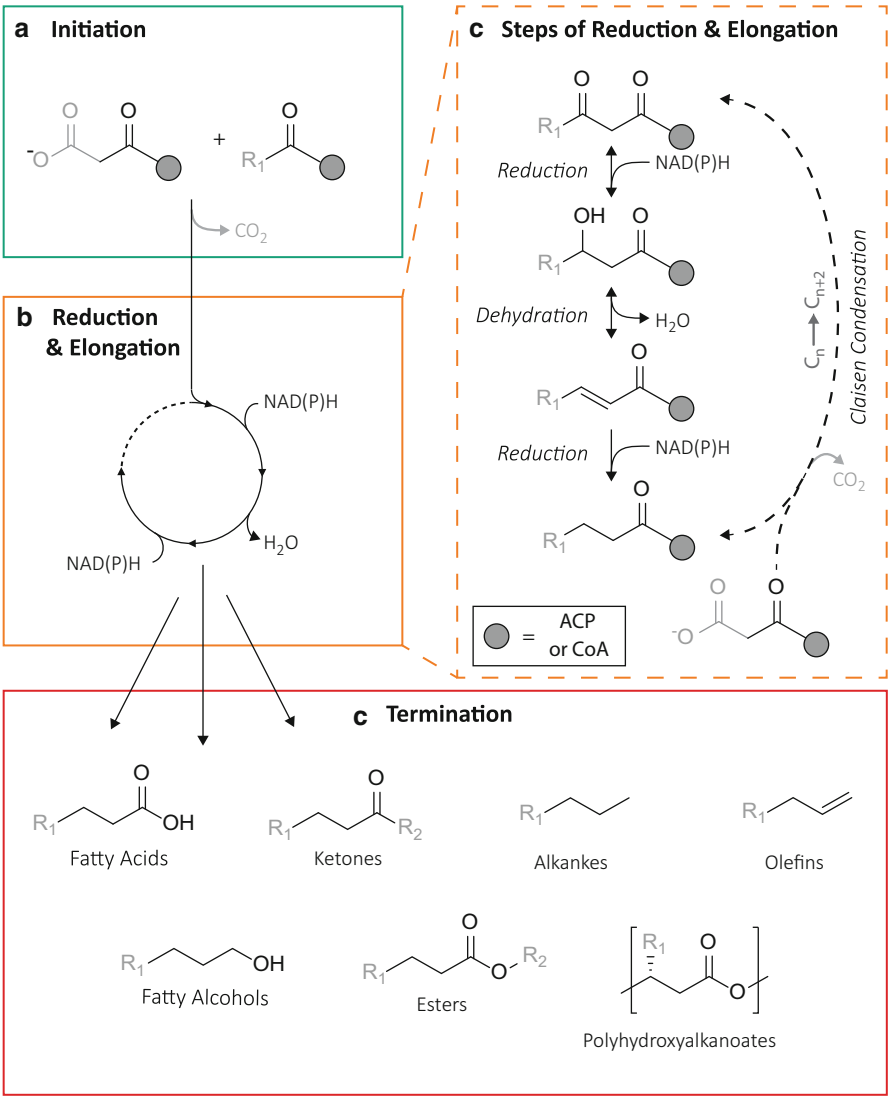

Fig. 1 Common themes for biosynthesis of fatty acids and related products

reactions. The initiation reaction can utilize different substrates whereas subsequent elongation reactions add two carbons from a conserved substrate (typically acetyl- or malonyl-thioesters). After each elongation, the β-keto thioester is reduced to a saturated acyl-chain, via β-hydroxy and *trans*-2-enoyl species, at which point it is a substrate for the next cycle of elongation (Fig. 1b, c). As the acyl-chain grows, it becomes a substrate for termination reactions that generate oleochemical products with a wide range of functional groups at the primary carbon (Fig. 1d). The last phase

of oleochemical synthesis is the modification of acyl chains by enzymes that introduce additional functionalities (e.g., double bonds, hydroxyl, cyclopropane, methyl) along the acyl-chain. While all "+2" pathways utilize these four phases, there are significant differences in how each individual pathway functions.

FAB and other "+2" pathways are subcategorized by a few key differences. The first distinction of "+2" synthesis pathways separates CoA-dependent pathways from ACP-dependent pathways. Most FAB pathways act on acyl-ACP intermediates with elongation monomers attached either to CoA or ACP. Fully CoA-dependent pathways include the β-reduction pathway used in *Clostridium acetobutylicum* butanol fermentation (Atsumi et al. 2008; Lütke-Eversloh and Bahl 2011), fatty acid elongation (Smith and Tsai 2007) in *Trypanosomes brucei* (Lee et al. 2006), and the wax fermentation pathway from the mitochondria of *Euglena gracilis* (Hoffmeister et al. 2005), which was used in part to engineer the reversal of the β-oxidation pathway in *E. coli* (Dellomonaco et al. 2011; Clomburg et al. 2012).

The second distinction is the type of Claisen condensation used to elongate acyl-chains. FAB pathways use the decarboxylation of malonyl-CoA as a thermodynamic driving force to push the Claisen condensation reaction in favor of longer products. In contrast, β-reduction pathways directly elongate chains with the Claisen condensation of two acetyl-CoA subunits with no thermodynamic driving force, often requiring these pathways to be coupled to NADH usage in the cell (Shen et al. 2011).

The final distinction of "+2" pathways concerns the organization of the enzymes within the cell. Type II systems are comprised of discrete enzymes for each step in the elongation/reduction cycle, such as the FAB pathway in *Escherichia coli.* Type I systems, on the other hand, contain all enzymatic activities in a single multidomain protein, or megasynthase, and are typically found in eukaryotes. Polyketide synthases, another type of megasynthase, use the same set of enzymatic reactions as FAB, but each elongation/reduction cycle can terminate in any of the four reduction states (β-keto, β-hydroxy, unsaturated, or saturated), allowing for wider chemical diversity (Smith and Tsai 2007).

In the remainder of this chapter, we will focus on metabolic engineering of FAB and β-reduction pathways to produce fatty acids and related oleochemicals in bacteria. Both of these pathways are well understood and have been used to produce a variety of fatty acids and oleochemical derivatives (Pfleger et al. 2015). While many of these principles also apply to higher organisms, the reader is referred to other reviews for detailed discussion of polyketide biosynthesis (Cummings et al. 2014) and oleochemical engineering in yeasts (Liu et al. 2013b), algae (Radakovits et al. 2010), and plants (Jetter and Kunst 2008).

3 General Metabolic Engineering Strategies for Producing Fatty Acids and Oleochemicals

The general goal of a metabolic engineering project is to produce product chemicals, such as fatty acids and their derivatives, with high titer, specific productivity (i.e., rate), and yield on low-cost carbon sources. These three performance metrics,

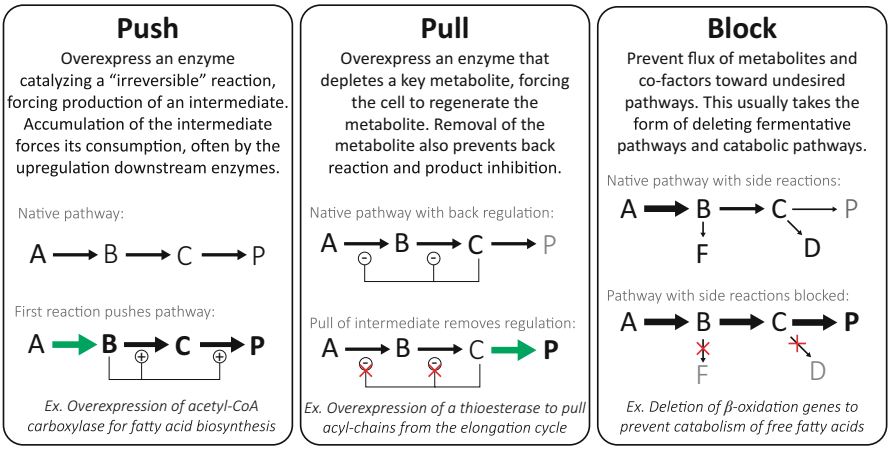

Fig. 2 Push-Pull-Block strategies

collectively called "TRY," are optimized though a combination of push, pull, and block metabolic engineering strategies (Fig. 2). The "push" strategy overexpresses pathway enzymes (best implemented on enzymes catalyzing irreversible reactions) to generate elevated levels of key intermediates. At a minimum, the elevated intermediate levels saturate downstream enzymes to increase pathway flux. When the intermediates are regulatory signals, cells can generate a feed-forward response in which the activity of downstream enzymes is upregulated in attempt to restore metabolic homeostasis. Conversely, the "pull" strategy overexpresses enzymes (again, best implemented on enzymes catalyzing irreversible reactions) to reduce backward pathway flux and deplete key regulatory intermediates such that earlier steps are upregulated. Finally, the "block" strategy deletes and/or downregulates genes encoding enzymes that allow carbon flux to go toward reactions other than the desired product. Deleting these competing genes is critical to producing a desired compound because it prevents product consumption and eliminates competing pathways that divert carbon flux away to undesired by-products (Ranganathan et al. 2012; He et al. 2014). As we discuss metabolic engineering projects to produce fatty acids and oleochemical derivatives, we will provide specific examples of "push-pull-block" strategies.

4 Chain Initiation

4.1 Fatty Acid Biosynthesis (FAB)

The first step in FAB involves a Claisen condensation of two short-chain acyl-thioesters to form the first β-keto acyl-ACP (Fig. 3a) (Heath and Rock 2002). In *E. coli*, acetyl-CoA is activated via a carboxylation, which is catalyzed by acetyl-CoA

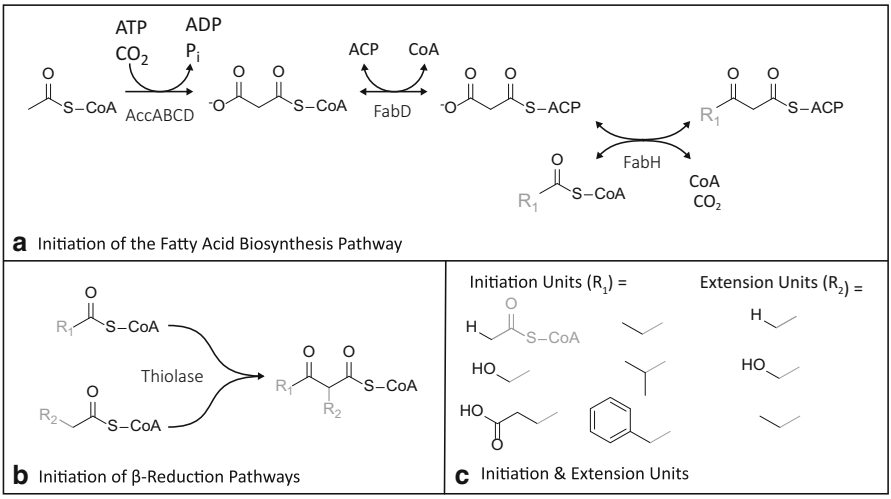

Fig. 3 Initiation steps of fatty acid biosynthesis and β-reduction pathways

carboxylase (encoded by *accABCD*) to form malonyl-CoA. The malonyl group is transesterified onto an acyl carrier protein (ACP) for use in both initiation and elongation reactions. For initiation, the newly-formed malonyl-ACP is condensed with acetyl-CoA to form acetoacetyl-ACP by a β-ketoacyl-ACP synthase III (KASIII, FabH in *E. coli*) (Cronan and Rock 2008). The activation of acetyl-CoA to malonyl-CoA and subsequent decarboxylation during the condensation provides a major thermodynamic driving force for this reaction and provides a "push" for acyl-chain synthesis through the FAB pathway (Weber 1991; Davis et al. 2000).

4.2 β-Reduction Pathways

In β-reduction pathways, chain initiation is a direct Claisen condensation of two acetyl-CoA subunits catalyzed by a thiolase (Fig. 3b). In bacteria, this reaction is best known in organisms that produce polyhydroxyalkanoates (PHA), where acetoacetyl-CoA is a precursor to the PHA monomer, β-hydroxybutyryl-CoA. The same reaction, catalyzed by Thl (Atsumi et al. 2008), is used by *C. acetobutylicum* in its butanol biosynthesis pathway. Most organisms, including *E. coli* (e.g., AtoB, FabA), express thiolases in β-oxidation pathways to catabolize organic acids including fatty acids. Here, thiolases remove an acetyl-CoA from a β-ketoacyl-CoA. In vitro kinetic and thermodynamic studies indicate thiolase reactions favor the catabolic direction (Clomburg et al. 2012). Therefore, the initiation and elongation reactions in β-reduction pathways do not have a thermodynamic driving force to "push" pathway flux and must be coupled with downstream enzymes providing a "pull" to produce oleochemical products (Shen et al. 2011). Later termination reactions, such as PHA

polymerization and or reduction to alcohols can provide the driving force for the formation of products through β-reduction pathways.

4.3 Alternative Chain Initiators

The initiation reactions also provide an opportunity to add diversity to the end of the elongating chain (Fig. 3c). In the FAB pathway, variants of KASIII permit condensation of malonyl-ACP with alternative acyl-CoA partners. This flexibility, coupled with biosynthesis or feeding of alternative acyl-chains (e.g., propionate), allows the production of odd-chain, branched-chain, and ω-1 hydroxy branched fatty acids (Choi et al. 2000; Wu and San 2014; Garg et al. 2016). The same enzymatic flexibility can be used in β-reduction pathways to produce ω-functionalized products by condensing various acyl-CoAs. One study demonstrated the production of 18 different products with ω-ends including phenyl-, carboxyl-, hydroxyl-, and isobutyryl functionalizations (Cheong et al. 2016). A challenge to applying this approach is identifying thiolases that incorporate the alternative monomers with high selectivity over acetyl-CoA. Most natural enzymes will utilize both acetyl-CoA and other CoAs leading to a mixture of normal straight chain and modified oleochemical products, but engineering efforts to enhance branch chain incorporation have succeeded (Bentley et al. 2016).

5 Chain Elongation

5.1 Elongation in FAB

In most bacteria, the cycle of fatty acyl-chain elongation and reduction (Fig. 5a) begins with acetoacetyl-ACP and ends with the synthesis of C_{16} or C_{18} acyl-chains that comprise membrane phospholipids (Zhang and Rock 2008; Cronan and Rock 2008). In each cycle, the β-keto position of a β-ketoacyl-ACP is reduced to a hydroxyl group by β-ketoacyl-ACP reductase (KR, e.g., FabG in *E. coli*) consuming one NADPH. Next, the β-hydroxy acyl-ACP is dehydrated by β-hydroxyacyl-ACP dehydratase (DH, e.g., FabZ and FabA in *E. coli*), creating a *trans* double bond between the α and β carbons. The resulting *trans*-2-enoyl-ACP is reduced to a saturated acyl-ACP by enoyl-ACP reductase (ER, e.g., FabI in *E. coli*), consuming one NAD(P)H. The next cycle begins when the saturated acyl-ACP is condensed with a malonyl-ACP molecule by β-ketoacyl-ACP synthases I and II (KAS, e.g., FabB and FabF in *E. coli*), releasing carbon dioxide and producing a new β-ketoacyl-ACP two carbons longer than the previous cycle. In sum, each cycle consumes one acetyl-CoA, one ATP, and two reducing equivalents (e.g., NAD(P)H). The pathway has also been reconstituted in vitro, and the kinetic properties of each enzyme have been characterized with at least one substrate. Pathway activity was shown to be maximized with equal molar amounts of FabA, FabB, FabD, FabF, FabG, FabH, and elevated amounts of FabI (10X), FabZ (10X), and holo-ACP (30X) (Yu et al. 2011).

Regulation of FAB is best understood in a few model bacteria – *E. coli* and *Bacillus subtilis* – where regulation occurs both transcriptionally and allosterically. Because FAB is an energetically expensive process, bacteria use transcriptional regulation to strictly control flux through FAB with regulator proteins, such as FadR in *E. coli*, DesT in *Pseudomonas aeruginosa*, and FapR in *B. subtilis* (Zhang and Rock 2009). From an engineering perspective, overexpression of FadR in *E. coli*, which is known to transcriptionally activate FAB genes *fabA* and *fabB*, leads to increased fatty acid production when expressed in combination with a thioesterase (Zhang et al. 2012b).

In *E. coli* when lipid synthesis slows, long-chain acyl-ACPs accumulate and allosterically inhibit ACC, FabH, and FabI (Zhang and Rock 2008). Expression of acyl-ACP thioesterases produces a pool of free fatty acids (FFAs), thereby depleting long-chain acyl-ACPs. This "pull" strategy removes the allosteric inhibition and increases flux through the FAB pathway. When coupled with a "block" of fatty acid catabolism (e.g., by deleting FadE or FadD in *E. coli*), thioesterase expression can lead to dramatically increased FFA titers (Lennen and Pfleger 2012). In some cases, chain-length specificity can be improved by increasing the level of its preferred substrates in the elongation and reduction cycle. For example, the production of octanoic acid via a heterologous plant acyl-ACP thioesterase was increased in *E. coli* by increasing the abundance octanoyl-ACP. To do this, FabF (KASII) was replaced with an enzyme variant incapable of elongating acyl-ACP species past C_8, thereby making long-chain lipid synthesis dependent on FabB (KASI). To produce octanoic acid, FabB levels were decreased through proteolytic degradation, which was mediated by a degradation tag fused to FabB and an inducible chaperone. Once cells were induced, elongation of medium-chain acyl-ACP was prevented, leading to increased flux to octanoic acid (Torella et al. 2013).

5.2 Elongation in β-Reduction Pathways

The biochemistry of the elongation and reduction cycle of the β-reduction pathways (Fig. 4b), while similar to FAB, is completely orthogonal, allowing production of oleochemicals independent of membrane lipid biosynthesis. While native β-reduction pathways in bacteria typically produce short-chain products (such as polyhydroxybutyrate, PHB, or butanol, only requiring a single turn of the elongation and reduction cycle), the reversal of the β-oxidation pathway allows the production of longer-chain products (requiring multiple turns). The orthogonal nature of the β-reduction pathways permits a wider diversity of enzymes to catalyze each step in the elongation/reduction cycle, leading to a range of pathways and products. In addition, the stoichiometry of a β-reduction cycle requires only one acetyl-CoA and two NADH saving at least 1 ATP per cycle compared to FAB. This advantage manifests as a significant increase in theoretical yield of products but comes with the loss of a thermodynamic driving force to elongate acyl-chains (Cintolesi et al. 2014). In the following paragraphs, we summarize several of the strategies used to develop

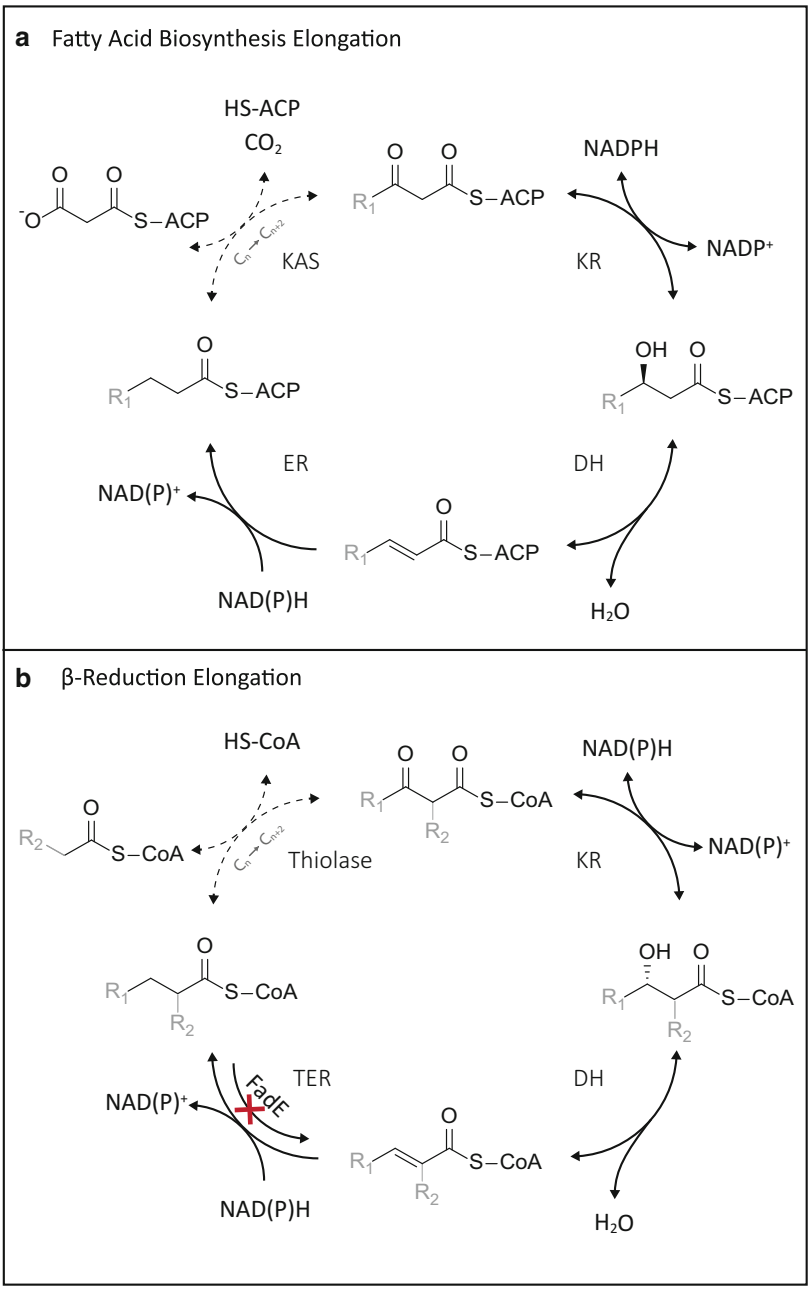

Fig. 4 Recursive elongation and reduction steps of fatty acid biosynthesis and β-reduction pathways. Abbreviations: (**a**) *KR* β-ketoacyl-ACP reductase, *DH* β-hydroxyacyl-ACP dehydratase, *ER* enoyl-ACP reductase, KAS β-ketoacyl-ACP synthases. (**b**) *KR* β-ketoacyl-CoA reductase, *DH* β-hydroxyacyl-CoA dehydratase, *TER trans*-2-enoyl-CoA reductase

β-reduction pathways for producing short-chain products (C_4) and longer chain-length products.

The butanol synthesis pathway within *Clostridium acetobutylicum* is a single-cycle β-reduction pathway for the synthesis of butyryl-CoA and two reductions to butanal and butanol. In *C. acetobutylicum*, the elongation pathway is catalyzed by (i) the thiolase Thl, (ii) the 3-hydroxybutyryl-CoA dehydrogenase, Hbd, (iii) crotonase, Crt, (iv) and the butyryl-CoA dehydrogenase and electron transfer flavoprotein complex Bcd-Etf (Fig. 4b). When this pathway is transferred to *E. coli*, replacement of the thiolase Thl with the native *E. coli* acetyl-CoA acetyltransferase AtoB increases activity (Atsumi et al. 2008). Further, replacement of the Bcd-Etf enzymes with a trans-enoyl-CoA reductase (TER) from *Treponema denticola* enables the operation of the butanol fermentation pathway to be coupled with anaerobic cell growth for further improvement of butanol productivity (Shen et al. 2011). A chimeric version of this pathway, where enzymes were used from three different organisms, has been shown to increase butanol yields while also demonstrating the ability to incorporate enzymatic diversity into β-reduction pathways (Bond-Watts et al. 2011).

Subsequent cycles of β-reduction pathways rely on enzymes with substrate specificity to longer chain-length acyl-CoAs. A second cycle of the *C. acetobutylicum* pathway, to produce hexanoyl-CoA, was demonstrated by the simple addition of the thiolase BktB from *Ralstonia eutropha* (Dekishima et al. 2011). Further improvements in medium-chain synthesis, including hexanoyl-CoA and octanoyl-CoA, were demonstrated by the replacement of the thiolase Hbd with the thiolase PaaH1 from *R. eutropha* (Machado et al. 2012). Each of these pathways relied on promiscuous acyl-CoA reductases and trans-enoyl-CoA reductases to provide a driving force to longer chain products. Another method to develop a β-reduction pathway focused mainly on the native biochemical machinery of *E. coli*, performing a functional reversal of the β-oxidation pathway. For butyryl-CoA synthesis, the elongation pathway was catalyzed by (i) AtoB, (ii and iii) FadB, and (iv) a TER from the mitochondria of *Euglena gracilis*. Overexpression of the FadA thiolase generated longer-chain products – up to C_{12} (Dellomonaco et al. 2011; Clomburg et al. 2012; Kim et al. 2015). These successful demonstrations motivate the development of additional high yield and highly selective pathways for accessing the full range of oleochemical species.

6 Chain Termination

Terminating the cycle of elongation and reduction is a key step in accessing desired classes of oleochemicals as well as selecting for a desired chain length. Chain termination breaks the thioester bond between an acyl-chain and the CoA/ACP and prevents further elongation. In this process, the carbonyl is either hydrolyzed, transesterified, reduced, or decarboxylated. The following sections describe the enzymes involved in each termination reaction and how they have been engineered in bacteria to produce different classes of oleochemicals.

6.1 Hydrolysis to Produce Free Fatty Acids (FFAs)

Heterologous expression of thioesterases (TE), typically from bacteria or plants, is the most common strategy to engineer FFA production in bacteria. TE hydrolysis often demonstrates a high degree of specificity toward particular chain lengths (Fig. 5). In addition to catalyzing the termination step of the pathway, the expression of a thioesterase in *E. coli* also creates a "pull" from the elongation and reduction cycle, resulting in the deregulation of FAB in *E. coli*. A "push-pull-block" strategy for producing FFAs is completed by "blocking" the β-oxidation from consuming the FFAs produced by the thioesterase and overexpressing acetyl-CoA carboxylase to produce malonyl-CoA, an intermediate dedicated to FAB. The combination of these strategies has allowed for the successful production of different chain-length fatty acids (Lennen and Pfleger 2012).

In plants, fatty acyl-chains are synthesized in the chloroplast and must be transported into the cytoplasm for incorporation into lipids and oils. To enable transport across the chloroplast membrane, acyl-chains are cleaved by thioesterases and reactivated by acyl-CoA synthetases in the cytosol for incorporation into lipids (Benning 2009; Kunst and Samuels 2009). Given the diversity in composition of plant oils, a wide range of plant thioesterases (Cantu et al. 2011) have been expressed in *E. coli* for production of FFAs. Unfortunately, many plant thioesterases have limited activity when heterologously expressed. The limited activity is sometimes associated with truncation of N-terminal localization and membrane insertion sequences which can lead to solubility issues. Low thioesterase activity can also be due to thioesterase expression levels, which must be tuned in order to maximize production without severely impacting cell fitness (Lennen et al. 2010; Zhang et al. 2012a).

Although some thioesterases have a broad chain-length specificity, most of the success using the FAB pathway for the production of FFAs has come through the expression of a highly specific thioesterase that produces medium- to long-chain fatty acids (Lennen and Pfleger 2012). While many acyl-ACP thioesterases have been characterized in vivo and their main substrate specificities reported (Voelker et al. 1997; Davies et al. 1991; Jing et al. 2011), the bacterial thioesterase 'TesA and the plant thioesterase BTE are among the most commonly used. A modified version

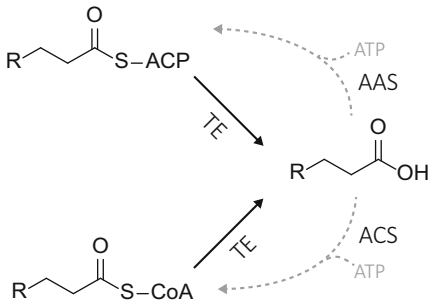

Fig. 5 Hydrolysis to form free fatty acids (FFAs). Abbreviations: *TE* Thioesterase, *AAS* acetyl-ACP synthetase, *ACS* acetyl-CoA synthetase

of the native periplasmic *E. coli* thioesterase, 'TesA (a TesA variant lacking the native N-terminal secretion tag), has been successfully used to produce C14:0 fatty acids (Cho and Cronan 1995; Zhang et al. 2012a). The plant thioesterase BTE from *Umbellularia californica* has been heterologously expressed in *E. coli* to produce C12:0 fatty acids (Voelker and Davies 1994; Lennen et al. 2010).

Thioesterases are not used in combination with β-reduction pathways to the extent that they are used in combination with FAB to produce FFAs, largely for two reasons. First, β-reduction pathways are not regulated by long-chain acyl-CoAs, so the thioesterase activity only serves as a route to produce FFAs. Second, it is difficult to utilize the substrate specificity of thioesterases to produce other acyl-CoA-derived oleochemicals because of futile cycles created by thioesterase and acyl-CoA synthetase (ACS) activity (dotted line, Fig. 5). That said, expression of the mammalian thioesterase mBACH in combination with a β-reduction pathway produced significant amounts of hexanoic acid (Machado et al. 2012).

A common strategy for producing specific oleochemicals combines expression of an acyl-ACP thioesterase to control chain length and a broad-specificity ACS to create a pool of acyl-CoA substrates for subsequent reduction, esterification, or decarboxylation. This approach costs the cell one ATP per product and depends on thioesterases with limited activity on acyl-CoAs to avoid the introduction of futile cycles. Analogously, any acyl-ACP synthetase activity, the ATP-consuming inverse of thioesterase activity, must also be removed to avoid futile cycles.

6.2 Reduction to Aldehydes, Alcohols, and Alkanes

Fatty aldehydes are a central intermediate in the synthesis of fatty alcohols and microbial alkanes (Fig. 6). Fatty aldehydes can either be separated as products, reduced further to produce fatty alcohols (consuming a second reducing equivalent),

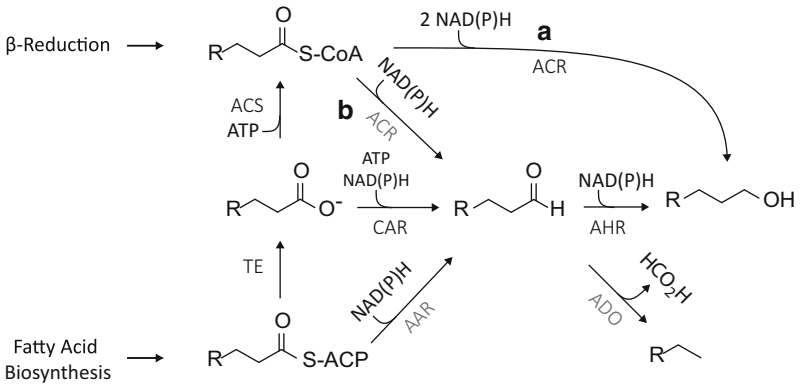

Fig. 6 Reduction to form aldehydes, alcohols, and alkanes. Abbreviations: *ACR* acyl-CoA reductase, *CAR* carboxylic acid reductase, *AAR* acyl-ACP reductase, *AHR* aldehyde reductase, *ADO* aldehyde deformylating oxygenase

or deformylated to produce alkanes. Because the chain length specificity of the reducing enzymes is often quite broad, the acyl-chain length distribution of the products is often similar to the acyl-chain length distribution membrane lipids in the cell. For this reason, a common strategy to produce specific oleochemicals is to leverage the specificity of thioesterases to control chain length, and then reduce the resulting fatty acids directly to aldehydes or reactivate them as acyl-CoAs for subsequent reduction.

Fatty aldehyde generation is the direct reductive cleavage of acyl-ACPs or acyl-CoAs by an aldehyde-forming acyl-ACP reductase (AAR) or acyl-CoA reductase (ACR), which consumes one reducing equivalent (often NADPH). Alternatively, a carboxylic acid reductase (CAR) can reduce FFAs made by thioesterases to fatty aldehydes at the expense of one ATP and one reducing equivalent (Akhtar et al. 2013). Although fatty aldehydes are desirable for their many commercial uses, their production in *E. coli* has proven difficult due to their toxicity and the large number of endogenous aldehyde reductases, which tend to reduce the aldehydes to alcohols (Kunjapur and Prather 2015).

Alkane biosynthesis was initially discovered in cyanobacteria and has more recently been heterologously expressed in *E. coli* for metabolic engineering purposes (Schirmer et al. 2010; Li et al. 2011). In both the natural and engineered pathways, aldehydes are generated by acyl-CoA reductase (ACR or FAR for fatty acyl-CoA reductase) and converted to alkanes one carbon shorter than the aldehyde. The aldehyde deformylation reaction is catalyzed by an aldehyde deformylating oxygenase (ADO) that requires molecular oxygen and a reducing equivalent to produce the alkane and formate. Medium-chain alkanes ($>C_8$) have been produced using this strategy in engineered *E. coli* (Choi and Lee 2013). A chimeric β-reduction pathway has been used to generate short-chain alkanes (C_3–C_5). This pathway uses endogenous thioesterases to generate FFAs followed by a broad-specificity CAR from *Nocardia iowensis* and an ADO from *Prochlorococcus marinus* (Sheppard et al. 2016). Reconstitution of ADO activities in vivo is still a challenge, however, due to sensitivity of reducing system to the supply of oxygen (Kallio et al. 2014).

The most common method of producing fatty alcohols is through the use of an alcohol-forming acyl-CoA reductase, which directly reduces an acyl-CoA to a fatty alcohols (Fig. 6a). This category includes AdhE2 from *C. acetobutylicum* (Fontaine et al. 2002) and MAACR (Maqu_2507) from *Marinobacter aquaeolei* (Wahlen et al. 2009; Willis et al. 2011). AdhE2, native to the Clostridial butanol pathway, has also been used to produce butanol in *E. coli* at high titers. When used in combination with a β-reduction pathway AdhE2 enabled production of hexanol and octanol (Machado et al. 2012). The enzyme MAACR has activity toward longer-chain products (Willis et al. 2011) and has been used to produce fatty alcohols from both FAB and β-reduction pathways. Using a β-reduction pathway, MAACR enabled production of a mixture of C_6–C_{10} alcohols (Kim et al. 2015). To obtain a narrow product distribution using FAB, the thioesterase BTE, an acyl-CoA synthetase (*E. coli* FadD), and MAACR were expressed in a strain lacking β-oxidation (Δ*fadE*), leading to production of largely C_{12} and C_{14} fatty alcohols (Youngquist et al. 2013).

A similar study using 'TesA, FadD, and MAACR in *E. coli* demonstrated production of fatty alcohols distributed between C_{12} and C_{18} (Liu et al. 2013a).

Fatty alcohols have also been produced using separate enzymes for the aldehyde production and the subsequent reduction to an alcohol (Fig. 6b). The acyl-CoA reductase Acr1 from *Acinetobacter calcoaceticus* produces fatty aldehydes (Reiser and Somerville 1997) and has been used to produce longer-chain alcohols (≥C12) in combination with aldehyde reductases (AHR) in *E. coli* (Zheng et al. 2012). The carboxylic acid reductase (CAR) from *Mycobacterium marinum* has been used in combination with an AHR from *Synechocystis* sp. PCC 6803 for the production of C_{12}–C_{18} fatty alcohols in *E. coli* (Akhtar et al. 2013). An acyl-ACP reductase (AAR) from *Synechococcus elongatus* has also been shown to reduce acyl-ACPs directly to aldehydes; this method was used to produce C_{12}–C_{18} fatty alcohols in *E. coli*, where the native aldehyde reductase AdhP was shown to reduce the aldehydes to alcohols (Liu et al. 2014).

6.3 Olefins (Alkenes)

There are several routes through which terminal alkenes (α-olefins) can be produced. First, the cytochrome P450 fatty acid peroxygenase enzyme ($OleT_{JE}$) from *Jeotgalicocus* sp. 8456 can catalyze the decarboxylation of a FFA to generate a terminal alkene via a radical intermediate (Fig. 7a) (Rude et al. 2011; Belcher et al. 2014). Second, an olefin synthase (Ols) from *Synechococcus sp.* strain PCC 7002, similar to a type I polyketide synthase, can produce 1-nonadecene from octadecanoyl-ACPs through an elongative sulfonation-assisted decarboxylation, resulting in an *n+1* terminal alkene (Fig. 7b) (Mendez-Perez et al. 2011). Lastly, the nonheme iron oxidase UndA identified in *Pseudomonas* species catalyzes the decarboxylation of C_{10}–C_{14} fatty acids to generate terminal alkenes through an

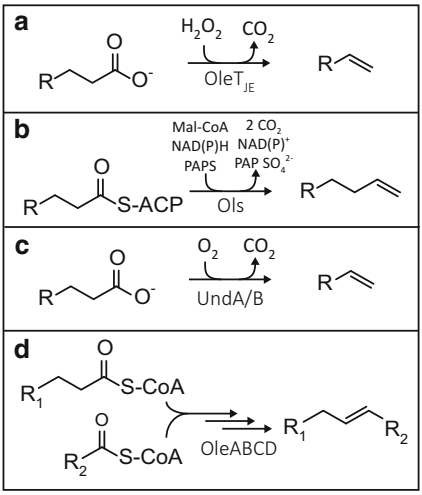

Fig. 7 Olefin synthesis pathways. Abbreviations: *Mal-CoA* malonyl-CoA, *PAPS* 3′-phosphoadenosine-5′-phosphosulfate , *PAP* 3′-phosphoadenosine-5′-phosphate

oxygen-dependent mechanism (Fig. 7c) (Rui et al. 2014). A similar membrane-bound fatty acid desaturase (UndB) from *Pseudomonas fluorescens* Pf-5 has also been used and was shown to catalyze the same oxygen-dependent decarboxylation reaction, which was used in an engineered *E. coli* to produce 1-undecene (Rui et al. 2015). In addition to α-olefins, a gene cluster from the bacterium *Micrococcus luteus* (OleABCD) is capable of condensing two fatty acyl-chains to ultimately produce long-chain internal olefins (Beller et al. 2010).

6.4 Esters

Esters (e.g., biodiesel) are typically formed by an acid catalyzed condensation between an alcohol and an organic acid or lipid. However, in vivo, the condensation typically takes place between an alcohol and an acyl-CoA. Fatty acid ethyl esters (FAEEs), in particular, can be produced in vivo from the condensation of an acyl-CoA with ethanol by the activity of a wax-ester synthase, such as AftA from *Acinetobacter sp*. strain ADP1, which natively fuses acyl-CoAs with long-chain fatty alcohols to produce waxes (Fig. 8a). AftA was used to produce C_{12}–C_{18} FAEEs when the acyl-CoAs were produced through the FAB pathway (Steen et al. 2010). An engineered strain expressing FadR, which senses presence of acyl-CoAs, to regulate expression of AftA and an engineered ethanol production cassette (Pdc) allowed for the production of FAEEs at high titer and yield (Zhang et al. 2012a). A similar system from *Saccharomyces cerevisiae* has been shown to produce a wide variety of esters using alcohol *O*-acyltransferases (ATFs) that condense acyl-CoAs and alcohols. Heterologous expression of Atf1 from *S. cerevisiae* in an engineered *E. coli* led to production of a wide range of esters, including isobutyl acetate (Rodriguez et al. 2014).

The production of fatty acid methyl esters (FAMEs) in vivo is mechanistically different than FAEEs. FAMEs can be produced by a bacterial fatty acid *O*-methyltransferase (FAMT) from *Mycobacterium marinum* using FFAs and S-adenosyl methionine (SAM) as the methyl group donor (Fig. 8b). This strategy was used to produce FAMEs at relatively low titers. One challenge to implementing this strategy is regeneration of SAM, which remains an opportunity for optimization (Nawabi et al. 2011).

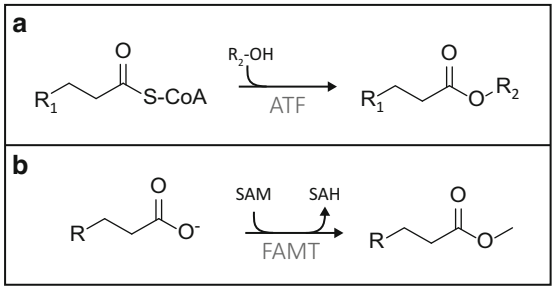

Fig. 8 Ester synthesis pathways. Abbreviations: *FAMT* fatty acid *O*-methyltransferase, *SAM S*-adenosyl methionine, *SAH S*-adenosyl homocysteine

6.5 Methyl Ketones

Methyl ketones can be generated by thioesterase catalyzed hydrolysis of a β-keto-acyl-thioester and spontaneous decarboxylation of the β-keto fatty acid (Fig. 9). Methyl ketones have been produced from fatty acid-producing strains by the expression of a CoA ligase (e.g., FadD) followed by β-oxidation to a β-keto-acyl-CoA via the activity of FadE and FadB. When β-oxidation is blocked by deleting thiolases (Δ*fadA*), the cell accumulates β-keto-acyl-CoAs which can be cleaved by thioesterases such as FadM. Using this method, C_{11}–C_{15} methyl ketones have been produced to high titers in fed-batch fermentation (Goh et al. 2012, 2014). The methyl ketone 2-pentanone has been produced using a β-reduction pathway similar to the butanol fermentation pathway in *C. acetobutylicium*. In this study, β-ketohexanoate was generated through using the 3-oxoadipate CoA-succinyl transferase PcaIJ from *Pseudomonas putida*. Instead of relying on spontaneous decarboxylation, this study used acetoacetate decarboxylase, Adc from *C. acetobutylicum*, to catalyze the decarboxylation of β-ketohexanoate to produce 2-pentanone (Lan et al. 2013).

6.6 Polyhydroxyalkanoates

Poly(3-hydroxyalkanoates) (PHAs) are a class of biopolymers that have similar properties to commercial plastics, such as polypropylene, but are synthesized from intermediates of the β-oxidation/reduction pathway. The simplest PHA, poly(3-hydroxybutyrate) (PHB), is produced by several species of bacteria, such as *Cupriavidus necator*. Pathways for PHB (Fig. 10a) production are dedicated toward that product and proceed as a partial β-reduction pathway (using PhaA and PhaB) to produce (R)-3-hydroxybutyryl-CoA, which is then polymerized by PhaC to form PHB (Madison and Huisman 1999).

Medium-chain-length (mcl) PHAs can be made either through exogenous feeding of fatty acids or via the FAB pathway through two enzymatic links. One strategy (Fig. 10b) uses an (R)-3-hydroxyacyl-ACP thioesterase (PhaG) followed by CoA

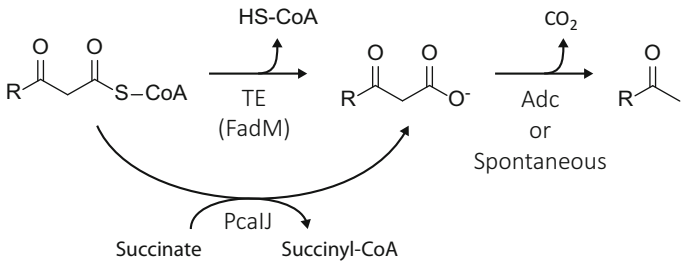

Fig. 9 Ketone synthesis pathways

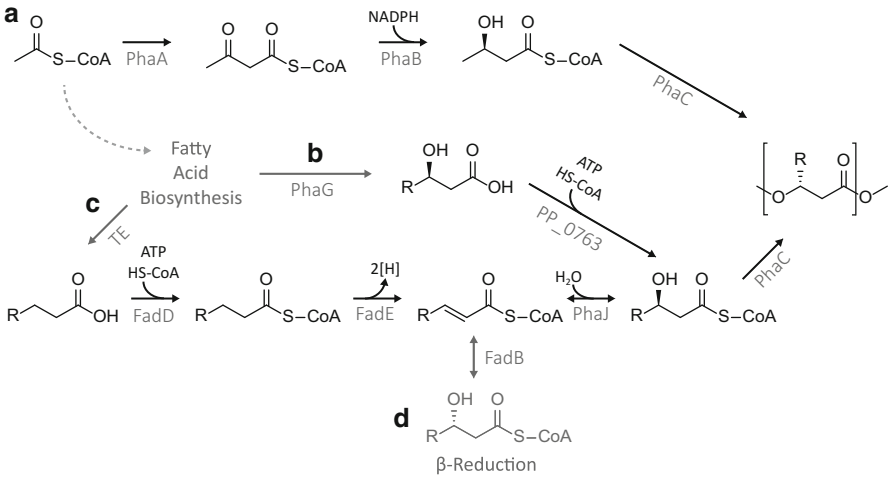

Fig. 10 Poly(3-hydroxyalkanoate) (PHA) synthesis pathways

activation with 3-hydroxyacyl-CoA ligase (PP_0763 from *Pseudomonas putida*) to form the (R)-3-hydroxyacyl-CoA (Wang et al. 2012). The second method (Fig. 10c) involves the expression of a thioesterase to hydrolyze a saturated acyl-ACP, followed by activation with an acyl-CoA synthetase (e.g., FadD), desaturation by FadE, and hydration by PhaJ to form the (R)-3-hydroxyacyl-CoA. The major advantage of this second strategy is the ability to control the polymer chain length with the use of a specific thioesterase (Agnew et al. 2012).

A β-reduction pathway has also been demonstrated to synthesize short- and medium-chain-length PHAs by direct synthesis of the *trans*-2-enoyl-CoA (Fig. 10d). The medium-chain-length *trans*-2-enoyl-CoA can be hydrated with PhaJ1 and polymerized with PhaC2 from *Pseudomonas aeruginosa.* A hetero-polymer of short- and medium-chain PHAs can be synthesized by the expression of the PHB pathway from *C. necator* along with PhaC2 from *Pseudomonas stutzeri* (Zhuang et al. 2014). As we have seen with other pathways, the enzymatic diversity allows for a variety of strategies to be used for the production of PHAs.

7 Post-Termination Modifications

A wide range of fatty-acid-related products are formed by the modification of fatty acids following the elongation and reduction cycle, such as hydroxylation, desaturation, and cyclopropanation (Cronan 2002; Buist 2007). While post-terminal modifications have not been used extensively for engineering purposes, a few studies illustrate the potential of such modifications. One study, for example, attempted to produce ladderane fatty acids, a type of fatty acid with a cyclized ω-end from anammox bacteria in *E. coli* by the heterologous expression of a

biosynthetic pathway from *Kuenenia stuttgartiensis*. While this attempt was unsuccessful, it demonstrates some of the elegant diversity of biosynthetic pathways and the difficulty to expressing exotic pathways in model organisms (Javidpour et al. 2016).

Two important classes of bifunctional chemicals, ω-hydroxyl fatty acids (ω-OHFAs) and α,ω-dicarboxylic acids (α,ω-DCAs), are monomers for polymer synthesis and can be produced through modification of the ω-end of the acyl chain. One study demonstrated the production of both of these chemicals in *E. coli* using FFAs generated in the FAB pathway. To make ω-OHFAs, a cytochrome P450 monooxygenase was used to oxidize the terminal carbon of FFAs. From the ω-OHFAs, α,ω-DCAs were produced by a two-step oxidation of hydroxyl group of ω-OHFAs by an alcohol dehydrogenase and an aldehyde dehydrogenase (Bowen et al. 2016).

8 Research Needs

Metabolic pathways and engineering strategies for producing a wide range of oleochemicals have been demonstrated at laboratory scale. Many commercial ventures are actively developing these strains for industrial scale cultivation. The major process challenges remaining to be solved include:

- ***Understanding regulation of FAB***. Regulation of fatty acid metabolism is well understood in *E. coli*, but not in many other industrially relevant organisms. A handful of transcriptional regulators have been identified by homology to known proteins (e.g., FadR, FabR), but many bacteria lack obvious candidate regulators. Strategies that have worked well in *E. coli* (e.g., thioesterase expression) have not generated the same results in other bacteria. This may be in part to different regulatory modes or to different strategies for controlling biosynthesis. Furthermore, the details on how oleochemical-producing enzymes (e.g., $FabH_{EC}$ or ACC_{EC}) are regulated allosterically remain elusive, thereby preventing enzyme engineering that circumvents the regulatory effects.
- ***Enzyme engineering to improve activity and selectivity***. Most oleochemical enzymes have a broad activity on different chain lengths. To leverage the higher yields possible through the β-reduction pathway, enzymes capable of processing specific acyl-CoA pools (e.g., a C_6-specific ACR for hexanol production) are needed to compile highly-selective pathways. While several studies have examined mutations that alter the activity of thioesterases (Mayer and Shanklin 2007; Hom et al. 2010), a general knowledge of how these enzymes control substrate preference remains elusive. Similarly, it is desirable to improve the overall activity of enzymes involved in oleochemical metabolism, allowing the cellular resources to be focused on oleochemical production and relieving the stress caused by protein overproduction.

- ***Increasing product TRY from low-cost feedstocks***. Maximizing TRY for each product class remains a work in progress. Yields of FFAs, fatty alcohols, and ethyl-esters have progressed beyond 50% of theoretical yield (TY) but remain below the yield performance of typical fermentation products (>90% TY). The highest reported yields of alkanes, ketones, and olefins are even lower. Bacteria are generally capable of rapid substrate uptake, which makes flux through FAB the target of rate improvements. As described above, enzyme engineering efforts to increase activity will help in improving the rate of product formation. Finally, the titers of many oleochemical products are capable of exceeding the g/L threshold but often require excessive feeds to do so. In order to minimize purification costs, strategies for producing oleochemicals in high-cell-density fed-batches or continuous culture need to be developed.
- ***Product secretion and separation***. Most oleochemicals are hydrophobic and have low aqueous solubility, which reduces purification costs relative to water soluble metabolites. Secretion rates can be increased by engineering membrane exporters to have increased capacity. While exporters of FFAs and other hydrophobic compounds have been identified (Dunlop et al. 2011; Lennen et al. 2013), specific transporters are not know for all oleochemicals. Another method of increasing the rate of product secretion is by sequestering the products in a second organic phase (such as dodecane); these biphasic fermentations simultaneously perform a crude product extraction and provide a "pull" by sequestering products away from the terminal enzyme reactions.
- ***Mitigating product toxicity***. Many oleochemical species can inhibit the growth and/or survival of their production hosts. For example, short-chain fatty acids have been shown to have dramatic effects on the growth of *E. coli* at fairly low concentrations (Royce et al. 2013). The specific cause of toxicity is not well understood but is commonly attributed to alteration of membrane function including fluidity, robustness, and leakage of proton motive force. To mitigate toxicity, products must be rapidly removed from the cell or the cell membrane must be engineered to be resistant. The expression of membrane pumps has been shown to improve strain survival and tolerance to many hydrophobic compounds (Dunlop et al. 2011; Jones et al. 2015). Similarly, expression of *cis-trans* isomerase in *E. coli* reduces sensitivity to octanoic acid presumably by changing the fluidity of the membrane (Tan et al. 2016). Additional work is needed to understand the modes of toxicity in order to develop optimal strategies for circumventing it.

Oleochemical metabolism comprises a powerful set of enzymatic conversions that can enable the sustainable production of many chemicals used in fuel, material, and consumer product applications. The fundamental genetics and enzymology of these pathways have been elucidated, and this knowledge is being used to design industrial biocatalysts. While promising, significant work remains to address metabolic, physiological, and economic barriers that currently limit the use of bacteria as biocatalysts for oleochemical production.

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Yarrowia lipolytica as a Cell Factory for Oleochemical Biotechnology

24

Kelly A. Markham, Lauren Cordova, Andrew Hill, and Hal S. Alper

Contents

Abstract

Yarrowia lipolytica is one of the more popularly studied nonconventional yeasts due to its innate abilities to accumulate lipids and utilize diverse carbon substrates. In this chapter, we discuss several recent oleochemical applications of *Y. lipolytica* that are currently under investigation. These applications include the production of single cell oil, various modified fatty acids, citric acid, lipase, and other compounds. Additionally, we cover the lipophilic nature of *Y. lipolytica* in catabolic applications

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including bioremediation of oil-polluted water and soil. Finally, we address future outlooks for the cost-effective production of these chemicals in *Y. lipolytica.*

1 Introduction

Y. lipolytica is a dimorphic fungus (Szabo 1999) widely studied for its growth on hydrocarbons and waste carbon sources (Fickers et al. 2005; Rymowicz et al. 2008; Papanikolaou and Aggelis 2009). This ability to utilize low-value carbon greatly enhances the industrial relevance of this host. This capacity is exemplified through the utilization of by-products of chemical processes. For example, traditional production of biodiesel from vegetable oils and animal fats produces large volumes of glycerol as a by-product (da Silva et al. 2009). *Y. lipolytica* can rapidly convert this crude glycerol into value-added chemicals including additional lipids (Papanikolaou and Aggelis 2002), citric acid (Papanikolaou et al. 2008), and succinic acid (Yuzbashev et al. 2010) at titers relevant for industrial fermentations. Although originally recognized for its oleaginous nature, *Y. lipolytica* has become an increasingly relevant host organism for many applications including food additives, livestock feed, production of organic acids, and bioremediation (Bankar et al. 2009; Groenewald et al. 2014). Aiding by a generally regarded as safe (GRAS) status (Groenewald et al. 2014), *Y. lipolytica* has been the focus of several industrial patents granted in the food additive space for the production of carotenoids (Bailey et al. 2010) and polyunsaturated fatty acids (Damude et al. 2006; Xue et al. 2012; Singh et al. 2014; Damude and Zhu 2015). To complement these strain-engineering efforts, the set of molecular and synthetic tools for this host has greatly expanded in recent years. Specifically, these applications are being expedited through the development of basic genetic tools for transformation (Davidow et al. 1985), overexpression (Juretzek et al. 2001), marker recycle (Fickers et al. 2003), knockout (Fickers et al. 2003), and CRISPR-Cas9- mediated genome editing (Gao et al. 2016; Schwartz et al. 2016).Additionally, wild-type strains have been fully sequenced (Liu and Alper 2014; Pomraning and Baker 2015).

At present, *Y. lipolytica* is being established as a host organism for the sustainable production of fuels and chemicals. Oleaginous yeasts such as *Y. lipolytica* serve as promising hosts for oleochemical production based on their capacity to convert inexpensive, renewable carbon sources into valuable drop-in replacements for petroleum products. In this chapter we specifically address how *Y. lipolytica* has been used for oleochemical biotechnology. Finally, we conclude on applications of this host not just for the production but also the catabolism of oleochemicals in bioremediation applications.

2 Biodiesel

As an oleaginous yeast, *Y. lipolytica* is characterized by its ability to generate intracellular lipids (especially when fed lipid substrates). These lipids can be chemically or enzymatically converted to biodiesel (fatty acid methyl or ethyl esters) through

traditional transesterification reactions and pathways (Zhao et al. 2010). In *Y. lipolytica*, fatty acid synthesis uses the Kennedy pathway (Dulermo and Nicaud 2011) and is unique compared to more conventional, non-oleaginous hosts such as *S. cerevisiae*. Specifically, this host utilizes enzymes such as ATP citrate lyases, *ACL1* and *ACL2*, which catalyze the production of acetyl-CoA by cleavage of citrate (Koch et al. 2014). Acyl chain synthesis for lipids is catalyzed by multifunctional enzyme complexes called fatty acid synthases (FAS). The FAS complex enables extension of fatty acyl chains via malonyl-CoA extender units. Once the growing fatty acid reaches a certain length, typically 16–18 carbons (Blazeck et al. 2014; Liu et al. 2015b), the activity of a thioesterase (TE) releases a free fatty acid (FFA) from the FAS complex (Beopoulos et al. 2008, 2009).Once synthesized, FFAs can be activated by coenzyme A (CoA) to form acyl-CoAs, a building block for cellular lipids such as triacylglycerides (TAGs) and phospholipids (PLs). These acyl-CoA elements are transferred to the backbone of glycerol-3-phosphate to ultimately form TAGs. TAGs are nonpolar, thus unable to be incorporated into membrane phospholipid bilayers and are instead stored in lipid bodies within the cell (Czabany et al. 2007).

A great deal of metabolic engineering effort in *Y. lipolytica* has focused on increasing lipid (i.e., TAG) production. Combinatorial approaches targeted lipogenesis and lipase regulators, in addition to knockdown of the tricarboxylic acid cycle, β-oxidation, and peroxisome formation. Overexpression of the native diacylglycerol acyltransferase (*DGA1*) (TAG biosynthesis) combined with *pex10* and *mfe1* deletion (prevention of β-oxidation and peroxisome formation) led to a maximum of 25.3 g/L lipids in *Y. lipolytica* (90% of dry cell weight, 76% theoretical maximum yield) (Blazeck et al. 2014). Similar efforts overexpressed *DGA1* and acetyl-CoA carboxylase (*ACC1*), the first committed step in fatty acid synthesis, to generate 17.6 g/L lipids (61.7% lipid content, 0.270 g/g maximum yield) (Tai and Stephanopoulos 2013). Another study identified the rate-limiting step in lipid synthesis as the delta-9 stearoyl-CoA desaturase (*SCD*) in lipid synthesis by reverse engineering an obese mammalian phenotype (Qiao et al. 2015). Overexpression of *SCD* with *ACC1* and *DGA1* led to 55 g/L TAG production reaching 84.7% of theoretical maximum yield (67% of dry cell weight) (Qiao et al. 2015). Transcriptomic analysis of an evolved, high-lipid *Y. lipolytica* strain identified that a mutant delta-9 desaturase (*mga2*) served to reduce tricarboxylic acid cycle flux and increase glycolysis flux (Liu et al. 2015). Overexpression of *DGA1* in this mutant strain led to lipid production at 25 g/L (86% of dry cell weight, 66% of theoretical yield) (Liu et al. 2015). An alternative approach deleted the lipase regulator *tgl3* and overexpressed the acyltransferases *DGA1* and *DGA2* from *Rhodosporidium toruloides* and *Claviceps purpurea*, respectively (Friedlander et al. 2016). Fed-batch fermentations of this engineered strain produced 85 g/L total lipids (77% of dry cell weight, 62.5% of theoretical yield) (Friedlander et al. 2016). Finally, the importance of ATP citrate lyase (*ACL*) on fatty acid metabolism under low nitrogen conditions was demonstrated via overexpression of the heterologous gene from *Mus musculus* resulting in greater than threefold increase lipid production in *Y. lipolytica* from 7.3% to 23% lipids in dry cell weight (approximately 1.7 g/L) (Zhang et al. 2014). In addition to rational metabolic engineering, evolutionary approaches have been used to enhance the lipid production of *Y. lipolytica*. Selection of floating cells after random mutagenesis identified a

Y. lipolytica strain capable of producing almost 40 g/L lipids (87% of dry cell weight, 76% of theoretical yield) (Liu et al. 2015). These extensive examples demonstrate that the complex fatty acid metabolism and regulation in *Y. lipolytica* can be improved with rational and evolutionary metabolic engineering strategies.

While the aforementioned metabolic engineering efforts have significantly increased lipid production in *Y. lipolytica*, they often relied on defined media with glucose as the main substrate at best (sometimes more complex media was used to achieve these titers). However, alternative carbon substrates decrease raw material costs and subsequently reduce process sensitivity to market fluctuation (Fontanille et al. 2012; Pfleger et al. 2015). One attractive feedstock is lignocellulosic biomass. This biomass source contains abundant hemicellulose containing xylose, a monomer that can be consumed by *Y. lipolytica* after engineering either native or heterologous enzymes for xylose assimilation (xylose reductase, xylitol dehydrogenase, and xylulokinase) (Rodriguez et al. 2016). Functional identification of native genes in *Y. lipolytica*, and subsequent overexpression, generated strains with comparable growth to glucose and lipid accumulation of 0.21 g/L (21% of dry cell weight) (Rodriguez et al. 2016). While these efforts resulted in low lipid accumulation, they demonstrated that *Y. lipolytica* naturally possesses the machinery to catabolize xylose. Alternate efforts to enable xylose catabolism involved the introduction of heterologous enzymes from *Scheffersomyces stipitis* in conjunction with starvation to generate a strain which produced 15 g/L lipids (29.3% of theoretical yield) (Li and Alper 2016). These same enzymes were overexpressed in conjunction with a native xylulokinase leading to 20.1 g/L of lipid accumulation (36% of dry cell weight) (Ledesma-Amaro et al. 2016). While initial efforts to enable xylose utilization have been successful, further optimization is required to improve lipid production rate and titers (especially when compared with glucose).

Beyond hemicellulose and xylose, hydrolysis of other sugar polymers has been explored as feedstocks for *Y. lipolytica*. For example, sugarcane bagasse was hydrolyzed to generate a mixture of xylose, glucose, and arabinose (4.9:1.4:1.0, respectively) which enabled production of 6.68 g/L lipids (58% of dry cell weight) (Tsigie et al. 2011). Molasses, predominately comprised of sucrose (glucose and fructose), has been utilized by *Y. lipolytica* through expression of a heterologous *SUC2* from *S. cerevisiae.* When combined with other rational lipid overproduction targets (specifically *mfe1* deletion and *DGA2* overexpression), an engineered strain generated 5.4 g/L intracellular lipids (nearly 50% of dry cell weight) (Gajdoš et al. 2015). Similar efforts to degrade the complex carbohydrate inulin into its fructose and glucose monomers generated 7.39 g/L lipids in *Y. lipolytica* (50.6% of dry cell weight) (Zhao et al. 2010). Collectively these examples show promise that *Y. lipolytica* can produce relevant titers of lipids from a variety of carbohydrate sources.

Beyond carbohydrates, glycerol is another desirable alternative feedstock for the production of oleochemicals. *Y. lipolytica* has the innate ability to utilize this waste carbon and produce valuable products as demonstrated by the continuous feeding of industrial glycerol generating 3.5 g/L lipids (43% of dry cell weight)

(Papanikolaou and Aggelis 2002). An alternative strain of *Y. lipolytica* was able to generate 4.72 g/L lipid from crude glycerol in batch reactor cultivation (21% of dry cell weight) (Dobrowolski et al. 2016). Metabolic engineering of *Y. lipolytica* has enhanced lipid production from glycerol. For example, reconstruction of TAG synthesis using four copies of *DGA2* in combination with fed-batch cultivation produced 9.9 g/L lipids (54% of dry cell weight) (Gajdoš et al. 2015). Likewise, a heterologous glycerol dehydratase was expressed under the control of a glycerol-induced promoter to generate 13 g/L lipids in *Y. lipolytica* (30% of dry cell weight) (Celińska and Grajek 2013). Glycerol has been further used as a supplementary carbon source. As an example, co-utilization of xylose and glycerol in a fed-batch reactor increased total lipid titer and lipid yield to 50.5 g/L and 42% of dry cell weight, respectively (Ledesma-Amaro et al. 2016). Likewise, growth on media containing both animal fat-based stearin and glycerol enabled production of 6.8 g/L lipids (54% of dry cell weight) (Papanikolaou et al. 2002). These numerous examples demonstrate that the production of lipids by *Y. lipolytica* can be achieved at relevant titers from a variety of carbon sources, effectively increasing the industrial relevance of producing biodiesel in a microorganism.

3 Designer Fatty Acids

While native fatty acids produced in *Y. lipolytica* serve as a drop-in replacement for diesel, the ability to enzymatically modify these abundant molecules allows for the production of specialized, value-added oleochemicals. One such group of modified fatty acids is polyunsaturated fatty acids (PUFAs). *Y. lipolytica* natively produces only the PUFA linoleic acid, but has been engineered to produce γ-linolenic acid (GLA, C18:3), eicosapentaenoic acid (EPA, C20:5), and docosahexaenoic acid (DHA, C22:6) (Quinn Zhu et al. 2015). Through simple, specific desaturation, linoleic acid becomes GLA, a dietary supplement that reduces chronic diseases by modulating immune response (Rothman et al. 1995). Traditional, natural sources of GLA include plant seed oils and some fungal oils that are either unsafe for human consumption or expensive to produce (Lu-Te Chuang et al. 2009). Endogenous *Y. lipolytica* linoleic acid was converted to GLA through heterologous overexpression of the Δ6-desaturase from *M. alpine* resulting in 69% conversion (Lu-Te Chuang et al. 2009). Further improvements in GLA titer were achieved by increasing the conversion of oleic acid to linoleic acid by overexpressing the Δ12-desaturase gene also from *M. alpine* (Lu-Te Chuang et al. 2009). Additional experiments have varied the proportion of PUFAs in *Y. lipolytica* by co-expressing heterologous desaturase genes (Chuang et al. 2010). The production of GLA demonstrates that *Y. lipolytica* can be engineered to make nonnative PUFAs.

Two PUFAs that have garnered extensive attention recently are EPA and DHA. The omega-3 fatty acids EPA and DHA are essential fatty acids with

well-known health benefits including proper fetal development (Ruxton et al. 2004; Lopez-Huertas 2010; Swanson et al. 2012). EPA and DHA are nutritionally obtained by consuming fish and fish oil, but can be more sustainably produced in microorganisms. In order to produce EPA and DHA in *Y. lipolytica*, the native fatty acid pool must first undergo elongation with additional malonyl-CoA precursors to produce C20 and C22 fatty acids. These precursors can then be converted to the desired products via further desaturase enzymes (similar to GLA described above). The first large-scale production of EPA was achieved through the overexpression of three copies of an efficient Δ17-desaturase gene, seven copies of a Δ8-desaturase gene, and five copies of a Δ5-desaturase gene paired with a mutant *pex10*in *Y. lipolytica* (Xue et al. 2013). This engineered strain produced EPA at 15% of DCW and 56.6% of total fatty acids (Xue et al. 2013). This strain was then converted into a DHA producer through overexpressing a $C_{20/22}$ elongase and Δ4-desaturase resulting in the production of greater than 5.6% DHA in the total fatty acids (Damude et al. 2014). These *Y. lipolytica* strains capable of producing EPA and DHA have been used in the commercial production of New Harvest EPA oil as well as feed for sustainably farmed Verlasso salmon (Zhu and Jackson 2015). These examples demonstrate that *Y. lipolytica* can be used to generate nonnative PUFAs essential to human health on a relevant, industrial scale.

In addition to PUFAs, other modified fatty acids have been produced in *Y. lipolytica.* Ricinoleic acid, the main component of castor oil, has been produced in *Y. lipolytica* to titers over 60 mg/g dry cell weight through deletion of β-oxidation, TAG acyltransferases, and Δ12 desaturase and overexpression of heterologous Δ12 hydroxylases from *Ricinus communis* and *Claviceps purpurea* (Beopoulos et al. 2014). Ricinoleic acid provides an intriguing chemistry for biodiesel (da Silva et al. 2006), yet also serves as an important starter molecule for further downstream molecules. As an example, *Y. lipolytica* serves as a promising host for the production of γ-decalactone formed from the β-oxidation of ricinoleic acid. Specifically, ricinoleyl-CoA can undergo four cycles of β-oxidation to produce 4-hydroxydecanoic acid which can then be cyclized into γ-decalactone (Waché et al. 1998; Braga and Belo 2015). Production of γ-decalactone has been improved using several strategies including uncoupling growth and production (Pagot et al. 1997); optimizing oxygen transfer rate, cell density, and oil concentration; and stepwise fed-batch cultivation resulting in 5.4 g/L titer (Braga and Belo 2015). This titer was further increased using intermittent fed-batch cultivation resulting in a titer of 6.8 g/L (Gomes et al. 2012). Collectively, these designer fatty acids are value-added products that have recently been demonstrated in the host *Y. lipolytica*.

Finally, the lipid pool in *Y. lipolytica* can be further engineered to produce hydrocarbons and other fatty-acid-derived molecules. These efforts take advantage of *Y. lipolyitca's* high native tolerance to n-alkanes, aldehydes, fatty alcohols, and carotenoid compounds. Many of these compounds (especially hydrocarbons) have

been produced in bacterial counterparts rather than fungi in general. Nevertheless, in one instance, *Y. lipolytica* has been engineered as a proof of concept to produce pentane. Production of 4.98 mg/L pentane was achieved through overexpression of the soybean lipoxygenase gene *Gmlox1* (Blazeck et al. 2013). Another proof of concept study overexpressed a fatty acid hydroperoxide lyase from green bell pepper in *Y. lipolytica* resulting in the production of 350 mg/L of C6-aldehyde (Bourel et al. 2004). In addition to alkanes and aldehydes, this host has been engineered to produce fatty alcohols. Specifically, the addition of a fatty acyl-CoA reductase from *Tyto alba*, *TaFAR1* resulted in greater than 636 mg/L intracellular and 53 mg/L extracellular hexadecanol (Wang et al. 2016). In a similar study, the overexpression of the fatty acyl-CoA reductase from *Arabidopsis thaliana* in a *pex10* knockout strain resulted in titers of 1-decanol exceeding 500 mg/L (Rutter and Rao 2016). These two examples demonstrate that *Y. lipolytica* could be engineered to produce moderate titers of medium-chain fatty alcohols – molecules that have potential applications as solvents, surfactants, lubricants, and fuels (Rutter and Rao 2016). Further work is needed in this area to increase titers to the g/L levels. Finally, the metabolic potential of *Y. lipolytica* to produce carotenoids has been explored through the production of lycopene (Matthäus et al. 2014; Nambou et al. 2015) and β-carotene (Grenfell-Lee et al. 2014). Collectively, these examples demonstrate the diverse potential of *Y. lipolytica* as a cell factory for oleochemicals.

4 Bioremediation and Lipase Production

Y. lipolytica not only has the ability to produce a wide variety of oleochemicals but also has the catabolic potential to utilize these same species. In this vain, *Y. lipolytica* can efficiently utilize a diverse assortment of environmental hydrophobic substrate contaminants including fatty acids, alkanes, and oils (Fickers et al. 2005; Bankar et al. 2009). Moreover, *Y. lipolytica* can produce biosurfactants (Amaral et al. 2006), which have been studied for their use in the oil and petroleum industries for decades and are currently used for enhanced oil recovery and remediation of polluted sites (Desai and Banat 1997). In this section we discuss the bioremediation applications of *Y. lipolytica*.

Offshore drilling is an effective way to obtain petroleum, but when oil spills occur on open water, they present a unique challenge to remedy. Pairing physical containing with biological degradation (with species such as *Y. lipolytica*) can mitigate environmental damage. In one example toward this end, a wild-type strain of *Y. lipolytica* was immobilized within a highly oil-absorbent polyurethane foam enabling stable sequestration and degradation of oil spilled on water (Oh et al. 2000). Likewise, *Y. lipolytica* has also been used for bioremediation of soil contaminated via oil spills (Bankar et al. 2009) in a process effectively monitored with geo-electrical methods (Zogała et al. 2005). Beyond oil spills,

Y. lipolytica can be used to reduce the chemical oxygen demand (COD) of oil processing in the effluent stream produced by various oil industries. For example, strains capable of reducing COD from olive mill wastewater (by 80%) (Scioli and Vollaro 1997) and palm oil (by 95%) (Oswal et al. 2002) have been isolated and studied. These examples (as well as those reviewed previously (Bankar et al. 2009; Liu et al. 2015)) demonstrate a unique bioremediation capacity of *Y. lipolytica*.

Beyond lipid catabolism pathways, *Y. lipolytica* expresses an abundance of lipases that aid in many of these bioremediation applications. Likewise, these lipase enzymes also garner significant commercial interest for use in products like detergents and biodiesel (Coelho et al. 2010).*Y. lipolytica* produces a host of lipase isoforms that can either be secreted or maintained intracellularly (Guerzoni et al. 2001; Fickers et al. 2004). Lipase production can vary based on several fermentation conditions including carbon and nitrogen sources (Fickers et al. 2004) and presence of metabolites (Pereira-Meirelles et al. 1997). Out of the 16 *Y. lipolytica* lipases encoded in that genome (Fickers et al. 2011), most studies have focused on the lipase encoded by the gene *LIP2* (Pignede et al. 2000). Applications of this lipase include the purification of ω-3 fatty acid esters (Casas-Godoy et al. 2014), phytosterol ester synthesis (Cui et al. 2016), and resolving racemic mixtures of 2-bromo-arylacetic acid (Bordes et al. 2009). To enable industrial applications, several attempts have been made to increase lipase productivity. For example, the use of multi-copy integration (Pignede et al. 2000b) and hybrid promoters (Nicaud et al. 2002) has increased production levels. Additionally, mutagenesis strategies have successfully improved the enantioselectivity (Bordes et al. 2009), activity (Wang et al. 2014) and thermal stability (Wen et al. 2012; Wang et al. 2014) of *LIP2*. Finally, *Y. lipolytica* has served as a host for heterologous lipases from *Candida antarctica* (Emond et al. 2010) and *Rhizopus oryzae* (Yuzbashev et al. 2012). Collectively, these studies highlight the potential for *Y. lipolytica* for both bioremediation and lipase applications.

5 Organic Acid Production

Y. lipolytica has the capacity to produce high titers of citric acid – a molecule with varied applications in the food and pharmaceutical industries (Goncalves et al. 2014) with approximately 1.6 million tons of production (Sauer et al. 2008). Under certain fermentation conditions (e.g., nitrogen starvation), the reduction of intracellular AMP shuts down the TCA cycle, leading to the accumulation of citric acid, isocitric acid, and *cis*-aconitic acid (Beopoulos et al. 2009a, b). Moreover, *Y. lipolytica*'s capacity for conventional bioprocessing (Rywinska et al. 2011) has an advantage over *Aspergillus niger* which is usually cultivated using submerged fermentation (Angumeenal and Venkappayya 2013). These traits have enabled *Y. lipolytica* to serve as an attractive host for citric acid biosynthesis (Papanikolaou et al. 2002,

2009; Kamzolova et al. 2003; Finogenova et al. 2005; Rywinska et al. 2009, 2012; Rywinska and Rymowicz 2010; Morgunov et al. 2013). To further increase titers, efforts have been made to alter the carbon source to include species such as n-paraffins (Crolla and Kennedy 2004), ethanol (Finogenova et al. 2002), glycerol (Rywinska et al. 2009), and plant-based oils (Kamzolova et al. 2008; Papanikolaou et al. 2008). Finally, limiting nutrient access (esp. nitrogen) (Morgunov et al. 2013) and further fermentation optimization led to citric acid titers as high as 154 g/L from glycerol (Anita Rywińska 2010). Further studies including repeated fed-batch (Moeller et al. 2011) and overexpression of genes such as pyruvate carboxylase (Fu et al. 2016; Tan et al. 2016) have further increased citric acid production potential in this host.

High citric acid flux in *Y. lipolytica* has also been exploited for a variety of other TCA-cycle-derived products. As an example, this organism has been engineered to produce itaconic acid, a versatile petroleum-replacement monomer in plastics and rubbers (Tate 1981; Tsai et al. 2000; Okabe et al. 2009). Heterologous expression of cis-aconitic acid decarboxylase (CAD1) from *Aspergillus* species (Bonnarme et al. 1995; Kanamasa et al. 2008) along with further modifications (co-expression of *CAD1* and *AMPD*) ultimately led to itaconic acid tiers of 4.6 g/L itaconic acid in bioreactor fermentation (Blazeck et al. 2015). Collectively, these studies demonstrate the ability of *Y. lipolytica* to produce high titers of organic acids like citric acid and itaconic acid.

6 Research Needs

As an oleaginous yeast, *Y. lipolytica* serves as an attractive host for oleochemical biotechnology. Recent developments in synthetic biology have enabled more rapid genetic engineering of this host which has led to a diversification of compounds including single cell oil, modified fatty acids, organic acids, and lipases. Though engineering efforts have proven successful thus far, as a nonconventional yeast, the synthetic biology tools available for this host lag behind the model host *S. cerevisiae* (Wagner and Alper 2016). Moreover, the dominant DNA repair mechanism in *Y. lipolytica* is nonhomologous end joining (NHEJ) (Kretzschmar et al. 2013). NHEJ complicates knockout and knockdown techniques and limits the ability to target gene overexpressions to specific loci. The dominance of NHEJ can be reduced by knocking out the responsible repair genes *ku70* and *ku80*, but this genotype is not ideal and can lead to strain instability (Kretzschmar et al. 2013). Beyond genome modifications, there are no characterized strong, inducible promoters available for use in *Y. lipolytica*, thus limiting the capacity for regulation in this host (Wagner and Alper 2016). Finally, the lack of stable episomal plasmids in *Y. lipolytica* reduces the speed of strain construction and flexibility (Wagner and Alper 2016). These limitations are all subjects of current studies and will ultimately enable *Y. lipolytica* to become a more attractive host.

In addition to new synthetic biology tools, future systems biology research is required to further elucidate the function of enzymes involved in each of the pathways described above. This enhanced knowledge base will speed the rate of *Y. lipolytica* engineering. Finally purification of oleochemical products from hosts such as *Y. lipolytica* still remains a large factor in the cost of producing these chemicals at a price on par with petroleum (Ledesma-Amaro et al. 2016). Ultimately, *Y. lipolytica* can become a cost-effective industrial host for the production of chemicals in a sustainable manner that can reduce our dependence on fossil fuels (Figs. 1, 2, and 3).

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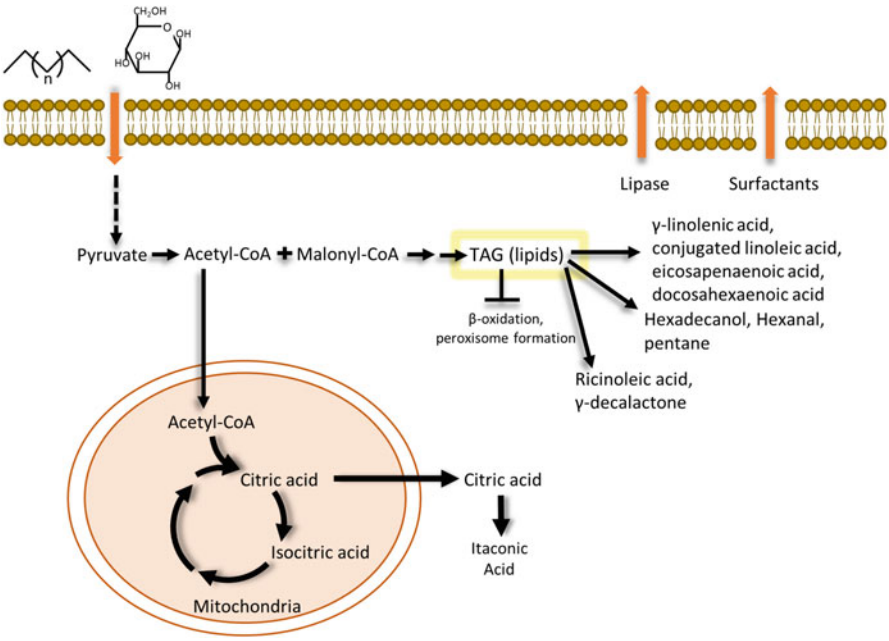

Fig. 1 *Schematic of Y. lipolytica metabolism and products*. Key products described in this chapter are depicted

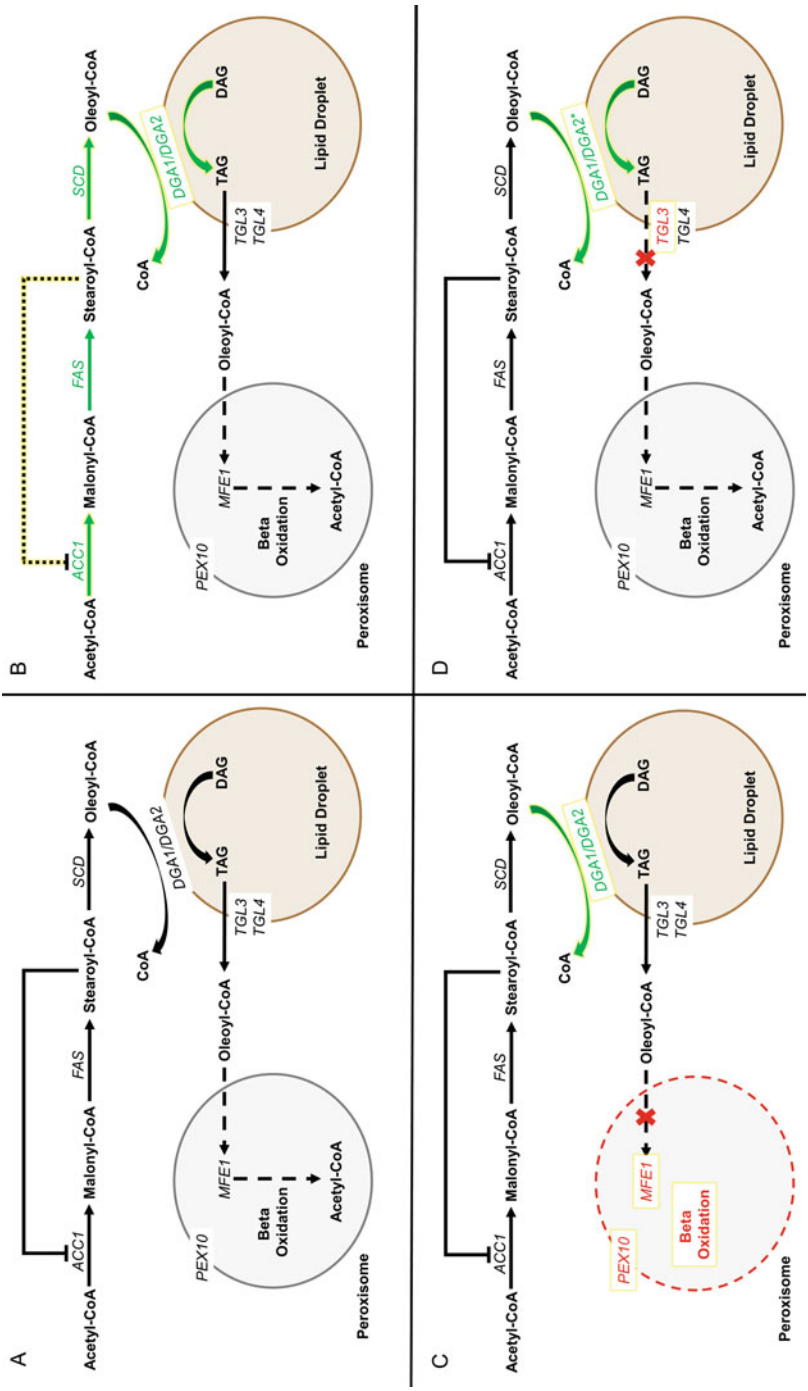

Fig. 2 *Metabolic engineering strategies to improve lipid accumulation in Y. lipolytica.* In native lipid metabolism in *Y. lipolytica*, acetyl-CoA is converted into malonyl-CoA by the acetyl-CoA carboxylase (ACC) enzyme that is inhibited by stearoyl-CoA. Malonyl-CoA serves as the building block for the fatty acid

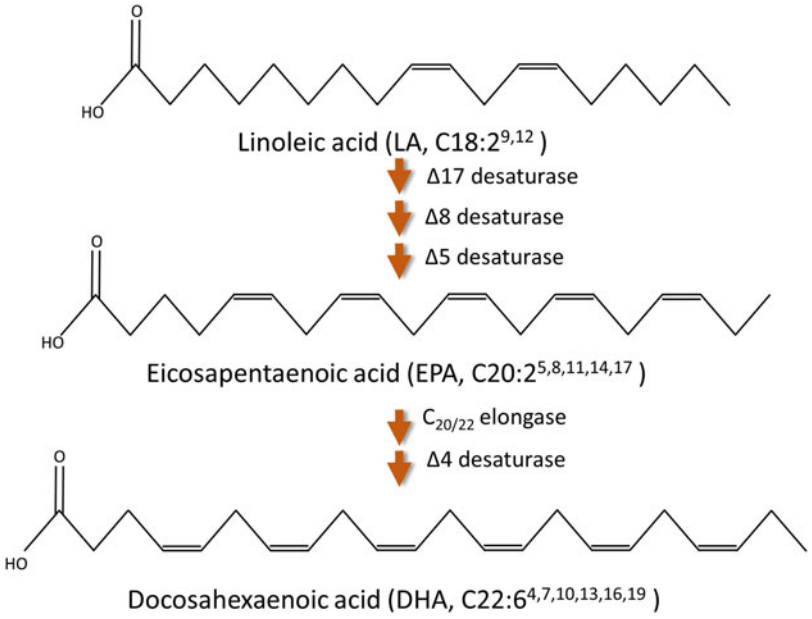

Fig. 3 *Conversion of native linoleic acid in Y. lipolytica to the omega fatty acids*. Overexpression of specific desaturases at carbons 5, 8, and 17, and one cycle of elongation generates the omega-3 fatty acid eicosapentaenoic acid (EPA, C20:3^{5,8,11,14,17}). Further elongation to 22 carbons and desaturation at carbon 4 to generate the omega-6 fatty acid docosahexaenoic acid (DHA, C22:6^{4,7,10,13,16,19}) (Xue et al. 2013)

Fig. 2 (continued) synthase (FAS) complex to produce saturated fatty acids (stearoyl-CoA) which is desaturated into oleoyl-CoA by the stearoyl-CoA desaturase (SCD) enzyme. Fatty acids are transferred from acyl-CoAs to diacylglycerol (DAG) to form triacylglycerol (TAG) by diacylglycerol acyltransferases (DGA1/DGA2) in lipid droplets. Acyl-CoA is released from TAGs through activity of TAG lipases (TGL3/TGL4). Acyl-CoA can then enter peroxisomes whose biosynthesis is promoted by peroxisomal biogenesis factors (PEX10). In the peroxisomes, fatty acids are catabolized into acetyl-CoA via beta oxidation, with the initial step catalyze by the peroxisomal multifunctional enzyme (MFE1). (**a**) One strategy for improving lipid production in *Y. lipolytica* involved overexpression of ACC1, SCD, and DGA1 to promote flux into fatty acid production and their accumulation into TAGs. Furthermore, overexpression of SCD1 alleviated accumulation of stearoyl-CoA to reduce feedback inhibition of ACC1 activity (Qiao et al. 2015). (**b**) A second strategy involved overexpression of DGA1 to increase TAG accumulation and impaired fatty acid catabolism through pex10 and mfe1 knockouts (Blazeck et al. 2014). (**c**) A third strategy involved increasing TAG production by overexpressing heterologous DGA1 and DGA2 enzymes and deleting TGL3 to reduce TAG degradation (Friedlander et al. 2016)

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Lipid-Containing Secondary Metabolites from Algae

25

J. G. Qin

Contents

Abstract

As secondary metabolites, algal lipids are composed of fats, phospholipids, steroids, and waxes, which are functionally important for cell structure and energy storage. On average, lipids account for 20–40% of algal dry weight with a maximum of 85% in some algae, which exceeds the lipid content of most terrestrial plants. The range of potential applications of algal oils is very wide. Polyunsaturated fatty acids in algae could be an importance source for human and animal nutrition and biofuels. However, a viable commercial production of fatty acid needs further selection and screening of oleaginous species, improvement of strains by genetic manipulation, optimization of culture conditions, and development of efficient cultivation systems.

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1 Introduction

Microalgae work as solar energy-driven factories that convert carbon dioxide to a variety of lipids (fats, phospholipids, steroids, and waxes) and provide the food base to support the entire animal population of the aquatic ecosystems. Algae contain a wide range of fatty acids in their lipid storage (Guschina and Harwood 2006). Of particular importance is the presence of significant quantities of the essential polyunsaturated fatty acids in algae (Ahlgren et al. 1992). There is increasing commercial interest in the exploitation of polyunsaturated fatty acids – since many algae are rich in these components.

The average lipid content in microalgae is 20–40% of dry weight, with a maximum of 85% of the dry weight as lipids, which exceeds the lipid content of most terrestrial plants (Borowitzka and Borowitzka 1988). Algal oils resemble fish and vegetable oils and can be important for human and animal nutrition and substitutes for biofuels (Becker 2004; Chisti 2007). Therefore, the awareness of algae, especially microalgae, as sources of lipid production would lead to the advance of commercial application of algal metabolites. The development of algal chemical industry to convert natural resources to industrial feedstocks will further enhance the range of explorations of important products from algae. The objective of this chapter is to review lipids containing secondary metabolites (fatty acids, phospholipids, steroids, and waxes) in algae with a focus on fatty acids and to look into their potential applications such as nutritional additives and biofuels, and further research directions.

2 Lipids in Algae

Lipid is sometimes used as a synonym for fats consisting of two kinds of smaller molecules: glycerol and fatty acids. Glycerol is an alcohol with three carbons, each bearing a hydroxyl group. A fatty acid has a long carbon skeleton, usually 16 or 18 carbon atoms in length. The fatty acids can each be joined to glycerol by an ester linkage form a triglycerol consisting of three fatty acids linked to each glycerol molecule. Fatty acids vary in length and in the number of location of double bonds in the structure of the hydrocarbon tails. If there are no double bonds between the carbon atoms composing the tail, then it is a saturated fatty acid. An unsaturated fatty acid has one or more double bonds formed by the removal of hydrogen atoms from the carbon skeleton. The nomenclature of fatty acids follows a particular convention. This is C x:y(n-z), where x denotes the number of carbon atoms, y denotes the number of double bonds in the chain and z denotes the carbon at which the first double bond appears numbering from the non-carboxyl (COOH) end. N is often replaced by ω (omega) in popular jargon; Thus, for example, docosahexaenoic acid (DHA) is C22:6 (n-3) fatty acid and eicosapentaenoic acid (EPA) is C20:3(n-3) fatty acid.

Lipids in algal cell function as membrane components, storage products, metabolites, and energy supplies. Lipids extracted with lipophilic organic solvents such as

ether, methanol, and chloroform are called total lipids. According to Reitan et al. (1997), commonly used algae in fish hatcheries such as *Isochrysis galbana, T. Iso*, and *Pavlova lutheri* (Prymnesiophyceae) contained lipids in the range of 11–22% dry matter. Within a species, the lipid composition of microalgae was suggested depending on light or nutrients under which they grow (Harrison et al. 1990). The accumulation of lipids in algae is hypothesized as a process of the steady synthesis of neutral lipids (Lombardi and Wangersky 1991) in combination with reduced cell division and protein synthesis following reduced availability of nutrients (Siron et al. 1989; Sukenik and Livne 1991). This hypothesis was true in *Isochrysis galbana, P. lutheri, P. tricornutum*, and *Chaetoceros* sp. where the reduction of nitrogen enhanced total lipids, but was not true in *Nannochloris atomus* and *Tetraselmis* sp. (Reitan et al. 1994). The species that did not accumulate lipids (*N. atomus* and *Tetraselmis* sp.) probably accumulated photosynthetic products in the form of carbohydrates. Reitan et al. (1994) also found an increased relative content of the monounsaturated fatty acid 18:1 and the saturated fatty acid 16:0, and a decreased relative content of polyunsaturated fatty acids in most algal species during nutrient limitation. In the species that accumulate lipids (*I. galbana, P. lutheri, P. tricornutum*, and *Chaetoceros* sp.), the content of *n*-3 polyunsaturated fatty acids increased with increasing nutrient limitation (Reitan et al. 1994). It seems that nutrient limitation at a certain stage plays an important role in regulating lipid storage and fatty acid profiles in algae.

3 Polyunsaturated Fatty Acids

The production of polyunsaturated fatty acids (PUFA) by marine and freshwater microalgae is the subject of intensive research and increasing commercial attention (Sijtsma and de Swaaf 2004; Wen and Chen 2003). Fish oil is a major source for the commercial production of these fatty acids, but since there is an increasing demand for purified PUFAs, some alternative sources are being sought. Some species of freshwater and marine algae contain large amounts of high-quality PUFAs and are currently widely used to produce PUFAs for aquaculture operations (Reitan et al. 1997). In aquaculture, the following three algal PUFAs are particularly important in promoting growth, stress resistance, and pigmentation for fish larvae (Qin 2008).

3.1 Eicosapentaenoic Acid (EPA)

The heterotrophic production of EPA (C20:5n-3) by microalgae has been recently reviewed in some detail including its distribution in different algae species, systems for mass cultivation of algae, factors influencing production of EPA, and improvement of microalgal strains for EPA production (Wen and Chen 2003). For a given species, the production of EPA generally depends on growing phase, temperature, and nutrient supplies.

Polyunsaturated fatty acid productions were studied in three marine microalgae *Nannochloropsis oculata* (Eustigmatophyceae), *Thalassiosira pseudonana* (Bacillariophyceae), and the Haptophyte *Pavlova lutheri* (Tonon et al. 2002). The time-course of production EPA and its incorporation into triglycerols differ between species and growing phases. In *N. oculata*, 90% of the total fatty acids were in triacylglycerols at the end of stationary phase but the EPA in this alga remained fairly constant during the exponential phase. In a diatom, *T. pseudonana*, the total fatty acid content increased significantly in stationary phase where 74% of the total fatty acids were in the triglycerol fraction. The amount of EPA partitioning into triacylglycerols increased from 16% to 67% after 150 h in the stationary phase. In contrast to these two species, the total fatty acids decreased upon transfer to the stationary phase in *P. lutheri* where only 52% of the total fatty acids were in triacylglycerols. These results indicate that the level of EPA accumulation is quite dynamic in the course of algal culture and caution should be taken when extrapolating the results from one species to the other.

Under a steady continuous culture, *Phaeodactylum tricornutum* produced two major fatty acids hexadecenoic acid and EPA, which accounted for 50% of total fatty acids (Jiang and Gao 2004). When temperature reduced from 25 °C to 10 °C for 12 h, EPA concentration increased by 120% compared with the control. When the temperature was steady, the growth phase seemed not significantly impacting EPA accumulation in this alga.

The production of fatty acids could be regulated by the condition of nutrient supply (Alonso et al. 2000). The EPA productivity in microalgae *Navicula saprophila, Rhodomonas salina*, and *Nitzschia* sp. was examined under photoautotrophic, heterotrophic, and mixotrophic conditions (Kitano et al. 1997). Under the photoautotrophic condition, EPA in total fatty acids was 20.1% for *N. saprophila*, 15.4% for *R. salina*, and 24.7% for *Nitzschia* sp. The mixotrophic condition in the presence of acetic acid significantly promotes the algal growth rate and EPA production of *Nitzschia* sp. (Kitano et al. 1997).

3.2 Docosahexaenoic Acid (DHA)

The heterotrophic marine microalgae *Crypthecodinium cohnii* can be a source of docosahexaenoic acid (C22:6n-3, DHA), another long-chain ω-3 PUFA family (De Swaaf et al. 1999). This alga accumulates lipids up to 20% of biomass and has very unique fatty acid profiles. The level of DHA in this alga is 30–50% of the total fatty acids. De Swaaf et al. (1999) showed that salinity >50% seawater was required to maintain good algal growth and lipid accumulation. The range of glucose of 25–84.3 g/l and a temperature below 30 °C were recommended by the authors. The potential use of ethanol as a carbon source for DHA was also examined in *C. cohnii*, with the highest value of DHA accumulation of 53 mg/l/h (De Swaaf et al. 1999). In the autotrophic alga, *Pavlova lutheri,* the DHA accounted for 27% of triacylglycerols (Meireles et al. 2003) and this alga has been used as an important DHA source for fish larvae (Qin 2008).

Thraustochytrids are common marine microheterotrophs, taxonomically aligned with heterokont algae. Recent studies have shown that some thraustochytrid strains can be cultured to produce high biomass, containing substantial amounts of lipid rich in polyunsaturated fatty acid (PUFA) (Lewis et al. 1999). According to Nakahara et al. (1996), *Schizochytrium* spp. produced DHA at a rate of 2.0 g/l per day. The lipid extracted from this alga was about 50% of the dry biomass and contained 93% triacylglycerols. The DHA content of total lipid fraction was 34% of the fatty acids. In a follow up study, the lipid content of *Schizochytrium* sp. strain SR21 in a medium containing 12% glucose reached 77.5% of dry biomass and DHA accounted for 35.6% of total fatty acids (Yaguchi et al. 1997).

The fatty acid profiles of three strains of *Schizochytrium mangrovei* isolated from decaying leaves showed that the percentage of DHA varied from 32% to 39% of total fatty acids, depending on the strain (Jiang et al. 2004). The compositions of algal fatty acids slightly changed between growth stages. In another thraustochytrid, *Thraustochytrium aureum*, triacylglycerol was also the dominant lipid and was composed of 40% DHA (Iida et al. 1996).

3.3 Arachidonic Acid (AA)

Although long-chain ω-3 fatty acids are quite abundant in microalgae, fatty acids of the ω-6 family like C20:3n-6 and C20:4n-6 (arachidonic acid, AA) are almost excluded from the lipids of freshwater algae and account for only a few percent of total fatty acids in the marine species (Bigogno et al. 2002). The green alga *Parietochloris incisa* (Trebouxiophyceae) isolated from the snowy slopes of Mt. Tateyama (Japan) contains 33.6% AA of the total fatty acids in the logarithmic phase and 42.5% in the stationary phase (Bigogno et al. 2002). In the logarithmic phase, AA was a major component and its proportion decreased significantly during the stationary phase. In contrast, triacylglycerols accumulated to larger amounts of AA in the stationary phase (from 43% to 47%). In *P. incisa*, AA and triacylglycerols were simultaneously accumulated allowing this alga to utilize AA from the triacylglycerols for membrane reconstruction during the adaptation to changing environments (Bigogno et al. 2002).

4 Biofuel from Algal Lipids

The use of microalgae lipids as a source of fuel has been proposed for decades, but it is now being taken seriously because of the high price of petroleum, and more significantly, the emerging concern about global warming that is associated with burning fossil fuels (Reviewed by Chisti 2007). Biofuels broadly refer to solid, liquid, or gas fuel derived from recently dead biological material, most commonly plants. Biodiesel refers to a nonpetroleum-based diesel fuel consisting of short-chain alkyl (methyl or ethyl) esters, made by transesterification of vegetable or algal oils. Biodiesel is produced currently from

Table 1 Comparison of sources of alternative biofuel feedstocks in the USA (From Chisti 2007)

Crop	Oil yield (L/ha)	Land area required (10^6 ha)	Percent of existing US cropping area needed[a]
Corn	172	1540	846
Soybean	446	594	326
Jatropha	1892	140	77
Coconut	2689	99	54
Oil palm	5950	45	24
Microalgae[b]	136,900	2	1.1
Microalgae[c]	58,700	4.5	2.5

[a]For meeting 50% of all transport fuel needs of the USA
[b]70% oil (by wt) in biomass
[c]30% oil (by wt) in biomass

plant and animal oils, but not commercially from microalgae (Metzger and Largeau 2005; Kulkarni and Dalai 2006).

Replacing all the transport fuel consumed at the current rate with oil crops, waste cooking oil, and animal fat is not realistically possible (Chisti 2007). For example, to meet only half the existing U.S. transport fuel needs by biodiesel would require unsustainably large cultivation areas for major oil crops (Table 1). Using the average oil yield per hectare from various crops, the cropping area needed to meet 50% of the U.S. transport fuel needs is calculated (Table 1). If oil palm, a high-yielding oil crop, can be grown, 24% of the total cropland will need to be devoted to its cultivation to meet only 50% of the transport fuel needs. Clearly, oil crops cannot significantly contribute to replacing petroleum-derived liquid fuels in the foreseeable future. This scenario changes dramatically, if microalgae are used to produce biodiesel. Between 1% and 3% of the total US cropping area would be sufficient for producing algal biomass that satisfies 50% of the transport fuel needs (Table 1).

Microalgae appear to be the only source of biodiesel that has the potential to completely displace fossil diesel. In comparison with other oil crops, microalgae grow extremely rapidly and many are exceedingly rich in oil. Microalgae commonly double their biomass within 24 h and biomass doubling times during exponential growth are commonly as short as 3.5 h (Janssen et al. 1999). Also, the oil content in microalgae can exceed 80% by weight of dry biomass (Metting 1996; Spolaore et al. 2006). Depending on the species, microalgae produce many different kinds of lipids and other complex oils (Banerjee et al. 2002; Guschina and Harwood 2006; Metzger and Largeau 2005), but oil levels of 20–50% are quite common (Table 2). Oil productivity depends on the algal growth rate and the oil content of the biomass. Microalgae with high oil productivities are desired for producing biodiesel. The advantage of using microalgae to produce biodiesel is that this operation will not compromise on the production of food, fodder, and other products derived from crops.

Microalgal oils differ from most vegetable oils in being quite rich in polyunsaturated fatty acids with four or more double bonds (Belarbi et al. 2000). For example, eicosapentaenoic acid (EPA, C20:5n-3; five double bonds) and docosahexaenoic acid (DHA, C22:6n-3; six double bonds) occur commonly in algal oils. Fatty acids

Table 2 Oil content of some microalgae (From Chisti 2007)

Microalgae	Oil content (% dry wt)
Botryococcus braunii	25–75
Chlorella sp.	28–32
Crypthecodinium cohnii	20
Cylindrotheca sp.	16–37
Dunaliella primolecta	23
Isochrysis sp.	25–33
Monallanthus salina	>20
Nannochloris sp.	20–35
Nannochloropsis sp.	31–68
Neochloris oleoabundans	35–54
Nitzschia sp.	45–47
Phaeodactylum tricornutum	20–30
Schizochytrium sp.	50–77
Tetraselmis suecica	15–23

and fatty acid methyl esters (FAME) with four and more double bonds are susceptible to oxidation during storage and this reduces their acceptability for use in biodiesel, but this need not be a significant limitation. The extent of unsaturation of microalgal oil and its content of fatty acids with more than four double bonds can be reduced easily by partial catalytic hydrogenation of the oil (Dijkstra 2006).

5 Phospholipids and Steroids and Waxes

Phospholipids are structurally related to fats, but they have only two fatty acids rather than three. The third hydroxyl group of glycerol is joined to a phosphate group that is negatively charged. Phospholipids show ambivalent behavior toward water. Their hydrocarbon tails are hydrophobic and are excluded from water. In contrast, the phosphate end forms a hydrophilic end and has an affinity for water. Because many species of red and brown algae have used as human food (Chapman and Chapman 1980), their lipid constituents have drawn the attention of researchers. Dembitsky and Rozentsvet (1990) studied the phospholipid composition of 14 species of red marine algae. The total amount of lipid varied from 0.5 to 2.6 mg per g dry weight. The amount of phospholipids varied from 9.8% to 21.3% of the total lipids. The major phospholipids appeared to be phosphatidylchohne (PC, 61.6–77.8%) and phosphatidylglycerol (PG, 10.4–23.3%). The authors suggest that red algae can be used as a source of large amounts of PC and PG. In contrast, phospholipids in two brown algae species (*Fucus vesiculosus* and *Ascophyllum nodosum*) were very similar (Jones and Harwood 1992). The major phospholipid was phosphatidylethanolamine but this represented less than 10% of the total acyl lipids. Despite the presence of conventional phospholipids in brown algae, Khotimchenko and Titlyanova (1996) identified a new amino acid-containing phospholipid from 30 species of brown algae belonging to eight different orders. The occurrence of this new amino acid-containing phospholipid has

chemotaxonomic value for brown algae. The amino- phospholipid is characteristic of all brown algae, and therefore, has a taxonomic value because it was not found in red algae (Dembitsky and Rozentsvet 1990) and green algae (Dembitsky and Rozentsvet 1989).

The steroids are lipids characterized by a carbon skeleton consisting of four interconnected rings. The most common steroids in algae are sterols, and algae produce a wide range of sterols (Borowitzka 1988). The majority of algae investigated contain sterols in the unsaponifiable lipid fraction. Cholesterol is a major sterol in some phytoplankton species that are used in aquaculture (Patterson et al. 1993; Tsitsa-Tzardis et al. 1993). Gladu et al. (1995) reported that *Nannochloropsis* sp. contained a high cholesterol and polyunsaturated fatty acid, which make it attractive as a potential aquaculture feed. In comparison, *Spirulina maxima* exhibited a higher cholesterol content (8.5%) than the two green algae *Scenedesmus* sp. and *Chlorella vulgaris* (l–2.5%) (Rzama et al. 1994). For most microalgae, the sterol levels appear to range from 1% to 4% of dry weight of algae (Nichols et al. 1984). The sterol composition varies during growth cycle and culture conditions (Borowitzka 1988).

Wax esters are the esters of fatty acids with long-chain alcohols. Saturated wax esters have been found in *Euglena gracilis* under anaerobic conditions (Ono et al. 1995). Wax esters were synthesized from paramylon, the reserve polysaccharide of this organism, and accumulated in the cells. Wax ester production was associated with anaerobic conditions and reduced oxygen concentrations. This wax ester production was also dependent on thiamine concentration, an essential growth factor of *Euglena*. The production of wax eaters from *Euglena gracilis* strain Z was enhanced through anaerobical incubation in the presence of unsaturated fatty acids after aerobic cultivation for 3 days on a glucose-peptone medium (Tani et al. 1987). In a marine cryptomonad *Chroomonas salina*, the *wax* esters present in lipid increased with culture age and were almost exclusively saturated and contained high proportions of 13:0 and (Henderson and Sargent 1989).

6 Research Needs

Algae contain lipids and fatty acids as membrane components, storage products, metabolites, and sources of energy. Algal fatty acids and oils have a range of potential applications. However, further research is required to scale up massive algal production and improve the production efficiency of algal biomass in various culture vessels, including the design of photobioreactors and harvesting skills for microalgae. The development of new systems will enable the growth of heterotrophic microalgae, which have significant economic advantages over photoautotrophic production. The understanding of life history of lipid producing algae is necessary to harvest algae at an optimal growing phase to maximize the production potential. The knowledge of synthetic mechanisms of various kinds of lipids is necessary to provide the foundation for further biotechnological study. Biofuel from algae is technically feasible, but the economics of producing algal oils need to improve substantially to make it competitive with petro fuel. To produce low-cost microalgal biofuel requires fundamental research through algal genetic and metabolic engineering.

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Chemical Feedstocks and Fine Chemicals from Other Substrates

26

K. Muffler, N. Tippkötter, and R. Ulber

Contents

Abstract

It is well known that classical energy sources such as petroleum oil and natural gas make up the fundamental materials, on which all modern industrial chemical parks are based on. According to the finiteness availability of these consuetudinary resources and due to the increasing demand for energy of developing countries and the related rise in prices of oil and natural gas, renewable resources must be considered as valuable alternatives. Discussions about climate changes with regard to alternatives of energy production are very fervid, but alternatives have to be examined from a matter-of-fact-based, economical, and scientific point of view. Within the frame of this contribution, we focus therefore on alternative

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sources with respect to their potential as future building blocks for chemical synthesis processes.

1 Introduction

Chemical industry processes are historically based on fossil resources. During industrial revolution energy sources like peat and such renewable biomasses as wood were substituted by coal and later on by natural gas and petroleum oil. The latter has been, until now, the main resource for raw materials and the energy supply for the private sector. Due to its very beneficial properties in terms of chemical synthesis processes, only a minor proportion of approximately 10% of this plentiful resource is used for such purposes, whereas 90% is utilized for energy production as well as transport. With regard to the increasing energy demand and oil consumption of developing countries, the limited availability of crude oil, and financially motivated trading operations, the price of oil rises steadily and peaked nearly 150 USD per barrel in 2008. It is assumed that most of the known so-called super giant oil-fields cross the oil peak, which comes along with a decrease in the discovery of novel oil springs (Campbell 2006). Therefore, alternatives have to be introduced to decrease the dependency on these transient fossil fuels. But one has to have in mind that alternative fuels and resources for chemical building blocks have to compete against classical fossil compounds.

Naturally occurring waste materials and by-products serve both the need for cheap supply sources and as renewable resources. Current promising sources are residual wood and plant parts, waste fats or oils, and crude glycerol, last mentioned arising in large quantities during the production of biodiesel. The sources of these substrates can be classified as oil plants, sugar beets/cane, and waste residues from agriculture, e.g., biomass, silage, oils, and whey. The ongoing research on utilization of such renewables is manifold, and much attention is paid on exploitation of these materials for production of several commodities, which is presented in Sects. 3 and 4. Within Sect. 2 we focus in particular on the generation of C1 carbonic compounds (methane and methanol), the production of which via the biomass feedstock is already feasible.

2 Biotechnological Generation of Methane and Methanol

Methane can be produced via several routes from renewable raw materials. The traditional conversion process by anaerobic digestion of biomass, originally developed for removal of organics from liquid wastes, is presumably the most widely used (Torkay 2000). Within this slow fermentation process, a couple of bacteria convert organic matter first to organic acids and afterward to methane. Furthermore, methane can be produced via syngas by biomass gasification, if the organic matter is heated in

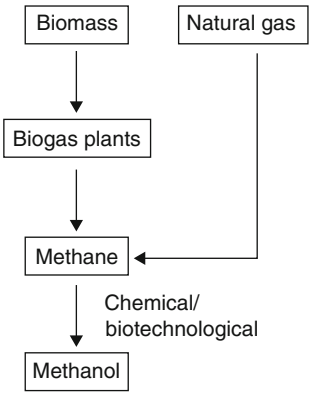

Fig. 1 Potential biotechnological production routes for methane and methanol

the presence of only low amounts of oxygen or anaerobic conditions, whereas a mixture of carbon monoxide and hydrogen is generated. Alternatively, the produced syngas can also be processed to methanol.

The beneficial aspects of methane and methanol in terms of their use in biorefineries can be summarized as follows (Busch et al. 2006):

Methane is available as a fossil raw material, and a biogenous generation via microbial degradation processes is possible.

Existing pipeline systems developed for transport of fossil fuels can be adopted to the special needs of methane/methanol.

Integration of methanol into the value added chain is feasible, and a conversion into paraffin hydrocarbons, olefins, aromatic hydrocarbons, and technical gases is possible.

Methanol can be used as fuel and gasoline substitute.

Methanotrophic microorganisms are available that can be applied for the production of basic and fine chemicals (Asinger 1986; Xin et al. 2004).

The flow sheet, presented in Fig. 1, summarizes the synthetic routes from biomass/natural gas to methanol via methane.

2.1 Methanol from Biogas

Anaerobic digestion from biomass is responsible for the generation of the so-called biogas, a mixture of mainly methane and carbon dioxide, owing to the breakdown of organic matter by mixed populations in the absence of oxygen. This is also a widely naturally occurring process, found in wetlands and swamps, whereas methane is generated due to fouling processes.

Biogas is composed mainly of methane (60–70%) and carbon dioxide (30–40%), whereas landfill gas commonly contains methane (50–55%), carbon dioxide (41–45%), and nitrogen (1–4%). The latter can also contain considerable amounts of halogenated carbons, depending on the waste, which was bailed (Hiller et al.

2006). As a minor compound hydrogen sulfide is generated, this limits the direct utilization of the gas without precedent removal of such sulfur content.

The application is not limited to freshly harvested biomass matter, and therefore, organic matter containing waste streams (e.g., pulp industries or sewage (Kleerebezem and Macarie 2003; Kortekaas et al. 1998; Seghezzo et al. 2006)) can also serve as an energy feedstock and as a renewable resource, respectively. The efficient performance of the operation requires that the digesting process is performed in a suitable biogas plant. As for the stoichiometry of biogas generation, 1 kg of organic dry matter results in 250 g methane.

The transformation process of organic matter, also described as biomethanation, within a biogas plant can be divided into four fundamental biochemical tasks: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. During the first step of biogas formation, the polymeric compounds such as polysaccharides, lipids, and proteins are degraded by hydrolytic (exo)enzymes. The liberated monomers are subsequently fermented within the acidogenesis, while interducts such as smaller subunits of carbonic acids, carbon dioxide, hydrogen sulfide, and ammonia are produced. The generated organic compounds are afterward transformed to acetate, carbon dioxide, and hydrogen. Finally, acetate is dismutated into methane and inorganic carbon by methanogenic microbacterial species, while this process is strictly confined to anaerobic conditions. Products other than acetate occurring during the first fermentative step are transformed by obligate hydrogen-producing acetogenic bacteria to acetate and hydrogen, whereas high pressures will reduce the methanogenesis, owing to thermodynamic reasons.

Methane generation can be expressed as follows:

$$CH_3COO^- + H2O \rightleftharpoons CH_4 + HCO_3^- \quad \Delta G^0 = -31.0 \text{ kJ/mol} \tag{1}$$

The performance of methane production depends on several factors. The utmost significant parameters were identified as pH, temperature, carbon-to-nitrogen ratio, oxygen concentration, carbon source/feedstock, and the amount and kind of inoculum (Nyns 2000).

Methanol production from biogas can be performed via the generation of syngas, in which further processing to the corresponding alcohol is described in more detail in the following section. While the application of the methane steam reforming process, methane is exposed to steam in the presence of a nickel catalyst at 800–1,000 °C and 20–30 atm. Thus, carbon monoxide and hydrogen are generated within an endothermic reaction (Kochloefl 1997). The carbon monoxide can further react with the steam within the so-called water-gas shift reaction. Both reactions can be described as follows:

$$CH_4H_2O \rightleftharpoons CO + 3H_2 \quad \Delta H_{298K} = +49.1 \text{ kcal/mol} \tag{2}$$

$$CO + H_2O = CO_2 + H_2 \quad \Delta H_{298K} = -9.8 \text{ kcal/mol} \tag{3}$$

The composition of the gas mixture depends strictly on reaction temperature, pressure, and the ratio of water to methane. Thus, the higher the temperature and the lower the pressure, the higher the syngas yield, whereas the necessary heat is provided by biogas combustion.

Another approach that uses the syngas route for the preparation of methanol makes use of partial oxidation of methane in the presence of insufficient oxygen at temperatures ranging from 1,200 to 1,500 °C (Pavone 2003). It results in a syngas, the composition of which is perfectly suited for methanol synthesis. But due to the exothermic CO_2 generation, the ratio of hydrogen and carbon monoxide shifts to more imperfect conditions. The reactions of this process can be given as follows:

$$CH_4 + 1/2O_2 \rightleftharpoons CO + 2H_2 \quad \Delta H_{298K} = -8.6 \text{ kcal/mol} \tag{4}$$

$$CO + 1/2O_2 \rightleftharpoons CO_2 \quad \Delta H_{298K} = -67.6 \text{ kcal/mol} \tag{5}$$

$$H_2 + 1/2O_2 \rightleftharpoons H_2O \quad \Delta H_{298K} = -57.7 \text{ kcal/mol} \tag{6}$$

Methods for the production of methanol from syngas are well known. The syngas route is a common approach for manufacturing the alcohol, whereas methane serves as the primary (90%) feedstock for the production of the annual demand of more than 32 million tons. Preparation of methanol from syngas can be carried out as presented by the following equations:

$$CO + 2H_2 \rightleftharpoons CH_3OH \quad \Delta H_{298K} = -21.7 \text{ kcal/mol} \tag{7}$$

$$CO_2 + 3H_2 \rightleftharpoons CH_3OH + H_2O \quad \Delta H_{298K} = -9.8 \text{ kcal/mol} \tag{8}$$

$$CO_2 + H_2 \rightleftharpoons CO + H_2O \quad \Delta H_{298K} = +11.9 \text{ kcal/mol} \tag{9}$$

With respect to thermodynamic considerations, the conversion to methanol is favored by an increased pressure and moderate temperature. In principle, synthetic gas can be certainly obtained by partial oxidation or reformation of fossil carbon-rich matter such as coal, coke, natural gas, petroleum, etc., but more sustainable is the utilization of a biomass feedstock. Nearly all kinds of renewable organic matter can be used, but the efficiency of the process strictly depends upon the origin and quality of the feedstock.

A sustainable and valuable tool for the direct conversion of methane to the corresponding alcohol can be provided by enzymes of mthanotrophic bacterial origin.

2.2 From Methane to Methanol: The Biotechnological Approach

The direct conversion from methane to methanol without taking the syngas route is a highly eligible process. As already mentioned in Sect. 2.1, a direct conversion by the

application of classical chemical catalysts is feasible, but the process generates a couple of by-products. To achieve a convenient conversion at moderate temperature, pressure, and selectivity, bacteria belonging to the group of methanotrophs could be exploited to establish a biochemical-based methanol manufacturing process. Such organisms are capable of utilizing methane as their sole carbon source, whereas different enzymes such as methane monooxygenase, methanol dehydrogenase, formaldehyde dehydrogenase, and formate dehydrogenase are responsible for the complete oxidation of methane to carbon dioxide (Xin et al. 2002). The first enzyme of this cascade is responsible for methanol generation. A direct utilization of the enzyme for a biotransformation process is currently limited with regard to the instability of the purified enzyme, the cofactor requirement, and its multicomponent nature. In such cases a biotransformation is preferred by application of whole cells, containing the necessary biocatalyst(s). But it has to be kept in mind that the desired product is in that case only an interduct that can be used by the cell and suffers a subsequent oxidation. To prevent further degradation of the valuable methanol commodity, the responsible enzyme (methanol dehydrogenase) must be inhibited. By supplementing cyclopropanol or EDTA to corresponding cells, such effect can be easily achieved, and during a biotransformation process, methanol accumulates in the extracellular environment (Furuto et al. 1999; Mehta et al. 1991).

An alternative and promising approach utilizes the inverse metabolism for the production of methanol from carbon dioxide in the presence of an excess of reducing equivalent (Obert and Dave 1999; Reda et al. 2008; Xin et al. 2007). Therefore, the greenhouse gas can be converted into valuable alcohol. But it has to be kept in mind that such a process depends on relatively high carbon dioxide concentrations, which have to be provided by traditional fossil fuels.

3 Crude and Fine Chemicals from Fats and Oils

Oleochemicals are hydrocarbons derived from plant oils. They are closely related to petrochemicals and hence can be integrated in the existing processes of the chemical industry. The oils and fatty acids, respectively, can be derived from several low-cost sources as waste product streams of the food and fuel industry or algae. Oil-derived products can be of use for several industrial applications. The fatty acids can, besides others, be used as source for plastic, soaps, alkyd resins, rubbers, and lubricants. In their methylated form, fatty acids and alcohols serve as cosmetics and cleaning agents. The fats' glycerol has applications as toothpastes, pharmaceuticals, and foodstuff or as a substrate for fermentation (Baumann et al. 1988).

Hydrolyzation of the fatty acids from oils can be done chemically by heterogeneous catalysis, enzymatically catalyzed with lipases, or by the use of lipolytic microorganisms. Microbial transformations of the gained fatty acids include hydration or epoxidation of the double bond, reduction of the carboxylic acid, and hydroxylations. Examples of industrially relevant microbial-derived products are wax ester and polymer coatings. Wax esters used in lubricants, plasticizers, and the cosmetic market can be produced by lipase-catalyzed esterification. The process was

shown to be 34% more energy efficient than the one based on petrochemicals. Epoxidized vegetable oils, e.g., those found in oxirane-rich linseed oil, can be used in cation-curable coatings prepared by exposure to UV radiation. The epoxidation of fatty acids is possible under mild conditions with lipase as a catalyst. This process currently needs to be enhanced by protein engineering of more stable lipases or the use of monooxygenases (Hatti-Kaul et al. 2007).

Microbial conversion of fatty acids is a source of stereospecific fine chemicals. Especially the stereo- and regioselective hydration reactions are of interest (e.g., for the food industry). The microbial enantioselective addition of water to unsaturated fatty acids can be done by several bacterial genera and was first observed in 1962 in form of the reaction of oleic acid to R-(10)-hydroxystearic acid (HSA). HSA is a precursor of γ-dodecalactone, an important flavor compound. Furthermore, *Pseudomonas* species *Nocardia*, *Rhodococcus*, *Corynebacterium*, and *Micrococcus* have been observed performing this hydration, as well (Biermann et al. 2000).

3.1 Dicarboxylic Acids

The group of dicarboxylic acids can be used for the production of, i.e., esters and polymers. Microbial ω-oxidation of fatty acids leading to the formation of dicarboxylic acids has recently been a topic of great interest. The yeast of the genus *Candida tropicalis* is capable of converting oleic, palmitic, stearic, and erucic acid to their corresponding dicarboxylic acids with yields up to 36%. By using a mutant strain of *C. tropicalis*, a transformation of oleic acid to its hydroxyl diacid with yields up to 76% is possible (Biermann et al. 2000).

Itaconic acid is an unsaturated dicarboxylic acid that can be widely incorporated in synthetic polymers. Substitution of fumarate and maleate was possible in several cases. For example, it is used as comonomer in resins, in synthetic fibers, adhesives thickeners, and binders. The biosynthesis of itaconic acid from carbohydrate by the fungus *Aspergillus itaconicus* was first reported in 1932 by kinoshita, the synthesis and the applied strains were further optimized in the later years and are nowadays primarily done with the species *Aspergillus terreus*. The fermentation of the fungi is currently done under phosphate-limited conditions with glucose or sucrose as carbon sources. Sources like starch, molasses, hydrolysates of corn syrup, and wood have successfully been used as substrates. The formation of itaconic acid is very sensitive to several medium components like Fe, Mn, Cu, Zn, P, and N. This limits the use of crude unrefined carbon sources. Sugarcane molasses needs to be treated with ion exchangers or similar techniques. Pure or sucrose-mixed glycerol has been used as feed. A newer attempt is to use citric acid as precursor for the itaconic acid (Willke and Vorlop 2001).

A further very promising building block for fine chemicals is succinic acid. Besides its present utilization in the food industry, the acid could potentially substitute maleic anhydride (Willke and Vorlop 2004). Succinic acid can be used as precursor for several relevant fine chemicals such as adipic acid, 1,4-butanediol,

tetrahydrofuran, N-methyl pyrrolidinone, 2-pyrrolidinone, succinate salts, and γ-butyrolactone. Furthermore, the production of the biodegradable polymer polybutylene succinate (PBS) is possible. Many different microorganisms have been screened for the succinic acid production. Especially *Anaerobiospirillum succiniciproducens* and *Actinobacillus succinogenes* have the capability to produce high amounts of the acid. Besides with glucose and sucrose, *A. succiniciproducens* was successfully fermented on glycerol with a raised yield. Furthermore untreated whey, lignocellulose hydrolysate, and corn steep liquor have been used (Song and Lee 2006).

3.2 Glycerol as Substrate

Besides the fatty acid component of the oil, glycerol can be used as microbial fine and bulk chemical production substrate. The chemical 1,3-propanediol (1,3-PD) has been of growing interest as an industrial chemical in the recent decades. The diol can be used as a component of several polymers. For example, 1,3-PD can be used as the diol component of polytrimethylene terephthalate (PTT), a polymer with properties comparable to those of nylon. Furthermore, a polymer prepared from terephthalic acid and 1,3-propanediol can be used as fiber or in apparel and carpet applications. It can be produced biotechnologically with several bacteria using glycerol as substrate. Besides others, the species *Citrobacter*, *Clostridium*, *Enterobacter*, *Klebsiella*, and *Lactobacillus* generate an excess of reducing equivalents in the form of NADH during anaerobic growth with glycerol as the substrate. The glycerol serves as an electron sink and undergoes a complex chemical reaction finalized by its NADH-dependent reduction to 1,3-propandiol (Nakamura and Whited 2003).

Glycerol arises in large quantities as a by-product of the biodiesel production. It is, and increasingly will be, a readily available substrate. For instance, the glycerol-water from the rape methyl ester production could be used for fermentations without further pretreatment except sterilization procedures (Willke and Vorlop 2001). The use of crude glycerol fractions is driven forward by the isolation of new 1,3-PD-producing strains. Especially *Klebsiella pneumoniae* and *Clostridium butyricum* produce significant amounts of the diol in crude glycerol medium. (Cheng et al. 2006; Gonzalez-Pajuelo et al. 2006; Papanikolaou et al. 2008).

Another promising, microbiologically produced bulk chemical is 2,3-butanediol. It is produced via a mixed acid pathway with further end products like ethanol, acetate, lactate, and succinate. 2,3-butanediol can be converted in 1,3-butadiene, a fine chemical for the production of rubber. By dehydration it can be converted to a liquid fuel additive methyl ethyl ketone.

A number of species can secrete the diol as end-product. *Aerobacter aerogenes*, *Aerobacillus polymyxa*, *B. polymyxa*, *S. marcescens*, and several *Klebsiella* species do so. Among these, *B. polymyxa* and *K. pneumonia* have potential for industrial-scale production. A variety of carbon sources based on hexoses and pentoses can be used to produce the diol. *Aerobacter aerogenes* can utilize xylose; *K. pneumonia* uses glycerol

and produces 1,3-propanediol and 2,3-butandiol depending on the pH value. Lactose, cellulose, hemicelluloses, and whey permeate have been used as substrate for the 2,3-butanediol production. More derivatives of this substance have a high potential for industrial utilization, as anti-freeze agent, solvent, or plastic component. Furthermore, it can be used as a flavoring agent in food products in its diacetyl form (Syu 2001).

4 Polyhydroxyalkanoates from Renewable Resources

Approximately 150 million tons of technical polymers are worldwide produced per year. Several of these products could be substituted by products derived from renewable resources. The required monomers like diols, polyols, and dicarboxylic acids can be readily produced via biotechnological routes. The production of the polymer polyhydroxyalkanoates (PHA) like polyhydroxy butyric acid (PHB) or poly hydroxy valeric acids is known since the 1920s (Willke and Vorlop 2004). The PHAs are of rising interest as they are biodegradable, biocompatible, and produced from renewable resources. PHAs are synthesized by various microorganisms as an intracellular carbon and energy storage system during reduced growth phases. For example, the bacterium *Cupriavidus necator* is capable of storing high amounts of PHA with typical lengths of three to five carbons. The storage of PHA occurs at nutrient-limiting growth conditions.

More than 150 different monomers can be obtained and offer a wide range of polymer properties. Besides the monomers, polymer microstructures can influence the polymer's properties. Block polymers are copolymers consisting of unique subunits covalently bonded together. The new structure inherits the properties of each block and thereby shows new characteristics not achievable by polymer blending. Examples of PHA-related block copolymers are poly(3-hydroxybutyrate) blocks balanced with blocks of poly(6-hydroxyhexanoate), poly (3-hydroxyoctanoate), monomethoxy-terminated poly(ethylene glycol) (mPEG), and poly(ethylene glycol) (Pederson et al. 2006).

Renewable substrates for the production of PHA/PHB are a topic of current research. The production of PHB with corn as a feedstock showed no environmental benefits as the amount of fossil fuels required to produce 1 kg of PHB exceeds the quantity required for the same mass of polystyrene. Corn is an energy-intensive feedstock in spite of the complex transport and process steps requiring the use of fertilizer, pesticides, and herbicides. *Ralstonia eutropha*, one of the commonly used PHA-producing bacterium, was successfully used for polymer production on several renewables as plant oils, fatty acids, animal fats (Taguchi et al. 2003). Their yield with, e.g., butyric acid as substrate is 0.65–0.95 $kg \cdot kg^{-1}$ and supersedes the yield gained with glucose (0.32–0.48 $kg \cdot kg^{-1}$). Nevertheless, the problem of restrained growth rate with substrates other than glucose or sucrose has to be overcome. Lactic and acetic acids have proved to be feasible carbon sources with a high assimilation rate of the PHA-producing bacteria (Tsuge 2002).

5 Lactose-Based Biotransformations

5.1 Whey and Lactose

Cheese whey is the aqueous fraction of the milk arising in large quantities as a by-product of the cheese-making process. For every produced kg of cheese, approximately 9 L of whey is gained. Lactose is the main solute of the whey making up to 5% of its weight. Disposal of whey as industrial effluent is not favorable and expensive due to its high oxygen demand (COD). The lactose from whey concentrates is nowadays used for dairy, bakery, and infant formulas. Many microorganisms thrive in milk-based media using the lactose as primary substrate. Excellent lactose utilization can be observed with lactic acid bacteria converting the lactose in the acid. The use of whey as fermentation medium is of great industrial interest, as besides lactose it already contains several minerals and vitamins required for microbial growth. By the addition of trace metals and increase of the nitrogen content, potent fermentation media can be obtained. The need for a higher nitrogen concentration can be circumvented by adding proteinases to the medium (Guimaraes et al. 1992). Furthermore, whey can be an effective additive to fermentation media. Addition of 1–2% whey protein concentrate or single whey proteins like α-lactalbumin or β-lactoglobulin can increase the cell growth for lactic acid bacteria (Bury et al. 1998).

Besides bacteria yeasts, primarily of the genus *Kluyveromyces*, play a significant role in the biotechnological use of lactose for the production of, e.g., biomass, ethanol, enzymes, and single-cell proteins (Adam et al. 2004). The use of *Kluyveromyces marxianus* offers several advantages such as a good growth yield and its acceptance as a safe microorganism. The production of β-galactosidase with *K. marxianus* (e.g., *Kluyveromyces marxianus* CCT 7082) is only one of the enzyme productions possible on whey lactose media (Manera et al. 2008). Optimizations of the β-glucosidase production with *K. marxianus* on cheese whey are currently driven forward. By application of fed-batch mode and optimized feeding, volumetric productivities of 291 $U \dot{c} L^{-1} \dot{c} h^{-1}$ have been reached (Rech and Ayub 2007). The fermentation of *Kluyveromyces marxianus* (MTCC 1288) on crude whey (non-deproteinized, nondiluted, and nonsterilized) for the production of ethanol has successfully been implemented in lab scale (Zafar and Owais 2006). The fermentation of whey to ethanol by *K. fragilis* has been described, as well. Here the problem of substrate (lactose) and product (ethanol) inhibition has to be circumvented. One solution is the application of immobilized cells in continuous reactors. The formation of ethanol by *S. cerevisiae* coimmobilized with β-glucosidase with lactose permeate as substrate has successfully been implemented. The process is designed with an evaporation membrane for energy saving ethanol separation (Lewandowska and Kujawski 2007). Furthermore, genetically modified *S. cerevisiae* and *E. coli* strains with encoded genes for the expression of lactose permease and β-galactosidase (e.g., from *K. lactis*) have been used for the lactose-based fermentation to ethanol or proteins (Adam et al. 2004).

5.2 Lactic Acid

Biodegradable polymers have been of great interest in the recent years. They can be applied in ecological packing materials and special applications like, e.g., wound dressing. Aliphatic polyesters possess the required properties like the degradability within less than a year and nontoxic hydrolyzation products. Among these, the polylactides have the most prominent role as building block for biodegradable foils. This can be explained by the ease of their production by fermentation with strains of *Lactobacillus* capable of growing on cheap raw substrates as whey. Lactic acids are produced in industrial production scale for their use in food products. Besides the lactic acid bacteria, the filamentous fungus *Rhizopus oryzae* is capable of accumulating lactate in starch and/or xylose. Lactic acid has recently been produced by fermentation on inexpensive substrates as lentil flour, kitchen waste barley hydrolysates, or liquefied cornstarch from cassava bagasse (Sauer et al. 2008). Furthermore, silage can be used as a source of readily available lactic acid throughout the year. The polymerization of lactic acid can be done by cationic or anionic polymerization or polymerization by the coordination-insertion mechanism based on the use of different alkoxide initiators (Kricheldorf 2001).

In 1997 Cargill and the Dow Chemical Company started a joint venture for the development of a commercial process for the industrial production of polylactic acid (PLA). With an investment of $300 million, a plant with a capacity of 14,000 tons was built in the USA. Future plants are planned to be capable of processing several other feedstocks such as agricultural waste (Jenck et al. 2004). For the established processes like the production of citric and lactic acid, the choice and availability of cheap substrates become increasingly crucial.

6 Research Needs and Summary

Today, the feedstock for raw material for industrial chemical processes is mainly based on petroleum oil. But within the near future, alternative reservoirs have to be extensively tapped on to avoid bottlenecks especially with regard to consumer products and pharmaceutical commodities, if the oil runs out. However one has to have in mind that alternative and sustainable feedstocks for chemical purposes can be represented only by a biomass feedstock.

Currently the prices of most bulk and specialty chemicals are too low for biotechnological routes to compete. It is estimated that competition begins at feedstock prices above 2$ per kilogram (Willke and Vorlop 2004). Nevertheless, the share of biotechnological produced chemicals is expected to increase from approx. 5% to 20% in the year 2010. The greatest impact is expected in the segment of fine chemicals with up to 60% of the products based on biotechnological processes. Interfacing with green biotechnology for enhanced crop properties and increased plant breeding can be expected (Hatti-Kaul et al. 2007).

Great attention is paid on the lignocellulose feedstock, which is extensively discussed and examined to be used as a sustainable raw material for ethanol production. In this chapter, we have presented a few current trends focusing on C1 carbonic compounds such as methanol and methane, fatty acids, and glycerol from plant oil and whey-based substrates that can be used as input compounds for a chemical refinery.

If one has a closer look to current activities of oil companies, it is obvious that the times are changing. As described in this chapter, several efforts to utilize sustainable biomass feedstocks for recovering fuel substitutes were carried out by these companies. But the exploitation and utilization of the biomass feedstock have to be implemented very carefully, to avoid such an already occurred competition of energy crops with food production, if first-generation biomass fuels were considered.

However, the complete substitution of fossil resources by biobased energy fuels and raw materials within the near future is quite improbable, considering the current process operation's dependency on large amounts of biomass feedstock. Therefore, socioeconomic trends must focus on a holistic approach, where fossil as well as biological resources have to be used in a complementary way.

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C1 Gas Refinery

27

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Abstract

C1 gas has been considered as a feedstock for biorefinery applications because CO, CO_2, and CH_4 can be easily obtained from synthetic and natural gases. C1 gas-fermenting microorganisms utilize these gases as sole carbon sources to generate cellular energy. Representatives of this class of microorganisms include cyanobacteria, acetogens, and methanotrophs, which have recently been engineered for the production of chemicals from such C1 gases. As it stands, the spectrum of target chemicals is limited to methanol, ethanol, butanol, isobutanol, isobutyraldehydes, acetone, 2,3-butanediol, lactate, butyrate, and terpenoids. In this chapter, we review the metabolic pathways for assimilation of C1 gases in cyanobacteria, acetogens, and methanotrophs. We also summarize the metabolic engineering strategies for developing host strains for the production of chemicals from C1 gases.

1 Introduction

The sustainable production of chemicals has gained increasing attention in recent decades because of problems related to climate change. A number of research groups have been engaged in developing new technologies for the production of chemicals from biomass, including decomposition methods for utilizing cellulosic raw materials and strain development for the chemical production from C5 to C6 sugars (Kamm 2007; Kim et al. 2015). In these processes, sugars serve as feedstock for sustainable chemical production using nonfood–based raw materials. More recently, feedstocks have expanded to include the C1 gases, carbon monoxide (CO) and carbon dioxide (CO_2) from synthetic gas, and methane (CH_4) from natural gas. Chemical production using C1 gases as feedstocks offers at least two advantages compared with sugar-based feedstocks: the broad distribution of CH_4 in nature and the low-complexity technology used to obtain synthetic gas from cellulosic biomass.

C1 gas-fermenting organisms primarily metabolize CO/H_2, CO_2, and CH_4 to obtain cellular energy through one of three C1 assimilation pathways: (1) the Calvin cycle, (2) the Wood-Ljungdahl pathway, and (3) the ribulose monophosphate (RuMP) or serine pathway (Clomburg et al. 2017; Durre and Eikmanns 2015; Jang et al. 2012; Liao et al. 2016). CO_2 can be used as a sole carbon source by both cyanobacteria and acetogens through the Calvin cycle and Wood-Ljungdahl pathway, respectively, whereas CO is metabolized together with H_2 only through the Wood-Ljungdahl pathway in acetogens. CH_4 can be used as a sole carbon source via the RuMP pathway or serine cycle in methanotrophs. Recently, these three types of microorganisms have been engineered to serve as hosts for the production of chemicals such as methanol, ethanol, butanol, isobutyraldehyde, isobutanol, acetone, 2,3-butanediol, lactate, butyrate, and terpenoids. The simple production of storage compounds, including lipids, fatty acids, and polyhydroxyalkanoates, is not been addressed here, because this topic has been well covered in recent reviews (Case and Atsumi 2016; Strong et al. 2015). In this chapter, we will describe in detail

the metabolic engineering strategies that can be used for the development of microbial strains as C1 gas refineries.

2 Cyanobacteria-Based Gas Refineries

Cyanobacteria naturally produce fatty acids, polyhydroxyalkanoates, and terpenoids from CO_2 through the Calvin cycle, one of the C1 assimilation pathways found in these bacteria (Ducat et al. 2011). In the first phase, CO_2 is converted into the carbon of ribulose 1,5-bisphosphate (RuBP) in a reaction catalyzed by ribulose-1,5-bisphosphate carboxylase/oxygenase. The next step is the reduction of RuBP to yield 3-phophoglycerate, which is used as a precursor for metabolite synthesis in cyanobacteria (Jablonsky et al. 2011). Building on this metabolic platform, researchers have extensively engineered cyanobacteria for the production of ethanol, butanol, isobutyraldehyde, isobutanol, 2,3-butanediol, and terpenoids. *Synechocystis* sp. PCC 6803 and *Synechococcus elongatus* PCC 7942, in particular, have been routinely used as hosts in metabolic engineering studies, because genome sequences of both strains, reported in 1996 and 2005, respectively, are known (Holtman et al. 2005; Kaneko et al. 1996).

2.1 Ethanol

Two cyanobacteria strains, PCC 7942 and PCC 6803, have been metabolically engineered to produce ethanol from CO_2. The ethanol-fermenting *Zymomonas mobilis pdc* and *adhII* genes, encoding pyruvate decarboxylase and alcohol dehydrogenase II – key enzymes in ethanol production – were introduced into *S. elongatus* PCC 7942 under control of the cyanobacterial *rbcLS* promoter. The resulting strain produced ethanol at 2.49 μg/OD/L/day from CO_2 (Deng and Coleman 1999). The same strategy was subsequently applied to strain PCC 6803, but replacing the *rbcLS* promoter with the strong promoter, *psb* AII. The engineered strain showed ethanol production of 0.24 g/OD/L/day from CO_2 (Dexter and Fu 2009). In 2012, the strain PCC 6803 expressing *Z. mobilis pdc* and *adhII* genes was engineered by disrupting the poly-hydroxybutyrate (PHB) synthesis pathway. In batch fermentation using the engineered strain, an ethanol titer of 5.50 g/L was achieved with a productivity of 0.21 g/L/day from CO_2 (Gao et al. 2012). In a later study, the PCC 6803 strain was further engineered using a strategy in which the glycogen synthetic *glgC* gene was also knocked out in addition to the disruption of the PHB pathway. Here, the *Z. mobilis pdc* and *adhII* genes were expressed under the control of the *nblA* promoter, which is inducible under nitrogen-starvation conditions. The surplus carbon flux achieved by knocking out the *glgC* gene was forwarded to ethanol production in the engineered strain through nitrogen starvation (Fig. 1). The resulting strain produced 2.96 g/L of ethanol at a specific rate of 0.24 g/gDCW/day and a volumetric production rate of 2.01 g/L/day (Namakoshi et al. 2016).

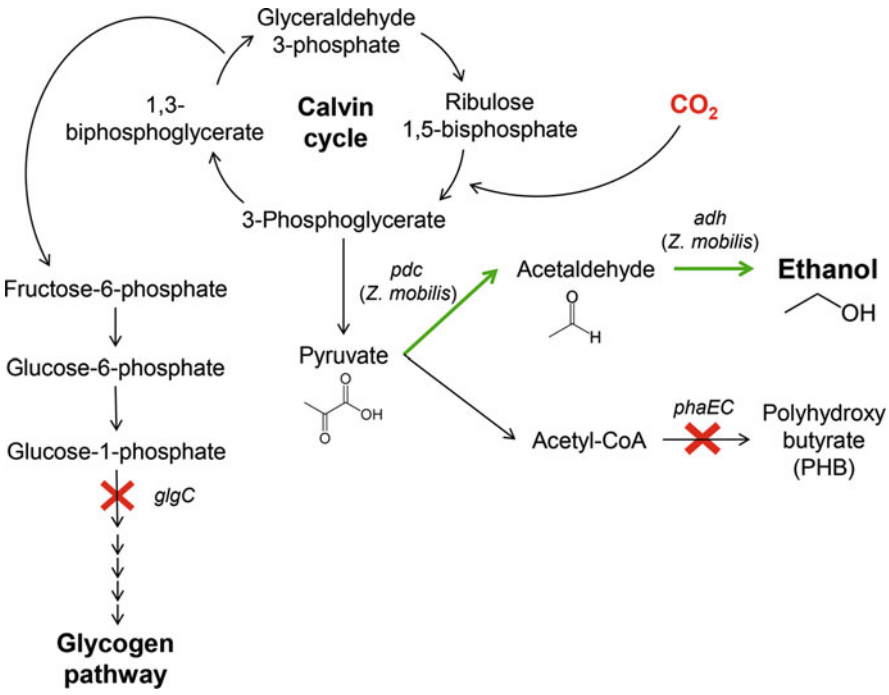

Fig. 1 The metabolic pathway for ethanol production in an engineered *Synechocystis* sp. PCC 6803 (Namakoshi et al. 2016). The green-colored arrow indicates heterologous expression. X indicates knockout. Gene and its coding enzyme: *pdc*, pyruvate decarboxylase; *adh*, alcohol dehydrogenase; *glgC*, glucose-1-phosphate adenylytransferase; and *phaEC*, enzymes associated with PHB synthesis

2.2 Butanol

A butanol-producing cyanobacterium was constructed using *S. elongatus* strain PCC 7942 through genomic integration of genes encoding enzymes of the butanol fermentation pathway, including *Treponema denticola* trans-enoyl-CoA reductase (*ter*); *Escherichia coli* acetyl-CoA acetyltransferase (*atoB*); and *Clostridium acetobutylicum* aldehyde alcohol dehydrogenase (*adhE2*), crotonase (*crt*), and 3-hydroxybutyryl-CoA dehydrogenase (*hbd*). The engineered *S. elongatus* strain produced 14.5 mg/L of butanol from CO_2 under anoxic conditions (Lan and Liao 2011). In a later study, an artificial pathway from acetyl-CoA to acetoacetyl-CoA via malonyl-CoA was constructed in the PCC 7942 strain in place of the endogenous pathway (acetyl-CoA to acetoacetyl-CoA), which is thermodynamically unfavorable. In the artificial pathway, the two-step reaction is catalyzed by endogenous acetyl-CoA carboxylase and *Streptomyces* sp. acetoacetyl-CoA synthase, encoded by *accABCD* and *nphT7* genes, respectively. The butanol synthetic pathway was finalized by the introduction of the chimeric enzymes acetoacetyl-CoA reductase (*phaB*), from *Ralstonia eutropha*; enoyl-CoA hydratase (*phaJ*), from *Aeromonas*

caviae; CoA-acylating butyraldehyde dehydrogenase (*bldh*), from *Clostridium saccharoperbutylacetonicum*; and NADPH-dependent alcohol dehydrogenase (*yqhD*), from *E. coli*. The resulting strain produced 29.9 mg/L of butanol from CO_2 in batch culture (Lan and Liao 2012). To improve butanol production in *S. elongatus*, Liao and colleagues (Lan et al. 2013) subsequently replaced the oxygen-sensitive *C. saccharoperbutylacetonicum* CoA-acylating butyraldehyde dehydrogenase with *Salmonella enterica* CoA-acylating aldehyde dehydrogenase encoded by the *pduP* gene (Fig. 2). Batch culture of this final strain resulted in butanol production of 0.40 g/L, with a productivity of 51 mg/L/day.

2.3 Isobutyraldehyde and Isobutanol

Isobutyraldehyde can be produced via the 2-ketoacid pathway and further converted to isobutanol (Atsumi et al. 2009). Isobutyraldehyde-producing cyanobacteria were constructed by introducing the 2-ketoacid pathway into *S. elongatus* strain PCC 7942 through integration of genes encoding *L. lactis* ketoacid decarboxylase (*kivd*); *Bacillus subtilis* acetolactate synthase (*alsS*); and *E. coli* acetohydroxy acid isomeroreductase (*ilvC*) and dihydroxy-acid dehyratase (*ilvD*). The *S. elongatus* PCC 6301 *rbcLS* gene encoding ribulose 1,5-bisphosphate carboxylase/oxygenase was also introduced (Fig. 3). Batch culture of the engineered strain produced 1.1 g/L of isobutyraldehyde with a productivity of 6.2 mg/L/h from CO_2 (Atsumi et al. 2009).

To expand the metabolic pathway to isobutanol from isobutyraldehyde in this strain, researchers from this same group introduced the *E. coli* gene *yqhD* and knocked out the *glgC* gene in the host (Fig. 4). Batch culture of the resulting strain produced 0.55 g/L of isobutanol from CO_2 (Li et al. 2014). In the metabolic engineering of the PCC 6803 strain for isobutanol production, the *L. lactis kivd* and *adhA* genes were integrated into the host's genome. In a batch culture equipped with a solvent (oleyl alcohol) trap, the production of isobutanol reached 0.30 g/L under mixotrophic culture conditions with 5 g/L glucose and CO_2 (Varman et al. 2013).

2.4 2,3-Butanediol

S. elongatus strain PCC 7942 was engineered for the production of 2,3-butanediol through genomic integration of the heterologous genes, *B. subtilis alsS*, *Enterobacter aerogenes* 2-acetolactate decarboxylase (*alsD*), and *Clostridium beijerinckii* alcohol dehydrogenase (*adh*) under the control of the *LlacO1* promoter. The resulting strain achieved 2,3-butanediol production of 2.38 g/L with a productivity of 9.8 mg/L/h from CO_2 (Oliver et al. 2013). In a study using *Synechocystis* sp. PCC 6803, the *Enterococcus faecalis als* gene and *Lactococcus lactis* acetolactate decarboxylase (*aldc*) and acetoin reductase (*butA*) genes were integrated into the genome (Fig. 5). Batch culture of the engineered strain resulted in 2,3-butanediol production of 0.43 g/L from CO_2 (Savakis et al. 2013).

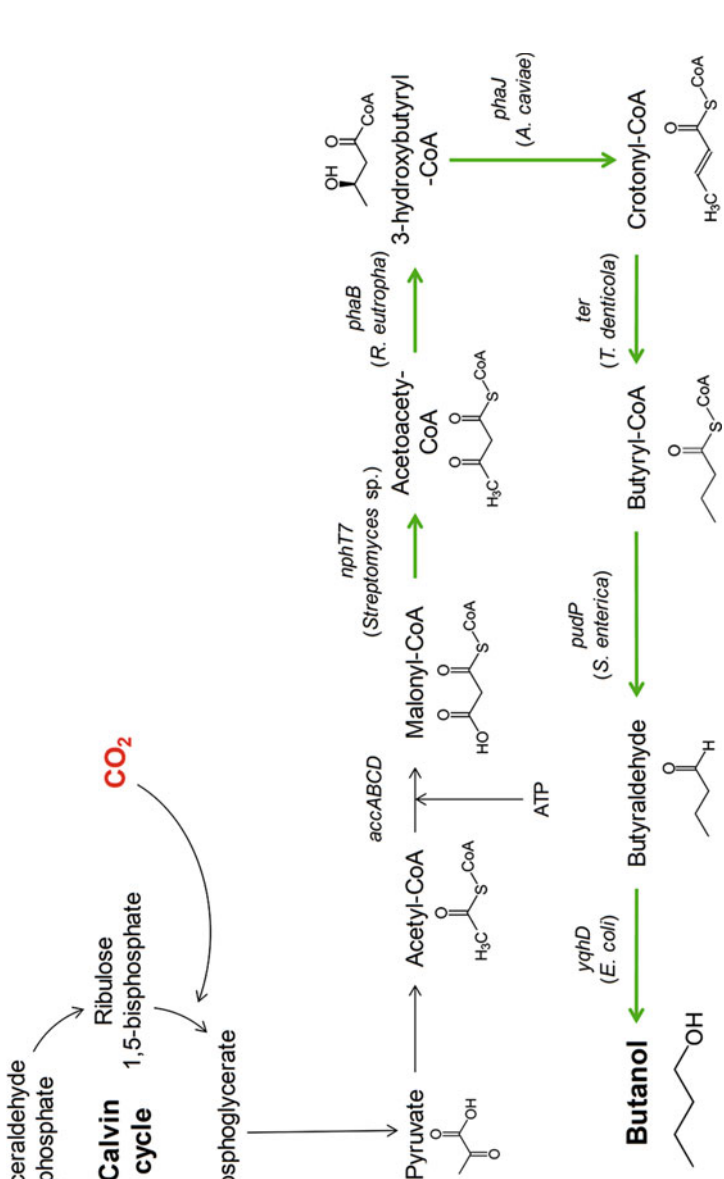

Fig. 2 The metabolic pathway for butanol production in an engineered *S. elongatus* PCC 7942 (Lan et al. 2013). The green-colored arrow indicates heterologous expression. Gene and its coding enzyme: *accABCD*, acetyl-CoA carboxylase; *nphT7*, acetoacetyl-CoA synthase; *phaB*, acetoacetyl-CoA reductase; *phaJ*, enoyl-CoA hydratase; *ter*, trans-2-enoyl-CoA reductase; *pduP*, CoA-acylating aldehyde dehydrogenase; and *yqhD*, NADPH-dependent alcohol dehydrogenase

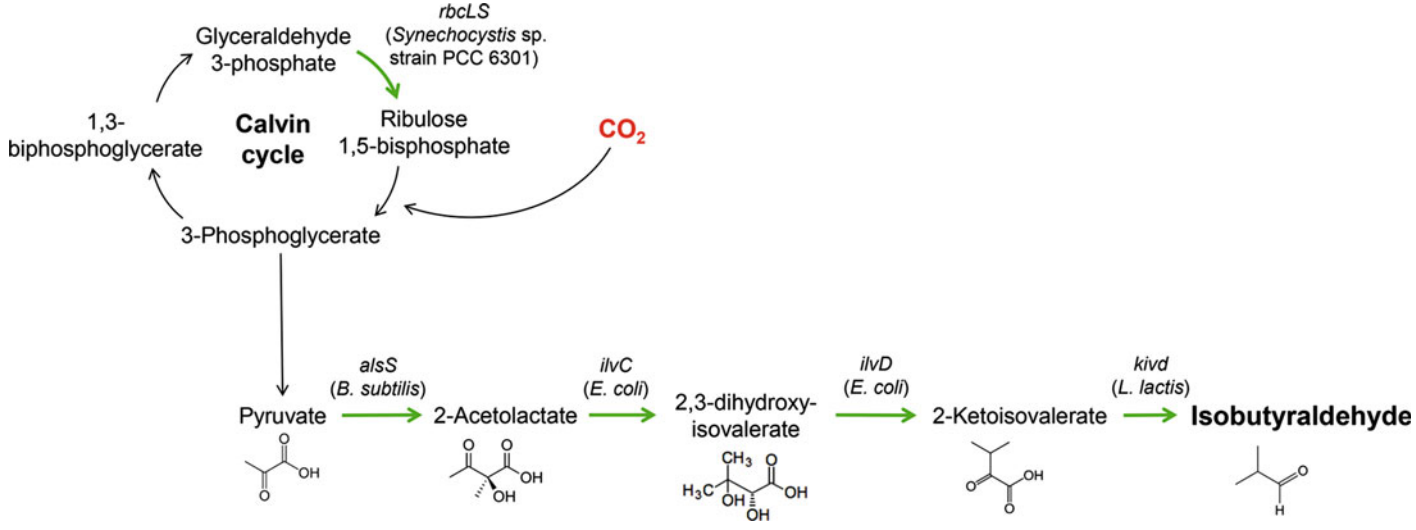

Fig. 3 The metabolic pathway for isobutyraldehyde production in an engineered *S. elongatus* PCC 7942 (Atsumi et al. 2009). The green-colored arrow indicates heterologous expression. Gene and its coding enzyme: *rbcLS*, ribulose-1,5-bisphosphate carboxylase/oxygenase; *alsS*, acetolactate synthase; *ilvC*, acetohydroxy acid isomeroreductase; *ilvD*, dihydroxy acid dehydratase; and *kivd*, ketoacid decarboxylase

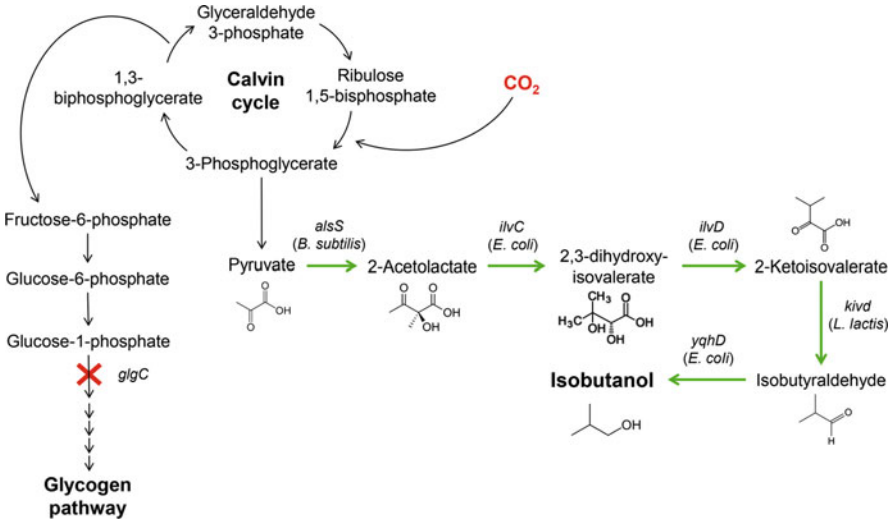

Fig. 4 The metabolic pathway for isobutanol production in an engineered *S. elongatus* PCC 7942 (Li et al. 2014). The green-colored arrow indicates heterologous expression. X indicates knockout. Gene and its coding enzyme: *alsS*, acetolactate synthase; *ilvC*, acetohydroxy acid isomeroreductase; *ilvD*, dihydroxy acid dehydratase; *kivd*, ketoacid decarboxylase; and *yqhD*, aldehyde reductase (NADPH-dependent alcohol dehydrogenase)

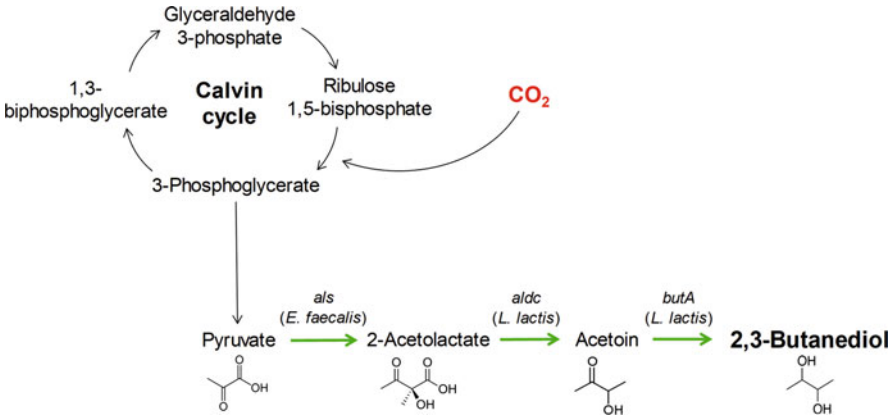

Fig. 5 The metabolic pathway for 2,3-butanediol production in an engineered *Synechocystis* sp. PCC 6803 (Savakis et al. 2013). The green-colored arrow indicates heterologous expression. Gene and its coding enzyme: *als*, acetolactate synthase; *aldc*, acetolactate decarboxylase; and *butA*, acetoin reductase

2.5 Terpenoids: β-caryophyllene and Isoprene

Metabolic engineering of *Synechocystis* sp. PCC 6803 for the production of β-caryophyllene and isoprene was reported by two different research groups. In nature, cyanobacteria harbor methyl-erythritol-4-phosphate (MEP) pathways, in which the intermediates dimethylallyldiphosphate (DMAPP) and isopentenyl diphosphate (IPP) are formed from glyceraldehyde-3-phosphate to yield terpenoids. A strain capable of producing β-caryophyllene was developed by introducing the gene for *Artemisia annua* (QHS1) β-caryophyllene synthase into the PCC 6803 strain. The resulting strain converted farnesyl diphosphate into β-caryophyllene at a rate of 0.03 μg/gDCW/h (Reinsvold et al. 2011).

For the production of isoprene, *Pueraria montana* isoprene synthase (*ispS*) was introduced into cyanobacteria *Synechocystis* sp. PCC 6803. The resulting strain converted DMAPP into isoprene, producing isoprene at a rate of 50 μg/gDCW/day (Lindberg et al. 2010).

3 Acetogen-Based Gas Refineries

Acetogens are native producers of acetate, ethanol, butanol, and 2,3-butanediol from synthetic gas through the Wood-Ljungdahl pathway. Recent studies have expanded the production portfolio of acetogens to include isobutanol, acetone, lactate, butyrate, and terpenoids through metabolic engineering. The Wood-Ljungdahl pathway is one of the efficient C1 metabolic pathways, in which two branched pathways supply methyl and carbonyl groups to yield acetyl-CoA (Jones et al. 2016). In the branch that supplies the methyl group, CO_2 is reduced to formate by formate dehydrogenase (Fig. 6). Formate, in turn, is reduced to methyl tetrahydrofolate

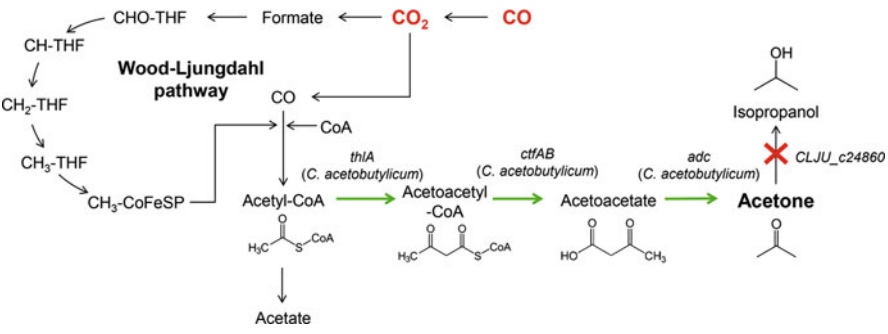

Fig. 6 The metabolic pathway for acetone production in an engineered *C. ljungdahlii* (Jones et al. 2016). The green-colored arrow indicates heterologous expression. X indicates knockout. Gene and its coding enzyme: *thlA*, thiolase; *ctfAB*, CoA transferase; *adc*, acetoacetatedecarboxylase; and *CLJU_c24860*, primary-secondary alcohol dehydrogenase

(THF) via formyl-THF and methylene-THF. The methyl group is transferred to corrinoid-iron-sulfur protein (CoFeSP) to yield methylated CoFeSP (Fig. 6). In the branch that supplies the carbonyl group, the carbonyl group is formed from CO_2 by carbon monoxide dehydrogenase (CODH). In the final step, which employs the acetyl-CoA synthase (ACS)/CODH complex, the carbonyl group is used to form acetyl-CoA accompanied by the consumption of a mole of methyl group and CoA. Acetogens have recently been engineered for the production of chemicals from synthetic gas or fructose. Major hosts used in these metabolic engineering studies are *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, *Acetobacterium woodii*, and *Moorella thermoacetica* (Banerjee et al. 2014; Hoffmeister et al. 2016; Jones et al. 2016; Kita et al. 2013; Liew et al. 2017).

3.1 Butanol, Acetone, and Lactate

An engineered *C. ljungdahlii* strain capable of producing butanol was developed using all the genes responsible for the butanol pathway in *C. acetobutylicum*. In this application, the *thlA-hbd-crt-bcd-bdhA-adhE2* gene cluster was expressed under the control of the *ptb* promoter in conjunction with the *adc* terminator. In batch fermentation, the engineered strain produced 0.15 g/L of butanol from syngas at the peak production stage (Kopke et al. 2010). In a recent study using wild-type *Clostridium carboxidivorans*, which natively produces butanol, 1 g/L of butanol production was achieved simply by optimizing media components (Phillips et al. 2015). This study also reported the production of other alcohols, including 3 g/L of ethanol and 0.9 g/L of hexanol (Phillips et al. 2015).

Acetone-producing acetogens have also been developed through metabolic engineering of *C. ljungdahlii* and *A. woodii* (Banerjee et al. 2014; Hoffmeister et al. 2016; Jones et al. 2016). In each case, the acetone pathway was constructed using the *thlA-ctfAB-adc* genes from *C. acetobutylicum*. The metabolically engineered *A. woodii* produced 0.76 g/L of acetone from CO_2 and H_2, while producing 3.19 g/L of acetone from 10.8 g/L of fructose (Hoffmeister et al. 2016). The engineered *C. ljungdahlii* produced about 0.8 g/L of acetone and 1.3 g/L from CO and fructose, respectively (Banerjee et al. 2014). More recently, the acetone operon was introduced into the secondary alcohol dehydrogenase knockout mutant of *C. ljungdahlii* (Fig. 6). The resulting strain produced 10.8 g/L of acetone from fructose with a theoretical yield of 74% (Jones et al. 2016).

The lactate-producing acetogen, *M. thermoacetica*, was constructed by genomic integration of the *Thermoanaerobacter pseudethanolicus* lactate dehydrogenase gene under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter. The engineered *M. thermoacetica* produced 0.61 g/L of lactate from fructose (Kita et al. 2013).

3.2 Ethanol and Butyrate

The acetogens *C. autoethanogenum* and *C. ljungdahlii*, native producers of ethanol and butyrate, respectively, have been metabolically engineered by two different

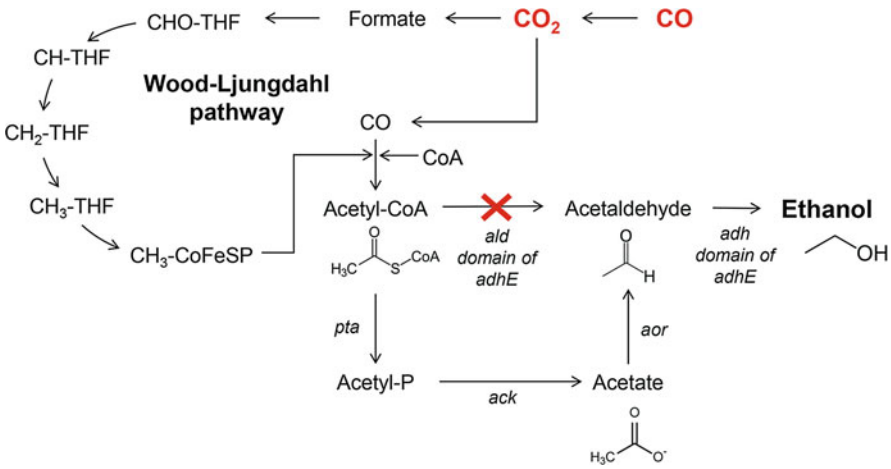

Fig. 7 The metabolic pathway for ethanol production in an engineered *C. autoethanogenum* (Liew et al. 2017). X indicates knockout. Gene and its coding enzyme: *pta*, phosphotransacetylase; *ack*, acetate kinase; *aor*, aldehyde:ferredoxin oxidoreductase; and *adhE*, bifunctional aldehyde/alcohol dehydrogenase

research groups for enhanced production of the corresponding compounds (Liew et al. 2017; Ueki et al. 2014). To construct the strain for enhanced ethanol production, Liew et al. (2017) knocked out the aldehyde dehydrogenase domain of *adhE1* in *C. autoethanogenum*. The engineered strain produced ethanol mainly through the acetyl-CoA/acetyl-P/acetate/acetaldehyde route, owing to the presence of the mutant *adhE1* gene (Fig. 7). The *adhE1* mutant produced 2.46 g/L of ethanol from CO, a production 1.83-times higher than that observed in the wild type (Liew et al. 2017). However, ethanol production from fructose did not differ between the *adhE1* mutant and wild type (Liew et al. 2017). For enhanced production of butyrate in *C. ljungdahlii*, the *C. acetobutylicum thl-crt-bcd-etfAB-hbd-ptb-buk* genes were expressed under the control of the *pta* promoter by integrating these genes into the genome while knocking out the *pta* and *ctf* genes (Ueki et al. 2014). The engineered *C. ljungdahlii* strain showed a butyrate production of ~1.3 g/L from CO_2/H_2 and ~2.4 g/L from fructose, values comparable to those of the wild type (~0.69 g/L and ~0.35 g/L, respectively) (Ueki et al. 2014).

4 Methanotroph-Based Gas Refineries

Methanotrophs have been considered as hosts for CH_4 refineries because they are capable of using CH_4 as a sole carbon source. In methanotrophs, the key metabolite, formaldehyde, is formed from CH_4 via methanol. Formaldehyde is further metabolized to yield cellular energy through either the RuMP or serine pathway in conjunction with tetrahydrofolate, pentose phosphate (PP), ethylmalonyl-CoA, and citric acid pathways. Gas refinery applications considered for methanotrophs include the production of methanol, lactate, and carotenoids.

4.1 Methanol

Although methanol can be produced from CH_4 in a single step mediated by methane monooxygenase, no efficient methanol-producing methanotrophs have been reported. Methanol accumulation of less than 1 g/L was observed in cultures of methanotrophs supplemented with methanol dehydrogenase inhibitors, such as phosphate and sodium chloride (Lee et al. 2004; Mehta et al. 1987). A recent study reported the production of 1.1 g/L of methanol through process optimization using high-cell-density cultures of *Methylosinus trichosporium* (17 gDCW/L) in optimized medium containing 400 mM phosphate, 10 mM $MgCl_2$, and 20 mM sodium formate (Duan et al. 2011). Relatively high concentration of phosphate was used for the inhibition of methanol dehydrogenase in the high-cell-density culture of the strain, but no cytotoxicity was reported.

4.2 Lactate

Methanotroph (*Methylomicrobium buryatense*) and methanogen (*Methanosarcina acetivorans*) were recently engineered for the production of lactate (Henard et al. 2016; McAnulty et al. 2017). In one study using methanotroph, *Lactobacillus helveticus* lactate dehydrogenase was expressed in *M. buryatense*, obtaining a strain that produced 0.8 g/L of lactate with a yield of 0.05 g/g of CH_4 in aerobic culture (Henard et al. 2016). In the other study using methanogen, which is not able to utilize CH_4 as a carbon source, a methyl-utilizing enzyme (methyl-coenzyme M reductase

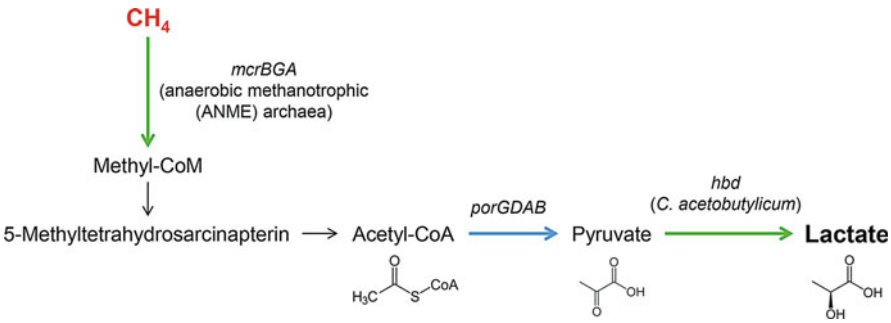

Fig. 8 The metabolic pathway for lactate production in an engineered methanogen *M. acetivorans* (McAnulty et al. 2017). In the engineered strain, methane can be assimilated through heterologous methyl-coenzyme M reductase encoded by the *mcrBGA* genes from anaerobic methanotrophic (ANME) archaea. The colored arrows indicate homologous overexpression (blue) and heterologous (green) expression. Gene and its coding enzyme: *porGDAB*, pyruvate synthase; and *hbd*, 3-hydroxybutyryl-CoA dehydrogenase

encoded by the *mcrBGA*) was introduced from anaerobic methanotrophic archaea in *M. acetivorans* (Fig. 8). A lactate-producing pathway was developed by overexpression of the *porGDAB* genes together with introduction of the *C. acetobutylicum hbd* gene. The enzyme encoded by *porGDAB* catalyzes the conversion of acetyl-CoA to yield pyruvate. In this study, the enzyme encoded by the *C. acetobutylicum hbd* gene was reported to catalyze the conversion of pyruvate to yield lactate in *M. acetivorans*. Endogenously, Hbd in *C. acetobutylicum* catalyzes the conversion of acetoacetyl-CoA to 3-hydroxybutyryl-CoA. The engineered *M. acetivorans* strain achieved a lactate production yield of 0.59 g/g of CH_4 (the exact production titer was not reported) (McAnulty et al. 2017).

4.3 Terpenoids: C40 Carotenoids

The *Methylomonas* sp. wild-type strain, a C30 carotenoids producer, was engineered for the production of C40 carotenoids by genomic integration of a gene cluster responsible for C40 carotenoids production (Sharpe et al. 2007). In this study, the transposon *Tn5* was used to integrate the gene cluster, without a promoter, into the locus of the *fliS* gene at nucleotide position 140, allowing expressing of the genes under the control of the promoter for the flagellin operon (*fliCGDS*). To block C30 carotenoids production, Sharpe et al. (2007) blocked the *crt* promoter in the C30 carotenoids pathway. The engineered *Methylomonas* sp. produced 2.0 mg/gDCW of C40 carotenoids from CH_4 (Sharpe et al. 2007). The study was expanded to the selective production of astaxanthin, another C40 carotenoid. In this latter study, the gene cluster responsible for astaxanthin production was further overexpressed by employing the strong hexulose-6-diphosphate synthase promoter in conjunction with two-copy integration of the gene cluster. The engineered strain showed astaxanthin production of 2.4 mg/gDCW (Ye et al. 2007). In another study, bacterial hemoglobin was co-expressed with the astaxanthin pathway in *Methylomonas* sp. to increase oxygen availability, which resulted in higher astaxanthin production compared with the parent (the exact production of astaxanthin was not reported) (Tao et al. 2006).

5 Research Needs

It is clear that C1 gas refineries are alternatives to oil refineries. However, the current technology for supporting C1 gas refineries is inefficient in terms of strain-development and gas-fermentation processes. The efficiency of chemical production will be further improved by expanding chemical portfolios through systems metabolic engineering of C1 gas-fermenting microorganisms. Further improvements can be expected through transplantation of the C1 metabolic pathway into microbial hosts that have previously been developed for the production of chemicals.

Acknowledgments This work was supported by grants from the C1 Gas Refinery Project (NRF-2016M3D3A1A01913250) and the Basic Science Research Program (NRF-2016R1D1A3B04933184) from the Ministry of Science, ICT and Future Planning (MSIP) through the National Research Foundation (NRF) of Korea.

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Chemical Production: Biohalogenation

28

Cormac D. Murphy

Contents

Abstract

Natural and synthetic organohalogens are used in a variety of applications, and with an increasing emphasis on sustainable methods of production, enzymes that can form carbon-halogen bonds are an attractive alternative to classical synthetic approaches. Since the discovery of $FADH_2$-dependent halogenases 20 years ago, there has been a dramatic increase in our understanding of biological halogenation reactions, with several distinct classes of halogenating enzymes identified. In this chapter, an overview of the various halogenases is given and examples of how these can be applied to the production of biotechnologically important compounds.

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1 Introduction

Organohalogens are used in a wide range of applications, including pesticides, plastics, fire-resistant materials, refrigerants, pharmaceuticals, and solvents, and thus much research effort is spent on the development effective methods for forming carbon-halogen bonds. Sustainable production of chemicals favors the use of enzymes where possible, and enzymes that catalyze the formation of carbon-halogen bonds have significant biotechnological relevance.

Chloroperoxidase from the fungus *Caldariomyces fumago* was the first halogenating enzyme to be discovered by Lowell Hager and colleagues (Shaw and Hager 1959), and for 40 years this class of enzyme was the only one known to catalyze the formation of carbon-halogen bonds, in this case through the production of free hypohalous acid. A new post-haloperoxidase era started with the discovery of PrnA, which catalyzes the regiospecific chlorination of tryptophan as the first step of the pyrrolnitrin biosynthetic pathway in *Pseudomonas fluorescens* (Hohaus et al. 1997). Subsequently, other classes of halogenating enzymes have been identified, including the first fluorinase from *Streptomyces cattleya* (O'Hagan et al. 2002). These discoveries have resulted in new strategies for the production of halogenated chemicals. This chapter provides an overview of the key applications of biohalogenation and identifies the future challenges for the technology.

2 Classes of Halogenating Enzymes

In broad terms, halogenating enzymes can be categorized according to the nature of the halogenating reagent formed in the active site. There are enzymes that employ an electrophilic halogenating species such as HOCl ($FADH_2$-dependent halogenases, haloperoxidases), a nucleophilic mechanism with halide ion (fluorinases and chlorinases that use *S*-adenosyl methionine as a substrate), or halogen radical (nonheme iron halogenases). Examples of the reactions catalyzed by the different classes are shown in Fig. 1.

3 Haloperoxidases

These are a class of enzymes that employ Cl^-, Br^-, and/or I^- to reduce hydrogen peroxide; the resulting hypohalous acid is, in all but the rarest cases, released from the enzyme active site where it can react with electron rich substrates. The initial work on *C. fumago* chloroperoxidase resulted in the development of a very convenient spectrophotometric assay with monochlorodimedone as the substrate for halogenation. This assay was used to identify haloperoxidases in many organisms but disguised the fact that haloperoxidases were too promiscuous to be involved in

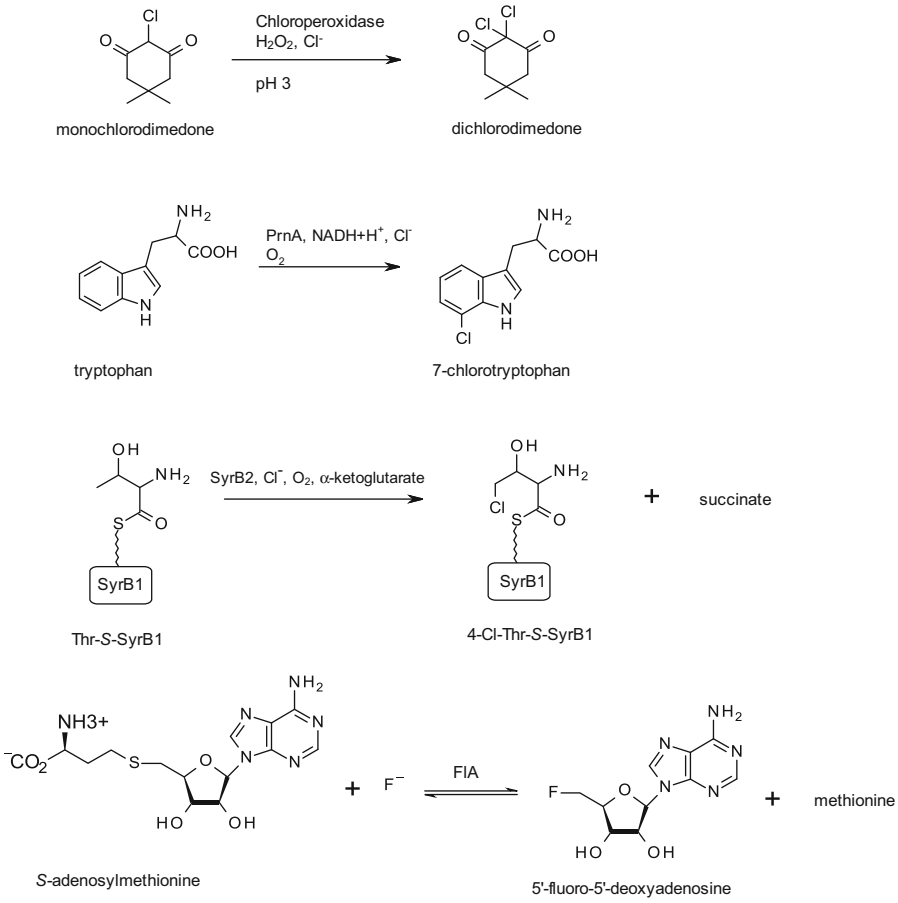

Fig. 1 Examples of enzyme-catalyzed halogenations. Enzymes: PrnA, $FADH_2$-dependent halogenase; $SyrB_2$, nonheme iron, O_2- and α-ketoglutarate-dependent halogenase; *FIA* fluorinase

halometabolite biosynthesis (Murphy 2003). Sequencing of halometabolite biosynthetic gene clusters (Hammer et al. 1997) and the discovery of other halogenase enzymes demonstrated that haloperoxidases do not normally have a halogenating function in nature. One notable exception to this is the vanadium chloroperoxidases Mcl24 and NapH1 (Bernhardt et al. 2011; Diethelm et al. 2014), which are found in *Streptomyces* spp. and are involved in the biosynthesis of the polyketide-terpenoid metabolites merochlorins and napyradiomycins, respectively. Both enzymes catalyze a regiospecific chlorination that enables subsequent cyclization of their substrates. Interestingly, neither enzyme can catalyze the chlorination of the classical chloroperoxidase substrate, monochlorodimedone, which clearly distinguishes them from other haloperoxidases.

4 $FADH_2$-Dependent Halogenases

A step change in our understanding of biological halogenation occurred with the discovery of an $FADH_2$-dependent halogenase, PrnA, which is responsible for the chlorination of tryptophan in the first step of pyrrolnitrin biosynthesis (Hohaus et al. 1997). Subsequent mechanistic studies revealed that hypochlorous acid was formed in the active site from chloride and flavin hydroperoxide; this is somewhat similar to haloperoxidases, which generate free hypohalous acid. However, unlike haloperoxidases, the HOCl reacts with an active site lysine enabling regioselective chlorination of the tryptophan substrate (Dong et al. 2005). A similar enzyme, RebH, from *Lechevalieria aeroclogenes* and is involved in rebeccamycin biosynthesis, was found to generate a lysine chloramine intermediate within the active site which was the ultimate chlorinating reagent (Yeh et al. 2007). Other $FADH_2$-dependent halogenases were recognized in biosynthetic gene clusters of halogenated natural products, and it has been determined that this class of halogenase is involved in the halogenation of electron-rich substrates such as phenyl and pyrrole (van Pee 2012). In vitro assessment of activity has proven difficult owing to lack of knowledge of the substrate or the requirement of some substrates to be tethered to a carrier protein.

5 Nonheme Iron, O_2- and α-Ketoglutarate-Dependent Halogenases

Secondary metabolites such as barbamide from *Lyngbya majuscula* (cyanobacterium) and syringomycin from *Pseudomonas syringae* contain chlorine on an aliphatic carbon, and the biosynthesis of these molecules involves a distinctive halogenase. The initial sequencing of the biosynthetic genes for these compounds revealed that flavin-dependent halogenases were not involved (Chang et al. 2002), but genes with homology to those coding for nonheme Fe (II), α-ketoglutarate-dependent enzymes were identified, and it was proposed that these were responsible for the chlorination reactions. Vaillancourt et al. (2005) first demonstrated in vitro activity of these distinct halogenase enzymes, by expressing *syrB1*/*B2* from the syringomycin gene cluster in *E. coli*. SyrB2 chlorinates threonine that is attached to SyrB1 (which contains adenylation and thiolation domains) via a radical mechanism in which decarboxylation of the α-ketoglutarate yields oxyferryl Fe(IV) = O, which enables hydrogen atom extraction from the substrate, leading to halogenation by rebound (Blasiak et al. 2006). In vitro and in silico analyses of biosynthetic gene clusters suggest that many of these halogenases only accept substrates tethered to a carrier protein. However, Hillwig and Liu (2014) identified a nonheme Fe (II), α-ketoglutarate-dependent halogenase in cyanobacteria that biosynthesize hapindole alkaloids and which accepted freestanding substrates.

6 Fluorinase and Chlorinase

Compared with chlorinated and brominated metabolites, fluorinated natural products are rare, mainly because fluorine-containing minerals, such as calcium fluorite, are insoluble, the small fluoride ion is heavily solvated in aqueous solution, and the redox potential of fluoride is too high for the generation of an electrophilic fluorinating species. Nevertheless, organofluorine compounds are known from some plants and bacteria, and in the actinomycete *Streptomyces cattleya*, which produces fluoroacetate and 4-fluorothreonine, the first fluorinase was identified (O'Hagan et al. 2002). The enzyme, FlA, catalyzes the nucleophilic substitution of methionine on S-adenosylmethionine (SAM) to generate the first fluorometabolite, 5′fluoro-5′deoxy-adenosine (5′-FDA), of the fluoroacetate/4-fluorothreonine biosynthetic pathway (Fig. 1). The crystal structure of FlA shed light on the mechanism of the reaction, in which water is stripped from the fluoride ion in the active site via hydrogen bonding to an active site serine and SAM binding, enabling it to react with the 5′-carbon of the substrate in an S_N2 manner (Zhu et al. 2007). With the *flA* gene in hand, fluorinase enzymes in other bacteria have been identified by homology searching and their in vitro activity demonstrated (Deng et al. 2014). Interestingly, an enzyme that catalyzes a cognate chlorination was identified in the marine bacterium *Salinospora tropica*, which produces the chlorinated polyketide/nonribosomal peptide salinosporamide (Eustaquio et al. 2008). The chlorinase, SalL, has a high degree of homology with FlA, but lacks a 23-amino acid loop in the N-terminal domain that is present in the fluorinase, and cannot catalyze the fluorination of SAM.

7 Halogenases in Biocatalysis

As almost all haloperoxidases lack regiospecificity and substrate specificity, the biocatalytic application of these enzymes is limited. Nevertheless, there are some examples that illustrate the usefulness of the enzymes to efficiently produce hypohalous acids for subsequent halogenation reactions. The vanadium-dependent chloroperoxidase from the fungus *Curvularia inequalis* was shown to be an excellent biocatalyst for the preparative scale bromination of thymol (Fernandez-Fueyo et al. 2015). The enzyme is much less sensitive to hydrogen peroxide, which is a main cause of instability in heme-dependent haloperoxidases; can tolerate the presence of cosolvents, such as ethanol; and displays exceptionally high catalytic activity, which enabled the complete conversion of 100 mM thymol to brominated products in 24 h with 100 nM enzyme.

Catalyzing the formation of C-F bonds is a considerable synthetic challenge; thus the discovery of a fluorinase (O'Hagan et al. 2002) opened the possibility of enzyme-catalyzed fluorinations. The biotechnological value of the fluorinase (FlA) is exemplified with its use in the preparation of fluorine-18-labeled compounds that might be valuable as positron emission tomography tracers. For example, by exploiting the

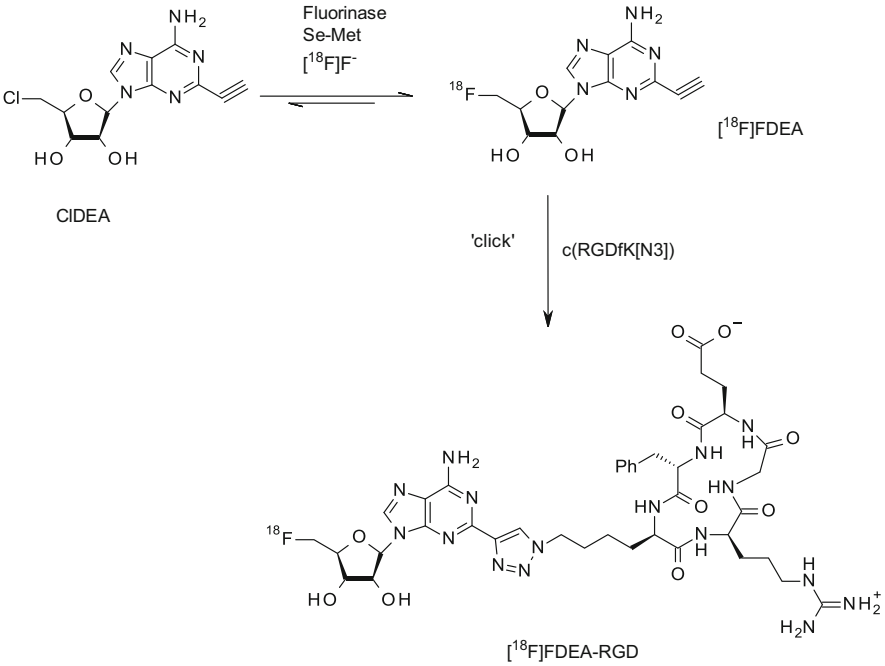

Fig. 2 Chemoenzymatic synthesis of fluorine-18-labeled RGD peptide. The fluorinase is employed to transhalogenate the starting substrate 2-ethynyl-5′-chloro-5′-deoxyadenosine (*Cl DEA*) via selenoSAM (not shown) to the F-18-labeled analogue, FDEA. The peptide is introduced using a Cu-catalyzed click reaction

transhalogenating ability of FlA, it has been demonstrated that [^{18}F]-5′-fluoro-5′-deoxy-2-ethynyladenonsine ([^{18}F]FDEA) can be formed from 5′-chloro-5′-deoxy-2-ethynyladenonsine in approx. 70% radiochemical conversion (Thompson et al. 2015). Subsequently, the [^{18}F]FDEA was used in a "click" reaction to couple the acetylene moiety with the azide on a cRGD peptide to generate a radiolabeled peptide (Fig. 2). The method does not require the radioactive fluoride to be azeotropically dried from the [^{18}O]water in which it is generated, and the final product is arrived at within 2 h, when an excess of FlA is applied.

The tryptophan halogenase PrnA, which chlorinates tryptophan to yield 7-chlorotryptophan in the first step of pyrrolnitrin biosynthesis in *Pseudomonas* spp., was the first halogenase to be discovered that did not belong to the chloroperoxidase class (Keller et al. 2000). Interestingly, both types of enzyme employ HOCl as the halogenating agent, but the crucial difference lies in the release of free HOCl from chloroperoxidase, which can spontaneously react with electron-rich substrates in the milieu, but not from PrnA, which also binds the tryptophan in the active site where HOCl is produced (Dong et al. 2005). The discovery of this regioselective halogenase, and the subsequent identification of similar halogenases, such as RebH in *Lechevaleria aerocologenes*, was biotechnologically exciting since

aryl halides are widely employed intermediates in the synthesis of pharmaceuticals and agrochemicals. However, scaling up the halogenation reactions catalyzed by tryptophan halogenases proved difficult owing to poor catalytic efficiency, meaning that large culture volumes were required to generate small amounts of halogenated product (Payne et al. 2013). Frese and Sewald (2015) demonstrated that by using a multifunctional cross-linked enzyme aggregate (combiCLEA) strategy to immobilize RebH, PrnF (flavin reductase), and ADH (alcohol dehydrogenase for coenzyme regeneration) enabled repeated reuse of the enzymes from moderate culture volumes and yielded gram quantities of 7-bromotryptophan.

8 Production of Novel Halometabolites in Cultures

It has been well established that by altering culture conditions that new halogenated secondary metabolites can be biosynthesized by microorganisms, for example, by replacing chloride for bromide salts in the medium, it is possible to encourage production of brominated analogues of chlorinated natural products (Clark et al. 2007). Furthermore, the enzymes in natural product biosynthetic pathways often display sufficient promiscuity to accommodate unnatural substrates. Thus, addition of halogenated biosynthetic precursors can lead to the production of new halometabolites with modified biological properties (Clark et al. 2011; Mahoney et al. 2014). De novo production of a new fluorinated metabolite was demonstrated by Eustaquio et al. (2010), who knocked out the chlorinase SalL in the marine bacterium *Salinospora tropica*, and expressed the *S. cattleya flA* in the resulting mutant. SalL catalyzes the first reaction in the biosynthesis of salinosporamide A, which is the chlorination of SAM producing the intermediate 5′-chloro-5′-deoxyadeonsine (Eustaquio et al. 2008). The reaction is analogous to that catalyzed by FlA; thus the expectation was that if SalL was replaced with FlA, fluorosalinsporamide A could be biosynthesized (Fig. 3), and this proved to be the case.

While many researchers see the production of a new halogenated metabolite as a means to obtain novel compounds with altered bioactivity, an interesting extension of the strategy was described by Deb Roy et al. (2010) who employed the PrnA enzyme involved in pyrrolnitrin biosynthesis to produce metabolites that could be subsequently easily chemically modified. Accordingly, *prnA* was cloned in *Streptomyces coeruleorubidus*, which biosynthesized pacidamycin, a nonribosomal peptide that includes tryptophan. The chlorinated derivatives thus formed (Fig. 4) were functionalized for cross-coupling reactions to generate a suite of semisynthetic pacidamycin analogues.

9 Discovery of New Halogenases

Genome sequencing efforts have led to the discovery of numerous putative halogenating enzymes, although few have been investigated in vitro. The genome mining approach has also led to the discovery of new fluorinase variants from different

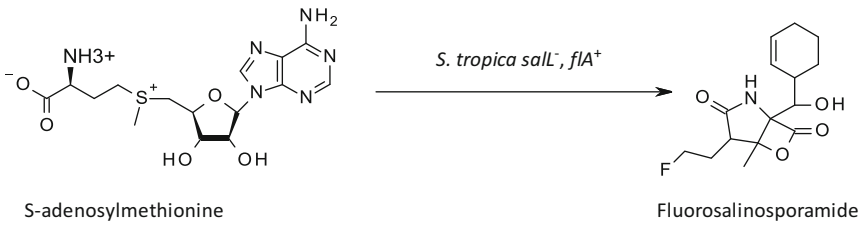

Fig. 3 Biosynthesis of fluorosalinosporamide by a mutant strain of *Salinospora tropica*

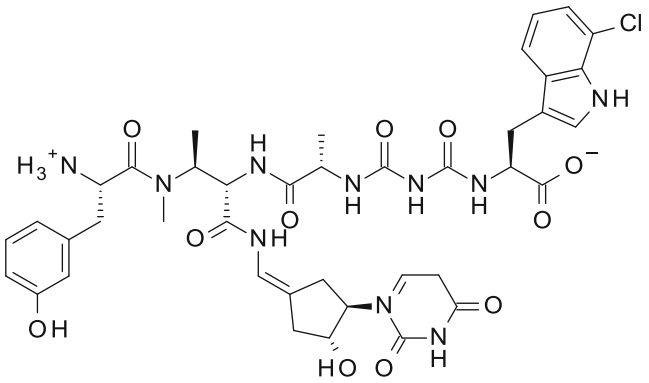

Fig. 4 Chloropacidamycin produced by *S. coeruleorubidus* expressing the tryptophan halogenase PrnA

bacteria (Deng et al. 2014), including one with enhanced kinetic properties and stability (Ma et al. 2016), which might be useful as a biocatalyst. The fluorinated natural product nucleocidin was originally isolated in 1957 from the bacterium *Streptomyces calvus* and has a distinctive structure including a 5′ O-sulfamoyl group and 4′-fluorine substitution on adenosine (Fig. 5). For decades it had not been possible to reestablish production of the fluorometabolite in *S. calvus* cultures; however, Zhu et al. (2015) discovered that a mutant *bldA* gene was responsible for the biosynthetic deficiency, and once a functional gene was cloned into the bacterium, nucleocidin production was restored. This is the first step in the identification of a fluorinating enzyme that is distinctive from that of *S. cattleya*.

10 Research Needs

As with other potentially useful enzymes, the key research needs are enzyme stability, improving catalytic efficiency, and broadening the substrate scope. There have been several studies investigating mechanisms to improve stability, for example, the fluorinase FlA has been immobilized on a polymer composed of glycidyl

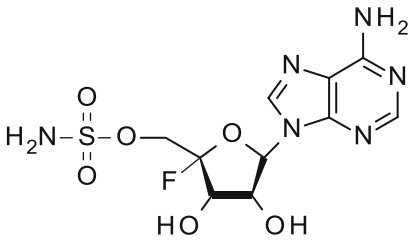

Fig. 5 The structure of nucleocidin

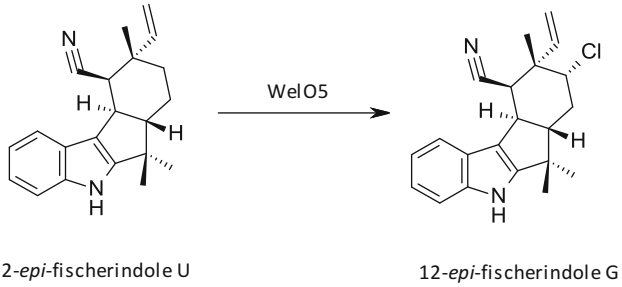

Fig. 6 Nonheme Fe(II), α-ketoglutarate-dependent-catalyzed halogenations of a freestanding hapindole-type substrate

methacrylate and ethylene dimethacrylate, which enabled repeated application of the enzyme in [^{18}F] FDA biosynthesis (Sergeev et al. 2013). Additionally, a fluorinase variant has been discovered in *S. xinghaiensis* that retained 87% of its initial activity after 4 days at 25 °C, and thus is relatively robust and may be more readily applied to upscaled processes (Ma et al. 2016). The other classes of halogenases are notoriously labile, and in order for these to be widely applied in biocatalysis, this issue must be addressed. One potential approach is to use a natural immobilization mechanism such as the producing strain cultivated as a biofilm, which are robust structures that afford additional longevity to bioprocesses involving other enzymes (Winn et al. 2012).

Two major limitations impeding halogenase applications are the narrow substrate specificity of the enzymes thus far identified and the requirement, for many halogenases, for substrates to be tethered to a carrier protein. In relation to the latter characteristic, the discovery of WelO5 (Hillwig and Liu 2014), which can chlorinate indole substrates that are not carrier bound (Fig. 6), is an exciting development and provides an enzyme that is more easily employed to introduce a halogen to a carrier-free substrate.

Strategies that are employed successfully to change the substrate profile of other enzymes have also been applied to halogenases in an effort to broaden the substrate specificity. For example, Payne et al. (2015) evolved RebH with a substrate walking approach to generate mutant enzymes, mainly through error-prone PCR, that could accept bulkier indoles and carbazoles as substrates. Significantly, Shepherd et al.

(2016) reported the successful switching of regioselectivity of a tryptophan 6-halogenase (SttH, from *Streptomyces toxytricini*) to favor 5-chlorination, by using a structure-guided mutagenesis approach to identify, and subsequently change, key residues involved in regioselectivity. Such strategies are likely to be successful for the tailoring of halogenases to custom biohalogenating catalysts.

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Rediscovering Biopolymers

29

M. Bassas-Galià

Contents

Abstract

The increasing concern for nondegradable plastic wastes effect as well as the need for new alternative materials to petrochemical plastics has triggered out much interest into biotechnological biopolymers. Polymers exist in all microorganisms and their environments, showing different biological functions and rather diverse and fascinating properties with a wide range of countless "potentially" biotechnological and industrial applications. Bio-based polymers are expected to triplicate their production capacity in 2021. Polyhydroxyalkanoates (PHAs), polylactides (PLA), polycaprolactone (PCL), starch- and cellulose-based polymers, or chitin (chitosan) are some examples of these biopolymers. In fact, PLA and PHA polymers together with bio-based PET polymers show the fastest rates of market growth. In this chapter various biopolymers and their applications, covering areas such as packaging, medicine, agriculture, tissue engineering, or

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pharmaceuticals, are discussed. Overall, this review shows biopolymers as a good and "natural" alternative to conventional and nonbiodegradable plastics.

1 Introduction: Biopolymers Versus Petrochemical Plastics

Nowadays one may consider that we live in the era of plastics. In the last 60 years, petrochemical derivatives have become one of the most widely used materials. Produced or derived from nonrenewable energy sources (like crude oil, natural gas, naphtha, or coal) plastics, as synthetic polymers, can be chemically manipulated to have a wide range of strengths and shapes and therefore a large number of applications. From packaging to medicine, it is this high versatility, technical properties, and relatively low cost of production that have contributed to their widespread use, and consequently, a global dependence. Up to 140 million tonnes per annum of a wide variety of petroleum-based synthetic polymers are manufactured worldwide and thus significantly contributing, with 57 million tons of plastic waste accumulation annually, to the pollution of the environment (Shah et al. 2008). Most of the conventional plastics, such as polyethylene, polyvinyl chloride, polypropylene, polystyrene, and polyethylene terephthalate, are resistant to microbial degradation, due to their "relatively new" presence in nature, natural enzymes capable of degrading such synthetic structures do not exist. At present, there exists an increasing trend to slowly start replacing these plastics for the so-called biodegradable ones. Polymers' biodegradability responds not only to the raw materials used for their production but also to the chemical structure of the material and their final degradation products. Despite the fact that the worldwide production of consumer plastics continues to be dominated by nondegradable petroleum based polymers, there are two main driving forces that have made biodegradable polymers economically more attractive: the environmental and economic concerns related to the final waste disposal and the unsustainable nature and increasing expenses petroleum production derivatives. However, along with the enormous burden these place on fossil resources, their disposal is difficult since many are made from nonbiodegradable materials, like polyethylene and polypropylene. Some petroleum-based polymers, termed "oxodegradable plastics," contain additives to facilitate their degradation; however, only very few (such as polycaprolactone (PCL)) are truly biodegradable (Shimao 2001). With the world entering a new era with new priorities and concerns for green energies and recycling, there is growing interest in new biodegradable polymers which can be produced efficiently and from renewable resources: a tendency reflected in the 30% annual increase in the consumption of biodegradable plastics (Leaversuch 2002; Philip et al. 2007). Given the changing views and an unavoidable reliance on plastics in a broad range of fields, biotechnologists have been prompted to design new polymer composites to replace their nonbiodegradable counterparts derived from fossil resources (Steinbüchel 2005). The increasing concern regarding the environmental effects of "petrochemical plastics" has led to place a greater emphasis on "natural" and biotechnological polymers (Jung et al. 2011). Such polymers are believed to be inherently

biodegradable and often biocompatible conferring thus considerable advantages over other conventional synthetic products in respect of both their eco-friendly appeal and low production costs (Williams et al. 1999; Zinn et al. 2001; Luengo et al. 2003).

According to the data reported at the 11th European Bioplastics conference in Berlin (Germany), the European Bioplastics' market update has revealed that the global bioplastics industry's production capacity was set to increase from around 4.2 million tonnes in 2016 to 6.1 million tonnes in 2021, packaging the largest market for bioplastic and representing almost 40% (1.6 million tonnes) of the total bioplastics share in 2016. The report shows an increase in the uptake of bioplastics in sectors such as consumer goods (22% or 0.9 million tonnes) or in the construction and building sector (13% or 0.5 million tonnes). Bio-based, nonbiodegradable plastics, such as polyurethanes (PUR) and bio-based PE and bio-based PET, are the main drivers of this growth, with PUR making up around 40% and PET over 20% of the global bioplastics production capacities. On the other side, production capacities of biodegradable plastics, such as PLA, PHA, and starch blends, are also growing steadily, and their production is estimated to increase from 0.9 million tonnes in 2016 to almost 1.3 million tonnes in 2021. This data illustrates an important trend, driven by changing consumer demands, to make plastic products more resource efficient and to reduce greenhouse gas emissions and the dependency on fossil resources. This trend is the result of substantial investments in research and development by the many innovative small and large companies that concentrate their strengths on the development of bio-based products designed with the circular economy in mind (http://www.plasticsnewseurope.com/article/20161130/PNE/161139990).

2 Biodegradable Polymers and the Importance of Biotechnology in their Production

As discussed in the previous section, the growing concerns about current petroleum-based production and the consequent accumulation of plastic waste in landfills and in natural habitats, together with the leaching of chemicals due to plastic disposal in the environment and the direct impact in animals and humans, draw the attention into bioplastics, derived from bio-based polymers (Thompson et al. 2009; Andrady 2017). Unlike the chemically synthesized polymers, the bio-based polymers are produced by living organisms, such as plants, fungi, or bacteria. Some microorganisms are particularly capable in converting biomass into biopolymers while employing a set of catalytic enzymes.

The two main criteria that define the classification of biopolymers or bioplastics are the source of raw materials and the biodegradability of the polymer. The term biopolymers include either biodegradable polymers (i.e., plastics made from fossil materials) or bio-based polymers (i.e., plastics produced from biomass or renewable resources). Biodegradable polymers can be made based on either renewable or nonrenewable resources (Fig. 1).

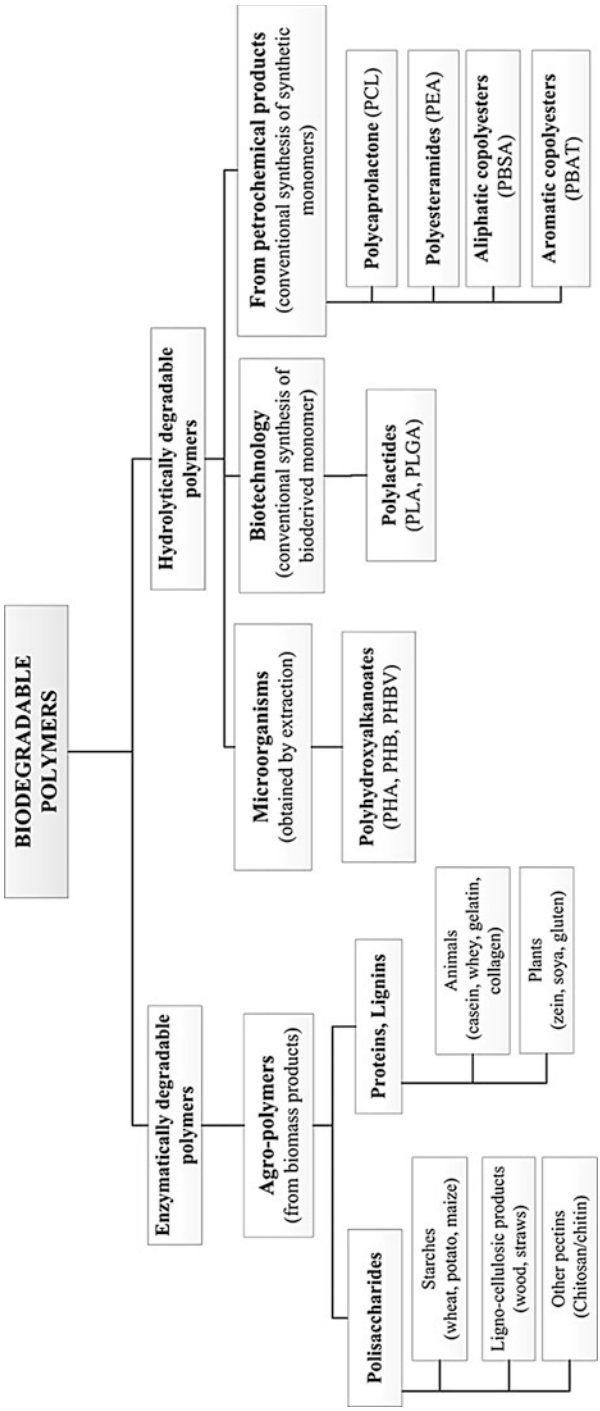

Fig. 1 Classification of biodegradable polymers: natural and synthetic

The American Society for Testing Materials (ASTM) and the International Standard Organization (ISO) has defined "degradable plastics" as those that undergo a significant change in their chemical structure under specific environmental conditions (Kolybaba et al. 2003). Biodegradable plastics may usually be decomposed not only from the action of microorganisms such as bacteria, fungi, and algae, but also by photochemical and enzymatic reactions. However, such characteristics also elicit increased instability and low performance of the polymer.

There are mainly three types of bioplastics in the commercial scale of production:

(i) Plastics derived from fossil carbon source but biodegradable
(ii) Plastics derived from polymers converted from biomass and biodegradable
(iii) Plastics derived from polymers converted from biomass but not biodegradable

The bioplastics available in the market are made from polymers such as starch-based polymers, polyhydroxyalkanoates (PHAs), polylactic acid (PLA), and other polymers derived from renewable sources (Table 1).

The polymers for bioplastics are usually produced in the biological fermentation processes using renewable and sustainable agricultural feedstocks, such as sugar, starch, oil, or lignocellulosic biomass. Agricultural materials were thought to be a good alternative as an energy source and a feedstock to produce biopolymers. Recently, the expectations in this area have considerably decreased due to the poor results obtained. Current researches in this direction are focused on the development of new technologies using model plant systems, industrial crops constructed via genetic modification routes (Poirier et al. 1992; Valentin et al. 1999; Matsumoto et al. 2009) to reduce the differences in the production costs between synthetic plastics and bioplastics, as well as to improve the physicochemical properties. Nowadays, most of the research on the production of biopolymers is still at the proof of concept or early research stage of development, while only a few have been

Table 1 Polymers for bioplastics on the market (Adapted from Elnashar 2011)

Polymer	Company	Scale (tonne/year)
Biodegradable but not bio-based		
Synthetic polyester	Ecoflex (BASF)	14,000
Polyvinyl alcohol (PVA)	Wanwei	100,000
Biodegradable and bio-based		
Starch-based materials	Novamont	60,000
Cellulose-base materials	Innovia films	30,000
Polylactides (PLA)	Natureworks	140,000
Polyhydroxyalkanoates (PHA)	Metabolix (ADM)	50,000
Bio-based but nonbiodegradable		
PDO from bio-based glycerol	Tate and Lyle (DuPont)	45,000
PE from bioethanol	Braskem (Dow)	200,000 (planned)
PVC from bioethanol	Solvay	360,000 (planned)
Polyamides (PA) from oils	Arkema	6000 (planned)

on commercial scales (Chen 2009). Most of these polymers are manufactured via the microbial fermentation routes using genetically modified microorganisms in order to improve the production yields of new bioplastics (Rehm et al. 1998; Madison and Huisman 1999; Olivera et al. 2001; Sandoval et al. 2005; Jung et al. 2010; Yang et al. 2010; Jung and Lee 2011). The development of sustainable processes using metabolic engineering has been essential to be able to transform microorganisms into efficient cells factories. Systems metabolic engineering approach has facilitated the creation of new metabolic enzymes/pathways or the enhancement of the existing ones in order to optimize the production of specific products. For instance, this technology can either be applied in the direct production of specific polymers (e.g., PLA and copolymers) in an innovative and efficient manner (Yang et al. 2010; Choi et al. 2016) or in the synthesis of monomers that later on can be further polymerized or used as synthons (Jang et al. 2012; Lee et al. 2012; Choi et al. 2015). Some examples of biopolymers produced by fermentation are certain polyesters, such as polyhydroxyalkanoates (PHAs), which are produced by a range of microbes under different nutrient and environmental conditions (Steinbüchel and Hein 2001; Bassas et al. 2006; Prieto et al. 2007; Verlinden et al. 2007). These polymers are accumulated as storage materials, allowing microbial survival under stress conditions (Sudesh et al. 2000). PHAs could be copolymerized with other polymers to achieve specific properties. PHAs derived plastics are considered as the best candidates to replace the current petroleum-based plastics due to their durability in use and wide spectrum of properties. The family of PHAs polymers is one of the most promising biodegradable materials to emerge in recent years. Up to date, there are more than 100 different monomers of PHA polyesters (Chen 2009). PHA-based bioplastics display similar properties to those of PE and PP, ranging in properties from strong, moldable thermoplastics to highly elastic materials to soft, sticky compositions. Polylactic acid (PLA) is another biopolymer (thermoplastic polyester) commonly used and produced by chemical polymerization of the D- and L- lactic acids obtained from fermentation (Madhavan Nampoothiri et al. 2010). PLA bioplastics show similar properties as those made from petroleum-derived polyethylene terephthalate polymer. Besides applications in plastics, PLA-derived bioplastics are used extensively in biomedical applications, such as sutures, drug release, stents, dialysis devices, orthopedic devices, and evaluated as a matrix for tissue engineering (Park et al. 2008; Yao et al. 2009; Shi et al. 2011). Even though PHAs and PLA could be considered as synthetic polymers, since they are not found in the nature, they are fully biodegradable (Stevens 2003).

Although the technologies were developed many years ago, large-scale production of polymers from biomass was not feasible because those technologies were too expensive. However, in recent years, the innovations from the research sectors, particularly those on biotechnology, have made some of the biological conversions able to compete with the existing fossil-based processes.

Another interesting approach in this field is the connection between molecular biology and polymer chemistry. This trend is especially evident with protein-based polymers, although initial applications for these types of engineered materials were focused mainly on medical or pharmaceutical needs due to the high production costs.

3 Biopolymers Applications

Biopolymers are likely to succeed in the market based just on sustainability or environmental considerations. Unfortunately, nowadays they can only compete with the petrochemical plastics if they show "special properties" that are lacking in the traditional plastics. Some of these biotechnological biopolymers have been used as hydrophobic coatings, specialty elastomers, medical implants, functionalized polymers for chromatography, microgranules used as binders in paints or blends that incorporate latex, and as a source of chiral compounds. Some of the most representative applications are described in the following paragraphs and are summarized in Table 2.

3.1 Packaging Applications

Currently, the use of biopolymers in packaging, especially in food technology, is increasing due to their biodegradability. It is estimated that 41% of all plastics are being employed for packaging applications, and moreover, almost half of these are used for food packing. In general plastic packaging materials are contaminated by foodstuffs and biological substances, which makes the recycling process most of the time not convenient economically (Siracusa et al. 2012). Owing to the large and growing consumption of plastic for these purposes, biodegradable polymers from renewable resources are receiving a considerable attention.

Among biodegradable polymers, ***PLA*** is one of the most promising, since it is easily produced in a large scale by the fermentation of carbohydrate feedstock from agriculture or food industry waste products. In addition, it is relatively inexpensive and has some remarkable properties that are equivalent or even better than those exhibited by synthetic plastics (transparency and cellophane-like mechanical features). PLA has been used in various forming processes, such as extrusion molding, injection molding, blow molding, extrusion foaming, fibers and nonwoven fabric, and mono-filament yarn (Obuchi and Ogawa 2010). For food packaging, the use of PLA is limited because of the ductility, thermal, and barrier properties (Martino et al. 2011; Arrieta et al. 2013). However, in order to enhance these properties, the use of additional substances, which must be approved as safe for food application, has been studied. For instance, Arrieta et al. investigated the effect of addition of limonene and showed that limonene acted as a plasticizer when incorporated in PLA films, increasing the elongation at break, and decreasing the elastic modulus PLA (Arrieta et al. 2013). As a result of the increase in the chain mobility, the barrier properties were reduced. Another example is the addition of antimicrobial substances to prevent food contamination by microorganisms (Jin et al. 2009; Mascheroni et al. 2010; Özge Erdohan et al. 2013; Lantano et al. 2014).

PLA is currently used to produce blowing films, which have been successfully applied as garbage bags and related products as well as molded objects such as disposable cutlery and plates, and food packing or shipping materials. Besides PLA,

Table 2 Classification of different commercialized biodegradable plastics. Polymers composition and applications

Polymer	Commercial name	Manufacturer	Applications
Cellulose acetate	Bioceta® Biocell	Mazzucchelli	Packaging films, tool handles, hairdressing items, perfume bottle cups, tooth brushes, shoe heels, toys, oil containers, textiles
Microbial cellulose	Biofill/ Gengiflex	BioFill Productos Biotecnologicos	Medicine (ulcers, temporary artificial skin, periodontal applications)
PLA	Natureworks® PLA	Cargill Dow / Natureworks	Film, rigid packaging
Polylactide/PLA	LACEA® PLA	Mitsui chemicals Inc	Packaging straps, electronic packaging, phone cards
PLA-based (blend of Ecoflex® and PLA)	Ecovio®	BASF	Packaging films, shopping and waste bags, agricultural mulch films, plant pots
PLA-based (PET)	Biomax®	DuPont	Waste bags, seed mats
Aromatic-aliphatic-copolyesters	Ecoflex®	BASF	Packaging purposes, hygienic disposable wrapping, food packing, agriculture films
Aromatic-aliphatic-copolyesters	Eastar bio®	Eastman	Disposable packaging, cutlery, bin liners
Starch/copolymer	Bioplast®	Biotec	Food containers, bags, cutlery, films
Starch blend with PLA/PHBV	Paragon™	Avebe	Packaging
Starch/synthetic polymer	Mater-bi®	Novamont	Packaging, disposable items, personal care and hygiene, toys, shopping bags, mulch films
P(3HB)	Biomer®	Biomer	Nonwoven cover stocks, hygienic products (nappies and sanitary towels), paramedical film applications in hospitals
P(4HB)	TephaFLEX®	Tepha Inc.	Medicine (surgical mesh)
P(3HB-co-3 HV)	Biopol®	Biomer	Bottles, oil containers, medicine
Several PHAs		Shandong Lukang Pharmaceutical	Raw materials and medical applications
P(3HB-co-3HHx)	Nodax®	Procter & gamble	Films, injection molded utensils, coated paper cup, plastic bags, manufacture biodegradable films used in agriculture
Soybean oil	Soyoyl™	Urethane soy systems co	Vehicle panels

(*continued*)

Table 2 (continued)

Polymer	Commercial name	Manufacturer	Applications
PCL	Tone®	Union carbide	Food-contact foam trays, loose fill, and film bags
PBS	Bionelle®	Showa Highpolymer	Mulch film, packaging film, bags, and hygiene products
Polyester amide	BAK®	Bayer	Films, bags, and containers

other polymers such as starch, PHAs, cellulose, and chitin or chitosan are also being employed for these purposes (Table 2) (Garde et al. 2000).

Starch is a polymer economically competitive with petroleum, which has been used for preparing compostable plastics for many decades (Avella et al. 2005). This natural polymer, produced by plants, is composed of linear amylose and branched amylopectin (Mohanty et al. 2000) and is one of the cheapest biodegradable materials available in the market. However, the polymer itself does not form films with adequate mechanical properties unless it undergoes specific treatments (plasticization) (Weber 2000; Glenn et al. 2014). Table 2 shows some examples of thermoplastic starch currently present in the market (Mater-Bi or Bioplast®).

PHAs are polymers produced directly by natural or genetically modified microorganisms. Depending on their monomer composition, which is related with the nature of the carbon source as well as the microorganism itself, these biopolymers will show different physicochemical properties and therefore might be applied in several potential fields (Steinbüchel and Valentin 1995; Garcia et al. 1999; Witholt and Kessler 1999; Bassas-Galià et al. 2015). Initially PHAs were employed to make everyday articles such as shampoo bottles and other packaging materials (Hocking and Marchessault 1994). The first consumer product made from PHA was launched in April 1990 by Wella AG. Over the last decade, applications have increased both in variety and specialization, for example, for motor oil containers, films, or paper-coatings. However, these kinds of materials possess some disadvantages compared with conventional plastics. To improve their properties several copolymers containing different hydroxyalkanoates units have been synthesized (Mohanty et al. 2000). For instance, polyhydroxybutyrate (PHB) or some of its copolymers, polyhydroxybutyrate-co-polyhydroxyvalerate (PHBV), have excellent gas barriers properties which can be exploited for food packaging applications as well as a wide range of nonpackaging uses (Mohanty et al. 2000). Paragon materials, based on thermoplastic starch in combination with PLA or PHBV, result in barrier coating, which has been employed successfully in cheese packaging or thermoformed containers (Tuil et al. 2000) (Table 2). However, modification of PHA properties such as addition of softeners is still needed to enhance the elasticity and make it more competitive with traditional petroleum-based plastics.

Cellulose is one of the most abundantly occurring natural polymers, consisting of a linear chain of d-glucose/anhydroglucose, and has excellent film-forming properties. It has been utilized for many decades in this field (Steinbüchel 2005);

nevertheless, due to its hydrophilic nature, insolubility, and crystalline structure, it is difficult to process for a bulk production. However, cellulose esters (cellulose acetate) are successfully used in biodegradable applications. This sort of polymer offers a wide variety of uses ranging from adhesive tapes to textiles and related materials (Table 2).

Chitin (and chitosan, commercially produced by deacetylation of chitin) is another naturally occurring molecule that represents the second most abundant polysaccharide resource after cellulose (Kittur et al. 1998; Ravi Kumar 2000; Elsabee and Abdou 2013). One of the main characteristics of this polymer is the formation of films with excellent gas barrier properties, therefore it is being used as an edible coating (Krochta and De Mulder-Johnston 1997; Swain et al. 2014). Moreover, chitin is also being successfully employed in food packaging applications due to its interesting antimicrobial features and its capability to absorb heavy metal ions (Makino and Hirata 1997; Chandra and Rustgi 1998) developed biodegradable film consisting of a combination of chitosan-cellulose-polycaprolactone polymers that may be used in modified atmosphere packaging of fresh products. Finally, materials based on proteins, with either plant or animal origin, have also excellent properties for using them in the packaging market, especially as edible coatings. For example, ***casein*** was employed as thermoset plastic during 1940s and 1950s and is still being used for bottle labeling due to its adhesive features. ***Collagen*** is a fibrous animal protein with excellent applications in food industry. This molecule is commonly employed as a raw material for the production of gelatin, a food additive with high potential for film and foam production (Weber 2000).

These biodegradable plastics are currently available in the market and produced from plant biomass (cellulose), raw materials (PLA), or by biotechnological processes (Ecoflex® or BAK®) using natural or genetically modified microorganisms like in the case of Biopol® (copolymer of PHBV). During the last decades, consumption of one-disposable products and the demand for safe and minimally processed "fresh" foodstuff (meat, fruit and vegetables, dairy products, ready meals, snacks, and dry or frozen foodstuff) increased. Owing to this, the industry must develop and select packaging systems that also offer marketing and cost advantages in the waste disposal. To succeed and justify their high costs, packaged products should preserve the quality and offer longer and better storage characteristics than conventional plastics do. Biota™ (PLA bottled water), Noble™ (bottled juices), and Danone™ (yogurts) are only some examples of PLA-derived food packing products.

The special characteristics of PLA together with its potential use in antimicrobial packaging make this polymer suitable for food packaging applications (Jin et al. 2008). Currently, an alternative to overcome the microbial growth in packaged food is the addition, directly or slowly released from packaging film materials, of antimicrobial proteins or bacteriocines that provide a continuous antimicrobial effect (Siragusa et al. 1999). Recently, a specific polymer capable of delivering nisin (a heat stable bacteriocin produced for some strains of *Lactococcus lactis* against gram positive bacteria often present as contaminants in food) (Jin and Zhang 2008) has been developed. Sodium caseinate films (Kristo et al. 2008) or methylcellulose and hydroxypropyl methylcellulose (Franklin et al. 2004) are some examples of

these kinds of materials. Salmaso et al. (2004) were the first to incorporate nisin in PLA polymers but first studies of nisin-PLA-based packaging did not appear until 2007 (Liu et al. 2007). Recently, a bacterial cellulose film containing nisin has also been developed and used on the surface of vacuum-packaged sausages to control the growth of *Listeria monocytogenes* and total aerobic bacteria (Nguyen et al. 2008). Nowadays, packages based on compostable materials, which are biodegradable and do not produce any negative effect during the composting process, are becoming widely used. This contributes to environmental preservation and the reduced use of nonrenewable resources.

3.2 Biomedical and Pharmaceutical Applications

The range of biomaterials available to use in tissue engineering as scaffolds, which support cell growth and later reabsorption in leaving viable tissue, has been limited. Somehow, this fact has slowed down the more widespread application in the field of tissue engineering, particularly in areas where there is a significant mismatch between the physical and the mechanical properties of target tissue and the availability of suitable biomaterials. Usually the ones with good properties are neither biodegradable nor biocompatible.

PHAs polymers and especially some of their copolymers seem to be good candidates as biomaterials for tissue engineering due to their excellent biodegradability and biocompatibility (Wu et al. 2009). It has been already described that PHB is compatible with mammalian cells and is reabsorbed at low rates, since it is hydrolyzed to natural occurring secondary metabolites. In 2007, poly (4-hydroxybutyrate) (P4HB) was approved by the FDA for clinical application as surgical sutures (Wu et al. 2009). Other applications of PHAs polymers are multifilament surgical sutures, wound dressings, pericardial substitutes (Sodian et al. 2002; Opitz et al. 2004), slow release drug delivery systems, disposal syringes, surgical swabs, and blister packs and strips. Moreover, PHB has been described to have piezoelectric properties. Therefore, it can be used as potential material for biodegradable fixative plates that could actually stimulate bone formation (Chen and Wu 2005). Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) was studied as an injectable implant system for postsurgical tissue adhesion prevention, showing better performance than PLA (Dai et al. 2009). PHBHHx as well as other PHAs can also be applied as various tissue implants like heart valves, blood vessels, cartilage or tendon, nerve conduits, artificial esophagus (Wang et al. 2008; Saxena et al. 2010; Qu et al. 2006; Mendelson et al. 2007). Another copolymer found to have good expectations in bone healing treatments is Degrapol®. Degrapol® is a block-copolyester urethane and is chemically synthesized from P(3HB)-diol and α, ω-dihydroxy-poly(ε-caprolactone-block-diethyleneglycol-block-ε-caprolactone) (Philip et al. 2007). In the early 1990s, PHAs became candidates as drug carriers due to their biodegradability and biocompatibility. Microspheres of PHB loaded with rifampicin were investigated for being used later as a chemoembolizing agent (Kassab et al. 1997; Zinn et al. 2001). The PHBV copolymers with varying

monomer ratios were used in the construction of controlled antibiotic systems to deliver tetracycline hydrochloride (TC) or neutralized TC in vitro release studies (Sendil et al. 1999). Nanoparticles made of PHA have been used for targeted delivery and controllable release of antitumor agents, inhibitors of metabolism, and even pesticides (Kılıçay et al. 2011; Lu et al. 2011; Dinjaski and Prieto 2015). However, due to the lipophilic nature of PHA, PHBHHx nanoparticles are difficult to use for encapsulation of hydrophilic drugs, e.g., insulin.

PHAs are considered enantiomerically pure polymers since they are mainly composed by [R]-3-hydroxycarboxylic acid. This group of biopolymers has been described to be good chiral synthons in the synthesis of antibiotics, vitamins, flavors, etc. The [R]-3-hydroxybutyric acid (R3HB) is used as a chiral building block for the synthesis of carbapenem antibiotics (Chiba and Nakai 1985). Furthermore Seebach et al. reported the use of these 3-hydroxyacids as chiral building blocks for the total synthesis of macrolides such as pyrenophorin, colletodiol, garamycin A1, and elaiophylidene (Seebach et al. 1986). Moreover, these compounds have also been described to have potential antimicrobial and/or antiviral activities (Ruth et al. 2007). PHAs can be easily depolymerized into an optically active, pure, and bifunctional hydroxy acid. PHB, for example, can be hydrolyzed to [R]-3-hydroxybutyric acid for being used afterwards in the synthesis of Merck's antiglaucoma drug Truspot® (Reddy et al. 2003). Along with [R]-1,3-butanediol, it is also used in the synthesis of β-lactams. PHA monomers and their methyl esters also have promise to be used as drugs or chiral intermediates for Alzheimer disease, osteoporosis, or Parkinson disease and even memory improvement (Zhang et al. 2013a; Cao et al. 2014).

Another example is the ***PLA*** that is considered as biodegradable and biocompatible in contact with living tissues. PLA can be abiotically degraded (i.e., simple hydrolysis of the ester bond without requiring the presence of enzymes to catalyze it). During the biodegradation process, the enzymes degrade the residual oligomers until final mineralization (biotic degradation). Since the basic monomer (lactic acid) is produced from renewable resources (carbohydrates) by fermentation, PLA complies with the rising worldwide concept of sustainable development and is classified as an environmentally friendly material (Dong and Feng 2007). Currently, PLAs have been produced on a semi-commercial scale for several companies (Evonik, Corbion Purac, etc.). The physical characteristics of PLGA, such as molecular weight, degradation rate, or reabsorption time, can be controlled by the synthesis method employed and by changing the chemical composition (Avgoustakis 2008; Lü et al. 2009). Its use in medicine has been extensively explored (Shimao 2001), for instance as implants, development of microspheres sutures, protein encapsulation and delivery, as well as drug delivery systems (Ikada et al. 1996; Castelli et al. 1998; Singh et al. 2001; Chandy et al. 2002; Hu et al. 2003; Caliceti et al. 2004; Ouchi et al. 2004; Zhang et al. 2006; Gupta and Kumar 2007). Because of its relatively strong mechanical properties, PLA has been used in many medical implants (Daniels et al. 1990) and it is approved by regulatory agencies in many countries. PLA, PGA, and their copolymer PLGA are the most widely used polymers in drug delivery system development, not only because of their biodegradability and biocompatibility but also for its relatively easy

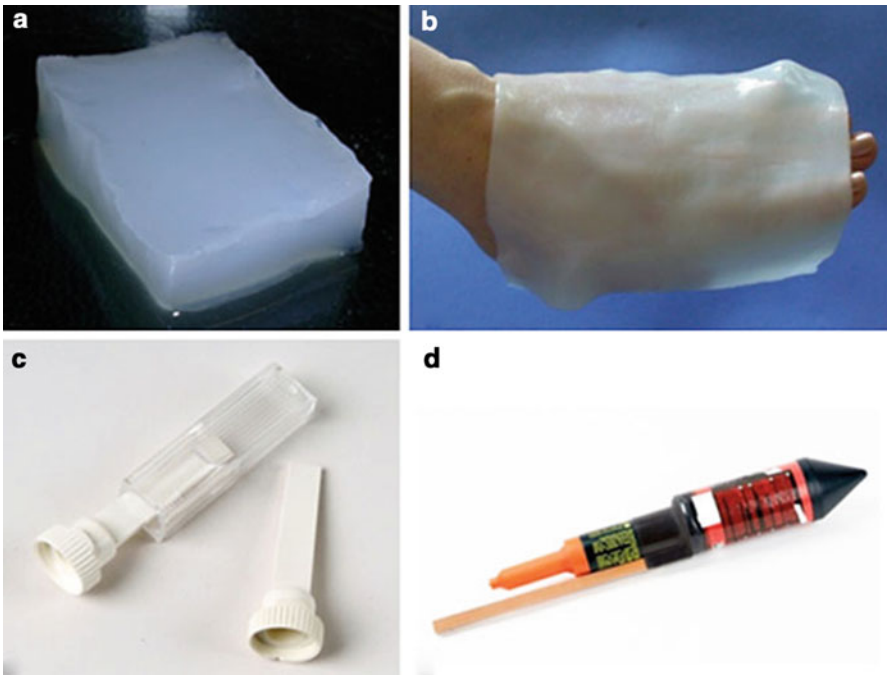

Fig. 2 (**a**, **b**) Bacterial cellulose block and films. (Professor Dr. Bielecki, University of Lodz); (**c**) Firework rocket made out of PHB (Biomer®, Germany); (**d**) ELISA sticks which are used for immunological tests (Biomer®, Germany)

production (Zhang et al. 2013b). Biomaterials such as PLA and PGA are required because they have suitable mechanical properties closely matched to target tissues and good biological interactions with cells (Farah et al. 2016).

Microbial cellulose (MC) is a polymer synthesized by *Acetobacter xylinum.* Due to the potential properties exhibited and its high value as a biotechnological product, it is considered as a very promising class of biopolymer, despite its applications not been fully developed (de Olyveira et al. 2012; Esa et al. 2014; Ul-Islam et al. 2015). The physical and mechanical properties of MC as well as its purity and uniformity suggest that MC can be used from the construction of high-quality audio membranes and electronic paper (Comiskey et al. 1998; Basta and El-Saied 2009) to fuel cells (Evans et al. 2003) and medical materials (Fontana et al. 1990). Many efforts have been done in exploring new skin substitutes and modern wound-dressing materials to be used in tissue engineering. Johnson and Johnson developed some approaches for the application of MC in the treatment of different types of wounds, since MC has a distinctive nanofibrillar structure that may become a perfect matrix as an optimal wound-healing environment (Fig. 2a, b). However, to our knowledge no data have been published. A Brazilian company, BioFill Produtos Biotecnologicos, has created a new wound-healing system based on MC produced by *Acetobacter.* Their line of products includes the following: Biofill® and Bioprocess® (used in therapy of burns,

ulcers, as temporary artificial skin), and Gengiflex® (applied in treatment of periodontal diseases) (Czaja et al. 2006).

3.3 Agricultural and Environmental Applications

Another promising application of biodegradable polymers is the use as a solid substrate for denitrification of water and wastewater treatment, in contrast with conventional processes in which the addition of liquid carbon sources is needed. Many different solid substrates (straw, wood, or some other polymers) have been tested with these purposes, but the *PHAs* seem to be the most suitable ones not only because they provide a source of reducing power for nitrogen removal, but also because they serve as matrices for the development of microbial films (Anderson and Dawes 1990; Boley et al. 2000; Hiraishi and Khan 2003). Moreover, PHAs (PHB – Biogreen®– or PHB/V –Biopol®) by themselves are microbial storage materials which may be catabolized for a broad variety of bacteria under denitrifying or anaerobic conditions (Abou-Zeid et al. 2001; Mergaert et al. 2001; Khan et al. 2002). However, not only PHA polymers are employed in these applications, but also other aliphatic polyesters such as poly-ε-caprolactone, *PCL* (Cellgreen PH®), which are economically more attractive, are being used for solid-phase denitrification, although the process rates obtained with these polymers are lower (Boley et al. 2000). Despite many advantages that the solid-denitrifying process offers, to become more attractive, the production costs of these polyesters must decrease and the reuse of waste bioplastics should be considered for these applications.

Many are the uses of biodegradable polymers within agricultural applications. For instance, the commercial copolymer Nodax™ (Table 2) may be degraded anaerobically, and hence, it is commonly used for urea fertilizers, herbicides or insecticides, or for the manufacture of biodegradable films (Philip et al. 2007). The copolymer PHBV is as well used in agriculture to release insecticides. Furthermore, another approach commonly used in agriculture is the use of PHA-producing bacteria inoculants to enhance nitrogen fixation in plants. Studies carried out on *Azospirillum brasilense,* which accumulates high amounts of intracellular PHA, have shown good results in this field (Fallik and Okon 1996). Ecoflex® films have also many uses in agriculture, such as cover sheeting that will be further degraded in the soil (Siegenthaler et al. 2012).

4 Research Needs

This chapter pretends to give a short overview on the most “new” and interesting biopolymers and their applications. Biopolymers have vast diversity, and therefore their applications are various and multiple. Biodegradable polymers have many important applications in food packaging, textiles, medicine, and agriculture. As it was described in the previous sections, there is a growing market in the medical field, in which biodegradable polymers are used as surgical implants, absorbable sutures,

implants for controlled drug release, or drug carriers. The fast growth of consumption is expected to continue depending on the availability of the biopolymers and of course the cost of production.

The appearance of these new and very promising biomaterials has generated great expectations concerning their applications, especially, since the appearance of Biopol®. At present, these biopolymers can hardly compete with the petroplastics in the most common uses. Only in very specific applications such as biomedicine and tissue engineering, where the cost of production could be assumed, these biopolymers are called to succeed. Despite the high cost of production, many of these bioplastics are already successfully commercialized. Cargill (USA), Galactic (Belgium), Treofan (the Netherlands), Corbion Purac (the Netherlands), Evonik (Germany), BASF, Procter & Gamble, and Metabolix are only few examples of companies producing biodegradable polymers. However, some of them (Boehringer Ingelheim, Germany, or Corbion Purac, the Netherlands) are mainly focused in the biomedical market (Avérous 2008). In the case of PLA, significant advances have been achieved in lactic acid (LA) production by improving the fermentation processes and trying to reduce the high cost of the biorefineries. Alternative fermentation strategies, such as the use of cheap and abundant biomass, starch, and lignocellulose, are being studied but requires the use of metabolic engineering in order to be able to use complex substrates without the need of exogenous saccharification pretreatments. The use of *PHAs* in the industry and further development has been impressive during the last decade, since they have been applied not only in the packaging, medicine, or pharmaceutical sector but also in a broad range of items such as toys, CDs, lighters, microphones, fireworks rockets (Fig. 2d), headphones, pressure sensors for keyboards, etc. (Philip et al. 2007). Moreover, PHA latex is being used for covering papers or cardboards to make water-resistance surfaces (Babel et al. 1990; Lauzier et al. 1993). The German company Biomer has the technology to produce PHB on large scale from *Alcaligenes latu*s and using sucrose as a sole carbon source. This polymer may be used to produce some daily items such as combs, pens, or bullets (Chen and Wu 2005). Metabolix, a US-based company, among others, markets metabolix PHA which is a blend of P3HB and poly (3-hydroxyoctanoate) and has been approved by the US Food and Drug Administration (FDA) for production of food additives. Metabolix has created a recombinant *Escherichia coli* K12 strain for this purpose, which is able to accumulate up to 90% of PHA in dry cell weight in 24 h (Clarinval and Halleux 2005). Now, this company is producing PHAs directly in switchgrass, a perennial plant that usually thrives on marginal land. The first evidence of PHA production in plants was reported by Poirier et al., who designed a transgenic plant of *Arabidopsis thaliana* capable of producing PHB (20–100 μg per gram), by introducing specific genes from *Ralstonia eutropha* (Poirier et al. 1992). Afterwards, many attempts have been done to improve the yield of PHA production as well as the downstream processes. By the 1990s many research groups (Nawrath et al. 1994; Mittendorf et al. 1998; Valentin et al. 1999) successfully developed transgenic plants that can accumulate PHB (up to 14% of the leaf dry weight), PHBV, or mcl-PHAs. Nowadays, the research in this area is focused on the production of PHA in crops, for example, tobacco, cotton, or corn

(Snell and Peoples 2002; Van Beilen and Poirier 2008; Bohmert-Tatarev et al. 2011). Although the cost of plant-derived PHA depends on several factors (crops used, yield of PHA, scale up and optimized downstream processes, etc.), it is expected, if the PHA production is 20–50% (w/w), the prices would become more competitive with their counterpart's oil-derived plastics.

As it has been described, there exists a wide range of natural and biotechnological polymers that are revealed to be a good alternative for nonbiodegradable materials. Additional efforts should be done to improve and enhance the production as well as to reduce costs. In the last years, research has been focused on the characterization and heterologous expression of genes involved in the biosynthesis of these polymers. The molecular biology revolution during the late 1970s provided new tools for biological research, which were successfully used to decipher genetic information and to understand further the principles of metabolic pathways at the genetic level. One step further brought scientists to the study of genes coding enzymes involved in biosynthetic pathways. The next natural step would be the application of protein engineering to determine whether the enzymes related to specific biosynthetic pathways can be modulated to obtain maximum efficiency for the polymer production.

To build our economy on a sustainable basis, we need to find a replacement for fossil carbon as chemical industry feedstocks (Andrady and Neal 2009). The demand of bioplastics is continuously rising, and the market is characterized by high and steady growth rates of between 20% and 100% per year. Partially bio-based PET is leading the field, which accounted for approximately 40% of the global bioplastics production capacity in 2013. According to the data published in the market study realized by the Germany's nova Institute, the bio-based polymer productions capacity is expected to triple from 3.5 million tonnes in 2011 to nearly 12 million tonnes in 2020 (www.bio-based.eu/market_study) being PLA and PHA the polymers showing the fasts rates of market growth. However, this is a challenging task that will require scientific advances and successful interactions among several areas of knowledge such as biotechnology, synthetic biology, or molecular biology.

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Abstract

The production of bio-based polymers has become increasingly important owing to accelerated fossil fuel depletion and environmental pollution. Bio-based polymers can be classified into three main groups. Bio-based polymers in the first group, e.g., polyhydroxyalkanoates (PHAs), are entirely synthesized by biological processes. Microbial host strains synthesize polymers using monomers produced by their inherent and recombinant metabolic pathways from renewable resources. Most currently available biopolymers belong to the second group, in which all or some monomers and/or monomer precursors for polymers produced by microbial fermentations are purified to polymer-grade products and employed for chemical polymer synthesis. Polybutylene succinate (PBS), polytrimethylene terephthalate (PTT), polylactic acid (PLA), and some bio-nylons belong to this group. Polymers in the third group are synthesized entirely by chemical processes. Specifically, polymers are chemically synthesized using monomers that are chemically produced from biomasses. In this chapter, we focus on the first and second groups and discuss current advances in the development of metabolically engineered microorganisms for the production of bio-based plastics. In particular, we clarify the technological feasibility of metabolic engineering strategies for the in vivo synthesis of PLA, PLGA, and their copolymers and for the production of bio-nylon monomers.

1 Introduction

Since the twentieth century, fossil fuel resources have been used to produce materials and chemicals to sustain everyday living, replacing the basic materials for daily living in the nineteenth century, including wood, wools, starch, and hides (Vink et al. 2004). Among these petro-based materials, plastics are highly versatile and have extensive practical applications. However, their widespread use has threatened environmental sustainability owing to the immense accumulation of waste that is not easily degradable or compostable. Although several solutions, including source reduction and thermal treatment by incineration, have been suggested to minimize the environmental impact of non-degradable polymers, these strategies are not considered ultimate solutions because they also cause serious harm to the environment (Khanna and Srivastava 2005). Accordingly, bioplastics produced by bio-based processes are strong candidates for the resolution of these environmental problems. They are potential substitutes for petroleum-based plastics because they can be designed to ensure complete biodegradation by modulating monomer types and compositions. Additionally, they can be produced by carbon-neutral processes based on biological and biochemical hybrid processes from renewable resources. Biochemicals, such as carboxylic acids, urethanes, diamines, amino carboxylic acids, and urea are key monomers for bioplastics, some of which can now be produced by bio-based processes employing recombinant microorganisms as host strains for fermentations (Lee et al. 2011; Jang et al. 2012; Chung et al. 2015; Tsuge et al. 2016).

Bioplastics can be classified into three major groups according to the synthesis method (Chung et al. 2015). In the first part of this chapter, polymers that are directly

synthesized by microbial fermentations are discussed (the first class of polymers). This class includes polyhydroxyalkanoates (PHAs), polylactic acid (PLA), and poly(lactate-co-glycolate) (PLGA). In the second part of this chapter, biopolymers produced by biological and chemical hybrid processes are discussed (the second class of polymers); in this class, monomers are first synthesized by biological processes, followed by the purification and polymerization of monomers by chemical processes. C3, C4, and C5 aminocarboxylic acids and diamines are discussed as representative monomers for biopolymers in the second class. The third class is not a focus of this review since these polymers are entirely synthesized by chemical methods.

2 In Vivo Synthesis of Bio-Based Polymers

2.1 Polylactic Acid (PLA) and PLA Copolymers

PLA is a representative bio-polyester; it is one of the most widely used biopolymers in the areas of films, packaging material, and fibers. PLA is synthesized by a hybrid biological and chemical process. Briefly, L-lactate is first produced by the microbial fermentation from corn starch and sugarcane wheat using lactic acid bacteria as host strains (Vink et al. 2004; Maharana et al. 2009). Polymer-grade purified lactic acid is then used for the synthesis of lactide, a cyclic dimer of lactic acid, which is used to synthesize high-molecular-weight PLA by ring-opening polymerization (Vink et al. 2004; Maharana et al. 2009). Recently, the direct synthesis of PLA by microbial fermentation, i.e., the in vivo synthesis of PLA, has been developed using PHA biosynthesis systems with metabolically engineered microorganisms (Yang et al. 2010; Jung et al. 2010).

PHAs are microbial polyesters that are synthesized naturally and accumulated as intracellular granules with a size of ~0.2–0.5 μm (diameter) in microorganisms in unfavorable growth conditions, such as nutrient-limited conditions, with excessive carbon sources (Lee 1996). To date, more than 150 kinds of 3,4,5,6-hydroxycarboxylic acids have been identified as monomer constituents of PHAs. The key enzyme for the biosynthesis of PHAs is PHA synthase, which accepts various hydroxyacyl-CoAs (HA-CoAs) as substrates for polymerization; these are provided by inherent and recombinant metabolic pathways in host strains. Since PHA synthase is only active toward (*R*)-HA-CoAs if a carbon at the hydroxyl group in HA-CoAs has chirality, only (*R*)-hydroxycarboxylic acids are monomers of PHAs. The substrate specificities of natural PHA synthases are highly dependent on the number of carbons in HA-CoAs and the position of the hydroxyl group. PHA synthase preferentially accepts 3-hydroxyacyl-CoAs (3HA-CoAs) as substrates compared with 4-, 5-, and 6-hydroxyacyl-CoAs, but cannot efficiently accept 2-hydroxyacyl-CoAs (2HA-CoAs), such as glycolyl-CoA, lactyl-CoA, 2-hydroxybutyryl-CoA (2HB-CoA), and 2-hydroxyisovaleryl-CoA (2HIV-CoA). Thus, PHA synthase has been engineered to exhibit increased activity toward 2HA-CoAs in order to develop a microbial system for the in vivo synthesis of 2-hydroxyacid containing PHA and to develop metabolic pathways that efficiently produce 2HA-CoAs, such as glycolyl-CoA, lactyl-CoA, and

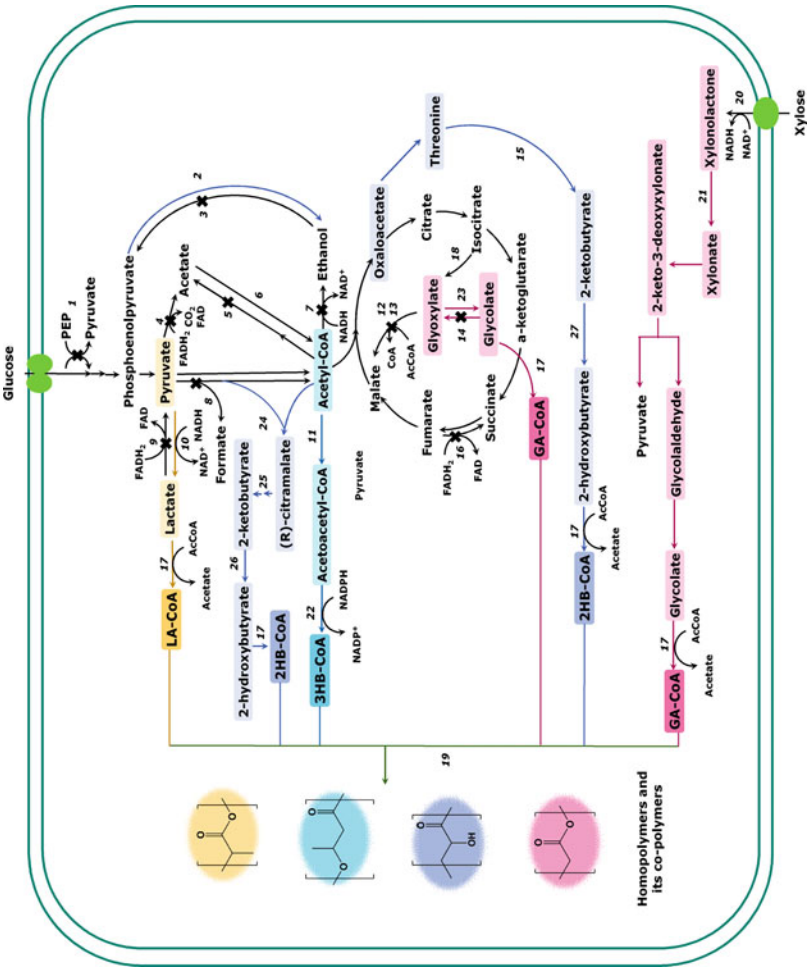

Fig. 1 Biosynthetic pathway for the synthesis of polylactic acid and poly(lactate-*co*-glycolate) and its copolymers. *1* ptsG, $IIBC^{Glc}$ glucose transporter; *2* ppc, phosphoenol pyruvate carboxylase; *3* pepck, phosphoenolpyruvate carboxykinase; *4* poxB, pyruvate oxidase; *5* ackA, acetate kinase; *6* acs, acetyl-CoA

2HB-CoA. We have recently developed metabolically engineered bacteria for the in vivo synthesis of PLA, PLGA, and PLA copolymers based on engineered type II PHA synthases from various *Pseudomonas* species and engineered *Clostridium propionicum* propionyl-CoA transferase (Pct), both of which are involved in the polymerization of HA-CoAs and the generation of HA-CoAs, such as glycolyl-CoA, lactyl-CoA, and 2HB-CoA (Yang et al. 2010, 2011; Jung et al. 2010; Park et al. 2012a, 2012b; Chae et al. 2016; Choi et al. 2016). *Pseudomonas* sp. PHA synthase I (PhaC1) engineered to accept 2HA-CoAs as substrates and *C. propionicum* propionyl-CoA transferase engineered to efficiently provide 2HA-CoAs to PHA synthase are key enzymes for the in vivo synthesis of PLA and PLA copolymers. A basic metabolic pathway consisting of PhaC1 and Pct for the in vivo synthesis of PLA and PLA copolymers was constructed in recombinant *Escherichia coli*. Subsequently, metabolic pathways of *E. coli* hosts were further engineered to supply enough substrates, e.g., lactyl-CoA and 2HB-CoA, for PhaC1 to enhance the production of PLA and PLA copolymers enriched in lactate monomers from renewable resources, as shown in Fig. 1. Removing of competing pathway for pyruvate (direct precursor of lactate) and acetyl-CoA (CoA donor) from *E. coli* chromosomal DNA increase the synthesis of lactate, as substrate for Pct. Additionally, lactate synthesis was enhanced by replacing the native promoter of *ldhA*, which encodes lactate dehydrogenase, with a strong *trc* promoter in the chromosomal DNA of the *E. coli* host strain; this provided Pct with more lactate and thereby enhanced lactyl-CoA synthesis. *E. coli* JLX10 (*ΔackA PldhA::Ptrc Δppc ΔadhE Pacs::Ptrc*) and *E. coli* JLXF5 (*ΔlacI ΔpflB ΔfrdABCD ΔadhE PldhA::Ptrc Pacs::Ptrc*), both of which are engineered from *E. coli* XL1-Blue, have been successfully used for the in vivo synthesis of PLA copolymers, such as poly (3-hydroxybutyrate-co-lactate) [P(3HB-co-LA)]. In addition, a PLA homopolymer was successfully synthesized in these two strains; however, the polymer content of PLA homopolymer was rather low (up to 4 wt%) (Jung et al. 2010).

2-Hydroxybutyrate (2HB) has also been examined to expand the monomer spectrum for the in vivo synthesis of 2-hydroxyacids containing polyesters in recombinant microorganisms from renewable resources. For the synthesis of 2HB, *Methanococcus jannaschii* citramalate synthase (CimA) was used to produce (*R*)-citramalate by condensing pyruvate and acetyl-CoA; (*R*)-citramalate was then further converted into 2-ketobutyrate (2 KB), the direct precursor of 2HB, by *E. coli* LeuBCD, which was finally used for the synthesis of 2HB by *Lactococcus lactis* subsp. *lactis* Il1403 D-2-hydroxyacid dehydrogenase (PanE) (Park et al. 2012b).

Fig. 1 (continued) synthase; *7* adhE, acetaldehyde-alcohol dehydrogenase; *8* pflB, pyruvate formate lyase; *9* dld, D-lactate dehydrogenase; *10* ldhA, lactate dehydrogenase; *11* phaA, β-ketothiolase; *12* glcB, malate synthase A; *13* aceB, malate synthase G; *14* glcDEFG, glycolate oxidase; *15* ilvA, L-threonine dehydratase; *16* frdB, fumarate reductase; *17* pct540, engineered propionyl-CoA transferase; *18* phaB, acetoacetyl-CoA reductase; *19* phaC1437, engineered PHA synthase; *20* xylB, xylose dehydrogenase; *21* xylC, xylonolactonase; *22* aceA, isocitrate lyase; *23* ycdW, glyoxylate reductase; *24* cimA, (*R*)-citramalate synthase; *25* LeuBCD, 3-isopropylmalate dehydratase; *26* panE, D-2-hydroxyacid dehydrogenase. Deleted genes are shown as × marks (Yang et al. 2010; Jung et al. 2010; Choi et al. 2016)

After in vivo synthesis of 2HB from glucose, 2HB was polymerized into 2HB-containing PHA by *C. propionicum* Pct and the engineered *Pseudomonas* sp. MBEL 6-19 PHA synthase, which are involved in the conversion of 2HB into 2HB-CoA and its polymerization.

2.2 Poly(Lactate-Co-Glycolate) [P(LA-*co*-GA)] and Its Copolymers

Glycolic acid (2-hydroxyacetic acid) is the smallest 2-hydroxy acid. Its polymers, including PLAG and PGA, are representative bio-polyesters and are similar to PLA in terms of biodegradability and biocompatibility. In particular, PLGA, a random copolymer of lactic and glycolic acids, has a controllable degradation rate depending on the molar composition. Accordingly, it is the most common biopolymer for medical applications, such as sutures [surgical stitches], drug delivery carriers, and scaffolds for tissue engineering (Huang et al. 2010; Makadia and Steven 2011). Currently, PGA and PLGA are synthesized by the ring-opening polymerization of glycolide and lactide using metal catalysts (Makadia and Steven 2011).

The synthesis of glycolate-containing polymers is possible, since engineered PHA synthase accepting lactyl-CoA as a substrate also shows activity toward glycolyl-CoA. The first incorporation of glycolate into PHA was achieved by feeding glycolate directly with dodecanoate to culture medium. *E. coli* LS5218 expressing *Pseudomonas* sp. 61-3 PHA synthase Ser325Thr/Gln481Lys mutant, *Megasphaera elsdenii* Pct, and *P. aeruginosa* enoyl-CoA hydratase produces poly (MCL-3-hydroxyalkanoates-*co*-17 mol% GA) (Matsumoto et al. 2011).

More recently, glycolate-containing polymers have been produced from unrelated carbon sources via two distinct biosynthetic pathways, i.e., the glyoxylate shunt pathway via the TCA cycle intermediate isocitrate (Li et al. 2016) and the Dahms pathway starting from xylose (Choi et al. 2016) (Fig. 1). To produce glycolate via the glyoxylate shunt pathway, the glyoxylate shunt was activated by overexpressing *E. coli aceA* and *aceK*, which encode isocitrate lyase and isocitrate dehydrogenase kinase/phosphatase, respectively. Then, glyoxylate was converted to glycolate by overexpressing *E. coli ycdW*, which encodes glyoxylate reductase. To prevent glycolate conversion to glyoxylate, glycolate oxidase was inactivated by deleting the chromosomal gene *glcD*, encoding a glycolate oxidase subunit. Glycolate was converted to glycolyl-CoA via *M. elsdenii* Pct and then polymerized by *Pseudomonas* sp. 61-3 PHA synthase Ser325Thr/Gln481Lys. By coexpressing *R. eutropha* PhaAB, a P(3HB-*co*-17 mol% LA-*co*-16 mol% GA) terpolymer was produced (Li et al. 2016).

The Dahms pathway for xylose metabolism is found in several bacteria, such as *Caulobacter crescentus* (Stephens et al. 2007). In the Dahms pathway, xylose is converted to pyruvate and glycolaldehyde, and glycolaldehyde is then converted to glycolate via aldehyde dehydrogenase (Stephens et al. 2007; Liu et al. 2013). To establish the Dahms pathway in *E. coli, C. crescentus* xylose dehydrogenase ($XylB_{ccs}$) and xylonolactonase ($XylC_{ccs}$) were expressed in *E. coli* XL1-Blue (Choi et al. 2016) (Fig. 1). Although *E. coli* XL1-Blue expressing $XylBC_{ccs}$ produced glycolate, cell growth and lactate production were reduced, and this was attributed to insufficient metabolic flux toward glycolysis. Based on an in silico

simulation, *E. coli* was engineered to utilize xylose and glucose simultaneously by deleting *ptsG*, which encodes a glucose phosphotransferase system subunit. Cell growth could be restored by the simultaneous use of two carbon sources, i.e., glucose and xylose. *E. coli* was further engineered to enhance metabolic flux toward lactate and glycolate by deleting several genes, including *adhE* (which encodes acetaldehyde-alcohol dehydrogenase), *pflB* (which encodes pyruvate formate lyase), *frdB* (which encodes fumarate reductase), *poxB* (which encodes pyruvate oxidase), *dld* (which encodes lactate dehydrogenase), *aceB*/*glcB* (which encode malate synthases), and the *glcDEFG* genes (which encode glycolate oxidase). Metabolic flux toward lactate was also enhanced by replacing the native promoter of the *ldhA* gene, encoding lactate dehydrogenase, with a strong *trc* promoter. The engineered *E. coli* strain coupled with the expression of $XylBC_{ccs}$, engineered *C. propionicum* Pct (Pct540), and engineered *Pseudomonas* sp. MBEL 6–19 PHA synthase (PhaC1437) produced P(50.3 mol%LA-*co*-48.1 mol%GA-*co*-1.6 mol%2HB) with a polymer content of 15 wt% of dry cell weight. The small fraction of 2HB generated from the *E. coli* isoleucine biosynthesis pathway could be removed by the deletion of L-threonine dehydratase or the addition of L-isoleucine, which allosterically inhibits L-threonine dehydratase, to the culture medium. Finally, engineered *E. coli* expressing $XylBC_{ccs}$, Pct540, and PhaC1437 produced P(70.5 mol% LA-*co*-29.5 mol% GA) to a polymer content of 36.2 wt% by fed-batch fermentation (Choi et al. 2016).

In vivo polymer synthesis has several advantages over chemical polymerization. First, it avoids residual monomers (lactides and glycolides) and metal catalysts, which complicate polymer processing and thus need to be thoroughly removed, particularly for biomedical applications. Second, the fermentative production process is much simpler than the chemical polymerization process, which requires the preparation of monomers and pre-polymers, with several purification steps. Lastly, the enantiopurity of the resultant polymers can be ensured because PHA synthases are specific to (*R*)-hydroxy acids.

3 Monomers for Bio-Based Nylon Synthesis

Polyamides are polymers synthesized by the polymerization of aminocarboxylic acid or copolymerization of dicarboxylic acid and diamine. They are used in a wide range of applications, including textile manufacturing, carpeting, and automotive parts (Lee et al. 2011).

The type and composition of monomers of polyamides greatly affect the material properties of the synthesized polymer. For instance, the incorporation of monomers with an aromatic structure can result in flexible amorphous polyamides, whereas the incorporation of aliphatic monomers in polyamides results in crystalline polymers. Nylon 6,6 and nylon 6 are representative polyamides that have industrial applications in textiles and motors; more than 4 million tons are produced per year. Nylon 6,6 is synthesized using six carbon hexamethylene diamine and adipic acid as monomers. In this process, the carboxylic acid group (COOH) at each end of each molecule reacts with the amine group (NH_2) at each end of the two chemicals. Nylon

6 is synthesized using caprolactam (a six-carbon substance) as a monomer via the formation of repeating units of the $(-NH-[CH_2]_n-CO-)_x$ chain, in which the polymerization of an acid at one end and an amine at the other end occurs. Other nylons, such as nylon 6,9, nylon 6,10, nylon 6,12, nylon 11, nylon 12, nylon 5,10, nylon 5,6, nylon 4, and nylon 4,6 have also been synthesized for potential industrial applications; they have different material properties depending on their monomer constituents and compositions (Jiang and Loos 2016). Although the majority of monomers for nylons are now produced by a petroleum-based chemical process, several monomers produced in bio-based processes employing microbial fermentations are currently being examined for the synthesis of bio-based nylons, including 1,3-propanediamine, gamma aminobutyric acid (GABA), putrescine, cadaverine, and 5-aminovaleric acid (Jang et al. 2012; Chung et al. 2015; Tsuge et al. 2016).

3.1 1,3-Diaminopropane (13DAP)

1,3-Diaminopropane/1,3-propanediamine (1,3-DAP) is a C3 diamine with potential applications in the pharmaceutical, agrochemical, and organic chemical industries. The biosynthesis of 1,3-DAP has been observed in *Pseudomonas aeruginosa* and *Acinetobacter baumannii* via the C5 and C4 pathways, respectively, but industrial 1,3-DAP production is limited by the host strains' pathogenicity (Chae et al. 2015). Thus, the biosynthesis of 1,3-DAP has been examined in recombinant *E. coli* expressing codon-optimized *A. baumannii dat* and *ddc*, encoding 2-ketoglutarate 4-aminotransferase and L-2,4-diaminobutanoate decarboxylase, respectively (Chae et al. 2015). Feedback inhibition caused by two major aspartokinases (encoded by *thrA* and *lysC*) was removed by introducing the mutations C1034T and C1055T in *thrA* and *lysC*, respectively. The flux toward phosphoenolpyruvate and L-aspartate was increased by replacing the native promoters of *ppc* and *aspC* with a strong *trc* promoter. Further deletion of *pfkA* significantly increased the concentration of 1,3-DAP. Fed-batch fermentation of the final engineered *E. coli* DP09 strain (W3110 Δ*lacI* $thrA^{C1034T}$ and $lys^{CC1055T}$ *Pppc:Ptrc PaspC:Ptrc* Δ*pfkA*) expressing *A. baumannii dat* and *ddc* along with *aspC* allowed the production of 13 g L^{-1} 1,3-DAP in glucose minimal medium (Chae et al. 2015).

3.2 Gamma Aminobutyric Acid (GABA)

Gamma-aminobutyric acid (GABA) is a C4 nonprotein amino acid and is widely used in the food and pharmaceutical industries (Shi and Li 2011; Park et al. 2013a). The key enzyme for GABA production is glutamate decarboxylase, which can convert L-glutamate, monosodium glutamate (MSG), and glutamic acid into GABA by their decarboxylation. Lactic acid bacteria have extensively been examined for the conversion of MSG to GABA by the whole cell conversion (Li and Cao 2010). Lactic acid bacteria efficiently convert MSG into GABA, but their inability to inherently synthesize L-glutamate and their requirement for complex medium for

growth limit the cost-efficient production of GABA at a commercial scale (Shi and Li 2011; Park et al. 2013a).

GABA production has also been examined in metabolically engineered *E. coli* overexpressing GAD by the whole cell conversion of MSG to GABA (Park et al. 2013a; Vo et al. 2014). Up to 5.69 g/L, GABA could be produced from 10 g/L MSG when *Pyrococcus horikoshii* GAD was expressed in *gabT* mutant *E. coli*, with a GABA conversion yield of 93% (Vo et al. 2014). High-level conversion of MSG to GABA has been examined by whole cell conversion using *E. coli* expressing GAD, highly concentrated to an OD_{600} of 100, as a whole cell biocatalyst (Park et al. 2013a). It was possible to produce 76.2 g/L GABA from 200 g/L MSG, with a GABA conversion yield of 62.4%. Additionally, bio-nylon 4 could be successfully synthesized by bulk polymerization using 2-pyrrolidone prepared from bio-based GABA employing Al_2O_3 as catalyst in toluene, with a yield of 96% (Park et al. 2013a).

Direct microbial fermentation from renewable resources is a potentially cost-effective method for GABA production. *Corynebacterium glutamicum* has potential as a host strain for the production of GABA from renewable resources owing to its ability to produce several amino acids, such as glutamate and lysine. Recombinant *C. glutamicum* ATCC 13032 expressing *Lactobacillus brevis* Lb85 *gadB2* (encoding GAD), *gadC* (encoding L-glutamate/GABA antiporter), and *gadR* (encoding a regulator) was reported to produce 2.15 g/L GABA from glucose (Shi and Li 2011). Similarly, the expression of *E. coli* glutamate decarboxylase enables *C. glutamicum* to synthesize 12.37 g/L GABA in optimized conditions (Takahashi et al. 2012). Since glutamate decarboxylase has optimal activity at a very low pH of around 4, which is not preferable for host strain growth, a balance between the growth of the host organism and the synthesis of GABA was established by the development of glutamate decarboxylase that is active at a higher pH range (up to pH 7). The introduction of a double mutation in *E. coli* GAD, Glu89Gln and Δ452–466, wherein 15 residues are deleted at the C-terminus, resulted in activity at pH values of up to 7 (Ho et al. 2013). Recombinant *C. glutamicum* expressing this GAD mutant under the strong synthetic P_{H36} promoter could produce GABA to a concentration of 38.6 g/L by fed-bath culture, when the culture medium was maintained at pH 6 (Choi et al. 2015).

3.3 Putrescine

Putrescine (1,4-diaminobutane) is a potential building block for pharmaceuticals, surfactants, additives, and other agrochemical applications. In the polymer industry, putrescine is condensed via adipic acid to produce nylon 4,6 (trademarked Stanyl by DSM, Heerlen, Netherlands). Nylon 4,6 possesses superior material properties, such as high melting temperature, mechanical strength, and solvent resistance. Similar to other petro-based chemicals, putrescine is produced by chemical process based on the hydrogenation of succinonitrile, which utilizes raw toxic materials and expensive catalysts and is operated under harsh conditions (Qian et al. 2009).

Systems metabolic engineering strategies in *E. coli* for the production of putrescine have been developed using ornithine as a precursor, in which the one-step

decarboxylation of ornithine results in putrescine production (Qian et al. 2009). To make more ornithine available for decarboxylation, the *argI* gene encoding ornithine carbamoyltransferase, a key enzyme responsible for the conversion of ornithine into urea, was deleted. Furthermore, putrescine degradation was eliminated by the deletion of the *speE* and *speG* genes, which encode spermidine synthase and spermidine acetyltransferase, respectively. The Puu pathway, which catabolizes extracellular putrescine, was eliminated by knocking out the *puuPA* genes (encoding putrescine importer and glutamate-putrescine ligase, respectively). Transcriptional repression of the *argA–E* genes was removed by replacing their native promoters with a strong *tac* promoter, along with the deletion of the *argR* gene, to support high metabolic flux toward ornithine synthesis. The decarboxylation of ornithine to putrescine could be enhanced by replacing the native promoters of the *speC* and *speF* genes, which encode two ornithine decarboxylase isozymes, as well as the *potE* gene, which encodes a putrescine/ornithine antiporter. Furthermore, the deletion of *rpoS*, responsible for the regulation of gene expression in *E. coli* under stressful conditions, enhanced the production of putrescine. *E. coli* XQ26 (W3110; *ΔlacI ΔspeE ΔspeG ΔargI ΔpuuPA ΔrpoS* P*argECBH*::P*trc* P*speF-potE*::P*trc* P*argD*::P*trc* P*speC*::P*trc*) expressing the *speC* gene produced 24.2 g/L putrescine in fed-batch fermentation (Qian et al. 2009). Similarly, the ornithine-producing strain *C. glutamicum* ORN1 (*ΔargF ΔargR*) expressing the *speC* gene was able to produce 6 g/L putrescine (Schneider and Wendisch 2010).

3.4 Cadaverine

Cadaverine, 1,5-diaminopentane, is a C5 linear aliphatic diamine. It is an industrially important platform chemical owing to its broad applications, e.g., as precursor for polyamides and polyurethanes, chelators, and additives. Specifically, cadaverine can be used as a monomer for bionylon 5, replacing petroleum-based monomers. Polymerization of cadaverine with other bio-based monomers, such as succinic acid and sebacic acid, allows the synthesis of completely bio-based nylons (Kind and Wittmann 2011). Similar to GABA, cadaverine can be produced by whole cell conversion using microorganisms expressing lysine decarboxylase, which is responsible for the direct synthesis of cadaverine from lysine by decarboxylation. According to a recent study, *E. coli* XL1-Blue expressing *E. coli ldcC* (encoding lysine decarboxylase) could be used as a whole cell biocatalyst for the high-level conversion of crude L-lysine solution from lysine manufacturer to cadaverine, with a 99.9% molar yield (Oh et al. 2015b).

Cadaverine can be produced by engineering the lysine pathway, which branches off from the TCA cycle, via oxaloacetate, as summarized in Fig. 2. A systems metabolic engineering strategy for the efficient synthesis of cadaverine has been developed by strengthening the lysine biosynthesis pathway in *E. coli* WL3110, a *lacI* mutant of W3110 (Qian et al. 2011). Metabolic pathways that utilize cadaverine were further inactivated by knocking out *speE*, *speG*, *ygjG,* and *puuPA* to eliminate the formation of aminopropyl cadaverine, *N*-acetylcadaverine, 5-aminopentanal, and

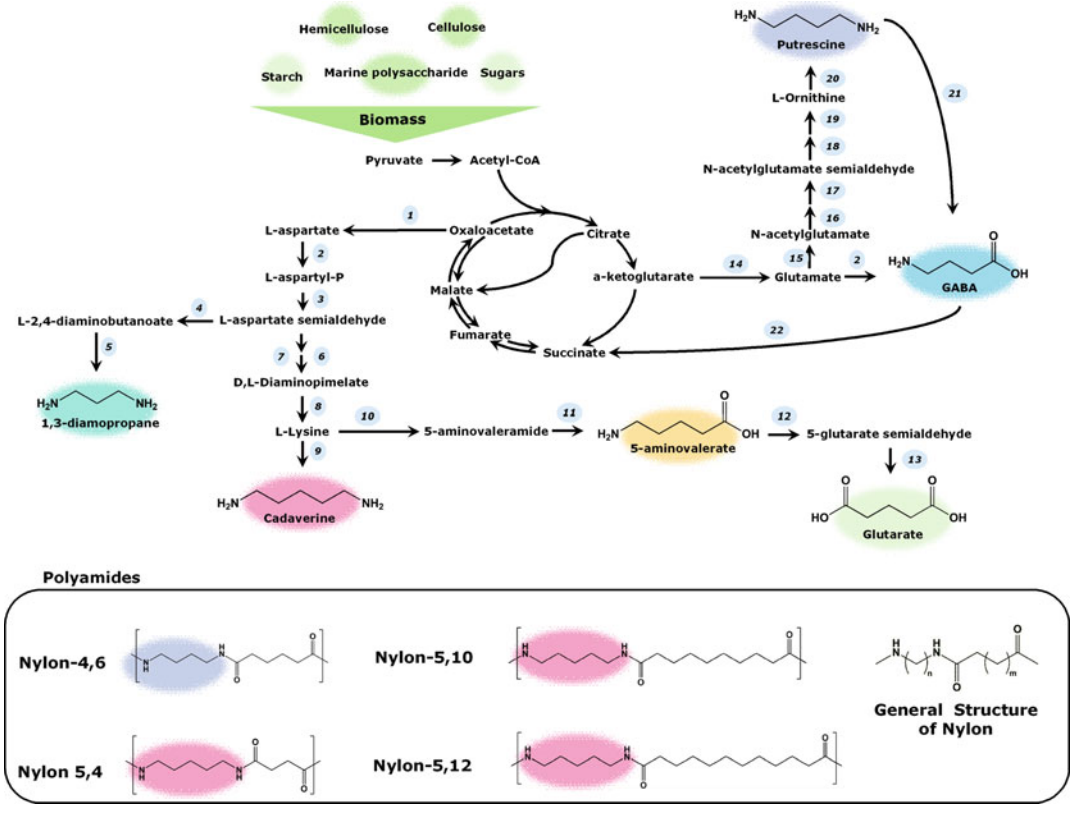

Fig. 2 Biosynthetic pathway for the biosynthesis of C3, C4, and C5 nylon monomers. *1* aspC, PLP-dependent L-aspartate aminotransferase; *2* lysC, aspartate kinase III; thrA, aspartate kinase I/homoserine dehydrogenase I, metL, aspartate kinase II/homoserine dehydrogenase II; *3* asd, aspartate semialdehyde

glutamylcadaverine, respectively. The *cadA* gene, which encodes lysine decarboxylase, was overexpressed in a multicopy plasmid to enhance the conversion of L-lysine to cadaverine. Next, threonine synthesis branching from L-aspartyl-*p* was weakened by replacing the native promoter of *dapA* with a strong *trc* promoter, since promoter substitution simultaneously increased the expression of *dapA* by eliminating the repression of the native *dapA* promoter caused by intracellular DAP and its direct precursor, L,L-diaminopimelate. Lastly, *C. glutamicum ddh*, which encodes DAP dehydrogenase, was inserted at the *iclR* locus to efficiently convert tetrahydrodipicolinate into DAP. The disruption of *iclR* increases the expression of the *aceBA* genes, encoding isocitrate lyase and malate synthase, in the glyoxylate shunt, which results in an increased supply of oxaloacetate and enhanced production of an aspartate-derived amino acid; accordingly, the *iclR* locus was chosen for the integration of the *C. glutamicum ddh* gene. *E. coli* XQ56 (*ΔlacI ΔspeE ΔspeG ΔygjG ΔpuuPA PdapA::Ptrc*) expressing *E. coli cadA* was able to produce 9.61 g/L cadaverine in a fed-batch culture for 30 h (Qian et al. 2011).

Since L-lysine serves as the direct precursor of cadaverine, recombinant *C. glutamicum* is a promising host strain for cadaverine production. It has successfully been employed as a host strain for the production of L-lysine to meet the annual industrial demand of 1.5 million tons (Mimitsuka et al. 2007; Kind et al. 2010b). Cadaverine synthesis in *C. glutamicum* was first demonstrated by the insertion of *E. coli cadA* in place of the *hom* gene, encoding homoserine dehydrogenase. A relatively low concentration of cadaverine of approximately 2.6 g/L was obtained in a useful proof-of-concept study that established the potential use of *C. glutamicum* as a work horse for the synthesis of cadaverine (Mimitsuka et al. 2007).

Fig. 2 (continued) dehydrogenase; *4* dat, 2-ketoglutarate 4-aminotransferase; *5* ddc, L-2,4-diaminobutanoate decarboxylase; *6* dapA, dihydrodipicolinate synthase; dapB, dihydrodipicolinate reductase; dapC, N-succinyldiaminopimelate-aminotransferase; dapD, tetrahydrodipicolinate succinylase; dapE, N-succinyl-L-diaminopimelate desuccinylase; dapF, diaminopimelate epimerase; *7* ddh, meso-diaminopimelate dehydrogenase from *C. glutamicum*; *8* lysA, diaminopimelate decarboxylase; *9* cadA, inducible L-lysine decarboxylase, ldcC, constitutive L-lysine decarboxylase; *10* davA, delta-aminovaleramidase; 11 davB, lysine 2-monooxygenase; 12 gabT, 5AVA aminotransferase; *13* gabD, glutarate semialdehyde dehydrogenase; *14* gdhA glutamate dehydrogenase; *15* argA, N-acetylglutamate synthase; *16* argB, acetylglutamate kinase; *17* argC, N-acetylglutamylphosphate reductase; *18* argD, acetylornithine transaminase; *19* argE, acetylornithine deacetylase; *20* speC/speF, biosynthetic/degradative ornithine decarboxylase, *21* ydcW, g-aminobutyraldehyde dehydrogenase, ygjG putrescine aminotransferase, ygjG, putrescine aminotransferase; puuA, glutamate-putrescine ligase; puuB, g-glutamylputrescine oxidase; puuC, g-glutamyl-g-aminobutyraldehyde dehydrogenase; puuD, g-glutamyl-g-aminobutyrate hydrolase; *22* yneI, NAD(+)-dependent succinate semialdehyde dehydrogenase, puuE, 4-aminobutyrate aminotransferase, gabD, NADP(+)-dependent succinate semialdehyde dehydrogenase, gabT, 4-aminobutyrate aminotransferase. Examples of nylon polymers having one biologically synthesized monomers include Nylon 4,6 (putrescine and adipic acid), Nylon 5,4 (cadaverine and succinic acid), Nylon 5,10 (cadaverine and sebacic acid), and Nylon 5,12 (cadaverine and dodecanedioic acid), wherein carboxylic acids are derived from chemical synthesis

Since it is important to achieve the stable expression of lysine decarboxylase at a high level for high production of cadaverine in recombinant *C. glutamicum*, synthetic promoters with various strengths have been examined for the expression of *E. coli ldcC*, which encodes lysine decarboxylase, in *C. glutamicum*. Recombinant *C. glutamicum* expressing *E. coli ldcC* under the strong synthetic P_{H30} promoter could produce 40.91 g/L cadaverine in 64 h (Oh et al. 2015a).

C. glutamicum DAP-3c with a superior cadaverine biosynthetic pathway was developed using systems metabolic engineering (Kind et al. 2010b). First, the conversion of L-aspartate to diaminopimelate in *C. glutamicum* was strengthened via the overexpression of *dapB* by promoter replacement with sod and by the duplication of the *ddh* and *lsyA* genes. A leaky mutation was introduced in the *hom* gene to attenuate the competing threonine pathway. To secure the oxaloacetate pool, the *pepck* gene was deleted. Flux toward oxaloacetate and L-aspartyl-*p* was enhanced by introducing point mutations in lysC (T311I) and pycA (P458S) and expressing them under a strong sod promoter. Lastly, codon-optimized *ldcC* expressed under the tuf promoter was integrated at the *bioD* locus. Unfortunately, 20% of the accumulated cadaverine was in the acetylated form. The deletion of diaminopentane acetyltransferase, which is encoded by NCgl1469, in the DAP-3c strain resulted in an 11% increase in yield, with a final yield of 223 mmol/mol glucose and no detectable acetylated form (Kind et al. 2010a).

C. glutamicum does not naturally synthesize cadaverine and can only tolerate up to 0.3 M. Additionally, lysine decarboxylase activity is inhibited by cadaverine (Mimitsuka et al. 2007). Using genome-wide transcription profiling, a permease, cg2893, was identified as the most highly expressed gene during cadaverine production. The overexpression of cg2893 along with the deletion of cg2894, a tetR-type repressor, contributes to an increase in production titers (Kind et al. 2011). Similarly, the expression of the cadaverine-lysine antiporter encoded by *cadB* from *E. coli* increases the secretion rate by 22% (Li et al. 2014). *C. glutamicum* DAP-16 (ATCC 13032 *Δpepck ΔlysE ΔNCgl1469 2xddh 2xlysA* hom^{V59C} $P_{sod}lysC^{T311I}$ $P_{sod}pycA^{P458S}$ $P_{sod}dapB$ $P_{sod}tkt$ $P_{sod}cg2893$ P_{tuf}fbp P_{tuf}ldcC^{opt} icd^{GTG}) produces 88 g/L cadaverine in a fed-batch fermentation (Kind et al. 2014).

3.5 5-Aminovaleric Acid (5AVA)

5-Aminovalerate (5AVA) is a C5 platform chemical that can be used in the synthesis of industrially important chemicals, such as glutarate, 5-hydroxyvalerate, and 1,5-pentanediol. It is also a precursor of δ-valerolactam (2-piperidone), a potential monomer of nylon 5. 5AVA exists as an intermediate of the L-lysine degradation pathway, known as the aminovalerate pathway, of *Pseudomonas putida* (Revelles et al. 2005, 2007). Lysine 2-monooxygenase, encoded by *davB*, and delta-aminovaleramidase, encoded by *davA*, are two key enzymes for the conversion of L-lysine to 5AVA via 5-aminovaleramide (Revelles et al. 2005, 2007). In *P. putida*, 5AVA is further converted into glutarate semialdehyde by aminovalerate aminotransferase, which is encoded by

gabT, and then glutarate semialdehyde is further converted into glutarate by 5-glutarate semialdehyde dehydrogenase via *gabD* (Revelles et al. 2005, 2007).

Even though 5AVA has been successfully synthesized from the fungal L-lysine α-oxidase with a high 5AVA yield (95% mol 5AVA/mol L-lysine) (Pukin et al. 2010), enzymatic synthesis of 5AVA seems not be suitable for large-scale production owing to the high production cost of enzymes and low 5AVA productivity. Recently, recombinant *E. coli* strains have been developed for the production of 5AVA from glucose. Recombinant *E. coli* XQ56 engineered to effectively synthesizes L-lysine from glucose was used as a host strain for the production of 5AVA from glucose via the expression of *P. putida davAB* genes (Park et al. 2013b). However, the concentration of 5AVA achieved in a fed-batch culture of *E. coli* XQ56 expressing the *davAB* genes was low (up to 0.5 g/L 5AVA) (Park et al. 2013b). Considering that *E. coli* XQ56 was successfully employed for the production of cadaverine from L-lysine, further metabolic engineering of the host strain and of key enzymes, including lysine 2-monooxygenase and delta-aminovaleramidase, is needed to enhance the production of 5AVA from glucose.

Similar to the large-scale production of GABA using recombinant *E. coli* expressing GAD as a whole cell biocatalyst, the high-level conversion of L-lysine into 5AVA was examined using an *E. coli* strain expressing *P. putida davAB* as a whole cell catalyst (Park et al. 2014). The engineered strain was grown to a high density, i.e., to optical densities at 600 nm (OD_{600}) of 30 and 60, by fed-batch cultures (Park et al. 2014). 5AVA could be produced at a concentration of 90.59 g/L from 120 g/L L-lysine with a molar yield of 0.942 by an *E. coli* strain expressing *P. putida davAB* grown to an OD_{600} of 60. Additionally, nylon 6,5 could be successfully synthesized by the bulk polymerization of ε-caprolactam along with δ-valerolactam prepared from microbially synthesized 5AVA (Park et al. 2014).

4 Research Needs

Despite extensive research on the development of bio-based polymers over the past few decades, further investigations are needed to achieve bio-based processes that are optimized with respect to carbon yield, product concentration, productivity, purification yield, and efficiency. Each of these properties should be extensively improved to make bio-based processes cost-effective and to enable their combination with chemical processes. Systems metabolic engineering strategies can play a significant role in the development of superior microbial host stains for cost-effective bio-based processes.

5 Conclusion

Several important factors affect the production of chemicals and polymers and accordingly should be considered in the development of microbial host strains, including carbon yield, product concentration, productivity, cofactor availability,

removal of by-product formation, fermentation efficiency, and product purification efficiency after fermentation. These factors determine the feasibility of bio-based processes and bio-chemical hybrid processes from technological and economical perspectives. The systems metabolic engineering strategies discussed in this chapter can be used to develop superior microbial host strains for the production of industrially important chemicals and polymers, while optimizing these factors.

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Applications of Microbial Biopolymers in Display Technology

31

Fabian B. H. Rehm, Katrin Grage, and Bernd H. A. Rehm

Contents

Abstract

Microorganisms produce a variety of different polymers such as polyamides, polysaccharides, and polyesters. The polyesters, the polyhydroxyalkanoates (PHAs), are the most extensively studied polymers in regard to their use in display technology. The material properties of bacterial PHAs in combination with their biocompatibility and biodegradability make them attractive substrates for use in display technology applications. By translationally fusing bioactive molecules to a gene encoding a PHA-binding domain, the appropriate functionalization for a given application can be achieved such that the need for chemical immobilization is circumvented. By separately extracting and processing the

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biopolymer, using it to coat a surface, and then treating this surface with the fusion proteins, surface functionalization for immunodiagnostic microarray or tissue engineering applications can be accomplished. Conversely, by expressing the fusion protein directly in the PHA-producing organisms, one-step production of functionalized beads can be achieved. Such beads have been demonstrated in diverse applications, including fluorescence-activated cell sorting, enzyme-linked immunosorbent assays, microarrays, diagnostic skin test for tuberculosis, vaccines, protein purification, and affinity bioseparation.

1 Introduction

The display of biologically active molecules is utilized for a range of applications such as diagnostics, biosensing, and microarray technologies. The substrate on which such display takes place is greatly deterministic of functionality and applicability. Common, well-established techniques include display on cell surfaces, ribosomes, and phage particles (Lee et al. 2003; Zahnd et al. 2007; Rakonjac et al. 2011). The use of microbial biopolymers as substrates has more recently been revealed in a variety of contexts. Although bacteria can produce a range of polymers, only a few of them have been considered for display technologies (Fig. 1). Bacterial cellulose, which exhibits various properties superior to plant-based cellulose with respect to display technology applications, has been limited to enzyme, bacterial cell and fungi immobilization (Wu and Lia 2008; Ullah et al. 2016). In contrast, bacterial polyhydroxyalkanoates (PHAs) have been extensively investigated as substrates for display technology applications, and thus will be the focus of this chapter.

2 Polyhydroxyalkanoates

PHAs are biopolyesters which serve as carbon and energy storage materials in a range of bacteria and archaea (Lenz and Marchessault 2005; Rehm 2010). During excess carbon availability, they are stockpiled as the amorphous core of PHA inclusions, surrounded by structural proteins (phasins), PHA metabolism-associated enzymes, and regulator proteins (Grage et al. 2009; Jendrossek 2009). Critical enzymes for PHA synthesis and inclusion assembly are the PHA synthases (such as PhaC) which catalyze the stereoselective conversion of the activated precursor (*R*)-3-hydroxyacyl-CoA (Rehm 2003). These CoA thioesters, depending on their carbon chain length, are synthesized from intermediates of fatty acid metabolism or directly from acetyl-CoA to polyoxoesters with the simultaneous release of coenzyme A (Steinbuchel et al. 1993; Rehm 2006) (Fig. 2). Unlike the other hydrophobically interacting PHA inclusion surface proteins, the PHA synthase remains covalently linked to the PHA inclusion core (Hezayen et al. 2002; Peters and Rehm 2006).

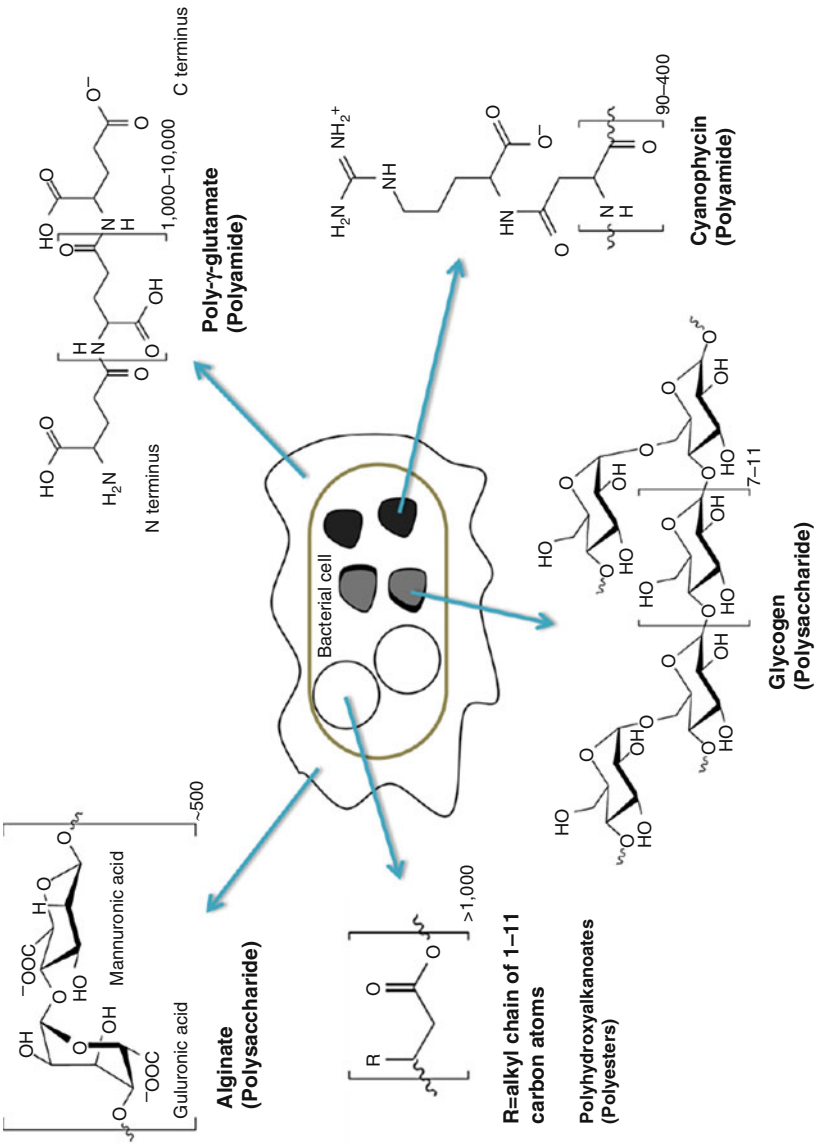

Fig. 1 Schematic of bacterial cell producing various intracellular and extracellular polymers. Only examples of the various classes of polymers are shown (Chemical structures of polymers were modified according to Rehm (2010))

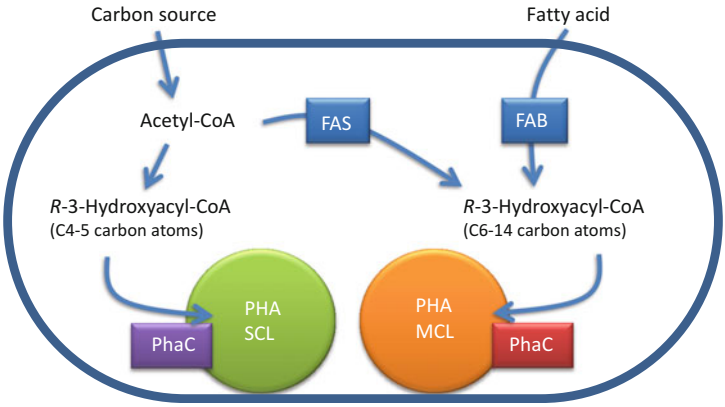

Fig. 2 Major steps in polyhydroxyalkanoate (PHA) biosynthesis. Arrows indicate enzyme catalyzed conversion reactions. *FAS* fatty acid synthesis, *FAB* fatty acid beta-oxidation, *PhaC* PHA synthase, *SCL* short chain-length, *MCL* medium chain-length

Although PHAs are all hydrophobic and water insoluble, they can drastically vary in composition and thus physical properties. Melting points can range from 50 °C to 180 °C and crystallinity can range from 30% to 70%, which is largely determined by monomer composition (Rehm 2010). As such, PHAs have been classified on the basis of monomer chain length. Medium-chain-length PHAs (MCL, C6-C14) are naturally produced primarily by pseudomonads, whereas short-chain-length PHAs (SCL, C3-C5) production is more widespread throughout bacteria and archaea (Anderson et al. 1990) (Fig. 2). While the common laboratory bacterium *Escherichia coli* does not naturally accumulate PHAs, it becomes a competent PHA producer upon introduction of the appropriate PHA biosynthesis genes (Schubert et al. 1988; Lee et al. 1994). The intracellular PHA inclusions may be isolated for polymer extraction and purification for processing into various materials, or maintained as functional shell-core beads (Fig. 3). The latter requires engineering of proteins attached to the PHA core in order to obtain functionality.

3 Microarray Applications of Polyhydroxyalkanoates

The PHA material properties enable film coating of solid surfaces suitable for microarray applications. Indeed, by exploiting the ability of PHA-associated proteins to specifically bind PHAs, and thereby overcoming specificity- or orientation-associated issues, PHA substrates for immobilization have been demonstrated as attractive for such applications.

The first relevant example of PHA as a protein micropatterning substrate was described in a study by Park and colleagues (2005). Poly(3-hydroxybutyrate) (P(3HB)) and poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) were independently produced, purified, and used to spin-coat glass substrate, producing PHA films.

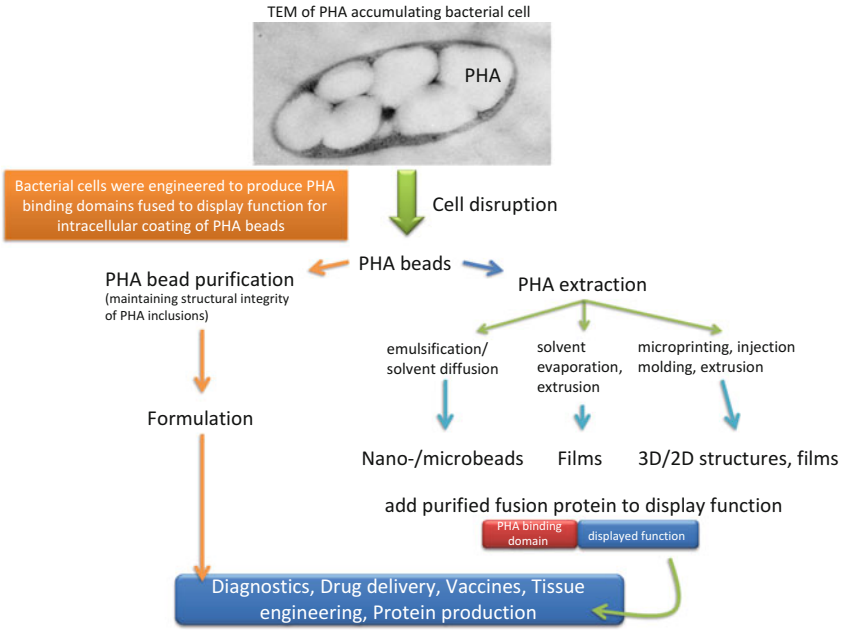

Fig. 3 Polyhydroxyalkanoate processing for display technologies. Comparison of in vivo and in vitro approaches towards the production of PHA-based material implementing display technologies

Subsequently, enhanced green fluorescent protein and red fluorescent protein, fused with the hydrophobic side chain-interacting substrate binding domain (SBD) of a PHA depolymerase, were micropatterned onto the film using microcontact printing. Confocal fluorescent microscopy clearly revealed the printed patterns after several wash steps and surface plasmon resonance spectroscopy using anti-green fluorescent protein polyclonal antibody further confirmed this specific fusion protein immobilization, additionally demonstrating the system as capable for examining protein-protein interactions.

Spurred on by these encouraging results, a further study aimed to demonstrate the possibilities of this system for protein microarray development centered on immunodiagnostic applications (Park et al. 2006). PHA depolymerase SBD fusions with the single-chain antibody variable region (scFv) against hepatitis B virus (HBV) preS2 surface protein as well as the severe acute respiratory syndrome coronavirus envelope protein (SCVe) were produced and microspotted onto P(3HB) films. Fluorescence-labelled HBV antigen and anti-SCVe antibody were then used to detect the interactions on the films. Fluorescence signals were detected only at the corresponding microspotted regions, indicating high affinity and selectivity and thus indicating the technology as appropriate for use in immunodiagnostics.

Subsequent investigation into whether such a platform could be applicable for clinical pathogen detection via immobilized DNA-protein complexes was

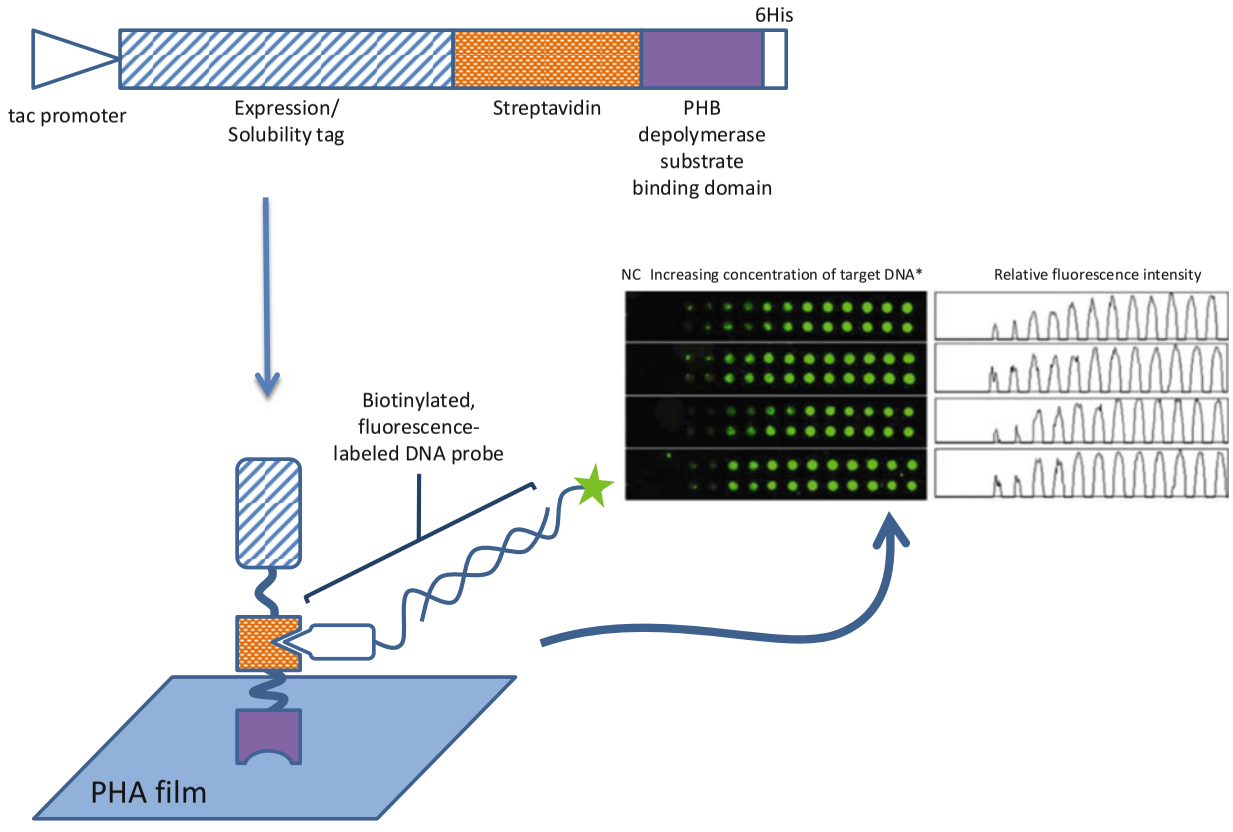

Fig. 4 PHA binding fusion protein attached to PHA films in microarray application. *NC*, negative control (no target DNA, i.e., DNA of bacteria to be detected); *Target DNA specific for bacterial species to be detected and homologous to immobilized DNA (Microarray data adapted from Park et al. (2009))

undertaken by Park et al. (2009) (Fig. 4). Pathogen-specific (*Acinetobacter baumannii*, *E. coli*, *Klebsiella pneuomoniae*, and *Pseudomonas aeruginosa*) biotin-labelled 15-mer DNA probes immobilized to core streptavidin fused to the PHA depolymerase SBD were microspotted onto polyhdroxybutyrate (PHB)-coated slides. By hybridizing differentially fluorescently labelled target DNAs to each pathogen-specific probe simultaneous detection of the corresponding pathogens added to the slide was demonstrated, further evidencing the potential of PHAs for use in immunodiagnostic microarrays.

4 Tissue Culture/Engineering Applications of Polyhydroxyalkanoates

The diverse, adjustable material properties of PHAs in concert with their biodegradability, biocompatibility, noncarcinogenicity, and low cytotoxicity have driven their development in the area of biomaterials research (Ali and Jamil 2016). On the basis of PHA surface functionalization by addition of PHA-binding protein fusions, several approaches to enhancing tissue engineering and culturing techniques have been undertaken.

By translationally fusing the PHA-binding phasin, PhaP, to the cell adhesion motif Arg-Gly-Asp (RGD) and applying this fusion protein to coat PHA surfaces, the effect on fibroblast growth was investigated (Dong et al. 2010). Under serum-free conditions, confocal laser scanning microscopy in combination with cell counting assays revealed a significant cell attachment increase on the PhaP-RGD-coated PHA films relative to the PhaP- (nonfused) and noncoated films, in a manner that is unlikely to be attributable to changes in PHA surface topology. Further cell proliferation assays revealed increased fibroblast proliferation levels on PhaP-RGD-coated PHA films. Thus, PHA surface functionalization, using a well-known cell adhesion motif, was demonstrated using aqueous solutions, avoiding toxicity of chemical immobilization techniques. Continuing this pattern of investigation, similar increases in cell adhesion and cell proliferation of human vascular smooth muscle cells (HvSMCs) were demonstrated using the PHA repressor protein (PhaR) fused to the specific integrin ligand peptide (KQAGDV) (Dong et al. 2012), and, more recently, PhaP fused to RGD or the laminin-derived IKVAV peptides used to coat PHA films demonstrated enhanced neural stem cell (NSC) attachment, proliferation, and better neurite outgrowths, without effecting NSC differentiation (Xie et al. 2013).

5 Diagnostic Applications: In Vitro and In Vivo

5.1 In Vitro Diagnostics

Fluorescence activated cell sorting (FACS) is a qualitative and quantitative technique used in biomolecule detection for in vitro diagnostics. Generally, antigen-displaying beads are used to bind antibodies which are then detected via fluorescent-signaling secondary antibodies when the bead suspension passes through the FACS machine flow cell. Current bead preparation techniques involve tedious antigen purification and chemical crosslinking. Therefore, more cost-effective, reliable means of producing antigen-displaying beads with comparable detection efficacy could greatly impact the accessibility of FACS. A series of studies have shown that one path towards such improvement may be via one-step production of surface protein engineered PHA beads.

The first demonstration of in vivo-produced PHA inclusions for FACS applications was described by Bäckström and coworkers (Bäckström et al. 2007). The authors generated fusions between either interleukin-2 (IL2) or the myelin oligodendrocyte glycoprotein (MOG) and the C- or N-terminus of PhaP via an enterokinase (specific protease) recognition site providing linker. Subsequently, the hybrid genes were expressed in *E. coli* and the PHA granules extracted. The resultant antigen-displaying PHA beads were then used for FACS using the corresponding fluorescently labelled monoclonal antibody which showed significant and specific antibody binding. Enterokinase treatment reversed this recognition indicating removal of the fusion partners/antigens. Finally, sera from mice immunized with MOG or ovalbumin (as a negative control) were analyzed using the MOG-displaying beads and FACS. Again, high specificity and sensitivity (antibody detection in sera diluted 1:100,000) were demonstrated, providing strong support for application of engineered PHA beads in FACS (Fig. 5). A subsequent investigation explored C-terminal PhaC-streptavidin fusions (Peters and Rehm 2008). The remarkably high streptavidin-biotin binding affinity has given rise to its use in range of biotechnological applications, thus making it a great candidate for further demonstrating the applicability of PHA beads in FACS. The study revealed a biotin binding capacity of 61 ng per μg of bead protein and demonstrated the detection of goat polyclonal biotinylated IgG in FACS using a secondary conjugated antibody. Most recently, PHA inclusions with GFP and MOG fused to PhaP or PhaC, such that each granule is simultaneously displaying two protein-based functionalities, were examined in the context of FACS, providing proof-of-concept for the biotechnological application of bifunctional PHA beads which may extend beyond FACS (Atwood and Rehm 2009).

The enzyme-linked immunosorbent assay (ELISA) is based on antigen-antibody binding on solid support either detecting antigen or antibody in samples. In vivo assembled engineered PHA inclusions displaying antigens or specific binding domains as described above were directly used to coat ELISA plates. Respective ELISAs showed specific and sensitive detection of the corresponding antibody or

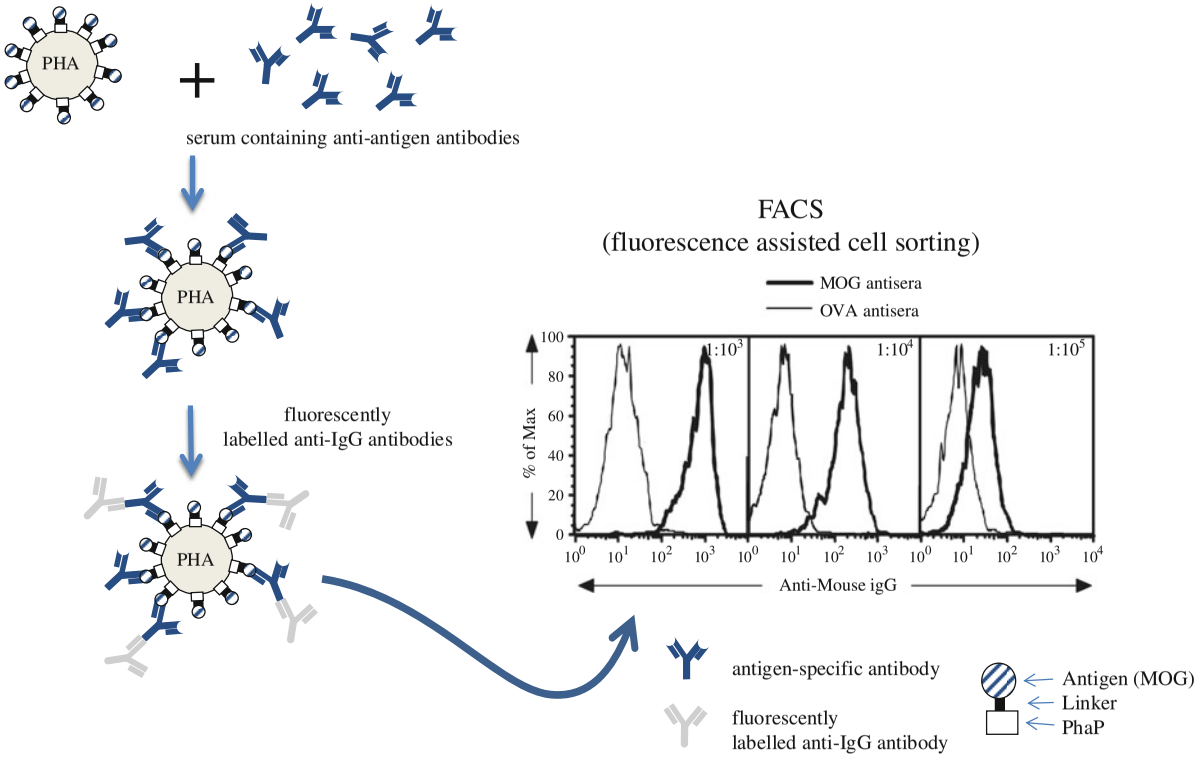

Fig. 5 In vivo manufactured antigen displaying PHA beads used in fluorescence activated cell sorting for specific antibody detection. PHA beads were incubated in sera of mice either immunized with MOG or OVA. After washing, bound antibodies were detected using a fluorescently labelled secondary anti-IgG antibodies combined with FACS. Anti-MOG antibodies were still detectable at a serum dilution 1:100,000. MOG, myeline oligodendrocyte glycoprotein; PhaP, phasin (structural PHA inclusion surface protein); OVA ovalbumin (FACS data representation was adapted from Bäckström et al. (2007))

antigen confirming the applicability of bioengineered PHA beads in ELISA (Peters and Rehm 2008; Atwood and Rehm 2009; Parlane et al. 2009).

5.2 Skin Test

One in vivo-produced PHA bead-based approach to in vivo diagnosis is a skin test for the detection of bovine tuberculosis (TB) (Chen et al. 2014). The major disadvantage of the currently widespread tuberculin skin test is the lack of detection specificity for pathogenic *Mycobacterium tuberculosis* complex members, that is, cattle exposed to nonpathogenic, environmental mycobacteria may result in false-positives. Thus, with the aims of improving specificity and lowering production cost, an alternative test was developed. Three immunodominant TB antigens, which are absent in most environmental mycobacteria, were fused to PhaC mediating production of triple-antigen-displaying PHA beads. These granules showed in vitro increased reactivity with antigen-specific TB antibodies when compared with granules displaying only one antigen. Assessment of triple-antigen-displaying PHA beads in the skin test (in vivo) showed specificity as all cattle experimentally infected with *Mycobacterium bovis* were detected while no false-positive reactions in cattle previously exposed to environmental mycobacteria were observed. A fourth antigen was added to the triple-antigen-displaying PHA beads to boost skin test sensitivity (Parlane et al. 2016). Dose response studies showed that very low amounts of mycobacterial antigens ($\geq$0.1 μg) were already sufficient for the skin test suggesting greatly increased immunogenicity of antigen-displaying beads versus soluble antigens. Hence, it was demonstrated that antigen-displaying PHA beads mediate an antigen-specific immune reaction upon injection into the skin, that is, serve as specific immune response stimulating antigen delivery systems.

6 Particulate Antigen Delivery Systems

The concept of in vivo assembly of nano-/microsized PHA beads displaying disease-specific antigen as particulate vaccines was investigated. Since PHAs are considered as biomaterials, when produced coated with pathogen-specific antigens they might serve as safe and efficient particulate vaccine. In general, the nano-/microsized particulate nature of a vaccine mimics the dimensions of a pathogen which boosts immunogenicity via enhanced uptake by antigen presenting cells (APC) (Shah et al. 2014). Immunodominant antigens from pathogens such as HCV and *M. tuberculosis* were displayed on PHA beads using PHA synthase engineering (Parlane et al. 2009, 2011). Purified antigen-coated PHA beads were injected into mice which resulted in strong and specific immune responses mediating protective immunity as assessed by challenge of vaccinated animals with the pathogen (Parlane et al. 2012; Martinez-Donato et al. 2016) (Fig. 6). It is noteworthy that these PHA bead-based vaccines stimulated both a humoral antibody (Th2) and cell-mediated (Th1) immune

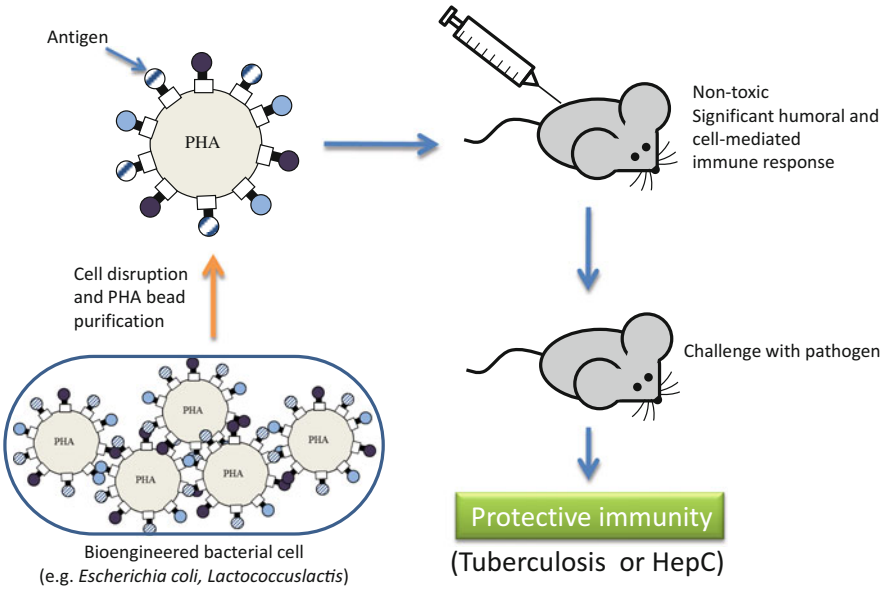

Fig. 6 PHA inclusions displaying disease-specific antigens as particulate vaccines

response, the latter being particularly relevant to protect against intracellular pathogens and is more challenging to achieve.

7 Recombinant Protein Production via Display on Polyhydroxyalkanoate Inclusions

Purification of recombinantly produced proteins, generally with the target protein fused to an affinity tag, can be a time-consuming and costly process since it often requires multiple chromatography steps which all need to be individually optimized, and additionally require cleavage and removal of the tag. Over the past 10 years, various approaches have been undertaken to make use of PHA and PHA-associated proteins for the development of alternative, simple and cost-effective protein purification systems. The general principle that most of these studies have followed is to produce the target protein as a translational fusion to a PHA inclusion-associated protein in cells that also make PHA and to then copurify the protein with the PHA beads. This can be done in a host that naturally produces PHA or in an organism that has been genetically engineered to make PHA (e.g., *E. coli*). Different methods have then been applied to release the target protein from the beads. Early examples used the phasin protein PhaP as an affinity tag and inducible self-cleaving inteins inserted between PhaP and the target protein to release the protein of interest. This method was applied by Barnard and coworkers to purify green-fluorescent protein (GFP) and β-galactosidase (LacZ) from *Ralstonia eutropha*, a natural PHB producer (Barnard

et al. 2005). GFP and LacZ could be successfully purified fused to either end of PhaP using a thiol-inducible intein. Similarly, several proteins (maltose-binding protein (MBP), LacZ, chloramphenicol acetyltransferase (CAT), and NusA) were tagged with PhaP and purified from recombinant *E. coli* with the help of a pH-inducible intein (Banki et al. 2005).

To strengthen the binding of the fusion protein to the bead surface to the point where significant leakage during the purification process could be avoided, multiple (two or three) phasins were used as a tag. Geng and coworkers chose a protein which is generally difficult to produce in bacteria due to the presence of several disulfide bonds (Geng et al. 2010). They fused recombinant human tissue plasminogen activator (rPA), a truncated version of tissue plasminogen activator, to PhaP and inserted a thrombin cleavage site as a linker. Active rPA could be released from isolated beads by thrombin treatment.

In another study which focused on diagnostic applications (aforementioned) the MOG and interleukin-2 were produced as PhaP-fusions containing an enterokinase recognition site (Bäckström et al. 2007). The successful removal of MOG or IL2, respectively, after enterokinase treatment was monitored by FACS analysis.

A slightly different approach to PHA-based protein purification is based on production of fusion proteins separate to PHA extraction and processing into beads. Various target proteins were fused to different granule-associated proteins such as PhaP or the regulatory protein PhaR, the fusion proteins recombinantly produced in *E. coli* and crude cell lysates incubated with beads processed from extracted PHA (Wang et al. 2008, Zhang et al. 2010). Release of the target protein was also achieved via intein-mediated self-cleavage. While this method is more labor-intensive than producing PHA and proteins in the same cell, advantageous might be the possibility to produce the tagged target protein in any host organism including eukaryotes. A simplified approach used the N-terminal part of PhaF to anchor a target protein to PHA beads in vivo, followed by bead isolation and release of the target protein (i.e., the entire fusion of target protein and PhaP) from the beads by detergent treatment (Moldes et al. 2004).

However, a general drawback of using PhaP (or PhaR) as a tag for protein purification is that these proteins are only attached to the PHA inclusion surface via hydrophobic interactions, so there is a risk that the fusion protein could detach during either the bead purification process or the tag cleavage process resulting in loss of target protein. In the natural system, phasins have the advantage of being the predominant protein on the surface of PHA inclusions (Wieczorek et al. 1995); however, in a recombinant system, proteins such as the PHA synthase can be overproduced to achieve a high density at the inclusion surface (Brockelbank et al. 2006; Mifune et al. 2009).

Grage et al. harnessed the covalent attachment of the PHA synthase to PHA inclusions by translationally fusing HcRed or an anti-β-galactosidase single-chain antibody fragment (scFv) (Martineau et al. 1998), separated by an enterokinase recognition site (Grage et al. 2011). Both target proteins were successfully released from purified beads by enterokinase treatment; however, cleavage efficiency was

relatively low. In an attempt to find a robust and inexpensive auto-processing module, Hay and coworkers used a modified soluble form of the cell surface sortase transpeptidase A (SrtA) from *Staphylococcus aureus* which had been engineered to self-cleave in the presence of Ca^{2+} (Mao 2004; Hay et al. 2015a). SrtA and the target protein were fused to the C-terminus of PhaC using an extended linker (Jahns and Rehm 2009). Using this technique, GFP, MBP, and antigen RV1626 from *Mycobacterium tuberculosis* could be released from isolated PHA beads at a high yield and purity (e.g., 6 mg/l of soluble GFP at a purity of about 98%) (Hay et al. 2015a).

8 Affinity Bioseparation Applications Using Display of Binding Domains

It may not always be feasible or desirable to produce the target protein fused to a bead-associated protein (and to coproduce it with the beads/resin). Hence PHA beads have also been engineered to serve as affinity resins for bioseparation, generally exploiting the possibility of densely displaying binding domains on nano-/microbeads. These resins can be produced in one step by fusing the binding domain of choice to the bead-associated protein and then isolating these functionalized beads from the production host (similar to the protein purification approach described above). The first example of this was the immunoglobulin G (IgG) binding ZZ domain of *Staphylococcus aureus* protein A translationally fused to PhaC (Brockelbank et al. 2006). The resulting ZZ domain displaying beads were isolated from *E. coli* and successfully purified IgG from human serum and mouse hybridoma supernatants, with purity and yield comparable to commercially available protein A sepharose (Brockelbank et al. 2006; Lewis and Rehm 2009).

Further PHA bead-based resins assembled by engineering PhaC displayed streptavidin which bound various biotinylated compounds such as enzymes, antibodies, and DNA (Peters and Rehm 2008). Grage and Rehm were able to purify β-galactosidase from a mixture of proteins using anti-LacZ scFv immobilized to PHA beads (Grage and Rehm 2008). An endotoxin-removing resin was developed by producing human lipopolysaccharide-binding protein (hLBP) fused to PhaP in *Pichia pastoris* (Li et al. 2011). After secretion and recovery from the culture supernatant, the hLBP-PhaP fusion was incubated with beads processed from extracted PHA, and the resulting hLBP-beads were tested for their endotoxin removal abilities under a variety of conditions. According to the authors, the beads performed better than commercially available endotoxin-removing gels (Li et al. 2011).

Recently, Hay and coworkers published a more extensive study that aimed at broadening the applicability of PHA bead affinity resins by identifying and testing several easily customizable affinity binding domains which they translationally fused to PhaC (Hay et al. 2015b). This study demonstrated that V_{HH} domains from camelid antibodies, designed ankyrin repeat proteins (DARPins) and OB-folds

(OBodies), could be densely displayed on PHA beads resulting in high affinity binding resins for purification of various target proteins. These binding domains were used to establish extensive libraries of variants enabling screening for binders specific for the target compound of interest (Binz et al. 2004; Harmsen and De Haard 2007; Stumpp et al. 2008; Steemson et al. 2014). PHA-based affinity resins showed a purification performance at least equal to current commercial offerings (Hay et al. 2015b). A recent surface topology study of the *R. eutropha* PhaC attached to PHA inclusions suggested new engineering strategies towards the development of PHA-based affinity resins with increased binding capacity via improved display (Hooks and Rehm 2015). This study identified several surface-exposed flexible regions of PhaC, which tolerated the insertion of the IgG-binding ZZ domain. One of the double ZZ domain insertions (i.e., ZZ inserted in two of the surface-exposed regions) showed greatly improved IgG binding capacity with some of the single insertions also showing improved IgG binding capacity when compared with terminal fusions. Overall, PhaC engineering such as N- or C-terminal fusions and/or insertions enabled efficient display of binding domains for interaction with target compounds resulting in purification performance suitable for application as bioseparation resin.

9 Research Needs

Microbial PHAs show great promise as polymers providing a support structure for display of a range of protein functions. Since PHAs can be composed of various constituent resulting in a diversity of material properties, it currently remains unexplored how these different PHAs perform in the context of anchoring binding domains for display. Additionally, research needs to address bioprocessing challenges to obtain PHAs of consistent structure and shape for improved implementation in various protein display applications.

10 Concluding Remarks

Although microorganisms are capable of producing a variety of polymers, the hydrophobic thermoplastic PHAs currently hold the greatest promise as support material to display protein functions. The PHA material properties allow processing into nano-/microbeads, films, and 3D structures, while PHA-related proteins provide specific PHA binding domains to anchor protein functions of interest. These approaches enabled implementation in microarray-based diagnostics as well as tissue engineering. Besides the binding of protein functions to isolated and processed PHA, recent research elucidated the concept of producing PHA inclusions within the

bacterial cell already coated with desired protein functions. The applicability of these PHA beads as vaccines, in diagnostics and as bioseparation resin was demonstrated.

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Rhamnolipids: Production, Performance, and Application

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Abstract

A circular bioeconomy requires the use of renewable resources to produce high-value specialty chemicals or pharmaceuticals, and also fine and bulk chemicals. Here, the surfactant market represents an ideal test case, because surfactants can cover diverse product classes ranging from fine to bulk chemicals and thus including large differences in purity and price. Biosurfactants produced by microbes from renewable resources are discussed for decades, and recently, sophorolipids arrived in the market, produced by fermentation of high-performing production strains and combined with simple product purification thus reaching low product prices.

Here, we review the current status of rhamnolipid research and applications. Molecular diversity of rhamnolipids and biochemical pathways involved in their synthesis are presented, and physicochemical parameters governing emulsification, foaming, and other properties of rhamnolipids are summarized, followed by applications in many different industries including the agro and pharma industry. We finish with a patent survey that covers rhamnolipid production and potential applications of these biosurfactants. We also tried to identify knowledge gaps that might limit a more rapid establishment of rhamnolipids in the markets.

1 Introduction

Bioeconomy requires to establish novel value chains to valorize waste streams to products for the chemical industry. The concept of biorefinery attempts to integrate biomass and waste conversion and the production of fuels, power, heat, and value-added chemicals. For the realization of a bio-based economy, biorefinery requires industrial scale production of bulk products for scale and high-value products for margin.

Microbial lipids, e.g., neutral lipids from oleaginous yeast, are discussed as alternative to plant-derived oils for the synthesis of biodiesel. While liquid fuels are by far the largest chemical products volume wise, easily guaranteeing large scales in a biorefinery, the contributions of microbial lipids are still under debate. Alternatively, biosurfactants represent an exceptional class of microbially produced lipids, which comprise surface active compounds produced by different fungal and bacterial microorganisms (Lang and Trowitzsch-Kienast 2002; Rosenberg and Ron 2013). This group includes secreted secondary metabolites of diverse composition and from different biosynthetic pathways ranging from small molecules like glycolipids, lipopeptides, and acylated amino acids to polymeric high molecular weight compounds (Smyth et al. 2010; Soberón-Chávez and Maier 2011; Thies et al. 2016). Beyond surface activity, biosurfactants often show beneficial properties like biodegradability, low toxicity (Johann et al. 2016), ability for metal ion complexation as well as antibacterial and antifungal effects (Banat et al. 2000; Marchant and Banat 2012a). In addition, the resource-efficient production from renewable resources is generally listed as benefit of biosurfactants.

Notably, some of these properties are reiterated by many authors, while experimental evidence is often scarce. This might be due to the large range of molecular structures that not only exists between the different classes but also in defined classes of biosurfactants. For example, rhamnolipids differ in the number of hydroxy fatty acid and rhamnose residues, in which the hydroxy fatty acids differ in length, and were even reported with additional decorations including methylation and acetylation (see Abdel-Mawgoud et al. (2010) for details). Consequently, analytical challenges result, which at least partly explain the low comparability of published studies.

While high biodegradability (low environmental accumulation) can be assumed for the structures including ester and glycosidic bonds, other properties like low toxicity and resource-efficient production seem more anecdotal than supported by detailed studies.

2 Rhamnolipids

2.1 Congeners and Biosynthesis

Rhamnolipids are currently considered the best-studied representatives of bacterial biosurfactants (Müller et al. 2012). Due to excellent surfactant properties, low toxicity, high biodegradability, and antimicrobial effects, rhamnolipids are discussed for various applications (Sect. 4), e.g., in cleaning agents, cosmetics, food industry, biocontrol, and soil remediation (Fracchia et al. 2014). A blueprint rhamnolipid consists of a hydrophobic domain with generally two molecules of hydroxy fatty acids forming a 3-(hydroxyalkanoyloxy)alkanoic acid (HAA), which is connected via a glycosidic bond to a hydrophilic part comprising one or two molecules of the sugar rhamnose, thus forming mono-rhamnolipids and di-rhamnolipids, respectively

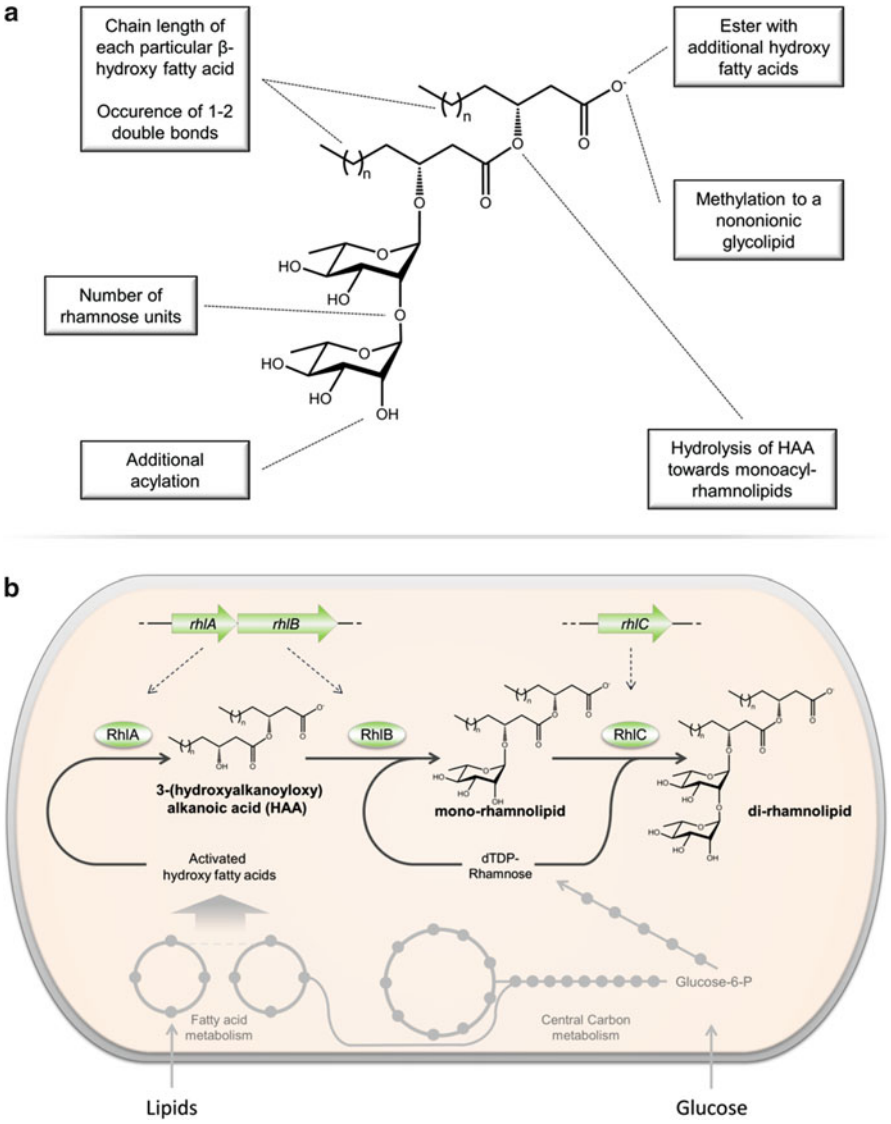

Fig. 1 Congeners and biosynthesis of rhamnolipids. (**a**) Molecular variations of rhamnolipids. Depicted is a typical di-rhamnolipid scaffold, and variable components are indicated. (**b**) Schematic illustration of rhamnolipid biosynthesis from primary metabolism-derived precursor molecules. Genetic organization of *rhlA*, *B*, *C*, and the architecture of the central carbon metabolism vary between different producer strains

(Jarvis and Johnson 1949; Ito et al. 1971). Many different variations of both commonly anionic glycolipids were described (Fig. 1a), most of them varying in the incorporated β-hydroxy fatty acids with respect to chain length and degree of saturation. An excellent overview on the congener diversity is given by Abdel-

Mawgoud et al. (2010). The reported lengths range between chains of 6 and 16 carbon atoms and 24 carbon atoms in a recent single report, with saturated chains dominating, but also occurring of chains with one or rarely two double bonds (Abdel-Mawgoud et al. 2010; Nie et al. 2010; Jadhav et al. 2011). Besides that, there are also congeners described, which occur in smaller amounts and deviate from the blueprint in their degree of acylation. They contain only one β-hydroxy fatty acid, here again a certain range of chains is possible (Lang and Trowitzsch-Kienast 2002), or, reported in a limited number of studies, even three chains or a further acylation/methylation (Hirayama and Kato 1982; Andrä et al. 2006; Abdel-Mawgoud et al. 2010). Natural rhamnolipid producers usually secrete a cocktail of different congeners with at least showing a certain range of fatty acids, as it is also common for other acylated metabolites (Youssef et al. 2005; Dwivedi et al. 2008; Thies et al. 2014). The production of mono- and di-rhamnolipid mixtures is also frequently observed.

At least two enzymes are required for the biosynthesis of rhamnolipids from the precursor metabolites dTDP-rhamnose and activated β-hydroxy fatty acids (Fig. 1b): (i) the acyltransferase RhlA for the generation of HAA and (ii) the glycosyltransferase RhlB for glycosidic bond formation (Abdel-Mawgoud et al. 2011). For a long time, it was assumed that these two proteins have to form a dimer to catalyze rhamnolipid synthesis: hence, they are often designated as subunit A and B of a rhamnosyltransferase, which is challenged by a very recent study that indicates an independent mode of action of both enzymes in vivo (Wittgens et al. 2017). For synthesis of di-rhamnolipids, a second molecule of activated rhamnose is added to mono-rhamnolipids by the rhamnosyltransferase RhlC (Burger et al. 1966). Further enzymes that may be involved in the biosynthesis of additional acylated variants or in the formation of monoacylated rhamnolipids were not elucidated until today. Regarding the latter variants, at least hints are reported that synthesis is rather obtained by the degradation of common rhamnolipid species due to HAA ester cleavage than by promiscuity of RhlB (Wittgens et al. 2017). Likewise unsolved is the mode of secretion of the intracellular biosynthesized molecules into the environment.

Both precursor molecules are supplied by the primary metabolism of the cells. dTDP-rhamnose, which is very common in Gram-negative bacteria, e.g., for the formation of LPS, is derived from glucose-6-phosphate within the sugar metabolism from. β-Hydroxy fatty acids in activated forms are part of the general fatty acid-related metabolic pathways, in de novo synthesis as well as β-oxidation. The stereochemistry of the HAA fatty acids (R-3-hydroxy-acyls (Schenk et al. 1997; Bauer et al. 2006)) resembles the conformation of intermediates of de novo synthesis (FAS II). On the other hand, there are studies, which show a direct influence of β-oxidation to rhamnolipid biosynthesis. Currently proposed is a contribution of both pathways to the R-3-hydroxy-fatty acid pool available for rhamnolipid biosynthesis by interlinks between degradation and synthesis pathways and by R-specific enoyl-CoA hydratases, which branch off intermediates from β-oxidation and convert them to the correct stereoisomer for HAA biosynthesis (Abdel-Mawgoud et al. 2014; Dobler et al. 2015).

2.2 Producer Strains

Microbial rhamnolipid production was initially discovered within the opportunistic human pathogen *Pseudomonas aeruginosa* (Jarvis and Johnson 1949), which is still the best characterized and most frequently applied organism for rhamnolipid production (Müller et al. 2010; Müller et al. 2012). Meanwhile, many more producers were discovered, several of them belonging to the *Pseudomonadales* with *Pseudomona*s sp. (like particular strains of *P. chlororaphis*, *P. fluorescens*, *P. putida*, *P. stutzeri*) or *Acinetobacter calcoaceticus* (Abdel-Mawgoud et al. 2010). Also other members of γ-proteobacteria are reported to produce rhamnolipids like *Pantoea ananatis* (Smith et al. 2016) and other *Pantoea* strains, *Enterobacter* sp. (Rooney et al. 2009; Hošková et al. 2013), *Thermus* sp. (Rezanka et al. 2011), and certain *Serratia rubidaea* strains (Nalini and Parthasarathi 2014). Still another genus with an apparently widespread distribution of rhamnolipid biosynthesis pathways is *Burkholderia* sp. (β-proteobacteria) with many strains capable of the production of rhamnolipids, e.g., *B. plantarii*, *B. thailandensis*, and *B. glumae* (Costa et al. 2011; Tavares et al. 2013; Funston et al. 2016; Elshikh et al. 2017). Remarkably, rhamnolipid synthesis was also discovered within members of completely different prokaryotes, namely, members of *Actinobacteria* (Kügler et al. 2015) and other Gram-positive bacteria (Lee et al. 2005).

The genetic organization of biosynthetic genes varies within rhamnolipid producers. In *P. aeruginosa*, they are split into two bicistronic operons, namely, *rhlAB* and *rhlC* together with a putative membrane protein of so far unknown function. In *B. glumae*, these four genes are clustered in one operon. Additionally, *B. thailandensis* and *B. pseudomallei* are reported to contain two copies of *rhl* operons within the genome, which both contribute to biosynthesis (Dubeau et al. 2009). BLAST analysis (Zhang et al. 2000) of published *Burkholderia s*p. genomes indicates that this is not uncommon amid this phylum as it predicts *B. cepacia*, *B. cenocepacia*, *B. ubonensis*, and *B. metallica* to have likewise two *rhl* clusters. Nonetheless, other strains of *Burkholderia*, e.g., *B. glumae*, harbor only one copy (Voget et al. 2015).

Furthermore, strains exist, which harbor only *rhlA* and *rhlB* and consequently produce exclusively mono-rhamnolipids, like *P. chlororaphis* (Gunther et al. 2005).

Natural biosynthesis is usually controlled by a complex network of regulatory pathways as is common for secondary metabolite production, with quorum sensing systems as a central component. A comprehensive description of the complex regulation of rhamnolipid biosynthesis in *P. aeruginosa* was recently published (Lovaglio et al. 2015).

Many different natural functions of rhamnolipids are proposed including solubilization and uptake of hydrophobic substances, improving surface motility, fighting competitors and predators, or contributing to virulence, and important roles during biofilm formation as summarized by Abdel-Mawgoud et al. (Abdel-Mawgoud et al. 2010).

2.3 Production Strategies

Due to their physical and chemical properties, rhamnolipids are of interest for a variety of biotechnological applications. Consequently, many studies were conducted to develop processes for biotechnological rhamnolipid production and to improve the biosurfactant yields. Despite its pathogenicity, many studies focused on *P. aeruginosa* reporting batch, fed-batch or resting cell process strategies with different carbon sources. Hence, *P. aeruginosa* is currently still the most advanced production strain for mid- and large-scale rhamnolipid production (Müller et al. 2010; Müller et al. 2012). Best results were obtained with hydrophobic carbon sources like plant oils or alkanes with frequently reported titers at a two-digit g/L scale (Leitermann et al. 2010), exceptionally high titers of up to 112 g/l were reported once in a patent application (Giani et al. 1995).

Nonetheless, in order to circumvent safety and regulation issues due to pathogenicity, several studies were conducted exploring processes with nonpathogenic producer strains, e.g., *P. fluorescens* and *P. chlororaphis* (Gunther et al. 2005; Toribio et al. 2011), or strains of *P. aeruginosa*, *Acinetobacter*, and *Enterobacter* claimed to be nonpathogenic (Hošková et al. 2013; Grosso-Becerra et al. 2016). Much attention in this context is furthermore payed to the genus *Burkholderia* containing many rhamnolipid producers that are non-hazardous for human beings (Hörmann et al. 2010; Toribio et al. 2010; Costa et al. 2011; Díaz De Rienzo et al. 2016; Funston et al. 2016).

A different strategy uses recombinant production strains, thus avoiding safety issues and furthermore circumventing complex metabolic regulation systems as depicted in Fig. 2. Typical *P. aeruginosa* fed-batch fermentations reveal a strong dependence of rhamnolipid production on the growth (Fig. 2a, b). After entering the stationary phase, the specific rhamnolipid production rate increases, until reaching its maximum and subsequently declines. In contrast, recombinant rhamnolipid synthesis is uncoupled from growth resulting in a constant-specific rhamnolipid production rate (Fig. 2c, d).

To achieve decoupled production in recombinant strains, the implementation of artificially controlled *rhl* genes in nonpathogenic natural producers may be one approach to increase their performance and/or modify rhamnolipid composition (Tavares et al. 2013). A more frequently applied strategy generates completely novel artificial production systems, where biosynthesis genes from natural rhamnolipid producers were introduced into suitable nonpathogenic host bacteria that are not capable of rhamnolipid formation by nature resulting in safe production strains with adjustable *rhlAB*(*C*) expression characteristics (Table 1). Applying this strategy, mono-rhamnolipid production was achieved by the introduction of *P. aeruginosa rhlAB* into *E. coli*, *P. fluorescens*, *P. oleovorans*, or *P. putida* (Ochsner et al. 1995; Kryachko et al. 2013). In particular the latter strain appears to be excellently suited for recombinant rhamnolipid production (Tiso et al. 2014, 2015). Consequently, there are several reports of successful strategies for rhamnolipid production with *P. putida*, in particular the certified safety strain KT2440 and derivatives thereof, yielding titers at g scale (Fig. 2) (Loeschcke and Thies 2015). Interestingly, introducing the genes from

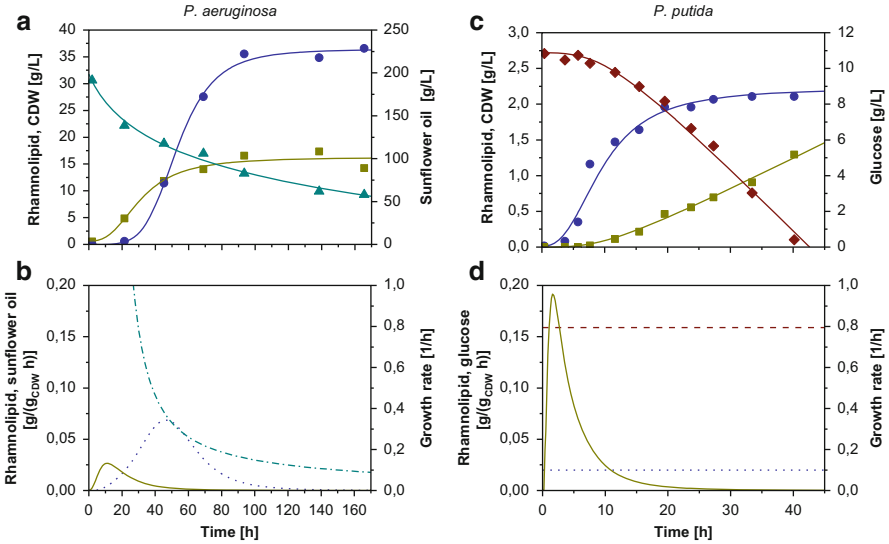

Fig. 2 Cultivation strategies using the native rhamnolipid producer *P. aeruginosa* (*left*) in comparison to the recombinant cell factory *P. putida* (*right*). (**a**) and (**c**): courses of CDW, RL, and substrate concentrations. *Blue dots* show RL titers, while *dark yellow rectangles* show CDW. *Red diamonds* represent glucose and *dark cyan* sunflower oil concentrations. The line show fitted trends. (**b**) and (**d**): RL production, growth, and substrate consumption rates during cultivation. The *dark yellow solid lines* show the growth rates, while the *dotted blue lines* show RL productivity. The *red dashed line* represents glucose consumption and the *dashed-dotted dark cyan line* shows the trend for sunflower oil consumption. The data for the *P. aeruginosa* cultivation was taken from Müller et al. (2010), while the data for the cultivation of *P. putida* was taken from Wittgens et al. (2011)

P. aeruginosa yielded the same congener distribution in *P. putida* (Behrens et al. 2016a, b, c). Different expression systems were evaluated in these studies for the expression of the ca. 2 kb operon, namely, the synthetic hybrid promoter P_{tac} (Ochsner et al. 1995; Wittgens et al. 2011; Setoodeh et al. 2014; Wittgens et al. 2017), completely different synthetic promoters (Blank et al. 2013a; Beuker et al. 2016a, b; Tiso et al. 2016; Wigneswaran et al. 2016), or the native regulation system via coexpression of the cognate autoinducer-dependent transcription factor/autoinducer synthase pair RhlR/RhlI from *P. aeruginosa* (Cha et al. 2008; Cao et al. 2012). To our knowledge, Beuker et al. (2016b) achieved the highest titer (14.9 g/L) with a recombinant system with a yield ($Y_{rhamnolipid/substrate}$) of 10 mg/g glucose applying synthetic promoter-controlled expression of *rhlAB* in *P. putida*.

Recombinant production of di-rhamnolipids appears to be less pursued; nonetheless, successful studies are also reported. One strategy is the expression of *P. aeruginosa rhlC* in natural mono-rhamnolipid producers as *P. chlororaphis* (Solaiman et al. 2015). Other studies describe the heterologous expression of all three rhamnolipid synthesis genes in *P. putida*. The expression of *rhlABC* from *P. aeruginosa* yielded up to 113 mg/l/OD_{600} using PHA-deficient *P. putida* GPp104 (Schaffer et al. 2012). Blank et al. (2013a) reported the production of both mono-

Table 1 Summary of approaches for recombinant rhamnolipid production (Updated from Loeschcke and Thies 2015)

Origin of genes[a]	Expression host[b]	Expression strategy[c]	Max titers[d]	Reference
Mono-rhamnolipids				
P. aeruginosa	*E. coli*	P_{tac}, *rhlAB*	<0.02 g/L	(Ochsner et al. 1995)
	E. coli	P_{lac}, *rhlAB*	0.005 g/L	(Kryachko et al. 2013)
	P. fluorescens	P_{tac}, *rhlAB*	<0.02 g/L	(Ochsner et al. 1995)
	P. oleovorans	P_{tac}, *rhlAB*	<0.02 g/L	(Ochsner et al. 1995)
	Burkholderia kururiensis	P_{tac}, *rhlAB*	5.67 g/L	(Tavares et al. 2013)
	P. putida KT2442	P_{tac}, *rhlAB*	0.60 g/l	(Ochsner et al. 1995)
	P. putida KCTC 1067	P_{native}(RhlRI), *rhlABRI*	7.3 g/l	(Cha et al. 2008)
	P. putida KT2440	P_{tac}, *rhlAB*, [d]Δ*phaC1*	1.5 g/l	(Wittgens et al. 2011)
	P. putida KT2440	P_{native}(RhlRI), *rhlABRI*	1.68 g/l	(Cao et al. 2012)
	P. putida KT2440	P_{tac}[d]/$P_{synthetic}$, *rhlAB* [d] Δ*phaC1*	up to [1]2.5 g/l	(Blank et al. 2013a)
	P. putida KT2440	P_{tac}, *rhlAB*	0.57 g/l	(Setoodeh et al. 2014)
	P. putida KT2440	$P_{synthetic}$, based on P_{rRNA}, *rhlAB*	0.02 g/l/ 0.08 g/l in biofilm reactor	(Wigneswaran et al. 2016)
	P. putida KT2440	$P_{synthetic}$, *rhlAB*	0.6 g/l	(Beuker et al. 2016a)
	P. putida KT2440[1] KT40CZC	$P_{synthetic}$, *rhlAB*	[1]3.2 g/l	(Tiso et al. 2016)
	P. putida KT2440	$P_{synthetic}$, *rhlAB*	14.9 g/l	(Beuker et al. 2016b)
+ Di-rhamnolipids				
P. aeruginosa	*P. chlororaphis*	*rhlC*	0.1 g/L	(Solaiman et al. 2015)
	P. putida KT2440 *P. putida* GPp104	P_{lac} *rhlAB*/*rhlABM*, *rhlABC*/*rhlABMC*	di-RL: 0.11 g/ l/OD_{600}	(Schaffer et al. 2012)
	P. putida KT2440-derived BOA-PP-002	$rhaP_{BAD}$, *rhlABC*, [d]*alkBGT* from *P. putida* Gpo1	>1.2 g/l	(Gehring et al. 2016)

(*continued*)

Table 1 (continued)

Origin of genes[a]	Expression host[b]	Expression strategy[c]	Max titers[d]	Reference
	P. putida KT2440	P_{tac}, *rhlAB/rhlABC/rhlC*	0.005 g/l (mono-RL) 0.004 g/L (mixture)	(Wittgens et al. 2016)
B. glumae	*P. putida* KT2440	P_{tac}, *rhlAB*(*C*)	0.08 g/l (mono-RL), 0.05 g/l (mixture)	(Blank et al. 2013a)

Source organisms whose corresponding biosynthetic genes were employed ([a]) are listed together with the applied production host ([b]) and the respective expression strategies ([c]). Here, promoters and genes are named. Additional strain engineering is indexed ([d]). Product yields are taken from the original publications. (*RL* rhamnolipid)

and di-rhamnolipids expressing *rhlAB*(*C*) genes not only from *P. aeruginosa* but also *Burkholderia glumae* PG1 controlled by P_{tac}, yielding 80 mg/l of pure mono-rhamnolipids and 50 mg/l of a mixture, respectively. Two strategies to achieve di-rhamnolipid production in *P. putida* were recently described (Wittgens et al. 2017). Here, biosynthesis was achieved either by the expression of an artificial operon of *P. aeruginosa rhlABC* or by feeding of mono-rhamnolipids to an *rhlC*-expressing strain, thereby proving uptake of the glycolipids by *P. putida*. Gehring et al. reported a strategy for di-rhamnolipids production in advance of an industrial process. They implemented *P. aeruginosa* like *rhlABC* genes controlled by a rhamnose inducible promoter in *P. putida* KT2440 (Gehring et al. 2016).

Metabolic engineering of producer strains in general was identified as useful strategy for process optimization. Hereby, the utilization of more suitable carbon sources can be implemented; moreover the host's metabolisms can be streamlined toward rhamnolipid production, e.g., by the elimination of competing pathways, boosting the primary metabolism or enhancing (recombinant) biosynthesis gene expression (Wittgens et al. 2011; Martinez-Garcia et al. 2014; Tiso et al. 2016).

3 Physicochemical Characterization of Rhamnolipids

Due to their surface activity, biosurfactants are, like oil-based surfactants, extensively discussed as emulsifiers, de-emulsifiers, cleaners, wetting, dispersing, and foaming agents (Moya Ramirez et al. 2015; Gudiña et al. 2016), in bioremediation of soil and sand (Van Dyke et al. 1991), and in the cleanup of hydrocarbon contaminated groundwater and enhanced oil recovery (Ron and Rosenberg 2001) (Sect. 4).

Compared to oil-based surfactants, biosurfactants are typically composed of a set of different molecules. Therefore the surface activity of, e.g., rhamnolipids described in literature varies as different production processes, and purification steps were used leading to different compositions.

Important physicochemical characteristics of biosurfactants for applications are their surface tension, critical micelle concentration, foaming behavior, wetting properties, and emulsification activity. Biological properties as, e.g., biodegradability, ecotoxicity, skin compatibility, and potential antimicrobial activity are also important for applications and are described in Sect. 4

In the following, methods are described to determine the physicochemical properties and the respective data reported for rhamnolipids. In most studies, only mixtures of rhamnolipids were analyzed, while congener properties are rare to date.

3.1 Surface Tension

The surface tension, usually represented by the symbol γ, is the cause of the behavior of liquids, such as water, to form energetically favorable spherical drops. The dimension is given in force per unit length [N/m]; in some older publications, [dyn/cm] is also used, where 1 mN/m corresponds to 1 dyn/cm. Pure water has a relatively high surface tension of 72.8 mN/m at 20 °C. The reduction of surface or interfacial tension, especially of water-based formulations, facilitates the wetting of solid surfaces and improves the stability of emulsions and dispersions. This is important for applications like printing, coating, cleaning, or the dispersion of pigments. There are several methods to measure the equilibrium or dynamic surface tension or interfacial tension, e.g., Wilhelmy plate method, Du Nouy ring method (Varjani and Upasani 2016), bubble pressure method, or drop volume method. For liquids with higher viscosity or if only very small amounts of liquid are available, the pendant drop measurement with drop shape analysis is a good alternative. The surface tension of surfactant solutions depends on the temperature and, for biosurfactants with ionizable groups, the pH value.

Rhamnolipids are reported to reduce the water surface tension from 72 to 35, 28 mN/m, or even lower values (25.9 mN/m) (Ma et al. 2016). The studies used different producer organisms, growth substrates, and fermentation processes and solutions with different pH values and concentrations of biosurfactant (Paulino et al. 2016; Varjani and Upasani 2016). Nevertheless, the values are in the range of sodium dodecyl sulfate (SDS), a very effective and often used surfactant.

3.2 CMC/CMD

Another important primary characteristic of a surfactant is the critical micelle concentration (CMC). It is defined as the concentration of surfactants above which micelles form and surfactant monomers and micelles exist in a dynamic equilibrium (Dominguez et al. 1997). It provides good insights into the nature of the surfactant's self-association. Low CMC values indicate that the minimum surface tension is reached with lower amounts of the surfactant, and therefore the surfactant is more efficient. The CMC value depends on temperature, pH, salt concentration, and presence of organic impurities. For surfactants with defined and known structure,

the CMC is often given in mol/L; for biosurfactants, the dimension mg/L is used. Especially during process optimization, the critical micelle dilution (CMD) is often used to characterize non-purified products. Here, a cell-free supernatant is diluted tenfold (CMD^{-1}) and 100-fold (CMD^{-2}) (Makkar and Cameotra 1998; Bordoloi and Konwar 2008) for measurements of surface tension.

The methods for CMC determination include measuring of conductivity, solubility, viscosity, light scattering, and surface tension (Song et al. 2015), of which the determination by the use of tensiometers is the most popular one. Typically, these measurements are performed with a Wilhelmy plate and an automated dosing accessory. The measured surface tension as a function of the logarithmic bulk surfactant concentration will result in a curve that can be fitted with two straight lines with the CMC at their intersection.

For rhamnolipids reported CMC values are between 230 mg/L for rhamnolipid mixtures with higher proportion of congeners with unsaturated fatty acids (Abalos et al. 2001) and 5 mg/L (Syldatk et al. 1985; Dubeau et al. 2009; Costa et al. 2010; Gogoi et al. 2016). The CMC value of 5 mg/L was reported by Nitschke et al. (2005) for di-rhamnolipid Rha-Rha-C_{10}-C_{10}, while the mono-rhamnolipid Rha-C_{10}-C_{10} reached 40 mg/L. Rhamnolipids obtained by Gudiña et al. (2015) showed CMC values between 10 and 200 mg/L; Ma et al. (2016) found 50 mg/L for their rhamnolipids. In general, low CMC values (11–20 mg/l) are observed in mixtures containing mainly mono-rhamnolipid with C_{10} fatty acids (Guo et al. 2009). The CMC strongly depends on the producing microorganisms. In single-strain cultures, Hošková et al. (2015) measured for rhamnolipid mixture produced by *P. aeruginosa* a CMC value of 56 mg/L, while rhamnolipids produced by *A. calcoaceticus* and *E. asburiae* revealed values of 15 and 21 mg/L, respectively. For comparison, the CMC for SDS in water is ten to hundred times higher with a value of 2100 mg/L.

3.3 Emulsification Activity

The emulsification activity or index (E24) is used to characterize emulsifying properties of biosurfactants, generally for oil-in-water (o/w) emulsions. It is often used as an indirect method to screen biosurfactant production (Thavasi et al. 2011). It was first described by Cooper and Goldenberg (1987) to measure the emulsifying activity of biosurfactants from *Bacillus* species. They vortexed for 2 min a defined volume of aqueous biosurfactant solution with a defined volume of a nonmiscible liquid of interest, in their investigation kerosene. They defined the emulsion index E24 as the height of the emulsion layer after 24 h, divided by the total height, multiplied by 100. Some authors extended the time to 30 days to study long-term behavior (Varjani and Upasani 2016). The values strongly depend on the experimental setup; nevertheless, the index gives indicative results concerning emulsifying behavior.

Investigations on rhamnolipids obtained from *P. aeruginosa* DN1 showed excellent emulsification activity in the order of 100% to several hydrocarbons (Ma et al. 2016). Gudiña et al. (2015) measured emulsification indices in the order of 60–70% using

n-hexadecane. Rhamnolipids produced by *P. aeruginosa* SP4 (Pornsunthorntawee et al. 2008) were found to exhibit excellent emulsification properties for vegetable oils (palm oil, soybean oil, coconut oil, and olive oil) but failed to emulsify short-chain hydrocarbons (pentane, hexane, heptane, toluene, and 1-chlorobutane).

3.4 Foaming Behavior

All surface-active substances tend to build foams when gas is introduced into the liquid phase. The foaming behavior of biosurfactants is vital for many industrial applications. For some applications, the formation of foams is desirable, e.g., in body care, culinary foams, flotation, and firefighting. However, in many applications foaming is unwanted and must be prevented, e.g., in printing and coating, cooling lubricants, liquid conveying, industrial cleaning, or during fermentation processes. Particular areas of application lead to different requirements for foamability, foam stability, moisture content, and the size of bubbles in the foam. Over the past decades, researchers have developed many empirical tests to evaluate foaming performance. Methods include the Rudin test used in the brewing industry (Rudin 1957), Bikerman test (Bikerman 1973), Ross-Miles method (Ross and Miles 1941), confocal microscopy method (Koehler et al. 2004), resistance-strengthening technology (Barigou et al. 2001), and others. The most common of these is the Ross-Miles method. The foamability of surfactant solutions and the stability of the foam produced are determined based on height measurement (Lunkenheimer et al. 2010). Several international standards were deviated from the method, of which ASTM D 1173 "Standard Test Method for Foaming Properties of Surface-Active Agents" is the one closest to the original publication.

During the fermentation of rhamnolipids, strong foaming is observed, which limits the yield and therefore increases production costs. Rhamnolipids are discussed as the dominant component that causes the severe foaming during fermentation (Zhang and Ju 2011). Alternatively, foam fractionation is suggested as in situ product removal method, hence taking advantage of the foamability of rhamnolipids (Siemann-Herzberg and Wagner 1993; Blank et al. 2013b; Küpper et al. 2013; Beuker et al. 2016a). In a recent systematic study, Long et al. (2016) demonstrated that purified rhamnolipids show a foaming behavior close to SDS, a very strong foaming agent, but only if additional stirring was applied. With the classical Bikerman test, the rhamnolipid showed a comparable foam stability as Tween 20, which is well known for its poor foam stability.

3.5 HLB/HLD

The hydrophilic-lipophilic-balance (HLB) number was introduced by Griffin and coworkers (Griffin 1949, 1954) to classify nonionic surfactants according to their emulsifying properties: the higher the HLB number, the more hydrophilic (water soluble) and the lower the HLB number, the more lipophilic (oil soluble) the

surfactant will be. The system was developed for ethoxylated surfactants, and the transferability of the concept to other surfactant structures is critically discussed in the literature. Nevertheless, it is an often used concept to select surfactants for specific formulations. In general, surfactants with HLB 4–6 are used to stabilize water-in-oil (w/o) emulsions, with HLB 8–12 for oil-in-water (o/w) emulsions, and with HLB 13–15 to formulate detergent solutions. The HLB can be calculated if the structure is known or determined experimentally by different methods, e.g., ^{1}H-NMR, dielectric constant, titration of phenol, gas chromatographically, or colorimetrically (Rabaron et al. 1993).

Another approach to select suitable surfactants for microemulsions is the hydrophilic–lipophilic deviation (HLD), a dimensionless number expressing the difference of affinity of the surfactant for the oil and water phases (Nardello et al. 2003; Witthayapanyanon et al. 2008).

No data on HLB or HLD numbers for rhamnolipids can be found in literature. Long et al. (2013) stated for rhamnolipids a HLB number of 10.9, unfortunately without details about experimental data or information about the investigated rhamnolipid.

For the application of rhamnolipids in, e.g., cosmetics or drug delivery products, knowledge about the HLB or HLD numbers is very important for a developer of formulations (Schmidts et al. 2010). Therefore, the determination of these numbers should be addressed in future works.

4 Applications of Rhamnolipids

The physicochemical properties described above suggest many possible applications for rhamnolipids also indicated by a high number of scientific publications as well as patents which have been summarized in excellent reviews (Lang and Wullbrandt 1999; Maier and Soberon-Chavez 2000; Irfan-Maqsood and Seddiq-Shams 2014; Paulino et al. 2016).

The first application for rhamnolipids after their discovery in 1963 (Burger et al. 1963) emanated in the late 1980s (Linhardt et al. 1989). Among the first patents, several described the production of L-rhamnose from rhamnolipids (Mixich et al. 1990). From this point on, rhamnolipids were used for a broad range of different applications, for various market sizes and requirements in terms of product purity. For example, different purities of rhamnolipids are suggested for the enhancement of crude oil recovery and in pharmaceutical applications. Up to 2010, rhamnolipids remained the only biosurfactants that have been approved for the use in food, cosmetic, as well as in pharmaceuticals (Toribio et al. 2010).

The annual worldwide consumption of chemical surfactants in 2012 was estimated to be in the order of 13 million tons (Marchant et al. 2014). In 2013, the biosurfactant market was around 350 thousand tons from which at least two thirds were artificially synthesized surfactants as methyl ester ketones and alkyl polyglucosides (Gran View Research 2015). Hence, the contribution of microbial

biosurfactants to the total surfactant market is lower than 2%, indicating a huge remaining market potential.

4.1 Applications with Environmental Concern

Among the most promising applications for rhamnolipids identified so far are those related to environmental concerns, such as bioremediation and enhanced oil recovery (Nitschke et al. 2005). Additionally, rhamnolipids find applications in agriculture.

4.1.1 Microbially Enhanced oil Recovery

Microbially enhanced oil recovery (MEOR) exploits specific traits of microorganisms to enhance oil recovery from oil wells, especially in tertiary oil recovery (Raiders et al. 1989). Two methods for MEOR exist. Ex situ MEOR uses culture broth gained from classical fermentations, which is injected into the oil reservoir. In in situ MEOR, the reservoir is inoculated with the bacteria itself (Paulino et al. 2016). Using ex situ MEOR is a selective method, which only requires small quantities of the surfactant and has a broad application range (oil type and reservoir conditions) (Irfan-Maqsood and Seddiq-Shams 2014). For both types of MEOR, lab experiments resulted in promising results. In situ experiments were, for example, carried out using *Pseudomonas aeruginosa* F-2 on oily sludge (Yan et al. 2012). The challenges are manifold, as many of the rhamnolipid producers are non-fermentative, but, like *P. aeruginosa*, can use alternative electron acceptors like nitrate and sulfur compounds, possibly contained in crude oil.

4.1.2 Bioremediation

There are different types of contaminations (mainly from anthropogenic origin) that can be remediated with the help of rhamnolipids with the most obvious being hydrocarbons, but the reduction of heavy metal contamination with rhamnolipids is also discussed (Nitschke et al. 2005).

Hydrocarbon Pollutions

Applying rhamnolipids for bioremediation has been tested intensively. Under laboratory conditions enhancement of the degradation of a whole range of different substrates could be shown. Hexadecane and octadecane were degraded using *Pseudomonas* strains (Desai and Banat 1997), *n*-paraffin, and phenanthrene (Maier and Soberon-Chavez 2000), and the herbicide atrazine by *Acinetobacter* (Singh and Cameotra 2014).

In principle, two methods are feasible for the remediation of contaminated soils: degradation of the pollutants and flushing/washing of soils.

Flushing of hydrocarbons from contaminated sandy loam and silt loam soil could benefit from the application of rhamnolipids (Van Dyke et al. 1993). The removal of aliphatic and aromatic hydrocarbons (Scheibenbogen et al. 1994), as well as polyaromatic hydrocarbons, and pentachlorophenol (Sachdev and Cameotra 2013) was

also increased by adding rhamnolipids. Flushing of soils enhanced by rhamnolipids furthermore showed removal of hexadecane, octadecane, phenanthrene, pyrene, polychlorinated biphenyls, a variety of PAH, and hydrocarbon mixtures (Maier and Soberon-Chavez 2000) as well as crude oil (Lang and Wullbrandt 1999).

For the remediation of oil spills like in the Gulf of Mexico in 2010, vast amounts of detergent are required. After the Exxon Valdez oil spill in 1989, it could be shown that rhamnolipid-aided large-scale in situ bioremediation by washing was successful for the removal of oil from contaminated Alaskan gravel (Harvey et al. 1990; Bragg et al. 1994).

Rhamnolipid-aided degradation of hydrocarbons was shown for hexadecane, pristine, tetradecane, creosote, and hydrocarbon mixtures (Maier and Soberon-Chavez 2000). In addition, natural breakdown of hydrocarbons in marine oil pollution was demonstrated to benefit from rhamnolipid application (Lang and Wullbrandt 1999).

Rhamnolipids may stimulate the indigenous bacterial population to degrade hydrocarbons at increased rates (Desai and Banat 1997). Mechanisms include increased hydrocarbon availability by forming rhamnolipid-fostered emulsions (Maier and Soberon-Chavez 2000). Another effect might be the interaction with the cell surface to make it more hydrophobic, which causes the cells to associate more easily with the hydrocarbons (Maier and Soberon-Chavez 2000). To explain the enhancement of oil degradation by washing, it has been speculated that above the CMC, hydrophobic compounds can diffuse into the center of the micelles (Paulino et al. 2016), which can then be flushed more easily. All in all, this leads to the efficient breakdown and removal of pollutants from contaminated areas aided by rhamnolipids. Thus, less surface active agents have to be introduced into the polluted areas (Nitschke and Costa 2007) diminishing the negative impact on nature even further. Bioremediation of sites polluted with hydrocarbons is therefore an important field of application for rhamnolipids, as here low requirements regarding purity exist and thus production costs remain low.

Metal Pollutions

Contaminations with metals and heavy metals are not easily remediated. Metal contamination in soils is, for example, caused by the use of metal salt-based fungicides. However, in higher concentrations, they can cause damage to plants, making it important to remove the metals from the soil. Another important field of application for the removal of metal pollutions is the bioremediation of former industrial sites.

Rhamnolipids have been shown to be able to remove Cd, Pb, and Zn from soil (Gautam and Tyagi 2006). Also metals such as Ba, Ca, Cu, Li, Mg, Mn, and Ni could be washed from soil (Sachdev and Cameotra 2013). A technique called micellar-enhanced ultrafiltration was applied for the successful removal of Cd, Cu, Ni, Pb, and Zn (El Zeftawy and Mulligan 2011). Rhamnolipid-enhanced flushing of soils was used for the removal of Cd, Cu, La, Pb, and Zn (Maier and Soberon-Chavez 2000).

Metal organic co-contaminated sites could be partially cleaned when rhamnolipids were added to the cleaning solution. In lab experiments, the degradation of naphthalene in a culture co-contaminated with cadmium was shown. In experiments using real soils, hydrocarbon degradation was enhanced in soils contaminated with cadmium and phenanthrene (Maier and Soberon-Chavez 2000).

The general mechanism for the removal of metals from soils is solubilization and subsequent washing. A proposed mechanism may involve a combination of complexation of the heavy metals with the rhamnolipid molecules and interaction with the cell surface to alter uptake of heavy metals (Ron and Rosenberg 2001; Gautam and Tyagi 2006). Complexes formed can easily be washed from the soil matrix (Christofi and Ivshina 2002). As in the hydrocarbon pollution, the micelle-forming capabilities help in heavy metal remediation. The polar heads of the surfactant in the micelles can bind to metals and facilitate desorption of the metal ions from the soil. The metal ions in emulsion can then be removed by flushing (Kiran et al. 2016). Apparently, also the remediation of sites polluted with metals can benefit from the addition of rhamnolipids. Moreover, rhamnolipid-aided remediation can also cope with co-contaminations, which make these glycolipids a powerful tool for a whole range of bioremediation applications.

4.1.3 Agriculture

The applications of detergents in agriculture are manifold, including their use as antifoam, superspreader, and active ingredient. Rhamnolipids can be applied on the soil or on the plant itself. The main impact of the rhamnolipids on plantations are their anti-pathogenic effects, which they feature regardless if introduced into the soil or sprayed on the plant.

The soil furthermore benefits by an improved quality, caused by the decrease in plant pathogens and an increase in nutrient availability (Paulino et al. 2016). Their positive effects on the rhizosphere were, for example, demonstrated by reducing damping-off disease in plants (Sharma et al. 2007a, b). As stated above rhamnolipids also help to improve soil quality by remediation of different pollutants, which is mainly done by solubilization of the hydrophobic molecules and increasing their bioavailability. The same mechanism acts on hydrophobic nutrients, thus increasing the uptake of these compounds by the plant. In addition, the wettability of soils can be increased by surfactants, leading thus to a better distribution of fertilizers.

On plants, rhamnolipids also serve as anti-plant pathogenic compounds by prohibiting growth of certain plant pathogens. Examples can be found in the successful treatment of *Nicotiana glutinosa* infected with tobacco mosaic virus or of *Nicotiana tabacum* for the control of potato virus X disease (Haferburg et al. 1987).

Rhamnolipids also show antifungal effects, which is why they are the active substance in the US EPA approved Zonix fungicide (Jeneil Biosurfactants Co., Saukville, USA) (Müller et al. 2012). They are effective against *Fusarium oxysporum* wilt disease in tomato plants (Deepika et al. 2015) and inhibit zoospore-forming plant pathogens that have acquired resistance to commercial chemical pesticides (Sachdev and Cameotra 2013). For example, zoospores of the

oomycete pathogen *Phytophthora capsici*, which is the causative agent of the damping-off disease of cucumber are lysed by rhamnolipids (Kruijt et al. 2009). Also the zoosporic plant pathogens *Olpidium brassicae*, *Phytophthora capsici*, *Plasmopara lactucae-radicis*, and *Pythium aphanidermatum* are effectively deactivated (Maier and Soberon-Chavez 2000). Furthermore, rhamnolipids can be used to prevent adhesion of microbes to roots and reduce bacterial biofilm formation (Haba et al. 2003). Another trait beneficial for agriculture is the insecticidal activity of rhamnolipids, for example, against the green peach aphid (*Myzus persicae*) (Kim et al. 2011).

Furthermore, rhamnolipids can mediate resistance against microbes by stimulating the plant immune system (Vatsa et al. 2010; Sanchez et al. 2012). This effect potentially facilitates the use of rhamnolipids as so-called priming agents. For priming, the surfactant is applied to the seeds prior to plantation. The plant subsequently develops immune responses against certain pathogens although the rhamnolipid is not present anymore. The mechanism of rhamnolipid action remains to be elucidated. However, rhamnolipids have more beneficial effects if applied to seeds. da Silva et al. (2015) showed an increase in germination rate of lettuce and corn, while soybeans showed increased seedling development.

A possible mechanistic explanation why rhamnolipids are more effective as chemical surfactants might be that rhamnolipids enhance the foliar (leaf) penetration of soluble molecules and the leaf wettability and surface properties (Liu et al. 2016). Thus, the plant can take up the biosurfactant more effectively.

In summary, rhamnolipids exert numerous effects when applied in agriculture with the main benefit being the use as anti-plant pathogenic agents. Since plant vermin causes tremendous losses, a huge market potential exists for rhamnolipids as plant protection agents.

4.2 Applications for Consumer Goods

Another broad field of rhamnolipid applications comprises its use as additive in consumer goods, namely, food, cosmetics, household detergents, and even medicinal products. Here, we briefly summarize some applications mentioned in the literature.

Rhamnolipids are applied in the food industry and also in medical fields because of their high potential to inhibit biofilm formation (for a mechanistic explanation, see "Biological Control") (Paulino et al. 2016). In the food industry, biofilms are encountered on processing devices, where they pose a potential threat for hygiene. In medical applications, biofilms growing, for example, on implants represent a major threat for the patients.

4.2.1 Food

Rhamnolipids are discussed as natural food additives, which can replace chemicals as they are of biological origin and can thus easily comply with the guidelines for natural organic foods. Rhamnolipids are reported to control consistency, which is important for the sensual enjoyment, delay staling, solubilize flavor oils (Nitschke

and Costa 2007), stabilize fats, and reduce spattering (Kosaric 2001). They have already been applied to improve texture and shelf life of starch-containing products (Nitschke and Costa 2007). In addition, rhamnolipids can modify the rheological properties of wheat dough and may be used to improve the consistency and texture of fat-based products (Kachholz and Schlingmann 1987). Properties of products such as butter cream, croissants, and frozen confectionery also benefit from the addition of rhamnolipids (Irfan-Maqsood and Seddiq-Shams 2014).

4.2.2 Cosmetics

Surfactants in general are widely applied in the cosmetics industry (Klekner and Kosaric 1993). As biosurfactants are believed to have a higher skin compatibility and only low skin irritation potential, they feature advantages over synthetic surfactants (Haba et al. 2003). Rhamnolipids are specifically mentioned in patents for the production of liposomes and emulsions (Maier and Soberon-Chavez 2000). Here, the pronounced emulsifying activity of rhamnolipids is beneficial for the texture of cosmetic products (Haba et al. 2003).

Rhamnolipids have already been applied as cosmetic additives (Maier and Soberon-Chavez 2000) and in health care products in several different formulations, for example, insect repellents, antacids, acne pads, anti-dandruff products, contact lens solutions, deodorants, nail care products, and toothpastes (Lourith and Kanlayavattanakul 2009). Also cosmetics as anti-wrinkle and antiaging products were produced in several dosage forms as commercial skin care cosmetics using rhamnolipids (Lourith and Kanlayavattanakul 2009). Rhamnolipids exert a broad range of functions including emulsifying and antimicrobial activity (e.g., in acne pads). The increased availability of rhamnolipids will foster further testing; hence, we expect many more applications in the future.

4.2.3 Pharmaceuticals

Rhamnolipids are in general suited for medical applications as they show antibacterial, antiphytoviral (Rodrigues et al. 2006), and excellent antifungal properties (Abalos et al. 2001). In addition, they are reported to stimulate the immune system of animals (Vatsa et al. 2010). They furthermore can mediate the disruption of established biofilms (Marchant and Banat 2012) or inhibit the adhesion of yeasts and bacteria to voice prostheses (Rodrigues et al. 2006).

Antimicrobial effects of rhamnolipids were demonstrated against *Serratia marcescens*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Staphylococcus epidermidis* (Haba et al. 2003), *Bacillus subtilis* (Wittgens et al. 2011), *Escherichia coli*, *Micrococcus luteus*, *Alcaligenes faecalis*, *Rhodococcus erythropolis*, *Bacillus cereus*, *Mycobacterium phlei* (Irfan-Maqsood and Seddiq-Shams 2014), and *Listeria monocytogenes* (Magalhães and Nitschke 2013); here, combination with nisin resulted in a synergistic effect.

Antifungal properties were shown against *Chaetomium globosum*, *Penicillium funiculosum*, *Gliocladium virens*, *Fusarium solani* (Haba et al. 2003), *Aspergillus niger*, *Chaetosphaeridium globosum*, *Penicillium chrysogenum*, *Aureobasidium pullulans*, *Botrytis cinerea*, *Rhizoctonia solani* (Abalos et al. 2001), and *Penicillium*

crysogenum (Irfan-Maqsood and Seddiq-Shams 2014). Mycelial growth of *Phytophthora* sp. and *Pythium* sp. was also impeded (Irfan-Maqsood and Seddiq-Shams 2014). Rhamnolipids are furthermore effectively inhibiting growth of the algae *Heterosigma akashiwo* and *Protocentrum dentatum* (Wang et al. 2005). However, mono-rhamnolipids showed low toxicity against *Aspergillus niger* spores during the germination and *Candida albicans* (Johann et al. 2016).

The antimicrobial effects of rhamnolipids were proposed to be caused by intercalation into the cell membrane resulting in increased permeability and subsequent cell death (Sotirova et al. 2008).

Rhamnolipids were specifically applied to enhance healing of burn wounds. They increase the wound closure time and, as they inhibit the activity of fibroblasts, decrease the collagen content in the wound, which leads to lesser scar formation (Stipcevic et al. 2006). In addition, they were successfully used to treat ulcers caused by the stimulation of bone marrow (Piljac et al. 2008). In vitro experiments also showed antitumor and antiproliferative properties, which might be caused by the reduction of surface tension of the culture medium (Paulino et al. 2016). In vivo experiments are thus crucial to confirm these effects. In human skin, rhamnolipids were demonstrated to induce the production of psoriasin, an antimicrobial protein (Meyer-Hoffert et al. 2011). Rhamnolipids were also applied to treat tuberculosis infections (Irfan-Maqsood and Seddiq-Shams 2014) and psoriasis (Piljac et al. 2008).

Pharmaceutical applications can also benefit from a trait described above. Rhamnolipids can be used to stabilize liposomes, which are pH sensitive and thus suited for transport of substances into cells (Sanchez et al. 2010) and have been successfully applied to produce microemulsions for drug delivery (Nguyen et al. 2010).

4.2.4 Household Detergents

Rhamnolipids as amphiphilic molecules are well suited for the use as detergents, e.g., in household cleaners. Detergents often end up in the environment, where they potentially harm ecosystems. The advantage of rhamnolipids over synthetic chemical detergents is their low ecotoxicity and furthermore their biodegradability, diminishing their impact on the environment even more.

Rhamnolipids are discussed as environmentally friendly cleaning agents (Randhawa and Rahman 2014) as was shown in cleaning soap mixtures (Ecover products); comparable results were obtained with commercial washing powders (Khaje Bafghi and Fazaelipoor 2012). The companies Evonik and Unilever filed patents for the use of rhamnolipids for textile washing (Parry et al. 2012; Kuppert et al. 2014).

4.3 Biological Control

As stated above, rhamnolipids are effective against a variety of microorganisms. This property renders them suited for a couple of biocontrol applications. As they are

capable of removing and even destructing biofilms of several Gram-positive and Gram-negative bacteria (Paulino et al. 2016), they were, for example, applied in the cooling system of an atomic power plant (Dusane et al. 2011) as well as for devices in the food and pharmaceutical industry (Paulino et al. 2016).

The prevention of biofilm formation effected by rhamnolipids was proposed to be caused by modifications of the surface hydrophobicity and interference in adhesive properties of the bacteria (Paulino et al. 2016). The destruction of already existing biofilms might be caused by the alteration of the biofilm environment and the removal of extracellular polymeric substances (Paulino et al. 2016).

4.4 Specialty Applications

Apart from the obvious applications based on the surface-active properties of rhamnolipids, a few applications have been published, for example, in the production of fine chemicals (Banat et al. 2000).

4.4.1 Fine Chemicals

Rhamnolipids can serve as precursor molecules for the production of fine chemicals, e.g., to synthesize enantiopure L-rhamnose for the production of high-quality flavor compounds and as chiral precursor for active agents or as hydrophilic carrier for the transport of insoluble drugs in humans (Linhardt et al. 1989). Further, to determine specific properties of solid surfaces, a pyrenacylester was synthesized from rhamnolipids to facilitate the use of pyrene (Ishigami and Suzuki 1997).

4.4.2 Nanoparticle Synthesis

Furthermore, biosurfactants can be used for high-performance nanomaterial production, since they easily form a variety of liquid crystals in aqueous solutions (Kiran et al. 2016). These nanoparticles could, for example, be used to deliver drugs (Dahrazma et al. 2008). Kiran et al. demonstrate successful synthesis of nanozirconia particles and silver nanoparticles aided by rhamnolipids, which proved to be more effective than chemical surfactants (Kiran et al. 2016). Rhamnolipid-mediated synthesis of NiO nanoparticles led to a decrease in nanoparticle size (Palanisamy and Raichur 2009).

5 Patent Landscape

5.1 Patent Analysis and Its Applications

In its core, patent analysis is a family of techniques that employs patent data to derive information about a particular industry or technology field. The advantage of using such data lies in the amount of structured and easily accessible information which patents contain clear identification of authors, jurisdictions, assignees, and technological domains. As a strategic tool, patent analysis can be used for monitoring emerging developments such as convergence between industries (Curran et al. 2010; Bornkessel et al. 2014). In more practical terms, patents carry business and legal

implications and are usually associated with considerable costs. These characteristics make them an indicator for high interest and potential investment, as well as a basis for various policy or corporate decisions.

For the purpose of this chapter, patent data is employed to build a landscape of technological areas and trends that are of importance to rhamnolipids and their application. Key industrial sectors and companies are also outlined. Results, however, should be interpreted with caution as legal practices, and patenting behaviors differ around the world and across industries. Additionally, the value distribution of patents is highly skewed; many have no industrial application, whereas few are highly valuable (Hall et al. 2005).

As there are various documents published at different stages during the patenting procedure, a few key terms ought to be defined: *patent application* refers to a pending request at a patent office for a patent to be issued, while *patent grant* is the intellectual property right granted by the respective patent office to the inventor. Furthermore, *patent family* denotes a group of documents published in different countries (or languages) but referring to the same invention. International Patent Classification (*IPC*) codes are a hierarchical system of symbols that classifies patents according to the different areas of technology to which they apply (all definitions follow the World Intellectual Property organisation (Trippe 2015)).

5.2 Patenting Activity and Assignee Structure

As mentioned in a previous section, the discovery of rhamnolipids happened in 1963 and led to actual application in the late 1980s. The time development of the patenting activity between 1985 and 2015 can be seen in Fig. 3. A pattern of peaks and fast

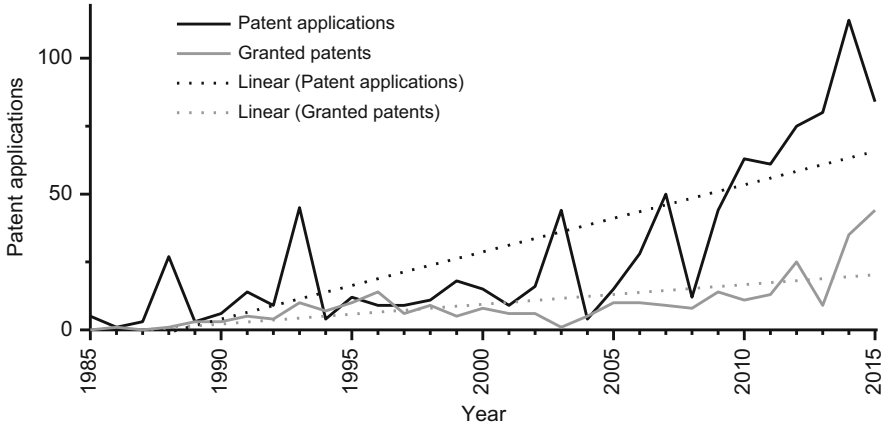

Fig. 3 Number of rhamnolipid patent documents per year between 1985 and 2015. N = 890 applications and 292 grants

decreases can be observed, with the first peaks being in 1988 and 1993 (27 and 45 filed applications, respectively), followed by a period with relatively low activity (the average number of patent applications between 1995 and 2001 is 12 per year). In 2003, the number of filed documents almost equals the peak from 1993, and in 1997 this number reaches 50. During the period 2009 to 2014, patent applications are steadily increasing, and it is in this time frame that 48% of all applications in the sample were filed. After the year 2014, however, the number starts to decrease again.

To aid the interpretation linear trend lines are introduced to demonstrate the general growth in patent documents. This overall positive development indicates an increasing interest and innovation effort in the synthesis and/or use of rhamnolipids.

The curve of granted (issued) patents somewhat follows the one in patent applications but with a significant delay, as the average time to obtain a grant for this specific sample is above 1500 days (more than 4 years). Furthermore, not all pending requests result with a patenting right – the average ratio between filed applications and issued patents in this sample is roughly 3:1.

A closer look at the patent assignees' structure reveals a total of 249 individuals, companies, universities, and research institutes that have applied for intellectual property rights on their innovations (110 of them with granted rights). The analysis of such data can give valuable indications of the most active "players" in the field of rhamnolipid synthesis and use. A list of those assignees that have ten or more patent applications is presented in Fig. 4. According to their core activities, they can be further divided into the following groups:

Chemistry and Biochemistry The patent data reveals that this is the most relevant industry sector for rhamnolipids and their production or application. Combined, the six companies hold a total of 217 patent applications and 62 grants (or 23% of all applications and 19% of all granted patents in the set). The most active assignee in the group, *Evonik*, possesses 59 pending and five issued patents. Second comes *Jeneil Biotech*, a company focused on food ingredients, with 56 applications and ten grants, followed by *Aventis Gmbh*, *Stepan Co*., and *Synthezyme LLC* (39, 28, and 20 filed applications, respectively). Last in the group is *DuPont's Danisco Inc.* with 15 applications and no granted patents yet.

Consumer Goods, Cosmetics, and Food The group includes *Unilever*, which is the most active patent assignee, holds a total of 75 applications and 23 granted documents. The multinational giant is followed by three companies with 11 filed applications each, namely, the sugar producer *Südzucker AG*, the cosmetics manufacturer and seller *Avon*, and *Clorox* that is famous for its cleaning products. Last, with ten applications each, come *Ecover* and *Procter & Gamble Co.* Combined, the companies from this group account for 128 filed and 43 granted patent documents (around 13% of the documents set).

Pharmaceuticals Two companies are listed here, the biopharmaceutical producers *Paratek Pharmaceuticals Inc.* and *Paradigm Biomedical Inc.* that focuses especially

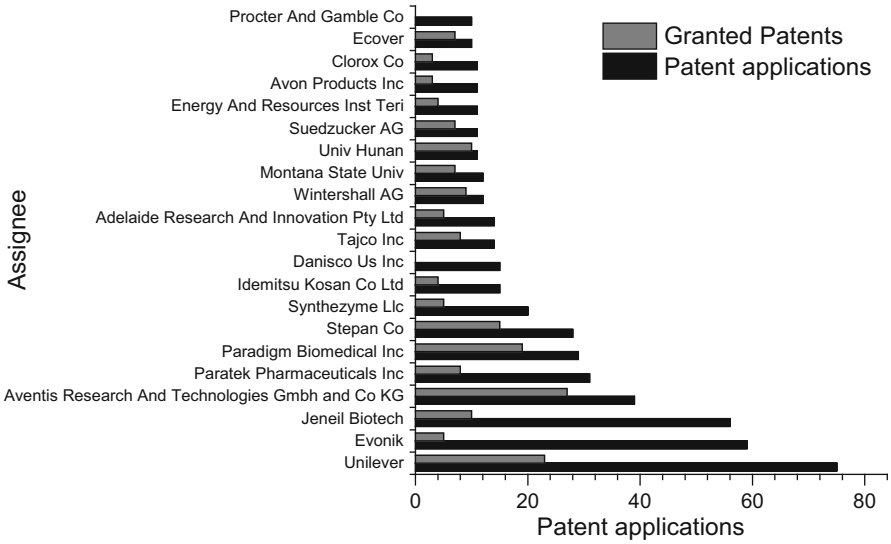

Fig. 4 Assignees with ten or more filed applications

on products derived from rhamnolipids. Together they have a total of 60 applications and 27 issued patents.

Academic Research Four of the most active assignees come from the academic field, namely, *Adelaide Research and Innovation Pty. Ltd.* (consulting services for the University of Adelaide), *Montana State University*, *University of Hunan*, and *Energy and Resources Institute TERI*. Together the group accounts for 48 patent applications and 26 granted documents. In fact, nearly 30% of all assignees in the whole sample represent universities or research institutes. Moreover, although a quarter of them have filed only one patent application, 45% of these requests have resulted in grants (as opposed to the 32% for the assignees that are companies or individuals).

Other The group features two companies from the oil sector – *Idemitsu Kosan Co. Ltd.*, *Wintershall AG* (subsidiary of *BASF*), and *Tajco Inc.* that comes from the automotive industry. They possess 41 applications and 21 issued patents in total (or 4% of the applications and 7% of the grants in the sample).

5.3 Application Areas Based on IPC Codes

As defined above, International Patent Classification (IPC) codes are a hierarchical system of language-independent symbols that define the technological field a patent relates to. An analysis of the codes most frequently associated with rhamnolipids

Table 2 Grouping of IPC codes and applicational areas

Applicational area	IPC codes	Meaning of the IPC code	Number of patent applications
Application in the field of chemistry and biochemistry			
	C07C	Acyclic or carbocyclic compounds	19
	C07D	Heterocyclic compounds	25
	C07H	Sugars and derivatives	140
	C12N	Microorganisms and enzymes	171
	C12P	Fermentation	229
	C12Q	Processes involving enzymes and microorganisms	32
	C12R	Biochemistry, enzymology, beer, spirits, wine, vinegar	109
Application with regard to environmental issues			
Oil recovery	C02F	Treatment of water/sludge	57
	C10G	Recovering of oils from shale, sand, or gases	14
Bioremediation	B09C	Treating contaminated soil	44
Agriculture	A01N	Preservation of bodies (human, animal, plants)	160
	A01P	Biocides and pest repellants	56
	C05D	Fertilizers	13
	C05G	Fertilizers	24
Consumer goods			
Food and feed	A23L	Food and preservation of food	11
	A23K	Animal feed	20
Cosmetics	A61Q	Cosmetics	120
Pharmaceuticals	A61K	Medical, dental, and toilet purposes	232
	A61L	Sterilizing materials, bandages, and dressings	26
	A61P	Medicinal preparations	110
Detergents	C11D	Detergent compositions	164

reflects the list of major applications given in Sect. 4. To illustrate this notion, the IPC codes were grouped according to these areas and can be seen on Table 2.

Applications with Respect to Environmental Concerns Innovations that refer to the emulsification abilities of rhamnolipids are mostly classified with the code C02F (corresponds to the area *treatment of waste water*) or B09C (corresponds to *remediation of contaminated soil*). Patents in this group feature, for example, environmentally friendly treatment of oil spills, or production methods for heavy metal biological absorbents. The assignee that has applied for the most patent documents in this application area is not a commercial entity, but the *State University of Montana*. Meanwhile, the use of rhamnolipids in the sphere of agriculture is denoted by classes A01P (*biocides and repellents*), C05D/C05G (corresponding to the area

of *fertilizers*), and A01N (preservation of bodies). *Jeneil Biotech* and its subsidiaries are the most active applicants in the subgroup.

Applications with Respect to Consumer Goods The patent data confirms the proposition that rhamnolipids cater for a variety of industrial demands in the area of consumer goods. Subclasses A61K, A61Q, A61L, A61P (assigned to patents in the field of medical science, hygiene, or cosmetics), and C11D (detergent compositions) account for a substantial share of patent applications. Additionally, class A23 (food or foodstuff) can be included, as it corresponds to the application of rhamnolipids in food preservation and in animal feed. Unsurprisingly, *Unilever* is the company with the most filed applications in this group.

Applications Referring to Biological Control No concrete IPC code corresponds directly to this area of application, but adding "biofilm" as a keyword in the search string results in 84 filed and 26 issued patents, the key IPCs being A61K, A61P, and A01N. The most prominent companies in this specific field are *Paratek Pharmaceuticals* and *Synthezyme LLC* with eight and five granted patents, respectively.

IPC Groups Referring to (Bio)chemistry and Related Activities In addition to the areas described so far, there are a few IPC codes that are assigned to the majority of rhamnolipid-related patents as they belong to the field of chemistry. Namely, these are the classes C07 and C12.

Finally, it is not only the application of rhamnolipids that attracts the attention of businesses and researchers but also their production. A manual scan of the 364 patent families obtained reveals that between 20% and 30% of them relate to innovative methods for the synthesis of rhamnolipids. Most of these patents focus on up- and downstream processing, different wastes as substrates for rhamnolipid synthesis, and medium compositions. In addition, high titers and yields are often claimed to be achieved, while only few patents mention the use of recombinant bacteria. It can thus be stated that apart from the application of these remarkable biosurfactants, also the production of rhamnolipids is a highly active field in the actual patent landscape.

6 Research Needs

At the moment, high production costs still represent the major drawback preventing a more widespread application of rhamnolipids and biosurfactants in general (Marchant and Banat 2012b). To foster the industrial use of rhamnolipids, production costs will have to be significantly decreased by further genetic engineering of the biocatalyst, optimization of fermentation procedures, and integrating affordable downstream processing.

Another challenge in the use of these alternative biosurfactants is that each organism produces a mixture of congeners with a range of different structures and therefore properties but is nonetheless metabolically limited to a particular set of congeners (Roelants et al. 2013). The production of rhamnolipid mixtures with a

specific composition adapted to desired physiochemical properties should be explored in the future by mixing lipids from different organisms and engineering producer strains or by producing tailor-made mixtures in vivo by choosing the respective strain biosynthetic operons and/or combining producers strains for production as already outlined by Hošková et al. (2015).

7 Concluding Remarks

A number of bacteria are able and applied to produce rhamnolipid biosurfactants, depicting a mix of different congeners. Remarkably, the rhamnolipid mixtures produced by different strains do not necessarily show the same composition but may differ regarding their particular set of rhamnolipid molecules, whereas, for example, most *P. aeruginosa* rhamnolipid mixtures constitute mixtures of mono- and di-rhamnolipids with predominantly C_{10}-chains (Déziel et al. 1999); *Burkholderia* sp.-derived mixtures seem to be dominated by di-rhamnolipids, which contain HAA with longer (around C_{14}) chains (Manso Pajarron et al. 1993; Dubeau et al. 2009; Díaz De Rienzo et al. 2016). Shorter chains are described for *P. desmolyticum*-derived species (Jadhav et al. 2011). The different fatty acid spectra may be attributable to specificities of *rhlA* (Blank et al. 2013a), and also supplied carbon sources may influence the final chain length. Furthermore, differences in the genetic organization may also be reflected in the variation of congener composition, in particular different ratios between mono- and di-rhamnolipids.

Obviously, the molecular structure and the particular mixture of congeners influence physicochemical properties like hydrophobicity or self-assembly behavior of the produced biosurfactant (Nitschke et al. 2005). These are connected to important parameters for potential use for industrial applications including surface tension, critical micelle concentration, foaming behavior, wetting properties, and emulsification activity. Furthermore, biological activity is of interest. In literature, data can be found on these properties for rhamnolipid mixtures showing the high potential of these biosurfactants. However, reliability of this data is often hampered by the use of congener mixtures and impure samples.

Based on the properties reviewed, the focus of this chapter shifts to suitable applications. These include the use of rhamnolipids in diverse field such as bioremediation, agriculture, and enhanced oil recovery as well as in the food, cosmetic, pharmaceutical, and detergent industry. Also biological control benefits from the use of rhamnolipids. Examples include the removal of biofilms. Again, the high amount of published applications shows that the scientific interest in rhamnolipids is high and that this high activity can lead to applications of industrial interest. However, reported applications have to be considered with care; as in most cases, no specific information about the exact composition of the applied rhamnolipids or the purity of the samples is available.

A comprehensive analysis of the patent landscape shows the transfer of scientific knowledge to actual applications. The main applications fields identified previously are also mirrored in the patents. Moreover, it can also be deducted from the patent

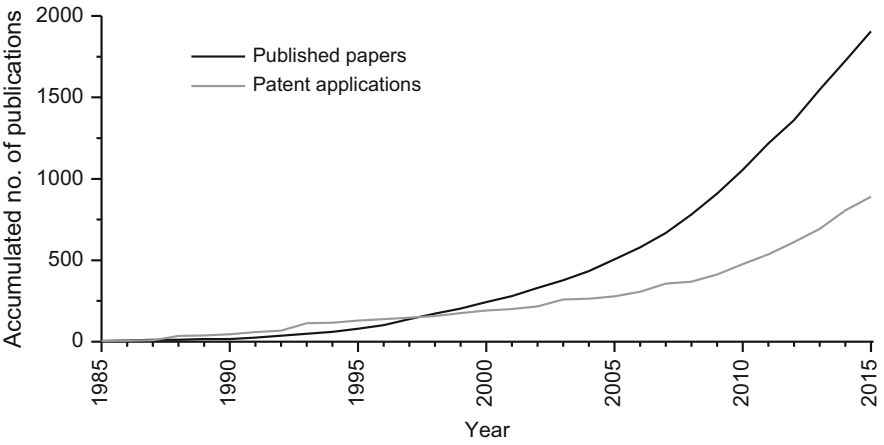

Fig. 5 Number of published scientific papers and patent applications accumulated

data that a lot of companies are considering rhamnolipids as viable alternative to petrochemical-derived synthetic surfactants. Fig. 5 shows that the number of patent applications follows the number of published scientific papers with a couple of years behind.

Following up on the path taken, further development toward cost competitiveness of production processes and the production of tailored rhamnolipid mixtures with a specific composition adapted to desired physiochemical properties for an ever-increasing field of applications will contribute to the promises of the envisaged bioeconomy.

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Protein Emulsifiers

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Eliora Z. Ron and E. Rosenberg

Contents

Abstract

The active components of the known high molecular weight biosurfactants are polysaccharides lipopolysaccharides, lipoproteins, or complex mixtures of these biopolymers. Recently a new group of bioemulsifiers has been identified, in which the active component is a protein. Thus, secreted outer-membrane proteins were shown to have strong emulsifying activity. These proteins will be used as examples for the potential in microbial protein emulsifiers.

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1 Introduction

Microorganisms synthesize a wide variety of high- and low-molecular-mass bioemulsifiers (Rosenberg and Ron 1997, 1999). The low molecular weight molecules – some of which are lipopeptides (Yakimov et al. 1995, 1998, 2000) efficiently lower surface and interfacial tensions and high molecular weight polymers that bind tightly to surfaces (Rosenberg and Ron 1997, 1999). The high molecular weight bacterial surfactants are produced by a large number of bacterial species from different genera and are composed of polysaccharides, polysaccharide-proteins, lipopolysaccharides, lipoproteins, or complex mixtures of these biopolymers (for review see Rosenberg and Ron 1999). The high molecular weight surfactants are less effective in reducing interfacial tension, but are efficient at coating the oil droplets and preventing their coalescence. These are highly efficient emulsifiers that work at low concentrations (0.01–0.001%), representing emulsifier-to-hydrocarbon ratios of 1:100–1:1000. These high molecular weight bioemulsifiers exhibit considerable substrate specificity. For example, some of them emulsify efficiently only mixtures of aliphatic and aromatic (or cyclic alkane) hydrocarbons but not pure aliphatic, aromatic, or cyclic hydrocarbons; others can also emulsify pure hydrocarbons but only of a high molecular weight. Several bioemulsifiers have been thoroughly studies. Among them are the bioemulsans produced by different species of *Acinetobacter* (Rosenberg and Ron 1998) that include the RAG-1 emulsan which is a complex of an anionic heteropolysaccharide and protein, whose surface activity is due to the presence of fatty acids that are attached to the polysaccharide backbone via O-ester and N-acyl linkages. *A. calcoaceticus* BD4, initially isolated and characterized by Taylor and Juni (1961), produces a surface active extracellular polysaccharide–protein complex and several bacteria produce lipoprotein emulsifiers. In all cases, the emulsifying activity was due to the polysaccharide or the lipid component. Recently it became evident that *A. radioresistens* produces a high molecular weight emulsifier (Alasan) in which the active component is a protein (Navon-Venezia et al. 1995, 1998; Toren et al. 2001, 2002a, b, c). This discovery opens up a range of interesting possibilities, as protein emulsifiers are well defined chemically as well as genetically.

2 Alasan

Alasan, produced by a strain of *A. radioresistens*, is a complex of an anionic polysaccharide and protein with a molecular weight of approximately 1×10^6 (Navon-Venezia et al. 1995). The polysaccharide component of alasan (apo-alasan) is unusual in that it contains covalently bound alanine. The proteins of alasan include three major proteins with apparent molecular masses of 16, 31, and 45 kDa. The alasan proteins appear to play an essential role in both the structure and surface activity of the complex, because apo-alasan had no emulsifying activity and does not show the large temperature-induced hydrodynamic shape changes that were

characteristic of alasan (Toren et al. 2001). Furthermore, treatment of alasan with specific proteases inactivates the emulsifying activity.

3 The 45 kDa Protein of Alasan

One of the alasan proteins with an apparent molecular mass of 45 kDa, is highly effective in stabilizing oil-in-water emulsions and in solubilizing hydrocarbons, including polycyclic aromatic hydrocarbons (Toren et al. 2002a, b). The 45-kDa protein is high similar to OmpA – one of the important outer-membrane proteins in Gram negative bacteria. The gene coding for this protein was cloned, sequenced, and expressed in *Escherichia coli* and the recombinant AlnA protein (35.77 kDa without the leader sequence) contained 70% of the specific (hydrocarbon-in-water) emulsifying activity of the native 45-kDa protein and 2.4 times that of the alasan complex, as purified from *A. radioresistens* and containing the polysaccharide and additional complexed proteins. In addition to their emulsifying activity, both the native 45-kDa protein and the recombinant AlnA were highly effective in solubilizing phenanthrene, ca. 80 g mg^{-1} of protein, corresponding to 15–19 molecules of phenanthrene per molecule of protein (Toren et al. 2002a).

4 The 45 kDa Protein of Alasan Is a Secreted OmpA

Although AlnA and the *E. coli* outer-membrane protein A (OmpA) have a high amino acid sequence homology, *E. coli* OmpA has no emulsifying activity. Comparison of the amino acid sequences of AlnA and *E. coli* OmpA reveals four hydrophobic regions in AlnA that are absent in *E. coli* OmpA. These four regions would be expected to partition to the hydrocarbon, if they were not prevented from doing so by the three-dimensional structure of the protein. Although the structure of AlnA has not yet been elucidated, by analogy with *E. coli* OmpA and other outer-membrane proteins (Beher et al. 1980), AlnA probably exists as a β-barrel structure from amino acids 6–161 with a short N-terminal arm (amino acids 1–5) and a long C-terminal arm (amino acids 162–327). Accordingly, the hydrophobic regions from amino acids 2–9 and 164–171 should be available to interact with the hydrocarbon. Thus, it is reasonable to hypothesize that all four groups could interact with the hydrocarbon. Amino acids 37–45 and 47–53, which appear on a loop extending out of the β-barrel, differ between *A. radioresistens* and *E. coli*, leading to the hypothesis that they are responsible for the emulsifying activity of AlnA. Indeed, deletions and substitutions (with the homologous OmpA sequence) in these regions, as well as in the other two hydrophobic regions of AlnA indicates that all four hydrophobic regions are necessary for emulsifying activity (Toren et al. 2002c).

For a biopolymer to stabilize oil-in-water emulsions, it must bind avidly to the oil and form a strong hydrophilic layer available to interact with the hydrocarbon. Thus, it is reasonable to hypothesize that in AlnA all four groups which are hydrophobic and extend out of the β-barrel interact with the hydrocarbon and are essential for

emulsification. However, it is interesting to note that most of the inactive mutated proteins still adhered avidly to hexadecane. These findings indicate that in addition to binding to hydrocarbons, the protein emulsifiers must form specific structures on the surface of the hydrocarbon that prevents coalescence of oil droplets.

5 Emulsifying Activity of the Acinetobacter Outer Membrane Protein A (OmpA)

The finding that *A. radioresistens* secretes OmpA and that this protein has emulsifying activity was unexpected. However, in view of the fact that *A. radioresistens* utilizes hydrophobic substrates as carbon and energy sources, it is conceivable that the presence of a secreted OmpA emulsifier may improve its ability to grow under certain conditions. Support for this possibility was obtained by the findings that production of an OmpA which has emulsifying capabilities and its secretion into the medium is a general property of Acinetobacter, and particularly of the oil degraders. Thus, it was found that OmpA is secreted to the medium in five oil-degrading Acinetobacter strains. Moreover, the *ompA* gene was cloned from two additional strains of Acinetobacter – ADP1, whose genome was recently sequenced and *Acinetobacter* sp. V-26. In both cases the protein purified from *E. coli* had emulsifying activity (Walzer et al. 2006). These findings suggest that secretion of OmpA with emulsifying ability is a general property of Acinetobacter strains and may be physiologically connected to growth on water-insoluble substrates.

6 Additional Proteins with Emulsifying Activity

The OmpA proteins of several Acinetobacter strains (*Acinetobacter* sp. V-26, ADP1, RAG-1, and BD4) are secreted and have emulsification activity. As these bacteria utilize hydrophobic carbon sources, the secretion of protein emulsifiers may be useful in enabling growth on these carbon sources. These findings raise the possibility that emulsifying activity can be found in outer-membrane proteins of other hydrocarbon-utilizing bacteria. Using the β-barrel of OmpA it was possible to develop a model for predicting the structure and hydrophobicity of OmpA-like proteins. The use of this model for scanning outer-membrane proteins of hydrocarbon-initializing bacteria indicated that, indeed, several OmpA-like proteins could be identified in oil-degrading bacteria and one of them OprG – an outer-membrane protein of the oil-degrading *Pseudomonas putida* KT2440 – was tested experimentally and shown to have emulsifying activity (Walzer et al. 2009).

The model used for predicting emulsifying activity of proteins was based on the OmpA protein and could therefore be used only for OmpA-like protein. However, it is expected that OmpA is not unique in its emulsifying activity and that this property can be found in other outer-membrane or secreted proteins, especially of bacteria that use hydrophobic substrates for growth.

7 Conclusion

In addition to the well known bacterial emulsifiers whose active components are polysaccharides or lipids, recent studies identified emulsifying activity of pure proteins. So far, such activity has been shown for several OmpA and OmpA-like proteins produced by oil-degrading bacteria. These proteins are secreted and emulsify hydrophobic substrates with high efficiency. The secretion of proteins with emulsifying activity may play an important role in bacterial life on hydrophobic substrates and oil.

The proteins emulsifiers open up a variety of possibilities for further understanding bacterial growth on oil and utilization of hydrophobic substrates. Moreover, protein emulsifiers can be used for a variety of biotechnological applications and their production can be made easy and cost-effective by the use of genetic manipulations.

8 Research Needs

There are research needs in several areas:

1. Identification of additional protein emulsifiers
2. Study of the role of these emulsifiers in bacterial growth on oil and in bacterial utilization of hydrophobic substrates
3. Development of production/fermentation technologies that will make the use of these emulsifiers cost-effective

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Distribution of Hydrocarbon Degradation Pathways in the Sea

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Contents

Abstract

Petroleum hydrocarbons are one of the most persistent and complex pollutants discharged to environment as a consequence of the human activity, significantly

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affecting marine and coastal habitats. Some members of microbial communities inhabiting marine ecosystems show the ability to use these hydrocarbons as a preferential carbon source. These compounds are metabolized through different catabolic pathways, leading to their total or partial degradation. Microorganisms are the first responsive component of marine ecosystem after an oil spill. Their contribution may be different depending on the environmental conditions, baseline community setup, and oil composition. Here, we describe how these hydrocarbon-degrading bacteria behave in the marine environment after an oil spill and report on main pathways involved in the degradation of the different hydrocarbons, with a particular focus in the Mediterranean and Red Sea and the Gulf of Mexico as examples.

1 Introduction

Oil is discharged in the sea as result of its extraction, transportation, manipulation and consumption (Oil in the Sea III 2003; Awal 2009). Crude oil constitutes an extremely complicated mixture composed by thousands of different organic compounds, being mainly (~97%) hydrocarbons (Marshall and Rodgers 2004; Benassi et al. 2013; Sammarco et al. 2013). According to their complexity, hydrocarbons can be classified in saturated, unsaturated, aromatic, and polycyclic aromatic hydrocarbons (PAHs, which include at least two benzene rings). The largest molecules are in the group formed by the so-called resins and asphaltenes (also known as a "polar fraction"). The proportion of each type of hydrocarbons varies depending on the class of crude oil or refinery derivatives (Fig. 1), giving the oil different physical and chemical properties that define their behavior and fate in the environment (Oil in the Sea III 2003; Fuentes et al. 2014).

Hydrocarbons are generally toxic and persistent molecules, and their release harms seriously the marine ecosystem. For instance, when a spill occurs all at once, like in case of accidental discharges, the oil slick covers the surface limiting the penetration of the light through the water, affecting to the photosynthetic processes. Moreover, direct contact, ingestion or inhalation of petroleum compounds may be either lethal for the marine fauna or significantly affect their growth or reproductive skills (Nogales et al. 2011; Montagnolli et al. 2014; Tsitou et al. 2015). Exposure to the petroleum compounds can also have long-term carcinogenic potential over the local human populations (Cirera et al. 2012; Montagnolli et al. 2014). Moreover, removal of released hydrocarbons from the marine ecosystem is an extremely difficult task. Firstly, oil released in the sea is under the influence of different physical and chemical factors, which cause the oil dispersion through the surface and water column (Yim et al. 2011; Stout et al. 2016). Secondly, heavy fractions of petroleum hydrocarbons, such as asphaltenes and heteroaromatics, have a long-term persistence in environment especially in sediments, making their removal a very complicated task (Reddy et al. 2002; Prosser et al. 2016). This forces us to improve thoroughly the cleanup methods to remove the oil spills. In the past decades, researches in the field of bioremediation have been focused in the ability of

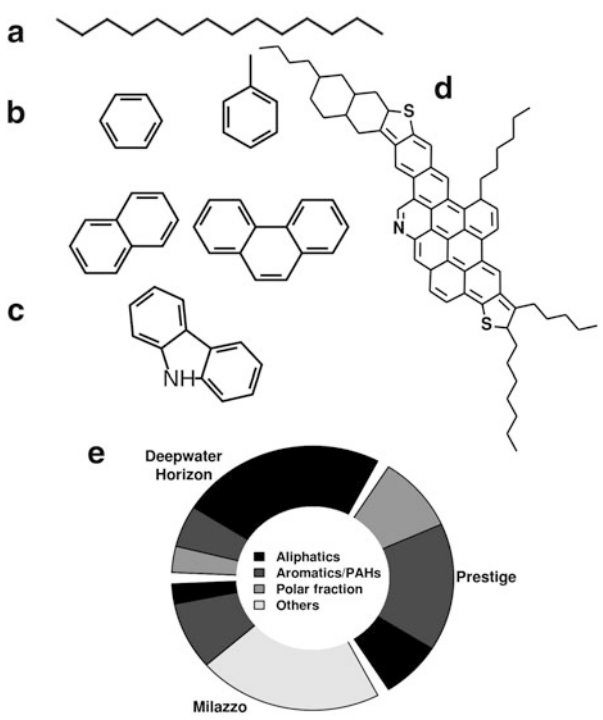

Fig. 1 Representative group of hydrocarbons in the crude oil and its overall composition. Examples of the different types of hydrocarbons are shown on the *left*: (**a**) aliphatic hydrocarbons; (**b**) aromatics, (**c**) complex PAHs-like. On the *right*, (**d**) possible structure of an asphaltene. On the *bottom*, (**f**) the different proportion of these groups of hydrocarbons in different oil-spilled areas: the Gulf of México (Deepwater Horizon), northwest coast of Spain (Prestige), and the harbor of Milazzo (Sicily)

some members of indigenous microbial communities to degrade hydrocarbons (Head 1998; Gillespie and Philp 2013).

Microbial marine communities respond to an oil spill by a rapid structural succession, leading to the emergence and dominance of species capable to use hydrocarbons as carbon source (Head et al. 2006; Kimes et al. 2014; Ma et al. 2015). These microorganisms absorb the petroleum compounds over the microbial surface. Then these compounds are transferred through microbial cell membrane and finally are degraded in the microbial cell (Xue et al. 2015). The first step in the hydrocarbon biodegradation is the molecule activation, consisting in the destabilization of the carbon-carbon link (C-C) in one of its carbon atoms. The resulting compounds are then transformed through specific catabolic pathways that converge in common intermediates. In turn, these intermediate compounds are degraded to simpler molecules that can be introduced into the general pathways, like the tricarboxylic acid cycle (TCA) (Díaz et al. 2013; Boll et al. 2014). In the majority, the different groups of hydrocarbon-degrading bacteria are specialized in the degradation of a particular range of compounds. Hence, microbial communities change in response to an oil spill disturbance, but also they keep changing during the time afterward, with a specific succession of members associated with degradation of the different types of hydrocarbons (Head et al. 2006; Kimes et al. 2014; King et al. 2015).

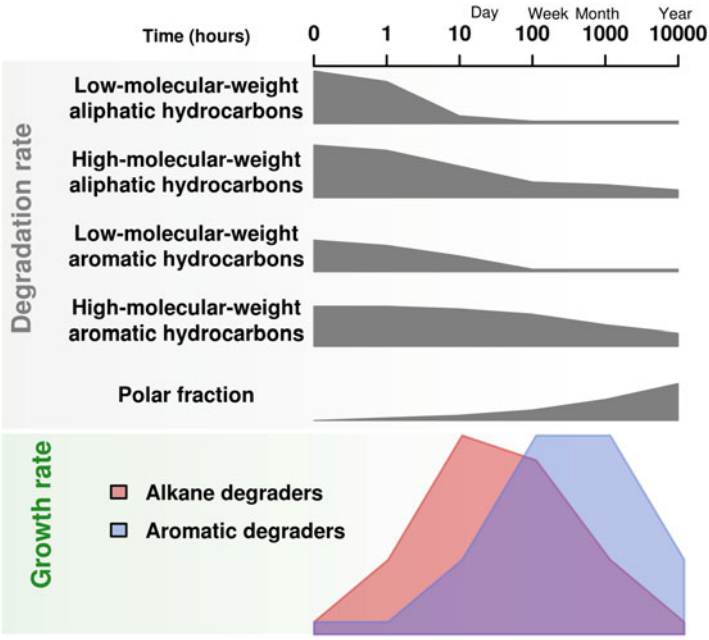

Fig. 2 Dynamic representation of the hydrocarbon degradation. On the *top*, the degradation rate of the different type of hydrocarbons shows the aliphatic hydrocarbons as the fastest group being degraded. On the *bottom*, the growth rate indicates that the alkane degraders grow up faster than those microorganisms specialized in the degradation of more complicated compounds (Adaptation from Head et al. 2006)

Growth rate of the hydrocarbon-degrading microorganisms in oil-polluted environments is conditioned by the complexity of the hydrocarbons and by their preference to degrade the different types of these compounds (Fig. 2). Most complex hydrocarbons show a slower degradation rate, and the microorganisms specialized in degrading them take more time to increase their presence (Head et al. 2006).

Catabolic pathways define the metabolic networks where the degradation of the oil compounds takes place. The size and richness of the degradation networks from the polluted sites depend on the environmental conditions, the composition, bioavailability, and toxicity of the complex mixture of hydrocarbons with different structures, the population structure and the adaptation and interactions of their components, and the metabolic capacity of the autochthonous marine microbiota in the process of biodegradation (Wang et al. 2016). In this chapter, we describe how the hydrocarbon biodegradation works and the main points that determine the size and diversity of catabolic networks in the marine milieu: (a) the biodegradation process, (b) the hydrocarbon-degrading bacteria, and (c) the structural succession of the microbial community after an oil spill. Finally, examples of how the catabolic pathways are differentially distributed in three geographically separated marine sites are exemplified. Through this analysis, we provide evidences of the distribution of

catabolic pathways, the differences of which most likely related to the different effect multiple factors exert in microbial community composition and the species responsible for the degradation of different pollutants.

2 Biodegradation of Petroleum Hydrocarbons

Hydrocarbons are degraded by indigenous microorganisms which can use these compounds as carbon source thanks to their specialized metabolism (Cappello et al. 2007a; Ron and Rosenberg 2014). After an oil spill, the diversity of the microbial community declines (Yakimov et al. 2005), while favoring in prosper of the hydrocarbon-degrading bacteria (HDB). In pristine waters, these microorganisms typically comprise less than 1% of the total bacterial population, but they proliferate rapidly when oil contamination occurs, amounting up to 90% of the microbial community (Kasai et al. 2002; Cappello et al. 2007a; Manilla-Pérez et al. 2010).

Once the biodegradation process begins, the occurrence and abundance of microbial groups, as well as quantities and diversity of enzymes they produce, change accordingly to the certain common patterns. Main influencing factors include the abundance and bioavailability of the different types of hydrocarbons in the sea (Head et al. 2006; Das and Chandran 2011) and the physical and chemical conditions such as temperature (Gutierrez et al. 2013a), oxygen (Kimes et al. 2013), and nutrient availability (Das and Chandran 2011; McGenity et al. 2012).

2.1 Principal Hydrocarbon-Degrading Bacteria (HDB)

Each microorganism possesses different metabolic abilities, allowing the degradation of different types of substrates. Among the HDB is the group of obligate marine hydrocarbonoclastic bacteria (OMHCB), which can only use hydrocarbons as sole carbon and energy source. To our knowledge, the marine environment is the only place where bacteria that preferentially utilize hydrocarbons as sole sources of carbon and energy are found. Most representative OMHCB belong to the class Gammaproteobacteria, highlighting the genera *Alcanivorax*, *Cycloclasticus*, *Oleispira*, *Thalassolitus*, and *Oleiphilus* (Harayama et al. 2004; Yakimov et al. 2007; Manilla-Pérez et al. 2010; Liu and Liu 2013). *Alcanivorax* is one of the most studied genera, being *A. borkumensis* the first sequenced OMHCB genome (Yakimov et al. 1998, 2007; Golyshin et al. 2003). Members of this genus grow in the presence of *n*-alkanes and branched alkanes, with the incapacity to use any carbohydrate or amino acid as the carbon source. The same case is for *Thalassolituus* (Yakimov et al. 2004), *Oleiphilus* (Golyshin et al. 2002), and *Oleispira* (Yakimov et al. 2003), with a high specificity for aliphatic alkanes (Yakimov et al. 2004). Members of the genus *Cycloclasticus* (Dyksterhouse et al. 1995) grow on minimal medium supplemented with PAHs like naphthalene, phenanthrene, or anthracene as sole carbon source (Harayama et al. 2004; Messina et al. 2016). Within Gammaproteobacteria, other

recent obligate polycyclic aromatic hydrocarbon-degrading specialists, that almost exclusively used them as sole sources of carbon and energy and live in association with marine phytoplankton, included bacteria from the genera *Polycyclovorans*, *Algiphilus*, and *Porticoccus* (Gutierrez et al. 2012a, b, 2013b, 2015a, b).

Beyond the OMHCB, other bacterial groups are able to metabolize hydrocarbons as one of their numerous carbon sources. For instance, within the same class of Gammaproteobacteria, we can note members of the genus *Neptumonas*, which are able to grow in rich medium and to degrade simple PAHs, like naphthalene (Hedlund et al. 1999; Harayama et al. 2004). The genera *Marinobacter* and *Pseudomonas* are widely known by their metabolic versatility to process both aliphatic alkanes and PAHs (Harayama et al. 2004; Tapilatu et al. 2010; Fathepure 2014). Within the class of Alphaproteobacteria, there are many genera with members showing similar catabolic abilities. Relevant examples are members of the genera *Sphingomonas* which are associated with the degradation of a large range of xenobiotic compounds, some of them being PAHs such as naphthalene, fluorene, anthracene, or phenanthrene (Luo et al. 2012); *Thalassospira* can degrade aliphatic hydrocarbons, while *Paracoccus* is able to use PAHs such as phenanthrene (Sauret et al. 2014).

Aside from Gamma- and Alphaproteobacteria which are the predominant members in marine ecosystems exposed to hydrocarbons, there are bacteria from other phyla capable to degrade petroleum compounds. For instance, the genera *Rhodococcus* and *Gordonia* (phylum actinobacteria) are able to degrade both alkanes and PAHs (Gallego et al. 2014; Tomás-Gallardo et al. 2014; Wang et al. 2014; Yang et al. 2014). Members of the genus *Planococcus* (phylum Firmicutes) can degrade alkanes of different length, monoaromatics, and PAHs (Li et al. 2006; Al-Awadhi et al. 2012).

All abovementioned bacteria develop their catabolic activities under aerobic conditions. However, even though it is much slower, hydrocarbon degradation under anaerobic conditions is also possible. Under anoxic conditions, microbial communities in oil-contaminated marine environments are usually dominated by Deltaproteobacteria (Widdel et al. 2010; Acosta-González et al. 2013; Kimes et al. 2013; Genovese et al. 2014), involved in the degradation of aliphatic and aromatic hydrocarbons, such as members from the orders Desulfobacterales and Desulfuromonadales, and the genus *Desulfovibrio* (Kimes et al. 2013). Other anaerobic degraders of PAHs such as benzene, naphthalene, and methylnaphthalene are also known (Meckenstock and Mouttaki 2011, Meckenstock et al. 2016).

Regarding archaea, their response after an oil spill is still to be clarified. The capability of these organisms of hydrocarbon degradation has been only demonstrated under aerobic conditions in hypersaline ecosystems. Members of the genera *Haloferax*, *Halobacterium*, and *Halococcus* are able to grow in minimal media supplemented with PAHs and alkanes of different length as unique carbon source (Fathepure 2014; Jurelevicius et al. 2014). Besides participation of methanogens in anaerobic degradation of hydrocarbon oxidation (Townsend et al. 2003; Jones et al. 2008; Hawley et al. 2014), neither the effect of oil release on marine archaea nor their contribution to the hydrocarbons degradation in oxygenated marine habitats is studied in details (Redmond and Valentine 2012).

2.2 Enzymes and Catabolic Pathways Involved in Hydrocarbon Degradation

According to the oxygen availability, there are two main strategies for hydrocarbon degradation: oxygen-dependent and anaerobic degradation (Díaz et al. 2013; Abbasian et al. 2015). Nevertheless, to destabilize the molecule of hydrocarbons, both pathways start with substrate activation (Fuchs et al. 2011).

In the presence of oxygen, the pivotal step is developed by various substrate-specific oxygenases which catalyze the insertion of oxygen and thus activation of otherwise very stable organic structure. Depending on quantity of oxygen atoms involved in activation, these enzymes are subdivided into two groups: mono-oxygenases, enzymes which catalyze the insertion of single oxygen atom into the substrate; and dioxygenases, catalyzing the insertion of two atoms of oxygen (Torres Pazmiño et al. 2010). In case of (poly)aromatic compounds, single activation event is not sufficient to break the complex structure, and once hydroxylated, intermediate products undergo a second oxidative attack to facilitate the cleavage of the aromatic ring (Fuchs et al. 2011).

Oxygenases are inactive in the absence of oxygen, so under anaerobic conditions activation of the hydrocarbon molecule is performed without the need of using oxygen atoms (Torres Pazmiño et al. 2010). Under anaerobic conditions, hydrocarbon molecules can be metabolized after hydroxylation, carboxylation, or activation by fumarate. After initial activation, hydrocarbons of different nature are transformed into few intermediates that are finally introduced in the central pathways (Boll et al. 2002; Fuchs et al. 2011).

Details on mechanisms and enzymes participating in anaerobic and aerobic degradation of aliphatic and aromatic compounds are given below.

2.2.1 Anaerobic Degradation

Anaerobic degradation of aromatic compounds is based on destabilization of the benzene ring through reductive processes. Hydroxylated aromatic hydrocarbons (2,3-dihydroxybenzene, 1,2,4-trihydroxybenzene, or 1,3,5-trihydroxybenzene) are transformed to resorcinol, hydroxyhydroquinone, or phloroglucinol, which are directly de-aromatized by dehydrogenases and/or reductases (Carmona et al. 2009; Fuchs et al. 2011; Boll et al. 2014). The remaining compounds suffer an anoxic oxidation to form CoA-thioester intermediates which are then de-aromatized by reductase-like enzymes. Here, the most important is the benzoyl-CoA pathway, through which a lot of substrates are degraded such as phenol, aniline, alkylbenzenes, and several hydrobenzoates (Philipp and Schink 2012).

Up to date, there are three different ways (Fig. 3) known to perform the initial step of the hydrocarbon degradation in the absence of oxygen. The first one is catalyzed by addition of fumarate, as it is described for anaerobic degradation of toluene. In this case, a molecule of fumarate is added to the substrate to form benzylsuccinate, by the benzylsuccinate synthase, which afterward is transformed to benzoyl-CoA, renewing the fumarate in the process (Boll et al. 2014). Naphthalene, phenanthrene, and aliphatic hydrocarbons can also be degraded by this strategy. In case of last

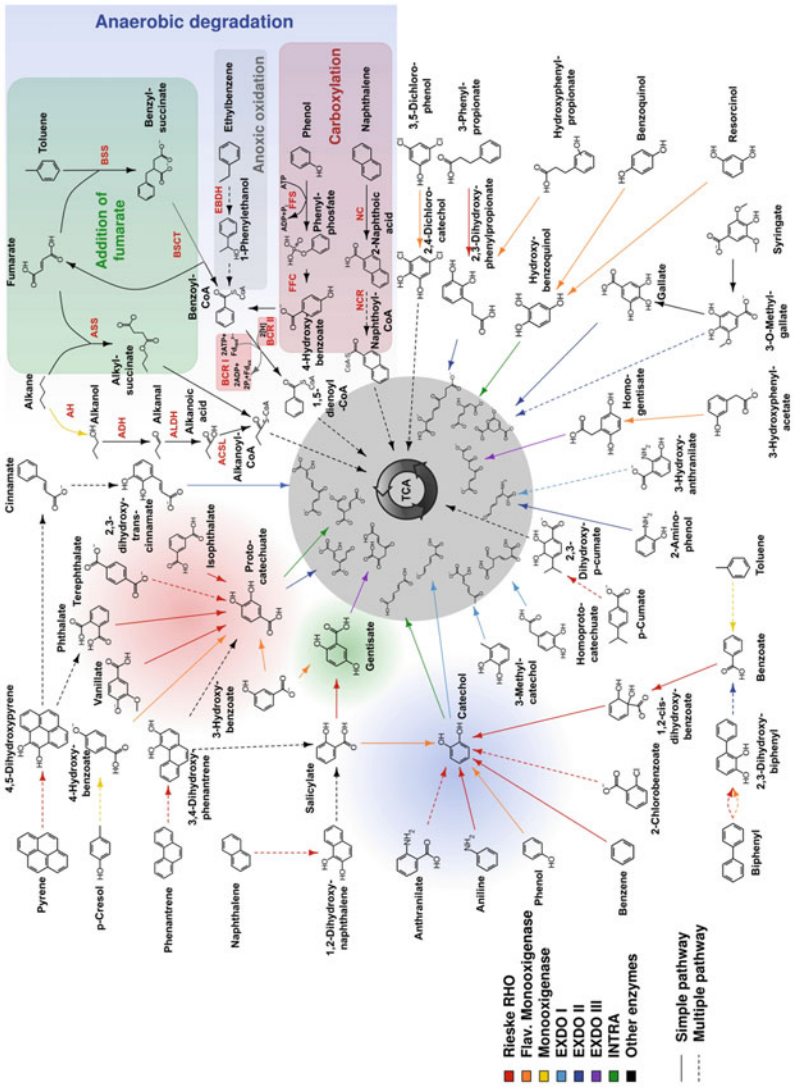

Fig. 3 Representative microbial aerobic and anaerobic degradation pathways. The main anaerobic strategies are summarized on the top right. Reactions ending in the transformation of benzoyl-CoA to 1,5-dienoyl-CoA supported by the benzoyl-CoA reductase (BCR) class I or II with or without ATP consumption,

compounds, an alkyl-succinate synthase adds fumarate to the alkane to form alkyl-succinate, which is then conjugated with coenzyme A (CoA) resulting in an acyl-CoA derivative which is finally processed in the β-oxidation pathway (Boll et al. 2002; Kniemeyer et al. 2007; Rojo 2009; Khelifi et al. 2014; Abbasian et al. 2015).

Second strategy is the O_2-independent hydroxylation. Aromatic compounds similar to ethylbenzene are processed by ethylbenzene dehydrogenase-like enzymes, which hydroxylate these compounds using water molecule instead of oxygen (Heider 2007; Szaleniec et al. 2014; Abbasian et al. 2015).

Third strategy is the carboxylation. Compounds like phenol are anaerobically degraded by this process, which is first phosphorylated to phenylphosphate by the enzyme phenylphosphate synthase, consuming a molecule of ATP. Then, phenylphosphate carboxylase is responsible for the carboxylation of phenylphosphate to produce 4-hydroxybenzoate, which, in turn, is conjugated with a CoA forming benzoyl-CoA (Fuchs 2008; Philipp and Schink 2012). Up to date, this activation method is the most probable pathway for the degradation of non-substituted compounds like benzene and PAHs such as naphthalene and phenanthrene. Theoretically, these compounds would be directly carboxylated to corresponding acids, forming afterward the CoA-thioester intermediates (Meckenstock and Mouttaki 2011; Boll et al. 2014; Luo et al. 2014; Abbasian et al. 2015).

Key step in the benzoyl-CoA de-aromatization is its reduction to 1,5-dienoyl-CoA. There are two different ways to perform this reaction: ATP dependent and ATP independent. The benzoyl-CoA reductase class I is responsible for the ATP-dependent strategy, using two molecules of ATP and two molecules of reduced ferredoxin as electron donor. The ATP-independent strategy is performed by the enzyme benzoyl-CoA reductase class II, without the consumption of ATP (Boll et al. 2014; Fuchs et al. 2011; Philipp and Schink 2012). Both pathways involve the formation of 1,5-dienoyl-CoA, which is afterward degraded through β-oxidation-like reactions, ring cleavage, and decarboxylation, finally resulting in the formation of TCA derivatives (Foght 2008; Carmona et al. 2009; Fuchs et al. 2011; Valderrama et al. 2012).

Fig. 3 (continued) respectively, are shown. Specific labels for enzymes involved in anaerobic degradation pathways are indicated as follows: *BSS* benzylsuccinate synthase, *BSCT* succinyl-CoA: benzylsuccinate CoA-transferase, *BCR I* benzoyl-CoA reductase class I, *BCR II* benzoyl-CoA reductase class II, *ASS* alkyl-succinate synthase, *EBDH* 1-phenylethanol ethylbenzene dehydrogenase, *NC* naphthoate carboxylase, *NCR* naphthoyl-CoA reductase, *FSS* phenylphosphate synthase, and *FFC* phenylphosphate carboxylase. Within aerobic steps, the main reactions feeding three of the main intermediates in aerobic degradation, catechol, gentisate, and protocatechuate are highlighted in *blue*, *green*, and *red* color gradients, respectfully. The different groups of oxygenases involved in aerobic degradation are shown by different colors in the arrows. Only enzymes for the aerobic alkane degradation pathway are indicated: *AH* alkane hydroxylase, *ADH* alcohol dehydrogenase, *ALDH* aldehyde dehydrogenase, and *ACSL* acyl-CoA synthase

2.2.2 Aerobic Degradation

In the presence of oxygen, alkane degradation is performed by monooxygenases (Fig. 3). These enzymes oxidize terminal methyl group leading to formation of the corresponding alcohols, which is successively oxidized into aldehydes and finally into a fatty acids. After incorporation of CoA, the fatty acid is processed in the β-oxidation pathway resulting in the final production of acetyl-CoA which is used in the TCA (Rojo 2009).

Classic degradation of aromatic hydrocarbons is performed by dioxygenases, the enzymes responsible for the ring cleavage. Most of degradation pathways converge into key intermediates like catechol or protocatechuate (Fig. 3). The dioxygenases determine the ring cleavage of these intermediates, and resulting products later are transformed into TCA intermediates (Díaz et al. 2013). In case of polyaromatics, their degradation is performed by the same strategy used in monoaromatics degradation (Vaillancourt et al. 2006). Hence, a first group of dioxygenases activates the molecule giving a dihydroxylated intermediate which is afterward catabolized by a second group of dioxygenases involved in the ring cleavage. Depending on the amount of condensed rings of PAHs, this cycle is repeated several times until the formation of the TCA intermediates occurred (Peng et al. 2008).

Cleavage of the benzene ring may occur in two different positions: *ortho*, i.e., between the two hydroxyl groups; or *meta*, contiguous to the hydroxyl groups (Fetzner 2012). Therefore, dioxygenases are divided into intradiol (INTRA) or extradiol (EXDO) dioxygenases, depending on the positions of oxygen insertion, respectively (Omokoko et al. 2008; Fuchs et al. 2011). There are also degradation pathways with the formation of non-catecholic intermediates (without hydroxyl groups in *ortho* or *meta* positions), as in the case of gentisate and homogentisate, which are processed by another group of extradiol dioxygenases (Fetzner 2012; Díaz et al. 2013).

3 Microbial Community Dynamics After an Oil Spill

Oil-mediated environmental changes result in quick responses by the microbial communities, adjusting their composition (Cappello et al. 2007a; Lanfranconi et al. 2010). The petroleum discharged in the marine ecosystem provokes drastic changes in these communities, decreasing their diversity. Mason et al. (2012) analyzed samples collected during the first weeks after the Deepwater Horizon spill in the Gulf of Mexico, from the deep-sea plume (~1,100 m) where a huge mass of petroleum and gas was released. A drastic change in the microbial composition was observed. On one side, this change is due to a selection in favor of the hydrocarbon-degrading bacteria (Harayama et al. 2004) and, on the other hand, due to the toxic effect of the petroleum hydrocarbons inhibiting the growth of other autochthonous microorganisms of marine ecosystems (Païssé et al. 2010). Moreover, changes in the taxonomic groups are different depending on the season and location of the oil spill (King et al. 2015). The environmental conditions such as temperature and oxygen concentration are also important factors contributing to the changes and

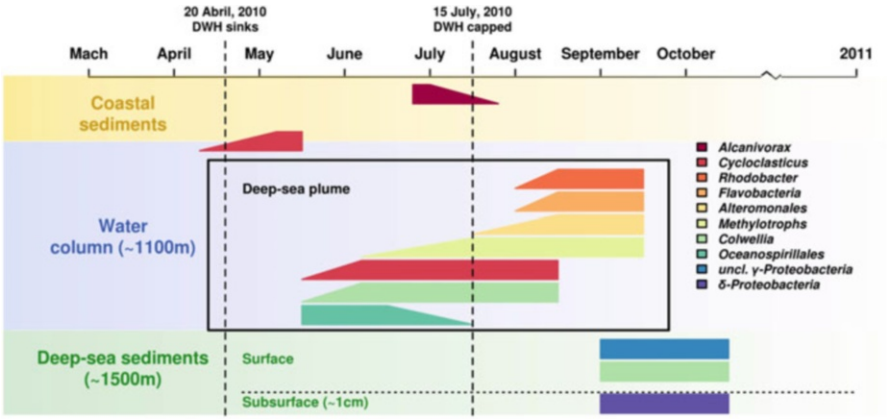

Fig. 4 Overview of the microbial response occurring after the Deepwater Horizon oil spill in the Gulf of Mexico. This representation is based on data from different studies performed on this oil spill (Adaptation from Kimes et al. 2014)

will define the best adapted taxonomic groups to develop (Redmond and Valentine 2012; Kimes et al. 2014). According to these environmental settings, it was found that up to 90% of the microbial population recovered from samples of the deep-sea plume is represented by uncultured Gammaproteobacteria of the order Oceanospirillales distantly related to the psychrophilic hydrocarbon-degrading organisms of the genus *Oleispira* (Fig. 4). Thus, these bacteria are associated with the hydrocarbon degradation (Mason et al. 2012; Kimes et al. 2014). Surprisingly, *Alcanivorax*, one of the most common genera in surface marine oil-contaminated samples, could not be detected in deep-sea plume samples of the Gulf of Mexico. Instead, there were sequences belonging to psychrophilic *Colwellia*. However, *Alcanivorax* was detected in sediment samples from the coast of the Gulf of Mexico. The colder water temperature in the deep-sea plume (approx. 2.0 °C) could explain the absence of mesophilic *Alcanivorax* in these samples, where psychrophilic bacteria such as the members of Oceanospirillales and *Colwellia* could be better adapted (Kostka et al. 2011; Redmond and Valentine 2012; Kimes et al. 2014).

Environmental changes caused by oil spill are not static. First, the environmental conditions could change depending on the season (water temperature); thus, the microbial response to an oil input can be performed by different taxonomic groups depending on the period of the year (Lanfranconi et al. 2010). Second factor, connected with the previous issue, is that the ecosystem keeps changing, while concentration and availability of the different hydrocarbons vary due to physical-chemical factors and biodegradation. The aliphatic hydrocarbons are degraded faster (Reis et al. 2013), while aromatics and PAHs are more persistent in the media (Harayama et al. 2004; Head et al. 2006), resulting in a dominant group succession in microbial community. Alkane-degrading specialists (e.g., *Alcanivorax*) are the first microorganisms to increase their population density (Gertler et al. 2009; Jiménez et al. 2011), while (poly)aromatic-degrading specialists (e.g., *Cycloclasticus*) rise

later (Head et al. 2006; Cappello et al. 2007b; Vila et al. 2010). Different studies over deep-sea plume in the Gulf of Mexico, performed at different periods of time, showed that Oceanospirillales was the dominant group in the population analyzed on May of 2010, i.e., right after the accident. These bacteria are associated with the degradation of aliphatic hydrocarbons. However, samples collected in June 2010 were dominated by bacteria of the genus *Colwellia*, involved in the degradation of both alkanes and aromatic compounds (Bælum et al. 2012), and *Cycloclasticus* (Fig. 3), known by its capability to degrade various (poly)aromatics. Later, in September 2010, the microbiota was still changing and dominated by a higher number of taxonomic groups, probably due to the lower amount of hydrocarbons (after dispersion and biodegradation) and the lowering of toxicity to marine microbiota (Bælum et al. 2012; Kimes et al. 2014).

Changes in the microbial community may also be followed at the functional level. The oil input provokes an increase in the abundance of genes related with hydrocarbon degradation (Lu et al. 2011; Mason et al. 2014), whose relative abundance depends on the environmental conditions (e.g., oxygen concentration and site temperature) and the type and concentration of released hydrocarbons (Acosta-González et al. 2013; Kimes et al. 2013). Lu et al. (2011) compared the genetic expression between samples from the deep-sea in plume (contaminated) and out of the plume (not contaminated) of the Gulf of Mexico. Hence, they found that samples from the plume showed enrichment in the expression for genes involved in the degradation of alkanes, cycloalkanes, BTEX (benzene, toluene, ethylbenzene, and xylene), aromatics, heterocyclic aromatics, and PAHs. The gene *alkB*, which codes for the alkane 1-monooxygenase responsible of the first step of aerobic hydrocarbon degradation, or the gene *bbs*, coding the benzylsuccinate synthase responsible of the anaerobic degradation of the toluene, constitute some examples of the genes whose expression level significantly increase in the plume. In addition, Lu et al. (2011) could notice a higher relative abundance of the expression of genes involved in the carbon and sulfur cycles and in iron reduction, the metabolic processes likely related with the hydrocarbon degradation.

The fact that the bacteria involved in alkane degradation increase their abundance faster than those degrading aromatics and PAH was observed both by 16S rRNA-based phylogenetic examining of microbial communities (Head et al. 2006; Cappello et al. 2007b; Gertler et al. 2009; Vila et al. 2010; Jiménez et al. 2011) and by analysis of gene content. Namely, genes essential for aliphatic hydrocarbon degradation exhibit a quicker response in expression than those related with the degradation of more complex compounds (Vila et al. 2010; Mason et al. 2012; Paissé et al. 2012). As example, in sediment samples from Pensacola beach, affected by the Deepwater Horizon oil spill in the Gulf of Mexico, Rodriguez-R et al. (2015) noticed a high relative abundance of the gene *alk*B in the samples collected in July 2010, decreasing in the October samples, when an increase in the relative abundance of genes involved with the degradation of aromatics was observed.

Above reports clearly demonstrated that the relative abundance of ubiquitous hydrocarbon-degrading bacteria and the expression of catabolic genes involved in hydrocarbon degradation as well as genes relevant for carbon, nitrogen, phosphorous,

sulfur, and iron cycling are modulated by variations in crude oil input (oil spills) in seawater and marine sediments. Drastic successions of those events were found in days or week time after an oil spill occurred (Mason et al. 2012, 2014). Following on from this, it has also been reported that the exposition to oil contamination on a site may affect the ability of marine microbial communities to respond more rapidly to a new spill, compared to microbial communities that develop in pristine sites (Sauret et al. 2012). In agreement with this, a recent analysis of numerous sites distributed over the Mediterranean and Red Sea also revealed that bacterial populations, established at chronically polluted sites, may respond more promptly to accidental oil spill compared to microbial populations thriving in pristine sites. This is most likely due to the fact that chronic pollution promotes long-term adaptation of hydrocarbon-degrading species and their consequent catabolic diversification due to exposition to multiple pollutants (Bargiela et al. 2015).

4 Hydrocarbon Degradation Pathways: Mediterranean Sea, Red Sea, and Deepwater Horizon as Case of Study

The information presented above exemplifies the fact that the structure of bacterial populations and the genomic material they contain are strongly influenced by oil spills or petroleum chronic pollution occurred in various marine sites. This influence is also modulated by multiple environmental factors (oxygen concentration, temperature, presence of nutrients, hydrostatic pressure, etc.). Altogether, the petroleum impact on the species distribution and their catabolic activities may differ in geographically separated marine sites, because of the different influence of environmental and geographical constraints and anthropogenic forces (hydrocarbon input and diversity of pollutants).

The identification of bacterial species that are altered during oil spill or as a consequence of chronic crude oil pollution is one of the essential analyses to be conducted. This can often be achieved through extensive high-throughput next-generation meta-sequence datasets of the 16S rRNA gene, and numerous available studies evidenced their identities. Using this technique, and as shown above, bacteria affiliated with the genera *Alcanivorax*, *Cycloclasticus*, *Oleispira*, *Thalassolitus, Oleiphilus, Polycyclovorans*, *Algiphilus*, *Porticoccus*, *Neptumonas*, *Marinobacter*, *Pseudomonas*, *Sphingomonas*, *Thalassospira*, *Paracoccus*, *Rhodococcus*, *Gordonia*, *Planococcus* and with the orders Desulfovibrionales, Desulfobacterales, and Desulfuromonadales, and archaea of the genera *Haloferax*, *Halobacterium*, and *Halococcus*, are good examples of prokaryotes whose abundance has been found to be significantly altered in marine sites after an oil spill. Significant differences in the distribution of some of these members have been also reported. Thus, as mentioned above, members of the genus *Alcanivorax* are most associated to epipelagic marine oil-contaminated samples, whereas members of *Oceanospirillales* and *Colwellia* are better adapted to deep and cold seawater (Kostka et al. 2011; Redmond and Valentine 2012; Kimes et al. 2014). Also, the abundance of *Alcanivorax* and other specialized hydrocarbonoclastic bacteria varies in oil-polluted sites on the coastlines

worldwide (Bargiela et al. 2015). Such differences are likely due to the unique environmental constraints characterizing the investigated sites.

More recently, metagenomics studies through extensive DNA sequencing were applied to analyze the alterations in microbial community structure, including analysis of gene contents to model the catabolic activities and metabolic network of microbial populations. As example, extensive data are available for marine sites such as the Deepwater Horizon oil spill in the Gulf of Mexico (Mason et al. 2012, 2014) and a number of geographically separated oil-polluted sites on the coastlines of the Mediterranean and Red Seas (Daffonchio et al. 2013; Bargiela et al. 2015). Their individual analyses have revealed the abundance of catabolic genes involved in oil-component degradation. However, comparative analyses of the distribution of catabolic genes (see Sect. 2.2 and Fig. 3 for examples) and the catabolic routes they support have not been performed. This can be now made by using the web-based AromaDeg resource (Duarte et al. 2014), which contains information on key catabolic gene families with functionally characterized references (Fig. 5) and an automatic reconstruction procedure recently developed by authors (Bargiela et al. 2015). Briefly, by using as input potential protein-coding gene sequences obtained by direct sequencing of DNA from microbial communities inhabiting different crude oil-contaminated sites, it is possible to reveal the differential distribution of hydrocarbon degradation pathways. By using this method and the sequence information of the different marine sites characterized by high level of oil contamination, particularly, the Red Sea, the Mediterranean Sea, and the Gulf of Mexico (for which extensive data are available), we were able to provide an overview of the catabolic pathways supported by microbial populations that develop in each of the three sites. Meta-sequences were obtained from public databases, with accession number as follows:

i. Two samples from the Deepwater Horizon oil spill, BM058 (Longitude, −88.4375; Latitude, 28.672222; Joint Genome Institute (JGI) project ID 403207; taxon IDs 2088090017 and 2081372002), and OV011 (Longitude, −88.4375; Latitude, 28.672222; JGI project ID 403191; taxon ID 2081372001). Both samples together were considered as representatives of the hydrocarbon degradation pathways in the deep Gulf of Mexico, although OV011 is located closer to the plume.
ii. Seven samples from coastal sites at the north and south of the Mediterranean Sea, located at coordinates from 44° 22′25.75″N and 8° 41′59.58″E to 31°9′31.20″N and 29°50′28.20″E (NCBI IDs PRJNA222659, PRJNA222657, PRJNA222658, PRJNA222660, PRJNA222661, PRJNA222665, and PRJNA222666; whole-genome shotgun projects deposited at DDBJ/EMBL/GenBank under the accession numbers AZIB00000000, AZIC00000000, AZIF00000000, AZID00000000 and AZII0100000, AZIE00000000, and AZIJ0100000). All seven samples were considered together for representing the hydrocarbon degradation pathways in crude oil chronically contaminated Mediterranean Sea.
iii. One coastal site at the Red Sea located at 30°22′42″N and 25°24′57″E (NCBI IDs PRJNA222667; whole-genome shotgun projects deposited at DDBJ/EMBL/

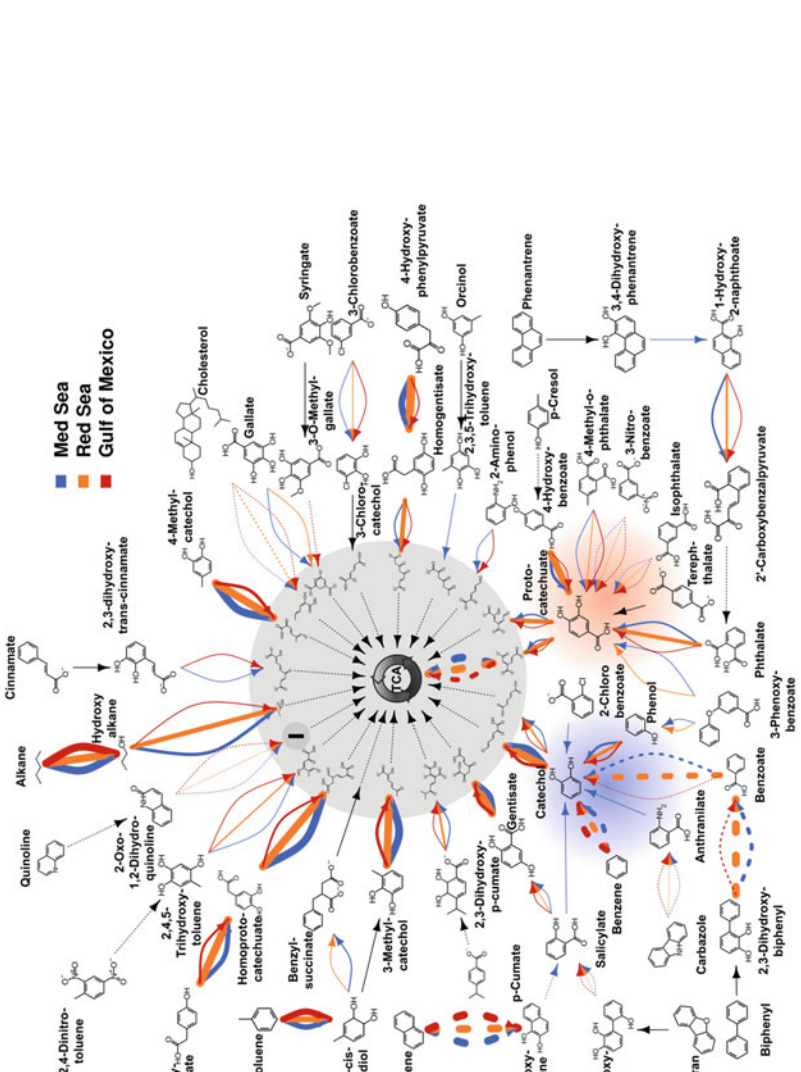

Fig. 5 Distribution of hydrocarbon degradation pathways in the Mediterranean and Red Seas and the Gulf of Mexico (area where the Deepwater Horizon accident took place). Sequences encoding enzymes involved in degradation reactions were identified, and their abundance level quantified, by submitting the

GenBank under the accession number AZIG0100000). This sample was considered for representing the hydrocarbon degradation pathways in crude oil chronically contaminated Red Sea.

Following the above protocol, each query sequence from the three sets of metagenomes representing the Gulf of Mexico, the Mediterranean Sea, and the Red Sea was submitted to web-based AromaDeg resource (Duarte et al. 2014). This web resource allows identifying sequences associated with catabolic enzymes performing an alkane or aromatic compound degradation reaction. These degradation reactions can be linked to conform a degradation network following an automatic procedure previously described (Bargiela et al. 2015). Figure 5 exemplifies the potential aerobic degradation networks of alkanes and aromatics via di- and trihydroxylated intermediates in the three marine areas (Mediterranean Sea, Red Sea, and Gulf of Mexico) that were examined as case of study and that are indicated by different color in the figure.

As shown in Fig. 5, microorganisms from each site are characterized by versatile degradation capacities, a high proportion of which are common to the three marine areas. As example, all three sites are characterized by a high abundance of sequences encoding AlkB monooxygenases, as well as enzymes supporting the degradation of catechol derivatives, toluene, and gentisate, to name few. Opposite, low-abundance sequences for all three sites are those encoding enzymes for the degradation of, e.g., quinolone and carbazole. This suggests that these sequences are high or low abundant, respectively, in crude oil-contaminated marine areas independent of the environmental constraints and whatever the population structure in those. The similar distribution (high or low) of many catabolic genes may be due to the so-called functional redundancy, which can reflect the evolutionary convergence of unrelated taxa so that variable combinations of species from different phyla could fulfill partial functional redundancy, and thus different bacteria perform similar functions in different sites (Bargiela et al. 2015). However, we do not only observed similarities in catabolic capacities but also some differences (Fig. 5), which exemplify the differential distribution of some degrading capacities in the sea. Some relevant examples are the degradation of cinnamate and aminophenol, for which no sequences were observed in the Red Sea sample, or the degradation of gallate, which seems to be rather irrelevant in the Gulf of Mexico. The observed differences may be due to the fact that each site is inhabited by a specific set of species that

Fig. 5 (continued) three sets of metagenomes representing the Gulf of Mexico, the Mediterranean Sea, and the Red Sea (see accession number in the text) to the web-based AromaDeg resource (Duarte et al. 2014), following an automatic reconstruction tool (Bargiela et al. 2015) that allows connecting degradation reactions to complete a network. Reactions associated to each of the three sites, herein used as cases of study, are indicated by different colors. The relative abundance (rel. ab) of catabolic genes supporting each of the degradation reactions in each of the samples is indicated by the thickness of the *arrow*. Rel. ab. is referred to the total number of genes in each of the metagenomes to avoid artifacts due to differences in sample size and sequence coverage

developed under the geochemical and anthropogenic constraints characterizing each site. Although some of the observed differences could be due to the limited sequence coverage in a given sample and the reconstructed pathways may be incomplete, the data presented in Fig. 5 allow a tentative view into the differential distribution of degradation pathways in some marine areas that are known to be subjected to extensive crude oil pollution.

5 Concluding Remarks and Research Needs

Crude oil pollution and the chemical diversity of its components, in combination with environmental constraints such as depth, oxygen concentration, temperature, nutrient input, and other physical and chemical factors, distinctly influence microbial populations and the biodegradation processes they mediate in response to accidental oil spills in seawater and seawater sediments. We have herein provided a revision of such changes at the level of population structures with particular attention in hydrocarbon-degrading bacteria and the enzyme arsenal they contain to catabolize crude oil components. We observed not only extensive dynamic variations within the same site as consequence of an oil spill but also between different marine sites. Actually there is a need to generate more sequence information that help us to decipher the overall distribution of degradation capacities in the sea. This will help in turn to develop bioremediation strategies by promoting degrading activities that are low abundant in sites where an oil spill occurred, supporting the complete degradation of all chemical components of the crude oil.

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Part III

Microbial Facilitation of Petroleum Recovery

3° Oil Recovery: Fundamental Approaches and Principles of Microbially Enhanced Oil Recovery

35

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Contents

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Abstract

Microbially enhanced oil recovery (MEOR) involves the use of microbes in petroleum reservoirs to enhance the amount of oil that can be produced. The microbes in MEOR are typically hydrocarbon-utilizing, nonpathogenic microorganisms that naturally occur in the reservoirs or are introduced. The microbes use hydrocarbons as a food source for their metabolic processes and excrete natural bio-products such as alcohols, gases, acids, surfactants, and polymers. These bio-products can change the physical-chemical properties of crude oils and/or modify oil-water-rock interactions that improve oil recovery.

An important requirement for devising an appropriate MEOR strategy is a clear understanding of the problem that needs to be addressed from a reservoir engineering point of view. *In situ* production of biopolymers is most suitable to address water channeling problems in heterogeneous reservoirs, while surfactant-, gas-, acid-, and alcohol-producing microbes may be more suitable for enhancing production from reservoirs where residual oil is trapped due to capillary forces. Ex situ production of chemicals, e.g., biosurfactants from bioreactors for injection in wells, offers the advantage of being more controllable.

1 Introduction

The world demand for energy is projected to grow in coming decades, and energy is the key to economic and social development in a context of a rapidly expanding world population. While the share of energy contributed by renewables is set to grow driven by their falling costs and a desire to reduce carbon emissions, most projections see fossil fuels remaining the dominant source of energy powering the world economy for the at least the next 20–30 years. For example, the BP Energy Outlook to 2035 forecasts the demand for oil to increase by more than 20%, driven by increasing demand from non-OECD countries (Fig. 1). Similar projections can be found in other forecasts (e.g., World Energy Outlook 2016, published by the International Energy Agency).

To ensure sustained oil supply, additions to conventional oil reserves must be in line with the increased demand. In mature basins, a major component of the addition to reserves is derived from "reserve or field growth" (McCabe 1998). Disruptive technological advances such as optimized hydraulic fracturing were stimulated by high oil and gas prices and have led to a dramatic increase of oil and gas production from unconventional rock formations such as shales and tight sandstone. In the USA, unconventional petroleum production accounted for more than 50% of total production in 2015 and is a significant contributor to the dramatic rise in petroleum exports from the USA since about 2008 (US Energy Information Administration). However, unconventional production is more costly, and it is not clear whether unconventional reserves will continue to grow in the current low oil price scenario and in oil provinces with more challenging political, social, and environmental environments.

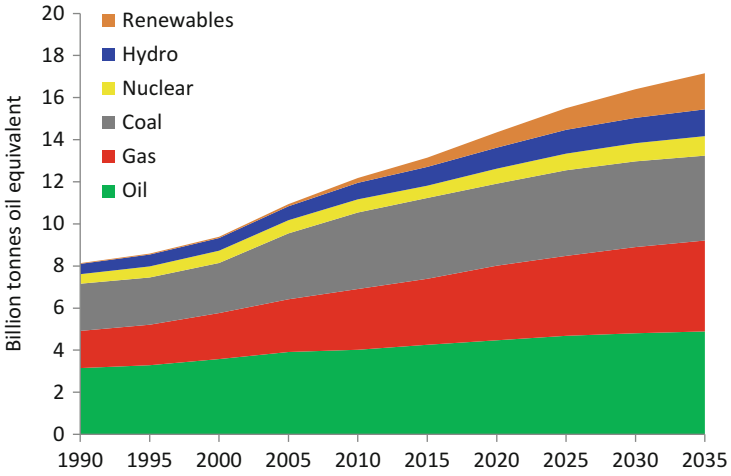

Fig. 1 Energy consumption by fuel (Data from BP Energy Outlook 2017)

While exploring for oil in new petroleum provinces and finding and exploiting conventional and unconventional oil in or near existing oil fields are important, enhanced oil recovery (EOR), sometimes called improved oil recovery (IOR) or tertiary oil recovery, will play a critical role in sustaining oil supply from mature fields. In the future, MEOR will offer an additional method for substantially improving oil recovery from mature fields.

MEOR is a technique where microbes are used to generate products such as acids, gases, biomass, and exopolymers, as well as surfactants and solvents that aid oil production. Not all of these metabolites are needed at the same time, and deciding which products will be most useful for enhancing oil production will depend on the factors limiting petroleum production in a particular reservoir. Due to their size and ability to grow under the conditions present in petroleum reservoirs, only prokaryotes (bacteria and archaea) are considered promising candidates for MEOR, whereas molds, yeasts, algae, and protozoa are not suitable (Van Hamme et al. 2003). The MEOR concept was mooted as early as 1920, and pioneering field studies were carried out in the USA in the 1930s and 1940s by Claude ZoBell and his group (e.g., ZoBell 1946). However, 90 years onward, the oil industry typically remains skeptical about the potential of MEOR to become a mainstream part of their EOR portfolio. This perception is nurtured by mixed successes and a lack of a full scientific understanding of the processes.

While it is clear that microbes may promote beneficial chemical reactions, such as the production of biosurfactants in a very specific and energy-efficient manner, a sound understanding of the underlying principles is important to predict site-specific effects of microbial activity on fluid flow in porous media and hence on the efficiency of oil production.

In this chapter, the underlying fundamental approaches and principles of MEOR are discussed, and some of the complex interrelationships between petroleum

microbiology and its physical and environmental constraints in petroleum reservoir bioreactors are outlined. In Liu et al. (this volume), experiences of the EOR industry are highlighted from a practical and economic viewpoint.

Since the first edition of this chapter, the concepts behind EOR including MEOR have evolved somewhat and now, with increasing pressure to reduce CO_2 emissions, oil recovery could be broadened to "energy recovery." There are a number of interesting approaches to "microbial enhanced energy recovery" (MEER) that we consider should be added to the arsenal. One is the conversion of residual petroleum to methane, and another is some very early work on the in situ bio-oxidation of oil and recovery of the energy at the surface via electrodes (Head et al. 2014, Head and Gray 2016) or by recovery of a reduced mobile species (electron shuttle) at the surface (http://www.ucalgary.ca/prg/research/project-1-syzygy).

2 The Petroleum Reservoir Biome

Microbial processes are important in petroleum reservoirs regardless of whether they are employed for EOR or not. Indeed, they have huge economic ramifications and, over geological timescales, are responsible for biodegradation of petroleum in relatively shallow and cold subsurface reservoirs. Recovery and refinement of biodegraded oil is generally more costly and less efficient than for undegraded oil, and therefore understanding and predicting biodegradation is an important aspect of petroleum exploration. A good overview of biodegradation in petroleum reservoirs can be found in Head et al. (2003), whereas Van Hamme et al. (2003), Röling et al. (2003), Magot et al. (2000), and Head et al. (2014) have provided excellent reviews on microbial consortia and their biochemical pathways. Head et al. (2014) have provided a detailed review of microbial communities in heavily biodegraded reservoirs in the Western Canadian Sedimentary Basin. *Epsilonproteobacteria* often dominate in these biomes, many of which are putative chemoautotrophic, nitrate-reducing sulfide oxidizers (Hubert et al. 2012).

Microbial life in the geosphere is constrained by chemical factors such as pH, Eh, electrolyte, and nutrient composition; by biological factors such as niche competition, syntrophy, and cytotoxicity of microbial metabolites; and by physical factors such as temperature, pressure, pore size, and pore geometry. Fredrickson et al. (1997) found that sustained bacterial activity required interconnected pores of at least 0.2 μm diameter. Research on the deep biosphere indicates that temperature is the dominant factor controlling the viability of microbial life (Jorgensen and Boetius 2007). According to these authors, microbes inhabit almost every environmental niche, and the biosphere may extend up to 4 km below the surface of the earth and in marine environments with average oceanic geothermal gradients (30–50 °C/km). Extremely thermophilic anaerobes that grow up to 120 °C have been isolated and cultured in the laboratory (Kashefi and Lovley 2003). The most thermophilic organisms belong to the domain archaea which, despite their morphological similarity, are evolutionary distinct from bacteria.

Careful sampling protocols are paramount for MEOR research. Although numerous highly specialized organisms have been described from petroleum reservoirs, there are cases where it suspected that these microbes represent exogenous bacteria derived from sea-water injection (Grassia et al. 1996). Thermophiles can survive in cold sea water, and several of the species described from petroleum reservoirs are identical to those found in submarine hot vents (Stetter et al. 1993). Evidence of hyperthermophilic archaea in continental oil fields from the East Paris Basin indicate that thermophiles may be widespread in the lithosphere. Microbially mediated degradation of petroleum in reservoirs is however, rarely documented in oil fields hotter than 80 °C (Head et al. 2003). This suggests that although certain hyperthermophiles may be present at high temperatures in petroleum reservoirs, their metabolic activity is low, and their potential for MEOR remains unproven.

MEOR is generally applied in mature fields, and the microflora encountered in these fields are unlikely to represent the pristine biome present before petroleum production. Initially, exogenous microbes are likely to have been introduced to the formation during water flooding with treated seawater, river water, or even formation water. Even without the introduction of exogenous microbes, the initial balance of the microbial population may have been shifted due to modified environmental factors. Although reservoirs that have not been waterflooded are considered to be the best models for studying indigenous microbes, new species may still have been introduced from drilling, well equipment operations, and damaged (leaking) tubing and casings. In addition, the petroleum industry uses biocides and nitrate to control unwanted bacterial activities that may lead to reservoir souring and corrosion problems. Although microbes normally recolonize treated reservoir sections, the microbial population dynamics of these sections will not be pristine.

Designing an appropriate MEOR approach requires a clear understanding of the production issues that limits the extraction of oil from the target reservoir. For this reason, the involvement of reservoir engineers is critical for devising and implementing a suitable MEOR solution (Bryant and Lockhart 2002; Gray et al. 2008). In addition, a detailed understanding of the microbiological communities in a particular reservoir is important. It is far more critical to understand the status quo of the microbial community before MEOR treatment rather than the pristine microbial diversity from decades ago, before petroleum production commenced. Any microbiological study needs to consider problems associated with oil reservoir sample recovery. Stringent sampling protocols should be used to minimize contamination. Monitoring the make-up of microbial consortia before and after MEOR applications is crucial for tracking community shifts responsible for MEOR effects.

In the last decade, the advent of relatively cheap DNA sequencing technologies has dramatically improved our ability to study entire microbial communities. Metagenomics, the sequencing and analysis of all the DNA extracted from a sample, has the potential to lead to a quantum leap in our understanding of the structure and more importantly the function of oil reservoir communities. In turn, this will improve our ability to control those communities to achieve the desired energy recovery outcomes.

3 Metabolites and Biomass Production Utilized for MEOR

This section will discuss the microbial production of metabolites and their influence on the producibility of crude oil. Once production rates of these metabolites are established, it may be possible to assess transport and fate and in particular biological degradation of these products.

3.1 Gas Production

Gases are common microbial metabolic by-products, and generation of biogenic methane has contributed to some of the world's largest natural gas fields (e.g., in West Siberia and in Trinidad). The most common gases generated by microbial metabolism are carbon dioxide, methane, hydrogen sulfide, and hydrogen, which is then rapidly consumed again by further microbial activity. Aerobic conditions are rare in petroleum reservoirs, which contain vast pools of hydrocarbons which would rapidly deplete oxygen in the presence of aerobic microbes. Ferrous iron and sulfur are typically abundant and also deplete free oxygen. Methanogenesis is a strictly anaerobic process and is responsible for the typical association of biodegraded oil and methane-rich gas in shallow petroleum reservoirs, and this process has recently been elucidated in more detail by studies on laboratory microcosms by Jones et al. (2008). Sea salt contains ca. 7.7 wt% sulfate, the most abundant anion after chloride. Although energetically less attractive as a terminal electron acceptor than nitrate, nitrite, or ferric iron, sulfate-reducing bacteria tend to dominate anaerobic environments with abundant sulfate, and as such they are common in petroleum reservoirs flooded with seawater and give rise to hydrogen sulfide if uncontrolled (Eckford and Fedorak 2002a, b; Gieg et al. 2011).

During petroleum production, the pressure in a reservoir often decreases due to the withdrawal of fluids, such that generation of microbial gases may have the potential to repressurize reservoirs for increased gas drive. However, to our knowledge no convincing cases of reservoir repressurization due to microbial gas production are documented in the literature, and repressurization of depleted fields may be due to numerous other factors.

In addition to repressurizing a reservoir, gases have the potential to alter pH of formation waters and to change viscosity, interfacial tension, and pour point of oils. Lowering of pH values by partitioning of CO_2 into formation water may have beneficial effects on the producibility of oil, in particular in fractured carbonate rocks (Tanner et al. 1991; Nagalakshmi et al. 2014) and in siliciclastic reservoirs where carbonate cements reduce permeability by blocking pore throats.

Several patents on extracting methane and hydrogen from biologically stimulated reservoirs have been filed. For heavy oils and tar sands, recovering energy in the form of gas rather than as higher hydrocarbons may present an economic and environmentally more sustainable pathway for extracting energy than retorting or steam assisted gravity drainage (Head et al. 2016). The extraction of methane is an approach that could

benefit from the body of work conducted on microbial stimulation of methane production in coal seams (Jones et al. 2010; Midgley et al. 2010).

3.2 Biosurfactants and Bioemulsifiers

Biosurfactants or bioemulsifiers are a heterogeneous group of amphiphilic molecules that contain both hydrophobic and hydrophilic groups. They reduce surface and interfacial tension and create micro-emulsions where hydrocarbons can solubilize in water or water can solubilize in hydrocarbons. Common biosurfactants are glycolipids, lipopeptides, fatty acids, phospholipids, lipopolysaccharides and glycoproteins. Many microorganisms are known to produce biosurfactants, and tables of surfactant-producing microorganisms can be found in Banat (1995) and Rosenberg and Ron (1999). Van Hamme et al. (2006) and Singh et al. (2007) published excellent companion reviews on physiological aspects of surfactants in microbiology and biotechnology as well as their applications.

Rosenberg and Ron (1999) grouped surfactants on the basis of their molecular weight. Low molecular mass bioemulsifiers are generally glycolipids or lipopeptides, whereas high molecular mass bioemulsifiers are amphipathic polysaccharides, proteins, lipopolysaccharides, lipoproteins, or complex mixtures of these biopolymers. According to these authors, MEOR effects of low molecular mass bioemulsifiers are due to lowering of surface and interfacial tensions, whereas high molecular mass bioemulsifiers are more effective at stabilizing oil-in-water emulsions.

Chemically synthesized surfactants have long been used in the EOR industry. However, these compounds are commonly expensive, not biodegradable and toxic. Biosurfactants have the attraction of being biodegradable and relatively inexpensive, and they can also be produced in situ in oil reservoirs. Although biosurfactants have potential to replace chemically synthesized surfactants, targeting biosurfactants for MEOR has drawbacks. The quantity and quality of biosurfactants are not only influenced by bacterial species but also by environmental conditions and nutrient supply (Banat 1995). This is an additional complication in producing a specific biosurfactant with a specific organism. Cytotoxicity of metabolites may limit microbial growth in petroleum reservoirs; biosurfactants such as surfactin have been studied and patented for their antimicrobial activity. While many microorganisms produce biosurfactants, only a few sufficiently reduce the interfacial tension between oil and brine for MEOR purposes. Many of these microorganisms are aerobic bacteria that are not suitable for in situ MEOR applications (McInerney et al. 2005). In a study funded by the US Department of Energy, however, McInerney et al. (2005) investigated the potential of *Bacillus* species to generate biosurfactants through screening, selection, and genetic manipulation. The importance of the 3-hydroxy fatty acid composition of lipopeptides for biosurfactant activity was investigated and its activity accurately predicted by multiple regression analysis. *Bacillus mojavensis* strain JF-2 was highlighted as a microbe that appears to be particularly suitable for lipopeptide surfactant production (Table 1).

Table 1 Summary of effects taking place in MEOR

Product	Microorganism	Application in oil recovery
Biomass	*Bacillus licheniformis*, *Firmicutes* sp.	Selective biomass plugging
	Leuconostoc mesenteroides	Viscosity reduction
	Xanthomonas campestris	Oil degradation, wettability alteration
Biosurfactants (emulsan, sophorolipids, peptidolipid, rhamnolipid)	*Acinetobacter calcoaceticus*	Emulsification, decrease of interfacial tension, viscosity reduction
	Arthrobacter paraffineus	
	Bacillus licheniformis	
	Clostridium pasteurianum	
	Corynebacterium fascines	
	Pseudomonas rubescens, *Bacillus mojavensis*, *Bacillus subtilis* subsp. *subtilis*, *Bacillus subtilis* subsp. spizizensis	
Biopolymers (alginate, xanthan, dextran, pullulan)	*Bacillus polymyxa*	Injectivity profile modification, mobility control
	Brevibacterium viscogenes	
	Leuconostoc mesenteroides	
	Xanthomonas campestris	
Solvents (*n*-butanol, acetone, ethanol)	*Clostridium acetobutylicum*	Oil dissolution, viscosity reduction
	Clostridium pasteurianum	
	Zymomonas mobilis	
Acids (acetate, butyrate)	*Clostridium* spp.	Permeability increase, emulsification
	Enterobacter aerogenes	
Gases (CO_2, CH_4, H_2)	*Clostridium acetobutylicum*	Increased pressure, oil swelling, decrease of interfacial tension
	Clostridium acetobutylicum	Viscosity reduction, permeability increase
	Enterobacter aerogenes	
	Methanobacterium sp., *Methanococcus* sp., Methanosarcina sp.	

Reproduced from van Hamme et al. (2003) with additional data from McInerney et al. (2005), Gray et al. (2010), Amani (2015) and Zhang et al. (2012)

3.3 Acids and Solvents

Microbial activity in petroleum reservoirs produces carboxylic acids of various molecular weights and gases such as CO_2 and hydrogen sulfide (H_2S) that can dissociate into water to form acids. Biodegraded oils commonly have high total acid numbers, which is defined as the amount of potassium hydroxide in mg that is needed to neutralize the acids in one g of oil. One of the main compound classes contributing to this acidification are high molecular weight naphthenic acids

(Meredith et al. 2000; Dias et al. 2014). In the water phase, low molecular weight carboxylic acids such as formic and acetic acids and dicarboxylic acids such as succinic acids are far more soluble and bioavailable. Solvents contributing to MEOR are mainly lower alcohols, volatile fatty acids, and ketones. Solvents and acids may reduce the interfacial tension between oil/rock and oil/water and may decrease oil viscosity, thereby promoting flow. Acids may also assist by dissolving carbonate precipitates from pore throats thereby enhancing permeability and porosity (Tanner et al. 1991).

3.4 Biopolymer Production and Biofilms

Many bacteria generate a biofilm of extracellular polymeric material that provides a means of attaching to solid substrates; the material may also serve as a growth medium and a means of cellular communication. Biofilms are commonly composed of exopolysaccharides (EPS), bound clusters of cells which may cause clogging of porous media. The clogging may be caused chemically, (e.g., by precipitation of carbonates due to the metabolite CO_2); physically, by trapping of particles in the biofilm; or biologically, by the biomass itself. Biopolymers and chemical precipitates produced by bacteria can be used to divert injection fluid to lower-permeability, higher-oil-saturation zones, leading to IOR. Such microbial plugging has been proposed as an effective, low-cost method of improving sweep efficiency by reducing permeability of water-swept regions of a reservoir. In some studies, bacterial cells were starved, to reduce their size and to increase their penetration depth into the reservoir formation, before adding nutrients to stimulate growth and biomass production (Cunningham et al. 2007). However, in situ production of biopolymers may also cause formation damage by clogging useful pores, and the potential of the bio-clogging approach of MEOR needs to be carefully evaluated against the risk of formation damage. Understanding the mechanisms associated with biomass plug development and propagation is needed for successful application of this technology. The most suitable candidates for this MEOR approach are heterogeneous reservoirs with water channeling problems. The visco-plastic behavior of biofilms during different fluid flow regimes is also a topic of interest for understanding MEOR processes (e.g., Ahimou et al. 2007). Shape and mechanical stability of the biofilm will be influenced by its rheology, which will then also affect both mass transfer and detachment processes (Gerlach and Cunningham 2012; Melaugh et al. 2016).

3.5 Bioelectrical Harvesting of Petroleum Resources

In recent years, there has been speculation on the recovery of energy from subterranean petroleum formations by microbial catalyzed electrochemical means. In one scenario, the in situ bio-oxidation of petroleum would occur at the surface of an electrode linked to a surface cathode (Head et al. 2014). In another scenario, the

energy transfer would be by means of highly mobile electron-shuttle species which is transported to the surface via a water column (http://www.ucalgary.ca/prg/research/project-1-syzygy). While these are very exciting prospects, it seems likely to us that the research effort required of overcoming the technical difficulties, time frame, and likely cost of implementation means that these approaches will be probably be overtaken by alternative zero or low emission technologies.

4 Approaches in MEOR

4.1 Ex Situ and In Situ Approaches

Petroleum reservoirs are examples of bioreactors that are difficult to observe and manipulate. For this reason, ex situ approaches where the microbial population, environmental conditions, and nutrient composition can be easily controlled have predominated in the field. In situ approaches where the indigenous microflora are stimulated with trace nutrients to use the residual oil as the carbon source producing the desired by-products are arguably simpler and cheaper and are more applicable where water channeling problems in heterogeneous reservoirs are mitigated by biopolymer production.

4.2 MEOR Treatment with Allochthonous Microbes

Most commercial MEOR service providers inject nutrients together with exogenous microbes. This approach allows selection of specific bacteria that have particularly useful properties in producing useful metabolites and biomass, the introduced microbes may not be able to compete effectively with the endogenous ones that are already adapted to the reservoir conditions. An additional difficulty is that co-injected nutrients in solution, such as phosphates and nitrates, tend to separate from co-injected bacteria and/or archaea which are typically between 1 and 2 μm in size. Therefore, when injected into a petroleum reservoir with low pore throat diameters, the more rapidly propagating nutrient front may nourish microbes already established in the reservoir such that injected microbes face an invigorated indigenous microbial consortium.

Despite the above-mentioned difficulties in seeding established microbial consortia with new species for stimulating MEOR, there are a number of case studies where this seems to have worked well. However, apart from a few selected case studies (e.g., Youssef et al. 2007, 2013), these claims are poorly documented, and no controls with “nutrients only” injections were carried out.

4.3 Anaerobic Versus Aerobic MEOR Approaches

Anaerobic processes may have greater potential for MEOR than aerobic approaches, because the microbiology of oil field water is vastly biased toward anaerobes.

A classical model of petroleum biodegradation viewed delivery of oxygen by meteoric water as critical for microbial activity in the subsurface (Palmer 1993). However, more recent work overwhelmingly came to see biodegradation as an anaerobic process (e.g., Head et al. 2003; Jones et al. 2008). Some studies on a number of oil fields in Western Canada showed that none of the oil field waters had high numbers of bacteria capable of growing under aerobic conditions (Eckford and Fedorak 2002a, b).

Nonetheless the presence of organisms, conventionally considered to be aerobic heterotrophs, e.g., *Bacillus* spp., *Acinetobacter* spp. and, *Pseudomonas* spp., in oil reservoirs continue to be reported in the literature (e.g., Zhang et al. 2012). Head et al. (2014) provide four potential reasons for this contrasting information: (i) contamination with oxygen and traces of aerobic microbes during sampling, transport, or storage, (ii) oxygen delivered to the reservoir due to meteoric water percolating into the reservoir levels or due to water injection, (iii) "cryptic" aerobic communities that may use in situ generated oxygen, or (iv) the capacity of the detected aerobes for aerobic growth.

A recent study on a number of oil fields in Western Canada showed that none of the oil field waters had high numbers of bacteria capable of growing under aerobic conditions (Eckford and Fedorak 2002a, b). Nevertheless, there are still advocates for aerobic MEOR processes (e.g., Sunde 1992; Belyaev et al. 2004; Kowalewski et al. 2006). Aerobic conditions, however, may have to be able to be established and maintained by air injection.

The potential of anaerobic microbes for MEOR is consistent with our current understanding of petroleum degradation. The efficient utilization of hydrocarbons under anaerobic conditions requires a consortium of organisms. The substrate pool is complex and requires a range of organisms to degrade it, and in many cases, their continued growth requires other organisms to remove their metabolic waste products. Case studies using both aerobic and anaerobic MEOR approaches will be discussed in Liu et al. (this volume).

4.4 Biotransformation of Crude Oil into More Mobile Forms

While most MEOR approaches assume that the effect of microbes flows from the addition of microbial metabolites to the crude oil or the oil field water in the petroleum reservoir, biotransformation of crude oil by biocracking has also been proposed and patented as a viable process (e.g., Maure et al. 2005). In this approach, *n*-alkanes and other major hydrocarbon constituents are "cracked" into smaller

molecules which improve the rheological properties of crude oil. This leads to a reduction of specific gravity, and an increase in the volume of residual oil in petroleum reservoirs, thereby facilitating producibility of the crude oil. The cracking of heavy paraffinic compounds is purportedly mediated by enzymatic action of symbiotic microorganisms and specific nutrients by alternating aerobic and strictly anaerobic cycles (Maure et al. 2005), although the details of the process were not discussed. The capacity to induce similar changes in crude oil properties were also documented for a *Rhodococcus* strain from the formation brine in the Daqing oil field (Zheng et al. 2012).

4.5 Reservoir Engineering Considerations

All microbial metabolites proposed for use in MEOR are from chemical families that are already used or are proposed for use in EOR (Bryant and Lockhart 2002). While biomass is not a metabolite, it may be compared to foams used in conventional EOR. The main difference, however, is the manner in which the chemicals are introduced into the reservoir (i.e., in situ generation), which has to be more efficient for MEOR compared to a conventional EOR process to make it an attractive alternative.

Once the problem is clearly identified, an appropriate MEOR solution can be formulated. Consideration of reservoir engineering principles will then help in pinpointing the specific technical challenges of a particular MEOR approach. Bryant and Rhonda (1996) classified production problems and possible microbial solutions, and this classification is summarized in Table 2. To make quantitative predictions of the field performance of these solutions, it is necessary to quantify parameters such as the rate of microbial growth, the mass of the target product per unit mass substrate consumed and added, and the concentration and fate of reaction products. These results will then determine appropriate shut-in times after nutrient and/or microbe injection (Bryant and Lockhart 2002).

Gray et al. (2008) provided a methodology for the systematic assessment of MEOR proposals using reservoir engineering principles. They classified MEOR processes into two broad categories, namely, the alteration of oil/water/rock interfacial properties, and changes to the flow behavior. Their analysis only considered a reservoir-wide displacement mechanism that could be used to change crude oil recovery on a large scale. They identified a number of problems for commonly postulated MEOR processes, such as the adsorption of biological surfactants to reservoir minerals, and concluded that plugging of high-permeability zones of fractured reservoirs has the highest prospects for MEOR.

While both Bryant and Lockhart (2002) and Gray et al. (2008) were fairly skeptical about the prospects of using microbes for EOR from a reservoir engineering point of view, both papers conclude that new integrated studies incorporating both reservoir engineering and microbiological principles have the highest potential to gain new insights into the high-risk/high-return research domain MEOR.

Thrasher et al. (2010) provide a methodology for screening candidate reservoirs for MEOR applications and methods for upscaling core-scale results of MEOR from

Table 2 Production problems and suitable MEOR processes

Production problems	MEOR process	Helpful microbes
Formation damage, low oil relative permeability	Well stimulation	Generally surfactant, gas, acid, and alcohol producers
Trapped oil due to capillary forces	Waterflooding	Generally surfactant, gas, acid, and alcohol producers
Poor sweep efficiency, channeling	Permeability modification	Microorganisms that produce polymer and/or copious amounts of biomass
Paraffin problems, scaling	Wellbore clean-up	Microorganisms that produce emulsifiers, surfactants, and acids. Microorganisms that degrade hydrocarbons
Unfavorable mobility ratio, low sweep efficiency	Polymer flooding	Microorganisms that produce polymer
Water or gas coning	Mitigation of coning	Microorganisms that produce polymer and/or copious amounts of biomass

Modified from Bryant and Rhonda (1996)

the laboratory to the successful implementation in the field. Matrix bypass events between injector-producer well are seen as particularly difficult to mitigate by biomass plugging alone.

5 Research Needs

Although the basic concepts of MEOR are understood, the current state of knowledge lacks a detailed understanding of the scale and detail of the causes and effects of MEOR. Ambitious research programs are required to close these gaps. When this occurs, MEOR will have enormous potential to become a cost-effective, mainstream tertiary recovery method. Such a research program should integrate findings in microbiology, geochemistry, and rock and fluid properties, including quantitative assessments of the interplay of these factors. A clear definition of reservoir production issues is critical for any MEOR study.

New techniques and paradigms in molecular biology and microbiology offer an exciting opportunity to advance research in the MEOR domain. Although traditional culturing techniques have yielded valuable information about microbial interactions with hydrocarbons in the environment, typically less than 1% of microbial diversity can be cultured. Therefore, culture-independent methods such as polymerase chain reaction (PCR), reverse sample genome probing (RSGP), and fluorescence in situ hybridization (FISH) have become increasingly useful for studying microbial diversity. The drawback with these methods is that they typically approach a taxonomic classification rather than a more detailed understanding of functionality. In addition, the specific probes and primers required to apply these techniques provide "filtered" views of microbial communities.

With the dramatic decrease in the costs associated with DNA sequencing in the last decade, metagenomic approaches are now the gold standard, providing both taxonomic and functional information at the same time. A recent study of the microbial populations in oil reservoirs of the Alaska North Slope demonstrates the power of this approach (Hu et al. 2016) to identify taxa and impute relevant metabolic function. We expect in the next few years to see these methods applied to field trials. Together with verification in microcosm studies, this will lead to the development of effective protocols for the field implementation of MEOR based on a knowledge of the metabolic potential present in the reservoir.

Major difficulties exist in upscaling insights from laboratory experiments to reservoir scale. To better understand the fundamentals of MEOR, integrated studies using systematic laboratory and field experiments linked with extrapolations based on modelling will be more useful than trial and error style injection of microbe – nutrient mixtures. One of the critical questions is whether it is possible to manipulate the physiology of microbes for a particular purpose. More work is also needed on biofilms and their manipulation for biosurfactants and polymer formation. The need for a robust field methodology for characterizing MEOR-related effects on reservoir properties is paramount.

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Oil Recovery: Experiences and Economics of Microbially Enhanced Oil Recovery (MEOR)

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Abstract

Decades of laboratory research and field trials of MEOR for a range of reservoirs worldwide have shown some encouraging results. With more effective bacteria and inexpensive nutrients, MEOR has the potential to become an economically feasible and technically viable mainstream component of the EOR portfolio. MEOR potentially offers an environmentally sustainable and cost-effective technology that can reduce or eliminate the need to use harsh chemicals and energy-intensive EOR methods in recovering the vast amounts of oil that remain trapped in aging fields. For example, residual oil in depleted oil reservoirs could be gasified via methanogenic pathway by invoking microbes, an innovative MEOR option. Integrated research programs and rapidly advancing insights into the diversity of microbial life and biochemical cycles have the potential to deliver breakthrough knowledge for understanding the effects of microbes that thrive in petroleum reservoirs in production time scales. At present, MEOR is yet able to provide reliable benefits that the petroleum industry requires from EOR techniques. However, due to the relatively low costs and low environmental impact of this tertiary recovery method, the potential reward for developing and implementing MEOR based on new fundamental understandings in microbiotechnology and reservoir engineering may be enormous.

1 Introduction

Microbially Enhanced Oil Recovery (MEOR) involves a suite of techniques that utilizes microorganisms and their metabolic products to improve the recovery of crude oil from reservoirs (Jimoh 2012; Fig. 1). The concept of MEOR is among the

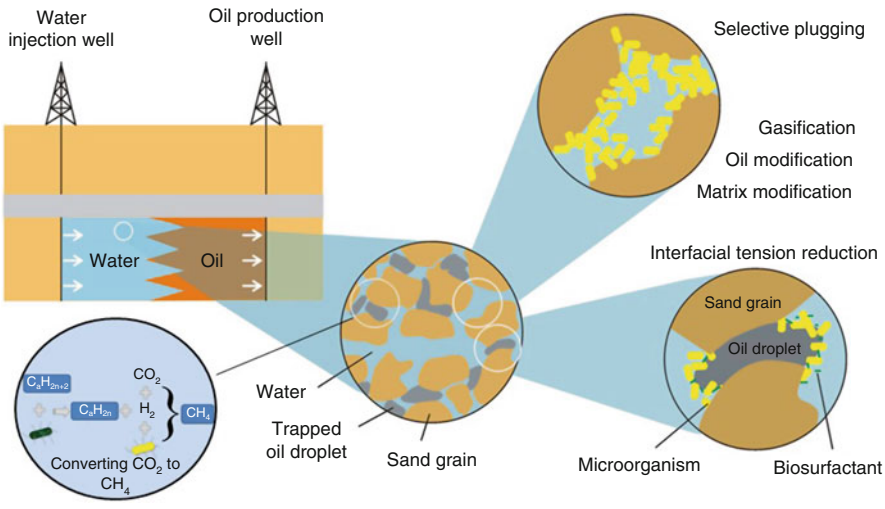

Fig. 1 Schematic illustration of microbial enhanced oil recovery processes

oldest of EOR strategies. Recently it has been extended to bio-gasification of residual oil in depleted reservoirs since the early twenty-first century (McIntosh et al. 2010). Residual oil in reservoirs can be bio-converted into methane to aid oil recovery. There are two pathways of bio-gasification in oil reservoirs. One is producing methane via acetic acid from residual oil (Larter et al. 2005; Gray et al. 2009), while the other is producing methane via CO_2 storage in depleted oil reservoirs using microbes (Fujiwara et al. 2006; Maeda et al. 2009; Wei et al. 2011). However, MEOR is not yet an accepted or routine procedure in the petroleum industry. One key reason may lie in our inadequate scientific understandings of the fundamentals and different approaches of MEOR including the processes and mechanisms, which were discussed in the previous chapter. However, any EOR strategy will need to be measured against practical experiences of the oil industry, and a careful evaluation of the risks, costs, and benefits is required.

Unlike conventional EOR methods, the MEOR approach targets more than one process simultaneously (Table 1), including (1) viscosity reduction and density modification by solvents, gases, and acids; (2) removal of paraffin wax by solvents and acids to enhance permeability; (3) removal of trace metal rims from pore throats by acids, solvents, alcohols, and biosurfactants to increase permeability; (4) oil-water and oil-rock Interfacial Tension (IFT) reduction by biosurfactants, solvents, and acids to improve oil flow; and (5) gasification of residual oil via methanogenic bacteria. This chapter provides a brief history of MEOR field trials, presents results from selected field applications of MEOR from the literature, and discusses the role of MEOR in the overall EOR portfolio. Special attention is given to the economics, potential contributions, and challenges of MEOR.

1.1 MEOR

The literature reports many successful MEOR field trials (Fig. 2), yet there is a high degree of skepticism in the industry as to the validity of some of the claims in the literature (Brown 2010). To a large degree, this skepticism is caused by the lack of detailed accounts of the published case studies. In addition, conditions may vary greatly from reservoir to reservoir. There have been suggestions of reservoir-specific customization of MEOR processes, but this has the potential to undermine the economic viability of MEOR.

Although the outcomes of MEOR are more difficult to predict than those of some of the conventional EOR methods (e.g., thermal EOR, gas injection, or chemical EOR using surfactants or polymers), the economic potential of MEOR could be huge considering that logistical costs of implementation may only approach the costs of conventional water flooding, especially for microbial stimulations using residual oil as the carbon source (Bryant and Lockhart 2002; Patel et al. 2015).

1.2 Reservoir Gasification via Microbes

Among all fossil fuel resources, only biogas can be potentially and sustainably regenerated. The development of a methane-producing system invoking indigenous

Table 1 Types of microbial processes for oil recovery

Process	Production problem	Type of activity or product needed
Production well stimulation to improve oil drainage into wellbores	Emulsion and paraffin, asphaltene	Demulsifiers, biosurfactants, solvents, acids, hydrocarbon degradation
Injection well stimulation to improve water injection rates	Buildup of residual oil, scale deposits	Biosurfactants, solvents, acids, hydrocarbon degradation
Injection well profile modification to improve injection efficiency and reduce high water production due to permeability variations	Water channeling into flushed high permeability zones, bypassing lower permeability oil-bearing zones	Biomass and polymers
Increase oil recovery to unlock oil bound by water/rock	High surface tension between oil and water, oil-wet rock	Solvents and biosurfactants
Reduce oil viscosity to allow oil to flow with less energy input	Slow flow rates	Biopolymers
Improve mobility ratio and sweep efficiency to allow more efficient displacement of oil by water	Thickened water issue	Biopolymers
Break up immovable residual oil into individual micro-droplets	Microbes are initially stimulated in situ with nutrient to allow multiplication and then migrate to mobilize residual oil in the process of searching for food source under a depleted nutrient condition	Microbial mobilization and altering the sweep efficiency
Sweeten produced fluids (reduce hydrogen sulfide)	Corrosion, iron sulfide scale, safety concerns	Stimulate denitrifying bacteria population

Modified after Mokhatab and Giangiacomo (2006), Zahnerc et al. (2010), and Patel et al. (2015)

microbes in depleted oil fields will benefit both the environment and the petroleum industry. One pathway for biogenic methane generation is via acetate acid in oil reservoirs with no CO_2 involved. Another bio-pathway for methane generation is through CO_2 reduction via some microbes such as *Methanobacterium sp*. and *Methanosaeta sp*., which are able to produce methane from CO_2 in oil reservoirs (Fig. 1). Therefore, CO_2 storage in depleted oil reservoirs could be recycled via the gasification pathway, which will benefit CO_2 Capture and Storage (CCS) projects. Normally, the targets of bio-gasification are depleted oil reservoirs, and the initial investment will eventually be paid back in a few decades due to the lag of the bioconversion process. Bioconversion of CO_2 in subsurface is related to the CCS-EOR, whose economic effectiveness is complicated but potentially high due to both the economical biological process and the implementation of a global carbon trading scheme.

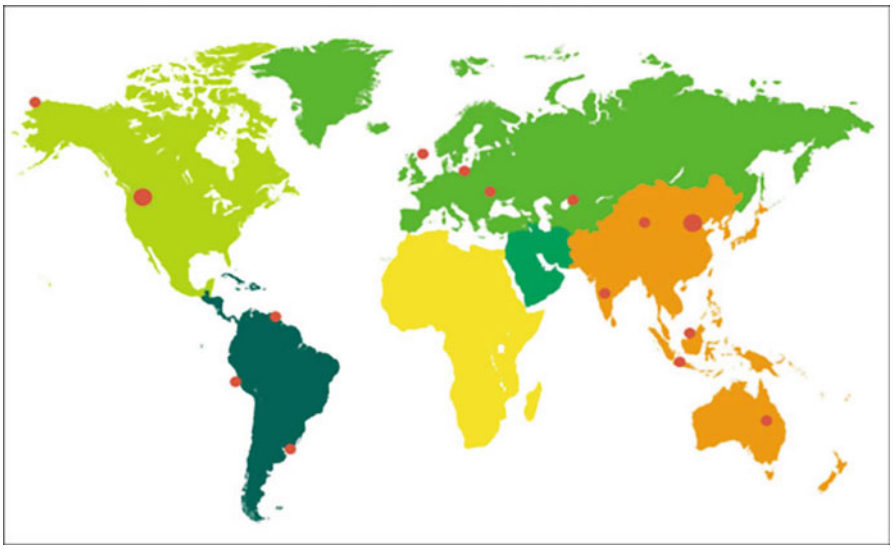

Fig. 2 Worldwide MEOR implementation. *Dots* sizes are meant to approximate number of field applications

2 History of MEOR Field Trials

Pioneering MEOR field studies were carried out in the USA in the 1930s and 1940s by Claude ZoBell with the group at the Scripps Institute of Oceanography in La Jolla, California (e.g., ZoBell 1946). A thorough review of early case studies is given in by Premuzic and Woodhead (1993) who outlined details and references for hundreds of field trials. In the 1960s and 1970s, many Eastern European countries such as Czechoslovakia, Hungary, and Poland carried out field MEOR trials, which were based on the injection of mixed anaerobic or facultative anaerobic bacteria selected on their ability to generate high quantities of gases, acids, solvents, polymers, surfactants, and cell biomass (Lazar et al. 2007; Patel et al. 2015). From the mid-1980s to the mid-1990s, there were many studies and field applications of MEOR, and a plethora of independent companies utilized various proprietary mixes of microbes and nutrients for wellbore injection. MEOR was demonstrated to work in the majority of reported cases, but it has also been shown to be ineffective in some other cases. Van Hamme et al. (2003) estimated that more than 400 MEOR field tests had been conducted in the USA alone, most of which were as single-well stimulation treatments on low-productivity wells onshore. It is also reported (Zahner et al. 2010) that MEOR was implemented in a full-field trial in southern California, USA, in 2010. An average of 6% incremental production has been achieved. Low oil prices in the late 1990s led to diminishing research in MEOR, especially for large-scale field studies designed to rigorously test performance. Several extensive trials of MEOR in Chinese oilfields (e.g., Daqing, Jilin, Xinjiang, Liaohe, Shengli, and

Dagang) were reported by Song et al. (2004). For example, application of *Pseudomonas aeruginosa* and its metabolic products in the Daqing oilfield is reported to have enhanced oil recovery by 11% by Li et al. (2002). Higher oil prices, more stringent environmental regulations including CO_2 emission for oil production, and advances in biotechnology have now spurred renewed interest in MEOR. The application of biotechnology in the oil industry has great potential, although it is still regarded as a "frontier" endeavor (Kotlar et al. 2004).

The application of gasification of residual oil by methanogenic bacteria has been studied in Canada (Jones et al. 2008) and Japan (Maeda et al. 2009). McIntosh et al. (2010) also traced the methanogenic bio-pathway from an oil well in the USA, which was injected with CO_2 for EOR in the 1980s and has shown that CO_2 was bio-converted into methane over a 30-year period.

3 Examples of MEOR Field Application

Although hundreds of MEOR field trails have been carried out worldwide, reliable data are sparse, as most of the applications are for single-well stimulations for low-productivity wells using unpublished proprietary mixtures of microbes and nutrients. The examples presented here were chosen to represent a variety of MEOR processes for addressing various reservoir engineering problems (Table 2). The full spectrum of MEOR processes are covered, ranging from biodegradation, viscosity and IFT reduction, and bio-gasification to bio-plugging using a variety of implementation mechanisms including single-production well stimulation ("huff-and-puff"), single-well water injection, and multi-well injection and production.

3.1 Oilfield in the USA

3.1.1 Bebee Field, Oklahoma, USA

In a study supported by the US Department of Energy, Youssef et al. (2007) tested whether two halotolerant *bacillus* strains (RS-1 and *Bacillus subtilis subsp. Spizizenii* strain NRRL B-23049), both of which produce lipopeptide biosurfactants, can metabolize and produce biosurfactants in the Bebee Field (Pontotoc County, Oklahoma) in sufficient quantity to stimulate MEOR. They applied huff-puff tests on five production wells that were produced from the Ordovician, Viola Limestone. Two wells received inoculums of the Bacillus strains and nutrients comprising glucose, sodium nitrate, and trace metals. Two wells received only nutrients, whereas one received only formation water in order to provide a base case control. The experimental conditions and procedures are thoroughly described in Youssef et al. (2007), including baselines for the abundance of microbial metabolites prior to field testing. Particular attention was paid to the 3-hydroxy fatty acid composition of lipopeptides described in Youssef et al. (2005), who concluded that the average concentration of lipopeptide biosurfactant in the produced fluids of the inoculated wells was approximately nine times greater than the minimum concentration

Table 2 Selected MEOR Field application examples

Country	Field	Description
Argentina	La Ventana, Tupungato-Refugio	Viscosity and IFT reduction
Australia	Alton	Single well stimulation; gas production; Interfacial tension (IFT) reduction
China	Daqing, Dagang, Jilin (Fuyu), Xinjiang, Liaohe	Hydrocarbon degradation, in situ stimulation, de-waxing, degradation, emulsion, IFT reduction, gas generation, single-well stimulation (huff-and-puff) and microbial injection, selective plugging of highly permeable zones, viscosity and IFT reduction, microbial injection, hydrocarbon degradation
India	Four fields by ONGC	Single-well stimulation, water-cut reduction
Indonesia	Ledok (offshore)	Single-well stimulation using indigenous
Malaysia	Bokor (offshore)	Single-well stimulation; profile control, demulsification, and plugging
Peru	Talara (offshore)	Multi-well injection, bio-cracking
UK	Beatrice, North Sea	Elimination of H2S
USA	Alaska, North Slope N Springfield, Indiana Oklahoma, Utah, various mature fields in Texas, Beverly Hills, Southern California, Gulf Coast Basin, Louisiana	Water-cut reduction, single-well stimulation, production profile control, water injection, plugging highly permeable zones, single-well stimulation, solvent production and de-waxing, single-well stimulation (huff-and-puff), viscosity and IFT reduction, altering the sweep efficiency of the injected water, methane bioconversion from CO_2
Venezuela	Lake Maracaibo	Multi-well injection and production, degradation, and IFT and viscosity reduction

required to mobilize tertiary oil from a low-permeability Berea sandstone core. Some by-products such as carbon dioxide, acetate, lactate, ethanol, and 2,3-butanediol were also detected in the inoculated wells. In contrast, only carbon dioxide (CO_2) and ethanol were detected in the nutrient-only control wells, with no elevated concentrations of lipopeptide biosurfactant.

Youssef et al. (2007) also presented microbiological and molecular data that showed recovery of the microorganisms injected into the formation, in the produced fluids of the inoculated wells. This indicates that exogenous microorganisms can be metabolically active in the presence of diverse, established populations of microorganisms that inhabited the reservoir before the field trial and thus demonstrates that biosurfactant-mediated oil recovery with a specific and selected microorganism is technically feasible. The volume of the soaked reservoir was small, and no significant increase in oil production was expected nor observed. Youssef et al. (2007)

concluded that this process would be economical at oil prices >$65. More definitive results, from which cost-effectiveness of this approach could be obtained, will require up-scaling of injections and trials on different reservoir – fluid combinations.

3.1.2 Beverly Hills, Southern California, USA

Zahner et al. (2010) injected nutrients in a mature waterflood well in Southern California, USA. A 30% oil production increase was observed in the offset producers with an overall production increase of about 6% of the total field production. The mature oilfield was discovered in 1952 and waterflooding commenced in 1967. By activating some species of microbes, the flow characteristics of the oil were altered. This resulted in the reservoir system to release additional oil to the active flow channels. The released oil, water, and microbes can interact to form a transient micro-emulsion which effectively altered the sweep efficiency of the injected water. The MEOR project was subsequently expanded to the full field by treating three water injection wells, each of which was treated for a total of nine times over a 7-month period. Two wells were further treated and the water cut was reduced after the treatment.

3.2 Oilfields in China

3.2.1 Dagang Oilfield, China

MEOR has been applied in the Dagang Oilfield for over 20 years (Feng et al. 2006). Field trials were conducted in four reservoir intervals in two blocks with 24 injection wells and 55 production wells. Both in situ stimulation of indigenous microbial species and injection of exogenous microbial were employed. Feng et al. (2006) reported that the in situ stimulation of indigenous microbes at 60 °C appeared to be more effective than injection of exogenous cultured microbes extracted from the formation fluids. A cumulative incremental oil production of 31,000 tons and 28,000 tons were achieved, respectively, during the two microbial treatments. The in situ stimulation had reduced average water cut by 55%. The major MEOR processes involved were biosurfactant production, emulsion, and gas generation. Nazina et al. (2007) studied the physicochemical conditions and microbiological characteristics of formation waters of the Kongdian reservoir of the Dagang Oilfield. Aerobic bacteria were detected mainly near the bottom of the injection wells, and these aerobic thermophilic bacteria were capable of oxidizing oil, with the formation of biomass and oxygenates such as volatile carboxylic acids and biosurfactants. They found that the reservoir is inhabited by microorganisms having great biotechnological potential.

3.2.2 Jilin Oilfield, China

In collaboration with the Jilin Oilfield Company, the Technology Research Center (TRC) of Japan conducted a field trial in the Fuyu Oilfield (Nagase et al. 2002). The reservoir has been produced through waterflooding for more than 20 years with water cut reaching 80–90% before the MEOR trial. The microbial strain, TRC-322,

Enterobacter, together with other microbes that produce CO_2, acid, and soluble polymers, was initially applied to the production wells. Microbes and nutrients were continuously injected after the huff-and-puff operation. Approximately 60% of the wells showed a reduction in water cut, but the effectiveness was limited. A different Enterobacter, CJF-002, which produces insoluble polymers, was injected into the oilfield using a huff-and-puff procedure at production wells and a microbial treatment at injection wells. Water cuts in the production wells were dramatically reduced shortly after microbial treatment at the injection wells. Oil production in the test area increased by more than 100% during the 7-month field trial, and the effect of plugging high permeable zones with bio-polymer lasted for over half a year. During these treatments, in situ growth of the microbes and metabolic activities were monitored and evaluated to identify and understand mechanisms of the microbial process. The study concluded that microbe screening and adjusting to particular environments based on laboratory investigations are keys to the successful field applications of MEOR.

3.2.3 Daqing Oilfield, China

The Daqing Oilfield in the Songliao Basin is the biggest oilfield in China and has produced oil since 1960. However, rapidly decreasing production rates have spawned many EOR field trials including MEOR. Nazina et al. (2003) claimed that the oilfield is inhabited by aerobic saprotrophic (including hydrocarbon oxidizing) bacteria that are able to produce oil-releasing metabolites (e.g., surfactants and exopolysaccharides) during growth on a wide range of substrates including hydrocarbons, lower alcohols, volatile fatty acids, and sugars. Zhang et al. (1999) reported incremental MEOR in 17 of the 25 treated wells, with a cumulative incremental production of over 3,000 tons. It was also suggested that an increasing number of live microbes in the produced waters show the potential to change the course of MEOR. However, there are some concerns about microbial viability after injection in the field. The concentration of live microbes before and after MEOR for five treatments on four wells using five-tube dilution for the Most Probable Number (MPN) method was studied, indicating a 1,000-fold increase in the abundance of microbes compared with the background concentration during MEOR stimulations. Zhang et al. (1999) reported that the main MEOR process occurring in the Daqing Oilfields is degradation of long-chain alkanes and reduction of viscosity.

3.3 Beatrice Oilfield, North Sea

The Beatrice Oilfield is located in the UK sector of the North Sea. The Titan Process (previously termed "BOS" for "Biological Oil Stimulation") was applied to the field in early 1992. In total, 11 cycles of treatment were preformed over a 3.5-year period. The field was on a well-established decline rate and was scheduled for abandonment in 1996. The field continues to produce to 2008. Some overlapping changes in production were implemented, which have complicated evaluation of MEOR such that quantifying its success is difficult; in addition, information on details on MEOR

other than microbiology is scarce. The Titan website (www.titanoilrecovery.com/northsea-results.html) has considered the trial to be successful, with a 10% increase in oil production coupled with elimination of hydrogen sulfide (H_2S) for two wells treated using single-well stimulation procedures.

3.4 Talara Offshore Oilfields, Northwest Peru

Maure et al. (2005) reported an MEOR field application on seven producing wells in the Providencia and Lobitos fields of Block Z-2 in the Talara Basin, Northwest Peru. The oils are predominantly paraffinic and therefore were targeted as promising candidates for biotreatment. This MEOR application demonstrates that biocracking can lead to systematically altered n-alkane profiles of the oils. Maure et al. (2005) presented information on applying enzyme biochemistry to accelerate redox reactions involving linear hydrocarbons, without providing biochemical detail. They claimed that by alternating aerobic/facultative hemicycles with strictly anaerobic cycles, the time-coordinated action of selected groups of microorganisms in oxidant and reductive environments results in substantial changes in oil composition. They also emphasized the importance of evaluating propensities of oils to act as carbon sources for nourishing microbial communities. It was further reported by Segovia et al. (2009) that MEOR was applied in the coast and the southern areas (Block-X) of the Talara Basin, Northwest Peru. An increment of 30% in oil production was achieved in two wells selected for the MEOR pilot project in a marginal oilfield. The investigation showed that the MEOR implementation was intimately linked to the paraffin content in the oil and its bio-treatability. An integral analytical methodology to precise ranking of the MEOR portfolio has been provided with the application of MEOR, which will extend the life of mature fields in in the Peruvian northwest area.

3.5 Reservoir Gasification Field Application

Currently, researches on bio-gasification have been focused on depleted oil reservoirs, oil shale, and coal mines. Although field applications were few, McIntosh et al. (2010) described the storage of CO_2 in a depleted oil reservoir in north-central Louisiana, USA, which had been used for CO_2-EOR in the 1980s, and the bioconversion of CO_2 into methane after almost 30 years. The natural gas and oil produced from the Paleocene to Eocene Wilcox Group coal beds and adjacent sandstones were investigated. It has been shown that microbial methane was generated in situ with the Late Pleistocene or younger aquifer waters. Depletion of the n-alkanes in the saturated hydrocarbons in the study area provides strong evidence for microbial degradation of the oils. Furthermore, the geological background also demonstrated that the oil may have migrated vertically into the shallow low-rank coals from the underlying Cretaceous source rocks via fracture systems. The methane accumulations in the Wilcox coal beds and sandstones are predominantly of microbial in

origin and were generated via CO_2 reduction. The production history of the Wilcox Group sandstone reservoirs, which were subjected to CO_2 flooding in the 1980s for enhanced oil recovery, leads to an intriguing hypothesis that CO_2 sequestration may actually enhance methanogenesis in organic-rich formations.

4 The Role of MEOR in the EOR Portfolio

4.1 Current Trends and Future Projection

At present, MEOR only forms a minute component of the overall EOR portfolio (Thomas 2008) which also includes (1) thermal processes, (2) gas injection (both miscible and immiscible), (3) chemical flooding such as polymer, surfactant, alkaline, or a combination (e.g., alkaline-surfactant-polymer/ASP, emulsion, and micellar treatment), and (4) foam flooding (Fig. 3). As an emerging alternative method, MEOR is currently largely confined to laboratory investigations and small-scale field applications, although single-well stimulations and borehole cleanups have been carried out widely in the petroleum industry, in particular for onshore US and Chinese wells. Khire and Khan (1994a, b) reported that MEOR has been applied to over 400 wells in the USA. Over 1,000 wells in a dozen oilfields in China (Chinese Ministry of Land and Resources; www.mlr.gov.cn) were also treated. Most major oil companies, including Shell, BP, Chevron, PetroChina, PETROBRAS, and PETRONAS are currently including MEOR as an option in their overall EOR portfolio. Much of the nearly 2,000 billion barrels of conventional

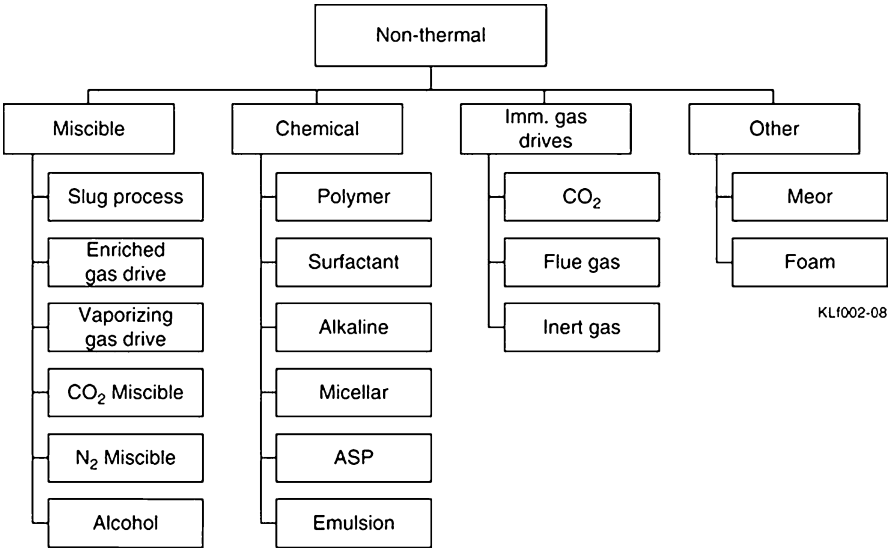

Fig. 3 Types of non-thermal EOR methods currently used by the petroleum industry

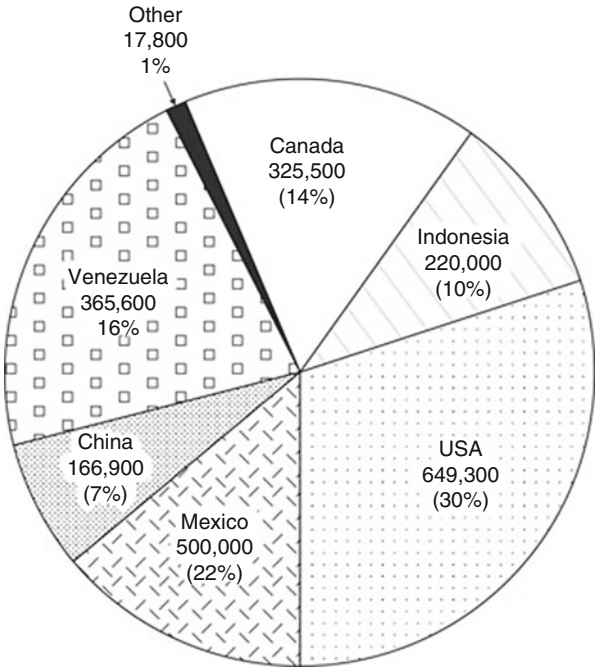

Fig. 4 Estimated worldwide EOR production barrels per day in 2006 with a total EOR production of 2.5 MMBL/D (Modified from Thomas 2008)

oil and about 5,000 billion barrels of heavy oil that have remained in reservoirs worldwide after conventional recovery may be recovered by EOR, including MEOR (Thomas 2008). The estimated worldwide EOR production in 2007 was about 2.5 million barrels per day (Thomas 2008), of which MEOR accounted for a negligible amount. However, a recent study by the Chinese Ministry of Land and Resources (www.mlr.gov.cn) indicates that about 50 billion barrels of oil in onshore Chinese oilfields may be suitable for MEOR. A portion of captured CO_2 will be stored in depleted oil reservoir or be used for CO_2-flooding. Therefore, the potential of methane production from bio-gasification will definitely be attractive when considering the CCS project blue plan. Currently the most EOR is carried out in the USA, Mexico, Venezuela, Canada, Indonesia, and China (Fig. 4), and these countries account for at least 99% of the world's EOR production. Among the EOR portfolio, thermal EOR accounts for the highest percentage (Fig. 5), followed by gas injection and chemical flooding, which is primarily used in some Chinese oil fields amounting for 200,000 barrels per day in 2006 (Thomas 2008).

4.2 Economics

While EOR allows the production of residual oil in place that otherwise could not be recovered and can help maintain production rates in mature fields, the main issues of conventional methods are cost-effectiveness and environment impact. EOR (tertiary

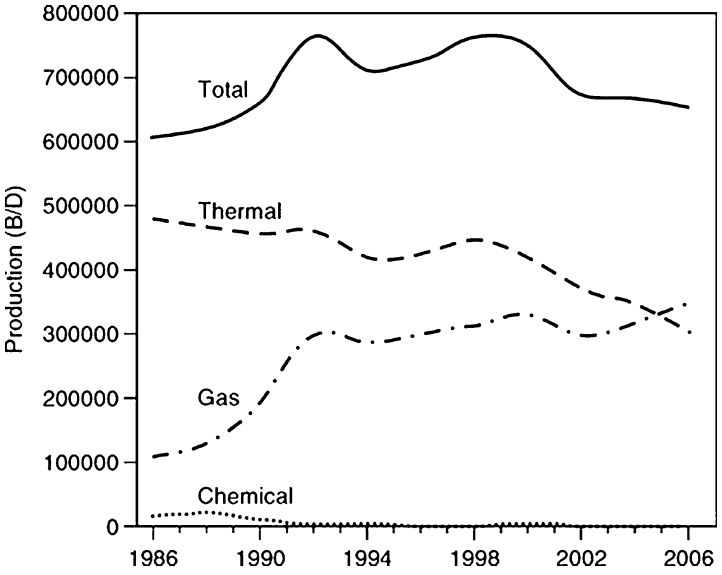

Fig. 5 US EOR application by type (Modified after Thomas 2008)

recovery) is much more costly than primary and secondary recovery. In the 1990s, when the oil price was about US$ 20 per barrel (Fig. 6), the cost for various EOR processes varied from $10 per barrel to over $50 per barrel (Fig. 7; Lake et al. 1992). Among the EOR methods, surfactant flooding is by far the most expensive ($20–$52 per incremental barrel in 1990), although it is the most effective, attaining recoveries of up to 75% of original oil in place (OOIP; Fig. 7). Although CO_2 injection can also be expensive, which depends on the CO_2 availability and locations of injection, the potential of methane producing via bioconversion will probably cover part of the investment. Thermal EOR can be costly and extremely energy intensive; it requires costly surface facilities, and incremental production increases costed $10–25 per barrel in 1990 (Fig. 7). Polymer flooding is one of the most cost-effective options among conventional EOR methods. Apart from the high cost of conventional EOR methods, environmental impact should also be considered in economic terms. For example, chemical flooding such as ASP can cause extensive scaling on drill pipes and damages to the production facility, which are often irreversible (Cao et al. 2007). Chemical flooding may also contaminate formation waters as well as surface environments. Thermal EOR is extremely energy intensive and can lead to considerable greenhouse gas emissions, which may have significant economic ramifications, especially in countries engaging in carbon trading schemes. MEOR may offer a viable EOR alternative both in terms of costs and environmental considerations. Giangiacomo (1997) evaluated the economics of conventional chemical and microbial treatments for paraffin based on an oil price of US$ 15/bbl. He showed that the ratio of the benefit to cost was approximately 1.03 for chemical flooding, but 1.48 for microbial treatment for every EOR dollar. The adoption of indigenous microbial

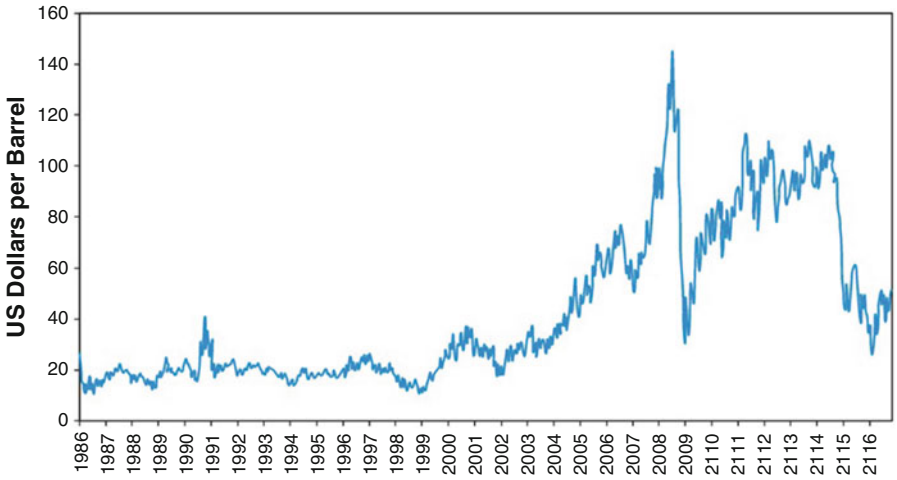

Fig. 6 World crude oil price from 1986 to 2016 (Source from US Department of Energy)

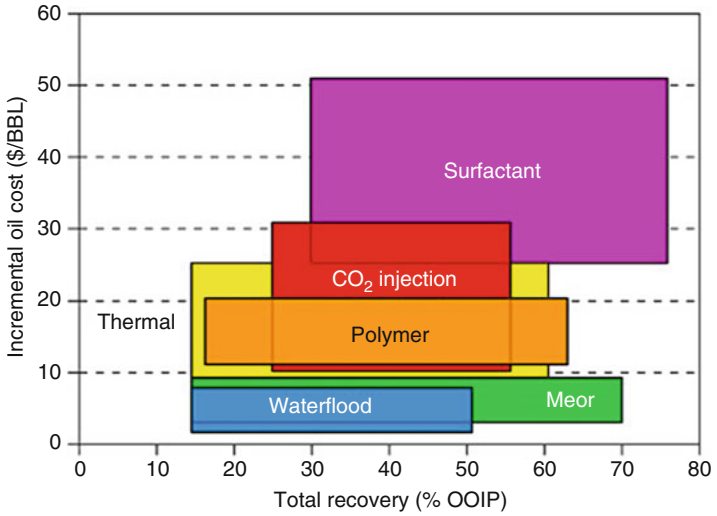

Fig. 7 Comparison of the cost of MEOR with other EOR techniques (Modified from Lake et al. 1992)

flooding is likely to be the most economically viable MEOR method, because it does not require facilities for culturing microbes. However, identification of extant microbial biomes and selection of appropriate nutrients and injection techniques to stimulate microbes are critical for these techniques.

In the selected field application examples mentioned above and listed in Table 2, increases for recovery in most cases were reported to be from 6% to 8% (Dagang Field, China; Feng et al. 2006) to 66% (La Ventana Field, Argentina, Maure et al. 2001) and in some cases over 100% (e.g., Fuyu Field, China; Nagases et al. 2002). In

addition to the increased oil production, decreased water production, increased gas to oil ratios, and improved injectivity are applied for some cases. In some onshore US oil fields, a single-well stimulation treatment could double the production rate for most wells, and this could be sustained for 2–6 months without additional treatments (Khire and Khan 1994a, b). For a recent application in Peru, the MEOR cost per barrel ranged from $1.30 to $7.92 (Maure et al. 2005). The investment/return ratio has been up to 1:9 at an oil price of US$40 per bbl for field trials in the Daqing, Dagang, and Shengli oilfields (Chinese Ministry of Land and Resources, http://www.mlr.gov.cn). The economic return ratio from reservoir bio-gasification is primarily controlled by the methane conversion rate. Methane generation from in situ bioconversion will be a new emerging MEOR-related technique to extend the life of depleted oil reservoirs by optimizing the CO_2 injection cost, methane recovery, and CO_2 tax credit. Therefore, a favorable CO_2 emission policy and/or an emission trading scheme will be a prerequisite for a viable in situ bio-gasification. With the post-2007 high oil prices (Fig. 6), the potential return on investment from MEOR during the high oil price period is much more attractive. Although the oil price fell subsequently since 2014, it appears that the oil price started to increase again at the end of 2016 with a price around $50 per barrel. Microbial activity can also be used for treating oil spills in onshore and marine environments around drilling rigs and has been routinely used in borehole cleaning and bioremediation. Some microbes are able to repair reservoir formations damaged by conventional EOR (e.g., polymer injection) processes (Feng et al. 2006).

4.3 Challenges and Potential

Despite numerous MEOR tests, considerable uncertainty remains, in particular regarding process performance. The major risk of MEOR at present is its reliability and reproducibility. While most reported cases indicate improvement in oil recovery, there are some cases where MEOR was reported to be ineffective. The biggest challenge in MEOR field application is in increasing its robustness with predictable outcomes. Unlike other EOR methods, MEOR may be limited by environment-specific factors of the reservoirs such as the reservoir type, reservoir temperature, formation water salinity, and prevailing indigenous microbial communities. The most serious challenge, however, is in ascertaining if the formation of microbial metabolite and biomass is sufficient to cause EOR effects in reasonable time frames. Although, at present, MEOR is still perceived by the majority of oil producers as a low cost but unreliable EOR strategy, it has huge potential in offering an environmentally low-impact, cost-effective, and energy-efficient approach in the future.

5 Research Needs

To enable a technically robust MEOR application with predictable outcomes, the areas that require further investigation are the following:

- Identification and development of microbial strains that can thrive in the hostile environments of petroleum reservoirs (e.g., at elevated temperatures or high salinities or both)
- Determining the rates of biochemical processes to ensure sufficient production of microbial biomass and metabolites
- Establishment of common baselines prior to successful MEOR strategies
- Development of rapid and innovative microbial screening methods, including sequencing and gene mapping techniques, leading to a gene collection database, and development of more advanced and robust MEOR simulation and prediction models by combining laboratory experiments, field trials, and numerical models
- Accelerating the methane bioconversion in depleted oil reservoirs and call for backup of tax credit for CO_2 capture storage

MEOR will therefore provide a rich field for fundamental and applied research for many years to come.

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Use of Biosurfactants in Oil Recovery

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Guoqiang Li and Michael J. McInerney

Contents

Abstract

Biosurfactant-mediated oil recovery has the potential to recover large amounts of crude oil that remain entrapped in oil reservoirs after current oil recovery technologies reach their economic limit. Lipopeptides (surfactins and lichenysins), rhamnolipids, and other glycolipids generate the low interfacial tensions and the appropriate rock wettabilities needed to mobilize entrapped oil. Biosurfactants are active over a wide range of temperatures, pH values, and salinities found in many oil reservoirs and are effective at low concentrations. A number of laboratory experiments show that biosurfactant-mediated oil recovery is effective in recovering large amounts of entrapped oil. Several field trials show that in situ

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biosurfactant production is possible and recovers additional oil. Biosurfactant-mediated oil recovery has been difficult to scale-up to a reservoir-wide technology due to the lack of understanding of how best to stimulate biosurfactant production in the reservoir. In addition, the relationship between biosurfactant concentration and oil recovery is still unclear. Ex-situ biosurfactant-mediated oil recovery where the biosurfactant is added to the injection fluids has not been implemented on a large scale, most likely due to the high production costs of biosurfactants. Multidisciplinary approaches are needed to move biosurfactant-mediated oil recovery from the laboratory to the reservoir.

1 Introduction

World economic growth will continue to be strong in the upcoming decades, and thus, the demand for energy will also be strong (Doman 2016). Total world energy consumption will rise from 580 ExaJoules (EJ) in 2012 to 860 EJ in 2040. An important question is how will we meet the future demand for more energy. Although renewable energy and nuclear power are predicted to be the world's fastest-growing energy sources, it is likely that liquid fuels – mainly petroleum – will remain the largest source of world energy (Doman 2016). Most of the growth in liquid fuel consumption will be in the transportation and industrial sectors where liquid fuels will continue to provide most of the energy consumed (Doman 2016). The demand for liquid fuels by the transportation sector is expected to increase by 62% by 2040. Thus, finding and producing sufficient amounts of petroleum in the future will be critical to sustaining world economic growth.

Petroleum is a non-renewable fossil resource derived from organic matter deposited eons ago in the lithosphere. When a well is drilled into an oil reservoir, oil and water are pushed to the surface by the natural pressure within the reservoir. As reservoir pressure dissipates, pumps are placed on the well to assist in bringing the fluids to the surface. This stage of oil production is called primary production (Youssef et al. 2009). Eventually, additional pressure must be added to the reservoir to continue to recover oil. Often surface water, seawater, or brine from a subterranean formation is injected into the reservoir to push the oil to production wells. This stage of oil production is called waterflooding or secondary oil production (Youssef et al. 2009). When the above exploration strategies reach their economic limits, only about one-third to one-half of the oil originally in place in the reservoir has been extracted, leaving behind a large amount of oil (known as residual oil) in the reservoir (Hall et al. 2003). The amount of residual oil in reservoirs worldwide ranges from 2 to 4 trillion barrels (0.3–0.6 Tm^3). Thus, there is a large resource of petroleum that could potentially supply future energy needs if technologies can be developed to recover entrapped oil.

Technologies to recover residual oil are called enhanced oil recovery (EOR) technologies. EOR includes three primary techniques: thermal recovery (hot water, steam, combustion), gas injection (N_2, CO_2, flue gas), and chemical injection (surfactants, polymers, solvents) (Alvarado and Manrique 2010; Sheng 2010).

Over one-half of the EOR-recovered crude oil in the USA is the result of gas injection with CO_2 injection being the most important. The remainder is the result of thermal oil recovery technologies. Chemical-based EOR technologies have been marginally economic due to high chemical costs (Alvarado and Manrique 2010; Sheng 2010). Another EOR approach is to use microbial technologies to enhance oil recovery (MEOR). Numerous laboratory and field studies have shown that microorganisms produce useful products such as biosurfactants that allow recovery of residual oil (Youssef et al. 2009). Microorganisms can produce these products from inexpensive and renewable nutrients injected into the reservoir. Thus, MEOR technologies have an economic advantage in that they do not consume large amounts of energy as do thermal processes, nor do they depend on expensive chemicals as many chemical processes do (Youssef et al. 2009). In addition, MEOR provides an ecofriendly approach to oil recovery compared to chemical EOR as the products of microbial metabolism are readily degradable (de Cássia et al. 2014).

MEOR has been investigated extensively in the laboratory and in the field and a number of excellent reviews are available (McInerney et al. 2009; Youssef et al. 2009; Harner et al. 2011; Shibulal et al. 2014; Siegert et al. 2014; Patel et al. 2015). In this chapter, we will discuss the use of biosurfactants for oil recovery. A recent book on biosurfactants provides an excellent overall resource (Sen 2010).

2 How Can Biosurfactants Help Mobilize Oil?

Over 80% of the production oil wells in the USA have low production rates (less than 1.6 m^3 of crude oil per day) and are at risk of abandonment due to their marginal economic returns (Youssef et al. 2009). However, oil production from marginal wells accounts for about 19% of USA domestic production. Thus, maintaining production in marginal wells will be important to meet future energy needs. To keep marginal wells economic, one must slow the rate of oil production decline or increase the rate of oil production in a cost competitive manner. Mobile oil may be present a short distance from the production well but cannot flow to the well because drainage channels have been blocked by particulates such as paraffin deposits or by areas of high water saturation (Youssef et al. 2009) (Fig. 1a). Removal of paraffin deposits and/or changing wettability in the near wellbore region through biosurfactant production would reconnect regions of high oil saturation, providing channels for the oil to drain into the well (Fig. 1a). There are a number of commercial microbial technologies where hydrocarbon-degrading bacteria and nutrients are injected into production wells to degrade paraffins and/or produce biosurfactants, which could change near wellbore conditions to allow better oil drainage. In fact, many commercial microbial paraffin removal technologies have been shown to slow the rate of decline in oil production and extend the operational life of marginal oil fields (Youssef et al. 2009). Injection of biosurfactants or their in situ biosurfactant production may also be effective in changing wettability in the near wellbore region (Al-Sulaimani et al. 2012). In fact, the injection of two biosurfactant-producing

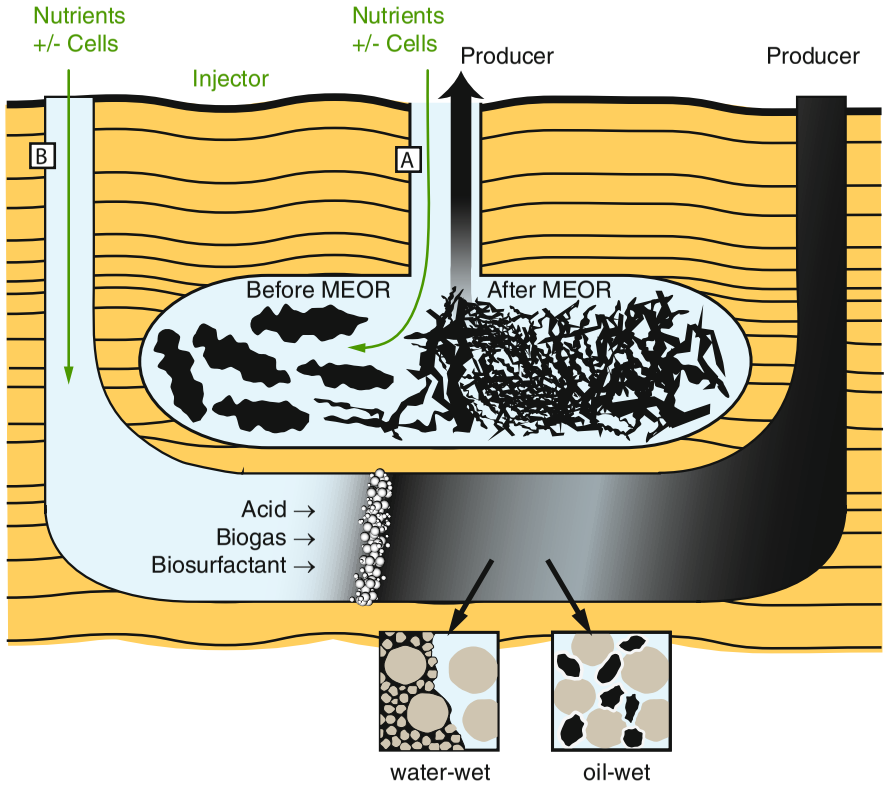

Fig. 1 Biosurfactant-mediated oil recovery. (**a**) The change in wettability by biosurfactants near the production well reconnects oil ganglia and increases oil drainage. (**b**) Biosurfactant production during waterflooding mobilizes entrapped oil. Insets: After waterflooding, large globules of oil exist large pores in water-wet regions and oil is found in small pores or in large pockets surrounded by water in oil-wet regions

microorganisms and nutrients into two production wells improved oil production (Youssef et al. 2013).

With larger, more productive oil fields, increasing the ultimate recovery factor is an important consideration. Large amounts of oil remain entrapped by capillary forces in reservoirs after water flooding. In water-wet regions of the reservoir, oil will be found as spherical globules in the center of the large pores and as ganglia of oil spanning multiple pores surrounded by water (Armstrong and Wildenschild 2012a) (Fig. 1b). In strongly oil-wet regions of the reservoir, oil will be found in small pores and in large pockets of oil surrounded by water. In both cases, mobilization of the entrapped oil will require an increase in viscous forces and/or a reduction of capillary forces in the reservoir. The viscous and capillary forces that entrap oil are expressed as a dimensionless ratio called capillary number ($N_{ca)}$ (Eq. 1):

$$N_{ca} = (\mu_w \bullet V_w)/(\gamma) \tag{1}$$

where μ_w is the viscosity of the recovery fluid (aqueous phase), V_w is the velocity of the recovery fluid (aqueous phase), and γ is the interfacial tension (IFT) between the oil and aqueous phases (Gray et al. 2008). Capillary numbers for a mature waterflooded reservoir are in the order of 10^{-7}–10^{-6} (Gray et al. 2008). Capillary number needs to be increased 100- to 1,000-fold in order to mobilize substantial amounts of entrapped oil. Typically, capillary number is increased by adding a polymer to increase the viscosity of the recovery fluid and/or by adding a surfactant or biosurfactant to reduce the interfacial tension between the oil and aqueous phases. Biosurfactant activity will mobilize entrapped oil in water-wet pores and will allow oil to drain from oil-wet regions (Fig. 1b) (Armstrong and Wildenschild 2012a, b). Many microorganisms produce biopolymers, which will increase capillary number by increasing the viscous forces. The combination of biopolymer and biosurfactant production could increase capillary number sufficiently for substantial oil recovery (Fernandes et al. 2016).

Armstrong and Wildenschild (2012a, b) used X-ray computed microtomography (CMT) to understand the mechanisms of MEOR operative at the pore-scale. Analysis of CMT images showed that biosurfactant-mediated MEOR altered the oil morphology, gave more oil-wet curvatures, and decreased the interfacial curvatures. As a consequence, large oil recoveries ranging from 44% to 80% were observed as a result of wettability and IFT changes (Armstrong and Wildenschild 2012a, b). Sarafzadeh et al. (2013) also found interfacial tension reduction and wettability alteration by biosurfactants important for oil recovery from carbonate cores. The change in capillary number due to interfacial tension reduction by the biosurfactant explained the observed oil recoveries. However, much lower residual oil saturations than predicted by changes in capillary number alone were observed, when both cells and the biosurfactant were used (Armstrong and Wildenschild 2012b). Thus, the clogging of pores with cells, which altered flow patterns, has a significant effect on oil recovery beyond that predicted by capillary number (Armstrong and Wildenschild 2012a, b).

3 Types of Biosurfactants

Diverse microorganisms produce surface-active agents (Youssef et al. 2009; Sen 2010; Santos et al. 2016). Biosurfactants are classified into five major categories based on their chemical structures: lipopeptides, glycolipids, phospholipids, neutral lipids, and fatty acids (de Cássia et al. 2014; Santos et al. 2016). The most common biosurfactants used in MEOR are lipopeptides (surfactin and lichenysin) and glycolipids (rhamnolipids, sophorolipids and trehalolipids) (McInerney et al. 2009; Youssef et al. 2009; Liu et al. 2015; Santos et al. 2016) (Fig. 2). The interfacial tension between oil and aqueous phases varies from 20 to 40 mN/m (Gray et al. 2008). A number of biosurfactants reduce oil-water interfacial tension to <1 mN/m (Table 1), which provides a 100-fold or greater increase in capillary number needed for substantial oil recovery. Some biosurfactant producers are also able to produce biopolymers that increase the viscosity of the aqueous phase, which further increases capillary number and oil recovery (Fernandes et al. 2016).

Many biosurfactants, in particular, surfactins and lichenysins, have low critical micelle concentrations (CMCs), 10–30 mg/L (Table 1). CMC is the concentration at which the biosurfactants form micelles and is the minimum concentration needed to mobilize entrapped oil (Youssef et al. 2009; Sen 2010). Many synthetic surfactants have higher CMC (>100 mg/L) than biosurfactants (Youssef et al. 2007a). Thus, low biosurfactant concentrations can be effective in mobilizing entrapped oil. In fact, microbial cultures where the biosurfactant concentration is at or slightly above the CMC recover large amounts of entrapped oil (Table 1).

Commercial of biosurfactant production is costly due to the low productivity of many biosurfactant-producing strains (Table 1), the use of expensive media components, and high downstream processing costs (Helmy et al. 2011; Banat et al. 2014; Geys et al. 2014).The use of low cost agro-industrial by-products such as whey, molasses, waste oils helps reduce nutrient costs (Banat et al. 2010; Makkar et al. 2011); however, complex substrates may have undesirable components that inhibit production or make downstream processing difficult. A number of investigators have used statistical approaches such as surface response methodology to optimize nutrient composition and operating conditions to improve biosurfactant productivity (Banat et al. 2010; Liu et al. 2005). Rotating disk, biofilm reactors (Chitoui et al. 2012), bubble less, membrane-aerated bioreactors (Coutte et al. 2010), and three-phase, inverse fluidized bed reactors (Nikolov et al. 2000) have been developed to provide adequate aeration without foaming and solid-state fermentation, where the biosurfactant producer is grown on a solid surface such as rice straw, reduces capital costs (Zhu et al. 2013). The combination of ultrafiltration with adsorption and ion exchange chromatography increased the recovery of biosurfactants from fermentation broths (Chen et al. 2008). It should be noted that there are some biosurfactant producers that produce very high concentrations of biosurfactants (Geys et al. 2014). For example, *Pseudomonas aeruginosa* produces 70–120 g/L of rhamnolipids when cultivated on vegetable oil (Giani et al. 1996) and *Starmerella bombicola*, the best

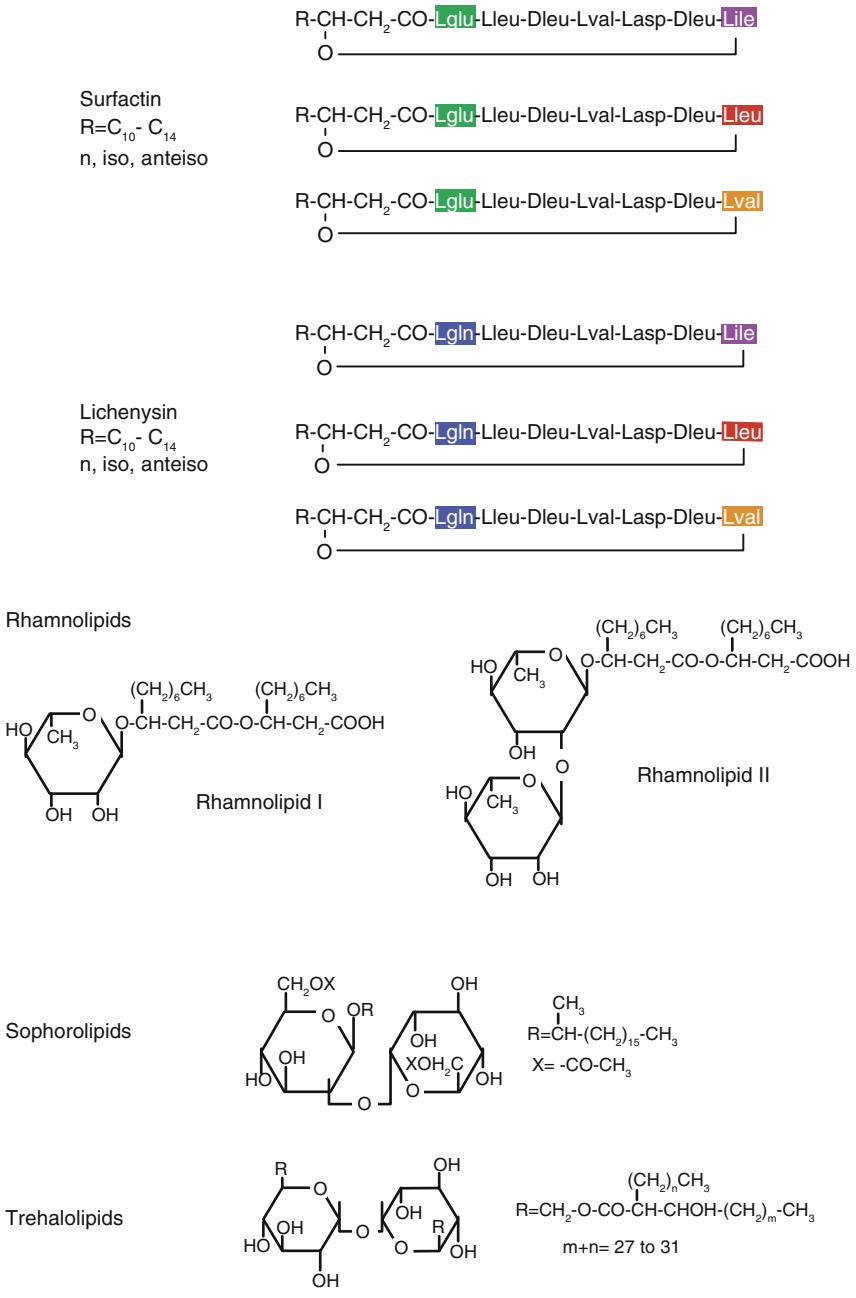

Fig. 2 Structures of lipopeptide, rhamnolipid, sophorolipid, and trehalolipid biosurfactants. Boxes highlight variations in amino acid sequence of lipopeptides

Table 1 Efficacy of biosurfactants commonly used for microbial oil recovery[a]

Biosurfactant	Microorganism	Lowest surface tension (mM/m)	Lowest interfacial tension (mN/m)	Critical micelle concentration (mg/L)	Additional oil recovery (%)	Yield (g/L)	Reference
Surfactin	*Bacillus subtilis* or *B. mojavensis*	28–30	0.006–0.3	10–35	40–80	0.5–1	Lin et al. (1994) and Youssef et al. (2007a)
Lichenysins	*Bacillus licheniformis*	28	0.3–0.5	10–19	37	1.1	Joshi et al. (2015) and Yakimov et al. (1999)
Lipopeptide	*Acinetobacter baylyi*	35	15	90	28		Zou et al. (2014)
Rhamnolipid	*Pseudomonas aeruginosa*	25–27	0.2–2	11–120	10–27	0.7–50	Amani et al. (2013) and Xia et al. (2012)
Glycolipids	*Rhodococcus* sp.	27–30	1	57	65–86	0.5–12.9	(Shavandia et al. (2011) and Zheng et al. (2012)
Glycolipids	*Enterobacter cloacae* and *E. hormaechei*	31	0.6–3.2		27–48	1.5–1.7	Darvishi et al. (2011), Rabiei et al. (2013), and Sarafzadeh et al. (2013)
Lipopolysacharide	*Alcaligenes faecalis*	20	<1		9	1.2 ± 0.05	Salehizadeh and Mohammadizad (2009)
Sucrose lipid	*Serratia marcescens*				90		Pruthi and Cameotra (2000)
Sophorolipid	*Candida bombicola*	33 ± 0.05	1.6 ± 0.3		27		Elshafie et al. (2015)

[a]The values differ depending on the strains, growth conditions, oils and porous media used in different experiments

studied sophorolipid producer, and produces 400 g/L sophorolipid when grown in a two-stage cultivation process (Daniel et al. 1998).

4 Strategies for Biosurfactant-Mediated Oil Recovery

Oil recovery occurs by the activity of microorganisms and/or their metabolites, such as biosurfactants, biomass, biopolymers, solvents, acids, gases, etc., which can be generated ex situ or in situ (Youssef et al. 2009). In ex situ MEOR approaches, microbes are cultivated in a fermentor on inexpensive nutrients and the microbes and/or their metabolites are injected into oil reservoir. In situ approaches involve the growth and metabolism of the indigenous or injected microbes in the reservoir to produce cells, metabolites, or a particular activity such as hydrocarbon degradation. Thus, there are three main strategies for using biosurfactants for oil recovery (Banat et al. 2000):

1. Production of biosurfactants in batch or continuous culture under industrial conditions, followed by their addition to the reservoir
2. Production of biosurfactant-producing microorganisms in batch or continuous culture under industrial conditions, followed by the injection of cells and nutrients into the reservoir
3. Injection of nutrients into a reservoir to stimulate the growth of indigenous biosurfactant-producing microorganisms

4.1 Injection of Ex Situ-Produced Biosurfactants

In addition to generating low interfacial tensions, biosurfactants must maintain activity under the environmental conditions present in oil reservoirs (Siegert et al. 2014). A number of studies have shown that lipopeptide biosurfactants and rhamnolipids are effective over a wide range of environmental conditions such as temperatures up to 80 °C, NaCl concentrations up to 15% and pH values from 5 to 10 (Youssef et al. 2009; Amani et al. 2013; Al-Wahaibi et al. 2014; Joshi et al. 2015).

Although many biosurfactants exhibit extraordinary interfacial properties, commercialization of biosurfactant-mediated oil recovery remains difficult and costly (Banat et al. 2014). The maximum concentrations produced during cultivation tend to be low (<2 g/L) (Table 1) although higher concentrations have been reported (Joshi et al. 2008; Xia et al. 2012). To our knowledge, there are still not any reports of ex situ field trial applications of biosurfactants. A promising approach is the use biosurfactants in conjunction with synthetic surfactants to reduce the amount of synthetic surfactants needed, providing cost savings (Youssef et al. 2007a; Al-Sulaimani et al. 2012).

4.2 Injection of Biosurfactant-Producing Microorganisms and Nutrients

If the biosurfactant-producing microorganisms or their activities are absent, then inoculation of the reservoir with exogenous biosurfactant-producing microorganism is needed. The use of large concentrations of exogenous microorganisms may also be an effective way to establish the appropriate activity quickly in the reservoir. The foremost consideration would be whether the exogenous biosurfactant-producing microorganism would grow under the environmental conditions present in the reservoir presence of competing indigenous population. However, many known biosurfactant-producing microorganisms grow under the environmental conditions present in many oil reservoirs (Youssef et al. 2009).

Another important critical factor is the transport abilities of the exogenous microorganism. Ideally, the injected microorganisms should migrate freely in the reservoir formation and have minimal adsorption to reservoir rock material. A field pilot conducted at Guan 69 Unit in Dagang Oilfield indicated that exogenous biosurfactant-producing bacteria migrated through the reservoir matrix at a speed about 1.7–4.2 m per day (Liu et al. 2005). The use of starved cells or spores could facilitate the migration of exogenous microorganisms (Youssef et al. 2009; Shibulal et al. 2014). While it may be problematic to inject microorganism large distances into the reservoir, it is possible to treat the near wellbore region with exogenous biosurfactant-producing *Bacillus* species (Youssef et al. 2007b, 2013).

4.3 Injection of Nutrients to Stimulate Indigenous Biosurfactant-Producing Microorganisms

To choose this strategy, one must first determine if the biosurfactant-producing microorganisms or their activities are present and then decide on how to stimulate these microbes and their activities. Often, this decision is based on the analysis 16S ribosomal RNA gene sequences or other genes with phylogenetic information (Kryachko et al. 2016; Li et al. 2014). In one field trial, phylotypes related to known biosurfactant producers in genera such as *Pseudomonas*, *Alcaligenes*, and *Rhodococcus*, were detected and their concentration in production liquids was closely related to the increase oil production and oil emulsification (Li et al. 2014). While phylogenetic analysis shows the types of microorganisms present, it can be difficult to infer metabolic function from phylogeny. The use target genes involved in biosurfactant synthesis such as *srf*A for surfactin, *lic*A for lichenysin, *rhl*R for rhamnolipid production would provide direct information on the potential for biosurfactant production in an oil reservoir. Such an approach showed that lipopeptide biosurfactant-producing *Bacillus* species, but not rhamnolipid-producing microorganisms, were present in Oklahoma reservoirs with a wide range of salinities (Simpson et al. 2011). Whether it can be concluded that biosurfactant producers are routinely present in oil reservoirs worldwide remains to be determined.

Once it is known that the indigenous biosurfactant-producing microorganisms are present, further tests are needed to confirm biosurfactant production and to develop a nutrient mixture to stimulate biosurfactant production selectively. The use of complex substrates such as molasses may provide a cost advantage over using more refined ingredients. However, the use of complex substrates makes it hard to control the process in situ. Systematic adjustment of C, N and P ratios and concentrations of other nutrients is a proven approach to stimulate biosurfactant production (Sen 2010). A simple, direct approach to stimulate in situ biosurfactant production in oil reservoirs has yet to be developed.

5 Success of Field Trials

Although a number of laboratory studies show the efficacy of biosurfactant production on oil recovery (Table 1) (Youssef et al. 2009), large-scale applications of biosurfactant-mediated oil recovery are rare due to the high cost of the biosurfactant or difficulties in controlling biosurfactant production within the reservoir. Sporadic reports of biosurfactant-mediated oil recovery have appeared in the literature. Earlier field trials have been extensively reviewed (Youssef et al. 2009); here, we summarize more recent field trial results (Table 2).

In the past two decades, a number of field trials of MEOR have been implemented in Chinese oil fields, including Dagang Oilfield, Daqing Oilfield, Huabei Oilfield, Shengli Oilfield, and Xinjiang Oilfield, (Liu et al. 2005; Huang et al. 2014; Li et al. 2014, 2015; Chai et al. 2015; Le et al. 2015). A well-documented trial involving hydrocarbon-degrading and biosurfactant-producing bacteria was implemented in a sandstone oil reservoir (Guan 69 Unit of the Dagang Oilfield in Hebei Province, China) (Liu et al. 2005). The injected, exogenous bacteria were detected in four of seven production wells after several months of injection. A slight decrease in the surface tension of the production liquids was observed and oil production increased over a 6-month period following the microbial treatment. About 9120 m^3 of additional oil was produced (Table 2). In another trial, a biosurfactant-producing, *Pseudomonas aeruginosa* P-1, and its metabolic products were injected into more than 60 oil-producing wells in Daqing oilfield, China (Li et al. 2002). About 80% of injected wells showed a significant decrease in the amount of water produced with a corresponding increase in oil produced.

We implemented two successful tests of biosurfactant-mediated oil recovery in a Viola limestone oil formation in Oklahoma (Youssef et al. 2007b, 2013). The first test showed that inoculation of oil wells with exogenous, biosurfactant-producing microorganisms is possible and in situ biosurfactant production was detected (Youssef et al. 2007b). The second test involved larger volumes of materials (tenfold greater quantities than the first test) to determine if in situ biosurfactant production simulated oil production (Youssef et al. 2013). Lipopeptide biosurfactants were detected in produced fluids of the two inoculated wells (20 and 28 mg/L, respectively) and the increase in microbial products in the production fluids corresponded

Table 2 Recent field trials involving biosurfactant-producing microorganisms

Mechanism	Microorganisms	Approach	Oil recovery (m^3)	Comments	Reference
Stimulate in situ hydrocarbon production	Indigenous *Pseudomonas* sp.	Treat injection wells with air and nutrients	2,200	Emulsification	Chai et al. (2015)
	Indigenous hydrocarbon degraders	Treat injection wells with H_2O_2 or oxygenated water with N and P	4,420	Emulsification; interfacial tension reduction	Nazina et al. (2008)
	Indigenous hydrocarbon degraders	Treat injection wells with air-saturated brine and minerals	16,200	Reduction in interfacial tension	Nazina et al. (2007)
	Oil-degrading and biosurfactant-producing microorganisms	Repetitive treatment of injection wells with nutrients and inoculum	9,122	All seven wells had increased oil production	Liu et al. (2005)
	Pseudomonas aeruginosa and its metabolic products	Not disclosed	7–14 m^3 per well	80% of wells had increased production	Li et al. (2002)
Stimulate biosurfactant production	*Bacillus* sp. RS-1 and *Bacillus subtilis* subsp. *spizizenii*	Treat producing wells with glucose-nitrate-metals and inoculum	53	20–28 mg/L of biosurfactant	Youssef et al. (2013)

directly with an increase in oil recovery. About 52.5 m^3 of additional oil (net cumulative increase) occurred during the first 60 days.

One of the more common approaches to MEOR is to stimulate hydrocarbon degradation by the controlled injection of air or H_2O_2 along with other nutrients (Liu et al. 2005; Nazina et al. 2007, 2008; Huang et al. 2014; Li et al. 2014, 2015; Chai et al. 2015; Le et al. 2015). Hydrocarbon metabolism often results in biosurfactant production. After the microbial process was initiated, products of microbial metabolism, biosurfactants and hydrocarbon-degrading microorganisms were detected in production fluids (Nazina et al. 2007, 2008). The water content of

production liquids decreased and the oil content increased, resulting in large amounts of additional oil (Table 2).

6 Research Needs

Research to date shows that biosurfactant-mediated oil recovery is technically feasible. That is, microorganisms produce biosurfactants that generate low interfacial tensions and recover large amounts of oils. Limited studies indicate that biosurfactant producers are likely present in oil reservoirs. Much more work is needed to understand how to control biosurfactant production in the reservoir in order for biosurfactant-mediated oil recovery to become a successful commercial approach to oil recovery.

1. More work is needed in media design and fermentation approaches to reduce nutrient costs and increase final biosurfactant concentrations. Very little work has been done to increase biosurfactant concentration or activity by genetic manipulation.
2. A greater understanding of the pore-level processes that occur during biosurfactant-mediated oil recovery is needed to understand how biosurfactants influence capillary forces and wettability and how multiple microbial mechanisms operate to enhance oil recovery.
3. More work is needed to develop nutrient and injection regimes to stimulate in situ biosurfactant production reproducibly. Fundamental information on the ecology of biosurfactant-producing microorganisms in oil reservoirs is critically needed as are the tools needed to monitor changes of biosurfactant concentration and metabolic activity of biosurfactant producers.

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Application of Microorganisms to the Processing and Upgrading of Crude Oil and Fractions

38

M. Ayala, R. Vazquez-Duhalt, M. Morales, and S. Le Borgne

Contents

Abstract

Biotechnology has been successfully applied at the industrial level in the medical, fine chemical, agricultural, and food sectors. Several applications of biotechnology in the oil and energy industry in the future can also be foreseen. The production of biofuels in large volumes is now a reality, although there are some concerns about the use of land, water, and crops to produce fuels. In the oil industry, biotechnology has found its place in bioremediation and microbial enhanced oil recovery (MEOR). There are other opportunities in the processing (biorefining) and upgrading (bio-upgrading) of problematic oil fractions and

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heavy crude oils. In the context of increasing energy demand, conventional oil depletion, climate change, and increased environmental regulations on atmospheric emissions, biotechnologies such as biodesulfurization, biodenitrogenation, and aromatic ring opening (biodearomatization) and upgrading of heavy oils (degradation of asphaltenes and removal of metals) recover interest. In this chapter we revise the status of current regulations regarding fuel properties that have repercussions on its environmental impact, such as sulfur and nitrogen content, cetane number, and aromatic content, mainly in the EU and USA. We describe the cumulative and highlight the recent scientific and technological advances of these biotechnologies; their advantages and limitations are also discussed. On this basis, the possibility of integration in oil production plants and future oil refineries and biorefineries for the production of oil, fuels, and chemicals is analyzed.

1 Introduction

Presently, the world depends on fossil fuels (oil, coal, and natural gas) for 81.1% of its total primary energy supply (TPES) (International Energy Agency 2015). Hydropower, biofuels, wastes, and others (solar, wind, etc.) only account for 2.4%, 10.3%, and 1.4% of the TPES, respectively. Even if the supply of natural gas has increased since 1973 (21.2%), oil is still the main energy source (31.3%). According to the 2015 World Energy Outlook of the OPEC, renewable energies will continue to grow untill 2040, although their overall share will remain low, with biomass remaining relatively stable (Organization of the Petroleum Exporting Countries 2015). Concerns about biofuels are the increased use of land and crops for fuel production versus food and associated environmental effects that have not been clearly quantified yet. As intense energy use is part of economic development, Latin American countries, India, Southeast Asia, and China will continue to increase their demand for energy in the form of fossil fuels. Meeting these increasing energy demands will require to discover new conventional oil deposits or exploit atypical unconventional resources in addition to the use of renewable energy and the implementation of energy saving policies (Alazard-Toux 2011; Demirbas et al. 2016). Unconventional resources cannot be produced, transported, and refined by conventional methods. They represent more than half of the world's oil reserves in the form of extra-heavy oil, bitumen, and tar sands (Demirbas et al. 2016). While the reserves of conventional oil have started to deplete, nonconventional resources are estimated to last at least one century more (Ahlbrandt 2006). Leading countries in terms of nonconventional reserves are Venezuela, Canada, and Russia (BGR 2015).

As the world is running out of conventional crude oils, unconventional heavy and highly viscous crude oils are being increasingly used as refinery feedstocks. Compared to conventional crude oils, unconventional materials have low API gravities and high viscosities (i.e., resistance to flow) (Fig. 1). API gravity (American Petroleum Institute gravity) is a measure of how heavy or light a petroleum liquid is compared to water; the lower the API gravity, the higher the density of the liquid.

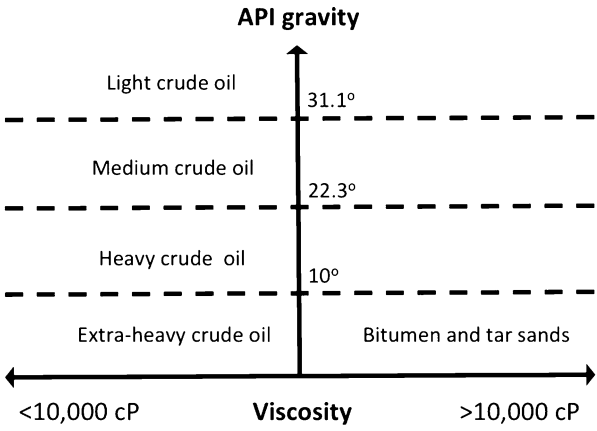

Fig. 1 Classification of oils, bitumen, and tar sands as a function of API gravity and viscosity (Adapted from Saniere 2011)

Additionally, these resources contain high amounts of undesirable compounds, namely, asphaltenes, heavy metals, sulfur, and nitrogen (Table 1) as well as paraffins and polar high molecular weight resins, compared to conventional crude oils. The viscosity of these oils together with the flocculation and deposition of asphaltenes, resins, and paraffin wax generate problems in recovery, separation, refining, and upgrading operations. The presence of impurities (sulfur, nitrogen, and metals) in refinery feedstocks contributes to the formation of coke and poisoning of refining and upgrading catalysts (Ancheyta 2016). Sulfur, nitrogen, and aromatic content in transportation fuels is responsible for SOx, NOx, and aromatic emissions as well as particulate matter (PM) and ozone formation, all being detrimental to the environment and human health (International Energy Agency 2016). Increasingly stringent environmental regulations have therefore been adopted all over the world to restrict the sulfur and aromatic content in transportation fuels. Moreover, the recent Paris Climate Conference (COP21) requires cutting greenhouse gas (GHG) emissions, chiefly CO_2 that mainly escapes during the combustion of fossil energies due to human activities (transport, residential, and industry).

In 2014, nearly two-thirds of the world oil consumption was in the transport sector (International Energy Agency 2015). According to the outlook presented by the International Energy Agency in 2012, in a "no dedicated policies" scenario, road travel is likely to double by 2050, with most growth coming from developing and emerging countries with a strong increase in road transport (Dulac 2012). Some solutions to reduce oil consumption for transport include the use of natural gas, liquid biofuels, and hybrid or hydrogen vehicles. Without doubt, significant research efforts should be continued to improve the processes for biofuels and hydrogen production and use in which microbial and enzymatic catalysis are already applied. In the meantime, as the availability of light oils is not expected to increase, the demand for energy is rising, and environmental concerns are increasing, there is no doubt that large quantities of oil- and petroleum-derived products will still be used.

There is a growing demand for gasoline, jet fuel, and diesel transportation fuels, which are produced from naphtha (boiling point <175 °C) and middle distillates

Table 1 Properties of different types of oils (Adapted from Quignard (2011))

Origin	USA	Russia	Nigeria	Saudi Arabia	Indonesia	Mexico	Canada	Venezuela	
Type	Extra-light	Light	Medium	Medium	Heavy	Heavy	Heavy	Heavy	Extra-heavy
API gravity	39.8	31.3	29.5	27.3	20.8	21.2	21.2	14.8	8.9
Sulfur (wt%)	0.33	1.39	0.18	2.89	0.20	3.35	3.69	2.81	5.61
Nitrogen (ppm)	0.10	0.21	0.12	0.18	0.35	0.38	0.35	0.48	0.66
Asphaltenes (wt%)	0.09	1	0.0	5.3	0.05	10.6	7.7	11.4	13.7
Ni+ V(ppm)	4	62	5	79	33	334	218	356	1,409
Distillate yield (wt%)									
At 175 °C	28.9	7.9	13.4	16.5	2.9	12.5	17.7	5.5	0.6
At 370 °C	67.3	51.4	64.0	44.6	25.4	37.6	36.0	28.4	16.0
At 550 °C	90.0	78.3	90.6	66.5	52.4	60.0	58.7	57.1	40.0

(boiling point <370 °C) (ExxonMobil 2016). As it can be seen in Table 1, non-conventional materials have lower yields of liquid distillates compared to conventional oils; moreover, sulfur tends to concentrate in middle distillates, so these types of oils will require deep conversion and substantial upgrading involving deep thermal and catalytic processes and significant hydrogen inputs to obtain fractions suitable for the production of transportation fuels and others products (Quignard 2011; Ancheyta 2016).

This situation is challenging for refinery operation all over the world. The development of new technologies will certainly contribute to the improvement of the refining of problematic feedstocks. The exploitation of unconventional hydrocarbons should take place in the context of technologies that limit the release of CO_2 into the atmosphere during production, refining, and use of these resources. Although microbial biotechnology has been successful in the medical, fine chemical, food, and agricultural fields, other industries may take advantage from its potential. In the oil industry, microbial applications have traditionally been limited to water treatment and remediation of aquatic and inland sites and MEOR. The benefits that could result from biotechnologies applied to oil and gas recovery, transportation, and processing are now well recognized (Kotlar et al. 2004; Bachmann et al. 2014; Kilbane et al. 2016).

The main advantages of biotechnologies are that they operate under milder conditions (lower temperatures and pressures) and are less energy and pollution intensive than traditional petroleum refining technologies (Le Borgne and Quintero 2003). The first review describing microorganisms capable of degrading hydrocarbons appeared in 1946 by ZoBell (Bacteriol Rev. 10:1–49, ZoBell 1946). Since then, the amount and depth of the information on the application of enzymatic and microbial catalysts to the processing and upgrading of oil and derived fuels have increased (Bachmann et al. 2014). The main current and foreseeable applications of biorefining are biodesulfurization (BDS), biodenitrogenation (BDN), aromatic ring opening or biodearomatization (BDA), and bio-upgrading of heavy crudes, with BDS being the most advanced area for which pilot plant scale has been reached (El-Gendy and Speight 2016).

2 Biodesulfurization

Sulfur is the most abundant heteroatom in crude oil with a content of 0.2–7.0% (wt) compared to nitrogen (<1.0%) and metals (<1,000 ppm) (Reynolds 1998). The combustion of sulfur-containing fuels leads to the emission of sulfur oxides (SOx) and particulate matter (PM) (Mohebali and Ball 2016). SOx react with water, oxygen, and other chemicals present in the atmosphere to form sulfuric acid, the primary component of acid rain which is harmful to human health, affects ecosystems, and causes the corrosion of materials, building stones, concrete, metals and paints, etc. PM is responsible for the typical black smoke associated with diesel vehicles. Gasoline vehicles also emit PM. Total PM emissions from diesel engines are proportional to the sulfur content of the fuel (Stanislaus et al. 2010). Although

PM does not have a defined chemical composition, they are known to contain volatile organic compounds (benzene, acrolein, etc.), polynuclear aromatic hydrocarbons (PAHs), and SOx that cause acute and chronic health effects and are classified as human carcinogens. Diesel engines are equipped with NOx absorbers, PM filters, and emission control systems (diesel oxidation catalysts, DOCs) that oxidize carbon monoxide, organic matter, and hydrocarbons. In the presence of sulfur, the effectiveness of DOCs is reduced, and high levels of sulfate are produced by oxidation of sulfur compounds in the DOC itself. Reducing sulfur levels in fuels to ultralow levels by environmental regulations can therefore reduce harmful emissions and improve air quality by directly reducing SOx and PM emissions and, indirectly, by increasing the efficiency of engine emission control devices (Stanislaus et al. 2010).

During the 1990s, several developed nations (USA, Canada, EU, and Japan) restricted the sulfur content in diesel to 500 ppm, and, in the 2000s, these standards were set to 10–15 ppm (ultralow-sulfur diesel, ULSD) (Stanislaus et al. 2010). Prior specifications were an order of magnitude higher: USA 5,000 ppm (prior 1993), Canada 1,530 ppm (prior 1998), and the EU and Japan 2,000 ppm (prior 1996 and 1997). Other countries have also adopted the requirement for ULSD, for example, Russia, Iran, Saudi Arabia, Australia, Taiwan, China, Mexico, Chile, Brazil, Argentina, Uruguay, etc. The current goal set by EPA under the tier 3 program mandates 10 ppm of sulfur on federal gasoline by 2017 (EPA 2014), while the Euro 5/6 legislations are pushing for virtually "sulfur-free" gasoline and diesel ($\leq$10 ppm) (The International Council on Clean Transportation 2016).

So, the quality of oil feedstocks is decreasing, and the content of sulfur in oils and distillates is globally increasing while, at the same time, the regulations on fuel quality are more and more stringent and the demand of fuels is globally increasing. As a result of the increasing demand for high-quality fuels, two important alterations in refineries operations are clearly detected (Swaty 2005). First, as the quality of refinery input decreases (Fig. 2a), so does the yield of lighter fractions with low-sulfur content. Thus, the capacity for processing higher boiling point fractions has been increased in order to meet the demand for light fuels (Fig. 2b). Second, the operation of conventional units must be adjusted to meet fuel specifications, especially when the feedstocks are highly contaminated with sulfur. For diesel, the development of novel catalysts has been the most successful strategy to improve the efficiency of desulfurization units, albeit at higher operational costs.

Hydrodesulfurization (HDS) is the technology used in refineries for the precombustion desulfurization of fuels. It is accomplished by the catalytic reaction of hydrogen with sulfur compounds in the presence of metallic catalysts at high pressures (30–130 atm) and temperatures (300–400 °C) to convert sulfur-containing hydrocarbons into H_2S. In fractions used to produce diesel, the HDS recalcitrant S compounds that have to be removed to reach ULSD levels are mainly dibenzothiophenes (DBTs) with alkyl substituents in the 4 and 6 positions close to the sulfur atom (Kabe et al. 1997; Monticello and Finnerty 1985). DBT and its derivatives have therefore been used as model sulfur compounds for the development of new technologies for ultra-deep desulfurization (Fig. 3). The removal of

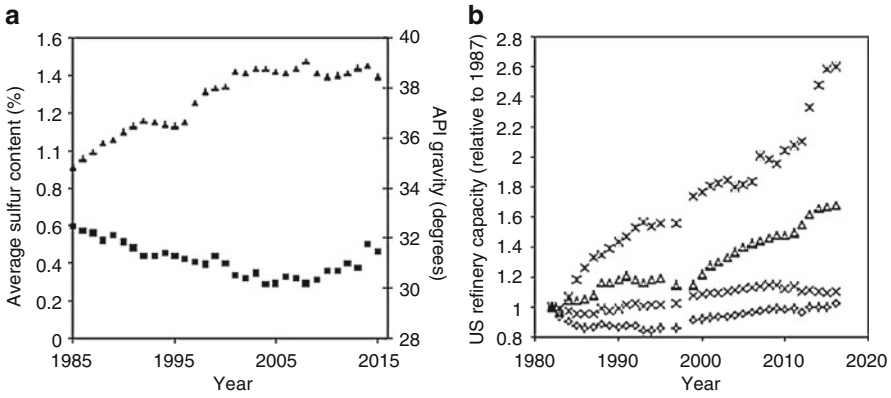

Fig. 2 **a** Quality of refinery input in the USA (Energy Administration Information. Official energy statistics from the US government) □ API gravity; Δ average sulfur content; **b** Refinery capacity in the USA (Energy Administration Information. Official energy statistics from the US government). (*diamond*) Atmospheric distillation; (multisign) catalytic cracking; (*triangle*) thermal cracking; (*asterisk*) hydrocracking

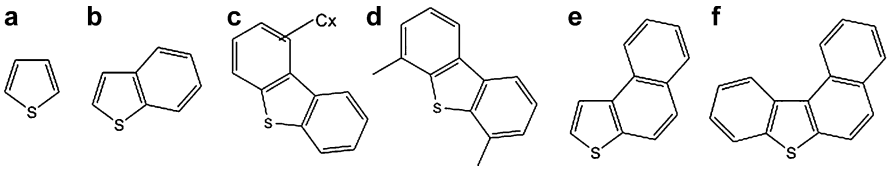

Fig. 3 Parental sulfur compounds found in crude oil. (**a**) Thiophene, (**b**) benzothiophene, (**c**) DBT and alkyl (Cx) derivatives, (**d**) 4,6-dimethyl DBT, (**e**) naphtothiophene, (**f**) benzonaphthothiophene

these refractory molecules is a challenge that has required the modification and revamping of existing low-sulfur HDS units. Higher temperatures and pressures and new catalysts are needed, leading to increased operation and capital costs, and CO_2 emissions. Moreover, the refineries will probably need to further adapt in the near future as other important properties of diesel as high cetane number or low-aromatic content will probably become mandatory by regulation, at the same time having to produce higher volumes of fuels from lower-quality feedstocks. There have been considerable research efforts on deep desulfurization in the last years, both from the academy and the industry. Stanislaus et al. (2010) and El-Gendy and Speight (2016) have extensively reviewed and discussed the advances and options. Briefly, concerning diesel, significant improvements of HDS catalysts and reactors have been obtained, and refiners have been able to revamp existing HDS units and processes that had been initially designed in the 1990s to produce 500 ppm sulfur fuels. High hydrogen consumption and the need to add more catalyst affect process economics; therefore, new non-hydrogenation alternatives as oxidative desulfurization (ODS), physical and reactive adsorption, and extractive desulfurization have been proposed. Among these, the most promising seems to be the ODS alternative

for which several oil companies are now at the level of process development and five of these processes are now at the commercialization stage (Stanislaus et al. 2010). In ODS sulfur compounds are oxidized to their corresponding sulfones, which can be removed, by extraction, adsorption, or other methods. The integration of ODS to existing conventional HDS units producing 500 ppm sulfur-containing diesel is estimated to be more cost-effective than revamping.

BDS alternatives have also been actively explored since the discovery of the bacterium *Rhodococcus erythropolis* IGTS8 (Kilbane 1992) and have recently regained interest. Extensive reviews on the subject have been recently published, and new research groups have emerged worldwide, contributing to the generation of new knowledge on the subject (Boniek et al. 2015; Mohebali and Ball 2016; El-Gendy and Speight 2016). This biotechnology lacks the formation of undesirable products or pollutants compared to physicochemical methods; however, it has not reached commercialization stage yet.

Early BDS studies showed that the enzyme chloroperoxidase (CPO) from the fungus *Caldariomyces fumago* can virtually oxidize the entire sulfur-containing species in straight run diesel containing 1.65% sulfur (wt) in the presence of hydrogen peroxide (Ayala et al. 1998). CPO catalyzes the oxidation of organosulfur compounds into sulfoxides that may be separated from the hydrocarbon-rich fraction by distillation or selective partitioning due to their increased polarity. After CPO treatment and distillation, there was a 20% hydrocarbon loss in the process. The oxidation of chemically diverse organosulfur compounds catalyzed by CPO highlights the potential of this enzyme for applications in the treatment of complex streams (Ayala et al. 2000). The largest and unsolved challenge with CPO has been that no heterologous, stable expression of this enzyme in a suitable host has been obtained yet (Conesa et al. 2002; de Weert and Lokman 2010). Thus, peroxide stability, substrate partition improvement, and overproduction issues for large-scale applications have been restricted to nongenetic approaches (Ayala et al. 2007).

Another approach was the use of the bacterium *Rhodococcus erythropolis* strain IGTS8 that can selectively remove the sulfur atom from the hydrocarbon structure of DBT through the 4S pathway, increasing the yield of hydrocarbon recovery, compared with the enzymatic alternative (Kilbane 1992). DBT is not degraded but only transformed into 2-hydroxybiphenyl (2-HBP), which partitions to the hydrocarbon phase (the fuel), while the sulfur is eliminated as inorganic sulfate in the aqueous phase containing the biocatalyst (Fig. 4). This cell-based biotechnology has been intensively investigated, and a number of other desulfurizing bacteria following the same metabolic pathway as *R. erythropolis* IGTS8 have been isolated; these include several strains of *Rhodococcus* and related bacteria as *Gordonia*, *Nocardia*, *Microbacterium*, and *Mycobacterium* from the Actinomycetales order, *Sphingomonas*, *Pantoea agglomerans*, *Stenotrophomonas* sp., *Brevibacillus*, and moderate thermophiles or thermotolerant bacteria as *Paenibacillus*, *Bacillus subtilis*, *Mycobacterium*, and *Klebsiella* sp. (Ayala and Le Borgne 2010; Morales and Le Borgne 2014; Akhtar et al. 2016; Mohebali and Ball 2016). Rhodococci and related bacteria have the ability to uptake and transform hydrophobic compounds as DBT and its derivatives because of the hydrophobic nature of their cell walls that contain mycolic acids with long aliphatic

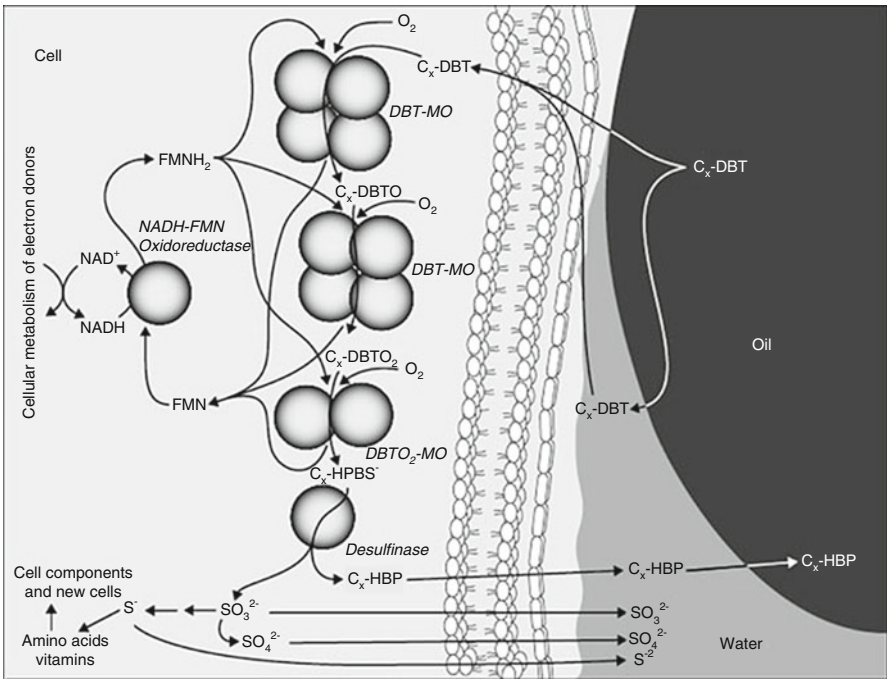

Fig. 4 Steps in the microbial desulfurization of oil fractions through the 4S pathway (Taken from Monticello 2000). Cx-DBT: DBT derivatives; Cx-HBP and Cx-HBPS: HBP and HBPS derivatives produced from Cx-DBT desulfurization; DBTO: DBT sulfoxide; DBTO2 : DBT sulfone; DBT-MO: DBT monooxygenase (DszC); DBTO2 –MO: DBTO2 monooxygenase (DszA); desulfinase DszB). The corresponding genes are *dszC*, *dszA*, and *dszB* in the so-called dsz operon (*dszABC*)

chains (Martínková et al. 2009). At higher temperatures, thermophilic strains could be advantageous for crude oil treatment due to lower viscosity. It would also be unnecessary to cool the fractions to ambient temperature, and thus BDS could be more easily integrated to a refining stream after HDS (Stanislaus et al. 2010).

Bacteria able to utilize sulfur-containing hydrocarbons are widespread in nature (Ayala and Le Borgne 2010). Three major pathways have been described for DBT utilization: the partial degradation pathway in which DBT is used as source of carbon but not as source of sulfur (Kodama pathway), the complete degradation pathway in which DBT is used both as source of carbon and sulfur, and, finally, the sulfur-specific pathway (4S pathway) (Mohebali and Ball 2016). Only the 4S pathway has been considered useful for the desulfurization of fuels since it does not destroy the hydrocarbon moiety of the molecule therefore retaining the calorific value of the fuel. It consists in three steps (Fig. 4): (1) the oxidation of the sulfur atom by the DszC monooxygenase to generate sulfoxides and sulfones, (2) the cleavage of a C–S bond by the DszA monooxygenase to generate 2-hydroxybiphenyl sulfinate (2-HBPS), and (3) the removal of the sulfinate group by the DszB desulfinase to generate sulfate and 2-HBP.

R. erythropolis IGTS8 has been the base of the BDS process proposed by Energy BioSystems Corporation (EBC) (named later Enchira). An intensively engineered bacterium was obtained: two orders of magnitude more active than the original strain (Monticello 2000). The copy number of desulfurizing genes was increased, the sulfate repression eliminated by promoter change, and the last gene of the metabolic pathway (*dszB*) deleted to eliminate this rate-limiting step (in terms of enzyme concentration) and allow the accumulation of 2-HBPS sulfinate, a potentially valuable surfactant. The DszC monooxygenase catalyzes the first and limiting step (in terms of oxidation rate and range of oxidized substrates) of the desulfurization pathway using molecular O_2 (Fig. 4). The activity of DszC was improved through directed evolution using gene shuffling and chemostat enrichment (Arensdorf et al. 2002; Coco et al. 2001). Simultaneous improvement of both desulfurization rates and range of oxidized substrates was obtained. A gain-of-function phenotype allowing the oxidation of 5-methylbenzothiophene was due to the single mutation V261F. The first two steps of the 4S pathway are catalyzed by the cofactor-dependent monooxygenases DszC and DszA (Fig. 4). The complexity of the 4S multi-enzymatic pathway and the requirement for cofactors impair the use of purified enzymes instead of whole cells. As shown in Fig. 4, the DszC and DszA enzymes use the $FMNH_2$ cofactor that is regenerated by the action of an NADH-FMN oxidoreductase (DszD) (Gray et al. 1996). Other genetic engineering strategies consisted therefore in overexpressing homologous or heterologous reductases for the efficient regeneration of cofactors (Hirasawa et al. 2001; Matsubara et al. 2001; Reichmuth et al. 2000) or coexpressing *Vitreoscilla* hemoglobin to increase cell internal oxygenation (Xiong et al. 2007). A recent investigation of the catalytic mechanism of DszD by combined quantum mechanics/molecular mechanics simulation methods opens up the possibility for rational protein engineering of this enzyme (Sousa et al. 2016). The presence of smaller or more positive amino acid residues instead of an active site Thr residue could lower the activation barrier for the rate-limiting step in the reaction of formation of $FMNH_2$ by DszD. Production of the desulfinase DszB (see Fig. 4) could be increased by mutating the untranslated 5′ region of *dszB* (Reichmuth et al. 2004). Removing the gene overlap between *dszA* and *dszB* led to a fivefold increase in desulfurizing activity (Li et al. 2007), while cells expressing a rearranged operon (*dszBCA*) presented a 12-fold higher activity (Li et al. 2008a). The desulfinase DszB is rate limiting for the whole pathway (in terms of quantity of enzyme expressed). The catalytic activity and thermostability of DszB were enhanced by two amino acid substitutions (Y63F and Q65G), and a double mutant (Y63F, Q65G) presented an improved desulfurizing activity (Ohshiro et al. 2007).

Conventional methods for isolating novel microorganisms and improving known biodesulfurizers by genetic and protein engineering strategies are time- and labor-consuming. Novel microorganisms were recently identified by using a bioinformatic approach consisting in mining genomic databases to identify DBT-desulfurizing bacteria (Bhatia and Sharma 2010). For this, the amino acid sequences of the Dsz enzymes were used to search for homologous proteins in microbial genomes databases. Thirteen novel desulfurizing bacteria belonging to 12 genera (*Burkholderia*,

Bradyrhizobium, *Methylobacillus*, *Magnetospirillum*, *Thermobifida*, *Azotobacter*, *Mesorhizobium*, *Oceanobacillus*, *Novosphingobium*, *Brevibacterium*, *Rubrobacter*, and *Ralstonia*) were identified. Interestingly, seven of these microorganisms lacked the DszB enzyme, which is rate limiting in the 4S pathway, and two of them were thermophilic (*Thermobifida* and *Rubrobacter*). Bacteria lacking the DszB enzyme accumulate 2-HPBS, a potentially valuable surfactant, as mentioned above. The potential advantage of thermophilic microorganisms has also been mentioned above. A novel and interesting approach based on evolutionary engineering to generate strains with an enhanced desulfurization activity has been reported by Pan et al. (2013). Bacteria assimilate very small amounts of sulfur for their growth and maintenance. These authors modified the *dsz* operon to include a synthetic gene encoding a peptide rich in sulfur-containing amino acids (methionine and cysteine). As the Dsz activity is repressed by the presence of sulfate and other readily available sulfur source as amino acids, desulfurizing strains depend on DBT desulfurization to obtain sulfur for growth. The increased demand of sulfur due to the presence of the sulfur-rich peptide exerts a selective pressure on strains growing fast on DBT as sulfur source. Evolutionary engineering provides new ways to improve industrially relevant microorganisms based on selectable characteristics (Winkler and Kao 2014) and does not need previous genetic knowledge; it is particularly suitable when multigene modifications are required as it seems to the case for desulfurizing microorganisms.

Petro Star and Diversa also worked in the development of a host harboring genetically engineered Dsz enzymes (Bonde and Nunn 2003a). The target was monooxygenase DszA, which is central in the desulfurization pathway, in order to engineer a host for desulfurization of DBT sulfones. Sulfones would be produced from the Petro Star Conversion Extraction Desulfurization (CED) process, a desulfurization processes involving oxidation and extraction. The CED process first extracts a fraction of the sulfur from diesel, then selectively oxidizes the remaining sulfur compounds into sulfones, and finally extracts these oxidized materials based on their increased polarity compared to the parental DBT compounds. The CED process is one of the ODS processes that has reached commercialization stage at this time. BDS would desulfurize the extracted sulfones and the desulfurized DBT hydrocarbon skeletons returned back to the fuels. No results were published concerning the performance of the obtained DszA variants. Recombinant desulfurizing and solvent-tolerant *Pseudomonas* strains were used to desulfurize DBT sulfones produced by chemical oxidation and solvent separation (ORNL 2000). In this process, 10% of the hydrocarbons were lost after solvent extraction; thus, BDS of the sulfones concentrated in the solvent was proposed to recover part of the hydrocarbon skeleton. The advantage of this process is that water would be added to the solvent extract and not directly to the fuel. DBT sulfones are more polar than DBT, and hence less hydrophobic *Pseudomonas* cells were suitable hosts in this case (Tao et al. 2006).

In the ODS, adsorption and extraction alternatives, ULSD fuels are produced. However, these methods imply the removal of organosulfur compounds leading to a decrease in the calorific value of the fuel and to the generation of waste streams

concentrated in sulfur compounds that could subsequently be treated using BDS to return the desulfurized hydrocarbons into the fuels or to produce valuable compounds (Kilbane and Stark 2016). Few reports of such combined physical-biological procedures can be found in the literature. For example, Li et al. (2006, 2008b, 2009) have reported the adsorption of DBT compounds on different adsorbents followed by the action of a *Pseudomonas*-desulfurizing strain to regenerate the adsorbents.

The BDS of real oil fractions (middle distillates, gas oil, and diesel) has been reported, and low to ultralow-sulfur levels (less than 50 ppm) were achieved depending on the initial sulfur content and whether the fractions had been previously hydrotreated, as reviewed by Mohebali and Ball (2016). Certain technical issues hamper the applicability of BDS; one of them is the biphasic nature of the process. An aqueous phase is needed in order to maintain a viable biocatalyst; thus significant quantities of water have to be added to the fuel. The values found in the literature generally indicate low oil-to-water volumetric ratios in the desulfurization of real fractions, ranging from 1% to 25% of oil in water. The highest oil-to-water ratios reported have been of 50% and 80% (Yu et al. 1998; Monot et al. 2002). *Rhodococci* cells are highly hydrophobic, and this is advantageous in terms of desulfurization rates because hydrophobic DBTs are efficiently transferred from the oil to the cells (Fig. 4); however, the mechanism by which DBT enters into contact with the Dsz enzymes is unknown (Monticello 2000). Nevertheless, these bacteria are difficult to separate due to their strong adherence to oil–water interfaces. Immobilization strategies were recently tested on nanosorbents and alginate to improve the BDS rate of recombinant *Pseudomonas* toward DBT in biphasic systems (Guobin et al. 2005; Li et al. 2008b; Shan et al. 2005). Cells coated with magnetite (Fe_3O_4) nanoparticles had the same biocatalytic activity as free cells and could overcome the mass transfer resistance of traditional entrapment immobilization processes (Guobin et al. 2005; Shan et al. 2005). Although improvements were obtained, the desulfurizing activity in these systems is still low. Other process issues that have been reviewed and discussed are biocatalyst production, bioreactor configurations, oxygen mass transfer and uptake rate, and substrate (fuel) mass transfer (Mohebali and Ball 2016). Concerning biocatalyst massive production, renewable carbon sources have been proposed (Alves et al. 2008; Alves and Paixão 2014); however, the main challenges are the repression of the *dsz* operon by sulfate and other easily assimilable sulfur sources as well as the growth inhibition by 2-HBP. The existence of bacteria as *Achromobacter* sp., isolated from an oil-contaminated soil, that degrade DBT following the 4S pathway and further transform 2-HBP to a less toxic compound, the 2-methoxybiphenyl, is therefore potentially interesting (Bordoloi et al. 2014).

DBT and its alkylated derivatives, mostly those with substitutions in the 4 and 6 positions (Fig. 3), are the most recalcitrant compounds to desulfurize if ULSD fuels are to be produced from 500 ppm sulfur-containing streams obtained from conventional HDS units. A clear advantage of BDS is that all the microorganisms following the 4S pathway can desulfurize these recalcitrant molecules, although the desulfurization rate is affected by the position and degree of alkylation (Mohebali and Ball 2016). Recent studies have shown that mass transfer issues also limit the desulfurization rate of alkylated DBTs by *Pseudomonas putida* in biphasic medium

(Boltes et al. 2013). While a complete conversion of DBT, 4-methyl DBT and 4,6-dimethyl DBT, was obtained in an aqueous medium, conversions of 38%, 19.5%, and 16.5% were obtained for the same compounds in a biphasic medium. Substrate competitive inhibition has been observed during the desulfurization of mixtures of DBT and 4,6-dimethyl DBT, representing a limiting factor for the desulfurization of real oil fractions (Chen et al. 2008). Zhang et al. (2013) have shown that the desulfurization rates decreased to 75.2 % in a DBT/4,6-dimethyl DBT mixture, 64.8 % in a DBT/4-methyl DBT mixture, and 54.7 % in a DBT/4-methyl DBT/4,6-dimethyl DBT mixture (considering that DBT desulfurization is 100%). These differences were due to an apparent competitive inhibition of substrates. Most of the studies with alkylated DBT have been performed with axenic cultures. Ismail et al. (2016) claim that microbial consortia have a better potential for a practical BDS application due to existence of synergistic or cooperative effects between all the species present. In this case, a consortium composed of *Sphingobacterium*, *Klebsiella*, *Pseudomonas*, *Stenotrophomonas*, *Arthrobacter*, *Mycobacterium*, and *Rhodococcus* was enriched from a petroleum hydrocarbon-polluted soil using DBT as a sulfur source. This consortium could desulfurize 4-methyl DBT and 4,6-dimethyl DBT in addition to DBT. The species richness and diversity depended on the substrate used, and the 4-methyl DBT culture presented the highest species richness and diversity. Microbial consortia may therefore be more versatile for a practical application.

Few reports concern BDS process design and cost analysis (Le Borgne and Quintero 2003; Soleimani et al. 2007; Alves et al. 2015). After more than twenty years of research by EBC and Enchira, several process innovations and an intensively engineered bacterium (Monticello 2000) were obtained. Eventually the project was continued until 2005 under the auspice of the US Department of Energy; Petro Star and Diversa worked in the development of a host harboring genetically engineered enzymes, but it appears that the biocatalyst activity was insufficient and the project ended without further progress to pilot plant scale (Kilbane 2006). It has been estimated that the BDS catalyst must have a sulfur removal activity within the range of 1–3 mmol DBT gr dry cell weight $^{-1}$ h $^{-1}$ in real petroleum fractions; thus, it may still be necessary to improve 500-fold the activity of current biocatalysts in order to attain a commercially viable process (Kilbane 2006). A recent publication including cost analysis study comparing two BDS process designs, upstream or downstream conventional HDS, in terms of energy consumption, GHG, emissions, and operational costs, showed that the BDS downstream HDS configuration was the best alternative to apply in an oil refinery (Alves et al. 2015). According to these authors, BDS integration downstream conventional HDS units may allow to obtain almost sulfur-free fuels with a much lower emission of GHG and CO_2. However, in this study, BDS costs and emission estimations were made considering the desulfurization of the model compound DBT, while HDS estimations were made on the basis of crude oil desulfurization.

The level of desulfurization activity achieved by genetic manipulation appears to be limited by unknown host factors related to the correct supply of cofactors and substrate transport (Kilbane 2006). Complex transcriptional factors involved in the

regulation of the utilization of organosulfur compounds as alternative sources of sulfur might also be involved (Tanaka et al. 2002). Diversa initiated the complete sequence determination of the *Rhodococcus* IGTS8 plasmid to gain a greater understanding of the genes required for BDS (Bonde and Nunn 2003b); however, the results have not been published. *Rhodococci* appear to have adopted a strategy of hyperrecombination associated with their large genome, and therefore these factors may be difficult to localize and understand (Larkin et al. 2005). The recent announcements of the genome sequences of two desulfurizing bacteria, *R. erythropolis* XP (Tao et al. 2011) and *Mycobacterium goodii* X7B (Yu et al. 2015), may provide new elements to elucidate some of the mechanisms limiting the desulfurizing activity and propose new strain improvement strategies. In *R. erythropolis* XP, the desulfurizing genes *dszABC* were found on a plasmid, while the NADH-FMN oxidoreductase gene *dszD* was located in the chromosome.

3 Biodenitrogenation

Nitrogen is not as abundant as sulfur in petroleum, and the nitrogen content in oil may vary from 0.1% to 0.9% (wt) (Speight 2014). After crude oil distillation, nitrogen concentrates into high molecular weight fractions in the form of poly-aromatic heterocycles. Emission standards have established that particular and light load transport vehicle emissions would not exceed 0.07 g of NOx's per mile in 2009 and, in March 2014, the EPA released the new standards for vehicles and fuels to be fully implemented by 2025. Both tailpipe and evaporative combined emissions of non-methane organic gas (NMOG) and NOx (MNOG+NOx) will have to be reduced from 109 mg mi^{-1} by 2017 to 30 mg mi^{-1} by 2025 (EPA 2014). The nitrogen present in middle distillates used to produce transportation fuels has to be removed in order to meet such standards (Bej et al. 2001).

Hydrodenitrogenation (HDN) of oil fractions is more difficult than HDS and requires more severe conditions and more hydrogen. A higher energy is required to break C–N bonds compared with C–S bonds (Szymanska et al. 2003). Besides, nitrogen removal requires the hydrogenation of aromatic rings prior to the attack of the C–N bonds. Nitrogen heterocycles are classified in basic compounds which are related to quinoline and usually comprise 25–30% (wt) of all nitrogen heterocycles, while nonbasic compounds are alkylated derivatives of carbazole and comprise 70–75% of nitrogen heterocycles (Fig. 5) (Mushrush et al. 1999; Laredo et al. 2002). Another problem associated with nitrogen compounds is that basic nitrogen compounds are known to inactivate HDS catalysts (Shin et al. 2000; Zeuthen et al. 2001). Nonbasic compounds can be converted to basic ones during the refining/catalytic cracking process, and thus, they also are potential inhibitors of HDS processes (Choi et al. 2004; Laredo et al. 2003, 2004). Moreover, the higher the sulfur content in petroleum fractions, the higher is the nitrogen content (Éigenson and Ivchenko 1977). So, denitrogenation is advantageous both from an environmental point of view (reduction of NOx emissions) and from an operational point of view (to avoid catalyst deactivation, corrosion of refinery equipments, and chemical

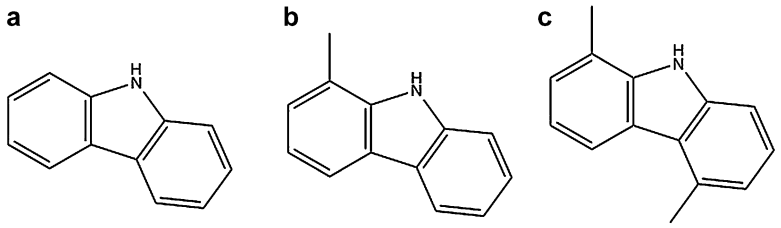

Fig. 5 Carbazoles. (**a**) Carbazole, (**b**) 1-methyl carbazole, **c** 1,5-dimethyl carbazole

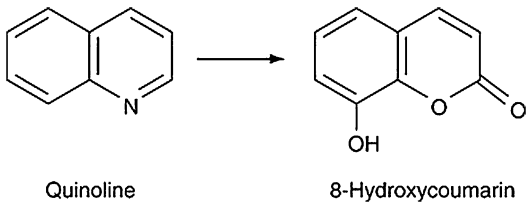

Fig. 6 Suggested pathway for the degradation of quinoline by *P. ayucida* IGTN9m

instability of refined petroleum). However, the physicochemical removal of recalcitrant heterocyclic organonitrogen compounds such as quinolines and carbazoles by HDN is expensive, hazardous, and nonselective which means that other constituents of petroleum can be modified (Bachmann et al. 2014) during the HDN process. The potential of BDN processes has therefore been explored, and several reviews about bioprocess-based technologies for the petroleum industry include detailed information on BDN (Bhatia and Sharma 2006; Singh et al. 2012; Bachmann et al. 2014).

The selective removal of nitrogen-containing compounds by *Pseudomonas* and *Acinetobacter* sp. cultures in raw shale oil was reported by Atlas and Aislabie (1992). Amines, nitriles, quinolines, and pyridines were converted to hydroxylated compounds, which are highly soluble in water. Kilbane et al. (2000) isolated a *Pseudomonas ayucida* strain (IGTN9m) that converts quinoline to 8-hydroxycoumarin without further degradation, and hence no carbon is lost in this case (Fig. 6). The 8-hydroxycoumarin is an intermediate in the degradation of quinoline by some *Pseudomonas* species, usually degraded by cleavage of the N-containing ring to produce aliphatic products (Morales and Le Borgne 2010). The production of 8-hydroxycoumarin by *P. ayucida* IGTN9m is therefore similar to previously reported degradation pathways. However, *P. ayucida* IGTN9m cannot use quinoline as a sole source of carbon, and such a partial pathway is unique to this strain. Resting cells of *P. ayucida* IGTN9m could selectively transform 68% of the quinoline from shale oil in 16 h. A mutant *P. ayucida* strain PTA-806 was obtained that could degrade quinoline under aerobic conditions (Kilbane et al. 2001, 2003). The obtained mutant could not remove all quinoline analogs, and the total nitrogen removal in the oil fraction tested was not more than 5% (wt). The calorific value of the treated fuels was maintained, which implies selectivity toward C−N bond cleavage. Quinoline is easily eliminated during hydrotreatment, and positive effects

on HDS efficiency have not been clearly observed after its removal; thus, studies have therefore focused on carbazole removal (Choudhary et al. 2008).

So far, no microorganisms able to selectively extract the nitrogen atom from carbazole have been isolated, so there is apparently no microbial pathway equivalent to the 4S pathway for this compound. The removal of the nitrogen atom from carbazole leads therefore to some degradation of the hydrocarbon skeleton and fuel loss (Fig. 7a). Microorganisms that degrade carbazole are phylogenetically diverse, and the most studied in relation to the BDN of carbazole are *Pseudomonas*, *Xanthomonas*, *Bacillus*, *Ralstonia*, *Klebsiella*, *Alteromonas*, *Neptunomonas*, *Cycloclasticus*, *Janthinobacterium*, *Acinetobacter*, *Marinobacterium*, *Achromobacter*, *Nocardioides*, *Burkholderia*, *Sphingomonas*, *Stenotrophomonas*, *Gordonia*, *Enterobacter*, and *Methylobacterium* (Morales and Le Borgne 2010, 2014).

In relation to the possible practical application of BDN, assays reported in biphasic media are of particular interest. A *Pseudomonas* sp. strain could degrade carbazole in a cyclohexane/water system (1:1) with a degradation rate of 1.2 mmol carbazole g dry cell $^{-1}$ min $^{-1}$ (Li et al. 2004). A *Burkholderia* sp. strain (IMP5GC) could degrade carbazole in an *n*-hexadecane/water system (1:1) with a degradation rate of 0.6 mmol carbazole g wet cell $^{-1}$ min $^{-1}$ (Castorena et al. 2006). This strain could also degrade carbazole and methyl-substituted derivatives in a gas oil/aqueous

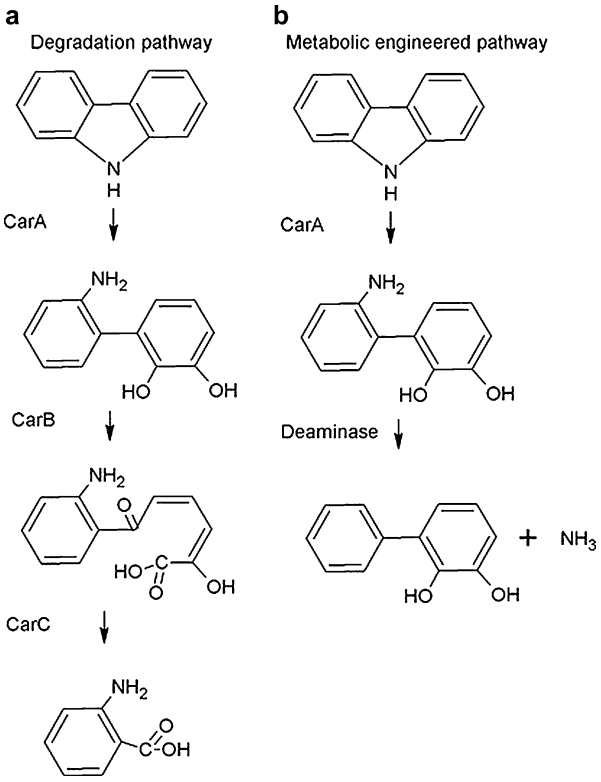

Fig. 7 Pathways for carbazole utilization. Natural (**a**) and genetically engineered (**b**) pathway

medium system (1:1). Carbazole was reduced about 90%, while 1-methyl carbazole and 3-methyl carbazole were reduced 53% and 68%, respectively, in 24 h with a biomass concentration of 4 g l^{-1}. The aforementioned bacteria completely mineralize carbazole to CO_2, and hydrocarbon loss is therefore expected. During the degradation of carbazole, the first enzyme of the pathway (CarA) converts carbazole to 2-aminobiphenyl-2,3-diol (Fig.7a) (Morales and Le Borgne 2010). The genes encoding CarA in *Sphingomonas* GTIN11 and *Pseudomonas resinovorans* CA10 were combined to create a truncated operon that only encodes the first step of carbazole degradation (Kayser and Kilbane 2004). The genetically modified microorganism could transform carbazole into 2-aminobiphenyl-2,3-diol in shale oil, crude oil, petroleum products, and coal tar. A degradation rate of 0.48 mmol carbazole g dry cell $^{-1}$ h^{-1} was obtained under resting cell conditions in an aqueous medium. The carbazole content of a petroleum sample could be reduced by 95% in petroleum/aqueous medium system (2:10) within 16 h. No specific degradation rates were reported. The same authors propose a strategy to avoid hydrocarbon loss; genes encoding mutant amidases capable of cleaving the C–N bond of 2-aminobiphenyl-2,3-diol could be inserted downstream the artificial *carA* operon; the goal would be to accomplish the cleavage of the final C–N bond and produce biphenyl-2,2′,3-triol, which could then be reintroduced to the fuel (Fig.7b). Natural isolates and genetically engineered strains capable of simultaneously using DBTs and carbazoles have been reported (Gai et al. 2007; Santos et al. 2006; Yu et al. 2006a). These microorganisms have not been used for BDS/BDN purposes yet, and therefore the influence of some process variables has not been studied yet.

There is only one report by Sugaya et al. (2001) that evaluates the influence of some process variables (cell activity, cell concentration, volumetric portion of crude oil in reaction mixture, and agitation) in the degradation of quinoline in petroleum using *Comamonas* sp. TKV3-2-1. Optimal conditions were 83% (v/v) of petroleum in aqueous medium with a cell density of 28.5 g l^{-1}. Under these conditions, the degradation rate was 1.6 mmol g cell $^{-1}$ h^{-1}. These conditions were considered for the design of a complete industrial BDN process including the following treatment stages: (1) a fermentation for cell production, (2) a bioreactor for quinoline removal, (3) a biomass separation by centrifugation and recirculation, (4) an oil separation for recirculation, and, finally, (5) the water treatment (Fig. 8).

Recently, research efforts have focused on operational issues such as processing temperature, biocatalyst concentration, and reusability as well as reactions in the presence of oil phases. Heavy oils require temperatures of 60–100 °C for processing as free-flowing fluids. Thus, microbes and enzymes that work at high temperature are required (Bhatia and Sharma 2006). Thermophilic carbazole-degrading microorganisms include *Anoxybacillus rupiensis* whose optimal degradation conditions were at pH of 7 in the presence of 0.5–1% of NaCl (wt) and temperatures between 55 and 65 °C. This bacterium was able to withstand 80 °C for 90 min (Fadhil et al. 2014). However, specific degradation rate was not reported, and assays were performed at low carbazole concentrations (up to 10 mM equivalent to 1.67 g l^{-1}) in an aqueous phase. The effect of high carbazole concentrations was reported with *Pseudomonas stutzeri* ATCC 21358 (Larentis et al. 2011) showing that, when the

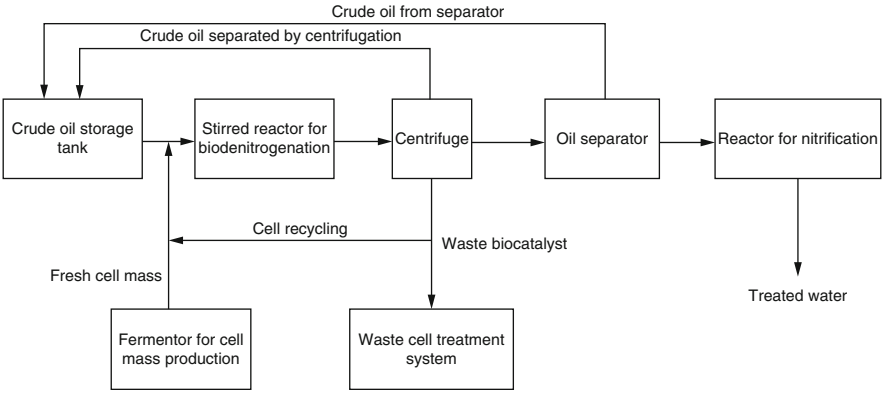

Fig. 8 Flow diagram for an industrial BDN process directed to quinoline removal in crude oil (Adapted from Sugaya et al. 2001)

carbazole concentration was higher (10 g l^{-1} vs. 1 g l^{-1}), the growth was ten times lower with considerable accumulation of anthranilic acid, which inhibited the growth of the bacterium. The authors consider that strain as promising for the application in biorefining of Brazilian crudes, which have a high content of nitrogenated compounds. Similar carbazole concentrations were used by Zakaria et al. (2016) that reported higher BDN abilities for *Bacillus clausii* BS1 than for *P. resinovorans* CA10, the model strain in BDN studies.

However, in the abovementioned works, the assays were done using carbazole as sole carbon and nitrogen source in mineral media. The use of a real fraction of petroleum such as light or heavy gas oil (HGO) has been explored by Maass et al. (2015) and Tang and Hong (2014). *R. erythropolis* ATCC 4277 was able to remove about 40 % of the nitrogen and sulfur present in HGO. The best result for nitrogen removal (43.2 %) was achieved in an aqueous biphasic system containing 40 % (v/v) of HGO (Maass et al. 2015). The authors consider that these results represent an advance in the development of a commercially and environmentally viable BDN industrial process. Tang and Hong (2014) reported an unidentified microorganism – bacterium HY9 – used in the denitrogenation of diesel oil. The nitrogen removal in diesel oil increased from 12.9% to 16.7% when 0.175 g of Tween 80 surfactant was added to 25 ml of diesel oil. The optimal conditions were 3 ml of bacterial suspension/100 ml of mixed liquor and 250 rpm shaking speed. Repeated denitrogenation batches had almost no effect on the nitrogen removal efficiency.

Finally, Zakaria et al. (2015) explored the reusability of *Bacillus clausii* BS1 coated by magnetic nanoparticles (magnetite, Fe_3O_4) to simplify the separation and purification of the final product and evaluate microbial longevity. The coated cells showed higher BDN rate toward carbazole than free cells, obtaining almost the complete removal (94.25%) of 1,000 ppm (1 g l^{-1}) of carbazole within seven days. In such a system, carbazole can be quickly transferred to the biocatalyst surface through the nanoadsorbent, resulting in the increase of biodegradation rate (Li et al. 2013). Besides, coated cells presented a higher BDN rate, storage and operational

stability, and lower sensitivity to toxic by-products, having the advantage of magnetic separation, which would solve some operational problems at the refinery level. The coated cells successfully retained their BDN efficiency during four batch cycles, while the free cells could be used only once, and lost ≈ 30% of their activity after the first batch cycle.

Compared with BDS, BDN processes suffer from the same limitations: low nitrogen removal activity and the need for an aqueous phase. Two additional limitations have to be considered for BDN to be economically attractive: (1) no microorganisms capable of selectively removing nitrogen without carbon loss have been developed yet, and (2) denitrogenation is an additional step that does not produce an upgraded fuel; therefore, reaction velocities should be even higher than for BDS.

4 Biodearomatization

In the USA, the current aromatic content in gasoline and diesel is around 25–30% and 35%, respectively. According to EPA, total aromatic content (benzene, toluene, styrene, xylenes) will not be regulated in gasolines, only the benzene content. Benzene is a known human carcinogen. Control of Hazardous Air Pollutants from Mobile Sources final rule, starting 2007, sets benzene content in all gasolines to 0.62%. According to EPA estimates, the levels set by this regulation will allow, by 2030, a 45% reduction of benzene emissions by passenger vehicles and a 38% reduction in benzene content of gasolines (EPA 2007). Due to transitions in the gasoline market (increased use of ethanol, removal of methyl tert-butyl ether (MTBE), fluctuations in crude oil prices), EPA does not consider to set a standard for aromatic contents at this time. Moreover, because the tendency is to elevate the content of ethanol, aromatic content will automatically reduce by dilution (by about 3–10% vol). Thus, EPA believes aromatic levels will fall in the future even without an aromatic standard (EPA 2007). Moreover, benzene (0.62% vol, current standard) has a 20-fold greater impact on toxic emissions from vehicles than other components, including aromatics (20–40% vol, depending on the refinery) (EPA 2008). On the other hand, in the EU, where diesel is the preferred vehicle fuel, it has been proposed that a total aromatic content of 10% would help reduce emissions (Swaty 2005). European Standard EN 590 sets a minimum cetane number of 51. Cetane number describes the ignition quality of a diesel fuel and could be improved by aromatic reduction, as discussed next.

An ideal diesel should be composed of 10- to 20-carbon linear aliphatic species. Thus, hydrogenation including a selective fragmentation of aromatic compounds could contribute to density decrease and cetane number increase of diesel (Rossini 2003). Figure 9 shows the evolution of cetane number for an aromatic molecule as hydrogenation and ring opening occur. In terms of environmental benefits, an inverse correlation has been observed between cetane number and particulate matter and NOx emissions (Leliveld and Eijsbouts 2008). Low-sulfur, low-aromatic, high cetane number diesel has been described as the fuel of the future. In fact, a recent

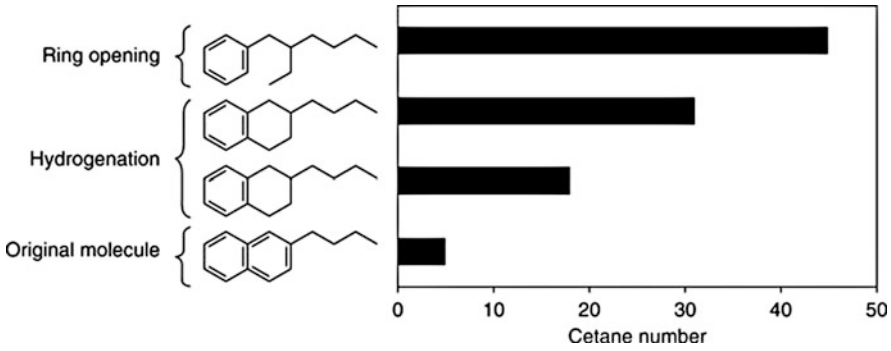

Fig. 9 Cetane numbers for selected aromatic compounds (Adapted from Rossini 2003)

report by EPA suggests that cetane number could be the best diesel fuel property to control because of its impact on emissions, particularly NOx (EPA 2003). In current refineries, catalytic hydrotreatment is the main process reducing the aromatic character of the mixture. However, the balance between hydrogenolysis and hydrogenation reaction reduces the efficiency of the process, generating low-molecular, volatile products that are excluded from the diesel fraction (Rossini 2003). Hydrogen consumption increases as more aromatic saturation is required; given that heavy oil will be increasingly used as refinery feedstock, a larger volume of highly aromatic streams is expected, leading to an increase in hydrogen consumption (Leliveld and Eijsbouts 2008).

In the field of petroleum biotechnology, it has been proposed that the naphthalene degradation pathway may serve as the basis to develop a biodearomatization (BDA) process (Fig. 10). The route for naphthalene assimilation starts with the double hydroxylation of one of the aromatic rings, catalyzed by the enzyme naphthalene 1,2-dioxygenase (step 1, Fig. 10); the dihydrodiol is further metabolized, through a cascade of oxidative and reductive steps, to salicylate. This metabolite may enter either tyrosine metabolism or the benzoate degradation pathway (Davies and Evans 1964). From the application perspective, by interrupting step 5 in Fig. 10, a more linear, oxidized hydrocarbon with the same carbon content as naphthalene could be obtained. The process would both reduce the aromaticity of the compound and generate hydrocarbons that could be hydrogenated under milder conditions. Suitable substrates for the enzymes involved in the pathway are unsubstituted and methyl-substituted di- and tricyclic polyaromatic hydrocarbons, as well as aromatic heterocycles such as DBT and carbazole. Tetracyclic and high molecular weight polyaromatic hydrocarbons (HMW-PAHs) are recalcitrant to biological treatment and do not follow the naphthalene degradation pathway described here, thus reducing the potential of whole-cell aromatic ring opening (Foght 2004).

The naphthalene degradation pathway has been characterized mainly in *Pseudomonas* species. However, partial or complete gene sequences from the pathway have been detected in gram-negative bacteria such as *Sphingomonas*, *Burkholderia*, *Ralstonia*, *Rahnella*, *Polaromonas*, and *Comamonas* and in gram-positive bacteria

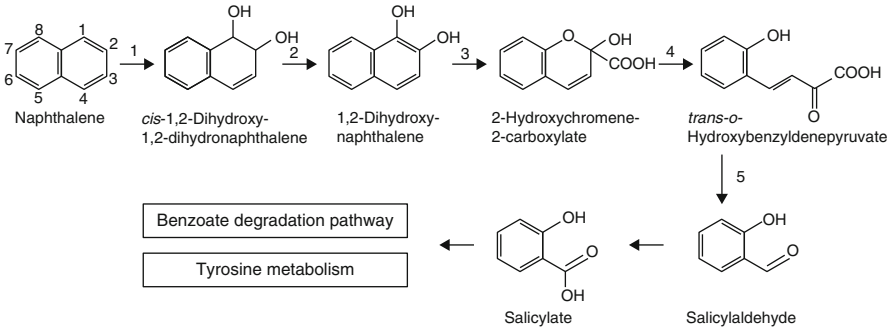

Fig. 10 Naphthalene degradation pathway

such as *Rhodococcus*, *Nocardioides*, and *Mycobacterium* (Ellis et al. 2006; Phale et al. 2007). Given that the genes are identified, both metabolic and protein engineering could be applied to enhance the biocatalyst properties, and the issues explored in BDS also hold in this case.

Specific examples of current research are reviewed by Foght (2004) and Kotlar et al. (2004). The gene encoding the hydratase-aldolase enzyme (step 5, Fig. 10) was interrupted in *Pseudomonas fluorescens* LP6a and *Sphingomonas yanoikuyae* N2, in order to generate mutants capable of oxidatively opening the aromatic ring, without degrading the resulting hydrocarbon. Resting-state cells of the *P. fluorescens* L69 mutant were active in a water–oil biphasic system and catalyzed the ring opening of model compounds such as naphthalene, methyl naphthalene, DBT, 4-methyl DBT, phenanthrene, and carbazole; the biocatalyst also reduced the aromatic content of authentic distillates. On the other hand, light gas oil (LGO) from a refinery was biotransformed with *S. yanoikuyae* N2 mutant in a system containing up to 50% LGO. C8-bencene and unsubstituted naphthalene, acenaphthene, DBT, and anthracene were fully converted. However, even a methyl substituent in the chemical structure of these compounds decreased the conversion of acenaphthene, DBT, and anthracene derivatives to 40% or less. These results emphasize the importance of having a biocatalyst with broad specificity, in order to convert mixtures of chemically diverse compounds.

The selectivity of the enzymes involved in naphthalene degradation is a challenging research area. The naphthalene 1,2-dioxygenase is able to catalyze the regioselective oxidation of substituted and nonsubstituted aromatic compounds (Ellis et al. 2006). However, the high selectivity of the reaction may become an obstacle when dealing with complex mixtures present in the oil industry. Molecular modeling analysis has suggested that even if the oxidation of the aromatic ring is thermodynamically favorable, some compounds may not be substrates for the enzyme; apparently, catalysis requires a specific positioning of the substrate within the active site (Wammer and Peters 2006). In naphthalene, there are two groups of equivalent hydrogen atoms: α-positions are carbons 1, 4, 5, and 8, while β-positions are carbons 2, 3, 5, and 7 in Fig. 10. For 2-alkyl naphthalenes, the only product is *cis*-

1,2-dihydroxy-dihydro-7-alkylnaphthalene; however, although for 1-alkyl naphthalenes the major reaction is dioxygenation of the ring to form *cis*-1,2-dihydroxy-dihydro-8-alkylnaphthalene, monooxygenation of the alkyl substituent to produce 1-hydroxyalkylnaphthalene also occurs. Moreover, the oxidation rate for α-alkylmethyl naphthalenes is lower than for their β-alkyl methyl counterparts (Wammer and Peters 2005). With 1,5-, 2,7-, and 1,8-dimethylnaphthalene, the only products are 1-hydroxymethyl-5-methylnaphthalene, 1-hydroxymethyl-5-methylnaphthalene, and 1-hydroxymethyl-5-methylnaphthalene, respectively. In this sense, it would be highly desirable to relax the selectivity of the enzyme by protein engineering in order to widen the aromatic substrate range. Moreover, there is limited knowledge about the kinetic behavior of enzymes catalyzing steps 2–4 (Fig. 10) when considering long-chain alkyl substituents in the polyaromatic skeleton. A novel area of opportunity is the biodiversity of enzymes now available through metagenomics. Metagenomic analysis of activated sludge and samples from extreme environments in which key dioxygenases of the naphthalene degradation pathway (1,2-naphthalene dioxygenase, step 1 in Fig. 10; 1,2-dihydroxynaphthalene dioxygenase, step 3 in Fig. 10) have been detected could represent another way to obtain versatile enzymes, amenable to BDA (Wang et al. 2012; Jadeja et al. 2014; Ufarté et al. 2015).

Refinery streams that could be upgraded by this technology include light cycle oil (LCO), with a low cetane number (15–25) and composed mainly by aromatics (75–90%, approximately 50% di-aromatic). However, conventional technologies (i.e., hydrocracking) to use LCO for gasoline (instead of diesel) have developed at a faster pace than the biotechnological alternative (Chong et al. 2015); no significant advances can be found in the scientific literature or patents (in the period 2005–2016).

5 Bio-upgrading of Heavy Crudes

Asphaltene is the heaviest and most polar fraction of crude oil. Asphaltenes are believed to consist of associated systems of polyaromatic sheets bearing alkyl side chains with a high content of O, N, and S heteroatoms as well as metals (V, Ni, and Fe) (Fig. 11). Asphaltenes are insoluble in *n*-heptane or *n*-pentane and soluble in benzene and toluene. Wax consists of (1) long-chain alkanes (C18–C36) known as paraffin wax and (2) naphthenic hydrocarbons or cycloparaffins (C30–C60). In reservoirs, paraffins and asphaltenes remain in equilibrium. When crude oil is extracted, this equilibrium is lost due to temperature and pressure changes, and as a consequence, asphaltenes and waxes tend to precipitate. The precipitate form deposits during extraction in oil wells, the blending of oils from different origins, storage and transportation, and refining of heavy fractions.

Waxes that comprise long-chain alkanes are degraded by a variety of bacteria including thermophilic bacteria (Sood and Lal 2008). Such microorganisms are good candidates for a biotechnological application to oil wells with paraffin deposition problems. Microorganisms have been found associated with bitumens with high

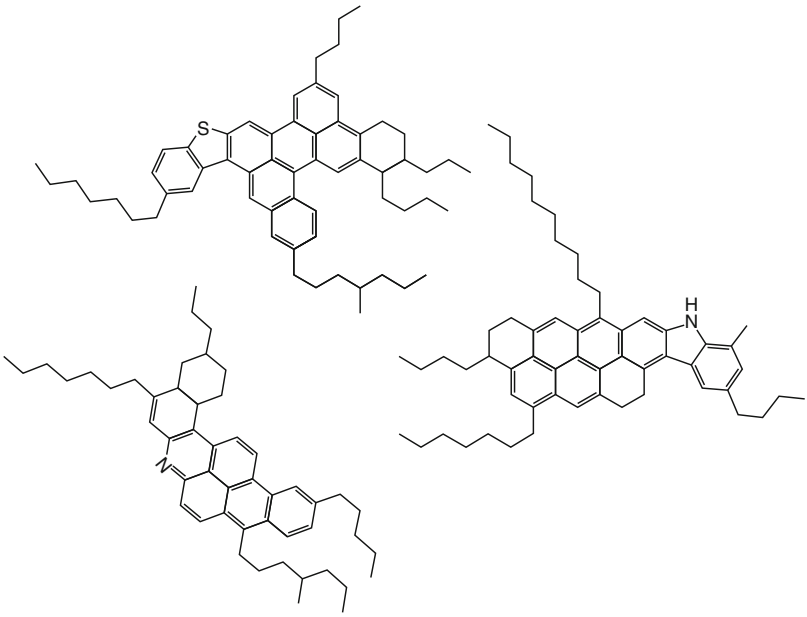

Fig. 11 Hypothetical structure of asphaltene molecules (Adapted from Groenzin and Mullins 2000)

asphaltenes contents (Wyndham and Costerton 1981). A molecular study by Kim and Crowley (2007) revealed a wide range of phylogenetic groups within the *Archaea* and *Bacteria* domains in natural asphalt-rich tar pits; interestingly, genes encoding novel oxygenases were also detected in such samples (Fig. 12). Until recently, there was no clear evidence that asphaltenes can be degraded or transformed by microbial activity; asphaltenic fraction is still recognized as the most recalcitrant fraction of oil. The microbial and enzymatic transformations of asphaltenes have been recently reviewed (Hernández-López et al. 2015a). There are reports, with rigorous experimental procedures, that clearly demonstrate the capacity of some microorganisms to transform asphaltenes.

Early studies found no bacterial growth and no changes in asphaltene content after bioconversion of heavy oils and asphaltenic fractions (Lacotte et al. 1996; Thouand et al. 1999). Few reports on oil biodegradation claimed that the degradation of the asphaltenic fraction was achieved by mixed bacterial cultures (Bertrand et al. 1983; Rontani et al. 1985). However, none of these reports described the analytical results of extractable materials recovered from appropriate sterile controls. An extensive screening involving more than 750 strains of filamentous fungi was carried out to select strains that could modify untreated hard coal (Bublitz et al. 1994; Hofrichter et al. 1997). Only six of the 750 strains tested acted noticeably on the hard coal. The cultivation of one of the most active white-rot fungi, *Panus tigrinus*, on wood shavings coated with asphaltenes led to a decrease of the average molecular weights of these hard coal-derived hydrogenation products (Hofrichter et al. 1997).

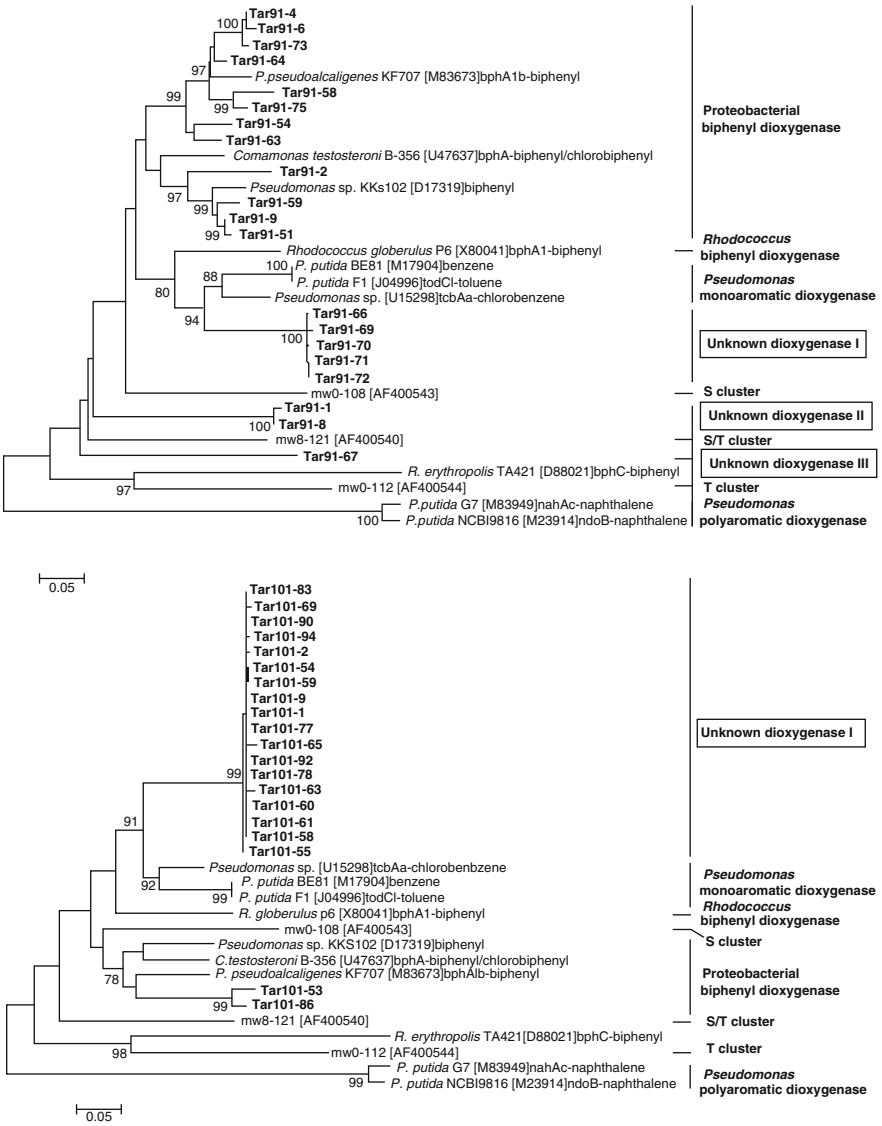

Fig. 12 Phylogeny of aromatic ring hydroxylating dioxygenases identified in asphalts of the of the Rancho La Brea (Los Angeles, CA) Tar Pits (Taken from Kim and Crowley 2007). (**a**) Pit 91, (**b**) Pit 101

Low-molecular weight compounds were found in a tetrahydrofuran extract; however, it remained unclear which enzyme was responsible for this. These results should be considered cautiously, because the asphaltene content was usually determined gravimetrically after *n*-alkane precipitation, and thus the reported changes could be attributed to the disruption of the asphaltenic matrix by the production of

surfactants during bacterial growth, liberating trapped hydrocarbons. Therefore, most of the asphaltene losses during microbial activity could be considered to be abiotic losses (Lacotte et al. 1996). In some cases, inappropriate analytical methods have been used. Recently, Jahromi et al. (2014) quantified asphaltene biodegradation by UV absorption in toluene solutions. In addition, in this work the culture medium contained yeast extract as nitrogen source, which is also a carbon source.

Biodegradation of recalcitrant compounds is faster and more effective when microbial consortia are involved when compared with single, pure, and axenic microbial cultures. In addition, microbial consortia are more resistant to fluctuations of environmental conditions, and they can transform recalcitrant compounds that are difficult or even impossible for individual microbial strains to degrade. A bacterial consortium was assayed for the biodegradation of an asphalt cement (Pendrys 1989). The enriched mixed culture included aerobic bacteria such as *Pseudomonas* sp., *Acinetobacter* sp., *Alcaligenes* sp., *Flavimonas* sp., and *Flavobacterium* sp. Different asphalt fractions were tested, and the polar aromatic and asphaltene fractions showed poor bacterial growth sustainability. It is important to point out that the asphalt cement is rich in asphaltenes but also contains other petroleum fractions. Another microbial consortium was able to grow in mineral medium supplied with asphaltenes as sole energy and carbon source (Pineda-Flores et al. 2004). The asphaltene biodegradation was demonstrated by monitoring the CO_2 production. The microbial consortium produced 800 μmol CO_2 in 13 days. Nevertheless, the two control experiments, cultures without asphaltenes and non-inoculated cultures, also showed CO_2 production of 200 and 300 μmol, respectively. It is important to point out that the inoculum for these experiments was obtained from cultures growing in crude oil, thus the high CO_2 production in the control cultures could be attributed to the presence of residues from crude oil in the inoculum. On the other hand, a consortium obtained from asphaltene-enriched cultures showed the highest asphaltene degradation rate so far (Tavassoli et al. 2012). Five strains were selected based on their higher capacity for asphaltene degradation as a sole carbon and energy source, including *Pseudomonas* spp., *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus cereus*, and *Bacillus firmus*.

There are very few reports on asphaltene degradation by pure and axenic bacteria and, unfortunately, most studies use gravimetric determination to evaluate asphaltene degradation. A halotolerant thermophilic bacterial strain, isolated from enriched cultures with asphaltenes as sole carbon source, reached asphaltene biodegradation rates of 96% after 21 days of incubation (Ali et al. 2014). A decrease in the average molecular weight of the asphaltenic fraction was observed after biodegradation, and bacteria seemed to oxidize thioether linkages of macromolecular structures to sulfoxides and sulfones as suggested by the IR spectra data. A bacterial strain, identified as *Garciaella petrolearia* by 16S rRNA full gene sequencing, was able to degrade different petroleum fractions including aliphatic, aromatic, and asphaltenes (Lavania et al. 2012). Unexpectedly, the degradation of the asphaltene fraction was higher than for the aromatic and the aliphatic fraction.

Asphaltene degradation by *Neosartorya fischeri* was the first report on the fungal degradation of petroleum asphaltenes with rigorously obtained preparations,

avoiding the presence of non-asphaltenic hydrocarbons (Uribe-Alvarez et al. 2011). This fungal strain was isolated from a natural asphalt lake from Orinoco, Venezuela. After culture, most of petroporphyrins were removed. This work clearly demonstrated that there are fungi able to metabolize and mineralize asphaltenes. Further studies showed that *N. fischeri* was also able to transform HMW-PAHs such as coronene, benzo(g,h,i)perylene, and indeno(1,2,3-c,d)pyrene (Hernández-López et al. 2016). The formation of hydroxy and ketone groups on the PAH molecules, together with the HMW-PAH transformation by microsomal preparations, suggested a biotransformation mediated by the cytochrome P450 system (CYP). In addition, the highly hydrophobic substrates are internalized in the fungal hyphae cells. A comparative microarray with the complete genome from *N. fischeri* showed three CYP monooxygenases and one flavin monooxygenase genes upregulated when *N. fischeri* was grown in asphaltenes as sole source of carbon and energy (Hernández-López et al. 2015b). These findings strongly support the role of CYPs in the oxidation of these recalcitrant compounds. Another fungus *Pestalotiopsis* sp. was reported as able to degrade asphaltenes used as sole carbon source. This halotolerant fungus was able to degrade 21.4% of asphaltenes after 15 days of incubation in pure water (Yanto and Tachibana 2013).

Enzymes have been shown to be able to transform petroporphyrins and asphaltenes in organic media where mass transfer limitations of these highly hydrophobic compounds are reduced. CPO and a chemically modified cytochrome c could catalyze the oxidation of petroporphyrin-rich fractions of asphaltenes (Fedorak et al. 1993; Garcia-Arellano et al. 2004; Mogollon et al. 1998). Notable spectral changes in the petroporphyrin-rich fraction of asphaltenes were observed, and the enzymatic oxidation of petroporphyrins led to the removal of 20–57% and 19–52% of Ni and V, respectively. According to FTIR spectra, the chemically modified cytochrome c catalyzed the oxidation of sulfur and carbon atoms of asphaltene molecules. On the other hand, as mentioned before, a monooxygenase family of CYPs has been reported for the first time to be involved in the first oxidation step during the fungal degradation of asphaltenes and high molecular weight hydrocarbons of more than five aromatic rings (Hernández-López et al. 2015b, 2016). CYP-mediated reactions for PAH transformation have been also reported in filamentous fungi (da Silva et al. 2004) and in fungi lacking extracellular oxidoreductases (Cerniglia and Sutherland 2010). The role of CYP in the biodegradation of asphaltenes and PAHs is also supported by the effect of specific inhibitors for CYP activity than can significantly inhibit phenanthrene transformation and asphaltene degradation (Ning et al. 2010; Yanto and Tachibana 2014). Recently, a specific CYP63A2 responsible for oxidation of PAHs has been described in the transformation of the six-ring benzo(g,h,i) perylene by *Phanerochaete chrysosporium* (Syed et al. 2013). Six PAH-responsive CYP genes inducible by PAHs were identified by genomic P450 microarray screening in this fungus (Syed et al. 2010). The enzymatic treatment of asphaltenes is an interesting alternative for the removal of heavy metals in order to reduce catalyst poisoning in hydrotreatment and cracking processes. On the other hand, the introduction of polar groups in asphaltene molecules could positively affect their sedimentation properties and improve their behavior.

The main challenges for an enzyme-based process applicable to the oil industry are well identified (Ayala et al. 2007). High activity in viscous organic media with low water content may be the most important issue for the application discussed in this section. Research should focus on creating a robust biocatalyst; a number of molecular and chemical tools such as protein engineering, chemical modification, and immobilization must be combined in order to enhance enzyme activity and resilience to both the presence of organic solvents and temperatures that are adequate for processing heavy fractions (normally above 100 °C). A second research field is process design. Probably the most important issue to be solved is mass transfer limitations or how to favor partition of a hydrophobic molecule from an oil phase to an intrinsically polar environment such as the active site of an enzyme.

6 Research Needs

Bio-based technologies for the processing and upgrading of fuels and heavy oils offer potential alternatives to conventional physicochemical technologies used in the oil industry. The main advantage of biocatalysts is that they operate under milder and less energy-intensive conditions; they do not consume hydrogen and emit considerably less CO_2 and GHG than conventional refining and petrochemical processes. The main applications proposed are desulfurization, denitrogenation, and reduction of aromatic, asphaltene, and metal content. Future specific targets are the biorefining and bio-upgrading of distillates and other petroleum fractions in refineries and the bio-upgrading of crude oil at the wellhead. However, these processes pose technical challenges plus new and complex scientific questions that must be overcome in order to reach commercial stage.

Technological challenges still comprise high activity and stability under process conditions (high temperature, low water content), adequate process configurations to allow high microbial activity, minimization of mass transfer problems, and enhancement of oil–water separation. Microbial processing of fuels clearly differs from other more common biotechnology processes. Although very intensive research and development has been conducted in BDS and many bacteria have been isolated and intensive genetic engineering improvement realized, the main challenge is still the design of a more active and resistant microbial catalyst. Even with intensive improvement of strains, the desulfurizing activity is still too slow compared with chemical reactions employed in a refinery (El-Gendy and Speight 2016). Any improvement in thermal stability, rate, or overall operational stability could be significant. In this sense, new concepts to be explored include genomes and metagenomes databases mining, the design of microbial consortia to deal with complex hydrocarbon mixtures and evolutionary engineering of microorganisms to achieve multi-target modifications.

Concerning desulfurization, the HDS, ODS, adsorption, and BDS technologies should be seen as complementary options where HDS would be the basic technology for obtaining low-sulfur fuels followed by one of these new technologies or a combination of them to remove the most recalcitrant sulfur species for ultralow-

sulfur fuel production. This is important in the context of increasingly stringent environmental regulations and conventional oil depletion, where both the fuel and the technologies to produce them should be cleaner. BDS and other bioprocessing technologies should also be explored for application at the wellhead to improve crude oil properties and refinery feedstock quality by lowering viscosity of the oil and decreasing the content in high molecular weight hydrocarbons, asphaltenes, sulfur, nitrogen, and/or metals (Speight 2011). The microbial and enzymatic cracking of PAHs and asphaltene molecules should not be excluded: the recent reports on microbial degradation of asphaltenes and the presence of microorganisms with new and previously undetected oxygenases in environmental metagenomes may be a potential source of biocatalysts that justify the exploration of the biotechnological alternative to upgrading heavy oils. Water is coproduced with the oil, and the process of water and oil separation is a routine field process. The biocatalyst could be added at this stage, and the only added step would be agitation of the oil and water mixture in the presence of the biocatalyst. The resulting aqueous stream could be reinjected as part of the oil recovery operations or used for the production of valuable chemicals. The concept of valuable chemical production has been recently reintroduced by Kilbane and Stark (2016). This idea had initially been proposed by EBC and Enchira to lower the cost of the entire BDS process by producing HBPS, a potentially valuable surfactant, instead of HBP as mentioned before. Sulfones are interesting molecules that could be used as building blocks for polymers, plastics, and antibiotic productions (Kilbane and Stark 2016).

The main obstacle for the implementation of new technologies is their cost. Not only scientific and technological advances will influence the use of biotechnology in the oil and energy industry (Kilbane 2016). The price of fossil fuels, politics, climate change, land and water use, resources availability, as well as the increased use of solar and wind energy will impact the panorama. Biotechnology could be integrated in the refinery of the future that could in turn become the base for the development of biorefineries (Speight 2011; Biernat and Grzelak 2015).

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Part IV

Global Consequences of Bioproduction of Fuels and Chemicals

Global Consequences of Bioproduction of Fuels and Chemicals: An Introduction

39

Andrew Hagan

Contents

Abstract

Computing power has increased dramatically in the last 30 years leading to new technologies and possibilities. This could be better described as an evolution rather than revolution. However, two factors are creating global revolutions. They are environment and sustainability and biotechnology. This chapter explores the link between the two and the potential of industrial biotechnology to contribute to addressing global challenges in a systemic way; looking at the global megatrends and challenges, sustainable development goals, planetary boundaries, GDP, economic and qualitative growth, circular economy, distributed manufacturing, health and nutrition, new related technologies, leveraging other emerging technologies,

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and ITC. It looks not just at direct impact but indirect secondary and tertiary effects. It hints at the potential of combining biotechnology with the increased computing power and move to the digital world. Finally, it highlights that there are potential disadvantages and risks in addition to the benefits and that these need to be mitigated.

1 Introduction

There has been much reference to experiencing a "Fourth Industrial Revolution" at this moment.[1] This generally refers to the digital and dematerialization that have been afforded by increased computing power. These could be argued to be incremental, but at least two aspects of the current time appear truly revolutionary. These include the environment/sustainability/climate change and also biotechnology. They are both fundamentally changing the way we think about things, the way we approach things, and indeed the way we live and they threaten to do so a lot more. Indeed, these two revolutionary aspects are very much intertwined. Industries and society will all be affected by digitization as well as environmental change and biotechnology.

Biotechnology and bioproduction of fuels and chemicals has a huge potential to mitigate key environmental aspects and will also affect many other aspects of our lives: financial economics, technologies, civil society, sustainability, and security among others.

2 Biotechnology, SDGs, and the Global Megatrends and Challenges

Bio-based products can help address some of the world's mega trends and challenges. These can be enunciated in different ways. At the World Economic Forum's Collaborative Innovation Platform, created by this author, the Chief Innovation Officers of some of the biggest global companies listed nine of them in 2009, yet they remain valid today. Biotechnology and bio-based fuels and chemicals can help address these as below (Fig. 1):

Additionally, bioproduction of fuels and chemicals help achieve the Sustainable Development Goals (SDGs), or "Transforming our world: the 2030 Agenda for Sustainable Development" and their 17 "Global Goals" with 169 targets between them.[2] Elaboration on how the bioproduction can contribute to addressing goals 1–3

[1] Some have referred to Industry 4.0 too, and the World Economic Forum has adopted this term for its work around digitization.

[2] Spearheaded by the United Nations through a deliberative process involving its 193 Member States, as well as global civil society, the goals are contained in paragraph 54 United Nations Resolution A/RES/70/1 of 25 September 2015. (1) The Resolution is a broader intergovernmental agreement that acts as the Post 2015 Development Agenda (successor to the Millennium Development Goals). The SDGs build on the Principles agreed upon under Resolution A/RES/66/288, popularly known as The Future We Want. (2) It is a nonbinding document released as a result of Rio + 20 Conference held in 2012 in Rio de Janeiro, in Brazil.

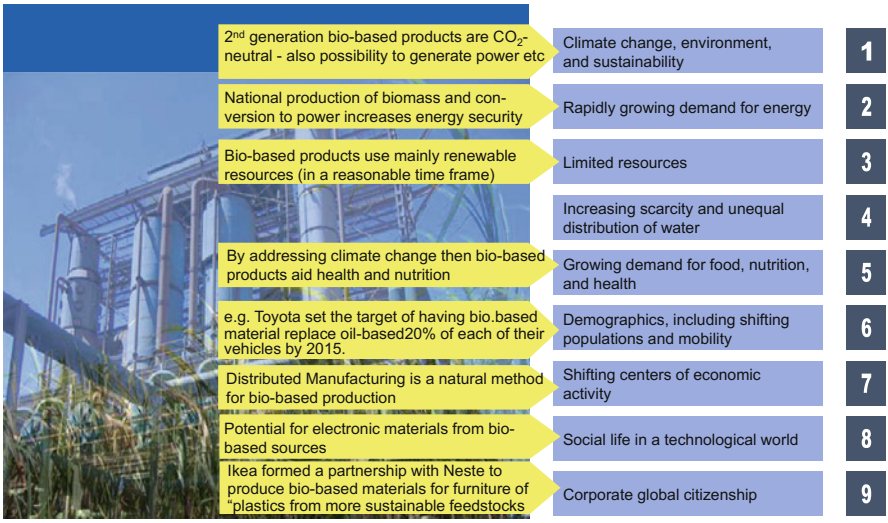

Fig. 1 Industrial biotechnology impact on global megatrends (Adapted and updated from work by the author when at the World Economic Form)

(No Poverty, Zero Hunger, Good Health and Well-being) is outlined below. They are linked to goals 7: Affordable and Clean Energy, 12: Responsible Consumption and Production, and 13: Climate Action. Arguably this, in turn, could also contribute to addressing goal 11: Sustainable Cities and Communities. Goals 8: Decent Work and Economic Growth and 9: Industry, Innovation, and Infrastructure are more goals that are strongly linked and could likely have a strong impact from the bioproduction of fuels and chemicals.

It is of course true that of the nine planetary boundaries proposed by Rockström at the Stockholm Resilience Centre, climate change is not the only one and, depending on how bioproduction is done can have a positive or negative effect and can vary significantly depending on the calculations used. At one extreme, GranBio have suggested at COP21 in Paris that using energy cane as a feedstock to produce ethanol has a negative carbon footprint with a carbon intensity of −9.67. Whereas people such as (Searchinger et al. 2009; Smith and Searchinger 2012) have proposed the opposite. There is a need for more research into a scientifically sound and accepted method of life cycle analysis.

Climate change and global warming are scientifically largely accepted and bio-based production can contribute to many industries' efforts to reduce impact. An example is given for the chemical industry by the World Economic Forum by this author and support from McKinsey in 2007. The Greenhouse gas abatement cost curve for the industry can be seen here. It highlights areas where biotechnology will have an impact: industrial feedstock substitution, cellulosic ethanol production for fuel, and enzymes and organisms (Fig. 2).

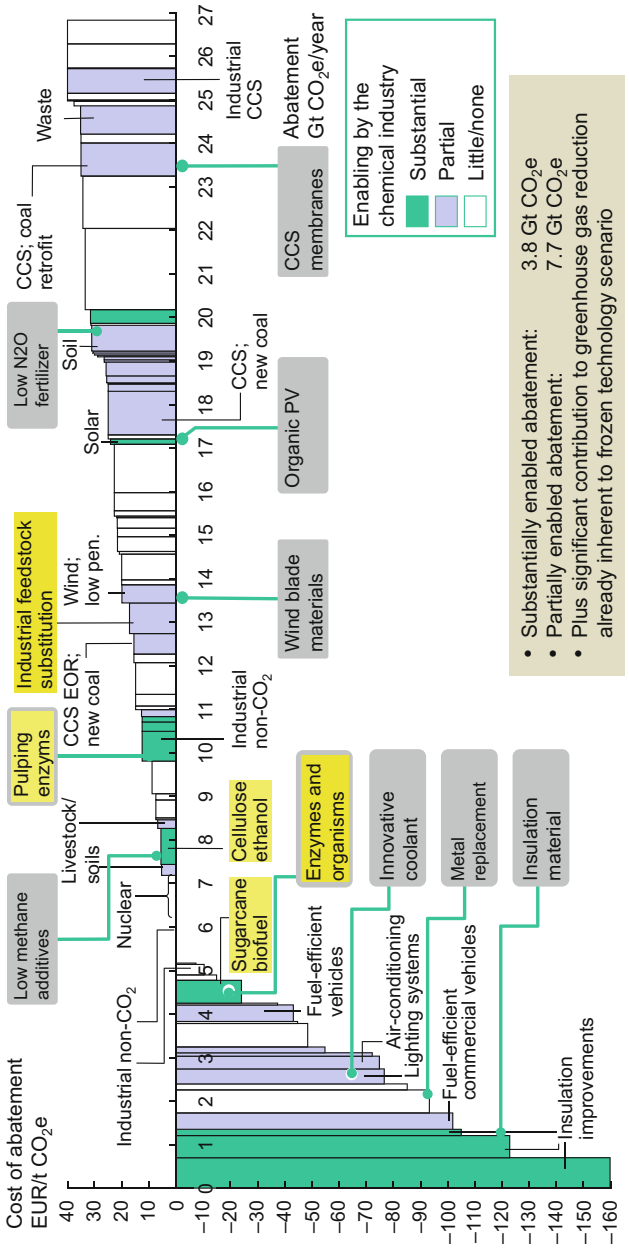

Fig. 2 Global greenhouse gas abatement cost curve for the chemical industry (Developed by the author for the World Economic Form with support from McKinsey)

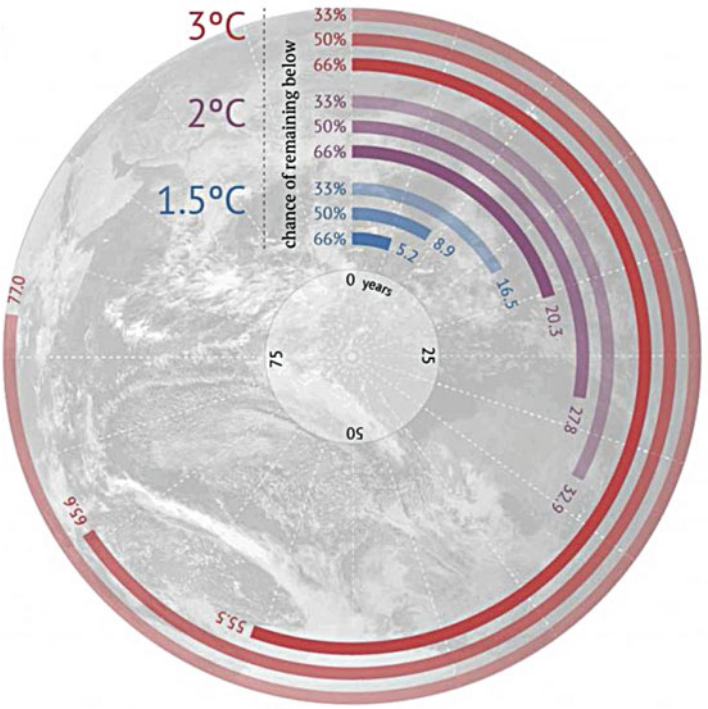

Fig. 3 Carbon probabilities stopclock under business as usual (Reproduced from the carbon Brief, 2016, www.carbonbrief.org/analysis-only-five-years-left-before-one-point-fire-c-budget-is-blown/amp)

The issue of climate change is an urgent one and one of the most clear existential threats. The Carbon Brief/NASA Goddard Space Flight Center has produced a powerful carbon countdown stopwatch image. It shows the number of years we have left from 2016 to remain below certain temperature increases with a given chance of doing so if we continue doing business-as-usual. For example, to remain below a 1.5 °C increase in temperature with a 66% chance of doing so would give until 2021 under a business-as-usual scenario. To remain below a 3 °C increase in temperature with a 33% chance of doing so would give until 2093 under a business-as-usual scenario. The Paris climate agreement from the COP15 discussions in Paris in 2015 aimed to enhance the implementation of the UNFCCC through, "Holding the increase in the global average temperature to well below 2 °C above pre-industrial levels and to pursue efforts to limit the temperature increase to 1.5°C above pre-industrial levels, recognizing that this would significantly reduce the risks and impact of climate change." To limit the average global temperature increase with a 66% chance to 1.5 °C and 2 °C above preindustrial levels under the business-as-usual scenario would then give us 4.2 and 30.3 years, respectively. Contributions

to abating this from bio-based production then help address an urgent existential risk (Fig. 3).

There is also the potential detriment in land system change, biodiversity and fresh water use. This is currently an underexplored area and the calculations of where the feedstock will come from and biomass to be used needs further investigation. There are many varied numbers on biomass in different regions and very little related work on effects on biodiversity, and especially scenarios if land use was changed; even from one crop to another.

However, on mitigating climate change, while there appears to be much acceptance that it can be a huge benefit this rests on it being a "renewable" source or energy. Notwithstanding all energy being renewable this suggests a time factor. This concept can be important with regard to GDP and is elaborated below.

3 Bio-Based Production and GDP/Economic Growth

The current global economic environment has little to no growth in the G7 countries. Their GDP in 2015 and 2017 have all been below 2% growth with the exception of the UK (2.2% decreased to 2.0 in 2017 so far) and the USA (2.6% decreased to 2.3% in 2017).[3] It can be argued that there is never any real growth except to recapture the value of destroyed capital such as through war and bubbles (dotcom, property, etc.). Destructive creation for GDP growth could also take the form of natural disasters. Nourim Shabnam reviewed work on natural disasters and economic growth and suggested the work is still in its infancy (Shabnam 2014). He does suggest that, "Given the complex relationship between natural disasters and economic growth, researchers need to examine the potential effects of natural disasters in three distinctive phases: short-run (for example, emphasizing political transitions through economic growth), medium- to long-run (for example, focusing directly on natural disasters and economic growth), and very long-run (for example, highlighting deeper indicators of growth)." He hints at a likelihood of a GDP growth as a result, especially in the very long-run. Existing papers on economic growth are overly complicated and make it difficult to conclude any clear mechanism of GDP growth. One proposal is that it comes from the destructive creation suggested above.

However, while it is difficult to make or prove the case for unrealized potential leading to GDP growth when the catch up is made there, it seems an area that could be a driver of GDP growth with examples such as the restraint and release of the Chinese economy in the last half century. It would be analogous to the physical sciences term of potential energy. Those two areas of destructive creation and unrealized potential are not about incremental increases nor productivity gains. They are much more fundamental.

[3]IMF data.

There has been a growing emphasis on the need to bring in environmental and ecological factors since the debate on growth began in earnest in the late 1960s. Perez-Carmona (2013) reviewed much work in 2013 from the Limits to Growth, (Meadows et al. 1972) Bruntland, (World Commission on Environment and Development 1987) Stern, (2006) and the Green Economy report (United Nations Environment Programme 2011) among others but with a large focus on the de-growth of Serge Latouche (2003, 2004, 2009) and the SSE (Steady or Stationary State Economy) of Herman Daly (1972, 1979, 1991).While some reports such as Stern have GDP growth as a fundamental assumption and target, others look to limit that growth and look more towards "qualitative" growth or "economic development." Latouche and Daly fall into the latter category and where "the SSE is, as the name indicates a state, while de-growth indicates motion. The discussion will ultimately rest in:

1. The physical quantities which economies need (population and man-made capital) for the good life in the long run;
2. How to decide on them, that is, biophysical limits, Daly's metrics, and Georgescu-Roegen's organic agriculture/population proposal;
3. How to achieve them, that is, Latouche's cultural change and a dynamic understanding of democracy; and
4. How to maintain an approximate steady-state."

Perez-Carmona acknowledges the criticisms of such proposals include that "their proponents want industrial societies to go back to the caves, or rather to the trees." That argument does neglect the focus and use of innovation and technology to achieve their goals. Yet innovation and technology have little clear effect on growth or not. This has been the subject of many articles such as by Wolf (2015) reviewing the technology progress in the last centuries or Maranda on countries in Europe (Maradana et al. 2017).

Daly's quantitative restrictions to address the Jevon's paradox with quantitative limits selected according to the most stringent necessity (depletion or pollution) and letting production/ consumption to adapt to the new prices are worth noting. However, of the few differences between the two proposals, one was in waste recycling which Latouche advanced but gave Daly reservations given the entropy law.

This appears to approach a circular economy argument. This is developed more by Stahel and others over the last 40 years, and he recently suggests a performance economy (Stahel and Clift 2016). This is "a concept which goes beyond most interpretations of a 'circular economy': the focus is on the maintenance and exploitation of stock (mainly manufactured capital) rather than linear or circular flows of materials or energy. The performance economy represents a full shift to servicisation, with revenue obtained from providing services rather than selling goods." Stahel and Clift suggest that such a focus on the quality, value, and use of stock would remove or lessen flows and GDP. However, that is not necessarily the case; value add and the flow of goods and services will still happen, particularly if utilizing "waste." That can necessarily add to value add and GDP.

When that is built in in a full and fully integrated cycle, then it should allow for GDP growth while using the same resources. A steady state of materials and resources, but with economic growth. This can be further developed with policy interventions. For example, a nation state wishing to lease its resources instead of selling them would ensure that resources are still in the state's portfolio balance. They would lease to add value. This concept is explored in an article, "The Licence to Mine," (Hagan A, Tost M, Inderwildi O, World Economic Forum, unpublished) by this author, together with Inderwildi and Tost, submitted seeking publication. Moreover, much emphasis is placed on so-called renewable energy or a "low carbon" society. Noting the Law of Conservation of Energy (energy cannot be created or destroyed, only changed from one form to another) and the first law of thermodynamics then all energy is renewable. The commonly accepted phrase of "renewable energy" then mainly refers to a timescale. Technically, oil can be regarded as a biofuel, just created over millions of years under extreme conditions in catagenesis.

The destruction of our environment and the untapped potential of biotechnology, from crop yields and human health and performance to bio-based products present cases for recapturing destroyed capital and recapturing unrealized potential. As such, bioproduction of fuels and chemicals offers promise for economic growth, financial, or otherwise. The white paper on The Future of Industrial Biorefineries (King et al. 2010) already put the global value chain business potential at almost US$300 billion (Fig. 4).

Moreover, if we nurture trees and the environment then it does not augment GDP. If we cut them down and sell them, then it does as outlined by Fioramonti (2017) in his new book. However, it is not a binary choice. We can easily do both and here we enter into the Circular Economy discussion. The stock would have to be managed, but it does not itself have to be static and mechanisms such as replacing can maintain while being used. This is significantly easier and quicker than letting nature replace oil and gas, which would take a long time as mentioned above.

However, industrial biotechnology can offer new mechanisms around this. Industrial biotechnology can help in oil recovery from areas that do not readily allow conventional methods to succeed by microbial enhanced oil recovery (MEOR). This refers to the use of microorganisms and their metabolic products, but there are other processes such as biodesulfurization, biodemetallation, biodenitrogenation, and biotransformation. That may contribute to the economy overall but does not necessarily lead to a circular economy (Bachmann et al. 2014).

Industrial biotechnology and metabolic engineering can also be used to produce gasoline (Choi and Lee 2013) and other petroleum derived products and chemicals. Although this field is still in its infancy, it does present a tool to significantly reduce the time to their manufacture adding value add and to GDP.

There are a number of suggestions on putting value on our resources and nature. Crude oil, minerals, and metals have this already, but less so for others. There have been suggestions on how to put a price on natural systems and ecosystem services (Turner 2007) including coral reefs (Burke and Maidens 2004) and even on

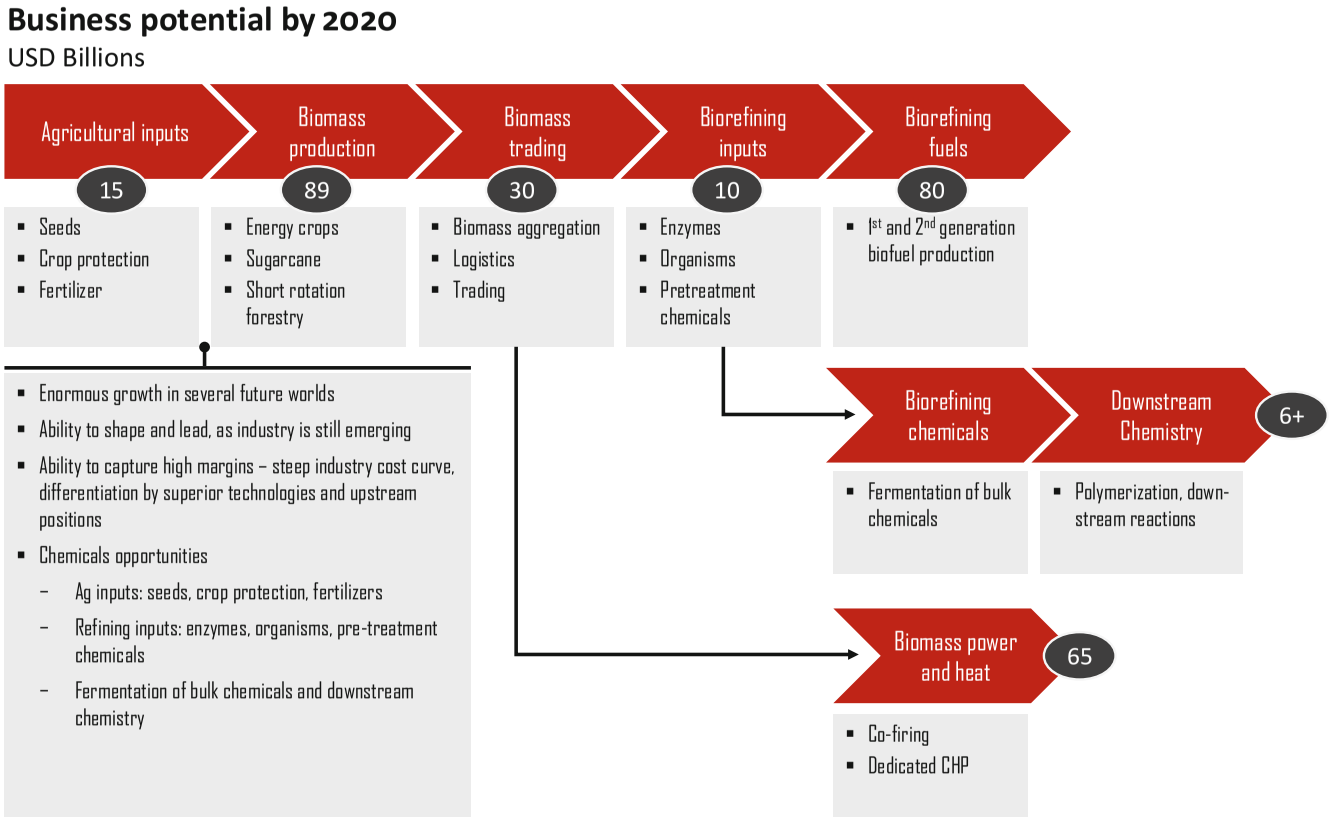

Fig. 4 Business potential of bioproduction value chain (Adapted from King et al., World Economic Forum, 2010)

individual species using public surveys on "willingness to pay" (Martin-Lopez 2008). There are now emerging experiments to create a commons transition using newly available tools harnessing the increased computing power and connectivity. In Ecuador and New Zealand, they are even looking at trying to give legal personhood to non-people such as rivers.

Such a financial economic figure does not speak of the employment gains and the secondary effects enabling delocalized and distributed manufacturing in remote locations such as rural communities or airports. EuropaBio says that industrial biotechnology already contributes 30 billion Euros to the economy and is responsible for 500,000 jobs. It also suggests that for every job in industrial biotechnology, four more are created indirectly. They expect the numbers to grow to between 900,000 and 1.5 million jobs with an accompanying economic contribution of up to almost €100 billion by 2030 (Debergh et al. 2016).

4 Distributed Manufacturing

There is also a global trend towards personalization and towards distributed manufacturing. The latter is driven by three factors: nationalism, severe weather, and fickle customers, which is sometimes a reflection, manifestation, or extension of the first of the drivers. Andre Wenger, founder and CEO of Authentise gives examples of this including Toyota's loss in manufacturing after the tsunami in Japan in 2011 severely reducing production (600 k units from production volume) due to centralized manufacturing (and little scope to switch), and Zara's switch to moving production (up to 50%) to different locations nearer to markets in season to reduce inventory waste (by 30%).

As Spencer Dale, Chief Economist of BP Plc. said, "Suppose people start buying EVs because of what it says about them, rather than the economics. All the economic modelling we do may get thrown out the window." People or fickle customers buy Teslas based on this. Buyers can customize their entire car online, on their own. They can choose the number of batteries and seats, add air filtration, smart air suspension, sound systems, towing gear – all in the space of 5 min. Then, 2 weeks later, the bespoke car is delivered.

Automobile company Daihastu and 3D printer manufacturer Stratasys said in 2016 that clients of Copen would be able to design and assemble customized 3D printed exterior panels. Recently, they announced that the large-scale customization system is fully prepared and is waiting to be tested. Customers can choose their own "skins" and change them within 2 weeks changing color and even exterior panel design.

Arguably even the decline of printed media can be attributed to the desire to tailor the product. This all means that being close to the market and customers is becoming more important. The recent impact of hurricanes such as Harvey on the US chemical installations has had a marked reduction of ethylene output. Political drives in the USA and UK have shown an increasing nationalism not to mention countries such as Poland and Hungary.

Bio-based production, including of fuels, allows a move to even remote locations and why it is of interest to the mining industry. Their mines are often in remote

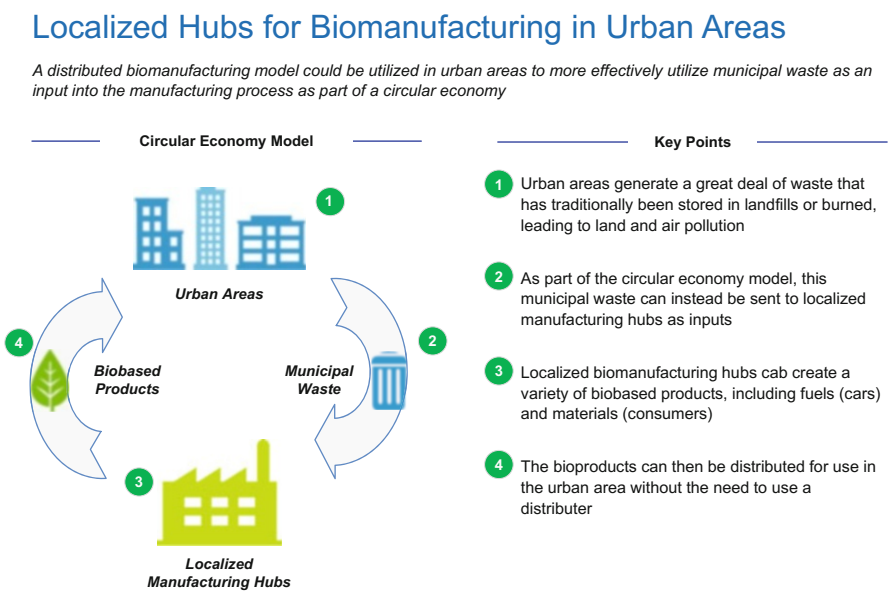

Fig. 5 Localized hubs for biomanufacturing in urban areas (Reproduced from this author's work at The World Economic Forum with support from Deloitte)

locations. With bio-based fuel production then outside a radius of 60–100 km in current conditions then the environmental impact of collection and transport to the production site starts to outweigh any advantages. For bio-based production of aviation fuel then this means that distributed manufacturing is helpful. Waste from many sources such as agriculture can also be used in such plants benefiting the environment and adding value thereby increasing GDP (Figs. 5 and 6).

5 Biotechnology and Health and Nutrition

The food and beverage industry is merging with the health and nutrition industry and industrial biotechnology will have a big impact on this. Regardless of the geography, the local agricultural produce will also change as a result of climate change and global warming as outlined in Haddad et al.'s "A New Global Research Agenda for Food" (Haddad et al. 2016). The dependency on this for food supply will be tested and likely collapse in many places if we continue in business as usual. Consequently, the access to affordable nutritional food will be diminished and likely increase already mounting health concerns such as some of the WHO's biggest issues; poor diet and nutrition and obesity. By contributing to mitigating these global warming effects, industrial biotechnology will be having a huge impact when you look at the knock-on effects and financial and economic concerns of society.

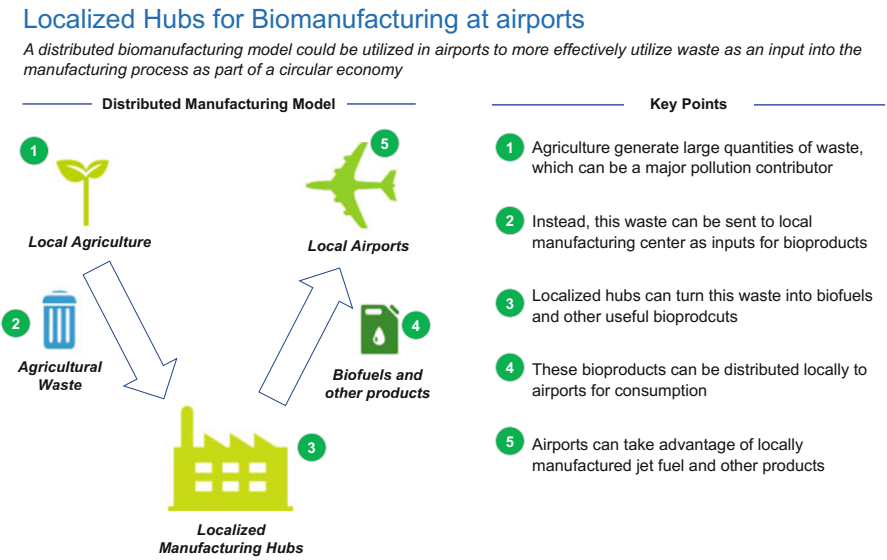

Fig. 6 Localized hubs for biomanufacturing at airports (Reproduced from this author's work at The World Economic Forum with support from Deloitte)

In terms of healthcare, this contribution can enable cost reduction. By one estimate the USA spent US$190 (Cawley and Meyerhoffer 2012) on obesity-related health care expenses in 2005, and it is growing in the developing industrial economy world. The WHO's number one risk for cause of mortality is poor diet and nutrition. This, in itself, has significant knock-on effects. According to 2015 Nobel Laureate for Economics, Angus Deaton, adult height is set in early childhood by net nutritional levels and changes little in adulthood, but it appears to have an impact on economic potential (Deaton 2007; Deaton and Arora 2009). Deaton says shorter people did not have the right nutrition to achieve their full genetic potential. For every 1 cm grown, he says, it brings a 1% increase in earnings. In the USA and the UK over the last 50 years, average height has increased by 1% per decade and with it, GDP has increased. According to Haddad et al., the economic consequences represent losses of 11% of gross domestic product (GDP) every year in Africa and Asia, whereas preventing malnutrition delivers $16 in returns on investment for every $1 spent.

Barbara Sahakian, professor of clinical neuropsychology at the University of Cambridge has linked good nutrition from birth through adolescence to prevention of mental health problems. Both affect societal and health burdens and diminish the potential of individuals and of a nation. As such, bio-based production of fuels and chemicals, by helping to mitigate climate change and its effect on agriculture, food and nutrition, contributes to the health and wealth of a nation.

6 Bio-Based Production, Oil-Based Production, and Price Volatility

In the past century, much of a nation's wealth has been created from the use of oil and gas taking advantage of its high energy density. However, there has been much volatility in the oil price and Ebrahim et al. suggest that volatility has several damaging and destabilizing macroeconomic impacts that will present a fundamental barrier to future sustainable economic growth if left unchecked (Ebrahim et al. 2014).

McNally (2017), in his book, *Crude Volatility: The History and the Future of Boom-Bust Oil Prices*, believes that global oil markets are entering an epic, structural shift to a protracted new era of wildly fluctuating crude prices. The reason is that the market now lacks a swing producer to level out production and demand shifts. He suggests, we are in for an extended period of prices oscillating "in a range well below $30 and above $100" per barrel with the crunch starting maybe as early as the 2020s (Fig. 7).

If McNally is right and the huge swings we have seen in the last decade, from $145/barrel to $26 in 8 years, are the new norm, then oil's reliability as a transportation fuel will increasingly be called into question and the search for alternatives will accelerate. Oil's reputation as a reliable transportation fuel depended not only on its abundance but also its stable pricing.

It is clear that since the USA started producing shale gas then OPEC has lost its ability to be a swing producer. Indeed, under President Trump's relaxing of drilling restrictions, Bloomberg (February, 2017) suggested that US exports are poised to surpass production in four OPEC countries in 2017. The issue of shale gas and other unconventional oil sources is examined in The Feedstock Curve (Inderwildi et al. 2014).

As King (2014) suggests, it is the elasticity that can reduce that risk and potential spikes. Therefore, alternative sources and feedstocks have a much broader economic effect. This makes a good case for shale gas and bio-based materials and sources. Indeed, the two can complement each other if gas powers a biorefinery or molecules above four carbon chains are needed. McNally also suggests that the oil price volatility could lead to a search for a more stable-priced fuel such as biofuels.

There is an increasingly linked systemic holistic effect and chemical routes to fuel become even more interesting. However, we must be cautious as presently gas is being sold at a price below cost of production due to lease contracts which encourage "drill or lose." By implication then, high oil prices could be seen to be subsidizing gas. Gas being used to make fertilizer influences biomass prices.

Even in times of a low oil price bio-based fuels and chemicals can benefit for several reasons. The RFS in the USA creates a separate market for renewable fuels and they compete with each other within that market. This is independent of oil price and so the focus is on volume not price. During a period of low oil price, and especially when combined with the growing political demand to reduce greenhouse gas emissions, the investment in refineries is less. If that is sustained, and trends such as car sharing or consumer demand for environmentally friendly and socially responsible products take hold pushing more demand away from petroleum or gasoline, then, eventually a substitute such as bio-based could be sought. Some

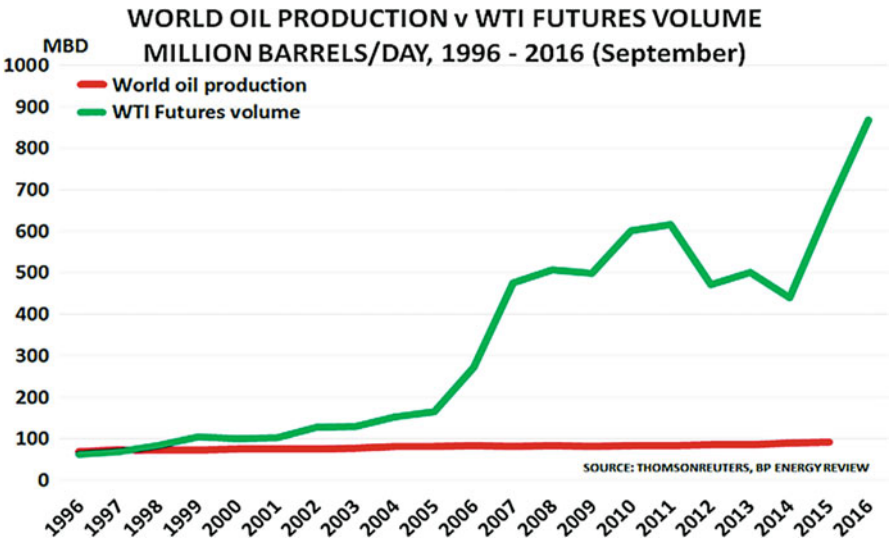

Fig. 7 World oil production V WTI futures volume

chemicals have significant advantages to producing by using a biotechnology route, fermentation, such as serono or PDO.

Bio-based production of fuels and chemicals then has a large potential to help the global economy by mitigating price volatility of oil and gas.

7 New Technologies Examples

The demand for environmentally "cleaner" products and fuels has driven new technologies and innovation and will continue to do so. One example is Daniel Nocera's (2013–2017) "artificial leaf."

Professor Nocera has genetically engineered bacteria to absorb hydrogen and carbon dioxide and convert them into alcohol fuel. Nocera was initially aiming for 5% efficiency – approximately five times better than plants. Now at the University of Chicago, he recently announced his bug, dubbed by his colleagues the "Bionic Leaf," converts sunlight ten times more efficiently than plants.

His group is now making isopropanol, isobutanol, isopentanol, from hydrogen from split water, and it is breathing in CO_2 using metabolically engineered bacteria. Nocera's artificial leaf had interest 5 years ago because the wafer of silicon and other elements can be dropped in water, exposed to sunlight, and it will continuously split the water into hydrogen and oxygen. Nocera has an even bigger vision for the basic technology. Beyond producing hydrogen and carbon-rich fuels in a sustainable way, he has demonstrated that equipping the system with a different metabolically altered bacterium can produce nitrogen-based fertilizer right in the soil, an approach that would increase crops yields in areas where conventional fertilizers are not readily

available. The bacterium uses the hydrogen and CO_2 to form a biological plastic that serves as a fuel supply. Once the microbe contains enough plastic, it no longer needs sunshine, so it can be buried in the soil. After drawing nitrogen from the air, it exploits the energy and hydrogen in the plastic to make the fertilizer. Radishes grown in soil containing the microbes ended up weighing 150% more than control radishes. This is another large global impact if bio-based production of fertilizer can be achieved.

8 The Need to Bridge and Leverage the Digital and Computing Power

To fully harness biology, it requires better understanding of the data, systems, and processes. Harnessing the recent increased computing power available will deliver even more of an impact from bio-based sources. That, in itself, brings needs and bespoke operating systems such as "antha," a language designed for biologists. Sadowski et al. highlight that building robust manufacturing processes from biological components is a task that is highly complex and requires sophisticated tools to describe processes, inputs, and measurements and administrate management of knowledge, data, and materials (Sadowski et al. 2016). They realize that "when experimenting with very large numbers of factors, which is most likely necessary, experiments are beyond the limit of what humans can reliably perform. Solving this problem ultimately requires automation, but the current state of the art in laboratory automation is very much geared towards the need to do a single process repeatedly and is not well suited to implementing designed experiments."

They argue that for bioengineering to fully access biological potential, it will require application of statistically designed experiments to derive detailed empirical models of underlying systems. This, in turn, requires execution of large-scale structured experimentation for which laboratory automation is necessary. This requires development of expressive, high-level languages that allow reusability of protocols, characterization of their reliability, and a change in focus from implementation details to functional properties.

Of the three important technologies they discuss, they suggest that the development and adoption of high-level machine languages for executable bioprocesses. Their reasons for this include: "(i) They provide an unambiguous medium of exchange and facilitate reuse (ii) They remove the artificial separation between description of a process and its executable form (iii) Abstraction and domain language based terminology enhance readability and remove unnecessary implementation details (iv) Machine writing of processes is facilitated, enables automated optimization and avoids human error (v) Practices for managing complexity in software development become available: version control, automated testing and validation, code validity checking, and others."

It is the combination of biotechnology with the new tools provided by developments in computing power and artificial intelligence that offers perhaps the greatest potential impact for biotechnology and bio-based production.

9 Security Risks

With every new innovation, technology, and solution will come unforeseen risks. Bio-based production and combinations of this and techniques such as metabolic engineering with new technologies will bring clear problems that need to be addressed urgently. One clear aspect that needs to be addressed and mitigated is international security.

When the financial markets price a performance in the value then that performance, when it occurs, changes little in the price. People were prepared for it. Sterling was not prepared for the UK's vote to leave the EU. The world is expecting many biotechnology security issues around big pharma companies and states and traditional methods. It has even reared its head in popular culture such as *Mission Impossible 2*, *Iron Man Three*, *12 Monkeys*, *The Congress*, *District 9*, *Gattaca*, *Planet of the Apes*, *The Stand*, *Transcendence*, and goes back to *Dr. Jekyll and Mr. Hyde*. However, little has been done to prepare.

Yet it is not the market-considered mainstream that we should be concerned about. Illusionists do not make a living by acting in the central gaze and criminals do not act in the policed structures of the Law. Therefore, it is the peripheral vision of the non-policed start-ups and SMEs and forgotten biotech behemoths and future tech that we should be wary of. Their main concern now must be symmetric threats such as cyber security, hacking, data theft, etc., and we hope they have counter measures should their R&D be stolen.

Scotland's whisky and beer industry alone has the biotechnology power to enable massive biotech experiments and development and particularly with mothballed facilities which are desperately pushed out, unregulated, to make returns on assets. An innovative would-be bio-terrorist would only have to have a trendy microbrewery project idea to gain their facilities.

Cash flow issues in SMEs and start-ups can be tempted to forget or neglect security concerns. Even a mainstream well-intentioned company on this scale will experience the inevitable issue of cash flow difficulties in early stages and pressure from investors for a return. This can easily encourage to be less-than-scrupulous in their approach to dual use or unintended negative consequences. They do not have the financial resources to dedicate resources to such "peripheral" activities for them.

This appears to be equally valid in education. Some institutions and societies take this very seriously such as South Korea and KAIST. There they have an ethics course before beginning education on synthetic biology/metabolic engineering. However, this is not the case in many world leading universities. Education in hacking yeast is easily come by both in higher education and online. We would not dream of helping even states develop acknowledged weapons of potential mass destruction but such potential weapons of the future we openly support all to master.

Detecting manufacture or storage of nuclear or chemical weapons is relatively straightforward due to the resources and capital required. Biotechnologies such as synthetic biology or metabolic engineering present a far more difficult challenge and can be used in a simple environment. It is an issue which the World Council on Industrial Biotechnology is giving much thought to in combination with a new

Centre for Emerging Technology Intelligence. They are driving much needed research on this important area.

10 Global Impact Conclusion

The vast potential and existing impact of bioproduction of fuels and chemicals to address climate change, the global economy, civil society, health, wealth, and nutrition as outlined above are fantastic and part of a genuine revolutionary aspect of society. Unforeseen consequences also need to be managed, but all ways to help the global growth and development and contribute to alleviating existential risks such as global warming make this a focus worthy of attention.

11 Research Needs

More investigation is required into:

Biomass/feedstock availability and then its effects on biodiversity
A sound and accepted life cycle analysis to measure the impact of bio-based production
How best to leverage newly available tools and technologies such as IT/computing power, sensors, big data, artificial intelligence, additive manufacturing among others
Identifying possible risks for international security and methods of how to mitigate them

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Can Biofuels Replace Fossil Fuels?

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Brent Erickson, Erick Lutt, and Paul Winters

Contents

Abstract

Because transportation fuel demand continues to grow and petroleum maintains a dominant position in the market, stable supportive policy is needed to enable biofuel to replace fossil fuels. Biofuel production and use grew rapidly in the first decade of the twenty-first century in countries that created welcoming business environments by implementing effective market mandates. Rapidly maturing technology has made biofuels cost competitive with fossil fuels. However, production and use slowed and consolidated as wavering public policy and economic uncertainty destabilized the business environment. Stable policies that help coordinate engine and fuel specifications, support the emergence of

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new feedstock commodity markets, and lower the cost of capital for biorefinery construction can help biofuel producers overcome remaining challenges.

1 Introduction: Biofuels in the Real World

In the first decade of the twenty-first century, biofuels were hailed as a solution to dependence on imported fossil fuels, greenhouse gas emissions that contribute to climate change, and pollution from gasoline additives such as methyl tertiary butyl ether (CEQ 2006). By the second decade, ethanol was accused as a source of higher carbon emissions than gasoline, a cause of land use change that would expand agriculture into virgin forest, and a bad deal for consumers through higher costs for fuels and vehicle operation (Cassidy 2014). Because of this misinformation, public policy support for biofuels waxed and waned in synchrony with the vagaries of public sentiment.

The financial signal created by public policy encouraged many entrepreneurial biotechnology companies to enter the biofuel sector (Somers 2007). Following years of research and development, several companies succeeded in commercializing technologies for competitively priced, commodity-scale biofuels. The worldwide transportation fuel market, however, remains reliant on petroleum (Dykstra 2016). The sheer scale of demand for liquid transportation fuels and the dominant position of the petroleum industry within the market are high hurdles for biofuels to overcome and completely replace fossil fuels. Nonetheless, biofuels can displace increasing volumes of petroleum fuels if fuel producers and engine manufacturers cooperatively adopt new technologies, farmers create functioning biomass feedstock markets, and financial markets accept sufficient risk to invest in new technologies. Effective, forward-looking policies that are stable over the long term can provide direction to coordinate these disparate markets.

This chapter asks whether biofuels – particularly advanced biofuels – can overcome remaining technological, market entry and financing challenges to displace increasing volumes of fossil fuels in the near future. Secondly, the chapter will explore what policies are needed to establish a level playing field in the transportation fuel market that enables biofuels to displace fossil fuels.

2 Commercial Development of Biofuels

Biofuel production and use have grown substantially around the world since the first decade of the twenty-first century. While the rate of growth in production and demand prior to 2010 was exponential, growth has slowed to a steady rate in the second decade. The number of countries producing biofuels has remained fairly stable, with investments concentrated in countries that offer "predictable business landscapes for entrepreneurs." (Pacini et al. 2013) The United States and Brazil,

together, represent nearly three quarters of worldwide liquid biofuel production and use.

In the United States, use of ethanol makes up 10% of the gasoline fuel market. However, the use of all biofuels in liquid transportation fuels (jet fuel, gasoline, and distillates/diesel for on-road, rail, air, and public transportation) is still below 7% (EIA 2016a). In Brazil, ethanol contributes up to 27% of the gasoline fuel supply. But biofuel use in all liquid transportation fuels remains much lower, as biodiesel contributes up to 7% of the distillate fuel supply (Barros 2015).

2.1 Platforms

Biofuel production technologies are generally categorized as either biochemical – utilizing fermentation microbes – thermochemical – utilizing pyrolysis and a chemical catalyst – or a combination, such as using a microbe to ferment gases or metabolize sugars to oils. The thermochemical approach is sometimes subdivided into fast and slow pyrolysis, depending on the temperature and amount of oxygen. Researchers have consistently demonstrated that the various production processes achieve maximum yields and economic feasibility when paired with specific feedstocks (Daystar et al. 2015).

2.2 Biofuel Types

Ethanol production in the United States expanded rapidly from 2000 to 2009 as the number of biorefineries nearly quadrupled. In 2000, there were 54 operating ethanol biorefineries in the United States, with nameplate production capacity of 1.75 billion gallons. By January 2010, there were 189 operating US ethanol biorefineries, with a combined nameplate capacity of 11.88 billion gallons (Renewable Fuels Association 2010). From 2011 through 2016, ethanol production in the United States increased, even as the number of operating biorefineries remained stable. According to the US Energy Information Administration, the number of operating ethanol biorefineries in the United States remained nearly unchanged from 2011 to 2016 (193 and 195, for each respective year). And yet, nameplate production capacity rose from 13.61 billion gallons in 2011 to 15 billion in 2016 (EIA 2016b).

After increasing rapidly from 2005 through 2010, ethanol biorefining in Brazil consolidated between 2011 and 2016, even as ethanol use as fuel increased. Between 2005 and 2010, 109 new ethanol biorefineries were built. In 2011, there were 418 operating ethanol biorefineries with a capacity of 42.8 billion liters (11.31 billion gallons). Actual production in 2011 was slightly less than half of capacity, at 20.2 billion liters (5.34 billion gallons). By 2016, Brazil was expected to have only 360 operating biorefineries with a total nameplate capacity of 38 billion liters (10.04 billion gallons), according to projections by the US Department of Agriculture (USDA) Foreign Agricultural Service's (FAS) Agricultural Trade Office in Sao Paulo. In aggregate, 49 ethanol biorefineries in Brazil were shut down between

2011 and 2016. However, production of fuel and use of existing capacity increased to a projected 28.15 billion liters (7.44 billion gallons) (Barros 2015).

In the European Union (EU), production of ethanol and use as a fuel has grown at a steady pace since 2009. In 2009, the EU had 66 ethanol biorefineries with an aggregate nameplate capacity of 6.23 billion liters (1.65 billion gallons); fuel production was 3.55 billion liters (938 million gallons). By 2016, there were 71 biorefineries with nameplate capacity of 8.48 billion liters (2.24 billion gallons) and fuel production of 5.25 billion liters (1.39 billion gallons) (Flach et al. 2015).

Ethanol producers rapidly built up production capacity through established technologies and feedstock markets; however, they also improved production efficiency as they built larger, newer plants. In 1982, producers achieved an average of 2.5 gallons of ethanol per bushel of corn; by 2014, the average increased to 2.8 gallons per bushel. Producers have deployed better designed enzymes and fermentation yeasts as well as optimized processes in order to reduce energy inputs and increase efficiency (Radich 2015). Additionally, producers have increased generation of coproducts, including corn oil, animal feed, electricity, and renewable chemicals (Gallagher 2015).

Biomass-based diesel production and use also increased during the second decade of the twenty-first century, in the United States, Brazil, and Europe, even while capacity consolidated. Biomass-based diesel includes both biodiesel (fatty acid methyl ester) and renewable diesel (hydroprocessed esters and fatty acids, or HEFA), which is a hydrocarbon rather than an ester. Renewable diesel is a more recently commercialized technology, based on hydrotreating renewable oils in the presence of a catalyst to remove oxygen. In the United States, the number of biodiesel biorefineries has consolidated, down from about 122 active producers in December 2009 to 94 in April 2016. At the same time, biomass-based diesel production has increased, with renewable diesel making up an increasing share. In 2009, US biodiesel producers generated 506 million gallons of fuel; however, in 2015 US producers generated 1.26 billion gallons, including 6.8 million gallons of renewable diesel (EIA 2016c, d).

Brazil has followed a similar pattern of consolidation in biodiesel capacity since 2011, with production and use increasing while the number of biorefineries declined. In 2011, there were 65 biodiesel biorefineries in Brazil that generated 2.67 billion liters (706 million gallons) of fuel. By 2016, analysts expected the number of biorefineries to decline to 57 while production increased to 4.11 billion liters (1085 million gallons) (Barros 2015).

The European Union, too, has consolidated production of biomass-based diesel in the second decade of the twenty-first century, with renewable diesel production gaining market share. The number of biomass-based diesel biorefineries in Europe peaked at 268 in 2012 and was forecast to decrease to 248 by 2016. However, capacity use among those biorefineries was projected to rise, with production reaching 12.59 billion liters (3.33 billion gallons) in 2016. Production of renewable diesel as a component of biomass-based diesel was projected to rise from 868 million liters (229.3 million gallons) in 2012 to 2.07 billion liters (547 million gallons) in 2016 (Flach et al. 2015).

Renewable jet fuels and butanol are the most recent biofuels to achieve commercial production, though at a limited scale. ASTM International, an international standard setting body, has approved four pathways for alternative jet fuels to be blended with petroleum fuels (up to a 50–50 blend), including alcohol to jet synthetic paraffinic kerosene (ATJ-SPK), HEFA renewable diesel, Fischer-Tropsch (FT) oils, and synthesized iso-paraffinic (SIP) fuel, which is derived from hydrotreating farnesene. Several biofuel producers have worked in partnership with aviation companies over the past 5 years to develop, test, and produce renewable jet fuels. AltAir in Downey, California, began commercial deliveries of biojet fuel to United Airlines at Los Angeles International Airport in 2016 (United Airlines 2016). Neste Oil in Porvoo, Finland, also began commercial deliveries of HEFA jet fuel to Oslo Airport Gardermoen for use by KLM Airlines in early 2016 (Neste 2016). In early 2016, Gevo in Luverne, Minnesota, began commercial deliveries of butanol to be used as on-road and marine engine fuel (Gevo 2016). Testing continues as companies seek ASTM approval for 100% renewable jet fuels.

2.3 Feedstocks

Cellulosic biofuels have also achieved commercial production at a limited scale, though most producers continue to experience technical disruptions, feedstock supply chain challenges, and financial uncertainty. Among companies that have constructed and operated first-of-a-kind large-scale cellulosic biorefineries, only a few have achieved consistent production as of July 2016, including Ensyn in Renfrew, Ontario, Canada, using wood biomass; Beta Renewables/Biochemtex in Crescentino, Italy, using agricultural residue; Raízen Energia in Piracicaba, Brazil, using sugarcane leaves; and Quad County Corn Producers in Galva, Iowa, using distillers grains from starch ethanol production. Other large-scale producers have begun production but continue to adjust production processes as they scale up toward nameplate capacity, including POET-DSM in Emmetsburg, Iowa, which uses corn cobs and stalks, and GranBio (Beta Renewables/Biochemtex) in Sao Miguel dos Campos, Brazil, which uses sugarcane leaves (Sapp 2016). DuPont completed construction of a 30-million-gallon-per-year biorefinery in Nevada, Iowa, and is starting up production using corn stover harvested from partner farms in the surrounding area.

Edeniq has developed technology to increase production of ethanol from corn kernels, including the fiber, and the process has been approved by the US Environmental Protection Agency (EPA) as a cellulosic ethanol production pathway. Edeniq, which was recently purchased by California-based Aemetis, has installed this technology at six existing US corn ethanol plants, including Mid-American Agri Products-Wheatland LLC, in Madrid, Nebraska; E Energy in Adams, Nebraska; Pacific Ethanol in Stockton, California; and Flint Hills Resources (FHR) in Fairbank, Iowa (Jessen 2016). FHR has licensed the technology for all seven of its ethanol biorefineries, located in Iowa, Nebraska, and Georgia. Aemetis plans to deploy Edeniq's technology at its 60-million-gallon-per-year ethanol biorefinery in Keyes,

California. And Siouxland Energy Cooperative has licensed the technology for its 60-million-gallon-per-year ethanol plant located in Sioux Center, Iowa. These companies must still register with EPA to produce and sell cellulosic transportation biofuel under the Renewable Fuel Standard program (RFS).

After completing construction of biorefineries, two companies suffered financial setbacks and stopped production. Abengoa built and in 2015 started up a 25-million-gallon facility in Hugoton, Kansas, that utilizes agricultural residues; in 2016, the company began a financial reorganization (Sapp 2016). And KiOR, after achieving production of several million gallons from woody biomass in Columbus, Mississippi, declared bankruptcy in 2014 (McCarthy and Doom 2014). A third company, INEOS Bio in Vero Beach, Florida, after beginning operations in 2014 using separated municipal solid waste, shut down production due to an unforeseen technical challenge. The company received an air permit from the state of Florida in September 2014 to install an emissions scrubber, but to date has not restarted production (VeroNews.com 2015).

Additional companies are commercializing advanced biofuel technologies for on-road and jet fuel applications, utilizing cellulosic material, municipal solid waste, and other nonstarch feedstocks. Several have announced plans to build first-of-a-kind biorefineries by 2020 (Schwab et al. 2016).

Companies developing algae as a feedstock for biofuels remain in the research and development or precommercial stage (Casey 2016). Many of the companies that set out to pursue algae biofuels have turned instead to producing nutritional supplements, food ingredients, or renewable chemicals, which can be commercialized with existing technology at smaller scale.

Renewable natural gas (biogas or methane) has been produced from landfills, methane digesters, and waste treatment plants for many years and used for electric power generation. Cars that run on compressed or liquefied fossil natural gas can use compressed or liquefied renewable natural gas as a direct replacement, and according to the US Energy Information Administration about 550 million gallons of compressed or liquefied natural gas is used in on-road transportation annually (EIA 2016a). There are currently 37 renewable natural gas producers in the United States registered as renewable fuel producers (EPA 2016a). The Renewable Natural Gas Coalition identifies another 14 producers expecting to start production by 2017 and projects the industry will generate 376 million gallons of fuel in 2017 (Cox 2016).

Biorefineries that have achieved consistent commercial production generally utilize readily available feedstocks with preexisting supply chains – e.g., agricultural crops, such as corn, sugarcane, and soy; or solid wastes, such as yard waste or animal fats. Companies developing cellulosic biorefineries or deploying new feedstocks – such as sorghum or fast-growing poplar – have had to build new supply chains. For example, DuPont signed a memorandum of understanding with USDA's Natural Resources Conservation Service to develop conservation plans with each farmer providing corn crop residues to its Nevada, Iowa, biorefinery to ensure sustainable harvest of the feedstock (USDA Natural Resources Conservation Service 2013). And POET-DSM has established a Responsible Stover Harvest program that limits stover collection to 20–25% of aboveground residues (POET Biomass 2014).

The technology for large-scale production of cellulosic biofuels and new fuel molecules continues to be perfected and readied for commercial deployment. But cellulosic and advanced biofuel producers – even those who have built first-of-a-kind biorefineries that have not achieved consistent production – still face substantial technology and feedstock supply chain risks. As a result, they represent higher financial and investment risk profiles. Decisive, stable policy is a prerequisite for directing investment and resources into commercializing these biofuels and expanding production.

3 Ongoing Challenges to Large-Scale Biofuel Production

Worldwide, demand for transportation fuel and mobility continues to grow, as shown in Fig. 1. The International Energy Agency's Oil Market Report forecasts that 96 million barrels of oil and liquid fuels will be used each day in 2016 – more than 35 billion barrels during the year (IEA 2016).

Despite rapid growth, renewable energy in general and biofuels in particular currently play only a supplementary role to fossil fuels in meeting world energy demand (Pacini et al. 2013). Companies continue to commercialize and improve technologies for biofuels. But for biofuels to displace increasing amounts of fossil fuels, the challenges of developing new feedstock commodity markets while ensuring sustainability, coordinating with automakers and engine manufacturers, and mitigating investment risk for new technology must be addressed through policy.

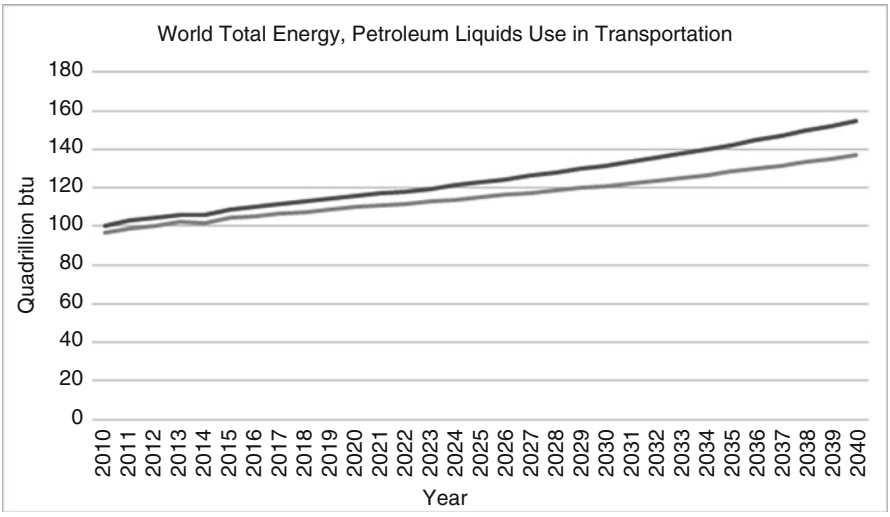

Fig. 1 World total energy, petroleum use in transportation

3.1 Feedstock Availability

In 2005, the US Department of Energy (DOE) assessed the potential for the United States to annually grow, harvest, and collect one billion tons of biomass and wastes. The agency estimated this amount of biomass would be needed to produce enough biofuels to displace 30% of then-current petroleum transportation fuel use (Perlack et al. 2005). DOE updated the biomass availability assessment in 2011, adding an evaluation of the economic feasibility of not just collecting one billion tons of biomass but also delivering it to biorefineries. The 2011 report estimated $60 per ton as the threshold price to encourage the harvest of biomass, with delivery to the biorefinery gate increasing the end cost (DOE 2011).

Significantly, use of transportation fuels (gasoline, diesel, kerosene jet fuel) in the United States is once again approaching peaks reached in 2007, after having declined between 2008 and 2013 as the country experienced a recession, slow economic recovery, and high oil prices. The amount of gasoline transportation fuel used in 2015, at 3.34 billion barrels, is nearly identical to the amount used in 2005, when the 30% replacement goal was established (EIA 2016e). The major difference is that in 2015 renewable fuel made up nearly 10% of gasoline use.

In June 2016, DOE issued a third assessment of the feasibility of producing one billion tons of biomass for biobased production (Langholtz et al. 2016). The agency estimates that the United States currently uses 365 million dry tons of agricultural crops, forestry resources, and waste to generate biofuels, renewable chemicals, and other biobased materials. The agency also projects that there is currently the potential to harvest and collect 325 million additional tons of forestry resources, agricultural residues, and waste (which includes biogas production) at $60 per ton. By 2040, the potential available additional biomass at $60 per ton increases to more than 800 million tons. While forestry resources and waste collection is expected to remain fairly stable, in DOE's projections, biomass availability is increased substantially by the addition of 411 million tons of new energy crops along with an increase in the harvest of agricultural residues (Table 1).

The DOE again estimates that delivery of the collected biomass to biorefineries will increase the end costs. At $84 per ton, the authors estimate that 45% (or 310 million tons) of the currently and potentially available biomass would be delivered to biorefineries, indicating that even higher prices per ton are necessary to drive the evolution of commodity markets for new sources of biomass, such as energy crops or algae.

Growing and harvesting switchgrass for $60 per ton, as an example of a new energy crop, is currently feasible. Moreover, production costs can be reduced as growers gain experience in cultivating switchgrass (Mitchell et al. 2016). And cellulosic feedstocks, because of their abundance, are expected to be the lowest cost feedstocks available in the future. In 2015, corn averaged $3.17 per bushel, which equates to $124.80 per metric ton (USDA National Agricultural Statistics Service 2016). But preestablished commodity markets for agricultural products and some waste streams make them economical choices for biofuel production, even at prices well above the DOE estimate of $84 per ton price.

Table 1 Summary of currently used and potential biomass collected at $60 per ton (million tons)

Feedstock	2017	2022	2030	2040
Forestry	154	154	154	154
Agriculture	144	144	144	144
Waste	68	68	68	68
Total currently used	365	365	365	365
Potential forestry	84	88	77	80
Agricultural residues	104	123	149	176
Energy crops		78	239	411
Additional waste	137	139	140	142
Total potential/additional resources	325	428	605	809
Total potential biomass resources	690	793	970	1174

Potential growers of cellulosic feedstocks must be certain of a stable market (in addition to a stable price) before committing to growing new energy crops and creating a functioning commodity market for delivering them to biorefineries. Cellulosic biofuel producers, such as POET-DSM and DuPont, as noted earlier, have worked directly with local growers while building new biorefineries to coordinate the establishment of harvests and delivery.

3.2 Sustainability

As the RFS came into effect in the first decade of the twenty-first century, environmentalists and other advocacy groups cautioned that growing and harvesting large amounts of biomass for biofuel could negatively impact the availability and cost of food crops and expand agricultural production into environmentally sensitive lands, such as South American rainforests, which would result in increased carbon emissions. The RFS program was designed to provide a limited demand floor for crop-based biofuels and to include periodic reviews of biofuel production's impact on crop availability.

An extensive 2015 review of the RFS program's impact by the USDA indicates that while the price of food crops rose the availability of those same crops also increased. Additional factors, including a steep rise in the price of oil that drove price increases for all commodities, also contributed to the increase in food crop and processed food prices. Further, while agricultural production expanded, the aggregate evidence indicates it did not expand into environmentally sensitive land (Riley 2015).

The rapid rise in biofuel production created new demand for corn and soy in the United States, contributing to the rise in prices for these staple crops. The price increase prompted planting of additional corn and soy acreage in the United States, which was done primarily on land previously planted to hay and other crops, according to the USDA analysis. Rapid yield improvements – through seed technology and planting techniques – enabled increased production on existing

agricultural land. USDA posits that reserve agricultural land may have been planted to grass, hay, or wheat, allowing other acres previously growing these crops to be planted to corn and soybeans (Riley 2015). Overall, aggregate acreage planted to principal crops in the United States following establishment of the RFS in 2005 remains below the aggregate acreage planted prior to the policy.

US exports of corn have declined since establishment of the RFS, even while worldwide demand for the grain as animal feed and food ingredient has increased. In response, corn production outside of the United States increased faster than within, again achieved through both an increase in area and an increase in yield, according to the USDA analysis. Corn area harvested in countries other than the United States rose 35% and average yields increased 37% by 2013, compared with a 2000 baseline. And again, USDA's review posits that the land planted to corn shifted from production of other crops or from hay (Riley 2015).

While land use change – including crop shifting – results in carbon emissions and is properly included in a lifecycle analysis, the evidence suggests that it is possible to avoid land use change in environmentally sensitive areas through appropriate policy. In 2016 (the year after publication of the USDA's review), corn acreage planted and harvested in the United States declined slightly, even as yields and productivity improved (USDA National Agricultural Statistics Service 2016).

Creating additional markets for agricultural products can both boost profitability for growers and crop producers and compliment production for existing food, feed, and fiber markets, rather than competing with them. Prior to establishment of the RFS, US corn producers had experienced a decade where corn production costs annually exceeded returns (Singh et al. 2014). Sustainable production of biofuels must include consideration of the economic benefit to growers as well as the environmental and social benefits to others.

3.3 Automobile and Engine Technology

In the United States, EPA, under the Clean Air Act (CAA), regulates both the production and use of transportation fuels and fuel additives and the tailpipe emissions from vehicle engines as a combined system. All new fuels, fuel additives, and vehicles (both engines and emission control systems) produced or imported into the United States must meet standards and guidance established by EPA. The agency's regulations and guidances refer to ASTM International standards – voluntary industry standards that coordinate the design and manufacture of automobile engines and emission control systems, fuel pipelines and pumps, as well as inform vehicle warranty and insurance programs.

EPA approved ethanol as both a transportation fuel and fuel additive and established guidance for its use with regulated engines and emission control systems. The approval and guidance refer to ASTM Standards D 4806 and D 5798, which establish the technical properties for ethanol used as a fuel additive and as E85 fuel. E85 fuel is approved for use only in flex fuel vehicles (FFVs) under the CAA. Biodiesel – esterified renewable oil – has different fuel properties from petroleum

diesel and different ASTM fuel standards. Diesel engines can run on 100% biodiesel with some additional maintenance requirements and the expectation of slight changes in performance. Many advanced biofuels – such as renewable diesel and jet fuel – are drop-in replacements for petroleum fuels, meeting existing ASTM standards for petroleum fuels.

EPA regulations under the CAA prohibit commercial use of any new fuel or fuel additive, or the increased concentration of a fuel additive, that is not "substantially similar" to the fuel used to certify automobiles and engines. To qualify as substantially similar to gasoline, for instance, fuels and additives must have all the physical and chemical characteristics of unleaded gasoline as specified in ASTM Standard D 4814, which sets volatility, corrosiveness, and oxidation stability limits for gasoline. EPA granted waivers of the substantially similar rule in 1978 and 1982 to allow ethanol to be used as an additive up to 10%. EPA similarly granted two waivers in 2010 and 2011, permitting ethanol to be used as an additive up to 15% in model year 2001 and newer light-duty motor vehicles. Fuel retailers may sell E15 fuel after registering with EPA and submitting a misfueling mitigation plan.

Because EPA's guidance until recently limited the use of ethanol as a fuel additive, automakers, refiners, and fuel retailers adapted their engine designs and warranties accordingly – creating a so-called blend wall. A comparison between Brazil and the United States illustrates the impact. In Brazil, more than 80% of new cars sold since 2007 have been FFVs, capable of operating on 100% ethanol or on gasoline with ethanol content between 22% and 85% (Associação Nacional dos Fabricantes de Veículos Automotores 2016). In the United States, of the 242.55 million light-duty cars and trucks on the road in 2016, only 19.61 million, just over 8%, are FFVs (3.72 million cars and 15.89 million trucks) (EIA 2016a)

In 2007 ASTM began to take necessary steps to change guidance on use of ethanol blends (Herman et al. 2007). And in 2014, EPA changed the certification fuel for new engines from E0 to E10, in effect changing the standard to which fuels and additives must be "substantially similar." (EPA 2016b) Accordingly, US automakers and engine manufacturers recently have begun to warranty new cars and engines for use of higher blends of ethanol and biodiesel (Renewable Fuels Association 2015).

In May 2010, EPA and the National Highway Traffic Safety Administration (NHTSA) issued joint greenhouse gas emission and corporate average fuel economy (CAFE) standards for light-duty cars and trucks in model years 2012–2016. Two years later, in August 2012, EPA and NHTSA extended and enhanced the joint standards for light-duty vehicles in model years 2012–2025. By 2025, automakers are expected to achieve an average fleet-wide emission level of 163 grams of CO2 per mile driven, which is equivalent to a fuel efficiency rating of 54.5 miles per gallon. The average emissions for model year 2014 (the latest data available) is just under 275 grams per mile (EPA 2016c).

In a draft Technical Assessment Report, EPA and NHTSA rate turbocharged, downsized and higher compression ratio engines as two of the more likely technologies that automakers will use by 2025 to meet the higher joint standards (EPA et al. 2016). The higher compression and turbocharged engines would necessitate an increase in the octane rating of certification fuels and in EPA's guidance for fuel

characteristics. And higher blends of ethanol in the gasoline – between 20% and 40% – can provide the needed octane (Splitter and Szybist 2014). These higher blends would provide additional room in the fuel market for renewable fuels, specifically ethanol, potentially achieving the 30% displacement of petroleum envisioned in the original 2005 Billion Ton Report. However, they would not in the near term completely displace petroleum with biofuels.

Automakers have many technology options to choose from in meeting the 2025 combined standards, including hybrid fuel technologies and plug-in electric engines. Deploying higher octane fuels, higher ethanol ratios, and high-compression engines to meet the higher emission and fuel economy standards would require coordination among several industries through voluntary standard setting organizations like ASTM. Clear policy direction by EPA and NHTSA could provide further impetus to the often competing groups engaged in this standard setting process.

With projected increases in demand for jet fuel as well as international regulatory pressure to reduce carbon emissions, the international aviation industry and the US military are actively looking to displace petroleum use and keep carbon emissions from growing. Environmental NGOs petitioned the US EPA in 2007 to reduce emissions from the airline industry. In 2014, EPA outlined a process to the United Nations' International Civil Aviation Organization (ICAO) to address the emissions. And in July 2016, EPA finalized a finding that airplane engines are a significant contributor to greenhouse gas emissions, which are a danger to public health (EPA 2016d). EPA will work with ICAO to formulate and adopt international standards to address the emissions by the end of 2016. As a strategy, the industry is looking to increase both engine fuel efficiency and use of drop-in biofuels.

In 2011, US President Barack Obama directed the Department of the Navy (DON), DOE, and USDA to work with private industry to create advanced drop-in biofuels that will power both the Department of Defense and private sector transportation, particularly aviation biofuels. This Memorandum of Understanding (MOU) between DON, DOE, and USDA launched a cooperative effort to support the development of a sustainable commercial biofuels industry and to foster mutual cooperation of the parties to achieve energy security and GHG reductions (Maybus et al. 2011).

A recent report from ICAO indicates that displacing 2% of petroleum with biofuels is achievable by 2020. However, reaching the goal of 2% fuel efficiency improvements per year will require technological improvements beyond those envisioned for deployment between 2020 and 2030. The report calculates that displacing 100% of petroleum use (which would be necessary to keep carbon emissions from growing) would require construction of 170 new biorefineries each year from 2020 to 2050, at a cost between $15 billion and $60 billion per year (Fleming 2016).

4 Ongoing Market Challenges

The price of oil has been volatile in the past decade, especially in comparison to the preceding two decades, as shown in Fig. 2 (EIA 2016f). Yet, oil maintains a dominant position in the fuels market. For biofuels to displace petroleum, they

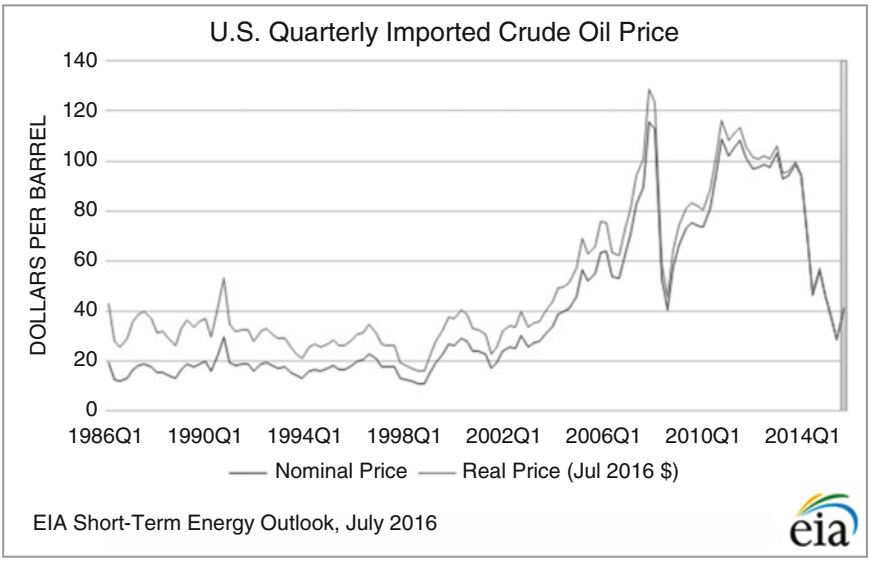

Fig. 2 US quarterly imported crude oil, nominal and real prices

must be cost competitive, and many commercial biofuels are now competitive. But advanced biofuel producers must also take account of the higher cost of capital for building capacity.

US ethanol production – including animal feed and corn oil coproducts – has been profitable for many years, even during the recent rapid decline in oil prices (Irwin 2016a). Ethanol remains the least-cost octane and oxygenate additive for gasoline, ensuring a low but relatively continuous level of demand. Ethanol as a primary fuel, such as E85, can also be cost competitive for flex fuel vehicle owners, depending on the relative price of E10 gasoline and the relative fuel economy of the particular vehicle. Websites such as E85Prices.com and GasBuddy.com enable US motorists to calculate the relative cost and find appropriate fuel retailers.

Cellulosic ethanol prices and costs vary – from approximately $2.17 per gallon to $4.55 per gallon – among the limited number of producers now in operation, with feedstocks representing about 40% of the total costs (Yu et al. 2016). Operating costs and costs of the capital to build the facilities also represent large shares of the final fuel price. Cellulosic ethanol remains more costly than conventional corn ethanol; but producers can continue to lower the price through economies of scale and lower feedstock prices. DuPont, for instance, was able to lower its feedstock costs by implementing its Corn Stover Harvest Program in cooperation with USDA and its partnering farms.

The price of soybeans determines the price of biodiesel in the United States. As a consequence, the price of biodiesel has fluctuated along with oil prices over the past decade. US biodiesel producers have benefited from a production tax credit, which improves their profitability. Unfortunately, according to Scott Irwin at the University

of Illinois at Urbana-Champaign, "losses appear to be the norm for the biodiesel industry outside of years when policy incentives combine to create a boom in biodiesel prices and profits." (Irwin 2016b) The primary reason this occurs is that the US biodiesel industry has too much built capacity and, relatedly, too little demand. Anecdotal reports indicate that renewable diesel is currently price competitive with petroleum diesel (Cannon 2016). The limiting issue with renewable diesel production is the cost and availability of waste feedstocks (Lane 2016).

The cost of capital is still a challenge for advanced biofuel producers, given the risks associated with first-of-a-kind commercial production facilities, emerging commodity markets for new feedstocks, and uncertainty about the long-term market uptake of fuels. Self-financing of first commercial facilities has been the primary strategy for advanced and cellulosic biofuel producers. But expansion of the industry will require additional investors with other sources of capital, including investment banks and large corporations with investment capital (Peters et al. 2015).

4.1 The Impact of Policy Instability

The RFS is designed to set a market floor for the production and use of renewable fuels in the United States, which increases to 36 billion gallons by 2022, according to the statutory language. EPA is charged with ensuring that the mandated renewable fuel volumes are used by annually setting percentage standards for conventional, advanced, biomass-based diesel and cellulosic biofuels that every petroleum refiner and importer must meet. EPA delayed issuing its 2013 annual rule until August 2013, when two thirds of the compliance year had passed. It then delayed issuing rules for 2014 and 2015 until December 2015. Biofuel use during those years fell far short of the statutorily set market floor. Moreover, the delays caused a shortfall of tens of billion dollars in capital investment necessary to expand and build new advanced biofuel production capacity (BIO 2016).

EPA clearly understands the need for stability in the RFS rulemaking process in order to drive investment. In its Response to Comments on the 2014–2016 RFS Proposal, EPA acknowledged that "the development of new technology won't occur unless there is clear market potential, and it requires multiple years to build new production, distribution, and consumption capacity and to develop the marketing effectiveness among consumers that is needed." (EPA 2015) Additionally, in its proposed 2017 RFS rule, EPA states: "Volume requirements over the longer term that are issued in a timely manner and which provide the certainty of a guaranteed and growing future market are necessary for the industry to have the incentive to invest in the development of new technology and expanded infrastructure for production, distribution, and dispensing capacity." (EPA 2016e)

In the European Union, the 2009 EU Energy and Climate Change Package includes a 10% minimum target for renewable fuel use in transportation by 2020. The 28 member states of the EU set national action plans that will raise biofuel use mandates from the current 5.75% minimum to 10% by 2020. The 2009 Renewable Energy Directive also stipulates that biofuels can fulfill the climate change package

only if they meet a minimum greenhouse gas reduction goal, which increases from 35% to between 50% and 60% after 2018 (Lieberz 2016). In April 2015, the EU Parliament voted to reform the Renewable Energy Directive to cap the use of food crop–based biofuels (such as corn ethanol or soy biodiesel) to 7%. The European Biofuels Technology Platform welcomed the 2015 vote as a way to settle a 3-year debate that had created uncertainty and blocked investment decisions (European Biofuels Technology Platform 2016).

Brazil currently requires fuel for gasoline engines to contain 27% ethanol and fuel for diesel engines to contain 7% biodiesel (Barros 2015).

In the United States, both biodiesel and "second generation" (either cellulosic or algae-based) biofuels are eligible for production tax credits. The tax credits were established in 2005 and 2008, respectively, with 4-year lifespans. The credits have been extended multiple times, often for 1 year and sometimes retroactively. As noted by Scott Irwin of the University of Illinois, the constant expiration of the biodiesel tax credit has undercut the profitability of biodiesel producers (Irwin 2016b).

In the EU, biofuel tax credits generally supplement existing mandates for use. The value of the tax credits is between €5.5 billion and €8.4 billion annually (Bourguignon 2015).

5 Displacing Increasing Volumes of Petroleum with Biofuels

Research and development of new biofuel molecules, new feedstocks, and new production technologies is ongoing. There are several impediments to translating this research and development into commercial-scale biofuel production, chief among them financing for demonstration-scale projects. A variety of public and private demonstration units have been built in the United States in the past decade to commercialize advanced biofuel technologies; those units could be retrofit to further research of new feedstocks, molecules and production processes at a pre-commercial scale, with adequate funding. At the same time, there is a need to translate the past decade's experience with large-scale biofuel production into improved research, modeling and demonstration of new production processes and feedstocks.

Researchers are continuing to develop genetic engineering techniques to enable microbes and enzymes to tolerate the toxicity inherent in producing fuel molecules from diverse or mixed sources of biomass. Companies continue to address the chemical and mechanical engineering challenges of processing biomass into a uniform substrate for conversion to biofuel. Farmers also have joined efforts to grow and harvest biomass resources that meet the needs of biofuel producers. Research is needed to analyze and coordinate the efforts of all stakeholders along the value chain to expand the number of successfully commercialized technology and feedstock combinations.

Biofuels today have displaced only a relatively small percentage of worldwide demand for transportation fuels. Consequently, petroleum maintains an overwhelmingly dominant position in the market to meet continually rising demand for transportation fuels. Despite the ongoing maturation of technology – both for biofuels and

vehicle technology to make use of them – biofuels have not reached a tipping point that would enable them to displace petroleum through market forces alone. Policy mechanisms are necessary to level the playing field.

The challenges that must be overcome in enabling biofuels to displace increasing amounts of petroleum in the near future are access to low-cost capital to build biorefineries, identification and commodification of additional feedstocks, and sustainability certification for technology and feedstock combinations. The experience of Brazil and the aviation industry demonstrate that market acceptance of biofuels by consumers and engine manufacturers will be based primarily on cost-competitiveness. In the near term, sufficient feedstocks, cost-competitive conversion technologies, and efficient engine technologies have been developed to enable biofuels to displace up to 30% of petroleum use in transportation in countries that provide stable business conditions for entrepreneurial companies.

The United States and Brazil stand out among countries providing the right business conditions, with EU member countries setting more conservative goals. The most effective policies demonstrated to date are strong, stable mandates for renewable fuel use. These have successfully directed capital to the commercialization of advanced and cellulosic biofuels; policy instability has been the biggest obstacle to their further commercialization.

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