Vijai Singh · Ajay Kumar Singh
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Chaitanya G. Joshi *Editors*

Engineering of Microbial Biosynthetic Pathways

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Foreword

I am happy to write an introductory massage for "Engineering of Microbial Biosynthetic Pathways," a timely volume on the rapidly evolving field of metabolic engineering.

The engineering of biosynthetic pathways has the potential to produce a large amount of valuable biomolecules by extension or modification of biosynthetic pathways in a wide range of organisms. Modified organisms have ability to produce a high amount of chemicals, drugs, enzymes, amino acids, antibiotics, biofuels, and so on. The recent advances in metabolic engineering along with fermentation and bioprocess technology have widened our understanding of industrial scale production of biomolecules in an eco-friendly manner. This book covers key areas of metabolic engineering that would allow the readers to easily understand the concepts and implement them.

This comprehensive book contains 17 chapters with several aspects of biosynthetic pathways from basic to advance level for laboratory scale to industrial scale production of biomolecules. It covers a number of major topics including an introduction to metabolic engineering, strain development and improvement, a biosensor for metabolite detection, metabolic engineering for the production of a wide range of industrially useful enzymes, proteins, organic acids, vitamins, antibiotics, therapeutics, chemicals, and biofuels, genome editing via CRISPR-Cas9 system, along with ethical and regulatory issues which have been presented using an easy-to-understand yet elaborative narration.

I am pleased to recognize the valuable efforts of Dr. Vijai Singh, Dr. Ajay Kumar Singh, Dr. Poonam Bhargava, Dr. Madhvi Joshi, and Prof. Chaitanya G. Joshi, who collaboratively brought out an excellent volume through the support from Springer Nature. I strongly believe that this book will be really useful for the students, researchers, scientists, stakeholders and policymakers, among many others.

Indrashil University
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Jhillu Singh Yadav

Preface

Engineering of microbial biosynthetic pathways has the potential to produce a broad spectrum of biomolecules by extension or modification of native pathways of microorganisms. It has recently gained a lot of scientific and industrial attention because of its capacity to overproduce targeted molecules in a renewable and sustainable manner. The production and optimization can be easily tuned or regulated by a wide range of synthetic biology, molecular biology, and genome editing tools. Though the natural microorganisms have the potential to produce required biomolecules, the production is not enough for commercialization purpose to fulfill the current market demands. Therefore, either assembly of biosynthetic pathways in heterologous hosts or redesigning the existing biosynthetic pathway in natural organisms is requisite.

Additionally, a pressing need has arisen to improve the quality of products for good health and management of diseases by using biomolecules that can be produced in a green manner. The recent advances in metabolic engineering along with fermentation and bioprocess technology have widened our understanding of industrial-scale production in a simple, cost-effective, and sustainable manner. This book covers a wide range of information from basic to advanced level for laboratory-scale to industrial-scale fermentation and production. The book is focused on different aspects of metabolic engineering, strain engineering, techniques for detection and extraction of metabolites, biosensors, fermentation methods, genetic engineering and synthetic biology toolboxes, and ethical, patents, and regulatory issues. This book also covers a wide range of topics on microbial production of vitamins, enzymes, therapeutics, antibiotics, biofuels, amino acids, and much more.

This book brings together the contributions of eminent scientists worldwide who have extensive experience in different aspects of microbial engineering and its applications. This book has been designed to benefit students, researchers, scientists, stakeholders, policymakers, and many more. It covers a rich literary text of excellent depth, clarity, and coverage which allows to easily understand and start research in this area. This book is a compilation of 17 chapters written by eminent scientists from five countries including China, India, Japan, South Africa, and the United Kingdom.

We hope that this book will not only provide a better understanding of the design of biosynthetic pathways but also trigger unanswered questions. Despite the fact that immense efforts have been invested to make this book user-friendly, we are aware that the first version always comes with bugs. This book will be an excellent basis from which scientific knowledge can grow, widen, and accelerate design as well as the construction of a metabolic pathway for high production of biomolecules. We would be delighted to receive suggestions to improve the book further.

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Vijai Singh

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Ajay Kumar Singh

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Poonam Bhargava

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Madhvi Joshi

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Chaitanya G. Joshi

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1 An Introduction to Design of Microbial Strain using Synthetic Biology Toolboxes for Production of Biomolecules

Gargi Bhattacharjee, Nisarg Gohil, and Vijai Singh

Abstract

Metabolic engineering has the potential to produce a wide range of biomolecules from renewable resources through the extension or modification of natural biosynthetic pathways in the microbes. Over the past two decades, it has been used for the production and optimization of number of biomolecules that can be easily tuned or regulated by synthetic biology and molecular biology toolboxes. Random mutagenesis or targeted genetic or genome engineering tools allow industrial strains to improve the productivity and yield of biomolecules or metabolites. In this chapter, we highlight the recent advances, challenges and future perspective in metabolic engineering towards industrial-scale production of biomolecules.

Keywords

Biosynthetic pathway · Metabolic engineering · Synthetic biology · Regulation · Optimization · Biomolecules

1.1 Introduction

In the past few decades, the dependence on the petroleum products for fuels and its by-products has immensely deteriorated the environment including the various life forms (Ramírez-García et al. 2019). Thus, it is imperative to find alternate solutions for the products arising from the environment-polluting industry. A wide range of chemicals or active biomolecules that are chemically synthesized require

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long steps which are laborious, time-consuming, expensive and have low activity. A pressing need has arisen to find an alternative way to produce high amount of biomolecules in order to fulfil the growing market demands. In order to replace the chemical synthesis of these products, its biological counterparts can be produced by microorganisms. Number of microorganisms have the natural ability to produce useful biomolecules in a cost-effective manner by utilizing cheaper carbon and nitrogen sources. However, one of the major issues of bio-based products is low output levels which affects the cost-effectiveness at the industrial scale.

Metabolic engineering is an area used for optimizing the regulatory and genetic processes in living organisms for increasing the production of target molecules. It regulates a number of biochemical reactions occuring within the cells with the aid of enzymes used for converting raw substrate into desired product (Yang et al. 1998; Stephanopoulos 2012; Gohil et al. 2017; Panchasara et al. 2018). These biomolecules are thus produced by organisms at industrial scale in a cost-effective manner. Some strategies that are used for overproducing biomolecules include: (1) overexpressing rate-limiting genes, (2) expression of heterologous biosynthetic pathways, (3) protein engineering, and (4) knock-out or knock-down of genes in competitive pathways (Ramzi 2018; Choi et al. 2019; Gohil et al. 2019). In the past decade, metabolic engineering has been explored for the production of number of industrial products through the extension of biosynthetic pathways in many organisms. *Escherichia coli* and *Saccharomyces cerevisiae* are the utmost common and well-studied microorganisms that are used for the production of various chemicals and metabolites.

Metabolic engineering provides solution to low output levels of microorganisms by extension or modification of pathways. These pathways can be transferred into microorganisms to obtain high yields of chemicals, biofuels and drugs. The extension or modification of pathways poses a technical challenge which can be addressed by novel state-of-art synthetic biology and genome engineering technologies. In this chapter, we highlight the recent advances, key issues and future opportunities in metabolic engineering for industrial-scale production of biomolecules in a sustainable manner.

1.2 Desired Characteristics of Strain Used for Production of Biomolecules

In order to produce industrial-scale biomolecules, strain should have characteristics such as (1) genetic stability which would allow microorganisms to produce for a longer time without loss of productivity, (2) efficient production of biomolecules, (3) microorganisms should be able to easily utilize simple nutrient than the expensive vitamins or growth factor, (4) utilize cheaper carbon and nitrogen sources, (5) the ease to carry out genetic manipulation in the microbe, (6) it should be non-pathogenic, preferably generally recognized as safe (GRAS), (7) product should be easily harvested from the medium, and (8) should produce limited by-products.

1.3 Methods Used for Strain Improvement

Wild-type strains can produce only a limited amount of biomolecules, therefore, it is hard to use them for industrial-scale production. Thus, a pressing need has arises to improve the performance of wild-type strains. A number of techniques/methods are adopted for improving the efficiency and performance of a strain. This can be done by using physical (e.g. UV light) and chemical methods (e.g. ethidium bromide, sodium azide, and ethyl methyl sulphonate) for generating mutation into the genome towards improving strain performance. However, it requires high-throughput assays or screening systems to detect hyper-producer strain.

Few other methods used for strain improvement include generation of genomic library, transposon mutagenesis, protoplast fusion and genetic engineering (Morgan 1983; Adrio and Demain 2006; Weber et al. 2017; Blount et al. 2018). For improvement of strain, it is important to perceive that the strains can be engineered or improved in a way that could increase genetic stability. As genetically stable strains can make high biomass, in result, the production can be increased (Lancini and Lorenzetti 1993). Transcriptional control via promoter engineering can improve the production of targeted compounds many folds by characterizing promoter strength, synthesizing promoter library and employing hybrid promoter approach (Sun and Alper 2017). Additionally, optimization of gene expression vectors is also very crucial for better metabolite production (Sun and Alper 2017).

1.4 Protein Engineering

Protein engineering is an important process for improving the protein quality that can eventually lead to enhanced the biomolecules production. Two of the general strategies used for protein engineering are rational protein design and directed evolution. Protein engineering has now moved far beyond the fixed number of amino acids coding for a particular protein, and expansion of genetic code or incorporation of unnatural amino acids for improving features of protein has already been attempted (Gohil et al. 2020). Rational protein design uses the knowledge of structure and function of protein and make suitable changes by site-directed mutagenesis. Though it sounds straightforward, it is not easy to predict the effects of mutation on a gene function (Marcheschi et al. 2013). In the directed evolution, a random mutagenesis using error-prone PCR or sequences saturation mutagenesis is applied on the protein and selection criteria are used for screening the desired traits. Several rounds of mutation and selection are used to find the desired features (Marcheschi et al. 2013).

A key to improving the cellular proteomics functions is through evolutionary engineering of either the carrier plasmid or the protein itself. The intensive protein-directed evolution may readily enhance the solvent tolerance of a strain and also endow them with unconventional functions (Cobb et al. 2013). Assembly methods with DNA fragments harbouring specific degenerate sequences or performing error-prone PCR are few of the widely preferred techniques to generate mutated proteins at

a particular locus or over a broad region, respectively (Yang et al. 2019). These methods are then followed by subsequent transformation and in vivo selection steps.

Designing heterologous metabolic pathways to create non-natural or non-endogenous products is often hit by the challenge of limited product yield, possibly arising due to poor enzyme activity under non-ideal conditions in the non-homologous systems. To overcome this challenge, a feasible solution is to engineer the enzymes to enhance their in vivo performance that suits the heterologous conditions, rather than upregulating the expression of protein (enzyme) to make up for the low activity. Optimizing enzymatic activity is apparently the most common objective to attain protein engineering endeavours (Singh et al. 2013). An approach to increase the in vivo activity of an enzyme is to mould the enzyme using tools of synthetic biology to adapt to the underlying conditions as faced in the production host. Directed evolution of enzyme hyperthermophilic geranylgeranyl diphosphate (GGPP) synthase from *Archaeoglobus fulgidus* to function at ambient temperature in *E. coli* is an example of this. The enzyme which is involved in the synthesis of the antioxidant astaxanthin (Wang et al. 2000) via the lycopene biosynthetic pathway was shown to increase the production of lycopene, a precursor of astaxanthin by 60–100% following directed evolution (Wang et al. 2000; Foo et al. 2012).

Lately, protein engineers have been working on extending the protein arsenal far beyond the limits of 20 naturally occurring amino acids. Scientists have been working on generating proteins with new functions and refined properties by assimilating non-natural amino acids into proteins (Gohil et al. 2020; Kohrer and RajBhandary 2013; Gao et al. 2019). These novel proteins are expected to play some very crucial roles in delineating functions that are not possible with conventional proteins. An interesting way to incorporate unnatural amino acids into proteins is through expanding the genetic code by introducing unnatural base pairs, which upon recognition by DNA polymerase may help in subsequent strand synthesis followed by its expression of proteins with novel characters. In this manner, the triplet codon system that accounts for 64 codons can be expanded to 256 codons if quadruplet system is taken into consideration (Gohil et al. 2020; Ovaa 2014; Diwo and Budisa 2019).

Functional expression of proteins may also add to improving their in vivo activity as the overexpression of a certain protein often tends to form inactive heterologous protein masses. Employing ‘back-to-consensus’ strategy to develop a mathematical model based on the principles of adaptive laboratory evolution and redistributing conserved amino acids boost production of metabolites by up to 80-fold in some cases (Yoshikuni et al. 2008).

1.5 Directed Evolution

It is possible to predict and design a protein if its catalytic mechanism, structure and conformation are known. The increasing number of protein structures that are being added regularly in the Protein Data Bank (PDB) have eased the rational designing of proteins which was actually difficult during the pre-genomic era. Despite this,

the protein dynamics being highly complex, any mutation distant from the active site can readily change the properties of protein (Foo et al. 2010; Petrović et al. 2018). For proteins whose structural and mechanical characteristics are unknown, a feasible approach is to go for directed evolution of those proteins. Directed evolution is cyclic process that mimics the natural selection process and steers gene diversification towards a more user-defined goal by selecting the functional gene variants (Packer and Liu 2015). The library size and high-throughput screening of mutants are some of the key determinants that drive the success of directed evolution. However, the unavailability of efficient screening methods demands limiting the size of the library to manageable numbers, so as to eliminate inactive mutants. In case the high-throughput screening of mutants is not possible, another approach is to make use of site saturation mutagenesis to identify target moieties through computational techniques such as homology-directed modelling. This allows to generate large number of mutants to select mutants of choice without the need of increasing the library size, thus facilitating effortless screening (Foo et al. 2012).

1.6 Design and Construction of Biosynthetic Pathways

Metabolic engineering has enabled to design and integrate potent biosynthetic pathways in microorganisms for the production of valuable chemicals and metabolites. For construction of de novo biosynthetic pathway, genes need to be identified from the environmental hosts and expressed into heterologous host. For the same, pathway prediction, analysis of yield and selection of parts are very crucial steps. The production of targeted molecule can be enhanced by introducing regulatory elements such as promoters, RBS, protein orthologs and regulators. Many synthetic biological tools and techniques, such as COnstraint-Based Reconstruction and Analysis (COBRA) (Becker et al. 2007), Biochemical Network Integrated Computational Explorer (BNICE) (Finley et al. 2009), golden gate assembly (Andreou and Nakayama 2018), Gibson assembly (Bordat et al. 2015), overlapping PCR (Bryksin and Matsumura 2010), CRISPR/Cas (Singh et al. 2017, 2018; Bhattacharjee et al. 2020), etc., have been developed to increase the metabolic flux towards targeted biomolecule. The resultant metabolic flux can be determined through a technique called metabolic flux analysis which examines the production and consumption rate of metabolites. In intracellular environment, it allows for metabolites quantifications. In this analysis, it is easy to find a pathway for upregulated or downregulated genes in order to enhance metabolites. It uses 13C to determine pathway fluxes with the help of nuclear magnetic resonance (NMR) and mass spectrometry. Moreover, the accessibility to various databases and repository for plasmids (e.g. Standard European Vector Architecture (SEVA)), standard biological parts (available at: http://parts.igem.org/Main_Page), and biological pathways (e.g. Kyoto Encyclopaedia of Genes and Genomes (KEGG)) have made the pathway engineering seamless (Trinh and Mendoza 2016).

1.7 Synthetic Biology Toolbox in Metabolic Engineering

Synthetic biology is a newly emerging field that employs the application of engineering principles to biology. Synthetic biology is used to develop a number of synthetic circuits for a wide range of applications including disease diagnostics, disease treatment and production of biomaterials, biofuels, and fine chemicals. Synthetic biology is not just about reshuffling the components found in nature (genes, promoters, RBS, etc.) to create newer functions but also about giving birth to entirely new breeds of components, albeit, as of now, its state-of-art is limited to only adding, deleting, altering or re-arranging DNA sequences using components that already exist (Singh 2014). Therefore, a need arises to develop next-generation synthetic circuits and technologies/methodologies that can solve some of the major issues of health, environment and energy.

Synthetic biology has led to tremendous explorations in the design of novel parts, devices and systems for better understanding of gene network and a variety of biotechnological applications (Singh 2014). Biobrick (http://biobricks.org) is one of the platforms to promote the idea of synthetic biology amongst students so that they can use the library for building gene cassette for biotechnological applications. With the advent of multiplex automated genome engineering, small regulatory RNA (Patel et al. 2018) and CRISPR-Cas9 techniques, the engineering of metabolic pathways has achieved a crucial breakthrough point.

1.8 Adaptive Laboratory Evolution

Adaptive laboratory evolution (ALE) is a method to study molecular evolution and adaptive changes in populations for a long period of time in vitro under specific growth conditions. It allows natural selection process to increase the overall fitness of the population (Dragosits and Mattanovich 2013). As for example, sodium tolerant mutants of *E. coli* can be generated by gradually increasing sodium concentration followed by continuous subculturing, which eventually allows and forces the strain to adapt the high sodium concentration environment (Wu et al. 2014). The potent genes that affect the fitness of mutants in the provided environment can be found by DNA sequencing (Wu et al. 2014). ALE has gained much scientific attention and is used widely for studying evolution process and generating industrial potent strains that can give high yield of products using cheaper resources. However, the downside is that this natural selection process is time-consuming and usually takes a few months to a year (Dragosits and Mattanovich 2013). In this regard, a simulator, ALEsim, was developed and validated to accelerate the time frame of ALE by designing and optimizing experiments (LaCroix et al. 2017). Industries prefer to use GRAS microbial cells for large-scale production of chemicals and other metabolites. However, the wild-type GRAS strains usually have less productivity or do not have adaptability for fermentation environmental stress conditions. ALE experiments have been performed by different research groups to push up the limits; such as *E. coli* and *S. cerevisiae* have been adapted to take up minimal medium of

glucose (Conrad et al. 2010), glycerol (Ibarra et al. 2002), lactate (Hua et al. 2007) and lactose (Dekel and Alon 2005), and withstand environmental stress of high temperature (Riehle et al. 2003), UV light (Alcántara-Díaz et al. 2004), freeze-thaw (Sleight and Lenski 2007, Sleight et al. 2008), osmotic stress (Stoebel et al. 2009), ethanol (Goodarzi et al. 2010), isobutanol (Atsumi et al. 2010), *n*-butanol (Reyes et al. 2012), pH (Dragosits et al. 2013), H_2O_2 (Dragosits et al. 2013), fluconazole (Selmecki et al. 2009) and high salt (Dragosits et al. 2013; Dhar et al. 2013).

1.9 Conclusion and Future Remarks

Microbes have been lauded as a sustainable source of a number of valuable substances with novel characteristics and functions. Naturally, these microbes produce meagre amounts of products in the form of secondary metabolites which in turn asserts their survival. However, these metabolites are produced only in very small quantities, sufficient to drive their survival but not enough to meet the ongoing industrial demands. Therefore, a pressing need arises to manipulate the microbes such that the metabolites are overproduced up to an industrially acceptable scale.

Though the commercialization of some biosynthetic molecules has been achieved in the past years, there remains a lot more number of products, the production of which have been limited due to the complexity and lesser explored aspects of their biochemistry and genetic engineering spaces. For the commercialization aspect, it becomes obligatory that these biosynthesized products compete with currently available products economically and also that they surpass the existing products in terms of sustainability and functionality. Devising means to engineer and develop strains can be a lucrative solution to produce substances endowed with the goodness of nature and that functions with the same synergy and modularity as the chemically-synthesized ones. Conventionally, genetically engineered strains improved by employing traditional mutagenesis practices are known to produce up to hundreds or thousands-folds of metabolites as compared to their originally isolated counterparts; a technique regarded as the 'brute-force' approach. Tremendous progress and strategic manoeuvring via advances in computational technologies, molecular and recombinant techniques and genetics have greatly uplifted the fermentation productivity and that has subsequently reduced the production cost as well. Reprogramming the genetic makeup of strains and introducing heterologous metabolic pathways have not only helped to achieve great titres but have also served other functions such as to eliminate undesirable by-products and analogues, unravel associated biosynthetic pathways, and discover novel antibiotics and other compounds.

Despite such advances, optimizing the engineered pathways to start giving titres up to acceptable range is easier said than done. The tight regulation within the biological system that controls and subsides the overexpression of metabolites often impedes the overproduction of desired compounds through means of negative feedback mechanisms. Additionally, introduction of heterologous genes and expression of foreign proteins may not work effectively or perform faultily, thus upsetting the inherent metabolic setting, causing overaccumulation of toxic compounds and leaving detrimental effects on the organism. Other than that, if the desired compound

is something which does not exist in nature but has been synthetically designed, then the substrate specificity of the natural enzymes plummets to a great extent. For all these reasons, the biosynthesis of metabolites on an industrial scale is far more challenging and a lot needs to be done to extract the most out of the engineered metabolic pathways and rise over the sub-optimal yields.

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2 Microbial Strain Engineering

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Abstract

The utilization of microorganisms for the production of chemicals at industrial scale requires improvements/changes at physiological, metabolic, and genetic levels. Natural or wild-type isolates produce minimal quantity of metabolites/compounds required as a matter of survival. Hence, to use these microorganisms at industrial level, different tools are required for strain improvement. These tools will improve the metabolite production of industrial importance. The strain improvement program traditionally employs classical mutagenesis approach followed by screening and selection of mutant strain. Today, in-depth understanding of genetics and recombinant DNA technology helps in strain improvement via metabolic and genetic engineering. These strain improvement approaches has increased the product yield with subsequent cost reduction. These approaches have also served other goals like reduction of undesirable products and elucidating the complex biosynthetic pathways. Further combination of different omics approaches like transcriptomics and proteomics with recombinant DNA technology has increased the prediction of accurate genes responsible for overproduction of metabolites/compounds.

Keywords

Mutagenesis · Physiology · Metabolic engineering · Genetic engineering · Omics

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2.1 Introduction

Microbes, the microscopically tiny miniatures, are the most abundant species present in the environment. Microbes are found in all the ecological niches and working as pillars of life on earth. Diverse microbes like bacteria, archaea, fungi, algae, protozoa, and viruses are in existence from at least 3500 million years ago and are supposed to be only present life forms on earth during that time. These life forms encompass the most phylogenetically diverse life on earth with many lineages. They dwell in every habitat including the terrestrial, aquatic, atmospheric environments (Sean and Jack 2015). Their presence reshaped many ecological, aquatic, and terrestrial niches including the extreme environments. Large diversity makes them suitable to live, adapt, and tolerant in many conditions like extreme salty environment, anaerobic conditions, limited water availability, extreme pH. Although, the microorganism grows in different niches, most important thing which makes them special in the way the metabolic pathways change for their existence in a particular environment. For example, although the microorganisms are microscopic with the addition of simple nutrients, they can grow on nutritive media and can be easily visualized by the naked eyes. This makes microbes to study them better. Additionally, microbes have a surface area where it can easily absorb the nutrients and release the end products. They also possess high metabolic activity making the system highly reproductive.

The presence of novel enzymes, high- and low-molecular-weight compounds, and metabolites makes microbes the best suitable source for industries to replace the chemically synthetic procedures with bio-based processes. In a real scenario, microorganisms can act as chemical factories for the production of commercially important compounds. Microbes, isolated from the natural environment and maintained in in vitro conditions, are mostly used for the industrial processes. Nowadays with the indispensable role of microbes in biotechnology, the use of microbes is increasing nearly in all the industries. The industrial sector like health care, pharmaceutical, food and beverages, agriculture, and chemical all are making efforts for generating bio-based/microbe-based process. The microbes, especially from diverse niches, can work as an important source for the discovering novel industrially important entities.

In any industrial process, it is important to consider that substitution to the chemical source needs as effective as any organic/synthesized molecule and also stable under different conditions. Microorganisms offer the best alternative as they are easy to handle and its maintenance is very much economically viable. The most challenging thing in the production of any compound using microorganism is to maintain the efficiency of the microbial line throughout the generations. However, in comparison to the mammalian and plant cells, microorganisms have a greater potential to produce/grow at high density within a short time frame. This makes the microbes feasible for large-scale industrial usage. The major disadvantage might be there is a frequent genotypic change in microorganism due to which microbes are highly variable and prone to the frequent mutations. Microorganisms could provide

everlasting solutions for environmental and societal issues. It can be used for the production of large volume commercially important drug, but the genetic stability with low fermentation economics needs to be prioritized.

For industries, the production of compounds requires to be at relatively lower cost with higher yields. Microorganisms that are used usually go through the strain improvement program for obtaining/screening the best suitable strain for commercial production of the industrially important compounds (Parekh et al. 2000).

2.1.1 Need and Significance for Strain Improvement

Microbes that live freely in the diverse ecological state are less likely to be suitable for the production of novel commercially important compounds. The metabolism of the wild-type isolate is inadequately adapted to the environment which is producing the enzymes and intermediates needed for surviving in a particular physicochemical condition. The regulation of metabolic and genetic machinery is controlled by the sequence of genes in genome. To improve these microbial strains, there is a prerequisite to alter the genes of metabolic pathways for overproduction of desired metabolites. In some cases, these changes lead to structural alteration in specific enzymes which increases the ability of enzyme to enhance its catalytic activity. Also, there are chances that due to alteration in a specific region of the gene (promoter), it can cause the deregulation of gene expression and metabolite overproduction. With the preset data set enzymes function, rate-limiting steps in metabolic pathways, environmental factors controlling growth help in designing screening strategies for the generation of industrially important mutant. However, the outcome of any strain development/selection depends on the kind and type of improvement we expect from the microbes (Elander and Vournakis 1986).

Fermentation economics is majorly dependent on product's manufacturing cost and the raw material prerequisite for production. Although lower fermentation cost can be availed by process designing like fermenter design and constructing material of fermenter, but the improvement in microbial strains offers the best opportunity for the cost reduction. Production enhancement through strain improvement for fermentation process is the prime factor which makes a major impact in fermentation economics.

Microbial engineering technique changes the genetic makeup of the microorganisms. It has played an exemplary role in biotechnology due to its unique features and ease of manipulation using recombinant DNA technology (Kou et al. 2016). For any strain to be used in industries, improvement is concerned with the development or modification for exploiting its properties for the production of compounds with less production cost and cheap raw material. The change in fermentation dynamics works well for optimizing the process for maximum production, but the strain improvement will give the desirable results at long time. The different methods for the strain improvement are given in Fig. 2.1.

Strain improvement/engineering encompasses creation of strains with the following properties.

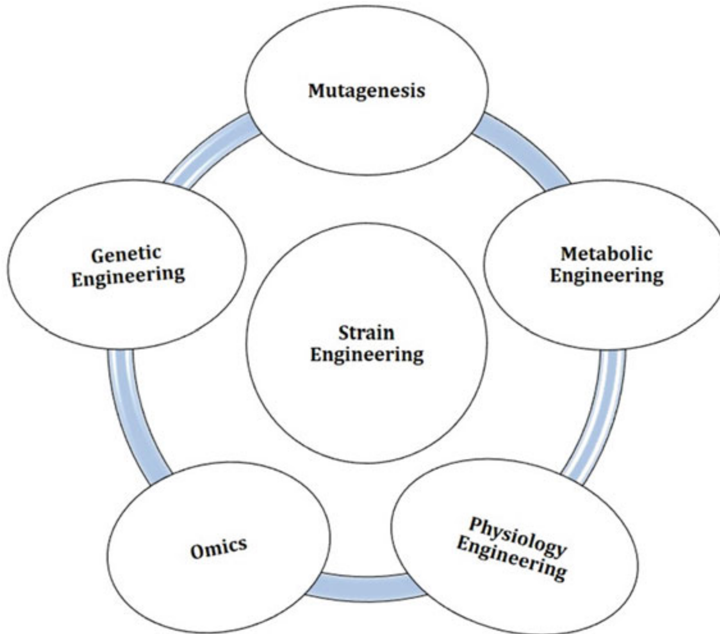

Fig. 2.1 Approaches of strain engineering

(a) Proficient assimilation of low-cost and complex raw materials.
(b) Removal of byproducts and change in the product ratios.
(c) Overproduction and excretion of native and foreign products.
(d) Short duration of fermentation time and easy scale-up.
(e) Tolerance of various metabolites produced (Parekh et al. 2000).
(f) Provide morphological changes in cell which is suitable for product separation.

The traditional empirical approach for the strain improvement is mutagenesis of the isolated/screened strain. The mutagenesis in this approach is random and is followed by the direct titer of large number of isolates. This approach has been successful in most of strain improvement program in pharmaceutical industries (Vinci and Byng 1999; Parekh et al. 2000). Although, the mutation is used to shift the proportion of metabolites produced in the fermentation broth to a more favorable distribution, elucidate the pathways of secondary metabolites, the yield of new compounds, etc. The major disadvantage of these techniques is a laborious procedure as a large number of isolates are processed in order to detect/screen the improved strain. The possibility to reduce the amount of work can be better understood by elucidating the biochemical and genetic mechanisms controlling the different metabolic pathways. Recently, most of the research and academic centers are developing the model organisms which can work effectively for system designing at industrial scale (Rowlands 1984). In context to the above lines, the present

chapter portrays different methods for strain engineering for improved production at industrial scale.

2.2 Mutagenesis

Current research trend focuses on influencing the regulation of specific biochemical pathways and strive for strain improvement to achieve the desirable conditions, metabolite, or phenotype. The strain improvement can either be achieved via a coherent metabolic engineering approach or by randomized mutagenesis strategy (Zhang et al. 2015). Whatever the case may be, a comprehensive understanding of genome and underlying metabolic mechanism of the microbial strain is of prime importance. In order to improve strain by using mutagenesis approach, the information of metabolic regulation mechanism, a well-defined rational plan and a robust method is required (Hu et al. 2017). One of the key challenges in strain improvement programs is involvement of enormous amount of time, cost, and need of skill labor arising due to the complications in understanding multifarious interactions of metabolic pathways. Furthermore, due to the poor understanding of underlying mechanism and pinpointing of precise sequence for manipulation, the radical metabolic engineering approach is always a time-consuming process (Lee and Kim 2015). On the other hand, random mutagenesis involving physical mutagen such as UV is rapid, relatively cost-effective, requires limited training and skill set, and is safer to handle compared to the chemical mutagen. However, physical mutagen generates mutations arbitrarily, and has a possibility to generate nonspecific or multiple mutations in genome.

2.2.1 Physical and Chemical Mutagenesis

The most common physical mutagen uses ionizing radiation such as gamma rays ($\lambda < 0.01$ nm), X-rays ($\lambda = 0.01$–10 nm), alpha particles, and non-ionizing radiation such as UV rays ($\lambda = 10$–400 nm) (Kodym and Afza 2003). The role of physical mutagen operates by two mechanisms, direct and indirect. The direct effect involves the direct ionizing of DNA strands owing to ejection or excitation of electrons to a higher energy level (Ravanat and Douki 2016) (Table 2.1). The indirect effects are produced by shifting of electrons to induce activate molecules, also known as free radicals ($OH^{\bullet}$ and $H^{\bullet}$) that arise from OH^{-} and H^{+}, resulting in base modification and/or single−/double-stranded breaks in the DNA (Morita et al. 2009). These free radical or reactive oxygen species (ROS) can result in the double strand breakages (DSBs) causing deletions and translocations. In some cases point mutation can arise owing to single-strand breaks (SSBs), or due to nitrogen base switchover for example, the conversion of pyrimidine bases to 5-(hydroxymethyl) uracil, 5-formyluracil, 5-hydroxycytosine, and 5-hydroxyuracil (Min et al. 2003).

DNA damage due to UV light exposure can be explained by two mechanisms. UVC ($\lambda = 200$–280 nm) and UVB ($\lambda = 280$–320 nm) are absorbed readily by

Table 2.1 Physical mutagenesis of some key microbial organism

Strain	Species	Mutagen type	Target metabolite	References
Bacteria	*Sporolactobacillus inulinus ATCC 15538*	UV mutagenesis	Lactic acid	Zheng et al. (2010)
	Streptomyces xanthochromogenes RIA 1098	UV mutagenesis	Compactin-resistant	Dzhavakhiya et al. (2015)
	Streptomyces griseoaurantiacus	UV mutagenesis	Production of endoglucanase and β-glucosidase	Kumar (2015)
	Streptomyces flavoviridis G-4F12	UV mutagenesis	6′-Deoxy-bleomycin Z	Zhu et al. (2018)
	Sporosarcina pasteurii (MTCC 1761)	UV mutagenesis	Urease and calcite production	Achal et al. (2009)
	Lactobacillus delbrueckii NCIM 2365	UV mutagenesis	Lactic acid	Kadam et al. (2006)
	Acidianus brierleyi	UV mutagenesis	Tolerance to $Cu2^{+}$ and a high bioleaching rate of chalcopyrite	Meng et al. (2007)
	Clostridium tyrobutyricum ATCC 25755	Heavy-ion irradiation ($^{12}C^{6+}$)	Ameliorate butyrate by 68%	Zhou et al. (2014)
	Dietzia natronolimnaea	Heavy-ion irradiation ($^{12}C^{6+}$)	Boost canthaxanthin	Zhou et al. (2013)
Fungi	*Penicillium janthinellum NCIM 1171*	UV mutagenesis + ethyl methyl sulfonate (EMS)	Boost cellulase	Adsul et al. (2007)
	Rhodosporidium toruloides 8766 2-31 M	UV mutagenesis	Lipid productivity	Yamada et al. (2017)
	Fusarium maire K178	UV mutagenesis + diethyl sulfate (DES)	Paclitaxel (taxol)	Xu et al. (2006)
	Aspergillus terreus CA99	Heavy-ion irradiation $^{12}C^{6}$	Increase lovastatin by four times	Li et al. (2011)
	Trichoderma viride	Heavy-ion irradiation $^{12}C^{6}$	Increase cellulase	Li et al. (2016)
	Aspergillus niger	Heavy-ion irradiation $^{12}C^{6}$	Increase cellulose	Wang et al. (2015)

Algae	*Scenedesmus* sp.	UV mutagenesis	Lipid productivity	Sivaramakrishnan and Incharoensakdi (2017)
	Nannochloropsis oceanica IMET1	Heavy-ion irradiation $^{12}C^{6}$	Boost biomass productivity and growth	Ma et al. (2013)
	Desmodesmus sp.	Heavy-ion irradiation $^{12}C^{6}$	Lipid productivity	Hu et al. (2013)
Cyanobacteria	*Spirulina* sp.	γ-Rays (^{60}Co)	CO_2 fixation	Cheng et al. (2017)

nitrogenous bases, resulting in the trigger of excited states and formation of pyrimidine dimers (Brash 2015). Whereas UVA (λ = 320–400 nm) and to some extent visible light (λ = 400–740 nm) interact via photosensitizers (produces chemical modification in another molecule by means of photochemical reaction) to initiate the DNA damage (Epe 2012).

Often, ionizing radiation results in the biological injuries to the cells and tissues. Therefore, before starting the mutation studies, the exposure and dose should be selectively controlled and maintained. Moreover, bacterial tissues are soft and hence needs lower dose of radiation and exposure. In most of the scenario, the dosing is often limited to 2–3 doses along with control; however, if the primary goal is to obtain a decent amount of mutants in surviving population, the optimum dose should be standardized to obtain the highest proportion of desirable mutants (Sauer 2001).

Unfortunately, either of induced or spontaneous mutations are of little use as the exact mechanism of mutation needs to be thoroughly understood for its repetitive and specific usage. From past many years, specific chemical mutagens like base analogs, deaminating agents, alkylating agents, and intercalating agents are used for the site-specific chemical mutagenesis to obtain desired mutant having industrial application. Certain chemical compounds may result in changes in DNA structure or its sequence, resulting in mutation. Such chemicals, which induce mutations, are known as chemical mutagens. Chemical mutagens or genotoxic compounds are natural as well as man-made. A strategy of using chemical agents to generate mutations in desired strains is known as chemical mutagenesis. Base analogs and DNA intercalating agents are two biggest class of chemical mutagens. A base analog can replace a DNA base during replication and can result in transition mutations. Whereas, intercalating agents are molecules that may get inserted in between DNA bases, resulting in frameshift mutation during replication. Other chemical mutagens may act by the generation of reactive oxygen species (ROS), deamination, alkylation, etc. (Table 2.2).

Chemical mutagens cannot perform a site-directed mutation and generally their effect is hence random that to at multiple sites of genome. Mutagenesis has been used since long in several microorganisms to enhance the performance and productivity arising from single or multiple gene traits (Giudici et al. 2005). A very good example of such strain improvement is production of penicillin antibiotic from *Penicillium chrysogenum* with huge increase of more than three orders of magnitude, attained after 65 years of research and development using multiple mutagenesis techniques (Demain 2010).

2.2.2 Mutation Signature

A mutation signature is specific mutation owing to the unique mutagenesis process (Brash 2015). The mutation signature helps to provide insight to screen out potential mutant colonies and can further be used for targeted therapies, in case of oncology (Forbes et al. 2017). However, the mutation signature currently is limited only in oncology studies but can also be extended for the microbial cells. One of the key

Table 2.2 Mode of action of physical and chemical mutagenic agents used for strain improvement

Agents	Mutagens	Mode of action	Type of mutation
Physical	Ionizing radiations (γ-rays, X-rays, α-particle)	Single- and double-strand DNA breaks, deamination, and dehydroxylation of nitrogenous bases	Point mutation
	Non-ionizing radiations (UV-A, UV-B & UV-C)	Pyrimidine dimers, mitotic crossing over; hydroxylation of nitrogenous bases and cross-linking DNA strands	Frameshift mutations, base pair substitutions, transversions, and deletions
Chemical	Base analogs (5-FU)	Thaimine analog tauntomerizes and pairs with guanidine	Transition
	DNA intercalating (EtBr, Proflavine)	Inserting an extra base opposite an intercalated molecule	Transition and frameshift mutation
	Alkylating agents (EMS, ENU, MNNG)	Ethyl or methyl group transfer to nitrogenous bases, cross-linking of DNA strands	
	Deamining agents (HNO_2)	Interstrand cross-linking of DNA, deamination of the amino group of adenine, and cytosine to an ether group	Transition

example of mutation signature is UV signature, where in the UV light is used to target the two pyrimidines (C or T) adjacent to induce CC → TT substitutions (Brash 2015). Another example involving mutation signature is the bacterial cells which undergoes stress during the radiation dose, owing to cellular and DNA damage. In such case, the cellular repair mechanism becomes active, for example, the *recA* is expressed due to DNA damage and thus expression of *recA* promoter is a suitable choice for screening out the potential mutant colonies (Min et al. 1999).

2.3 Engineering Physiology of Microbes

For industrial application of any microbe, microbial physiology plays a major role for the identification of production hosts and in designing strategies for strain improvement. The metabolic activity gives reflections of the physiological responses/adaptation to the external environments in which microorganisms are growing. The physiological performance of the microorganism is a type of selection criteria for its industrial usability. It is also influenced by the change and combination in components present inside cells with respect to the external environments, viz. the conditions prevailing during the fermentation process. Important physiological characteristics like fitness, tolerance, and robust nature ensure the industrial value of microorganisms. In this concern, the engineering of the microbial physiology is important to make the process more industrial viable (Zhang et al. 2009).

Fig. 2.2 Steps in physiological engineering

For physiological engineering, the most important thing to understand is the mechanism of cells sensing and adapting to the environmental changes. Also, it is important to critically analyze the specific adaptation of few organisms in extreme conditions like high pH, temperature, and solvents. For successful engineering of microbial physiological functions, below-mentioned steps can become helpful in designing an industrial strategy for the strain improvement (Fig. 2.2).

1. Define the desired physiological characteristics.
2. Candidate screening.
3. Selection of host strain.
4. Engineering within host strain.

2.3.1 Desired Physiological Characteristics

The selection of the target strain possessing desired physiological characteristics is based on the bioprocess used for compound/metabolite production. The factors involved in the selection of process are mainly the market demand and cost of process parameters like methods for strain improvement (upstream) and for purification (downstream). For example, sulfuric acid is used for ethanol production from the corn straw. During the process, to avoid contamination, acidic fermentation at high temperature is prerequisite (Shaw et al. 2008). Also, with acidification at high temperature, the microbial cells should also perform simultaneously hydrolysis of the sugar to produce ethanol (Wisselink et al. 2009).

2.3.2 Candidate Screening

Strains with more desired characteristics are screened using tools like directed evolution, non-specific mutagenesis, and stress-induced adaptations (Foster 2007; Galhardo et al. 2007). The screened strain will be ideal/model candidate for generation of library for candidate strains for further screening. With much collection of screened strains, the library contains strains which are having mutation at different sites or have engineered genes important for the industrial applications. In practical scenario, the targeted strain will possess the desired physiological characteristics by using the different genetic tools and mutation strategies. However, once the

collections of screened microbial strains are available, the efficiency of the process to extract the desired screen heavily relies on high-throughput screening process.

2.3.3 Selection of Host Strain

Once the strain having desired characteristics is identified, further it is important to understand the metabolic regulatory network/pathways along with the genes by genome-wide annotations. However, the other microbial physiological characteristics like fitness, tolerance, and robust nature depend on complex cell components. Therefore, it is utmost important that during designing an physiological engineering strategy, it is better to understand first the underlying physiological characteristics of screened microorganism (Lee et al. 2006; Jeffries et al. 2007). This gives a clear idea for selection of suitable host. The host which shows/exhibit the most complex physiology and which is fully genetically characterized can be the best suitable host for physiological engineering.

2.3.4 Engineering into the Host Strain

After successful screening of strain and identifying the host strain, the remaining part is expression or infusion of the desired characteristics by various engineering approaches. If the host microbe is strong and fit, the first target to be engineered should be microbial regulatory metabolic capabilities. The host should also be adaptable to the engineered changes so that the expression of the desired product can be easily screened. Another approach can be the reverse engineering. In this type, the host is not fit and robust, but it is highly metabolically active. The promising route to proceed with such strain is to elucidate/decode the evolutionary mechanism of stress-tolerant microorganisms. The rationale for selection of stress-tolerant microorganisms is that the physiological characteristics can be transferred to host strain by the DNA altering genes, transposons, or by genome shuffling (Foster 2007).

2.4 Metabolic Engineering

Metabolic engineering is intended for direct improvement in formation of product or its cellular properties via alteration/modification in particular biochemical pathways or by intruding the new set of specific regulatory genes with aid of recombinant DNA technology (Stephanopoulos 1999; Nielsen 2001). Metabolic engineering has emerged as new designing/engineering in which microorganisms are capable of novel compound production. Technique is amalgamation of control of fluxes with molecular tools and at the same time quantifies the fluxes with analytical methods for getting the desired genetic alteration. The metabolic engineering has revealed that the flux associate with a metabolic pathway is not a single rate-limiting step; instead

it is dependent on many steps involved in the biochemical pathways (Kacser and Acerenza 1993).

2.4.1 Methodologies and Tools for Metabolic Engineering

Before designing the different strategy/methodologies for metabolic engineering, it is important to identify the key fundamental essential requirements:

1. Detailed information of biosynthetic pathway of compound to be produced.
2. Set of genes encoding the enzymes and its regulatory pathways.
3. Methods to transfer and express the desired gene in the host organism.
4. Different tools for in vivo and in vitro gene mutation.

Metabolic engineering normally starts with the genetic alteration which is followed by characterization of gene expression and further analysis of change in the metabolic pathway of the mutant. Many strategies are used for designing a pathway for the metabolic engineering. Few major strategies are as follows:

2.4.2 Engineering of Biosynthetic Pathways

For engineering the biosynthetic pathway, the first step is to understand the key components of pathways and its regulatory points.

(a) Increase the number of genes coding for rate-limiting steps in biosynthetic pathway (Cremer et al. 1991).
(b) Increase/amplify the genes responsible for the branch or end point enzyme which we can give direction to the intermediate compound to move the process.
(c) Infuse heterologous enzymes with unusual structures which can allow them to bypass regulatory step.
(d) Infuse heterologous enzymes having diverse mechanisms which are functionally more advantageous (Ikeda et al. 1994).
(e) Inclusion of the enzyme which is divergence point for the central metabolic pathway. This will lead to increase the flow of carbons in biosynthetic pathway.

2.4.3 Central Metabolism Engineering

Central metabolic pathways are the main suppliers of energy and precursors for biosynthesis of many essential compounds. Engineering the central metabolic pathways is very complicated as it is regulated globally, and the identification of regulatory network pattern is yet to be resolved completely. For example, the omission of phosphenolpyruvate carboxylase from the biosynthetic pathway has led to increase the production of threonine by 40% in *E. coli* (Hermann 2004).

Another example is of increase in lysine production in host strain *C. glutamicum*. 250% increase in lysine is reported by overexpression of pyruvate carboxylase and aspartate kinase (Koffas et al. 2003). The pentose phosphate pathway which aids in the synthesis of aromatic amino acids is responsible for major supply of NADPH, ribose-5-phosphate, and erythrose-4-phosphate. By amplification using error-prone PCR for transketolase gene, it increases the production of erythrose-4-phosphate which leads to mutant having higher tryptophan production capacity (Ikeda and Katsumata 1999).

2.4.4 Transport Engineering

Mutants that have modified transport systems can continue to thrive at low intracellular level of the product. Such mutants are not subject to feedback control. For example, there is significant increase in tryptophan and threonine yields using a *C. glutamicum* and *E. coli* mutant generated by transport engineering (Ikeda and Katsumata 1995). Mutants having dynamic efflux system and weak uptake system can overexpress the amino acid without deregulation of biosynthetic pathways (Ikeda 2003).

2.4.5 Engineering the Whole Cell

Majority of metabolic pathways and flux associated with the central metabolism is extensively studied and elucidated in few microorganisms. However, the regulation of flux and physiology is not reported in the microorganisms which are of industrial interest. With much development in genetic tools, currently it is difficult to predict possible product outcome of metabolic pathways when it is redirected. For example, the intersection between the glycolysis and TCA cycle is most important for the regulation of amino acid synthesis. Moreover, the control of pathway flux is not controlled but shared. In this context, the inverse metabolic engineering, physiology engineering, and systems biology have become important tools for designing an industrially important mutant (Koffas and Stephanopoulos 2005). Novel approaches like functional genomics and genomic breeding are trending as these tools can identify and remove the unwanted mutants from the process (Petri and Schmidt-Dannert 2004).

2.5 Genetic Engineering

The production level of desired compound from the natural wild type isolate is always too low for the industrial applications. Henceforth, the strains are improved using different engineering methods. Among all, the most important is genetic engineering. In normal approach, by single step improvement, there is no significant improvement in the product yield. In comparison, the genetic approach is specific for

microbial engineering of industrial importance (Verdoes et al. 1995). The following points needs to be considered for constructing the genetically engineered strain.

(a) Screening of the desired microbial strain.
(b) Cloning of desired gene required for the synthesis of desired protein/metabolite.
(c) Amplification for creating multiple copies of genes.
(d) Investigation of the expressed product.

2.5.1 Screening

The molecular basis of selection of specific strain depends on the specific enzyme activity of the microbial strain (Van Gorcom et al. 1990). Once the specific strain is selected, the desired enzyme/protein of interest is cloned from this strain. Many methods are reported for gene cloning using different fungal species as host organisms (Timberlake 1991). Routinely, the "reverse genetics" is used for insertion/cloning of desired genes. This method works with very simple principle of elucidation of genomic or cDNA from the isolated proteins from the cell extracts. Briefly, the desired protein from the culture filtrate is purified and amino acid sequence of purified protein is resolved. From the resolved amino acid sequence, oligonucleotides are designed. These nucleotides are used for screening of cDNA library. Further, DNA is amplified by PCR for further experimentation (Choi et al. 1993; Gomi et al. 1993).

2.5.2 Gene Expression

Host strains which are used for overproduction are mostly constructed by insertion of multiple copies of gene of interest. However, the direct selection is not possible of strains having multiple copies, henceforth the indirect selection of the strain having multiple copies is done using a selectable marker. This selectable marker is inserted in same vector as gene of interest. The transformed mutants have desired sequences which are stable by integration with genome by homologous or nonhomologous recombination. After insertion of the gene sequence, it is most important that the gene is expressed at desired level. For example, in certain cases it was found that even though strains possess multiple copies of gene but the expression level is not significant. Therefore, for improvement of gene expression level, it is necessary to design a system in which the expression levels of gene of interest can be controlled or modified for higher yields (Verdoes et al. 1995).

2.5.3 Enzyme (Over)Production and Posttranscriptional Control

It is important to study a correlation between copies of gene of interests and protein production. A previous finding suggests that with high copy numbers protein

production levels are lower than expected. The amount of protein expressed in the culture medium not only influences levels of expression but also the protein degradation pattern was observed in a study conducted for overproduction of pectin lyase enzyme (Kusters-van Someren et al. 1992).

Another approach can be possibly to associate posttranscriptional mechanisms for tuning the expression of multiple genes in the operons. Pfleger et al. (2006) has successfully demonstrated the library of tunable intergenic regions (TIGRs). TIGRs consists of many control elements like mRNA, RNase cleavage sites, and couple of sequestering sequences. TIGRs was able to modify the processes of transcription, mRNA stability, and initiation of translation. Combination of RNAse site and TIGRs in particular cleavage sites has helped to fragment (decouple) the coding region stability helping the self-regulating expression variations. This method was successfully utilized for the optimization of flux for mevalonate pathway using host strain *E. coli*. Using this method, significant increase of sevenfold increase in mevalonate production was reported. This overexpression was observed due to counteractive mechanism of HMGS and tHMGR reduction activity. Therefore, it is utmost important to understand that combinatorial strategies for strain improvement will be able to rescue the phenotypic variant for the beneficial changes in strain improvement. Also, the combination will make the system much more understandable and clearer for designing of novel tools for strain engineering.

2.6 Omics for Strain Engineering

With the advancement in DNA sequencing technologies, the data obtained from DNA sequencing is much more rapid, reliable, and specific. Nowadays, for most of the model organisms, complete genome sequences are there which increase the postgenomic research in the strain development field. With help of complete data set, it is easy to predict the probable outcome of the genotypic changes to be incorporated for the strain improvement. Transcriptomics and proteomics allow parallel analysis of mRNA and protein expression levels using the DNA microarrays and two-dimensional gel electrophoresis system or mass spectrometry methods. The other approach is metabolomics which quantifies the metabolites and intermediates using the analytical technique mass spectrometry or nuclear magnetic resonance spectrometry. The study of flux in the metabolic pathways; fluxomics allows the quantification of metabolic fluxes based on balancing of metabolites or isotope analysis.

2.6.1 Genome Analysis

Comparative genome analysis is a simple but very useful technique for the identification of desired genes that can be inserted or deleted for achieving the desired phenotypic change. Using the genome analysis, easy comparison is possible for the wild type and mutant/engineered strains. In one approach, the only essential or active

genes helpful for the metabolic functions are retained while the unnecessary genes are deleted without any genomic or metabolic burdens (Kolisnychenko et al. 2002). This technique used is called minimum strain development. The minimum strain development suggests that only the metabolically active gene sets are present in the host microorganisms. Ohnishi et al. (2002) has compared the genome sequence of the wild-type *corynebacterium* strain to locate the genes having point mutation and are also responsible for the overproduction of L-lysine. Even though, we have succeeded in engineering of few industrially important strain at genome scale, but the initiation in this field has led us to understand the engineering of local reactions in the biosynthetic pathways and possibly can lead to significant improvement in the performance of screened microorganism (Lee et al. 2005). The other major advantage of genome analysis is that using the data of whole genome in silico metabolic models can be developed which can give the prediction of expected gene expression levels.

2.6.2 Transcriptome Analysis

With development of throughput DNA microarrays, the accuracy has increased for quantification of the changes at the gene transcription levels by monitoring the relative changes in mRNA level in multiple samples. Comparing the transcriptome profiles of wild-type and mutant-type strains at different time points/culture conditions helps to identify/locate the regulatory networks and probable target genes to be altered/manipulated. The new information and understanding in this way can be further used to improve the performance of microorganism. For example, transcriptome profiles of *E. coli* used for the production of human insulin-like growth factor was analyzed by high cell density culture method. From the data sets of nearly about 200 genes, only those genes involved in the amino acid or nucleotide biosynthetic pathways were targeted. Among these genes, the amplification of prsA and glpF genes has shown increased production of IFG-I_f production from 1.8 to 4.3 g/L. These two genes were encoding for phophoribosyl pyrophosphate and glycerol transporter. The cited example suggests that the strategy for targeted engineering-based approach using the global information will allow the identification of gene which would be helpful for the construction of superior strain giving high yield at industrial scale.

2.6.3 Proteome Analysis

Proteome analysis, a prevailing tool for the comparative analysis of two or more protein spots, needs to be done showing varying intensity under different genetical and environmental conditions. Also, proteome analysis is much more useful as the central metabolic activities are correlated or mediated via proteins. Henceforth, proteome analysis will give us edge to understand more regulatory networks of metabolic pathways. The major disadvantage of proteome analysis is that every

protein spot is not identified yet, and therefore the information gathered after proteome profiling may be less compared to the transcriptome profiling. Best example for the proteome analysis is proteome comparison of two *E. coli* strains. The first strain was gathering the biodegradable polymer 3-hydroxybutyrate and the other is wild-type/native *E. coli* strain. This comparison has helped in the identification and understanding of the importance of protein Eda (2-keto-3-deoxy-6-phosphogluconate aldolase) in poly(3-hydroxybutyrate) production by mutated/engineered *E. coli* strain (Han et al. 2001). Another classic example is of comparison of *E. coli* strains overproducing the human leptin. Interesting observation was that the expression of few enzymes was decreased significantly, indicating the possible limitation in serine biosynthetic pathways. At quantification levels, the content of serine in leptin is about 11.6% which is significantly higher than the serine content 5.6% found in proteins expressed in *E. coli* (Han et al. 2003). The above-cited examples indicate even though there is limited information in proteome profiling/analysis, it can result into successful designing of a new strategy for strain improvement.

2.6.4 Fluxome and Metabolome Analysis

2.6.4.1 Metabolome Analysis

The varied and dissimilar chemistry of different metabolites and availability of only a limited number of detectable chemicals/standards turns the whole cell metabolome profiling realistic. These analyses were possible due to the development of the high-throughput quantification methods like NMR, gas chromatography (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and MALDI-TOF. Usually, the number of metabolites in cells are always fewer than the number of genes expressed in specific conditions which give edge to metabolome analysis compare to the transcriptomic and proteome analysis. For example, the low-molecular-weight metabolites in *S. cerevisiae* is estimated to be only 560 which is very less compared to genes or proteins expressed. Also few researchers have tried to integrate the metabolome and transcriptome analysis for construction of mutant strain giving the high yield. Furthermore, Wittmann and Heinzle (2001) has used the metabolome profiling to understanding of flux distribution in *Corynebacterium glutamicum*.

2.6.4.2 Fluxome Analysis

For better understanding of cellular metabolic status, it is important to go for metabolic flux analysis. As intracellular flux cannot be easily quantified, they are usually measured using the bioinformatics approach. For calculation of fluxes, practical data like substrate uptake and secreted product are constraints for proper analysis. The isotope analysis/experiments can provide important information on the intracellular fluxes. Usually, for the isotope analysis, the 13c carbon is labeled uniformly on substrate in the process. Whenever, the substrate is metabolized in the cell, isotope distribution can be located, and intracellular flux ratios can be calculated (Wittmann and Heinzle 2001).

2.6.5 Combined Omics Approach

The real integration of all omics approaches is still not a realistic. There are several reported approaches where different combination of omics approach was tried for improvement of strain. For example, the combination of transcriptome and proteome was carried out in *E. coli* strain for overproduction of L-threonine. The results show that genes involved in the TCA cycles, amino acid biosynthesis, and glyoxylate shunt were upregulated compared to the downregulated ribosomal proteins. Due to this combination, significant overproduction of L-threonine was reported (Lee et al. 2003).

Another classic example is of improvement of *Aspergillus* strain for production of lovastatin. In this, a combination of transcriptome and metabolome analysis was carried out. First, the libraries were constructed of the desired strains having desired gene expressions. These screened strains were further characterized by the metabolome and transcriptome profiling. By using these combination approach, the resultant mutant strain was able to secret 50% more lovastatin compared to the wild/native strain (Askenazi et al. 2003).

2.7 Conclusion

Microorganisms have natural tendency to produce compounds of industrial importance. The power of microbial culture needs to be appreciated due to fact that even simple molecules which are similar to synthetic compounds are produced by microorganisms using different fermentation process. Strain improvement programs are completely required for commercial production of compounds at industrial scale. To obtain these desired properties, it is utmost important to define the right host strain having specific physiological, biochemical, and genetical functionalities. Furthermore, advances in high-throughput screening and omics approaches have enabled rapid isolation of mutant strains having desired expression profiles. We believe that this review will provide the valuable insights for identifying and designing strain optimization strategies to improve product yield and reduce the fermentation economics.

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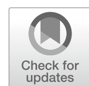

3 Techniques for Detection and Extraction of Metabolites

Payal Gupta, Sonam Gupta, and Vikas Pruthi

Abstract

Metabolites are the small low-molecular-weight biological molecules that are key players in energy conversion and biosynthesis reactions. Both qualitative and quantitative investigation of the entire set of intracellular and extracellular metabolites extracted from growing cells at a specified time of their growth or reproduction cycle is called metabolomics. However, analysis of metabolites is extremely challenging due to factors like metabolites' reactivity, structural diversity, and broad concentration range. This chapter outlines the past, present, and future development in various extraction and detection protocols of metabolites. These improvements are made majorly in fast sampling as well as quenching of cellular activity, along with quick extraction of the intra- and extracellular metabolites. Furthermore, it will also briefly describe metabolite quantification using modern hyphenated analytical protocols, which chiefly involves use of chromatographic platforms (LC, GC, CE) coupled to mass spectrometry and nuclear magnetic resonance spectroscopy (NMR).

Keywords

Metabolomics · Mass spectrometry · NMR · LC-MS · Quenching

3.1 Introduction

Low molecular weight (>1500 Da) organic and inorganic molecules which are intermediate or end products of enzyme-catalyzed metabolic reactions occurring inside cells during metabolic pathway are known as metabolites (Fiehn 2002). The

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metabolites do not follow a fixed structural template like DNA, RNA, and proteins, and thus have diverse physical properties (Lu et al. 2017). Indeed, a group of analytical tools are required for studying complete range of metabolites and metabolic networks (Bino et al. 2004). In light of biological dimension, metabolites can be classified as primary or secondary depending upon their role in cell physiological processes, while chemically, water soluble and insoluble (Lu et al. 2017). The solubility is key performer in studying about metabolite's structure and function, like primary water-soluble metabolites play crucial role in the conversion of nutrients into usable energy, provide the basic building blocks for synthesis of biomolecules and turn over rapidly along with simultaneous execution of the vast majority of metabolic flux (Whitfield et al. 2004).

The field which deals with the identification and characterization of both endogenous and exogenous metabolites is called metabolomics. It is a global metabolite profiling scaffold which involves application of high-resolution analytic techniques along with chemometric statistical tools (Zhang et al. 2012). This will generate an integrated picture of both endogenous and exogenous metabolites including both organic (nucleic acids, peptides, amino acids, vitamins, carbohydrates, polyphenols) and inorganic species. For separation, identification, and accurate measurement of these small molecules, innovational technologies of high resolution are required like nuclear magnetic resonance (NMR), mass spectrometry (MS), high and ultra-performance liquid chromatography (HPLC/UPLC), gas chromatography (GC), CE, liquid chromatography (LC) (Holmes et al. 2008). For representing the functional phenotype of a cell, tissue, and organ, detailed study of small molecules is performed which enables the utilization of biomolecule as biomarkers (Arakaki et al. 2008). Also, for getting more deeper insight into the factors, like dietary and lifestyle, responsible for manipulating specific disease, measurement of metabolites is recommended (Patejko et al. 2017). The major reason for quantification and evaluation of metabolites is its ability to reflect both the normal and pathological biological processes and even pharmacological response to any therapeutic intervention and thus widely accepted in clinical practice for diagnosis (Van der Werf 2003; Villas-Boas et al. 2007; Canelas et al. 2008). Metabolomics has also been useful in defining metabolites which are related to both prognosis and diagnosis of diseases and thus would enhance our understanding to pathophysiology of a disease (Wishart et al. 2016). Hence, the application of set of metabolomics technique provide direction for advancement and integration of robust as well as reliable protocols involved in biomass sampling, cultivation of microbes, isolation/extraction, and quantification of these metabolites.

The brief outline of procedure involved in cellular metabolite studies is depicted in Fig. 3.1. Firstly, microbial metabolic activity is rapidly quenched by using different approaches like traditional one where sample's temperature is changed instantly ranging from < -40 °C to very high >80 °C, or by varying sample pH from highly alkaline to very low acidic. Secondly, the method of filtration or centrifugation is preferred for separating quenched cells from the medium, followed by extraction of intracellular metabolites using organic solvents at high or low temperature from biomass. Later under vacuum, organic solvents are evaporated

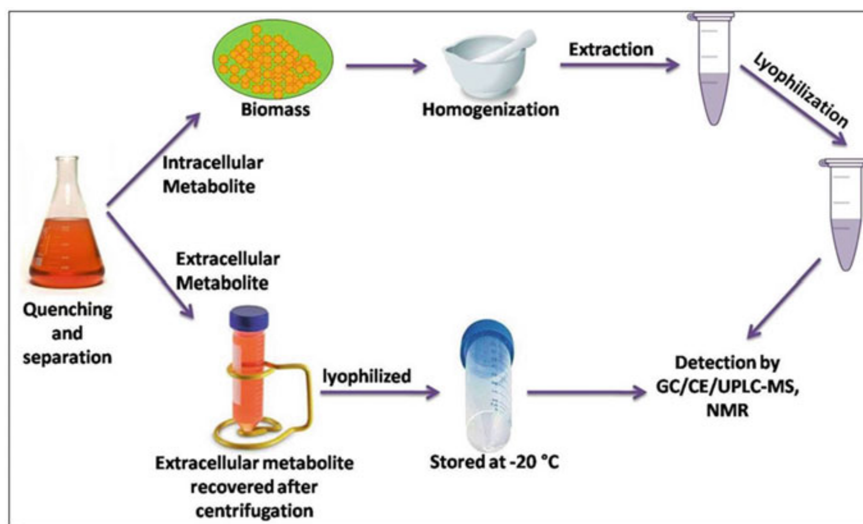

Fig. 3.1 Schematic representation of an overview of steps involved in the extraction and detection of metabolites

while the outstanding residue is dissolved in small amount of milli-Q water followed by centrifugation. Finally, the supernatant containing metabolite is stored at low temperature until analyzed by suitable analytical method.

This chapter focuses on the practical challenges that are encountered during analysis of primary metabolites. Initially, we will discuss quenching and extraction steps that are universal in metabolite preparation and critical for accurate measurement. This is followed by in-depth discussion of techniques used for the detection of metabolites which include liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), nuclear magnetic resonance (NMR), and enzyme assays.

3.2 Extraction of Metabolites

Accurate detection and measurement of metabolites inside the cell is quite challenging in metabolomics research, therefore different methods for efficient extraction of metabolites from biological sample is crucial (Mashego et al. 2006). Idyllically, the data obtained from metabolomics shows the detailed image of the metabolic condition of cells at a point of time when cells are harvested. However, sometime many important metabolites quickly get metabolized (<1 mMs^{-1}) in the presence of some external factors like light and temperature (Villas-Boas et al. 2007). As a result, during sample preparation, the amount of these metabolites would get altered thus producing results that differ from the exact one of population's true metabolic state. This difference in metabolite levels would be minimized or completely eliminated

by quenching the cell's metabolism before or during sampling so as to acquire accurate metabolomics results (Canelas et al. 2008; Da Luz et al. 2014).

The quenching step is often performed while studying the intracellular metabolites as they present inside the cell compartments and thus their extraction is necessary for their quantification. However, sometimes due to factors like temperature, light as well as metabolite interaction, the level and composition of extracellular metabolites in the medium also differ from the real one. Thus, to circumvent such difference in the results of metabolites, the cellular biomass should be quickly separated from growth media and should also be rapidly treated with quenching solution so that the degradation of metabolites could be stopped (Villas-Boas and Bruheim 2007; Patejko et al. 2017). Moreover, the metabolite sample should always be stored at a low temperature of about <20 °C to avoid any damage due to temperature and light (Villas-Boas et al. 2007).

3.2.1 Quenching Methods

As soon as the importance of quickly quenching the cells' metabolisms and avoiding its leakage is recognized, the accurate measurement of both extracellular as well as intracellular metabolites could be obtained, and a variety of quenching methods have been developed.

3.2.1.1 Perchloric Acid

It is one of the oldest quenching method where sampling is performed by directly adding the acidic solution of perchloric acid into the culture broth (Harrison and Maitra 1968; Strange et al. 1963). Although this approach was efficient but not followed widely because this method provides a mixture of both intra- and extracellular metabolites. Since it damages the cell envelope which results in leakage of intracellular metabolites into the outside medium (Faijes et al. 2007). The quantification of such intracellular metabolites extracted using this approach shows great variability because highly concentrated media components interfere during chemical analysis and degradation of pH-sensitive metabolites (Maharjan and Ferenci 2003).

3.2.1.2 Liquid Nitrogen Method

The problem of previous method is partially addressed by Saez and Lagunas (1976), where they developed a method which quenches the cellular metabolism as well as extracts out the extracellular metabolites separately, but it involves two different steps (Saez and Lagunas 1976). Firstly, using fast filtration biomass is collected followed by quick immersion in liquid nitrogen which enables separation of living cells from culture media. Also, liquid nitrogen decreases the rate of cell metabolism as well as the turnover of metabolites due to its low temperature and thus provides additional time for the extraction of metabolite. Second step involves extraction of intracellular metabolite from biomass using different pH solutions. Although, this is one of the most widely used method but not suitable for the analysis of fast turnover compounds such as NADH, glutamate, pyruvate, ATP, and many others because it

takes more than 10 s per sample to actually quench cell metabolism (Saez and Lagunas 1976).

3.2.1.3 Methanol Method

This quenching method was proposed by de Koning and van Dam in 1992, since then this method is considered as the gold standard method of quenching microbial cells (Dekoning and Vandam 1992). In this method, microbial culture is added into methanol solution (60% v/v) or kept at low temperature of about −40 °C. Then, centrifugation is performed to separate cell biomass from the culture medium. This method is advantageous due to its low-temperature strategy which is beneficial in arresting cellular enzymatic activity in less than a second. Next, cells were processed for intracellular metabolite extraction. Even though cold-methanol solution is very efficient and its use continues to be popular these days, there are some issues related to its usage which include little cell leakage and variation in biomolecules content (Wittmann et al. 2004; Villas-Bôas et al. 2005a; Bolten et al. 2007; Villas-Boas and Bruheim 2007; Canelas et al. 2008). For example, it has been found that the concentration of free amino acids was reduced by 90% when cold-methanol solution was used as quenching agent (Bolten et al. 2007).

Additionally, the majority of the quenching methods known till date have been developed as modification of cold-methanol solution along with involvement of liquid nitrogen; however, their success depends largely on the study organism or type of cells.

3.2.2 Extraction of Extracellular Metabolites

The obligatory condition in metabolomics studies is correct estimation of substrate as well as extracellular metabolite concentrations in the culture medium. For quantifying the true values, the time between sampling and quenching of the cell metabolism is considered most crucial because the high turnover rates of metabolites present inside cell in the order of 1–2 s like glucose-6-phosphate and ATP (Dekoning and Vandam 1992). Therefore, for capturing the correct in vivo snapshot of the metabolite pool and cell's metabolic state ideally, the time gap between collection and quenching of sample should be lesser compared to the turnover rates for such metabolites. The two common methods preferred for biomass separation or sampling are centrifugation and filtration. Further, for quick sampling of biomass, the type of culturing device used is also crucial, i.e., culture flasks or bioreactors (Pinu et al. 2017). Additionally, little modification can be combined with aforementioned separation methods and culturing devices for fast sampling include use of pre-cooled glass tubes which contains 4 mm in diameter cooled glass beads or stainless steel spheres (Theobald et al. 1993). While others prefer to add liquid nitrogen to the medium followed by thawing and centrifugation (Verduyn et al. 1992; Diderich et al. 1999; Van Hoek et al. 1999). Researchers find all methods suitable depending upon the experimental organism, metabolites of interest, and many more.

Although, accurate preparation of metabolites is very essential for getting correct picture of metabolites; however, sample concentration is also a crucial aspect of metabolomics because the metabolites present in sample are diluted. Thus, to improve the analytical tools' detection limits, the samples are concentrated (Villas-Bôas et al. 2005a; Smart et al. 2010). The most common method applied for concentrating metabolite samples involves removal of water using lyophilizer or freeze dryer. Conversely, organic solvent evaporating systems or vacuum drying methods could also be performed, but care should be taken as metabolites are susceptible to thermal degradation. Indeed, solvent evaporation technique is preferred for concentrating non-aqueous extracellular samples (Pinu et al. 2017).

Even though, the extracted and concentrated samples of metabolites are quenched but need to be stored under appropriate conditions before analysis to ensure the occurrence of no reaction during storage. However, few necessary precautions which have to be taken to avoid any unwanted changes in metabolites during the storage. These storage conditions depend on the types and stability of metabolites. Therefore, the storage of metabolite samples in dark and cool place (−20 °C or −80 °C) is suggested so that metabolite degradation by light and heat could be avoided. This also ensures the possibility that metabolites have retained their original properties. However, still some metabolites lose their characteristics even though they are stored at low temperature by getting oxidized easily which may result in change in their entire features. Thus the most suitable way to protect metabolites from degradation is storing them under vacuum (Villas-Boas et al. 2007). Although, there are few metabolites which remain unaffected by exposure to temperature and are considered as stable which could be stored even at room temperature in dry powder form so that reproducible and high-quality data could be generated.

3.2.3 Extraction of Intracellular Metabolites

The extraction approach for intracellular metabolites differs based on the complexity of structural polymers which are present in microbial cell envelope and also on composition as well as the extent of cross-linking among the polymers and with other cell-wall components. Variety of mechanical and non-mechanical/chemical methods are generally followed for the disruption of cell envelope (Villas-Boas et al. 2007). However, both methods have their own advantage and disadvantage which need to be considered before applying them. Ideally, different approaches together should be used for obtaining optimum performance depending on the cell-wall composition and structure (Villas-Boas et al. 2007).

3.2.3.1 Non-mechanical Lysis

For intracellular metabolite extraction, chemical agents are widely used as non-mechanical method where metabolites extracted in the organic solvents distribute themselves depending on their partitioning coefficients, solvent temperature, solubility between two phases. Use of chemical agents also help in concentrating the metabolites in a single phase which is very crucial for metabolite studies

(Villas-Boas et al. 2007). However, care should be taken while selecting the chemical solvent for extraction as the rate of metabolite extraction varies with the nature of chemical. Although, the selection of chemical solvent and extraction method largely depends on the kind of microbe and the set of metabolites of interest. Most extraction methods involving use of chemicals extract only a particular metabolite species (like, amino acids; fatty acids); hence, emphasis has been given to necessitate the use of multiple extraction methods in order to obtain a complete picture of possible intracellular metabolite (Canelas et al. 2009; Duportet et al. 2012; Park et al. 2012; Kim et al. 2013). Usually, organic solvents (both polar and nonpolar) are used for the extraction of intracellular metabolites because they work by attacking cell wall and membrane proteins as well as lipids. This solvent interaction with cell wall and membrane components generates pores and results in the release of metabolites which are present inside the cell (Villas-Bôas et al. 2005a, 2007). In next section, the most common extraction protocols for preparing intracellular samples are given in the following.

Boiling Ethanol

The method involving the use of boiling buffered ethanol (75% v/v) is widely acceptable, rapid, and simple protocol for the extraction of intracellular metabolite. Briefly, the microbial cells that were quenched earlier are now exposed to the buffered ethanol at 80 °C. This boiling deactivates microbial cell wall enzymes and proteins and thus increases cell disruption, which promote the extraction of intracellular mainly, water-soluble metabolites. Before analysis, the mixture of ethanol and water is evaporated, and the pellets thus obtained are resuspended in water. The main advantage of this method is its excellent reliability (Villas-Boas et al. 2007). However, poor recovery of many metabolites of different classes, like phosphorylated metabolites tricarboxylic acids and nucleotides, is the limitation of this method (Maharjan and Ferenci 2003; Villas-Bôas et al. 2005a). Besides, this metabolite extraction method could not be applied for isolation of thermo-labile metabolites, and the reduced metabolites are susceptible for oxidation (Villas-Boas et al. 2007).

Cold Methanol

It is simple, fast, and an extensively used method for the extraction of intracellular metabolites from different microbial cells like bacteria (Maharjan and Ferenci 2003; Park et al. 2012), yeasts (Bolten and Wittmann 2008; Kim et al. 2013), and filamentous fungi. This is a very influential method that utilizes single organic solvent for the extraction of metabolite and that too can be effortlessly removed from the extracts by evaporation. Furthermore, the steps involved in the extraction process are usually carried out under very low temperature of approximately lees than −20 °C, and hence it is appropriate for heat-susceptible metabolites. However, the key drawback of this method is its incapability in completely inactivating enzyme and thus there is probability of alteration in intracellular metabolite pools. This method overcome the disadvantages of previous boiling ethanol method as it shows excellent reproducibility and good recovery of metabolites ranging from polar

to mid-polar but that is not true for nonpolar metabolites (Villas-Bôas et al. 2005a). Occasionally, for enhancing the cell permeability cold methanol extraction is performed along with freeze–thaw cycles or sonication.

Buffered Methanol–Chloroform–Water

Folch et al. (1957) by using this buffer first time reported lipid dominating intracellular metabolites extraction from animal tissue. Later, similar buffer was employed for the extraction of polar metabolite from yeast at low temperature ranging from −40 to −20 °C. It is a highly recommended method for the extraction of temperature-sensitive, nonpolar, and polar metabolites from yeasts, bacteria, and filamentous fungi. The involvement of toxic and carcinogenic chloroform assisted in denaturing of complete set of enzymes present in the microbes and inhibited further chemical reactions. Although, it is a painstaking and extremely long method, and the buffers which is used for extraction also may lead to difficulty for different analytical techniques, but it is an excellent method for the recovery of phosphorylated and thermo-labile compounds (Villas-Bôas et al. 2005a, 2007).

Hot Water

Since 1950s, high-temperature water has been used for bacterial amino acids extraction which later has also been applied for microbial metabolite extraction (Gale 1947; Bagnara and Finch 1972). It is an extremely straightforward and easily executable method along with the ability to quench enzyme activity due to utilization of hot water.

3.2.3.2 Mechanical Lysis of Cell

Number of cell-disruption protocols where cells were broken using mechanical force like microwave, grinding, French press, and ultrasonics are widely followed for the extraction of metabolite from animal and plant cells. Although, these methods are not recommended for the metabolites extraction from microbes; however, two different methods that have been applied for the metabolite extraction from microorganisms include pressurized liquid extraction (PLE) and supercritical fluid extraction (SFE).

Supercritical Fluid Extraction

In this method, mid-polar to nonpolar metabolites are extracted from microbial cell by using a supercritical fluid such as carbon dioxide (Cocks et al. 1995). For increasing the applicability toward extraction of polar metabolites from microbes, methanol or ethanol is also included along with carbon dioxide as a modifier (Lim et al. 2002). Sometimes, nitrous oxide and xenon could also be applied in place of CO_2. The advantage of this method lies in its requirement of small volume of solvent and sample as well as less time requirement.

Pressurized Liquid Extraction

This method is exclusively performed for the extraction of microbial secondary metabolites. But this method did not find any application in the metabolomics

research as the metabolite extracted by this method is very concentrated and hence, not appropriate for high-throughput screening (Gomez-Ariza et al. 2004). On the flip side, only temperature-stable metabolites can be extracted using this method (Villas-Boas et al. 2007).

3.3 Detection of Metabolites

For the quantitative analysis of extracellular and intracellular metabolites, enzyme-based assays were used earlier (Theobald et al. 1993, 1997). But these traditional assays have some drawbacks like large sample volume requirement with detection of very few metabolite per assay, low sensitivity etc. Therefore, for detection of even trace of metabolites more sensitive and reliable analytical methods have been developed which include GC, UPLC/HPLC, and LC (Fig. 3.2). The chromatographic techniques enable partition of metabolites depending on their chemical and physical properties, and then the separated compounds analyzed for mass detection with MS and NMR spectroscopy (Dunn et al. 2005; Dunn and Ellis 2005). The advantages associated with these analytical techniques have increased its applicability and utility which includes high sensitivity of up to picomole, small sample requirement for analysis and the simultaneous quantification of many metabolites belonging to different pathways like tricarboxylic acid cycle, pentose phosphate pathway, and glycolysis (van Dam et al. 2002; Villas-Bôas et al. 2005b).

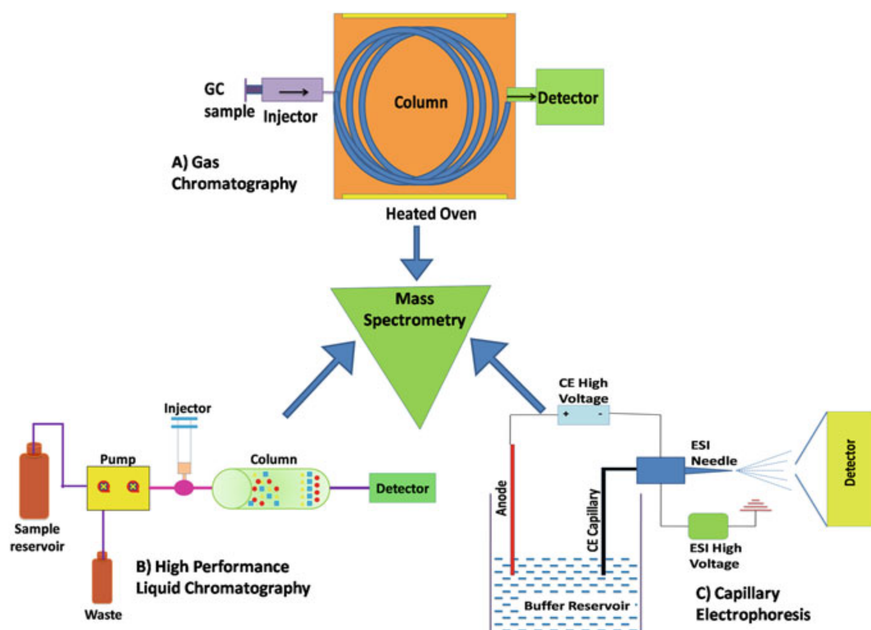

Fig. 3.2 Different analytical techniques that are used for metabolites separation coupled with mass spectrometry for detection

3.3.1 Mass-Spectrometry (MS)

MS often coupled with chromatography techniques are drawing attention in high-throughput metabolomics. In past few decades, MS has been extensively developed due to which it enjoys the distinguished status in separation science. MS works by analyzing ions produced using ion source by converting sample into ions. These ions generated from sample were then separated based on different trajectories of moving ions due to different mass/charge (m/z) ratios in the presence of electrical and/or magnetic fields and then quantitatively detected. Till date, MS is the most potential technique which is able to measure with high precision, and to produce a wide variety of metabolites during cellular processes. Features that made MS a popular choice for researchers are broad dynamic range, reproducible quantitative analysis, mass accuracy, and the proficiency of evaluating extremely complex biofluids (Lee et al. 2010). MS can analyze biological samples either directly by simply injecting the sample without prior purification or sometime following chromatographic separation. Although, direct-injection MS is extremely fast for metabolic fingerprinting; however, it has some drawbacks like co-suppression and low ionization efficiencies. To overcome the issue, MS is often used as a key hyphenated technique instead alone in metabolomics.

The advantage of using MS in metabolomics is its ability to outlook the effect of nutritional status, time, stress, and environmental perturbation on hundreds of metabolites. Currently, efforts have been made toward MS-based identification of metabolic biomarkers because MS facilitates the finding as well as functional annotation of biomarkers and reconstruction of metabolic networks (Hiller et al. 2010; Weston 2010).

3.3.2 Gas Chromatography-MS

Gas chromatography-mass spectrometry is a versatile technique that can measure a broad spectrum of primarily water-soluble metabolites (Legido-Quigley et al. 2010). Its strength is measurement of low-molecular-weight and volatile analytes, including small species that typically uncharged. For some compound classes, especially for essential oils and volatiles, GC and GC-MS are the only universally applicable analytical methods (Mohler et al. 2007). Besides, for broad metabolic profiling, GC-MS must be chemically derivatized before analysis to increase metabolite stability and volatility. This is typically achieved by the trimethylsilylation derivatization reaction on samples that have been completely dried. The reaction occurs at room temperature in the presence of pyridine as the catalyst (Goodpaster et al. 2011). The extent of derivatization is impacted by solvent and sample cleanliness, and dryness (Gao et al. 2008). In GC, the sample solution is injected into the instrument where it gets mixed with a carrier gas stream (helium or nitrogen) which transports the sample in the form of gas into a separation tube known as the “column.” The various components due to their differential partition in the mobile gas phase and stationary liquid phase get separated in the column. The component which separates

in gas comes first to detector while those partitioned into liquid phase comes out later. A standard sample with known concentration is usually injected into the instrument if a sample with an unknown concentration is to be measured where the concentration of an unknown compound is calculated based on the area and retention time of standard sample peak.

GC-MS is the preferred analytical technique for the measurement of many metabolites like very-short-chain fatty acids and alcohols, hydroxy acids, sugars, and monophosphorylated sugars as well as sterols that are hard to measure by other chromatography-MS (An et al. 2010). For general metabolomics, the strengths of GC-MS include outstanding chromatographic peak sharpness and extensive mass spectral libraries for peak identification (Castillo et al. 2011). The effectiveness of GC as a separation method reduces the pressure over MS analysis, leading to cost-effective and robust detection with single quadruple. However, the requirement of hot injection for evaporation of molecules in GC disturbs the quantification of thermolabile compounds, such as di- and triphosphates (including ATP or NADPH) despite derivatization. For example, the guanidinium group of arginine decomposes to yield ornithine (Psychogios et al. 2011). Nevertheless in metabolomics, all signals are recorded, including decomposition products, which can puzzle biochemical interpretations or lead to structures that are absent from chemical libraries such as PubChem (Wu et al. 2010a, b).

GC-MS uses hard electron ionization which complicates the identification of unknown compounds because this type of ionization yields many highly reproducible fragments and rearrangement ions and also responsible for the absence of molecular ions from spectra. Indeed, identification of known compounds by using this ionization source is straightforward as it is based on comparison to mass spectra and retention indices of authenticated standards which have already been deposited in databases. But when chemical ionization approach is applied for GC-MS, molecular ion peaks could easily be seen as it is a softer technique (Legido-Quigley et al. 2010).

3.3.3 Capillary Electrophoresis-MS (CE-MS)

CE-MS is emerged as an influential and promising separation technique which is extremely suitable for the analysis of charged and polar metabolites. In CE, separation of compounds is based on the differences in intrinsic electrophoretic mobilities of compounds, which is dependent on their charge and size. Moreover, as the fundamental mechanism of separation of CE and other chromatographic-based techniques is different, and hence will receive a complementary view of the metabolite composition in a biological sample (Ramautar et al. 2011; Andreas et al. 2015; Kok et al. 2015). In last few years for metabolomics studies, CE-MS is being a choice of study due to its improved concentration sensitivity as a result of advancement in novel interface designs (Zhao et al. 2012; Lindenburg et al. 2015; Ramautar 2016; Zhang et al. 2016). Since the majority of primary metabolites are intrinsically polar, CE-MS serve as a promising adjoining microseparation platform in

metabolomics (Britz-McKibbin 2011). In CE, firstly metabolites are separated based on their size and charge, and then selectively detected by using MS which monitor ions over a large range of m/z values.

Although in metabolomics the use of CE–MS is still relatively low as compared to other analytical techniques because of its technical complexity and other constraints such as low sensitivity, method robustness, and migration time variability (Kuehnbaum and Britz-McKibbin 2013; Kohler et al. 2016). Despite, CE-MS possesses a number of crucial advantages over other separation techniques due its ability to perform the reproducible and global profiling of original peptides and metabolites (endogenous) found in a clinical setting (Kami et al. 2013; Pejchinovski et al. 2015; Pontillo et al. 2015; Harada et al. 2016). For biological samples, the first global metabolic profiling using CE-MS was introduced by Soga et al. (2002, 2003). CE-MS has been applied for the analysis of a wide variety of targeted and non-targeted metabolites which include inorganic and organic acids, vitamins, thiols, nucleotides and nucleosides, carbohydrates, and peptides.

3.3.4 Liquid Chromatography-MS (LC-MS)

Diverse analytical methods are available for quantifying extracted metabolites. The most sensitive detection, and thus the broadest metabolome coverage, is achieved by MS-based methods (Theodoridis et al. 2011; Kuehnbaum and Britz-McKibbin 2013; Milne et al. 2013; Junot et al. 2014). Among MS techniques, LC-MS is the most versatile. In LC-MS, analytes are separated on column, ionized at an ion source, separated by a mass analyzer, and detected. Specificity is achieved through the combination of retention time from the column and the MS signature. In metabolomics, to obtain optimal coverage one must employ multiple LC-MS approaches (Patti 2011). Like, reversed-phase chromatography using a C18 column is performed for efficient separation of fatty acids and lipids, but ideally not for the analysis of polar metabolites due to poor retention on column. However, the retention of negatively charged metabolites can be improved by including a cationic ion-pairing agent in the running buffer (Coulier et al. 2006; Luo et al. 2007). These also improve the peak shape for phosphate-containing metabolites (Lu et al. 2008, 2010). But use of ion-pairing agent have a drawback as it takes days to wash out of an LC system, and also suppresses ionization of positively charged metabolites.

Further, the consistent and detailed measurement provided by LC-MS forms the grounds for successive data processing and multivariate data analysis. LC-MS-based large-scale metabolomic technologies are gaining popularity for their application in the diagnosis of human disease by analyzing metabolites in biological samples (Lv et al. 2011). Various biostatistical tools (PLS-DA, PCA) were adopted for classification of metabolites which were separated from different tissue and as a result, approximately 112 hydrophilic metabolites were identified within 8 min of run.

3.3.5 Nuclear Magnetic Resonance

Nuclear magnetic resonance is the most common spectroscopic technique which uniquely identifies and concurrently quantifies a large variety of organic compounds in the micromolar range. The application of this analytical technique in the emerging field of metabolomics provides a "holistic view" of the metabolites under a specific conditions, and hence it is best-fitted as well as beneficial for metabolomic studies (Wu et al. 2010a, b). Conventionally, for metabolomics study of biofluids, NMR is the most widely adopted technique because of its ability to analyze complete biomaterials in the hostile way along with providing the rich structural information. Hence, NMR-based metabolomics allow extensive research of low-molecular-weight metabolites in biological samples with significant improvements. High-resolution NMR is an ideal technique biofluids or tissue extracts' metabolite profiling and thus, there has been much interest in the establishment of a biomarker for a disease using high-throughput NMR techniques. In drug discovery and development research, NMR could prove to be more beneficial by providing detailed information about the structural transformation of a compound which occurs due to metabolism (Zhang et al. 2010). The features responsible for inclination toward NMR for metabolic studies are its simple and automated operation which is non-selective and non-destructive. Here, the sample that is analyzed in single run could be reused, and provides useful structural information that prove to be an asset in the characterization of each components of complex mixtures (Malet-Martino and Holzgrabe 2011).

Although, for comprehensive metabolite profiling NMR has been found to have relatively low sensitivity; it is currently become a most widely used diagnostic tool due to its specificity. Currently, NMR has also been applied for the study of metabolites associated with a particular disease like those involved in Alzheimer's disease, prostate cancer, etc. (Jordan and Cheng 2007; Barba et al. 2008). Using NMR-based metabolomics approach, Jung et al. have investigated the link of altered metabolic pattern in plasma and urine of cerebral infarctions patients and also discovered a metabolic biomarkers associated with stroke (Jung et al. 2011). The differential metabolites present in the plasma of stroke patients are characterized by increased amount of lactate, pyruvate, glycolate, and formate, while decreased amount of glutamine and methanol. On the other hand, the metabolite profile of urine of stroke patients shows decrease in citrate, hippurate, and glycine. These detected biomarkers were associated with anaerobic glycolysis and folic acid deficiency. Generally, metabolomics of urine of human being is considered as an important source of information related to the health and therefore it is the most suitable source of studying the status of the global system. This is indicative of the fact that in future magnetic resonance methodologies will be dominating in disease management.

3.4 Conclusion

Although, huge advancement has been made in metabolomics in the last two decades; however, still there is no universally applicable methodology for immediate quenching of cellular metabolic activity, extraction, and analysis of all metabolites of interest. Further, these challenges are exacerbated due to the high degree of chemical diversity such as polar and nonpolar metabolites. Also, the current procedures are so strongly organism-specific that they could not be applied to any other organism without prior optimization. The main problem which remained to be unsolved is leakage of intracellular metabolites into the surrounding medium during quenching and another is the loss of metabolites during extraction which needs to be corrected for increasing the reproducibility of results.

Investigation of the metabolome with analysis of all possible measurable metabolites in the sample could be achieved by metabolic analysis. Based on the type of sample, both targeted and/or non-targeted approaches can be used for supervising hundreds of metabolites at a given time. However, this requires high-throughput and high-end techniques that enable measurement of relative changes in compounds under a wide dynamic range, rather than estimating the absolute concentrations of compounds. The analytical techniques generally useful for these purposes include GC or HPLC/UPLC as separation modules which are coupled with MS for fast and accurate detection of separated metabolites. The cutting-edge analytical technologies enabled the measurement of metabolites and estimation of the changes in metabolite concentrations accurately with precision under defined conditions and thus, have highlighted the effects of perturbations in pathways of interest. Occasionally, researchers also prefer to use more than one analytical method which is complementary to each other, in order to circumvent the pitfalls of one technique and also to avoid improbability to incorrectly measure the metabolites.

The standardization of analytical tools and extraction methods is the prime requisite for metabolite profiling. Therefore, it appears to be more crucial to develop new or modify existing techniques that are dedicated to a particular class of metabolites, i.e., sugar intermediates, organic acids, amino acids, and cofactors. From the above discussions, it is clear that for having the comprehensive metabolite profiles of biological samples, single analytical technique is not sufficient and a combination of different techniques needs to be used for acquiring as much information as possible.

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4 Genetically Encoded Biosensors and Their Applications in the Development of Microbial Cell Factories

Yaokang Wu, Guocheng Du, Jian Chen, and Long Liu

Abstract

The genetically encoded biosensors, which could transform the input of specific metabolic concentrations into output of gene expression levels, have been developed by hacking the sensing and regulatory systems of the cell such as allosteric transcription factors (aTFs) and riboswitches. In this chapter, we first introduce the classification and functional mechanism of genetically encoded biosensor. Furthermore, the applications of biosensor in the development of microbial cell factories including high-throughput screening and dynamic metabolic engineering are reviewed. Finally, the future perspectives on biosensors and their applications are discussed.

Keywords

Biosensor · Allosteric transcription factors · Riboswitch · Synthetic biology · Microbial cell factory · High-throughput screening · Dynamic metabolic engineering

More and more microbial cell factories have been constructed for the production of valuable products such as biofuels, chemicals, materials, and nutraceuticals using renewable biomass sources (Cordova and Alper 2016; Liu et al. 2017a, b; Luo et al.

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2019; Zhou et al. 2018), and this process has been further facilitated by the development of synthetic biology (Ng et al. 2015). Microorganisms have the ability to sense the change of a wide range of metabolites and modulate related pathways accordingly. This process is achieved by their sensing and regulatory systems such as allosteric transcription factors (aTFs) and riboswitches. With the aid of synthetic biology, the genetically encoded biosensors, which were designed and built by engineering the native sensing and regulatory systems of cells, have been widely applied in the high-throughput screening and metabolic regulation of the microbial strains (Koch et al. 2019; Michener et al. 2012). In this chapter, we focused on the constructions and applications of biosensors derived from allosteric transcription factors (aTFs) and riboswitches, and divided them into two categories, namely the protein-based biosensors and the RNA-based biosensors. Other types of biosensors, including the Förster resonance energy transfer (FRET)-based and two-component regulatory system (TCRS)-based biosensors that have not been used widespread in the development of microbial cell factories, will not be discussed here (refer to reviews (Greenwald et al. 2018; Ravikumar et al. 2017)).

4.1 The Classification of Genetically Encoded Biosensors

4.1.1 Protein-Based Biosensors

4.1.1.1 The Functional Mechanism of Protein-Based Biosensors

The protein-based biosensors were usually constructed by engineering aTFs, which could interact with specific small ligand molecules and change the activity of corresponding promoters (Fig. 4.1a) (Table 4.1) (De Paepe et al. 2017). The aTF typically consists of two function domains, namely the N-terminal ligand-binding domain (LBD) and the C-terminal DNA-binding domain (DBD). The binding of

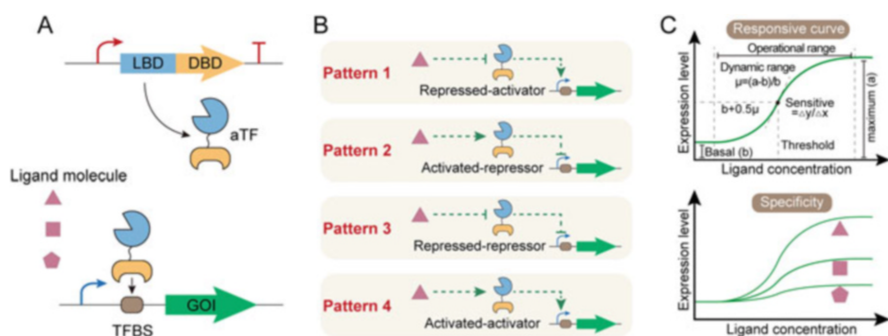

Fig. 4.1 The functional mechanism and fundamental characteristics of protein-based biosensors. (**a**) The two function domains of protein-based biosensors. (**b**) The four general patterns of the aTF-mediated transcriptional regulation. (**c**) The fundamental characteristics of protein-based biosensors

Table 4.1 List of the protein-based biosensors

Ligand	Origin	Host	Application	Refs.
L-Arginine	ArgP (*E. coli*)	*E. coli*	N/a	Binder et al. (2012)
L-Tyrosine	TyrR (*E. coli*)	*E. coli*	Dynamic metabolic engineering (DME)	Chou and Keasling (2013)
L-Phenylalanine	TyrR (*E. coli*)	*E. coli*	High-throughput screening (HTS)	Liu et al. (2017a, b)
FA/acyl-CoA	FadR (*E. coli*)	*E. coli*	DME	Zhang et al. (2012)
Malonyl-CoA	FapR (*B. subtilis*)	*E. coli*	DME	Xu et al. (2014)
Acyl-CoA	FadR (*E. coli*)	*E. coli*	HTS	Xiao et al. (2016)
Adipate	PcaR (*P. putida*)	*E. coli*	HTS	Dietrich et al. (2013)
Glucarate	CdaR (*E. coli*)	*E. coli*	HTS	Rogers et al. (2015)
Glucarate	CdaR (*E. coli*)	*E. coli*	HTS	Zheng et al. (2018)
Acrylate	AcuR (*Rhodobacter sphaeroides*)	*E. coli*	N/a	Rogers et al. (2015)
Ferulic acid	FerC (*Sphingobium*)	*E. coli*	N/a	Machado and Dixon (2016)
Itaconate	ItcR (*Yersinia pseudotuberculosis*)	*E. coli*	HTS	Hanko et al. (2018)
Myo-inositol	IpsA (*C. glutamicum*)	*E. coli*	DME	Doong et al. (2018)
Muconic acid	CatR (*P. putida*)	*E. coli*	DME	Yang et al. (2018)
Ectoine	AraC (*E. coli*)	*E. coli*	HTS	Chen et al. (2015)
NADPH	SoxR (*E. coli*)	*E. coli*	HTS	Siedler et al. (2014a)
N-acetylneuraminic	NanR (*E. coli*)	*E. coli*	HTS	Peters et al. (2018)
Benzoate/2-hydroxybenzoate	NaHR (*P. putida*)	*E. coli*	HTS	van Sint Fiet et al. (2006)
Benzoate	BenR (*P. putida*)	*E. coli*	HTS	Uchiyama and Watanabe (2008)
3,4-Dihydroxy benzoate	PcAU (*Acinetobacter*)	*E. coli*	HTS	Jha et al. (2014)
Kaempferol	QdoR (*B. subtilis*)	*E. coli*	HTS	Siedler et al. (2014b)

(continued)

Table 4.1 (continued)

Ligand	Origin	Host	Application	Refs.
Quercetin	QdoR (*B. subtilis*)	*E. coli*	N/a	Siedler et al. (2014b)
Naringenin	FdeR (*Herbaspirillum seropedicae*)	*E. coli*	N/a	Siedler et al. (2014b)
Naringenin	TtgR (*P. putida*)	*E. coli*	N/a	Rogers et al. (2015)
Vanillin	QacR (*Staphylococcus aureus*)	*E. coli*	N/a	De Los Santos et al. (2016)
Resveratrol	TtgR (*P. putida*)	*E. coli*	HTS	Xiong et al. (2017)
p-Coumaric acid	PadR (*B. subtilis*)	*E. coli*	HTS	Siedler et al. (2017)
Benzoate	Chimeric aTF	*E. coli*	N/a	Juárez et al. (2018)
Pinocembrin/naringenin	FdeR (*Herbaspirillum seropedicae*)	*E. coli*	N/a	Trabelsi et al. (2018)
Vanillin/syringaldehyde	EmrR (*E. coli*)	*E. coli*	HTS	Ho et al. (2018)
Naringenin/apigenin/luteolin	FdeR (*Herbaspirillum seropedicae*)	*E. coli*	HTS	De Paepe et al. (2019)
3-Dehydroshikimate	CusR (*E. coli*)	*E. coli*	HTS	Li et al. (2019)
Arabinose	AraC (*E. coli*)	*E. coli*	N/a	Rogers et al. (2015)
Fucose/gentiobiose/lactitol/sucralose	LacI (*E. coli*)	*E. coli*	N/a	Taylor et al. (2015)
Cellobiose	CelR (*Thermomonospora fusca*)	*E. coli*	N/a	Kwon et al. (2018)
1-Butanol	BmoR (*Pseudomonas butanovora*)	*E. coli*	HTS	Dietrich et al. (2013)
Phenol	DmpR (*Pseudomonas*)	*E. coli*	HTS	Choi et al. (2014)
3-Hydroxypropionate	PrpR (*E. coli*)	*E. coli*	HTS	Rogers and Church (2016)
3-Hydroxypropionate	AcuR (*R. sphaeroides*)	*E. coli*	HTS	Rogers and Church (2016)
Lactam	ChnR (*Acinetobacter*)	*E. coli*	N/a	Zhang et al. (2017)

Formaldehyde	FrmR (*E. coli*)	*E. coli*	HTS	Woolston et al. (2018)
Choline	BetI (*E. coli*)	*E. coli*	DME	Saeki et al. (2016)
Ammonium	GlnR (*Lactococcus*)	*E. coli/ P. putida/ synechocystis*	DME	Xiao et al. (2017)
Putrescine	PuuR (*E. coli*)	*E. coli*	HTS	Chen et al. (2017)
Anhydrotetracycline	TetR (*E. coli*)	*E. coli*	N/a	Rogers et al. (2015)
Erythromycin	MphR (*E. coli*)	*E. coli*	N/a	Rogers et al. (2015)
Erythromycin	MphR (*E. coli*)	*E. coli*	HTS	Kasey et al. (2018)
Copper	MarR (*E. coli*)	*E. coli*	N/a	Hao et al. (2014)
Zinc	ZntR (*E. coli*)	*E. coli*	N/a	Watstein et al. (2015)
Arsenite	ArsR (*E. coli*)	*E. coli*	N/a	Merulla and Van Der Meer (2016)
L-lysine/L-arginine/L-histidine	LysG (*C. glutamicum*)	*C. glutamicum*	HTS	Binder et al. (2012)
L-leucine/L-isoleucine/L-methionine/L-valine	Lrp (*C. glutamicum*)	*C. glutamicum*	HTS	Mustafi et al. (2012)
L-lysine/L-arginine/L-histidine	LysG (*C. glutamicum*)	*C. glutamicum*	HTS	Schendzielorz et al. (2014)
Shikimic acid	ShiR (*C. glutamicum*)	*C. glutamicum*	HTS	Liu et al. (2018)
Malonyl-CoA	FapR (*B. subtilis*)	*S. cerevisiae*	DME	David et al. (2016)
Malonyl-CoA	FapR (*B. subtilis*)	*S. cerevisiae*	N/a	Dabirian et al. (2019b)
Acyl-CoA	FadR (*E. coli*)	*S. cerevisiae*	HTS	Dabirian et al. (2019a)
Cis, cis-muconic acid	BenM (*Acinetobacter*)	*S. cerevisiae*	HTS	Skjoedt et al. (2016)
Muconic acid	ARO9 (*S. cerevisiae*)	*S. cerevisiae*	HTS	Leavitt et al. (2017)
S-adenosylmethionine	MetJ (*E. coli*)	*S. cerevisiae*	HTS	Umeyama et al. (2013)
NADH	GPD2 (*S. cerevisiae*)	*S. cerevisiae*	N/a	Knudsen et al. (2014)
Xylose	xylR (*B. xylosus*)	*S. cerevisiae*	HTS	Wang et al. (2016)
3-Hydroxypropionic acid	LysR (*P. denitrificans*)	*P. denitrificans*	N/a	Zhou et al. (2015)
Pamamycin	PamR2 (*Streptomyces alboniger*)	*S. alboniger*	HTS	Rebets et al. (2018)

aTF on the transcription factor binding site (TFBS) of the promoter will increase or decrease the affinity of RNA polymerase (RNAP) to it, and the conformation changes of aTF induced by specific ligand will affect its binding to the promoter thus building a relationship between ligand concentration and promoter activity (Wan et al. 2019). Among the four general patterns of the aTF-mediated transcriptional regulation, patterns 3 and 4 were most employed due to the positive correlation between the input and output (Fig. 4.1b) (Mannan et al. 2017).

The two fundamental characteristics, namely responsive curve and specificity, were often used for the evaluation of the protein-based biosensor (Fig. 4.1c) (De Paepe et al. 2017). The responsive curve represents the relation between the input of ligand concentration and the output of promoter strength, which can be obtained by fitting the input and output into the Hill function as shown below:

$$y = y_{\min} + (y_{\max} - y_{\min}) \frac{x^n}{K^n + x^n} \tag{4.1}$$

where y is relative expression activity of the promoter ($y_{\min}$ and $y_{\max}$ are the minimum/maximum activities), x is the ligand concentration, K is the threshold, and n is the cooperativity (Meyer et al. 2019). And many important parameters of the biosensor could be acquired from the curve including basal, maximum, operational range, dynamic range, threshold, and sensitivity (Fig. 4.1c). Specificity determines the responsive of the biosensor to different ligand molecules.

4.1.1.2 Designing and Tuning Protein-Based Biosensors

In order to build a protein-based biosensor with favorable responsive curve in a host, specific aTF should be expressed properly, and applicable synthetic promoter needs to be designed and constructed. Sometimes, molecular modification on the aTF may be implemented to improve or change the specificity of biosensor (De Paepe et al. 2017). That is to say, the tuning of protein-based biosensor mainly focuses on aTF level and promoter level.

Tuning at aTF Level

To construct a protein-based biosensor responsive to a specific molecule, corresponding aTF must be chosen by consulting literatures or retrieving the databases such as RegulonDB (Gama-Castro et al. 2011), BRENDA (Placzek et al. 2017), and RegPrecise (Rodionov et al. 2013). Besides, transcriptome sequencing and analysis can also be used to identify specific aTF (Li et al. 2019). However, there may not be aTF in nature which responds to certain molecules. So the engineered aTFs responded to new non-natural ligands must be constructed, which could be achieved by the combination of rational design and directed evolution (Koch et al. 2019; Libis et al. 2016). For example, five amino acid positions located in the effector binding pocket (P8, T24, H80, Y82, and H93) of the L-arabinose-responsive aTF AraC were selected for simultaneous saturation mutagenesis, and the mutants that responded to mevalonate, triacetic acid lactone, and ectoine, respectively, were obtained by fluorescence-activated cell sorting (FACS)-mediated

negative–positive dual screening (Chen et al. 2015; Tang et al. 2013; Tang and Cirino 2011). The computational design method is often used to reduce the design space. As an example, the Rosetta software was used in combination with single-residue saturation mutagenesis and error-prone PCR (epPCR)-based random mutagenesis for the construction of LacI mutants responding to fucose, gentiobiose, lactitol, and sucralose, respectively (Taylor et al. 2016). In addition, chimeric aTFs have also been built by fusing DBD and LBD from different proteins, and it is worth mentioning that the LBD could come from proteins other than aTF as long as it has demonstrable binding affinity to the ligand. For instance, benzoate-responsive aTFs were constructed by connecting benzoate LBDs to different DBDs with optimized linkers (Juárez et al. 2018).

The fundamental characteristics of the protein-based biosensors can also be optimized by introducing molecular modification into or tuning the expression level of the aTF. For example, the specificity of aTFMphR (that is derepressed by several naturally produced and semisynthetic macrolide antibiotics including erythromycin (ErA), josamycin, oleandomycin, narbomycin, methymycin, and pikromycin) to erythromycin was enhanced through epPCR and FACS; and its sensitivity was improved by introducing random mutagenesis to ribosome binding site (RBS) fortuning its expression level (Kasey et al. 2018).

Tuning at Promoter Level

To build a protein-based biosensor in a host, synthetic responsive promoters need to be designed and constructed by inserting the TFBS into the promoter of this strain because the native promoter regulated by the aTF may lose its activity there. For example, FA/acyl-CoA-responsive promoters were built by inserting the TFBS of aTF FadR into a phage lambda promoter and a phage T7 promoter, respectively, and TFBS of LacI was added into the constructed synthetic promoters to eliminate leaky expression (Zhang et al. 2012). In addition, the fundamental characteristics could be modulated by changing the starting engineered promoter or the position and numbers of the TFBS. As an example, Siewers and coworkers have constructed several malonyl-CoA biosensors in *Saccharomyces cerevisiae* by inserting the TFBS of aTF FapR (FapO) into five native promoters, and improved the dynamic range and reduced the basal by adjusting the position and numbers of FapO (Dabirian et al. 2019b).

4.1.2 RNA-Based Biosensors

4.1.2.1 The Functional Mechanism of RNA-Based Biosensors

The RNA-based biosensors could be constructed by engineering the cis-acting metabolite-responsive riboswitches, which consist of ligand-binding (aptamer) domains that could bind with specific ligand when its abundance exceeds a threshold and expression platform that control the gene expression by interacting with various gene expression apparatus (Table 4.2) (Serganov and Patel 2007). In the natural world, riboswitches responsive to numerous small molecules including ion, purines,

Table 4.2 List of the RNA-based biosensors

Ligand	Origin	Host	Application	Refs
L-lysine	Lysine riboswitch (*E. coli*)	*E. coli*	HTS	Yang et al. (2013)
L-lysine	Lysine riboswitch (*E. coli*)	*E. coli*	HTS	Wang et al. (2015)
L-tryptophan	L-tryptophan riboswitch (artificial)	*E. coli*	HTS	Yang et al. (2013)
L-tryptophan	L-tryptophan riboswitch (artificial)	*E. coli*	N/a	Jang and Jung (2018)
Thiamine 5′-pyrophosphate (TPP)	TPP riboswitch (*E. coli*)	*E. coli*	N/a	Nomura and Yokobayashi (2007)
TPP	TPP riboswitch (artificial)	*E. coli*	N/a	Wieland et al. (2009)
TPP	TPP riboswitch (*E. coli*)	*E. coli*	N/a	Muranaka et al. (2009)
TPP	TPP riboswitch (*E. coli*)	*E. coli*	N/a	You et al. (2015)
Theophylline	Theophylline riboswitch (artificial)	*E. coli*	N/a	Lynch et al. (2007)
Theophylline	Theophylline riboswitch (artificial)	*E. coli*	N/a	Win and Smolke (2007)
Theophylline	Theophylline riboswitch (artificial)	*E. coli*	N/a	Wieland and Hartig (2008)
Theophylline	Theophylline riboswitch (artificial)	*E. coli*	N/a	Lynch and Gallivan (2009)
Theophylline	Theophylline riboswitch (artificial)	*E. coli*	N/a	Wachsmuth et al. (2013)
Theophylline	Theophylline riboswitch (artificial)	*E. coli*	HTS	Eckdahl et al. (2015)
Theophylline	Theophylline riboswitch (artificial)	*E. coli*	N/a	Page et al. (2018)
Flavin mononucleotide (FMN)	FMN riboswitch (artificial)	*E. coli*	HTS	Meyer et al. (2015)
Flavin mononucleotide (FMN)	FMN riboswitch (*Fusobacterium nucleatum*)	*E. coli*	N/a	Rode et al. (2015)
Vitamin B12	Vitamin B12 riboswitch (*Propionibacterium freudenreichii*)	*E. coli*	N/a	Zhu et al. (2015)
Theophylline/ tetramethylrosamine/fluoride/ dopamine/thyroxine/2,4-dinitrotoluene	Artificial riboswitches based on related aptamer	*E. coli*	N/a	Espah Borujeni et al. (2016)
S-adenosyl-L-homocysteine (SAH)	SAH riboswitch (*Ralstonia solanacearum*)	*E. coli*	HTS	Su et al. (2016)

Neomycin thiamine/TPP/*S*-adenosyl methionine (SAM)/adenine (ade)	Artificial riboswitches based on related aptamer	*E. coli*	N/a	Endoh and Sugimoto (2015)
Purine	Purine riboswitch (*B. subtilis*)	*E. coli*	N/a	Stoddard et al. (2013)
Glycine	Glycine riboswitch (*B. subtilis*)	*E. coli*	N/a	Ketterer et al. (2016)
Ammeline/azacytosine	Adenine riboswitch (*Vibrio vulnificus*)	*E. coli*	N/a	Dixon et al. (2010)
Naringenin	Naringenin riboswitch (artificial)	*E. coli*	N/a	Jang et al. (2017)
Naringenin	Naringenin riboswitch (artificial)	*E. coli*	N/a	Xiu et al. (2017)
N-acetylneuraminate	*N*-acetylneuraminate aptazyme (artificial)	*E. coli*	HTS	Yang et al. (2017)
Neomycin	Neomycin riboswitch (artificial)	*E. coli*	N/a	Weigand et al. (2008)
Atrazine	Atrazine riboswitch (artificial)	*E. coli*	N/a	Sinha et al. (2010)
Pyrimido[4,5-d]pyrimidine-2,4-diamine (PPDA)	PPDA riboswitch (artificial)	*E. coli*	N/a	Kent and Dixon (2019)
5-hydroxytryptophan/3,4-dihydroxyphenylalanine	Artificial riboswitches	*E. coli*	N/a	Porter et al. (2017)
Ni2+/Co2+	NiCo riboswitch (*Clostridium botulinum*)	*E. coli*	HTS	Furukawa et al. (2015)
Glucosamine-6-phosphate (GlcN6P)	GlcN6P riboswitch (*B. subtilis*)	*B. subtilis*	DME	Niu et al. (2018)
L-lysine	L-lysine riboswitches (*E. coli* and *B. subtilis*)	*C. glutamicum*	DME	Zhou and Zeng (2015a)
L-lysine	L-lysine riboswitch (*E. coli*)	*C. glutamicum*	DME	Zhou and Zeng (2015b)
GlcN6P	GlcN6P riboswitch (*B. subtilis*)	*S. cerevisiae*	HTS	Lee and Oh (2015)
Theophylline	Theophylline riboswitch (artificial)	*S. cerevisiae*	N/a	Klauser et al. (2015)
Theophylline	Theophylline riboswitch (artificial)	*E. coli/Acinetobacter baylyi/Acinetobacter baumannii/Agrobacterium tumefaciens/Streptococcus pyogenes/B. subtilis*	N/a	Topp et al. (2010)

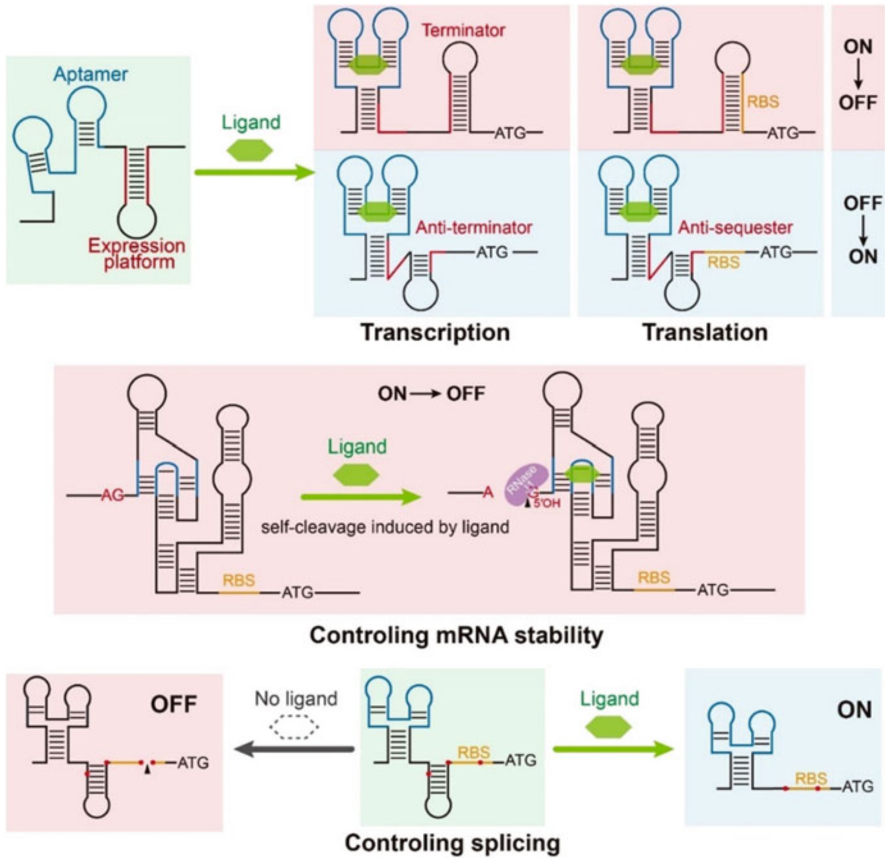

Fig. 4.2 The functional mechanism of riboswitch

and their derivatives, amino acids, phosphorylated sugar, and so on have been found, and they could modulate gene expression by controlling transcription, translation, mRNA stability, and splicing (Fig. 4.2) (Serganov and Nudler 2013). Because the regulations on genes expression are achieved by modulating the secondary structure of mRNAs, RNA-based biosensors possess faster responses compared with the protein-based biosensors. In addition, they have a good transplantable character on account of the protein-free control process (Topp et al. 2010). For example, the glucosamine-6-phosphate riboswitch of *B. subtilis* was directly used for high-throughput screening of *N*-acetylglucosamine high-producing strain in *S. cerevisiae* (Lee and Oh 2015). The RNA-based biosensors also function in a dose-dependent manner, hence their fundamental characteristics for evaluation are the same as the protein-based biosensors mentioned above (Chang et al. 2012).

4.1.2.2 Designing and Tuning RNA-Based Biosensors

Duo to functional mechanism of riboswitches, the RNA-based biosensors are easily to be designed and built by adding the natural or engineered riboswitches into mRNAs (usually on 5′ untranslated region (UTR)), and the responsive characteristics could be tuned by modifying their sequences in aptamer region or expression platform and getting the mutants using high-throughput screening (Jang and Jung 2018; Page et al. 2018).

The dynamic range of the RNA-based biosensors can be improved by changing their promoter or RBS, and the anti-RBS sequence on the riboswitch also needs to be modified if it functions in an RBS sequestering manner. For example, RBS sequence on expression platform of the pyrimido[4,5-d]pyrimidine-2,4-diamine (PPDA) riboswitch was exchanged with the *E. coli* consensus RBS sequence (AGGAGG) for enhanced maximum of the biosensor firstly, and then high-throughput fluorescence-activated cell sorting (FACS)-based selection/counter selection methodology was used to identify anti-RBS sequences that give riboswitches with optimal OFF and ON states. Introducing these modifications improved the maximal expression and dynamic range of the biosensor by 8.2-folds and 80-folds, respectively (Kent and Dixon 2019). As an another example, Jiang et al. improved the dynamic range of a L-tryptophan riboswitch-based biosensor by changing its promoter and copy number (Jang and Jung 2018). To modulate the operational range of the riboswitch-based biosensor, the aptamer region can be modified to change the affinity between ligand and riboswitch. For instance, the dose–response curve of a L-tryptophan riboswitch-based biosensor was shifted toward higher ligand concentrations by exchanging a low affinity aptamer (Jang and Jung 2018).

The ligand specificity of the RNA-based biosensors may be enhanced or changed by modifying the aptamer regions (Robinson et al. 2014). For instance, the specially responsive ligand of the natural adenine riboswitch was turned to ammeline or azacytosine by introducing site-directed mutagenesis at U47 and U51 sites on the aptamer region that are responsible for the interaction with the ligand molecule (Dixon et al. 2010). In addition, "non-natural" synthetic riboswitches could be designed and constructed using corresponding aptamers found in the natural world or built artificially (Darmostuk et al. 2014; Kinghorn et al. 2017; Sun and Zu 2015). For example, an L-tryptophan riboswitch was built by selecting the N_{10} sequences connecting L-tryptophan aptamer region that had been reported previously with RBS and dual selection module (tetA-sgfp) in vivo (Yang et al. 2013). In addition, a statistical thermodynamic model has been proposed for the aptamer-based artificial riboswitch design (Espah Borujeni et al. 2016). In another example, self-cleaving ribozyme-based artificial riboswitches have been built by linking the *thiM* aptamer domain from *E. coli* into stem III of a fast-cleaving hammerhead ribozyme (HHR) (Wieland et al. 2009).

It is worth mentioning that new artificial RNA aptamers that bind to specific ligands could be easily constructed using the technology called systematic evolution of ligands by exponential enrichment (SELEX) in vitro (Darmostuk et al. 2014), and then the new aptamers will be used for the building of corresponding riboswitches (Jang et al. 2017). Alternatively, riboswitches responsive to new ligand can be also

constructed by directly introducing a random-sequence library into the aptamer domain of a native or ready-made riboswitch and then conducting multiple rounds of dual genetic selection and FACS screening in vivo. Using this method, theophylline riboswitch that possesses a 2.3-fold dynamic range was obtained from the native ThiM#2 riboswitch (Page et al. 2018).

4.2 The Application of Genetically Encoded Biosensors

4.2.1 The Application in High-Throughput Screening

Because the metabolic networks and their regulations are very complex in the cell, high-throughput screening (HTS) is often used to obtain the best producer from the mutant libraries of enzymes or pathways (Lim et al. 2018). The genetically encoded biosensors could couple the target products' concentrations with expression levels of the reporters, and then the best producer can be obtained by adaptive evolution or FACS.

4.2.1.1 Screening by Adaptive Evolution

To carry out adaptive evolution, appropriate reporter needs to be chosen to link cell growth with product concentration. For the biosensors whose expression levels are positive correlation to the concentrations of ligands, resistance maker could be used. For example, a tetracycline resistance protein TetA was used as the reporter of the aTF-based biosensor for directed evolution of a heterologous biosynthetic pathway of 1-butanol in *E. coli* (Dietrich et al. 2013). As the biosensors whose expression levels possess negative correlation with the ligand concentrations, negative selection marker needs to be used. For example, cytosine deaminase that has a cytotoxicity was used as the reporter of the GlcN6P riboswitch-based biosensor for the screening of the best mutant of the key pathway enzyme GFA1 for N-acetylglucosamine (GlcNAc) synthesis in *S. cerevisiae* (Lee and Oh 2015).

4.2.1.2 Screening by Fluorescence-Activated Cell Sorting

The genetically encoded biosensors could also be applied for FACS by using fluorescence protein as the reporter. For instance, the yellow fluorescence protein (YFP) was acted as the reporter of a lysine biosensor in *C. glutamicum*, and then FACS was conducted for screening of pyruvate carboxylase variants created by error-prone PCR that enable improved L-lysine production from glucose (Kortmann et al. 2019).

4.2.2 The Application in Dynamic Metabolic Engineering

The genetically encoded biosensors also have wide applications in dynamic metabolic engineering, which is capable of dynamically coordinating the metabolic flux in a feedback manner and can avoid the adverse effects on cells caused by metabolic

modification such as metabolic imbalance and accumulation of intermediate products (Lalwani et al. 2018; Shen et al. 2019; Xu 2018). Here, we divide these applications into three categories according to the regulation processes, namely dynamic pathway activation, dynamic pathway repression, and dynamic dual control (simultaneous activation and repression).

4.2.2.1 Dynamic Pathway Activation

Dynamic pathway activation can be used to redirect the flux from the native metabolism toward the target product by introducing a biosensor responsive to prevalent intermediate at the key branch points in the metabolic networks of the cell. For example, amalonyl-CoA biosensor was employed to alter the metabolic flux from central carbon metabolism into a heterologous 3-hydroxypropionic acid (3-HP) synthetic pathway by controlling the expression of the malonyl-CoA reductase derived from *Chloroflexus aurantiacus*, which enabled the dynamic switching between growth phase and production (David et al. 2016).

4.2.2.2 Dynamic Pathway Repression

The competitive pathways of target product were often knocked-outed to force more metabolic flux into the pathway of interest, while sometimes these competitive pathways may be necessary for the cell growth. In this situation, dynamic repression can be employed to redirect the flux toward target product. For instance, the lysine-OFF riboswitch was used to control the expression of citrate synthase (*gltA*), which is the key metabolic point of tricarboxylic acid (TCA) cycle, in a L-lysine-producing *C. glutamicum* strain, thus dynamically channel flux from central carbon metabolism into L-lysine synthesis (Zhou and Zeng 2015a). Similarly, a GlcN6P-OFF riboswitch was set as an intermediate metabolite biosensor that dynamically repressed the competitive pathways, namely peptidoglycan synthesis pathway and glycolysis pathway, in a GlcNAc-producing *B. subtilis* strain (Niu et al. 2018).

4.2.2.3 Dynamic Dual Control

To achieve the better and more precise control of the metabolic networks in a microbial cell factory, dynamic activation and repression on multiple targets simultaneously, which is widespread in the natural world, may be needed. This process can be realized by designing and building biosensors that possess opposite regulation effects. Xu et al. have constructed malonyl-CoA activating and repressing biosensors regulated by the aTF FapR, and controlled the malonyl-CoA source pathway (ACC) and the malonyl-CoA sink pathway (FAS) by the malonyl-CoA activating and repressing biosensors, respectively, which avoided the accumulation of intermediate product malonyl-CoA and balanced metabolism between cell growth and target product fatty acids formation (Xu et al. 2014). In another example, lysine-ON riboswitches were built by engineering a native lysine-OFF riboswitch from *E. coli*, and lysine-ON and lysine-OFF riboswitches were applied for the control of lysine transport protein and the key competitive pathway, namely TCA cycle, respectively (Zhou and Zeng 2015a).

Except for the double sensor mediated dynamically dual control, single biosensor-based dual control, which could be achieved by coupling the biosensor with some regulation tools acted as NOT gates, have also been reported. Yan and coworkers have presented a bifunctional dynamic control system based on biosensor and antisense RNA (as RNA), which can be used to upregulate and downregulate multiple genes simultaneously, and applied this system to achieve the dynamic flux distribution between native metabolism and the muconic acid biosynthetic pathway (Yang et al. 2018). In addition, the CRISPRi based NOT gate was also coupled with a biosensor to achieve the autonomous dual-control of metabolic flux in *Bacillus subtilis* (Wu et al. 2020).

4.3 Conclusions and Perspectives

Genetically encoded biosensors have been widely applied in the construction of efficient microbial cell factories. However, the building process of novel biosensors responsive to specific macular, which is the premise of all subsequent operations, is still time-consuming. Hence the computer-aided methods need to be further explored for accelerating biosensor design in the future. In addition, the biosensors-mediated feedback and dynamic regulation of the metabolic networks can be combined with the rising co-culture engineering strategy, which has been proved to be more advantageous in the synthesis of many products (Jones and Wang 2018), to achieve the coordination control of population dynamics. Furthermore, biosensors may also be used in the regulation of engineering spatial organization of metabolic enzymes, which can enhance flux into interested pathway and reduce their interactions with cellular background metabolism (Lee et al. 2012), for the reconstruction of the cell metabolism in space and time dimensions simultaneously.

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Metabolic Products of Mixed Culture Fermentation

5

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Abstract

Mixed culture fermentation provides a great avenue for the production of various metabolites of commercial use. It has clear economical and process advantages over pure culture fermentations. However, some limitations still exist as the consortium of bacteria drives the process kinetics. To overcome such limitations, the bio-processes as well as syntrophic relationship of the microorganisms must be taken into account to control the process meticulously. In this chapter, we have focused mainly on mixed culture fermentation, complexities associated to it, control and regulation of the process and several parameters which play a vital role in stable operation of the mixed culture fermentation process. This chapter

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aimed at understanding the functionality and importance of solid-state fermentation, along with submerged fermentation.

Keywords

Mixed culture · Metabolic products: fermentation · Metabolism regulating parameters · Solid-state fermentation

5.1 Introduction

Production of fermentation products through anaerobic conversion of organic substances is one of the potential biotechnological processes. In general, fermentation is a biological catabolism process and controlled through the substrate-mediated redox reactions. More specifically, the generation of adenosine triphosphate (ATP) takes place in the absence of commonly used external electron acceptor such as O_2, NO_3^-, or CO_2 (El-Mansi et al. 2006). Although, substrate works as primary sink for the electrons, but excess electrons may be utilized for the production for elemental hydrogen through reducing protons. In such processes, the conversion of potential energy to cellular energy is facilitated by oxidation reactions, especially in the form of ATP. This mechanism is propagated in literature as substrate-level phosphorylation. Literature review revealed that requisite energy can also be leveraged by fermentation cells via alternate ways of electron transfer, i.e. cations and protons-mediated process, and other electron transport chains (Konings et al. 1994).

To date, accurate prediction of fermentation products is quite strenuous, especially for the mixed culture-based systems. Industrial fermentation processes are typically performed using pure cultures and aimed at the production of high-value products. For bulk chemical production, the pure culture process seems less attractive due to their elevated financial requirements, particularly associated with the controlling mechanism of the culture performance and its strictly sterile working conditions in order to prevent contaminations. Important equipment investments are necessary for these processes at industrial scale. In addition to this, pure culture fermentations require generally the use of pure and therefore more expensive substrates. The risk of contamination of the culture furthermore remains since an unstable pure microbial culture is used. Mixed cultures comprise of a consortium of stable and mixed microbial species, typically found in nature. The use of less pure substrates (even wastes or by-products) is possible with the subsequent cost implications. Mixed culture fermentations (MCF) did not find wide application at industrial scale because they present still important limitations. Further, great variation in qualitative and quantitative characteristics of MCF is observed in some studies, which suggest that maintaining the optimum balance between mixed culture microorganism required a thorough understanding of behaviour of associated microorganisms.

5.2 Production of Metabolic Products from Mixed Culture Fermentations (MCF)

From the industrial processes, large amounts of residues are produced as waste. The further utilization of these wastes by the biotechnological industry is quite limited. The major obstacle identified as presence of diversified organic compounds in these waste residues. Production of energy carriers (Claassen et al. 1999) or other valuable products by mixed culture fermentations would bring utility to those useless wastes or by-products and also enable interesting downstream integrations. MCF is a potentially interesting technology for validation of these streams and generation of specific products. Products that can potentially be obtained by MCF include mixtures of volatile fatty acids, alcohols, lactate that may serve as building blocks in other processes. Several interesting applications exist for MCF processes.

(a) Production of biodegradable polymers such as 3-hydroxyalkanoicacids (PHAs) and poly-3-hydroxybutyricacid (PHB) has been extensively investigated (Lee 1996). PHAs can effectively be produced by mixed cultures of bacteria by imposing a strong selection pressure on the mixed culture (Reis et al. 2003).
(b) Biological hydrogen production by MCF has a large research interest (Benemann 1996) due to its potential application as energy carrier. The yield of hydrogen depends on composition of formed fermentation products and their stoichiometric quantities.
(c) Solvent fermentations, for the production of alcohols and acetone, butanol or propanol by using clostridial cultures, have been of increasing interest in the past few decades (Dürre 1998). These processes have been proven effective in the development of sustainable additives to gasoline. To date the extent to which mixed culture can be applied to the production of specific solvents remains largely unclear.
(d) Furthermore, the carbohydrate fermentation is a crucial step in valorization of solid waste streams as well as anaerobic digestion of applied wastewater. In anaerobic digestion processes of wastewater, the initial fermentation of carbohydrates, known as acidogenesis, leads to a wide variety of products that are subsequently methanized by using other microbial consortia. The interest of carbohydrate fermentations in the framework of all these applications motivates for modelling these processes from the perspective of control of the product formation (Singh et al. 2016).

If anaerobic bioconversion process is carried out in multiple steps, various intermediate by-products can be separated out through favourable pathways of individual substrate conversion. Such specifically designed bioreactors warrant an enhanced biodegradation of organics along with the increasing yield, selectivity and improved qualities of product. With this vision, two-stage processes are marked by obvious advantages over single-stage anaerobic systems, as two-stage processes can guarantee an enhanced production of gaseous products, i.e. methane and hydrogen, and other desired products/outputs such as bioplastics, bio-flocculants, bio-polymers, bio-pesticides, biosurfactants and energy. Therefore, by offering an

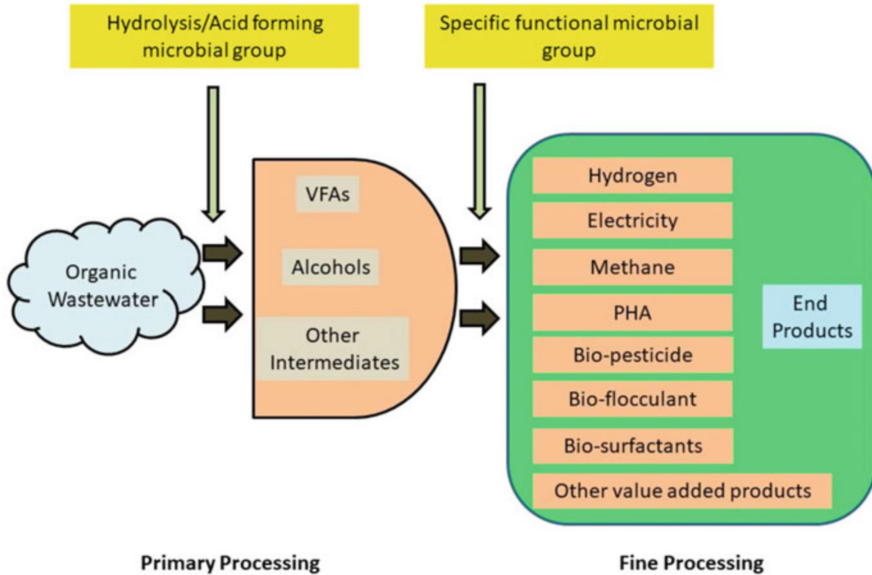

Fig. 5.1 Schematic of fermentation process showing possible biochemical end products recovered from wastewater

appealing avenue, it can provide a promising platform, probably a practically viable and economically feasible, for future applications. However, there are also various challenges which remain as a hurdle in such path-breaking developments (wen-wei Li et al. 2011). Figure 5.1 represents a schematic depicting the biochemical products oriented possible pathways and energy recovery from an anaerobically treating two-phase wastewater system.

5.3 Complexities Reported in Mixed and Pure Culture-Based Microbial Fermentation Processes

Generally, microbial communities of anaerobic systems do not utilize the external electron acceptors. Instead, these microbial populations are expected to generate the larger part of their ATP through a well-known mechanism designated as "substrate-level phosphorylation". Further, in the absence of substrate-level reduction, utilization of excess electrons can be facilitated by the substrate reduction mechanism which lead to the production of light weight compounds such as short chain fatty acids, sugar alcohols, and some other alcohols. Some researchers also reported that hydrogen gas can also be produced through such electron transfer mechanism. Thus, fermentation processes are mediated through coupled substrate-level oxidation–reduction mechanisms. More specifically, oxidation reactions (or substrate-level phosphorylation) provide the required catabolic energy, whereas reduction reactions are supposed to be favouring the sinking of electrons. Moreover, excess electrons

can be sunk through hydrogen, however; thermodynamically/energetically seems to be unfavourable, especially at high partial pressure of hydrogen (Rodríguez et al. 2006).

One of the considerable examples of pure culture-based processes is production of bioethanol from starch by using a model organism *Saccharomyces cerevisiae*. This process is rapid in nature and can be inoculated with well-acclimatized cultures. This pure culture-based system is able to generate desired quality and quantity of products, even in the presence of inhibitory/competing species. Thereby, high yield of products can also be expected in the absence of expensive sterilization equipment (Lin and Tanaka 2006).

On the other side, in pharmaceutical production, stringent guidelines of production such as complete elimination of contaminating/secondary microorganisms, are imposed by Food and Drug administration of United states and some medical agencies of European countries. With respect to uncontrolled and high-rate fermentation of sugars through mixed cultures, microbial contamination can be expected via undesirable side reactions of various microorganisms. This behaviour can largely be attributed to the insufficient knowledge about the control of mixed culture-based systems (Ciani et al. 2010). More specifically, lack of knowledge about the factors affecting product spectrum is mainly responsible for failure of mixed culture system. In spite of these challenges, mixed culture-based systems are reported to have few secondary products such as propionate and lactate (Eng et al. 1986; Horiuchi et al. 2002). To date, biotechnological products such as acetate, butyrate, and ethanol have been reported to be produced from glucose, in controlled mixed culture fermentation processes (Temudo et al. 2007). Besides, published research studies lead to the development of fermentation models, which can be accurately used for the prediction of specifications of products at set pH range and with low substrate concentration (Rodríguez et al. 2006).

5.4 Factors Affecting Regulation of Metabolism in Mixed Culture-Based Systems

To date, various factors have been reported to affect the functioning and control of mixed culture system. Some of the important factors include pH, temperature, hydraulic loading, organic loading and type of substrate (Himmi et al. 2000; Voolapalli and Stuckey 2001; Batstone et al. 2002). The variation in aforementioned parameters can directly affect the product spectrum as well as microbial diversity. Each of this factor has the tendency to impose the effect on cellular metabolism or ecology of the systems. Results of such interaction have been reported to be producing wide ranges of products.

5.4.1 Role of pH and Its Effect

The pH of media plays a vital role in qualitative and quantitative characteristics of mixed culture fermentation system. In particular, the rate of dissociation of microbially produced organic acids is reported to be dependent on pH levels of media. Further research investigations revealed that the dissociation rate can directly affect the production of ATP, required for the cell maintenance (Rodríguez et al. 2006). With respect to undissociated organic acids, favoured by low pH conditions, diffusion of these forms of acids into the cell takes place passively. This led to the requirement of increased rate of active transport (ATP-consuming) to facilitate the removal of acid products. Such conditions of energy requirements may direct the cells to adopt a secondary ATP generation mechanism, i.e. shifting from acetate pathway to butyrate pathway with lower ATP yield at low pH conditions. The stoichiometric conditions of butyrate pathway have also shown lessen impact on pH per mole of glucose. In this way, the active energy transport requirements are reduced. This shift in metabolic pathways (acetate to butyrate) for mixed culture systems has also been reported in scientific literature by various researchers (Temudo et al. 2007). Some authors also reported that at low pH conditions, mixed culture can also maintain ATP generation through acetate pathways by increasing the pH neutral ethanol (Ren et al. 1997). Regardless of this, it is still a matter of research that what type of conditions causes mixed cultures to produce butyrate even at low pH in some cases, and ethanol in some another studies. This metabolic behaviour change may be linked with the type of used substrate. Temudo et al. (2007) observed such behaviour with glucose as sole carbon source, while possible results were reported by the Ren et al. (1997) for the combination of sucrose and molasses. Additionally, one of the exceptional results was also published by Horiuchi et al. (2002), in which higher yield of propionate was reported at a pH of 8, with fermentation of glucose through mixed cultures. However, these results have not been replicated till now.

5.4.2 Effect of HRT and OLR

Hydraulic retention time (HRT) and organic loading rate (HRT) are well-known operational parameters of biological systems. The change in these parameters is also found to be associated with metabolic activities of pure and mixed culture systems. Both OLR and HRT ensure the availability of substrate in continuously operated biological systems. So far, OLR effect, which is considered as an important primary variable and represents a combined effect of applied substrate concentration and HRT, has not been investigated much in fermentation processes. However, it is considered as principal lumped variable which determines the maximum cellular flux or available feed for microbial communities. Therefore, many scientists and engineers have argued about the effect of OLR in mixed culture systems. We, as an author, also into the perusal of OLR effect, as a controlling factor in fermentation metabolic processes. Till now, researchers have reported the effect of OLR on mixed

culture, mainly in the anaerobic digestion process and defining the limits of methanogenic activities. Eng et al. (1986) reported that increased OLR of sucrose in an aerobic digestion process can led to the high yield of lactate and propionate. This subsequently changes the yield of methane and H_2, probably due to inhibited dissociation of these products. The research studies of Voolapalli and Stuckey (2001), based on shock loads of glucose and sucrose, revealed that VFA production can increase in such case. However, a difference in acid products in not discussed in this study. Further examinations were also conducted at increased OLR with constant substrate concentration. Agler et al. (2012) reported that production rate of fermentation products such as butyrate can be increased by varying the HRT with constant OLR conditions (maintained by varied substrate concentrations). These findings revealed that both the HRT and OLR can affect the metabolic pathways of mixed culture systems. Further, it should also be investigated whether there will be a difference between varying OLR with constant HRT and varying HRT with constant OLR conditions. Overall, it has been observed that very limited published work is available in this direction and thus there is ample opportunity to conduct research on this topic (Pandey and Sarkar 2017).

5.4.3 Role of Substrate Type

Like pH and loading rates, choice of substrate has also an impact on metabolic activities of fermentation processes. The experimental investigations on the effect of substrate type in fermentation processes are well-documented by various researchers (Ren et al. 1997; Temudo et al. 2007; Lu et al. 2013). The results of these studies revealed that there may be a difference in the yield of ethanol and butyrate, especially at low pH conditions. In particular, the degree of reduction of substrate depends on its type, which consequently led to the production of reduced EMCs with differing quantities. As shown in Fig. 5.2, each substrate has a different tendency to enter the fermentation metabolism. Hence, each substrate offers a different pathway for the production of metabolic products and their quantities. Furthermore, inhibitory effect can be observed during some particular pathways for some substrates. One of such examples, reported by Cameron et al. 1998, include inhibition of sugar for the 1,3-propanediol pathway. The research studies, based on pure culture, have shown extensively that product spectrum can vary significantly according to the choice/type of substrate. One of such study was conducted by Lewis and Yang (1992), in which varying product spectrum was achieved by *P. acidipropionicion* glucose, lactose and lactate. Further examination of this study revealed that in case of lactate as fermentation substrate, highest and lowest production was observed for propionate and acetate, and succinate, respectively. Yu et al. (2007) also demonstrated that glucose and lactose have different abilities of affecting fermentation gene expression for *Clostridium acetobutylicum*. Detailed analysis of this research study revealed that genes, responsible for lactose fermentation, can be expressed in the sole presence of lactose. On the contrary, when a mixture of glucose and lactose is provided, genes responsible for lactose fermentation were not expressed until a sufficient amount of

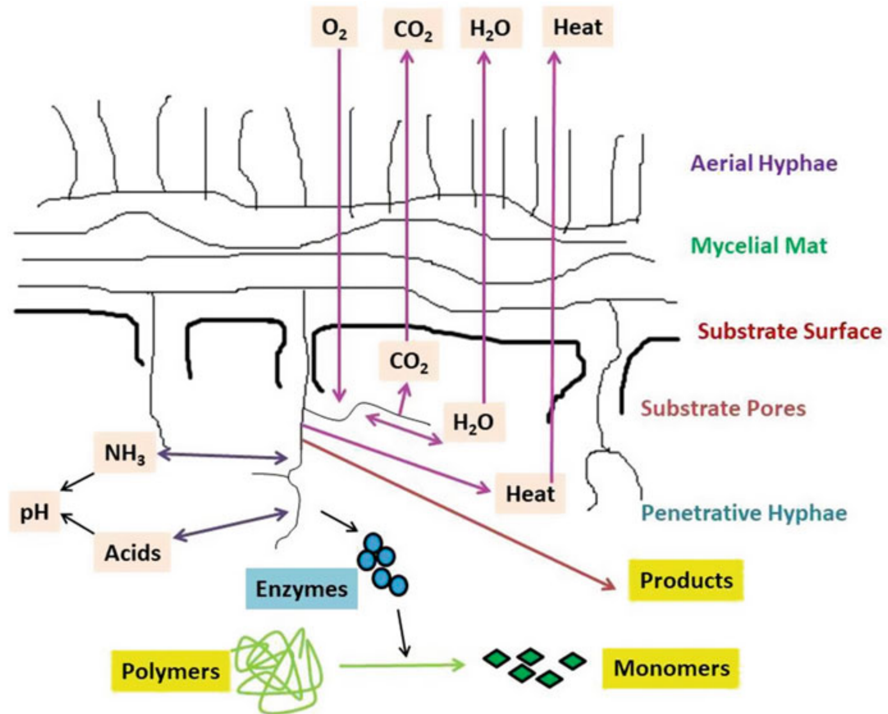

Fig. 5.2 Microscale processes occurred during solid-state fermentation

glucose had been consumed. It is clear that a change in product spectrum is dependent upon substrate types as well as its composition. Further in-depth examinations revealed that choice of substrate mainly affects the NADH:NAD+ ratio of *C. acetobutylicum* and *E. faecalis*, respectively (Girbal and Soucaille 1994; Snoep et al. 1991). However, these results were indecisive, as change in ratio of substrates was a direct or indirect relation with relative redox state of the substrate. Such observations may be attributed to the fact that catabolic pathways are microorganisms as well as substrate-specific. One of such cases include bioethanol industry, where fermentation of xylose was observed to be negligible in the presence of strains of *S. cerevisiae* (Jeffries 2006; Ha et al. 2011). Such behaviour of a particular species may be linked with the role of selective pressure maintained in the mixed culture system. Recent published works also revealed that substrate plays an important role in the growth of microbial communities of anaerobic digesters (Zhang et al. 2014).

5.4.4 Effect of Temperature

Like other operational parameters of biochemical systems, temperature is one of the inevitable parameters which has profound effect on biodiversity of systems as well as product spectrum. In particular, the temperature conditions provide selective pressure for psychrophiles, mesophiles, thermophiles at <20 °C, 30–40 °C, and >50 °C, respectively (Pervin et al. 2013). With reference to chemical reaction engineering principles, temperature has a direct relation with the activation energy of biochemical reactions and thus alters the thermodynamic equilibrium. The experimental investigations of studies, based on ethanol production using *S. cerevisiae*, revealed that substrate consumption rate and product yield increased with increase in temperature. On the contrary, the yield of volatile by-products (e.g. acetaldehyde, isobutanol, VFAs) was found to be inversely proportional to the degree of temperature (Bakoyianis et al. 1997; Galanakis et al. 2012). The studies performed using mixed culture reported that fermentation rate increases with the increase in the temperature of system. However, as compared to temperature, the individual product responses, i.e. VFAs and H_2, only were found to be strongly dependent on pH of the system (Infantes et al. 2011). Detailed examination of such type of studies revealed that high temperature mainly favours the oxidative reactions or production of hydrogen over reductive reactions (Batstone et al. 2002). Another study reported that thermodynamic effect of temperature was found to be associated with cellular membrane permeability. The same was evidenced by Bischof et al. (1995) in their research study, performed by using fluorescent dye at different temperatures using two types of animal cells. Infantes et al. (2011) also reported that membrane permeability increases with temperature, which significantly reduces the substrate consumption at elevated temperature and low pH conditions. Unfortunately, no published literature could be found mentioning the relationship between temperature and membrane permeability of fermenting microorganisms.

5.4.5 Control with Electro-Fermentation

The application of requisite negative potential, through a particular electrode, to fermenting cultures has been proven effective in desired product spectrum. Such approach was found to be effective in achieving the high yield of reduced products. Some of examples include increased yield of propionate and ethanol by *Propionibacterium freudenreichii* and *Clostridium thermocellum* and *S. cerevisiae*, respectively (Emde and Schink 1990). In this regard, two mechanisms of electron uptake by cells are suggested. These are known as direct and indirect transfer of electro-fermentation. In direct electron transfer mechanism, interaction of redox active components (EMCs and/or nanowires) which are present in outer membrane of cell is reported in literature too. This mechanism is particularly hypothesized for anode-driven systems (Bond and Lovley 2003; Reguera et al. 2005; Gorby et al. 2006). Further, cathode-driven systems are also reported to be governed by this mechanism (Rabaey and Rozendal 2010; Lovley et al. 2011). Unfortunately, authors

could not find any published work highlighting the applicability of this mechanism in fermentation processes.

On the contrary, indirect electron transfer refers to the transport of electrons between microbial cultures and the electrode. This transport is facilitated by redox active shuttling molecules through the diffusion process in culturing medium. The shuttling molecules generally works as electron mediator in this process. Some of these examples include secondary metabolites such as phenazine, flavins, hydrogen produced at cathode surface, and added synthetic molecules (Marsili et al. 2008). Some of the reported synthetic electron mediators for cathodic systems are anthraquinone 2,6-disulfonic acid, methyl viologen, and neutral red (Emde and Schink 1990). Noting that control of mixed culture system is one of the major hurdles in successful implementation, synthetic mediators may play a crucial role in the regulation of extracellular electron transfer in fermentation processes. This may be attributed to their selective ability for desired value of reduction potential. As, each carrier may have a distinct stoichiometry, the process control can be governed by the energy levels at which the electrons enter into the cells. Dennis et al. (2013) reported that cathodes may be used for desired product spectrum in mixed culture fermentation, whereas, mediators with negative reduction potential were found to be associated with higher yield of products in pure culture systems (Emde and Schink 1990). These advanced strategies, i.e. use of mediators of increased negative reduction potential, can explore dimensions in making more thermodynamically favourable reductive metabolic pathways.

5.5 Two-Phase Anaerobic Reactors for Mixed Culture Fermentation (MCF)

In many cases, when the hydrolysis and fermentation processes are rate-limiting steps, anaerobic wastewater treatment process carried out in two stages is considered to be effective. The two-stage anaerobic reactor is comprising of two reactors in series where the first stage includes processes of hydrolysis/acidogenesis and another for specific product generation. However, an insignificant acetogenesis and methanogenesis also take in the first stage. By separation of stages, the control over optimum environmental conditions becomes easy and this enables to solve the problems associated with the microbial activities and their growth kinetics by facilitating an optimized environmental condition for each group of bacteria in each reactor (Pandey and Sarkar 2019a). Separation of stages of microbial processes increases the overall stability of the anaerobic treatment which is difficult to achieve in traditional delicately balanced single-stage anaerobic reactors. So far, various researchers have been reported the successful application of two-stage anaerobic reactors, which entails the benefits of treating waste under thermophilic and mesophilic conditions of temperature mesophilic. However, very low solid containing wastes are preferable for using two-phase digestion processes.

Till now, many modified versions of two-stage anaerobic digestion processes have been proposed, in which acidogenic and methanogenic processes are separated

and the first stage was optimized for thee agricultural and food process wastes, municipal food waste (Kim et al. 2004), demonstration of promising utilization of feedstocks such as glucose and production of hydrogen. As the less NO_x is produced in this process, it gets cleanly combusted than the methane gas. As per the reports of Cooney et al. 2007, methane produced using two-stage anaerobic digestion has more stability and is significantly more effective than the methane produced in single-stage anaerobic reactor.

Two-phase anaerobic wastewater treatment can be operated in thermophilic or mesophilic conditions of temperature. High rate of biogas production adversely affects sludge settle ability as it carries over biomass excessively and finally it gets washed out. This phenomenon could be overcome by using either granulation of sludge, encapsulation of biomass, and/or biofilm formation. Nevertheless, operation under high temperatures negatively affects the granulation of sludge as the degree of mineralization of sludge is comparatively high in thermophilic reactors. Thus, a mesophilic range of temperature increases the process stability as compared to thermophilic conditions (Pandey and Sarkar 2019b). The production of extracellular polymeric substance is adversely affected by mineralization which ultimately restricts firm and dense sludge granulation. As a result of this, insignificant granulation or even degranulation takes place when mesophilic sludge inoculum is utilized as seed. Thus, with systems achieving higher concentrations of biomass, low efficiencies of treatment are expected. Van Lier (1996) reported that suspended as well as fixed-film growth reactors have encountered such problem at thermophilic temperature conditions. Further limiting the application of thermophilic anaerobic reactors industrially due to excessive biomass washout, environmental sensitivity, waste heat availability, feed characteristics variation resulting in unsatisfactory treatment performance, and degraded effluent quality.

For an enhanced propionic acid metabolism, microbial proximity, configuration of the reactor, nutrient supplementation, and characteristics of the substrate are equally important factors to be addressed (Speece et al. 2006). The rapid consumption of hydrogen by homoacetogens or methanogens is important for the oxidation for anaerobic degradation intermediates. Bioreactors such as up-flow anaerobic sludge blanket reactor, expanded/static granular sludge bed reactor, biofilm reactors (anaerobic filter or fluidized bed reactor), and membrane bioreactor provide an excellent opportunity for the proximate growth of a diversified microbial community. Therefore, to maintain the low hydrogen partial pressure, high-rate anaerobic systems are considered to be extremely efficient.

5.6 Solid-State Fermentation

Fermentation process in which solid matrix is used as substrate with very less water is known as solid-state fermentation (SSF). The substrate should however essentially be moist enough for the growth and metabolism of microbes. In this process, the solid matrix can itself be a source of nutrients. In many cases the substrate does not

provide nutrients, but it is impregnated with proper nutrients to support the proper growth of the microorganisms (Hoelzle et al. 2014).

The scheme of micro-processes involved in SSF has been shown in Fig. 5.2. The solid substrate acts as a support to the mycelial mat where the fungal hyphae develop. The development of hyphae protrudes from the mycelial mat into two directions. Hyphae protruding towards gaseous space are called aerial hyphae, and hyphae protruding towards moist pores of the substrate are called penetrative hyphae. When the moisture level is normal, the penetrative hyphae and mycelial mat remain in contact with water, whereas the aerial hyphae remain in contact with air. As shown in Fig. 5.2, substrate surface and inner pores support the major metabolic process. Some of the metabolic processes can also occur in the exposed region of mycelium, e.g. aerial hyphae, and it also facilitates the transport of the substances from the penetrative hyphae to aerial hyphae. Mycelium also produces hydrolytic enzymes which diffuse through the solid matrix and further helps the hydrolysis process by catalysing the macromolecules degradation into simple monomers. These simple monomers are easy to assimilate further by the fungus. In this process oxygen is consumed, and many fermentation products are produced along with O_2, CO_2, H_2O, and heat. The transport of gases and moisture takes place due to the development of gradient between the substrate subsurface and aerial hyphae. The major problem with the SSF process is development of heat near the substrate surface. The removal of heat either takes place via conduction or evaporation, loss of heat. The evaporation process also acts to balance the water in the system. The water is consumed during hydrolysis reaction and the same is produced back during the respiration process. The decrease in pH is also an important factor which arises from the exchange of ammonia and production of organic acids near the substrate surface. During the metabolic process, many products of interest are also released on the surface of solid matrix which needs further separation and downstream processing. Along with all these processes, many other factors, reactions, and physicochemical changes can also influence the process of SSF.

The primary aim which makes SSF a promising technology is that it allows microorganism to remain very close to the substrate as the nutrients remain in highest concentration at the solid matrix surface. It allows microbes to get nutrition easily and also favours them in a way that it resembles to the natural habitat of the microbes which adds on to their ease in growth. Biotechnology industries have now started looking forward to this technology as this provides a great avenue for the production of value-added by-products like biofuels, industrial chemical, food, secondary metabolites, and pharmaceutical products. SSF gives advantage over submerged fermentation which is the most attractive feature of it. The major applications of SSF process lie in the field of bio-pulping, bioleaching, bioremediation, etc. SSF process can directly utilize wastes generated from agro-industries as substrate which is an additional advantage as these residues can be used efficiently without contributing to environmental pollution.

The SSF technology is very much suitable for the production of secondary metabolites as solid support provides a great support for the growth of mycelium of the microorganisms. On the other hand, submerged fermentation has to be highly

Table 5.1 Examples of secondary metabolites produced by specific solid-state fermentation

Product	Microorganism	Substrate	References
Cephalosporin	*Streptomyces clavuligerus*, *Cephalosporin aermonium*	Barley	Jermini and Demain (1989)
Aflatoxin	*Aspergillus niger*	Cassava	Barrios-González et al. (1990)
Ergot alkaloids	*Claviceps fusitormis*, *C. pupea*	Bagasse	Hernández et al. (1993)
Mycotoxin corn	*Aspergillus flavus*	Wheat, oats	Hesseltine (1972)
Penicillin	*Penicillium chrysogenum*	Bagasse	Barrios-Gonzalez et al. (1988)
Tetracyclines	*Aspergillus*	Sweet potato	Yang and Ling (1989)
Zearalenone	*Fusarium moniliforme*	Corn	Hesseltine (1972)

viscous in order to favourably support the production of secondary metabolites which further interferes with the transfer of oxygen. Furthermore, the secretion of secondary metabolites and filaments attached to the microbes may further increase the viscosity of the media which causes detrimental effect on the overall process. SSF technology allows for better circulation of oxygen and has many other benefits over submerged fermentation (SmF) process (Singh et al. 2018). Some secondary metabolites produced by SSF process have been tabulated in Table 5.1.

5.6.1 Biological Features

The advantage of SSF processes lies in providing a natural habitat to the microorganisms. Microbes like *Ascomycetes*, *Basidiomycetes*, and *Deuteromycetes* are found in nature on wet substrates in terrestrial habitats. SSF is widely as well as preferably used for the production of mould cheese in food industries, for which conidiospores of *Penicillium roquefortii* or *P. camemberti* are required. SSF is also used for the production of protection agents for plants which use *Coniothyrium minitans*. SSF also facilitates the option of growing microorganisms in mixed culture which is advantageous for many processes. A broad spectrum of enzymes is secreted by fungal consortia during their growth, which causes synergetic increase in the activity of individual enzyme. Thus, SSF has a potential to increase the productivity of the target metabolite (Singhania et al. 2009). In addition to this, SSF favours to the possibility of controlling the water activity as a selection parameter during the co-cultivation of fungi as different fungi have different water demands. In food industry, SSF finds its widespread and important application as these processes demand use of mixed cultures which is essential for a particular flavour of the food produced. For example, during the fermentation process of bamboo sprouts, a wide array of metabolites is produced along with 29 different volatile substances which are responsible for its aromatic properties.

5.6.2 Ecological Features

SSF process is devoid of free aqueous phase which boons this process with a minimum utilization of water which accounts for the very low amount of wastewater produced during the process. SSF is a highly environmentally friendly process as it produces low waste as compared to SmF process. Additionally, as the SSF process is carried out using less water, the chances of contamination of the system by bacteria and yeast are minimized. This further reduces the demand of energy-intensive sterilization processes. SSF process can directly use the wastes from agricultural processes as a source of nutrition, and plant residues are also frequently used for the production of enzymes and organic acids as a carbon source, which makes the SSF process environmentally friendly and sustainable.

5.6.3 Engineering Features

SSF process has yet to find its widespread application in western countries as it lacks standardization, low amenability, and limited reproducibility of the results. Due to lack of understanding of the processes involved and bacterial activity in consortium, the scale-up of SSF process is hard to achieve (Pandey et al. 2016). The control of the process at industrial scale of operation is a difficult task as gradients like humidity, temperature and substrate concentration cause adverse effects on the overall process. Parameters like oxygen level, temperature, and moisture content are interrelated and contribute to the difficulty in regulation of these parameters. Aerobic reactions also occur during the growth of the microorganisms, which liberates heat and ultimately causes rise in temperature. Excess heat is detrimental for the enzymatic activities involved in the SSF process as enzymes produced during fermentation process get denatured easily and become inactive at the end of the process.

5.6.4 Economic Viewpoint

As described previously, the SSF technology has many environmental and biological benefits which from economic point of view are very much advantageous to use. The SSF technology has been estimated to be 100 times more efficient than the SmF for the production of crude enzyme cellulose. There are multiple reasons for SSF to be a highly efficient technology. The substrate required to run SSF process is very cheap, and in fact organic wastes can be used very efficiently for the economic benefits. As the utilization of water by this process is very low, thus SSF process does not get contaminated easily and hence it saves the cost for the sterilization, energy equipment, and instruments. For the same reason, it also saves cost in subsequent downstream processing. Due to the characteristics of solid substrates for containing very high concentrations of enzymes, the cost for concentrating the enzymes during product purification is also saved.

5.7 Conclusions

A wide range of fermentation metabolic products of economical and commercial value can be produced from organics present in waste. Mixed culture fermentation requires little control over pure culture fermentation; however, better understanding of the various physicochemical and biological parameters plays a key role in successful operation of the process. Parameters like pH, organic loading, hydraulic loading, substrate type and temperature play key role in the regulation of a metabolic process, and these become even more critical in case of mixed culture fermentation as bacteria involved may have different response to the varying physicochemical parameters. However, solid-state fermentation has a clear advantage as it provides the bacteria a natural habitat and better control over the process. Scale-up of SSF process is still a challenge for researchers as it causes detrimental effect on product formation. Thus, for a controlled and effective operation, a better understanding is essential.

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Recent Advances in Genetic Engineering Tools for Metabolic Engineering

6

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Abstract

Metabolic engineering has evolved exponentially due to rapid progress in the development of omics (proteomics, lipidomics and glycomics) technology and synthetic biology toolsets. Particularly, omics technologies, synthetic biology and metabolic engineering are interdependent in terms of principles of technology and their biological applications. In this article, we showcase synthetic biology toolsets currently used for the metabolic engineering of microbes and focus on some of the recent advances in the field. Specifically we have elaborated on recent progress in metabolic engineering, the associated synthetic biology toolsets, microbiome metabolic engineering (MME) and the potential industrial metabolic engineering applications. As a case study for the industrial application of

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metabolic engineering, we have reviewed the use of synthetic biology toolsets to bioengineer microbes and re-engineer their metabolic pathways for customised industrial applications. Some of the examples included in this article are as follows: (a) metabolic engineering of cyanobacteria, (b) metabolic engineering of *Bacillus subtilis* and (c) metabolic engineering in synthetic yeast for industrial applications. As a future perspective, this chapter presents a case for the broader use of synthetic biology toolsets across metabolic engineering applications.

Keywords

Microfluidics · CRISPR-cas9 · Metabolic modelling · Bioengineered microbes · Phage therapy · Microbiome and industrial synthetic biology

6.1 Introduction

Synthetic biology (Thimiri Govinda Raj et al. 2019), nanobiotechnology (Thimiri Govinda Raj and Khan 2018, 2019; Thimiri Govinda Raj et al. 2020), and metabolic engineering are the three main research areas that are currently driving applied biotechnology research (Nxumalo Z and Thimiri Govinda Raj DB 2020). Metabolic engineering emerged as a nascent field during the 90s through the intersection of what was then the genetic engineering and applied molecular biology disciplines. Particularly, the efforts of two MIT research groups can be credited with catalysing the emergence of the field (Bailey 1991; Stephanopoulos and Vallino 1991). This was followed by an international conference in 1996 which led to the publication of a seminal literally resource (Metabolic Engineering Academic Press, 1998) and the founding of the journal, Metabolic Engineering. Based on these developments and ongoing international discussion, the metabolic engineering field was ultimately defined by the use of recombinant biotechnology to rewire metabolic pathways for the enhanced production of commercially relevant substances (e.g. fuels, chemicals, biopharmaceuticals) (Kumar and Prasad 2011). Since both metabolic and genetic engineering fundamentally rely on altering gene expression, there has been considerable effort to distinguish the field of metabolic engineering through the prioritisation of metabolic pathways and regulatory networks as compared to focusing on individual genes and proteins. In brief, metabolic engineering preceded the systems biology and synthetic biology era with a main focus on understanding the logical architecture of metabolic pathways and approaches in order to optimise their function. As the system biology field evolved, it became clear that metabolic engineering would require a more nuanced approach to build stable functional systems. While it is theoretically possible to improve the production capacity of a particular pathway by simply modifying genes within that pathway, these modifications can also result in various negative off target effects on cellular homeostasis and stability. Hence the metabolic engineering field includes genetic engineering, molecular biology, chemical reaction engineering, cell biology, and

pathway optimisation. As reported by (Stephanopoulos (2012), the fundamental foundation of metabolic engineering includes (a) design and implementation of pathways that convert a specific substrate to a commercial target product, this includes ranking of pathways based on selected methods; (b) thermodynamic feasibility studies on potential pathways; (c) evaluation of pathway validation and pathway flux analyses; (d) pathway kinetic analyses; (e) reverse metabolic engineering; (f) the effect of synthetic genetic networks and gene circuits.

Microorganisms have not naturally evolved to meet requirements for the industrial-scale production of specific molecules. For this reason, one of the main goals of microbial metabolic engineering is to transform these naturally occurring microbial systems into efficient cell factories for the production of industrial products (Jakociunas et al. 2015). In particular, this approach to biomanufacturing has been widely explored in industrial fermentation processes for the production of chemicals such as 1,3-propanediol, farnesene and other terpenoids (Klein-Marcuschamer et al. 2007). Additional advancements in metabolic engineering have also facilitated the industrial biosynthesis of fuels such as isobutanol as well as other hydrocarbons (Lennen and Pfleger 2013). Likewise, the development of complex biological circuits that are able to sense and respond to specific environmental cues has demonstrated an increasing relevance for metabolic engineering approaches to human health applications (Sheth et al. 2016). In this book chapter, we focus on recent developments in the application of microbial metabolic engineering approaches to human health and biomanufacturing.

6.2 State-of-the-Art and Recent Developments in Genetic Engineering

Genetic engineering refers to the direct and deliberate manipulation of an organism's DNA using various genetic technologies which fall under the broader scope of biotechnology. These technologies span from methods for inserting or deleting DNA fragments to precision editing of genes. In nature, subsets of organisms can share DNA through vertical or horizontal gene transfer in order to reproduce and evolve. Genetic engineering allows us to create transgenic organisms by combining DNA from different species into a recombinant DNA molecule. With this approach, scientists have been able to elucidate the function of genes, improve disease resistance and the durability of organisms and produce high-value proteins on a larger scale. In 1972, the first recombinant DNA was created by Paul Berg, which leads to the creation of the first transgenic organism in 1973 (Jackson et al. 1972; Cohen et al. 1973). They developed a technique called molecular cloning, which used microbial restriction endonucleases (type II nucleases), DNA polymerase and DNA ligase to cut and join two DNA molecules from different origins into one circular DNA plasmid. The recombinant DNA was then transformed into the bacterium *Escherichia coli*. Since then, microbes have played a pivotal role in genetic engineering as vectors that shuttle DNA to other higher organisms. Microbes have also been engineered as biological production systems for industrial manufacturing of

important pharmaceuticals, biofuels, metabolites and other biomolecules. For example microbes have an innate ability to synthesise nanoparticles, which can be used to make safer and more environmentally friendly materials for environmental remediation, drug delivery, medical equipment and many other consumer products (Iravani and Varma 2019). Through genetic engineering, we can control the size, shape and composition of these microbially produced nanomaterials (Boedicker et al. 2018).

There have been many advancements in genetic engineering tools which are all borrowed from microorganisms. Although the type II restriction endonucleases are still extensively used for microbial genetic engineering, new programmable nucleases were developed which bind and cut specific DNA sequences with improved efficiency and accuracy. These nucleases cause double-strand breaks (DSBs) in an organism's DNA that the cell then naturally repairs through homology-directed repair (HDR) and non-homologous end-joining (NHEJ) processes. NHEJ can result in the insertion and/or deletion of random DNA sequences, while HDR generally relies on the insertion of specific donor or template DNA sequences (Mansour et al. 1988; Capecchi 1989). This new generation of nucleases include homing endonucleases (HE), zinc-finger nucleases (ZFN), TALENs and CRISPR-associated protein (Cas). Programmable nucleases also allow for in vivo manipulation of DNA when the organism cannot be cultured or the DNA molecules are too large for in vitro manipulation (Chandrasegaran and Carroll 2016).

HEs are a lot like restriction endonucleases, but they can recognise much larger DNA sequences of >14 base pairs (bp) which increases the accuracy of cutting a specific genetic sequence or target region (Hafez and Hausner 2012). However, HEs are difficult to tailor because the DNA-binding and endonuclease activities are located on the same domain of the enzymes. ZFN and TALENs are both fusions of a FokI nuclease subunit from Flavobacterium okeano koites and protein-guided DNA-binding subunits that can be separately tailored to target specific DNA sequences (Kim et al. 1996; Christian et al. 2010). These endonucleases can, therefore, be tailored more efficiently and thereby increase targeting accuracy. The most revolutionary of these technologies is the modified type II CRISPR-Cas9 genome-editing system, which originated from *Streptococcus pyogenes*. This unique Cas9 nuclease uses a designer RNA-guide which can be easily tailored to cleave specific DNA sequences with single-nucleotide accuracy (Jinek et al. 2012). Development of the CRISPR-Cas9 system has resulted in improvements to the speed, affordability and efficiency of genome engineering approaches in microbes. Recently, the CRISPR-Cas9 system was used to simultaneously delete (knockout) multiple genes in thermophilic filamentous fungi to increase the production of plant biomass-degrading lignocellulases (Liu et al. 2017). Lignocellulases degrade lignocellulosic biomass to produce biofuels. CRISPR-Cas9 has also been used to activate silent bacterial biosynthetic genes in Streptomyces species which resulted in the production and discovery of novel metabolites (Zhang et al. 2017).

Advances in genetic engineering have also made it possible to manipulate whole microbial communities in situ. Probiotics (live bacterial strains) can be genetically engineered and introduced into a microbial community like the gut microbiome for added health benefits. These probiotics can be engineered to induce a modified immune response or to secrete beneficial proteins like the human interleukin-10 to

reduce inflammation (Hassan and Curtiss 1994; Steidler et al. 2000). Most recently there have been advances in the genetic engineering of bacteriophages which are viruses that naturally infect bacteria and co-opt bacterial machinery through modifications to the bacterial genome. Natural phages are highly diverse, have a narrow host range, large genomes and produce bacterial toxins, which limits their use as genetic engineering tools. However, phages have now been engineered in yeast to deliver specific genes and target different host ranges (Ando et al. 2015). With this approach, phages can deliver antibiotic sensitivity genes to specific bacteria or deliver CRISPR-Cas9 directly to bacterial communities and eliminate antibiotic-resistant strains (Sheth et al. 2016).

Recent developments in genetic engineering have opened exciting platforms to improve our health and the environment we live in. Microorganisms are central to genetic engineering whether it is as a source of genetic engineering tools, as vectors or as factories/effectors that manufacture specific products. We envision that advancements in the genetic engineering of microbes will undoubtedly shape the future we live in and impact various aspects of human life from health to environmental remediation and various industries.

6.3 Microbiome Metabolic Engineering (MME)

The human microbiome refers to the collection of symbiotic, pathogenic and commensal microorganisms (e.g. bacteria, archaea, viruses and fungi) that co-inhabit discrete sites across the human body (Lederberg and McCray 2001). Recent estimates suggest that the number of bacterial cells slightly outnumber their human counterparts in the average human body. Strikingly, these microbes encode for over three million genes (compared to the ~23,000 genes encoded by the human genome), suggesting a rich source of functional diversity (Qin et al. 2010; Sender et al. 2016). This has led to the microbiome being referred to as our 'second genome' (Grice and Segre 2012; Cani 2018). Unlike our base genetics however, this 'second genome' can be readily and specifically modulated by a number of non-invasive acute interventions (Dethlefsen and Relman 2011; Maurice et al. 2013). This malleable property of the microbiome together with its significant impact on various aspects of human health have positioned the microbiome as the next big frontier for therapeutic innovation. With the advent of high-throughput sequencing and proteomics technologies, microbiome profiling has revealed that the composition and metabolic activity of bacterial communities are strongly associated with human health and clinical outcomes (Vázquez-Baeza et al. 2018; Chankhamjon et al. 2019). Specifically, it has become very apparent that the dysregulation of the microbiome (dysbiosis) is causal to many disease phenotypes, resulting in substantial efforts to fine tune the abundance and metabolic activity of specific microbial species or populations for improved health outcomes (Lynch and Pedersen 2016; Zmora et al. 2016; Integrative, H.M.P. 2019). The ability to engineer the composition and function of the host microbiome through additive (e.g. probiotics, faecal matter transfer), subtractive (e.g. antibiotics, phage therapy) and modulatory

(e.g. xenobiotics, prebiotics) approaches thus represent attractive strategies for novel therapeutic intervention (reviewed by Mimee et al. 2016; Sheth et al. 2016). Additive and subtractive approaches in particular tend themselves to metabolic engineering applications.

6.3.1 Additive Approaches

Additive approaches are based on augmenting the native microbiome with individually selected strains or collections of bacterial species. The gut microbiome is highly amenable to this type of intervention as it is readily accessible to the introduction of beneficial bacterial species in the form of ingested probiotics and faecal matter transfer (FMT). Gut microbiome replacement or augmentation by faecal transplantation is a method for the correction of dysbiosis that has shown success, particularly in the treatment of inflammatory bowel disorders, *C. difficile* infection and autism spectrum disorder (Kang et al. 2019; reviewed by Ooijevaar et al. 2019). However, this method does involve certain risks involved with the introduction of bacterial species from an allogeneic donor, which have resulted in the death of immunocompromised individuals as a result of pathogenic colonisation of the gut (Wang et al. 2016; Ooijevaar et al. 2019). A more targeted approach to microbiome engineering involves the addition of beneficial microbial species that have been engineered to sense and respond to specific environmental cues. This strategy in particular holds great promise for the development of next-generation therapeutics. Examples of engineered commensal bacterial strains (engineered probiotics) that may be utilised to sense, correct or combat specific human pathologies are summarised in Table 6.1. While this list is not exhaustive, it aims is to highlight the potential range of applications for additive microbiome engineering based on synthetic systems. These vary from biosensors that can actively monitor risk of cancer (Danino et al. 2015) and prevent high blood pressure (Jeffrey et al. 2016); to effectors that address pathogenic challenge (Hwang et al. 2017), cancers (Ho et al. 2018), nutrient deficiencies (Wassef et al. 2014); metabolic disorders (Chen et al. 2014; Durrer et al. 2017; Kurtz et al. 2019) and those that modulate the host inflammatory response and associated disorders (Mohamadzadeh et al. 2011). Notably, the stable colonisation of the human gut by exogenous bacterial species introduced through additive approaches remains challenging as the host microenvironment may not support the long-lived occupancy of these modified bacterial species. Therefore, more natural in situ methods that are able to modulate microbial populations may be less intrusive to the natural dynamics of microbiome populations, allowing for the safer and more stable augmentation of microbial populations within the host (Table 6.1).

Table 6.1 Engineered probiotics

Primary metabolic modification(s)	Chassis	Application	References
Phenylalanine lyase insertion	*Lactobacillus reuteri*	Enhanced conversion of dietary phenylalanine to tyrosine; phenylketonuria (PKU) treatment	Durrer et al. (2017)
Arginine repressor deletion (ΔArgR); argA215 insertion	*E. coli* Nissle 1917	Enhanced arginine biosynthesis to reduce systemic NH3: Treatment of hyperammonaemia disorders (*Phase I clinical trial success*)	Kurtz et al. (2019)
Insertion of *aeruginosa*-specific S5 pyocin and anti-biofilm enzyme, DspB	*E. coli* Nissle 1917	Detection and elimination of pathogenic *Pseudomonas aeruginosa* in vitro	Hwang et al. (2017)
Insertion of angiotensin-(1–7)	*Lactobacillus paracasei*	Prevention of the progression of pulmonary hypertension	Jeffrey et al. (2016)
Insertion of *N*-acyltransferase	*E. coli* Nissle 1917	Reduced obesity in response to high-fat diet	Chen et al. (2014)
Insertion of 4 enzymes from β-carotene biosynthetic pathway	*E. coli* MG1655*	Probiotic delivery of vitamin A to host tissues	Wassef et al. (2014)
Insertion of tryptophan decarboxylase	*Bacteroides thetaiotaomicron*	Targeted relief of constipation through tryptamine-induced fluid secretion in colon	Bhattarai et al. (2018)
Deletion of phosphoglycerol transferase	*Lactobacillus acidophilus*	Treatment of inflammatory intestinal disorders through enhanced IL-10 production	Mohamadzadeh et al. (2011)
Insertion of luxCDABE (genomic), IPTG-inducible lacZ (stabilised plasmid)	*E. coli* Nissle 1917	Probiotic biosensor for detection of hepatic cancers	Danino et al. (2015)
Insertion of INP–HlpA and YebF-I1	*E. coli* Nissle 1917	Prevention of carcinogenesis by targeted conversion of ingested metabolites from broccoli into anti-cancer compounds in colorectal cancer cells	Ho et al. (2018)

6.3.2 Subtractive Approaches

Subtractive therapies seek to eliminate bacterial species in the microbiome associated with disease and poor clinical outcomes. In contrast to classical subtractive approaches like antibiotic treatment, bacteriophage (phage)-mediated therapies represent an exciting strategy for microbiome engineering which has experienced

renewed interest in the wake of increased antimicrobial resistance to antibiotics (Cooper et al. 2016). As the natural predators of bacteria, phages are an integral component of the microbiome that shape bacterial populations through their bacteriolytic function (Hsu et al. 2018). Importantly phages usually only target a single or small subset of bacterial species thus providing greater specificity than broader spectrum antibiotics which also increase the risk of adverse side effects like opportunistic *C. difficile* infection. Similarly to bacterial vectors, phages are also amenable to a certain degree of genetic manipulation, thus allowing for the rational design of phages with augmented function and properties (reviewed by Pires et al. 2016). The insertion of effector molecules targeting bacterial defence mechanisms like quorum sensing, outer membranes and biofilms can be utilised to improve killing potential (Pires et al. 2016). Likewise host tropism can be modified to redirect lytic activity towards desired bacterial strains and the surface domains on phage capsids can be further modified to confine phage activity to discrete sites within the host (Barr et al. 2013). Indeed a cocktail of genetically engineered phages was recently administered intravenously to successfully treat a 15-year-old cystic fibrosis patient infected with *M. abscessus* (Dedrick et al. 2019). Notably, lysogenic phage cycles may also be utilised to specifically modify the genomic content of bacterial host cells through the integration of phage DNA into that of the host (Pires et al. 2016). In the absence of lytic activity, this approach provides the possibility for large-scale and stable in situ modifications to the host microbiome, including the introduction of complex biological circuits like those utilised in engineered probiotics. As with engineered probiotic approaches however, the application of engineered phage therapies to treat diverse human pathologies is highly dependent on and currently limited by the availability of suitable synthetic biology tools and concerns related to the safety of such systems.

6.3.3 Sourcing Synthetic Biology Tools for Microbiome Engineering

Genetic engineering to expand the functional repertoire of the human microbiota requires the development of highly characterised genetic parts from which complex programmable circuits can be constructed using principles of abstraction (Endy 2005; Andrianantoandro et al. 2006). Importantly, these engineered microbes should be able to sense their environment and produce a calibrated effector response through the coupling of stimulus-dependent genetic regulatory elements to effector modules in the circuitry. While the catalogue of effector parts is relatively large, the availability of well-characterised inducible elements that would regulate the activation of effector modules in a stimulus-dependent manner in situ remains quite poor. Essentially, the lack of genetic 'switches' has been a large bottleneck in the development and application of engineered microbes as the activation of their genetic programming can often only be activated by synthetic molecules such as IPTG in proof of concept demonstrations.

Microbes have persisted through the arc of evolution and as a result gained immense genetic diversity and function (Smanski et al. 2016). The microbiome is therefore potentially replete with regulatory elements that respond to a wide range of host-specific stimuli. However, the identification of these elements remains challenging. Perhaps the most obvious way to mine the microbiome for these regulatory modules is through the use of high-throughput RNA-Seq to identify genes that are switched on under certain stimulatory conditions, from which promoter regions can then be inferred. Alternatively, screening strategies for regulatory elements inspired by those developed in mammalian systems, such as STARR-Seq (Arnold et al. 2013), could be adapted for genome-wide screens for stimuli-specific inducible elements that include promoters, enhancers, and riboswitches. In addition to this, the use of combinatorial selection strategies could streamline the screening process and quickly identify inducible regulatory genomic elements. It is however important to note that the retrieved candidate sequences might have species-specific function (Mutalik et al. 2013). We would therefore recommend that the screen be performed in the microbial chassis intended for genetic functionalisation from the beginning or the sequence be functionally validated if used orthogonally. By broadening the library of inducible regulatory elements, genetic circuits can be designed to be responsive to various stimuli, allowing for the human microbiota to be engineered to carry out expanded functions in numerous contexts, ranging from the detection of disease biomarkers to the metabolism of nutrients as self-regulatory genetically engineered machines.

For the construction of such biological systems, abstraction is a key design principle that is necessary for the stratification of a system into layers of reduced complexity (Endy 2005; Andrianantoandro et al. 2006). Integral to this is the characterisation and tuning of individual parts, such that their function is predictable and complementary in the entire system. The use of naturally occurring genetic parts (as discovered by functional screens) may not be ideal in bespoke synthetic genetic circuits that are designed for alternative purposes (Mutalik et al. 2013). For example a disease-specific inducible promoter element may not display sufficiently fast transcription kinetics to elicit a binary response or the response itself may be too weak to be effective. Similarly, effector proteins may display insufficient fidelity or processivity that is necessary for efficacious activity. Therefore, the ability to tune the activity of these parts is essential.

Directed evolution has emerged as a powerful tool to re-engineer biological parts for customised functional characteristics (Arnold 1998; Turner 2009; Beaudry and Joyce 1992). Perhaps the most crude method of this technique is to generate libraries of variants, which subsequently have to be individually tested for desired function. Such diversification methods classically involved the production of a boutique collection of mutants by site-directed mutagenesis. This method is labour-intensive and severely limited in creating a variant with enhanced function. Subsequently, large-scale mutagenesis strategies have been developed to produce libraries of greater diversity and coverage, through the use of error-prone PCR (Copp et al. 2014) and more recently, CRISPR/Cas9 base editors (Hess et al. 2016). These libraries are then functionally screened to retrieve variants that display the desired

characteristics of the part, which can then undergo further rounds of mutagenesis and screening to fine tune its activity. While undoubtedly very powerful, this method for directed evolution remains cumbersome and requires multiple rounds of diversification and screening.

Phage-assisted continuous evolution (PACE) is an advancement of directed evolution that makes use of the highly mutagenic replication process of the M13 bacteriophage (Esvelt et al. 2011). Numerous rounds of evolution can occur in a short space of time without human intervention, due to the fast life cycle of the M13 bacteriophage. PACE can integrate the diversification process with the screening assay in a single culture system, resulting in the rapid and continuous evolution of genetic parts. Conventionally, PACE achieves continuous selection by linking the evolution of a protein function to the expression of phage protein III, which strongly mediates phage infection. Using this strategy, Esvelt et al. (2011) was able to evolve the activity of the T7 RNA polymerase such that its enhanced activity conferred the increased ability of the phage to infect bacteria and propagate through enhanced phage protein III expression. We envision that the next generation of PACE will be adapted to decouple the continuous selection from the expression of phage protein III, allowing for broader selection strategies to be implemented. For example promoter elements can be evolved by linking their activity to the expression of a bacterial selection marker, allowing for the retrieval of variants with very specific characteristics, such as discrete expression kinetic curves and dose responses, by altering the selection conditions. This could be a very powerful method for tuning the parts for the construction of genetic circuits and the production of engineered microbes.

6.3.4 Biocontainment and Safety

The application of engineered microbes for human health has remained controversial due to the potential risks associated with the ingestion of living genetically modified organisms. It is therefore highly important to include biocontainment measures in the design of the genetic circuits to control the viability of synthetically modified microbial populations. Ideally, bacterial biosensors should constantly be present for the effective surveillance of disease biomarkers. In such cases, population limiting kill switches can be engineered to synchronise with the density of the bacterial population by coupling quorum sensing modules to the expression of bactericidal molecules. When the population reaches a threshold, the kill switch will activate and result in the elimination of most of the population, but allow for a founder population to endure for the next generation (Din et al. 2016). Conditional replication strategies, adapted from therapeutic lentiviral vectors, could also be adopted to regulate engineered microbial populations (Turner et al. 2009). With this method, the engineered microbe is maintained at a relatively low number, but rapid cell division is induced in the presence of a stimulus (such as a disease biomarker), essentially creating an amplifier for the engineered function. With the loss of this stimulus, the engineered microbial species will return to its lowly

abundant basal state over time. In addition to engineering in these population control measures, it is still necessary to include a master kill switch into any synthetically modified organism to acutely control its viability. Ideally, such switches should be integrated into highly conserved or essential genomic regions within the bacterial genome to ensure stability.

6.4 Current and Future Industrial Applications of Metabolic Engineering

Currently, several industrial applications of metabolic engineered microbes have been used in the biotechnology field. In the current era of genomics and synthetic biology in particular, several teams are engineering microbes both at the level of whole genomes and at the levels of more simplistic metabolic engineering for laboratory and industrial applications. The most commonly used model microbes for metabolic engineering are the bacterium *E. coli* and yeast. In addition several research groups are also working on modifying commensal microbes such as *C. glutamicum*, *P. pastoris*, *P. putida*, *Z. mobilis*, *Y. lipolytica*, *B. subtilis*, *cyanobacteria species*, *B. subtilis*, *Streptomyces* sp. and *Clostridium* sp. for industrial applications. In this book chapter, we have focused on two examples as case studies for the industrial application of metabolically engineered microbes; however, there are several other instances where research groups have modulated bacteria and yeast metabolism using metabolic engineering approaches.

(a) *Metabolic engineering of Bacillus subtilis*: *B. subtilis* is a well-characterised gram-positive bacteria which has been identified as an effective vector for recombinant protein production. By using current methods of systems biology and synthetic biology technologies, a thorough analysis of *B. subtilis* productive capacities and the subsequent optimisation thereof through metabolic engineering has been attempted by several research groups (Atalla and Schumann 2003). Here, we elaborate the recent strategies for high-performance capacity and applications of *B. subtilis* using synthetic biology toolsets. In addition, we propose potential opportunities and appropriate strategies for the progress of *B. subtilis* as an effective microbial cell factory. Key advantages of using *B. subtilis* are its superior properties that are essential for industrial applications. *B. subtilis* has been developed as an efficient expression system and is a major workhorse for recombinant protein production. Developing cell factories requires specific advantages such as native cellular features required for industrial application, well-established bioengineering strategies, state-of-the-art synthetic biology technologies. However, strain developments of *B. subtilis* for industrial application require specific strategies that can improve the use of *B. subtilis*. Firstly, bioinformatic characterisation of *B. subtilis* which includes transcriptomics, proteomic and metabolomic analyses needs to be well developed. Although, several publications on *B. subtilis*-fed batch culture conditions have been reported, most of these datasets are based on small-scale culture

conditions which cannot be used as a route map for large-scale industrial applications. Hence, it is essential to investigate the relevant cellular responses and circuits under industrial conditions using omics technologies. Secondly, systems and synthetic biology technologies for *B. subtilis* are underdeveloped and limited in effectiveness compared to those available for *E. coli*-based genome-editing strategies. A key example of this is the current challenges faced in applying CRISPR-Cas9 technology to the multiplexed, automated genetic modification of *B. subtilis* (Westbrook et al. 2016). Thirdly, *B. subtilis* is well documented for having a high capacity for native protease secretion, yet the effective production of heterologous enzyme by *B. subtilis* for industrial applications remains challenging, hence the optimisation of the *B. subtilis* cell factory chassis for efficient industrial applications. Lastly, *B. subtilis* also has a number of adverse properties such as foam production, sporulation and high maintenance that restrict its industrial application. Hence, there is a necessity to construct versions of the *B. subtilis* chassis which exclude these unwanted properties. We hope that this article will further enable bioengineers to more effectively utilise synthetic biology strategies for the optimisation of *B. subtilis* cell factories.

(b) *Metabolic engineering of synthetic yeast*: One of the most widely used cell factories for metabolic engineering is the yeast, *Saccharomyces cerevisiae*. *S. cerevisiae* has been bioengineered to produce products with high production capacities. Particularly with the recent development of biosynthetic pathway optimisation and synthetic biology tools (CRISPR) in order to improve and advance metabolic engineering in yeast. Whole genome engineering that can effectively rewire all ~6000 genes in *S. cerevisiae* would provide a new route map for the yeast metabolic engineering. Despite the fact that CRISPR knockout-based genome-scale screening (Gilbert et al. 2014; Konermann et al. 2015) has been reported in yeast, its impact on metabolic engineering and industrial applications has not been fully realised. In yeast-based genome engineering, efficient genome editing can be achieved only by the combination of endogenous HDR and CRISPR-cas9-based editing.

Most instances of metabolic engineering in yeast are mainly limited to examples like biosensors, GPCRs, riboswitches, and promoters that can be integrated into a genome-driven metabolic engineering pipeline. The combination of automated workflows with a microfluidic platform represents an effective solution for the high-throughput screening of the desired phenotypes in engineered systems. Another recent development in metabolic engineering of yeast is the development of yeast cell factories based on other species and not just *Saccharomyces cerevisiae*. This approach has been effective in producing some products such as fatty acid-derived compounds and organic acids at low pH. Using synthetic biology tools that are designed for other types of yeast such as *P. stiptis* (Cao et al. 2017) and *Y. lipolytica* (Schwartz et al. 2016) has shown promise as replacement to *S. cerevisiae* for synthetic biology applications.

6.5 Conclusion

Synthetic biology seeks to optimise the engineering process of biological systems through the characterisation and design of novel genetic parts and improvement of the methods and tools that support the engineering process. This also includes ensuring that engineered biological systems conform to specific technical and safety standards. This will allow biological engineers to assemble and improve the production of engineered organisms at a faster pace. In this review, we have discussed some of the recent advances and applications of synthetic biology in metabolic engineering. Despite progress in the past decade, there has been varied success in metabolic engineering efforts for the industrial application of microbial cell factories. This is mainly due to the innate complexity of metabolic and regulatory networks within microbes. Despite the fact that several microbial species have been well studied with regard to their industrial applications, a more complete understanding of systemic metabolic regulatory networks, even within these microbes, is still lacking. There is thus a clear need to apply genome-scale metabolic engineering approaches to fully understand the regulatory networks of these industrially relevant microbes. Another recent advancement in metabolic engineering has come from the application of mathematical modelling strategies to extract novel biological information from complex experimental datasets. Particularly in this era of OMICS biology, where it has become easier to produce large datasets with increased dimensionality, mathematical modelling is likely to play a larger role in the future of biological research. The main challenge for the broader application of mathematical modelling to improve our understanding of living biological systems however remains the reproducibility of phenotypes which are dependent on processes that are intrinsically complex, diverse and variable. Despite these challenges however, it is clear that the use of machine learning holds great potential for the optimisation of metabolic engineering strategies and the improved performance of engineered microbial systems (Heijnen and Verheijen 2013).

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Recent Developments in Synthetic Biology Toolbox

7

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Abstract

Synthetic biology is a new and evolving branch of science, which has extensive application in the surrounding as well as human life. This field deals with the knowledge gain from the living system by the means of bioinformatics or other relevant field and try to regulate or restructure the pathways and the system of the higher organism in much simpler microorganism. It actually translates the knowledge gain from an organism or system in simpler system for the benefit of the nature. Evolution in the field of biotechnology, easy fast and high-throughput and accurate technologies available for DNA sequencing, and synthesis, has made it very easy to design and structure a specific pathway, which is useful in a particular organism in a model organism, which in field of synthetic biology is termed as chassis. Synthetic biology brings together different areas such as engineering, molecular biology, cell biology, biotechnology, bioinformatics, and system biology in such a way that they all together forms this new area with vast application in various fields. Synthetic biotechnology has its application in almost all fields such as from cellular programming to drug designing to biofuels production. In this chapter, we will be mainly dealing with the application part of the synthetic biotechnology in development of biosensors, for new drug discovery, tools for application in agriculture, for secondary metabolism, for chemical production, and for biofuel production.

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7.1 Introduction

Synthetic biology is an emerging field which deals with the regulation, synthesis as well as reprogramming of genes as well as various cellular metabolic pathways either in the same host or manipulating and constructing new pathways in heterologous host. However, sometimes it also represents and construct new genetic circuits, or enzymes even new biological entities. SB brings together different areas such as engineering, molecular biology, cell biology, biotechnology, bioinformatics, and system biology in such a way that they all together forms this new area with vast application in various fields. The basic difference between SB and molecular biology along with cell biology is that it relies more specifically on understating and focusing on the small components or important steps in metabolic/cellular pathways that can be synthesized, modeled as well as can be used for the assembly of whole pathway with desired control over new synthesized pathway. SB also assembles small parts (of biological origin and system) and devices (engineering side) into a larger system for solving a specific problem. It is an application-driven field endeavoring to put on a cogent engineering methodology to restructure the biological systems, for producing treasured and innovative biological functions. It can be assumed as a natural evolution of biotechnology as opposed to being a separate field although it does offer a novel and exciting approach. As a comparatively new field, it sits at the intersection of engineering principles and biological design. The overall aim of the synthetic biology is to gain a better understanding of coordinating and regulating the genes as well as pathways in living cells (Andrianantoandro et al. 2006). For expression of a gene, various elements such as promoter, transcription factor binding sites, and terminators are necessary and important. However, if expression of a gene is to be done in a heterologous host or just the regulation of gene in the same host is required, then a different component or element that is vector also comes in the picture, which is also very important. In particular, the engineering of synthetic systems involves practical and rationale design of disparate synthetic parts that all must work together to obtain the desired function.

Synthetic biology has two main approaches "top-down" and "bottom-up." In the top-down approach, an existing functional cell or organism sometimes referred as biology is modified or reengineered, while in the second bottom-up approach, synthesis of functioning circuits and pathways occurs with the help of DNA and other biomolecules. The second approach starts from the scratch and ends in the final synthesis of a complex pathway or gene cluster. Top-down approach was predominated in the early stages of this field, which allowed scientists to test designed biological circuits in a "chassis." Here in SB, the term chassis is used for a cell or organism (functioning and self-replicating) in which the engineered DNA or biopart is entrenched for producing the desired device or system, e.g., *E. coli*. Utmost top-down biology experiments can be categorized into two ways. The first

one is by combining short elements of a system from various organism in a single cell or organism (modified or engineered organism) to perform a desired task, while in the other way the complex system of an organism or cell is simplified (Fig. 7.1).

Latest progresses in SB have provided various tools for specific regulation of expression of gene of interest, by synthetically engineering promoters for specific genes along with posttranscriptional modification and either degradation or stabilizing the product (Lee et al. 2016). Synthetic biotechnology has its application in almost all fields such as from cellular programming to drug designing to biofuel production. In this chapter, we will be mainly dealing with the application part of the synthetic biotechnology toolbox in the development of SB tool for biosensors, for new drug discovery, tools for application in agriculture, for secondary metabolism, for chemical production, and for biofuel production.

7.2 Development of Synthetic Biology Tool for Biomonitoring/Biosensors

The term "biosensor" or biomonitors denotes a wide variety of devices with the biological common component providing recognition of a certain highly specific target analyte, and converting this detection in an easily detectable, quantifiable response which can be fed to an electronic device for signal processing, data storage, etc.

7.2.1 Types of Biosensors

The biological component in many biosensors is either an enzyme, antibody, or whole cell. Based on various biological components, biosensors can be categorized into various classes such as whole-cell biosensor, transcription factor-based biosensors, nucleic acid-based biosensors, and transcription-independent protein-based biosensors (TIPBS). These biosensors on the basis of their construction and organization methods can be further subdivided, which will be covered in the following section.

7.2.1.1 Whole-Cell Biosensor (WCB)

Whole-cell biosensor (WCB) sometimes also referred as bioreporter (Belkin 2003) in which living cell is used as a biological component. These cells have the ability to detect the target analyte via receptors and are able to generate a detectable response, most commonly by inducing expression of a reporter gene. This device uses the whole prokaryotic or eukaryotic cell (experimentally modified for increased transducer capacity or sensitivity toward analyte) as a single reporter, integrating both bio-receptor and transducer elements into the same cell.

If we are using WCB, we can perform two types of bioassays, i.e., turn off and turn on assays (Belkin 2003). In turn off assays, the sample toxicity can be estimated from the degree of inhibition of a cellular activity, or a decreased expression of specific reporter gene. In the second assay that is turn on assay, a promoter of a

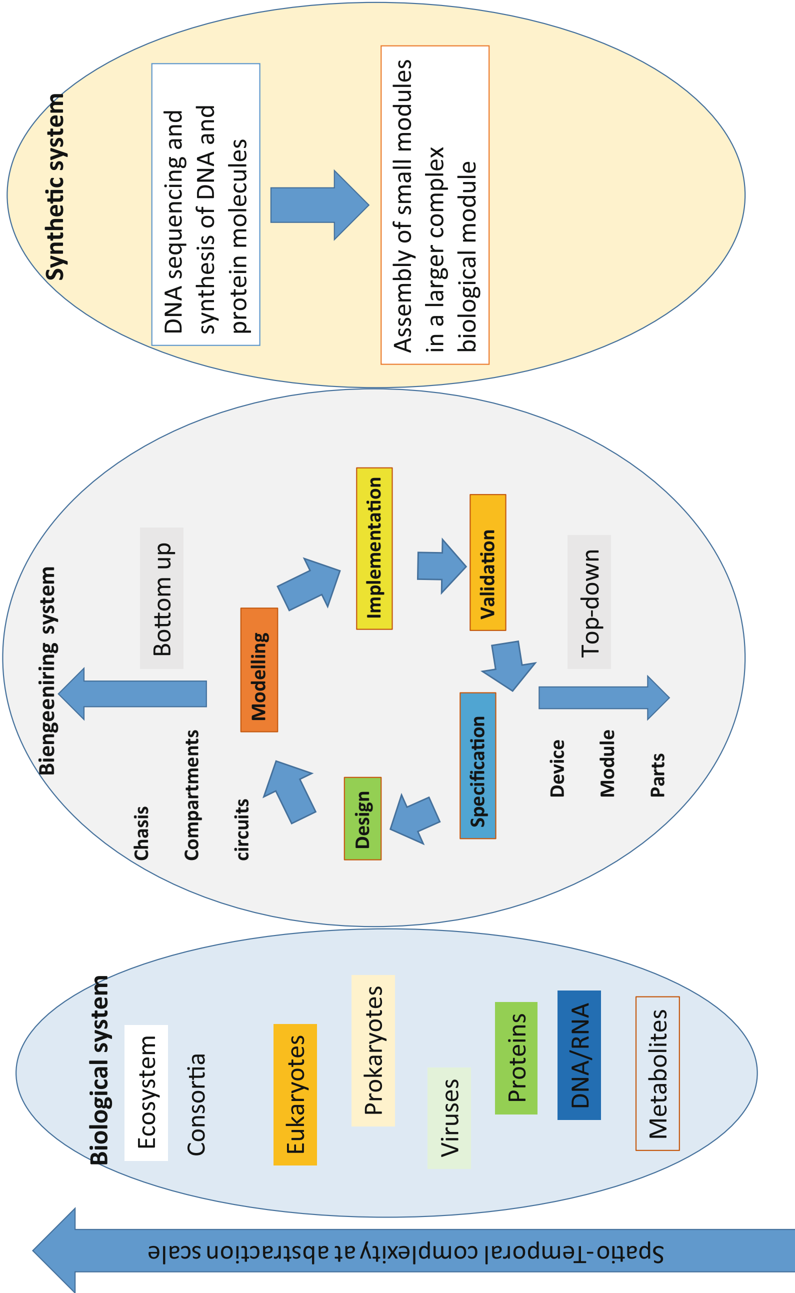

Fig. 7.1 Different component of synthetic biology showing top-down and bottom-up approach (Ph.D. Thesis: Henary Poicare)

specific gene (which is specific to a given pollutant or target analyte) is fused with the reporter gene. So in this assay the degree of expression of reporter gene is directly proportional to the presence of analyte or pollutant. By comparing both of these assays, we can say that turn off assays are more nonspecific because the signal decreases as a result of a broad range of cytotoxic effects, while turn on assays, based on an inducible gene expression, are usually more specific as induction of the gene reporter only takes place when the pollutant is present. Their specificity will therefore depend on the degree of the gene promoter specificity to be opened by an exclusive pollutant of a chemically related group of pollutants. With respect to specificity, WCBs can be divided into effect- and compound-specific sensors (Yagi 2007). Changes in a physicochemical condition (e.g., pH, temperature, or osmotic changes) or pollutants that give rise to a specific type of toxicity (e.g., oxidative stress or protein damage) stimulate effect-specific biosensors. Compound-specific biosensors respond to only one type of pollutant or compounds with similar chemical features (e.g., any heavy metal).

There are two types of biosensors based on their expression level. One with constitutive expression system, while the other one is with inducible system. A constitutive system comprises a promoter of a specific gene which has higher expression under normal condition, which results in a high basal expression level of the reporter gene. During exposure to the signal (target analyte), the basal expression level of reporter gene is decreased, so the reduction in expression of reporter gene is inversely proportional to the concentration of analyte. In second type of biosensor, inducible biosensors inducible promoter is fused with the reporter gene. So when there is an analyte, its presence will induce the expression of the reporter gene. For such type of biosensors, it is essential to have promoter which is specific for the analyte which needs a detailed study of the promoters.

7.2.1.2 Transcription Factor-Based Biosensors

Transcription factor-based biosensors (TFBs) respond to the ligand by utilizing the transcriptional and translational machinery of cell for the generation of output signals. The ligand binding to the biosensors causes changes in confirmation of the TFBs thus disturbing its binding with the promoters of the gene and finally the expression of specific gene is converted into the output signal. These TFBs further subdivided into the following groups.

Native Transcription Factor-Based Biosensors

If the transcription factors, which are native to their origin and not synthetically designed, are used for the development of TFBs, then this system is referred as native transcription factor-based biosensors. These are easy to construct and therefore widely used in the field of synthetic biology for para-hydroxybenzoic acid (Williams et al. 2017), NADP/NADPH (Siedler et al. 2014), muconic acid (Leavitt et al. 2017), fatty acids (Zhang et al. 2012), etc.

Heterologous Species Transcription Factor-Based Biosensors

If the host does not have the specific transcription factor for the specific target ligand, the transcription factor of different species can be used for the generation of TFBS.

However, these systems are referred as heterologous species transcription factor-based biosensors. For example, Dietrich et al. (2013) developed a biosensor for butanol which could be used in *E. coli*. This device uses a sigma factor (BMOR) and promoter sequence (PMBO) of *Thauera butanivorans* (Dietrich et al. 2013) and used tetracyclin-resistant-GFp fusion gene (TETA-GFP). PBMO, the promoter sequence, was placed at the 5 prime end of tetracyclin-resistant-GFp fusion gene, while BMOR gene was expressed under its own promoter (PBMOR promoter) (Dietrich et al. 2013). When there is butanol, it binds with BMOR and this ligand gene product (butanol-BMOR) causes activation of PMBOR, and it will further result in increased expression of TETA-GFP. When there is limited supply of butanol, there will be limited expression of GFP fusion protein in comparison to the condition when there is plenty of supply of butanol resulting in excessive expression of GFP fusion protein.

Modular Transcription Factor-Based Biosensors

If we are utilizing modular, transcription factors for the generation of TFBS, these devices will be named as modular transcription factor-based biosensors. These are a little bit tricky in engineering comparing to the TFBS discussed above and are more versatile. In this case, we have to take care of the various domains of the protein which must bind to ligand as well as induce transcription of specific gene. The domains either can be fused with transcriptional activation domains and DNA-binding domains or can be expressed. In the absence of the ligand, these domains are disconnected resulting in lesser or limited transcription of the output gene. However in a condition when the ligand is present, the domains are presented at the promoter resulting in increased expression of the target gene (Carpenter et al. 2018).

This approach was utilized by Chou and Keasling (2013) to engineer isopentenyl-pyrophosphate (IPP) biosensors for use in *E. coli* and *S. cerevisiae*. In this biosensor, the araBAD promoter (PBAD) and its native transcriptional regulator Ara C were used in *E. coli*. In its native perspective, when arabinose is present, it binds with AraC, resulting in a conformational change that results in its association with PBAD and induction of transcription of the gene. However, in case of the abovementioned biosensor, the DNA-binding domain of AraC is joined via a linker to a known IPP-binding domain that is IPP isomerase (Idi). Due to crystallographic data, it was assumed that in the presence of IPP, dimerization of IPP-binding domain occurs. In a condition when there is low concentration of IPP, the AraC DBD-Idi fusion protein is free to bind to PBAD resulting in the expression of mCherry-encoding reporter gene. However, in a different scenario when the concentration of IPP is higher, it causes dimerization of Idi and thus blocking the ability of the AraC DBD from inducing transcription from PBAD, and reducing the expression of mCherry.

7.2.1.3 Nucleic Acid-Based Biosensors

This is a different class of biosensors, which utilizes the nucleic acids (DNA/RNA) as a functional unit or compartment of the biosensors. These nucleic acid sequences have binding affinity toward target ligand. The binding of the target ligand to the

nucleic acid sequence (DNA/RNA) alters its structure and conformation which is utilized by various mechanisms to generate an output signal (Carpenter et al. 2018).

Aptamer-Based Biosensors

Aptamers are single-stranded DNA or RNA molecules having affinity toward target ligand (Tuerk and Gold 1990). In general, the binding of the aptamer to the target ligand brings a conformational change in the aptamer. In some of the cases the conformational change results in secondary structure change which could be either from a linear to stem-looped structure, or from a hair-pin to ligand-coordinated structure. The sutural changes in the aptamers upon binding with the ligands are used to generate the signals.

In one example, McKeague et al. (2014) used an DNA aptamer for Ochratoxin A (OTA) for the generation of aptamer-based biosensor. This DNA aptamer has a predominantly stem-looped structure which is modified via ligand binding (Song et al. 2008). In their biosensor, the approach that they had followed depends on the signals that are generated by binding of DNA to the syber green. In a condition when there was no OTA, the aptamer will remain in stem loop structure thus having various sites for the binding of the syber green generating a high signal in the form of fluorescence. However, in presence of OTA, there is conformational change in the aptamers resulting in lesser sites available for syber green binding thus finally reducing the fluorescence.

Riboswitch-Based Biosensors

Riboswitch is a regulatory segment of a messenger RNA molecule that in the presence of specific small molecule or ligand results in a change in production of the proteins that are encoded by the mRNA. These are composed of two domains, one which has binding site with the ligand while the second domain is a response domain which is used for the generation of signals after binding to ligand (Findeiss et al. 2017). In the presence of ligand, which binds with the binding domain of riboswitch, conformational or structural changes in the domain is induced which further result in the change in response domain resulting in induction of signals. Most of the time, the response domain is a messenger RNA (mRNA) transcript, and the change in structure leads to either activation or reduction of gene expression/ translation (Findeiss et al. 2017).

Muranaka et al. (2009) developed thiamine pyrophosphate (TPP) riboswitch developed for *E. coli*. In this particular case, the ligand binding domain (TPP-binding domain) is located at 25 bp upstream of the RBS of tetracycline resistance (TETA) gene. After transcription, the TPP-binding domain folds back onto the RBS and blocks access of the ribosome, preventing translation of TETA. However, in the presence of TPP, the TPP-binding domain binds with the TPP making RBS free for the access of ribosome thus resulting in transcription and translation of TETA, and the *E. coli* containing this biosensor are allowed to grow in the presence of tetracycline.

7.2.1.4 Transcription-Independent Protein-Based Biosensors (TIPBs)

This class of biosensors are very wide in terms of their working, i.e., how they detect an analyte and how they produce the output signals. Main components of a type of biosensors include both receptor and response domains. The analyte is received by the receptor domain, and the signal is produced and transmitted by the response domain (Carpenter et al. 2018).

Integrated Transcription-Independent Protein-Based Biosensors

These are the biosensors in which receptor domain/s, which has binding site/capacity to specific ligand, are expressed as a fusion protein with either little or no linker domain to a response domain. In these biosensors when ligand binds to the receptor domain, it causes conformational change in the respective domain which is directly transmitted to the response domain. Then response domain leads to the induction of signals (Stein and Alexandrov 2015).

Nagai et al. (2001) had developed integrated TIBP-based biosensor for calcium utilizing expression of GFP-fused calmodulin. In this biosensor, GFP was expressed with both calmodulin and M13 domains which has binding sites for the calcium. In the absence of calcium, the GFP is not correctly folded due to the disturbance resulting from the calmodulin and M13 domain. However, in the presence of calcium, both of these domains M13 and calmodulin bind with the calcium thus allowing the correct folding of GFP which results in the production of signals in the form of fluorescence.

Semi-Modular Transcription-Independent Protein-Based Biosensors

These are the biosensors in which there is a linker present in between the receiver and response domain. In these types of biosensors, the conformational changes in the receiver domain after binding with the ligand is transmitted to the response domain through linker resulting in the induction of signal.

7.2.2 Applications of Biosensors

Biosensors are a major application of synthetic biology. They have their application in human heath as well as they can also be used for the detection of any type of heavy metals or pollutant in any type of the sample, which could be either water or soil. Their ability to detect specific and accurately a wide range of molecules makes them highly relevant to a choice of industrial, ecological, medical, and scientific applications.

7.2.2.1 Biosensors for Detecting Heavy Metals Pollutant from Various Samples

Heavy metals are those metals which possess specific gravity either five or five times more than that of water. These are among the most abundant, toxic, and persistent inorganic environmental pollutants (Hill 2004). Human activities such as mining and other industrial anthropogenic activities are responsible for increasing the heavy

metal pollution in the environment. Heavy metals cause their toxicity by increasing reactive oxygen species production. Further, these species (ROS) produced are responsible for significant alterations in nucleic acids, proteins, and lipids (Leonard et al. 2003) which ultimately can result in cell death by necrosis or apoptosis.

Wan et al. (2019) engineered and optimized ultrasensitive *E. coli*-based biosensors for detecting arsenic and mercury contamination in water. This group had developed a modular cascaded signal amplifying methodology and combined this methodology with basal background tuning approaches. By this approach, they were able to improve the sensitivity and output dynamic range of cell-based sensors. The camera of cell phone could also capture the output signals that are produced by the biosensors. This feature made this biosensor portable, handy, and it also decreased the cost of the biosensor.

Biosensors for Diagnostic and Detection Devices

In many Gram-negative bacteria, cell-to-cell communication system (quorum sensing) is maintained or established by acyl-homoserine lactone (acyl-HSL) and it results in biofilm production. Research in acyl-HSL-based quorum sensing is achieved by simple methods devised to detect AHLs with the help of bacterial biosensors that recognize exogenously produced AHLs and produce output signals (Steindler and Venturi 2007). For example, Wen et al. (2017) designed a modular DNA-encoded biosensor in cell-free protein expression systems that can be used to detect a bacterial biomarker of *Pseudomonas aeruginosa* infection from samples of cystic fibrosis patient. This pathogen produces two types of the biomolecules (AHLs), *N*-butyryl-homoserine lactone (C4-HSL), and *N*-3-oxo-dodecanoyl-homoserine lactone (3OC12-HSL). With the help of this specific biosensor, *Pseudomonas aeruginosa*-specific QS molecules can be detected in patient samples (sputum, urine and blood).

7.3 Development and Application of Synthetic Biology Tool in Agriculture

The application of synthetic biology in the field of the agriculture has some serious challenges. These challenges include genome size of the plants (wheat, a hexaploid >15 Gb genome), ploidy level of the crop, propagation, transformation of plants that is still very difficult. CRISPR/Cas9-mediated gene editing (Puchta 2016) has boosted the field of plant biotechnology. It is also important for primary industries dealing with genetic manipulation of food crop to take prior permission from the government; as well, they should also aware of consumer attitudes (Goold et al. 2016).

Improvement of the nitrogen fixation in the field by the plants can be achieved by various applications of synthetic biology (Rogers and Oldroyd 2014). While plants are unable to fix atmospheric nitrogen. However, the microbes are capable of fixing atmospheric nitrogen, particularly rhizobia in legumes. By engineering the plant microbiome, the nitrogen fixation in crop-associated microbial species can be

improved (Rogers and Oldroyd 2014). Similarly, considerable efforts are underway to introduce direct nitrogen fixation into higher plants (Allen et al. 2017) and to introduce novel symbiotic associations with nitrogen-fixing bacteria (Rogers and Oldroyd 2014). Photosynthesis is a very unique feature of the plants that also drives agriculture and has numerous opportunities for plant synthetic biology. RubisCo which is the key enzyme (responsible for CO_2 fixation) of the photosynthesis is inherently inefficient, with a theoretical maximum efficiency of ~11% but typically not exceeding a few percent (Ort et al. 2015). However, the introduction of carboxysomes of cyanobacteria into the chloroplast could potentially overcome the inherent suboptimal activity of RuBisCO (Giessen and Silver 2017). Thus the capacity and efficiency for fixation of atmospheric CO_2 can be achieved by synthetic biology ultimately increasing the agricultural productivity.

On the other side, the nutritional value of the foods can also be stimulated such as development of carotenoid-enriched functional crops and oilseed crops with increased levels of omega-3 fatty acids. The carotenoid profile of tomato seeds has been shown to be modified by altering the plastids (D'Andrea et al. 2018). This study had shown that this pathway could be manipulated to breed fruit crops with designed carotenoid content. The synthetic control of plastid identity is an ambitious approach that has been proposed to develop new carotenoid-enriched crops (Llorente et al. 2017). The nutritional value and quality of seeds of canola-producing oils have been improved by metabolic engineering. *Arabidopsis thaliana* (a model plant) had been shown to enhance the quality of seed oil by using seven enzymes from five different organisms, the yeasts *Lanchancea kluyveri* and *Pichia pastoris* and the algae *Micromonas pusilla*, *Pyramimonas cordata*, and *Pavlova salina* (Petrie et al. 2012).

Land usage is also an increasing problem for agriculture, which also can also be solved by the application of synthetic biology in the same field. As population is increasing day by day, limiting the availability of land for agriculture. Along with the population industrialization and increasing pollution is also responsible for reducing the available land for the agriculture. Two potential strategies to address this issue are bioremediation using microbes, and engineering plants to grow in non-arable land. The first, bioremediation, is a technique in which the ecological system including microbes is modified to change the environment. Extension of this concept to rational engineering approaches pertinent to agriculture is also being undertaken by researchers to convert microbes such as *S. cerevisiae* and *E. coli* into potential bioremediation agents (Ravikumar et al. 2017). These are capable of bioremediation of heavy metal contamination, degradation of toxic aromatic compounds, and biomass-based sugars. However, in the second strategy the plant itself is genetically modified so that it can grow in unfavorable condition. This is possible by the sequencing of different cultivars as well as the organisms or the plants growing in the extreme condition. Reverse engineering of traits such as arsenic accumulation and tolerance gene of aquatic plant *Ceratophyllum demeresum* into crop plants such as rice had shown remediation in areas of the world affected by heavy metal stress, and potentially will aid in the regeneration of non-arable land (Shri et al. 2014). A comprehensive strategy to harness the potential of synthetic biology will deliver the next-generation of improved agricultural crops.

7.4 Development of Synthetic Biology Tools for Secondary Metabolites Production

Secondary metabolites are the intermediates of the primary metabolism of the cells. These molecules do not play very important role in the primary metabolism. However, these molecules possess various biological activities such as antimicrobial, antifungal, and antiviral activity. These molecules are also responsible for immune defense system of plants. Secondary plant metabolites are classified according to their chemical structures into several classes such as phenolics, alkaloids, terpenes, lipids, and saponins. Microbial synthesis overcomes many of the obstacles hindering traditional chemical synthesis and plant metabolic engineering, thus providing an alternative avenue for exploring plant specialized pathways. In the coming section, we are going to discuss synthetic biology tools for various secondary metabolites production.

7.4.1 Production of Lipids Via Synthetic Biology

Improvements in synthetic biology and enabling technologies like sequencing as well as synthesis of DNA and analytical techniques have speeded up the cycles for protein and metabolic engineering to that extent where these can be organized for the biosynthesis of a particular molecule.

Lipids extracted from plants have various applications such as the cocoa tree (*Theobroma cacao*), which is utilized for industrial purposes. The lipids extracted from this plant are the basic component of cocoa butter. The main composition of this triacylglycerol product is 1-palmitoyl-3-stearoyl-2-oleoylglycerol, 1,3-dipalmitoyl-2-oleoylglycerol, and 1,3-distearoyl-2-oleoylglycerol. Wei et al. (2018) cloned and expressed the biosynthesis gene of TAGs (glycerol-3-phosphate acyltransferase, lysophospholipid acyltransferase, and diacylglycerol acyl transferase) from *T. cacao* into *S. cerevisiae* through Gibson assembly. Table 7.1 represents few secondary metabolites that are synthetically produced in engineered host.

7.4.2 Alkaloids

Alkaloids are known to have various roles in human health, ranging from the relief from pain (opioids) to treatment of a disease like cancer (vinblastine and vincristine). Opioids belongs to an important class of medicines including morphine and codeine. Morphine is a pain killer, member of opioids synthesized naturally by various animals and plants. It actually works on central nervous system of patient and decreases the sensation of the pain. Synthetic biology can be helpful in the overproduction of such type of pharmaceutically important molecules as we have the sequence of the genes of morphine and other specific alkaloid molecule. If we are able to overproduce even a molecular intermediate of a metabolic pathway, it can change the flux of the system and have a capacity to mold the flux toward the higher

Table 7.1 Secondary metabolites produced in engineered host (Cravens et al. 2019)

Compound	Titer	Engineered host	Utility
Taxadiene	1 g/L	*E. coli*	Precursor of taxol (widely used as anticancer drug)
Strictosidine	0.5 g/L	*S. cerevisiae*	Monoterpene indole alkaloid PNP branch point
Reticuline	160 mg/L	*E. coli*	Alkaloid PNP branch point
Naringenin	110 mg/L	*E. coli*	Flavonoid PNP branch point
Amorphadiene	>40 mg/L	*S. cerevisiae*	Precursor to antimalarial drug (artemisinin)

Table 7.2 Engineered strain for biosynthesis of reticuline

Module	Name of module	Purpose for synthesizing modules/components of modules
Module 1	Precursor overproduction module	Increase accumulation of L-tyrosine and 4-hydroxyphenylacetaldehyde (4-HPAA)
Module 2	Tetrahydrobiopterin module	Expression of four proteins from *Rattus norvegicus*—(1) sepiapterin reductase, (2) 6-pyruvoyl tetrahydrobiopterin synthase, (3) quinonoid dihydropteridine reductase, and (4) pterin carbinolamine dehydratase
Module 3	(S)-norcoclaurine module	Expression of four proteins: (1) mutant of tyrosine hydroxylase; (2) catecholamines and the dihydrofolate reductase both from *R. norvegicus*; (3) DOPA decarboxylase from the bacteria *Pseudomonas putida*, and (4) norcoclaurine synthase from the plant *Coptis japonica*
Module 4	(S)-reticuline module	For the expression of five plant proteins: (1) norcoclaurine 6-*O*-methyltransferase, (2) coclaurine-*N*-methyltransferase, (3) 4′-*O*-methyltransferase, (4) cytochrome P450 reductase (1–4 from *P. somniferum*), (5) *N*-methylcoclaurine hydroxylase from *Eschscholzia californica*

production of the desired compound. One such type of example is a yeast strain produced by Galanie et al. (2015) that can produce (S)-reticuline, a key biosynthetic intermediate of various downstream benzyl isoquinoline alkaloids including the morphine. This strain was developed by customized genetic modules with better carbon flux through tyrosine to (S)-reticuline. In this modified strain, the biosynthetic pathway of reticuline had been divided into four genetic modules containing 17 biosynthetic enzymes. Details of the modules are described in Table 7.2.

7.4.3 Nonribosomal Peptides (NRP)

Nonribosomal peptides belong to a class of peptide secondary molecules. These are primarily synthesized by microorganisms. NRP synthetases (NRPSs) is the enzyme

which is utilized for the synthesis of the NRPs. This particular enzyme differs from ribosome in the action of production of peptides as it does not depend on mRNAs. These enzymes are very specific ass one type of enzyme is responsible for the production of specific NRPs. So if we change the structure of NRP synthetase, we can produce an entirely different NRPS. Awan et al. (2016) expressed the whole biosynthetic pathway for benzylpenicillin synthesis in the yeast *S. cerevisiae*. The biological pathway for benzylpenicillin synthesis consists of five enzymes (encoded by pcbAB, npgA, pcbC, pclA, and penDE) (Fig. 7.2).

7.4.4 Flavonoids

Flavonoids consist of a variety of application in health practices. They possess various activity including antioxidant and antimicrobial activities (Skrovankova et al. 2015). Breviscapine is a flavonoid which is extracted from the vegetal tissues of *Erigeron brevisca* and is used in Chinese medicine. The major components of breviscapine are apigenin 7-*O*-glucuronide and scutellarin. Liu et al. (2018) group was able to produce apigenin 7-*O*-glucuronide and scutellarin from glucose in yeast.

7.5 Development of Synthetic Biology Tools for Biofuel Production

Biofuels are fuels that are produced from biomass, rather than a fuel which is produced by geological processes involved in the formation of oil. The production of biofuel is less time and energy consuming in comparison to fuels that are produced by geological activities. As time passes on, the extraction of fuels from the natural resources are decreasing, so there is a need of looking into other options also for the production of fuels. Here comes the synthetic biology which provides various model organisms such as *Escherichia coli* microalgae and *Saccharomyces cerevisiae* for the production of biofuels. In this section, we will discuss the application of yeast and microalgae for biofuel production as well as the problems associated while using yeast and microalgae for the same.

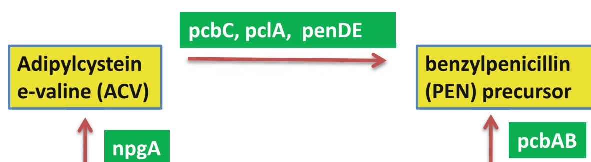

Fig. 7.2 Schematic diagram showing various genes involved in biosynthetic pathway for benzylpenicillin synthesis in the yeast *S. cerevisiae* by Awan et al. (2016)

7.5.1 Synthetic Biology Tools for *S. cerevisiae* for Biofuel Production

7.5.1.1 DNA Assembly Tool

A very first step in SB is the assembly of DNA sequence of biological pathway into an expression cassette. To complete this goal, a very quick and highly efficient method which is competent of linking genes in a particular pathway in a long chain is required. As in a pathway, various genes are under the control of various promoters and terminators exist, so the method must offer a very high capacity and efficiency so that a very long nucleotide sequence with various modules can be combined. There are mainly three ways available for DNA assembly. The first one involves various integrative sequences, e.g., sites for restriction enzyme, integrase (ligase), and clonase. The second one uses the homologous recombination principle, while the third one is de novo DNA synthesis. Here we will focus mainly on HR-dependent DNA assembler.

Saccharomyces cerevisiae has advantage over *E. coli* system for assembly of large fragments of DNA inside the cell. If we are focusing on *E. coli* system for the assembly of DNA fragments, then we have to first clone in a cloning vector with expression cassette, then only we can assemble the sequences. However, the presence of high HR activity of *S. cerevisiae* bypasses these steps, and the assembly of large DNA fragments directly in the chromosomes of yeast is possible. In yeast, it is possible to integrate the in vivo-assembled constructs into the target site in the genome. This can be achieved by designing the 5′ 3 ends of the construct to be homologous and to flanking chromosome sequences of the target insertion, and it must be coupled with selection marker.

7.5.1.2 Genome Editing Tool

The foundation of SB is the pathway assembly for the production of value-added products such as biofuel. Pathway assembly is the basis of synthetic biology for synthesizing value-added products. There exists a strain-dependent variation in the efficiency of various pathways. The efficiency can be increased or improved with the help of SB. For example, the xylose utilization pathway consists of mainly five components, i.e., XYL1, XYL2, XYL3, PHO13, and ALD6. This pathway was inefficient in the strain of *S. cerevisiae*. However, ALD6 and PHO13, two of the genes of this pathway, were mutated on the chromosome by evolutionary engineering as well as optimal expression of all the three XYL genes for generating a highly xylose fermenting strain (Kim et al. 2013). Therefore, for construction of optimized strains, mutations of target genes on chromosomes are necessary. Among various tools available for genome editing, few such as transcription activator-like effector (TALE) nucleases (TALENs), clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) and Zinc-finger nucleases (ZFNs) are the best studied and applied.

7.5.2 Application of Yeast in Biofuel Production

Yeast (*Saccharomyces cerevisiae*) serves as a chassis in SB due to its small sequenced genome, small life cycle, and easy handling in laboratory. It was only few decades back when engineered yeast was used for the production of biofuels from cellulosic sugars. Since then, tremendous efforts have been made to *utilize* this chassis as efficient model for cost-effective biofuel production. However, there are constrains while using yeast as a model system for this purpose. These challenges are as follows: to broaden the substrate ranges from just sugars to biomass, to develop new synthesis pathways for producing advanced biofuels, and to minimize product inhibition by toxic. So further research has been made to address these problems and solving these problems for biofuel production. To increase the production of biofuels, the substrate utilization was broadened so that substrates other than cellulosic sugars can be utilized.

7.5.2.1 Utilization of Various Substrate for Increased Biofuel Production

It took a very hard work and efforts to expand and increase the substrate range for biofuel production by genetic, metabolic, and protein engineering. The efforts had been made to construct heterologous pathways in yeast for efficient fermentation of non-glucose carbon sources such as galactose, cellobiose arabinose, and xylose. This was possible just because of the discovery of various metabolic enzymes and transporters for utilization of nonglucose sugars. The next step was to improve the expression level of each unit of metabolic pathways via SB such as by utilizing strong and constitutive promoters as well as terminators. Along with this, various cofactors had also been modulated by protein engineering for efficient and broaden substrate utilization for biofuel production. All the necessary and important factors are shown in Table 7.2. With this table, it can be concluded that modulation of the expression of rate-limiting enzymes and optimization of heterologous metabolic pathways are the keys for improving the substrate utilization of sugars other than glucose sugars (Tsai et al. 2015).

7.5.2.2 Advanced Biofuel Production by Engineered Yeast

In the present scenario, ethanol is a biofuel, which can be produced at a huge scale. However, there are few problems associated with the utilization of ethanol as liquid biofuel. So, there was a need for the production of biofuels which is almost parallel to petroleum-based fuel. Metabolic pathways to produce various biofuels such as isobutanol, 1-butanol, isoprenoids, and fatty acid ethyl esters have been reconstituted in yeast (Table 7.3).

Table 7.3 Various approaches for broadening substrate utilization for biofuel production (Tsai et al. 2015)

S. no.	Various approaches for broadening substrate utilization	Impact of the strategy
1	Protein engineering of xylose reductase of *Candida tenuis*	Increase in the ethanol yield by 42% Decrease in xylitol by 51%
2	Overexpression of *Clostridium* phytofermentans XI for codon Optimization	0.43 g g − 1 ethanol yield 0.03 g g − 1 h − 1 sp. ethanol productivity 0.07 g g − 1 h − 1 sp. xylose uptake
3	Overexpressed *Piromyces* xylose isomerase, *S. stipites xylulokinase*, and pentose phosphate pathway	1.87 g g − 1 h − 1 sp. xylose uptake 0.41 g g − 1 ethanol yield (after evolutionary engineering)

7.6 Development in Synthetic Biology Tools for New Drug Discovery

Synthetic biology is redefining the field of drug discovery in the same way as organic chemistry did in pharmaceutical industries a century ago (Trosset and Carbonell 2015). “Since ages nature is used as a source of human medicines, but this process is arduous and very expensive”. Often the large-scale production of these compounds being laborious, complex, and non-profitable has led the pharmaceutical industries to abandon these natural medicinal compounds. Being able to biosynthesize these compounds from the source organisms to genetically friendlier hosts would change the scenario. SB is an attempt to apply the concepts of engineering to transform the biological cells into an industrial biofactory. These organisms can be engineered to have either act as biosensors or help us understand the intricacies of living systems or have desirable novel biosynthetic capabilities (Neumann and Neumann-Staubitz 2010).

Initially the application of SB in drug discovery was to enhance the creation of novel chemicals that have properties similar to well-known natural products used for medicinal purposes (Atanasov et al. 2015). The current advances in molecular biology, protein engineering tools, and genome editing are leading a pathway toward the discovery of biological systems that can produce controlled phenotypes using molecular switches. Genetic circuits support the different steps in drug discovery as well as use microorganisms for bioproduction of drugs (Breitling and Takano 2015) in pharmaceutical research.

The advances in the field of DNA technology like inexpensive DNA synthesis, the next-generation DNA sequencing technologies, and well-characterized genetic modules have drawn attention for synthetic biology (Neumann and Neumann-Staubitz 2010). The principles of molecular biology can be applied to reprogram the microorganisms not only to produce the desired drugs but also to deliver it at the

site of action. Here is an overview of how recent development in synthetic biology has revolutionized the field of pharmaceutical biotechnology.

7.6.1 Basic Concepts of SB for Drug Development

An archetypal synthetic cell is composed of three basic elements, namely an inducer of gene expression, gene circuit to control the expression of specific genes, and reporter genes to control the output signals related to a disease phenotype (Trosset and Carbonell 2015). To enable the gene expression of targeted compound or for exploration of chemical space of natural products, the gene circuits from enigmatic biosynthetic units of a microorganism can be integrated into host microorganism. Optogenetic biosensors, e.g., lux operon, protein photo sensor like green fluorescent protein can be used to understand drug's mechanism of action through a designed disease model, induce a drug delivery mechanism at a specific site or under specific condition, and validate drug targets. For studying the drug resistance or persistence mechanism or optimize secondary metabolism or fight toxic effects in bacteria, synthetic quorum sensing can be used. Protein engineering is used to modify enzymes or shuffle biosynthetic modules to explore chemical diversity of secondary metabolites (Table 7.4).

7.6.2 Mining NPs Space

Biosynthetic machinery of microorganisms, fungi, and plants (Mattern et al. 2015) produces NP derivatives with therapeutic interest. Polyketides (Cummings et al. 2014) non-ribosomal peptides (NRPs), isoprenoids, terpenoids, flavonoids, and alkaloids (Cummings et al. 2014; Trosset and Carbonell 2015) are some of the widely used NPs. The basic idea of using a cell as a biofactory started in early 1900s, when scientist Katz and Leadlay showed that the antibiotic erythromycin was synthesized by a unique gene cluster which forms a giant biosynthetic unit made

Table 7.4 Synthetic biology tools in various steps of drug discovery

Synthetic biology	Drug discovery
Genetic circuits in host organisms	Increase flux of secondary metabolic pathways
Protein engineering—modify enzymes or shuffles biosynthetic modules	Explore chemical diversity of secondary metabolites
Optogenetics biosensing	Target validation Drug mechanism of action Disease models Drug discovery
Synthetic quorum sensing: cell–cell communication	Overcome drug resistance Optimize secondary metabolites Fight toxic effects

of 28 protein modules (Donadio et al. 1991; Walsh and Fischbach 2010). These biosynthetic units could be further isolated and applied to modify at the genetic level to produce NP derivatives into host organism (Newman and Cragg 2012). Bioinformatics tools, such as antibiotics and Secondary Metabolite Analysis Shell (antiSMASH) and Secondary Metabolite Unknown Regions Finder (SMURF) (Wohlleben et al. 2012; Doroghazi et al. 2014), which are used for large-scale genome or metagenomes sequencing of microorganisms have extended the discovery of such biosynthetic gene clusters (Cacho et al. 2015). Even though most of the biosynthetic gene clusters remain cryptic or silent under usual conditions, lots of effort have been made to boost or reactivate silent gene expression through the design of ligand-controlled aptamers, synthetic transcription factors (TFs) (Leavitt and Alper 2015), or riboswitches (Shao et al. 2013) "knock-in" promoter replacement strategy (Harvey et al. 2015).

7.6.3 In Vivo Combinatorial Libraries

Combinatorial libraries are widely used in pharmaceutical industries for drug discovery. However, owing to perceived failures with this technology, different in vivo compound libraries can be produced for the drug discovery. Such libraries would have several advantages like maintenance, and amplification would be simply done by cultivating cells. Since, genetic information of the cell is directly linked to the compound, it can be used to screen the compound. Thirdly, limitations of in vitro assays for enzymatic activity can be overcome by an intracellular genetic selection. Also, greater level of sensitivity of the drug for its target could be achieved when the selection is performed in context to the living cell. Finally, problems with uptake and solubility of the compounds could be avoided.

Antibiotics like valinomycin and fungal toxins like amatoxins, phalloidin are few of the natural compounds which are modified cyclic peptides providing resistance to cellular degradation and restricts conformational freedom, thus potentially improving binding affinity and specificity (Katsara et al. 2006). Polyketides are another important class of natural products prominently produced by actinomycetes, (e.g., erythromycin, anthracyclines, indolocarbazoles, isoprenoids, and epothilone). Their individual modules have been split and recombined to form active enzymes which catalyzed the formation of polyketides and their precursors like epothilone C and D (anticancer drugs), aklanoic acid (which is a precursor to several antitumor polyketides like aclacinomycin A and doxorubicin) in *E. coli*. This approach thus can be used to develop a potential compound with novel biological activity.

7.6.4 Metabolic Engineering for Drug Production

The most significant and well-known use of SB is for the production of artemisinin, which is an antimalarial drug, traditionally made from materials naturally produced by the sweet wormwood tree. Scientists at Amyris altered the genome of yeast,

adding six genes that enabled it to produce artemisinic acid, a precursor component that can be converted into the medication thus generating this therapeutic faster and cheaper than its natural source. Similar approach has been used for the production of isoprenoid paclitaxel (also known as taxol), a drug used for chemotherapy. As its chemical synthesis and natural extraction from Pacific yew is difficult, and inefficient modular approach was used for its production, achieving a titer of 1 g/L in *E. coli*.

7.7 Conclusion

In this chapter, many of the thought-provoking applications from the area of synthetic biology are discussed. In most of the cases, these are the representatives of a handful of strains with relatively simple interactions. However, these advances have future application using the new emerging tools in the field of synthetic biology, molecular biotechnology, and mathematical modeling. Upcoming advances in the area of synthetic biology have the potential to renovate fields of bioproduction, medicine, environmental engineering, and bioprocessing. Synthetic biology has a vast application in the field and has a potential to solve various problems in the field of biotechnology via mathematical modeling as well as applying the engineering in biotechnology. Additionally in future we also have to check the integration and response of the synthetically designed strain on the pressure of the evolution whether these strains are stable enough during the course of evolution. Recently emerging bioinformatics and computational tools have shown a futuristic word with modified organisms with enhanced value-added product as well as engineered strains. However, in reality these need to be further checked.

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8 Ethical, Patent, and Regulatory Issues in Microbial Engineering

Sumer Singh Meena and Anee Mohanty

Abstract

Engineered microbial systems are finding application in various sectors and are predicted to revolutionize variety of fields such as health care, food and agriculture industries, paper, textile, and environmental remediation. With the rapid advancements in technology for production of genetically modified organisms, there is also ever-increasing debate on social, ethical, and legal implications of such technologies. For these cutting-edge technologies to be commercialized and become the basis of successful business ventures, there is a need to protect these inventions through regimes of intellectual property rights (IPR). In addition to legal protection, the innovation must meet the regulatory framework keeping in mind the societal concerns. Ethical concerns are intertwined with genetically modified microorganisms and questions like are scientists "interfering with nature" or "playing god" are being widely debated. This chapter elaborates on the evolution of patent system for biological material, patent landscape of microorganisms across the globe, various ethical concerns, and the way forward.

Keywords

Genetically modified microorganisms · Intellectual Property Rights (IPR) · Patents · TRIPS · IDA · Bioethics

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8.1 Introduction

The history of exploiting microorganisms for production of food and beverages dates back to thousands of years. There are credible molecular evidences now that ancient civilizations in China, Egypt, Mesopotamia, and Greece have been using microorganisms for fermentation of grains and fruits (McGovern et al. 1996, 2004; Samuel 1996; Valamoti et al. 2007). Louis Pasteur's work on lactic acid and alcohol fermentation and later microorganisms as causative agents of diseases was a milestone discovery in microbiology (Gal 2008; Pasteur 1857) which led to search for newer fermentation techniques and improving the efficiency for production of various economically important products. In the twentieth century, scientific advancements made it clear that microbial genome possesses enormous potential to synthesize hundreds and thousands of complex molecules with diverse application. Now various industries are centered around microbial products starting from vaccines, antibiotics in health-care industry to harvesting renewable sources of energy from microbial biomass and food processing industries using an array of microbial enzymes and whole-cell microorganisms as biocatalysts (Vitorino and Bessa 2017).

Synthesis of bioactive compounds in living systems are often controlled by complex regulatory pathways comprising of dozens of genes. Thus, production and isolation of industrially important compounds in economically efficient ways would require manipulation and optimization of metabolic networks and if required addition of new genes or entire pathways to reprogram the microbial systems (Ginsberg et al. 2014). In the last couple of decades, breakthrough in genetic engineering and next-generation sequencing technologies are enabling the scientists with required methodology and genome information for engineering prokaryotic and eukaryotic cells. Microbial engineering holds enormous potential to change human lives; as cost of health care would reduce, greener and cheaper sources of renewable fuels would fulfill the energy requirement of the ever-increasing population, and resource recovery from waste material and higher yield from agriculture would be facilitating sustainable living (Smanski et al. 2016).

The ability to apply engineering principles to living systems for redesigning the existing microorganisms or the pursuit to even construct completely novel biological entities or designing tailor-made living entities is raising serious legal, ethical, and social issues. This chapter aims to elaborate on the various aspects of intellectual property rights, ethical, biosafety, and regulatory issues pertaining to microbial engineering.

8.2 Patent-Related Issues in Microbial Engineering

Microbial engineering involves manipulating the microbial cells for creating new products or processes. The engineered microorganisms are designed to enhance the yields of industrial chemicals such as enzymes, vitamins, amino acids or alcohol, biopolymers, or other disease-fighting agents such as vaccines, antibiotics, or simply

probiotics for enhanced immunity by colonization of human guts. Exploitation of microbial systems for value-added products are benefiting plethora of industries such as pharmaceuticals, biomedical, agroindustry, energy sector, and food processing industries. Thus engineered microbial systems designed to solve specific problems with practical application need to be commercialized to earn revenue and bring economic prosperity to the inventor and nation. The profitability of such ventures will also provide much required impetus to R&D activities in related fields.

In order to commercialize a product first, it needs to be protected from unfair copying and competition. Therefore, the novel engineered microorganisms are subjected to intellectual property rights (IPRs) which will not only be rewarding for the inventor but also will pave the way for exploring the unrecognized and untapped commercial utility of such inventions. IPRs grant legal protection to the creation of human mind and could be inventions of scientific, literary, or artistic nature, for specified amount of time to its creator or inventor. There are various types of IPRs like copyrights, industrial designs, trademarks, and patents, and depending on the nature of innovation, it can fall into one of the categories.

IPRs concerning a novel variety of genetically modified microorganisms, plant species, animal cell lines, genes, and any process of isolation or modification of biomolecules like DNA, RNA, proteins, and metabolites fall into the category of patents. Patents are legal rights conferred to inventors for fixed number of years after satisfying few conditions such as novelty, industrial application, nonobviousness, and full disclosure (Webber 2003). This legal right gives monopoly to the patentee to manufacture, sell, and import the patented product or process for a stipulated period of time. Ever since the rapid development in biotechnology, legal rights pertaining to novel life forms have been under serious debate partly because of differences in sociocultural and economical standings of nations. Though all the major technologies in the past that pushes the frontier forward have been faced with certain amount of resistance and tension, genetic modification of organisms is mostly perceived as commodification of life and an attempt to play with nature. Therefore, there is wide variation in patent laws across the globe, and this is even more apparent when it comes to patenting life forms. However, patenting of live forms especially microorganisms is not new as patents were granted to novel variety of yeasts used in brewing and baking industries in 1833 in Belgium and in 1843 in Finland (Webber 2006).

8.2.1 TRIPS Agreement: Patenting Microorganisms

The extent of legal protection to engineered life forms varies across different countries. For any biological material to be eligible for patent application, it still has to fulfill the basic criteria of patentable subject matter, i.e., inventive step, nonobviousness, and industrial utility as applicable to any other technology. So, any new organism or genetically modified microbe must be different from the already existing form in terms of character and/or usage. As stated earlier, there is a wide variation in patenting methodology adopted in different countries, one such

variation is mere isolation and purification of microorganisms or their genes cannot be patented in some countries, whereas in USA it can still be patented. As the profits from bioprospecting are rising tremendously over the years, the inconsistencies in patent laws have led to heated debate over ownership rights of patents (Wynberg and Laird 2007).

To reduce the distortion in patent laws and to enable seamless movement/trade of products and processes arising of biological patents, TRIPS (Trade-related Aspects of Intellectual Property Rights) agreement was signed by the member countries of WTO. It is aimed at bringing coherence in IPR policies of all member countries (Matthews 2003). TRIPS was negotiated in Uruguay Round of the General Agreement on Tariffs and Trade (GATT) between 1989 and 1990 and is administered by the WTO (Drahos 2002). It is mandatory for all the member states of WTO to enforce the minimum standards of IP protection, and noncompliance could lead to punitive action like sanction/fines. The developing nations were having some apprehensions that scope of patentability is too wide and may impede the social and economic welfare of less affluent nations.

Section 27 of TRIPS states that "patents shall be available for any invention, whether products or processes, in all fields of technology, provided that they are new, involve an inventive step and are capable of industrial application." The section reiterates the basic tenets of patentability of be novelty, nonobviousness, and industrial application, but the noteworthy thing is it brought all fields of inventions at par with each other as far as patenting is concerned and made non-discrimination its underlying principle.

With this TRIPS brought microorganisms also under the ambit of patent protection and therefore many nations modified their domestic patent laws and have now started patenting of microorganisms. From 2005, all the member countries had to allow microbial patenting if was not allowed earlier (Sekar and Kandavel 2002).

8.2.2 Budapest Treaty, Deposition of Microorganisms, and IDA

As patent laws globally require full disclosure of invention during patent application in such a way that person skilled in the art could comprehend the full invention and its working. In case of microbial patents or patenting of their genetic material or any compounds derived from them, drawings or mere description would be highly insufficient, and the need for microbial samples to be deposited in a culture collection for patent application was felt internationally. Therefore, Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure was signed in 1977 and was enforced in 1980 and is administered by WIPO (World Intellectual Property Organization). As of June 2019, there are 82 countries which are party to this treaty, and it led to the creation of IDA (International Depository Authority). IDA ensures that a patent applicant needs to deposit the biological material in only one of the recognized culture collections and eliminated the practice of depositing in every country where the patent is filed. Examples of IDAs are American Type Culture Collection (ATCC), the European

Collection of Cell Cultures (ECACC), Japanese Federation of Culture Collections, and the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Microbial Type Culture Collection and Gene Bank (MTCC) in India. The IDAs accept a wide range of biological materials which include microbial cultures (e.g., viruses, algae, fungi, bacteria), animal cell lines, hybridomas, plant cell lines, genetic material (including DNA fragments), vectors along with the host used for expression of a gene, spores, etc. (www.wipo.int/budapest/en/idadb). These culture collections also alleviate the problems related to storage, transportation, handling of diverse biological samples and could very well be transformed into biological resource centers with legal mandate for benefit sharing internationally (Sekar and Kandavel 2004).

8.2.3 Status of Microbial Patenting in Developed Countries

Article 27.3(b) of TRIPS agreement does not allow member states to exclude microorganisms from patents (Kothamasi et al. 2011). However, the agreement neither defines the term “microorganism” nor elaborates on the scope of protection. Another important thing is under Article 27.1 of TRIPS, subject matter must be an invention and not mere discovery. So, this raises a very pertinent question of whether mere isolation of microorganisms that exist in nature comes under patentable subject matter. Across the globe there seems to be more or less an agreement that any substance occurring in nature should not be patentable without significant human intervention.

8.2.3.1 US Patent System

This varies widely with some countries like USA taking a liberal stand on this by allowing patents on microorganisms which were isolated and purified from a consortia in laboratory and their existence was not known previously (Martin and Vermeylen 2005). US patent regime for biological materials have also gone through transitional phase and initially did not allow for patenting of living organisms. Only after the landmark judgment of supreme court of USA in the *Diamond v. Chakrabarty* case (Kevles 1994; Robinson and Medlock 2005) in 1980, the status changed to “all inclusive” or “include anything made by man under the sun.” This was one of the first well-known case of engineered microbial system to get a patent. The applicant had genetically modified a bacterium of the genus *Pseudomonas* with catabolic plasmids capable of degrading oil spills. Initially the United States Patent and Trademark office (USPTO) studying the claims accepted that the bacterium does not occur in nature, but the application was rejected on the ground that subject matter was “living organism.” An appeal against USPTO decision was filed in Supreme Court, and the court decided in favor of the applicant in an epoch marking verdict. The court ruled that the issue is not between living and nonliving nature of subject matter but between product of nature and human made invention (Chakrabarty 2010). Almost three decades later, US patent system has undergone various changes

and now take a liberal stand to bring a variety of living materials under the ambit of patent laws.

Here another pertinent question to ask is whether patenting of engineered microbial systems would also entail the gene responsible for designing the microbe patentable? In the case of Association for Molecular Pathology v. Myriad Genetics, Inc., 569 U.S. 576 (2013), Justice Clarence Thomas, of Supreme Court pronounced the verdict and held, "A naturally occurring DNA segment is a product of nature and not patent eligible merely because it has been isolated, but cDNA is patent eligible because it is not naturally occurring" (Ingram 2014). Biotechnology and pharmaceutical companies are taking advantage of this rule and patenting cDNA which is an edited version of original gene.

As a leading country in biotechnological innovation, the number of patents in US concerning microbial systems have increased many folds in past few years. With new issues with bio-patents, the legal system also needs to be modulated to be more accommodating. In 2013, American Invent Act was introduced in US which changed the status quo from first-to-invent to first inventor-to-file (Schafer 2013) to encourage filing of more patents in time-bound manner. This law also enlarges the scope of post grant opposition by third parties to increase transparency.

8.2.3.2 European Patent System

European nations have unified patent policy which is mostly derived from two documents, European Patent Convention (EPC) and the Biotechnology Directive, 1988 (Kranakis 2007). A unique feature of European patent system which is not present in American system is inclusion of a "public order and morality" clause under Section 53(a). Some other inventions are also not allowed for the purpose of patenting which are listed under Section 52 of EPC. The national IP laws of European nations also have their own approach when it comes to granting of biological patents.

8.2.3.3 Status of Patenting Microorganisms in Developing Countries: Case Study of India

Indian patent system is quite similar to European system and takes a firm stand by excluding preexisting phenomena, and creation of nature cannot be patented (Senan et al. 2011). The Indian Act of 1970 regulates the patent protection in India. This act has undergone many amendments over the years especially after signing the TRIPS agreement to meet its obligation for free and fair trade (Ganguli 2004). Patent amendment act of 2002 allowed patenting of microorganisms. Another major change that took place in 2005 was the deletion of Section 5 of Indian Patent Act, 1970, which allowed for only process patent paving the way for product patents. Indian patent system still does not allow patenting of process of production of plants, animals, or plant and animal as a whole or part of it only exception being the microorganisms. However, this could create confusion as the word "microorganism" has neither been defined clearly in TRIPS agreement nor by Indian patent system. Overall, the amendments made to the Indian patent system have been successful in removing the red tape and attracting more patent filing involving microbial processes

and engineered microbial systems, and this would augur well for the economy of the country (Balachandra Nair and Ramachandranna 2010; Mishra et al. 2019).

8.3 Ethical Concerns

Ability to alter life forms, even creating novel life forms and making them into profitable business ventures, raises many questions of legal, ethical, and societal concerns. The most contentious issues out of all is the issue of "owning life" which also leads to questions over use, transfer, and dissemination. The following section would deal with all such issues pertaining to engineered microbial systems.

8.3.1 Societal Concern and Public Trust

It is not surprising that a powerful technique like genetic engineering having the potential to create novel life forms would raise ethical security and safety concerns. The major concern being these technologies might blur the line between what is natural and what is not.

8.3.2 Biosafety and Bioterrorism Concern

As these engineered microorganisms could be created for various purposes, concerns about creating engineered pathogenic microorganisms for bioterrorism purposes also loom large in the psyche of common people. These biosecurity concerns were triggered in early twenty-first century when scientists were able to create Influenza virus (Tumpey et al. 2005) and cDNA of polio virus (Cello et al. 2002) in lab. If these technologies are easily accessible, then "biohackers" could use them to unleash terror attacks by creating more virulent strains of pathogen.

Another health concern of engineered microbial system is the potential use of these bugs in therapeutic clinical trials. Human body harbors many microorganisms in various anatomic site, and their role in digestion and immunity is increasingly becoming clear. In this regard, therapeutic microbiota enrichment like fecal microbiota transplantation (FMT) have shown promising result for treating recurrent *Clostridium difficile* infection (Van Nood et al. 2013). Success of these studies led to the investigation of FMT studies for treatment of other diseases like inflammatory bowel disease, treatment of graft versus host disease in hematopoietic cell transplant patients, reduction in intestinal carriage of multidrug-resistant organisms (Woodworth et al. 2017). U.S. Food and Drug Administration (FDA) is closely monitoring these cases and is bringing regulations and requirements to study the potential risks associated with such studies and the long-term effect of altering the microbiota.

8.3.3 Accidental Release and Environmental Implication

Potential release of these engineered microorganisms can lead to severe environmental and health hazards. Unlike air or water pollution which might dissipate over time, engineered microbes if released to environment will multiply and could pose grave threat to naturally occurring microbes in soil, water, air, or other parts of ecosystem. Our experience of introducing plant or animal species to new geographical areas in the past has created many ecological disasters, e.g., fungus introduced from Asia to North America killed half of its chestnut trees (Steiner et al. 2017), and many such examples of creating invasive species exist. Therefore, thorough assessment of genetically modified microorganisms and their effect on environment needs to be assessed before their release (Clark 2006) as they might have unpredictable and emergent properties.

It is felt that in the rat race of patenting life, the multinational corporations might have the tendency to only conduct short-term trials which may be highly inadequate as the long-term effects will be unknown. To avoid such scenarios, many patent systems across the world have "public health" clause in the patent laws, and inventions which are likely to be injurious to public health do not qualify for patents. Regulations should be put in place for thorough evaluation of whether the invention has undergone detailed biosafety trials or not, and these safety check points would go a long way in mitigating the fear in the minds of general public.

8.3.4 Economical Concern: Costlier Health Care or Food Products Only Available to Rich

Patents are in nature negative legal rights which bar others from using the patented technology and grant monopoly to the inventor for a stipulated amount of years. According to some human rights activists, this might increase the gap between rich and poor where patented technologies that are costlier are only available to rich out of reach for the poor, disadvantaged minorities, women, and underdeveloped world. These fears were raised by few nations after TRIPS agreement where it was felt that bringing everything under the IPR regime will make quality health care unaffordable to people in least developed parts of the world. To address these concerns, Doha Declaration on the TRIPS Agreement and Public Health was adopted by the WTO Ministerial Conference of 2001 in Doha which kept essential medicine and life-saving drugs out of the patent regime and gave flexibility to member nations to deal with any public health crisis.

Similar apprehensions exist for application of engineered microbial systems in agricultural industries. Farmers unconsciously act as selection agents in the development of microbial resources/germplasm, but their role goes unrecognized as multinational corporations acquire the rights of microbial resources, and there is no equitable profit-sharing mechanism in place (Kothamasi et al. 2011).

Regulatory mechanisms should be put in place such that ownership and commercialization of genes, genomes, and genetic information which might result in new food or health-care products is not out of reach for some sections of the society.

8.4 The Way Forward

Arguably, microbial engineering holds the potential for new industrial revolution with the production of cheaper drugs, disease-resistant plant variety, increasing soil fertility, enhanced crop growth, greener fuels, bioactive compounds for preservatives, color, fragrance enhancer, bioremediation of recalcitrant compounds to just name a few. However, proper regulatory mechanisms should be put in place to avoid any malicious attempt to misuse these technologies. Various checks and balances like screening of companies and thorough biosafety documentation before granting patents, creating awareness about biosecurity issues among scientists and general public alike, formation of professional society, including civic society members as stakeholders in decision-making process for granting patents for engineered microbial systems, will go a long way. There might not be an appropriate "one size fits all" approach with diverse systems and technologies in place, so regulatory bodies must take into account a wide range of perspectives about risk, economic impact, scientific progress, and moral reasoning in dealing with commercialization of genetically modified microorganisms.

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9 Microbial Production of Vitamins

Indra Mani

Abstract

Vitamins are important nutrients for humans and animals. Their requirement is rising worldwide in different areas such as therapeutic and food industries. Vitamins are naturally produced by several microorganisms. However, its biosynthetic mechanisms are well coordinated as its requirements are just in tracer quantities and the synthesis of some vitamins is limited to few microorganisms. Recently, large scale productions of vitamins have been more concentrated on microbial fermentation as compared to chemical synthesis. Because many steps are required in the chemical synthesis of vitamins, which makes it expensive. So, industrial microbial fermentation is utilized to produce large amounts of vitamins to accomplish the global yearly demands. Further, recent advancement in the fields of systems and synthetic biology would provide an opportunity to engineer the microbial metabolic pathway for the increased production of vitamin. It can lead over to chemical synthesis methods, but scientific difficulties might persist. Further scientific and regulatory concerns remain, which need to be resolved before extended to the user.

Keywords

Vitamins · Microbial · Biosynthesis · B_{12} · *E. coli*

9.1 Introduction

For the normal functioning of organisms, vitamins play a vital role in various physiological reactions. On the basis of the solubility nature of vitamins, it has been classified into two groups such as water-soluble (B, C group) and fat-soluble

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(A, D, E, and K). Microbes and plants are producing these different types of vitamins naturally; however, animals get the vitamins from these sources. Intriguingly, vitamins are needed in a small amount in various pathophysiological and other conditions because it acts as a coenzyme in different metabolic reactions in all organisms. Vitamins are sensitive to heat, light, pH, and oxygen, and its content decreased during food processing and preservation (Vandamme and Revuelta 2016). Presently more than 10 tons of vitamin B_{12} are produced per year from different bacterial species (Martens et al. 2002). The worldwide production of fermented vitamin has been increased from 5 to 75% from 1999 to 2012 (Schwechheimer et al. 2016).

Two fungi *Candida maltose* R42 and *Botrytis allii* NRRL 2502 have been utilized for the microbial transformation of vitamin D_3. 1α-Hydroxyvitamin D_3 was produced as a metabolite (Ahmed et al. 2014). The study has shown that CYP105A1 can convert vitamin D_3 (VD_3) to its active form 1α,25-dihydroxyvitamin D_3 (1,25D3). A site-directed mutagenesis method has utilized to construct double variants (R73A/R84A and R73A/R84V) of CYP105A1. An activity of the double variants has shown 100-fold higher as compared to the wild type of CYP105A1 (Yasuda et al. 2017).

Vitamin K [Menaquinone-8 (MK-8)] is comprised of a polar head group and a non-polar side chain. For the production of vitamin K, *Escherichia coli* has been utilized. Overexpression of *E. coli* DXR, IDI, or IspA has enhanced MK-8 quantity up to twofold. However, MenD or MenA has significantly enhanced MK-8 quantity than the wild type (Kong and Lee 2011). Previously, a study has demonstrated that overproduction of menaquinone (MK) was achieved using mutated *Bacillus subtilis*. Menaquinone has been produced by menadione-resistant mutant 30% more as compared to its parent strain (Sato et al. 2001). Some bacterial strains can synthesize various vitamins such as vitamin B_1, B_2, B_3, B_5, B_6, B_7, B_9, and B_{12} as shown in Table 9.1.

9.2 Microbial Production of Vitamin B_1, B_2, B_3, and B_5

Vitamin B_1 (Thiamin) biosynthesis is mainly regulated by thiamine pyrophosphate (TPP) riboswitches in bacteria and a transcriptional repressor in archaea (Hwang et al. 2017). TPP is a vital cofactor in amino acid and carbohydrate metabolism (Eggersdorfer et al. 2012). A riboswitch-based biosensor enabled the discovery (Genee et al. 2016) and metabolic engineering (Bali et al. 2018) of thiamine transporters, and an improved thiamine production in *E. coli* overexpressing thiFSGHCE and thiM or thiD combined with transposon mutagenesis (Cardinale and Sommer 2017).

Vitamin B_2 (Riboflavin) is used in food industry as food colorant and also as food supplement. *E. coli* RF05S-M40 strain has been utilized for the production of vitamin B_2 and the study demonstrated a 12-fold (2702.8 mg/L) higher production than other strains of *E. coli* RF01S (Lin et al. 2014). Initially, riboflavin manufacturing has been enhanced by an amalgamation of conventional mutagenesis

Table 9.1 Vitamins-producing bacterial strains

S. no.	Vitamins	Microorganisms	References
1.	B_1 (thiamin)	*Lactobacillus rhamnosus* strain GG (LGG) *Bifidobacteria* *Leuconostoc* *Bacteroides fragilis*	LeBlanc et al. (2017) Hou et al. (2000) Kneifel et al. (1992) Magnúsdóttir et al. (2015)
2.	B_2 (riboflavin)	*L. lactis* LGG *Bacteroides fragilis* *Clostridium difficile* *Lctobacillus plantarum* *Ruminococcus lactaris*	Burgess et al. (2004) LeBlanc et al. (2017) Magnúsdóttir et al. (2015) Juarez Del Valle et al. (2016) Russo et al. (2014)
3.	B_3 (niacin)	*Ruminococcus lactaris* *Clostridium difficile* *Helicobacter pylori*	Deguchi et al. (1985)
4.	B_5 (panthothenic acid)	*Bacteroides fragilis* *Ruminococcus lactaris* *Ruminococcus torques*	Magnúsdóttir et al. (2015)
5.	B_6 (pyridoxin)	*Bifidobacterium longum* *Collinsella aerofaciens* *Bacteroides fragilis*	Deguchi et al. (1985) Magnúsdóttir et al. (2015)
6.	B_7 (biotin)	*Campylobacter coli* *Lactobacillus helveticus* *Bacteroides fragilis*	Shah and Patel (2014) Magnúsdóttir et al. (2015)
7.	B_9 (folic acid)	LGG *Bacteroides fragilis* *L. Plantarum WCSF1* *Fusobacterium varium* *Prevotella copri* *B. adolescentis* DSM 18350	LeBlanc et al. (2017) Magnúsdóttir et al. (2015) Santos et al. (2008) D'Aimmo et al. (2012) Rossi et al. (2011) Strozzi and Mogna (2008)
8.	B_{12} (cobalamin)	*Prevotella copri* *Bacteroides fragilis* *L. fermentum* CECT 5716 *Fusobacterium varium*	Deguchi et al. (1985) Magnúsdóttir et al. (2015) Cardenas et al. (2015) Lee and O'Sullivan (2010)

and genetic engineering. A study has suggested that *Bacillus megaterium* can be utilized for the production of biotechnologically important molecules. Another study has demonstrated that the microbial fermentation of riboflavin can be achieved through genetically modified *Ashbya gossypii (A. gossypii*). The RIB genes contribute to the production of riboflavin in *A. gossypii*. An RIB-gene-modified *A. gossypii strain* has produced 5.4-fold more riboflavin than the wild type (Ledesma-Amaro et al. 2015). *B. subtilis* is widely used as vitamin B_2 (riboflavin)-producing strains. A biosynthetic mechanism of riboflavin in *B. subtilis* has been well-established, and a

study has demonstrated it through the combined approaches of metabolomics and transcriptomics and ^{13}C metabolic flux analysis under various dissolved oxygen (DO) tension states. In ResD-ResE system, DO has been utilized as the signal receiver to analyze the differences between riboflavin synthesis and biomass (Hu et al. 2017).

Vitamin B_3 (Niacin) occurs in three forms that are enzymatically changed into the important cofactors (Rajman et al. 2018). An industrial fermentation process for vitamin B_3 is still not established (Chand and Savitri 2016) although biocatalytic methods exist that use 3-cyanopyridine as a first material that is hydrolyzed to niacin by a nitrilase or hydrated to niacinamide by a nitrile hydratase (Chuck 2009). Vitamin B_5 (D-Pantothenic acid) is widely used, and its microbial production mainly depends on the pantothenate synthetase (PS) enzyme. A recent study has utilized different phylogenetically dissimilar PS-encoding genes, from *B. subtilis*, *E. coli*, *Bacillus thuringiensis*, *Bacillus cereus*, *Enterobacter cloacae*, and *Corynebacterium glutamicum (C. glutamicum)* to overexpression in *E. coli.* The maximum specific activity (205.1 U/mg) and turnover number (127.6 s^{-1}) have been shown by *C. glutamicum* (Tigu et al. 2018). A notion of phylogenetically distant based study should offer support to other researchers that are thinking similar planned work.

9.3 Microbial Production of Vitamin B_6, B_7, and B_9

Vitamin B_6 (pyridoxine) is a biologically very important nutrient, which acts as a cofactor for several enzymes. However, vitamin B6 is synthesized by microorganisms and plants. For the production of pyridoxine, *B. subtilis* has been utilized. The strain produced 14 mg/L pyridoxine in a small-scale production assay. On the other hand, by improving the growth environments and co-feeding of deoxyxylulose and 4-hydroxy-threonine, the yield has been improved to 54 mg/L (Commichau et al. 2014).

Vitamin B_7 (or biotin) is a vital cofactor for carboxylation reactions. Biotin intermediate pimelic acid is produced by two different ways (Lin and Cronan 2011). Previously, efforts for engineering biotin synthesis strains using random mutagenesis and antimetabolites encountered insufficient achievement. The maximum biotin titer described is 500 mg/L with *Serratia marcescens* after 10 days of fermentation (Streit and Entcheva 2003). Vitamin B_9 (Folic acid) is the common name of folates that play a vital function as cofactors in one-carbon transfer reactions. Folates are contributed to the metabolism and biosynthesis of different biomolecules such as hormones, lipids, DNA, and proteins. A recent study has shown the production of folic acid through a fungus *A. gossypii.* Engineered strains of *A. gossypii* has produced a 146-fold vitamin B_9 as compared to the wild type (Serrano-Amatriain et al. 2016). However, folic acids are mostly synthesized through chemical methods.

9.4 Microbial Production of Vitamin B_{12}

Vitamin B_{12} is an important nutrient, which is essential for vital metabolic activities in humans. Presently vitamin B_{12}-producing lactic acid bacteria (LAB) have been considered significantly because of the generally recognized as safe (GRAS) position. Recent study has demonstrated the production of vitamin B_{12} (adenosylcobalamin) from the engineered *E. coli* strain. A study has shown about 250-fold increase in the production of vitamin B_{12} using recombinant *E. coli* strain (Fang et al. 2018). It has presumed that adenosylcobinamide (AdoCbi) is synthesized through the attachment of (R)-1-amino-2-propanol (AP) to AdoCby to yield AdoCbi in a single step reaction, which is catalyzed by a two-component system (designated as α and β in *Paracoccus denitrificans*) (Fig. 9.1).

A very significant coenzyme vitamin B_{12} (cobalamin) in the cell metabolism has been broadly used in therapeutic and food industries. The broad biosynthesis of VB_{12} requires about 30 genes; nevertheless, overexpression of these genes did not result in an estimated rise in VB_{12} production (Cai et al. 2018). *Propionibacterium*

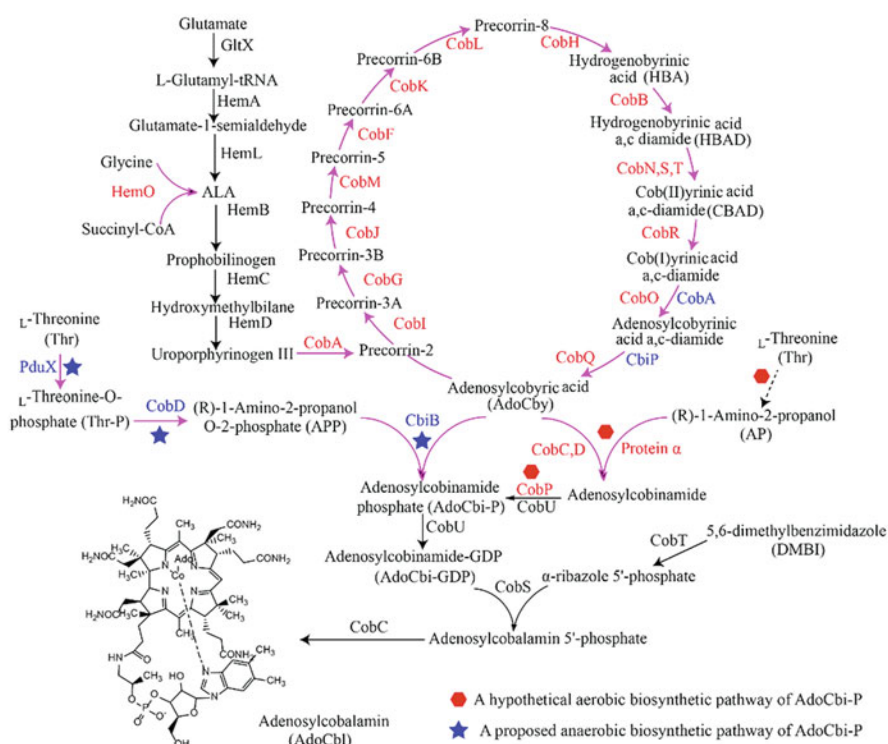

Fig. 9.1 Biosynthetic pathway of adenosylcobalamin. Endogenous enzymes from *E. coli* are shown in black. Enzymes from aerobic bacteria such as *Brucella melitensis*, *Rhodobacter capsulatus*, *Sinorhizobium meliloti*, and *Rhodopseudomonas palustris* are shown in magenta. Enzymes from *Salmonella typhimurium* are shown in blue. Ado represents the abbreviation of adenosyl (Fang et al. 2018. *Adapted with permission*)

freudenreichii has been utilized for overexpression of fusion enzyme BluB/CoBT2. This enzyme is responsible for the biosynthesis of the 5,6-dimethylbenzimidazole, which plays a vital role in the biosynthetic pathway of vitamin B12 (Deptula et al. 2015). *Gluconobacter oxydans* NBRC3293 strain produces 2,5-diketo-D-gluconate (2,5DKG) from D-glucose via D-gluconate and 2-keto-D-gluconate (2KG). This compound acts as an intermediate for the production of vitamin C (Kataoka et al. 2015).

Liquid chromatography-selected reaction monitoring mass spectrometry assays (LC-SRM-MS) has been utilized to investigate the proteins involved in vitamin B_{12} production from marine microbial populations. Use of this technique is helpful to analyze the nutritional status of microbial community members with respect to vitamin B_{12} production (Bertrand 2018). For the production of vitamin B_{12}, *Sinorhizobium meliloti* has been utilized using the novel mutation technique of atmospheric and room temperature plasma (ARTP). In this study, a riboswitch element has been used from *Salmonella typhimurium*, and it provides a convenient high-throughput assessment technique for increasing high VB_{12}-yield strains (Cai et al. 2018). Recent new technologies such as DNA microarray, proteomic, and metabolic investigations have been utilized to enhance the production of riboflavin using *A. gossypii* (Kato and Park 2012). *Ketogulonigenium vulgare* (*K. vulgare*) strain has been utilized for the overproduction of 2-keto-L-gulonic acid (2-KGA) that is a precursor of vitamin C. L-sorbosone dehydrogenase (SNDH) is one of the key enzymes for the biosynthesis of 2-KGA. As per whole genome sequence analysis of *K. vulgare*, it has been demonstrated that two genes were encoding sorbosone dehydrogenases, one derived from the chromosome (named as sndhg) and the other from the plasmid (named as sndhp) (Chen et al. 2016a). For the improvement of the production of 2-KGA, in silico approach has been utilized. In the study, L-sorbose dehydrogenases (SDH) genes of *K. vulgare* has been modeled. For molecular docking, six SDHs have been used for the prediction of binding mode with cofactor pyrroloquinoline quinone (PQQ). After docking, these genes were overexpressed in *K. vulgare* HKv604 and found significant enhancement (7.89–12.56%) (Chen et al. 2016b). Previously genomics- and proteomics-based studies have investigated SDH and SNDH from *Gluconobacter oxydans* T-100 strain. These two enzymes have the ability to convert D-sorbitol to 2-keto-L-gulonate (2-KLGA). Significant production from D-sorbitol to 2-KLGA (130 mg/mL) had been achieved through recombinant *Gluconobacter* using fermentation (Saito et al. 1997).

Akkermansia muciniphila involves in the degradation of mucus sugars into oligosaccharides. After degradation and release of oligosaccharides, it becomes available for various intestinal microbes for microbial synthesis of vitamin B_{12} and other organic molecules (Belzer et al. 2017). Squalene is a triterpene compound and usually found in numerous organisms such as bacteria, fungi, algae, plants, and animals. It acts as a precursor for the synthesis of vitamins (Ghimire et al. 2016). *Bacillus megaterium* has been utilized to produce a large scale of vitamin B_{12}. After providing an essential supplement, it has reached up to 204.46 μg/mL of the B_{12} production as compared with control (0.26 μg/mL) (Mohammed et al. 2014).

Several studies have utilized *Lactobacillus* and *Enterococcus* for the microbial production of vitamin B_{12}. A study has utilized five *Enterococcus* strains isolated from infant feces for the production of vitamin B_{12}. *Enterococcus faecium* LZ86 has shown the highest B_{12} production (499.8 ± 83.7 μg/L), among all five strains of *Enterococcus* (Li et al. 2017a). Similarly, another study has demonstrated vitamin B_{12}-producing *Lactobacillus* strains and their characteristics in tolerance to environmental stresses, gastric acid, and bile salts. Two isolates *Lactobacillus plantarum* LZ95 and CY2 exhibited great extracellular B_{12} production of 98 ± 15 μg/L and 60 ± 9 μg/L, respectively (Li et al. 2017b). Anaerobic biosynthesis of the lower ligand of vitamin B_{12} 5,6-dimethylbenzimidazole (DMB) has been investigated in the obligate anaerobic bacterium *Eubacterium limosum* (Hazra et al. 2015).

Propionibacterium freudenreichii subsp. shermanii has been grown on the spent media previously used by lactic acid bacteria (LAB) for the production of vitamin B_{12}. A study has demonstrated that utilized media could be reused for the production of *Propionibacterium* and metabolites, depending on the LAB strain that was earlier grown. Media remediation is needed to improve the production of vitamin B_{12}, particularly by immobilized cells (Gardner and Champagne 2005). This investigation presents a possibility of reutilizing the used media generated by the producers of LAB or producers of fermented vegetables. It is an attractive procedure from cost-effective and eco-friendly positions.

9.5 Concluding Remarks

Vitamins are not synthesized by humans and animals, and therefore it is required from other sources. Trace amounts (~1 μg/day) of vitamins are required for the nourishment of humans. However, vitamin deficiency is a critical problem of micronutrient malnutrition affecting billions of individuals globally. Consequently, the supplement of some vitamins into food has been adapted as compulsory in several nations, therefore, adding to a rising need of vitamin. Vitamin B_{12}-producing *Enterococcus faecium* strain LZ86 and *Lactobacillus plantarum* LZ95 have essential probiotic properties, and may help as a good candidate for vitamin B_{12} enrichment in the food industry. Furthermore, there is a need to identify a better microbial strain, which can produce large quantities of vitamins to fulfill the current demands. To identify the better microbial strain, we can explore the different omics approaches such as metagenomics, metatranscriptomics, metaproteomics, and metabolomics.

Competing Interests: There is no competing interest.

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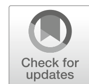

Bacterial Production of Organic Acids and Subsequent Metabolism

10

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Abstract

Adequate understanding of a microbial-mediated process is the key to its successful operation. It involves the study of microbes, process metabolism, substrate degradation, and by-product formation. As compared to aerobic process, the anaerobic process is more complex as it involves multistep interdependent stages of metabolism in series. Thus an anaerobic process is carried out by syntrophic microbial consortium which altogether makes the metabolic reactions thermodynamically feasible. Among all the bacteria, methanogens are highly vulnerable and sensitive to variations in the conditions of environment and thus they are considered rate-limiting agents for the overall process. These methanogens thus demand a meticulous process control for the stable operation.

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In this chapter, various metabolic stages of anaerobic processes and related microbiological aspects are presented in detail.

Keywords

Microbial production · Organic acids · Anaerobic processes · Acidogenesis · Methanogenesis · Metabolic products · Shock imposition · Hydrogen partial pressure · Microbial diversity · Syntrophic relationship

10.1 Introduction

Anaerobic production of organic acids is a multistep biological process having complex combination of various processes involving several groups of microorganisms which collectively act to convert organic carbon into organic acids. Fundamental understanding of these various processes, their energetics, and microorganisms involved and their interdependence shall be essential to critically analyze and design the process requirements to overcome the limitations commonly faced by any anaerobic processes. Production of organic acids using microbe-mediated anaerobic acidogenesis process is a sustainable approach. However, the various by-products produced along with organic acids affect the overall stability of the process. Thus, to control these various by-products becomes highly important for the acidogenesis process. Therefore, it is highly desired to control the operating conditions practically in order to selectively produce various organic acids and other by-products. pH has been reported as the indicator parameter by various authors as it is great influencer in shifting and driving the bioprocess in a particular direction (Zoetemeyer et al. 1982). Further, the pH trends not only present the progress of acidogenesis in anaerobic processes but also an important parameter for the selective production of a specific organic acid during the acidogenesis. It can be easily understood from the scenario that pH plays a vital role in selective production of a variety of organic acids. Thus the understanding of the significance of pH on the organic acid production during anaerobic process is of utmost important.

10.2 Anaerobic Biotechnology

Anaerobic processes are broadly categorized into four sub-processes which are well known as hydrolysis, fermentation/acidogenesis, acetogenesis, and methanogenesis, respectively. Process of anaerobic metabolism starts with hydrolysis which basically refers to the formation of simpler/low molecules weight compounds such as oligomers and monomers from its precursor molecules of complex/high-molecular-weight organic compounds. These simpler compounds have the tendency to be easily consumed/absorbed inside their cells of fermenting bacteria. More specifically, simple sugars, amino acids, and long-chain fatty acids (LCFA) and glycerol

are produced from proteins, carbohydrates, and lipids, respectively. While oxidation of LCFA obligately requires an electron acceptor, sugar and amino acid are capable of getting oxidized in the absence of electron acceptors such as oxygen, nitrate, nitrite, sulfate through a process called fermentation (Pandey and Sarkar 2017; Stanbury et al. 2013). The most common factors responsible for different pathways in fermentation of sugars such as glucose, include pH, concentration of substrate, and dissolved hydrogen (Rodriguez et al. 2006; Murto et al. 2004). Such factors are responsible for quantitative as well as qualitative production of different end products, i.e., hydrogen (H_2), volatile fatty acids (VFAs), alcohols. Furthermore, energy associated with different pathways is also reported to be the function of these factors (Pandey and Sarkar 2019a; Thauer et al. 1977; Rodriguez et al. 2006). The process of fermentation begins with conversion of glucose, a 6-carbon (C6) molecule, into pyruvic acid (C3) in an oxygen-independent pathway where the electrons released from glucose molecule are taken up by NAD^+ which in turn reduces to NADH. The Gibbs free energy for the conversion of NAD^+ to NADH is positive, meaning that energy must be taken from the organic molecule being oxidized. In order to keep the process running sustainably, NADH should be re-oxidized back to NAD^+ so that it can be reused again. Re-oxidation of NADH into NAD^+ involves extraction of electrons and passing them onto another electron acceptor or to another carrier, with a concomitant release of chemical energy which may be converted to other useful forms. When the electron acceptor such as oxygen is absent, nitrate or sulfate, the electron is channelized to reduce protons to form H_2. Pyruvate formed from the glucose molecules can be oxidized to acetate through reaction with acetyl coA enzyme while ferredoxin ferries the electrons to H_2. Other pathways are possible in which much reduced end products such as butyric acid, propionic acid, valeric acid, lactic acid, and ethanol may be produced. Eventually, these end products which have more than two carbon atoms are again metabolized to form acetate through a process known as acetogenesis where along with acetate, H_2 is also produced. Furthermore, there are two possible different pathways for the conversion of acetic acid into methane. In one of pathways where methanogenesis occurs directly is known as aceticlastic methanogens. In this pathway, CH_4 and CO_2 are directly produced from the carboxyl and methyl groups of acetic acid. Hydrogenotrophic methanogenesis is an alternate pathway for methane production. CO_2, produced as a by-product of the fermentation, hydrolysis, or acetogenesis process reacts with H_2 to form methane gas; this process is known as hydrogenotrophic methanogenesis. It is also possible that through a process known as syntrophic acetate oxidation, acetic acid can be oxidized to carbon dioxide and hydrogen, which are further transformed into CH_4 via hydrogenotrophic methanogenesis. The CO_2 and H_2 thus formed can also led to the production of acetic acid through a process called homoacetogenesis. Compared to all the other processes that the anaerobic degradation of organic compounds consists of, the methanogenesis process, more specifically the aceticlastic methanogenesis where decomposition of acetic acid takes place to form methane, is more sensitive to changes in temperature, organic matters composition, pH, reactor configuration, and organic loading rate. In case of anaerobic degradation of wastewater containing

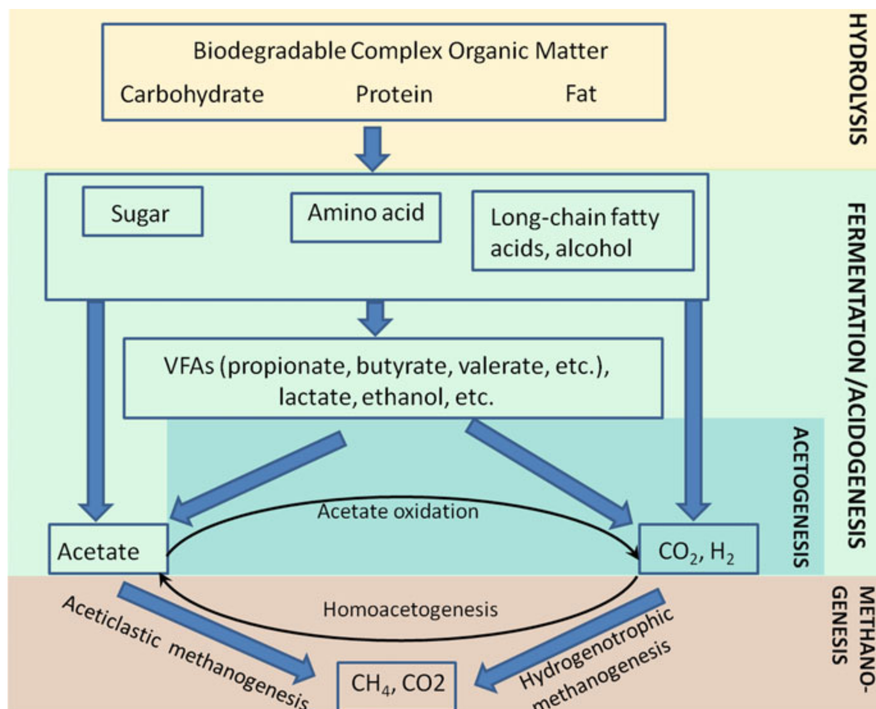


Fig. 10.1 Diagram showing complete progression of an anaerobic treatment process carried out in steps by different classes of anaerobic bacteria

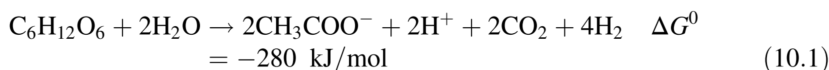
simpler organic molecules, the aceticlastic methanogenesis is the slowest process compared to other processes; hence, it is the rate-limiting process in the anaerobic treatment of wastewater. However, in case of wastewater containing particulate organic material or complex soluble substrate hydrolysis becomes the slowest step, making it the rate-controlling step. Being major pathway, aceticlastic methanogenesis contributes to methane production up to 72–77% (Khanal 2011; Wang et al. 2013). Figure 10.1 shows the different metabolic pathways of anaerobic degradation.

10.3 Thermodynamic Basis of Various Processes Constituting Anaerobic Production of Organic Acid

Redox reactions, accomplished by the microorganisms, helps in maintaining the energy requirements for cell growth and maintenance. In most, if not all, of the biological processes, the electron(s) are removed from the primary donor molecule and are ferried to a terminal electron accepting molecule via one or more number of electron carriers. The donor molecule thus gets oxidized whereas the electron acceptor gets reduced, while the electron carrier being a simple transporter does

not undergo any net change. The transfer steps are associated with a free-energy release that is captured by the cells of the microorganisms in the form of energy carriers. In anaerobic treatment process, first group of bacteria (hydrolytic bacteria) converts the complex/high molecular compounds into monomers/low-molecular-weight compounds such as glucose, amino acids, glycerol, and fatty acids. Second group of bacteria (fermentative or acidogenic bacteria) further convert these monomers through redox reactions.

For each molecule of glucose, four moles of H_2 is produced along with the formation of two moles of carbon dioxide as per the following equation:



$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COO^- + 2H^+ + 2CO_2 + 4H_2 \quad \Delta G^0 = -280 \text{ kJ/mol} \tag{10.1}$$

The change in free energy (ΔG) of the reaction is given by

$$\Delta G = \Delta G^0 + \text{RT} \ln \frac{[CH_3COO^-]^2 p_{CO_2}^2 p_{H_2}^4 [H^+]^2}{[C_6H_{12}O_6]} \tag{10.2}$$

where, [] stands for the molar concentration of the component and p stands for the partial pressure. The high negative value of ΔG^0 indicates that the reaction is spontaneous and significant amount of energy would be possible to be extracted from the reaction even when the reactants and products are present in unit concentrations. However, it is also evident from eq. 10.2 that Gibbs free-energy change, ΔG, is directly proportional (fourth power of the partial pressure of hydrogen) to the partial pressure of hydrogen. So, the system where hydrogen scavenging activity is not present or has been compromised, reaction 10.1 may get inhibited and hydrogen ion concentration increases. The maintenance of low hydrogen partial pressure is accomplished by hydrogenotrophic organisms, such as hydrogenotrophic methanogens which produce methane as per the following reaction:

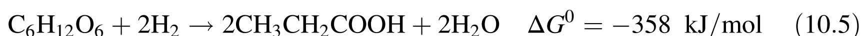
$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad \Delta G^0 = -136 \text{ kJ/mol} \tag{10.3}$$

As per reactions 10.1 and 10.3, it may also be observed that accumulation of other reaction end products such as acetate also may cause rise in the ΔG value, making the forward reaction less favorable. However, impact of accumulation of acetate shall not have that great an impact on the forward reaction as accumulation of hydrogen would have. Therefore, evacuation of acetate is also important, but more important is the evacuation of hydrogen from the system. Acetate eventually gets converted to CH_4 by the aceticlastic microorganism as per the following reaction:

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad \Delta G^0 = -31 \text{ kJ/mol} \tag{10.4}$$

The above reaction pathway is known as aceticlastic methanogenesis. Maintenance of low concentration or low partial pressure of hydrogen by hydrogenotrophic microorganisms results in faster fermentation rates (Schink 1997; Kuntze et al. 2008;

Zhang et al. 2009). At the attainment of the threshold, the H_2 production being faster than its consumption, fermenting organisms should slow down its H_2 production. This causes a shift in fermentation reaction in such a way that metabolites which are in more reduced form such as propionate (reaction 10.5), butyrate (reaction 10.6), ethanol (reaction 10.7), and lactate (reaction 10.8) are produced. In fact, the Gibbs free-energy changes for the reactions are more energetic than the acetate reaction, the highest being for propionate (−358 kJ/mol), butyrate (−255 kJ/mol), ethanol (−226 kJ/mol), etc. For lactate fermentation, ΔG^0 is lower than that for acetate fermentation (−198 kJ/mol). The relevant reactions are indicated below as follows:



$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \quad \Delta G^0 = -358\ \text{kJ/mol} \quad (10.5)$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad \Delta G^0 = -255\ \text{kJ/mol} \quad (10.6)$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2OH + 2CO_2 + 2H_2 \quad \Delta G^0 = -226\ \text{kJ/mol} \quad (10.7)$$

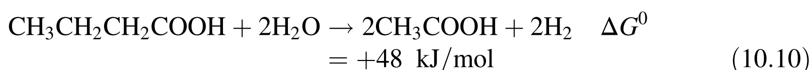
$$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH \quad \Delta G^0 = -198\ \text{kJ/mol} \quad (10.8)$$

At low partial pressure of hydrogen (<10 Pa), electrons are released as hydrogen molecules following reaction 10.1, through which more acetate, hydrogen, and CO_2 will be produced rather than formation of butyrate or ethanol, or other more reduced products following the other fermentation reactions. In a well-balanced anaerobic system, it is must to achieve low hydrogen partial pressure through hydrogen scavenging microorganisms; the mass diffusion of carbon and electrons takes place exclusively through the acetate, carbon dioxide, and hydrogen pathway. Whereas, the minor role is played by the reduced fermentation products such as fatty acids. Lactate is formed when the concentration of the substrate or glucose is significantly high. Normally such a situation is not encountered during wastewater treatment processes. Therefore, while undergoing anaerobic decomposition of wastewater containing COD mainly due to the presence of intermediate metabolites of treatment process such as glucose and other volatile fatty acids (acetate, propionate, butyrate, etc.). Please note that out of the VFA intermediates, formation of propionate does not produce any H_2, rather consumes two moles of H_2. Fermentation of butyrate produces two moles of H_2 as compared to four moles H_2 produced during the fermentation of acetate. Thus, it may be concluded that the role of the H_2 scavengers is important for the sustainable production of VFA intermediates like acetate and butyrate, however not so much for the production of propionic acid. Thus, the products from the first stage, i.e., fermentation, generally consist of approximately acetate (51%), H_2 (19%), and other reduced products like alcohols, higher VFA, or lactate (Angelidaki and Sanders 2004). The reduced intermediates such as VFAs would become extremely important if elevated concentration levels of hydrogen are observed. These levels can be attributed to the increased concentration of substrate for fermentation, inhibition of hydrogenotrophic methanogens probably due to pH drop to a level of <6.0, and availability of toxic substances.

The methanogenesis process cannot directly consume the fermentation products or VFA having carbon atoms three or more, alcohols having more than one carbon atom, fatty acids having branched chains or aromatic fatty acids. These complex compounds first need to be oxidized into acetate and hydrogen through acetogenesis stage before the onward conversion of the products to methane through either aceticlastic or hydrogenotrophic methanogenesis pathway. Propionate is considered as a dominant volatile fatty acid and a precursor to about 35% (by mol) of total methane produced during anaerobic digestion.

Below reactions show the reaction pathways through which propionic and butyric acids are oxidized to acetic acid.

$$CH_3CH_2COOH + 2H_2O \rightarrow CH_3COOH + CO_2 + 3H_2 \quad \Delta G^0 = +72 \ \text{kJ/mol} \tag{10.9}$$



$$CH_3CH_2CH_2COOH + 2H_2O \rightarrow 2CH_3COOH + 2H_2 \quad \Delta G^0 = +48 \ \text{kJ/mol} \tag{10.10}$$

The changes in free energy of the above reactions are calculated as per the following equations:

$$\Delta G = \Delta G^0 + \text{RT} \ln \frac{[CH_3COOH] p_{CO_2} p_{H_2}^3}{[CH_3CH_2COOH]} \tag{10.11}$$

$$\Delta G = \Delta G^0 + \text{RT} \ln \frac{[CH_3COOH]^2 p_{H_2}^2}{[CH_3CH_2CH_2COOH]} \tag{10.12}$$

It can be inferred from reactions 10.9 and 10.10 that the change in Gibbs free energy, i.e., ΔG^0, is mainly positive. The positive value indicates that under unit activity (concentration) of the reactants and the products, under standard conditions the forward reaction is not spontaneously possible. Further, the negative values of change in Gibbs free energy (ΔG) favor the forward reaction. Eqs. 10.11 and 10.12 suggest that in order that the overall value of ΔG becomes negative, it is required that concentration or partial pressure of H_2 should be as low as possible. In other words, sustaining the low values of hydrogen partial pressure can shift the reaction stoichiometry of propionate and butyrate degradation into acetate. The eqs. 10.9 through 10.12 also suggest that due to greater amount of hydrogen produced, oxidation of propionate is more susceptible to the changes in partial pressure of H_2, as compared to butyrate. Calculations show that for the oxidation of propionate to acetic acid to take place, the partial pressure of H_2 should be kept between 10^{-4} and 10^{-6} bar (Azbar et al. 2001). Such low pressure of H_2 can be maintained through hydrogenotrophic methanogens, and methane formation will take place via reaction 10.3.

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad \Delta G^0 = -136 \text{ kJ/mol} \tag{10.3}$$

Aceticlastic methanogens cleave the acetic acid produced during the fermentation and acetogenesis process and convert it into methane and carbon dioxide according to reaction 10.4:

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad \Delta G^0 = -31 \text{ kJ/mol} \tag{10.4}$$

10.4 Interspecies Hydrogen Transfer and its Implications

From the reactions and eqs. 10.9 through 10.1, 10.2, 10.3, and 10.4; it may be concluded that in order to achieve the oxidation of VFAs into acetic acid, the driving force between acetogenic bacteria and hydrogenoptrophic methanogenic bacteria is interspecies hydrogen transfer. This is a kind of symbiotic relationship between two or more otherwise dissimilar group of bacteria in the interest of gaining energy from the degradation of a common substrate and is known as syntrophic relationship (Schink 1997). Such association helps the species involved to gain energy from reactions, which are not thermodynamically feasible under standard/normal conditions. One such example of syntrophic association is the anaerobic oxidation of propionate. The complete mineralization of propionate in anaerobic condition requires syntrophic participation of three groups of bacteria, namely (1) acetogenic bacteria for conversion of propionate to acetate, (2) hydrogenotrophic bacteria for scavenging of hydrogen from the acetogenesis reaction to convert it into methane, and (3) aceticlastic bacteria which help to convert acetate into methane. Individually, even at low hydrogen concentration, the degradation of propionate would not generate enough energy for the sustenance of the acetogens unless both the processes, i.e., degradation of propionate with hydrogenotrophic and aceticlastic methane production, take place simultaneously. Same can be observed in reactions 10.9, 10.3, and 10.4. Combination of these reactions results in a net ΔG^0 value of about −56 kJ/mol which is approximately equivalent to the energy required for generation of a molecule of ATP from ADP. Therefore, each of the syntrophic partners at the end of degradation get a share of energy of approximately one-third of ATP if it is equally shared. However, it was later proven that there is no such minimum quantum of energy requirement; the reactions can run at the availability of smallest possible energy also. (Jackson and McInerney 2002). Figure 10.2 illustrates a schematic on the importance of hydrogen transfer in various stages of anaerobic treatment.

Working under a severe energy limited condition demands that the participating microorganisms must organize themselves in order to overcome the inconveniences in growth. The minimum requirement is efficient evacuation of hydrogen gas from the producer (acetogens), and to ensure its maximum availability to the consumers (hydrogenotrophic methanogens). In other words, the flux of hydrogen from the producer to the consumer should be the maximum. Fick's first law of diffusion states

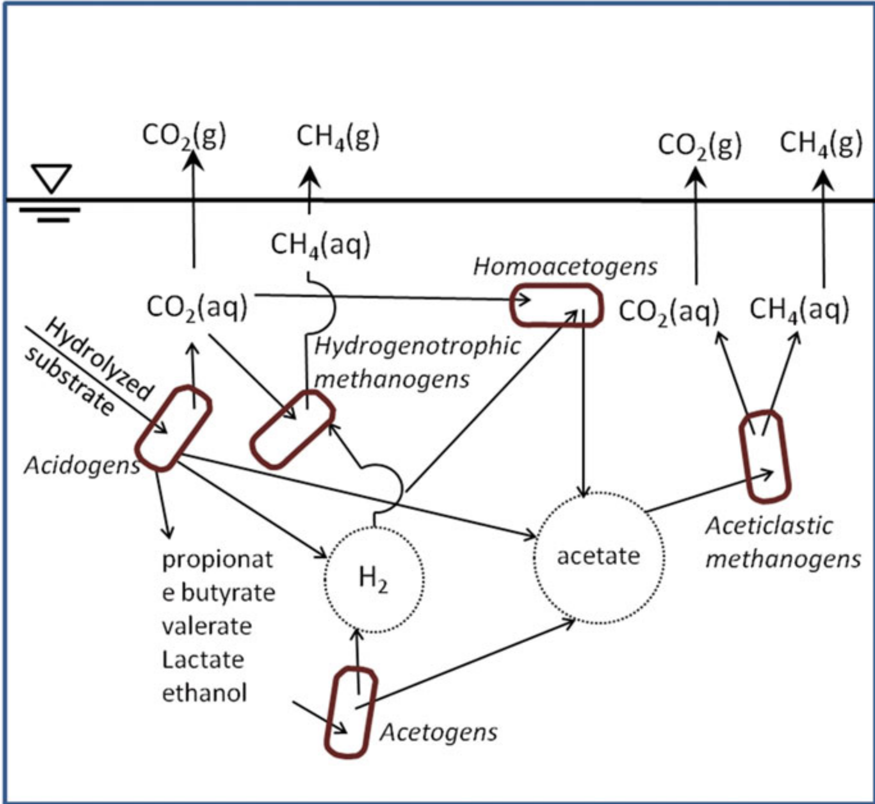


Fig. 10.2 Schematic showing the importance of interspecies hydrogen transfers in various processes in anaerobic treatment

that the hydrogen flux from producer to the consumer can be formulated as the following equation:

$$J_{H_2} = A_P \cdot D_{H_2} \frac{[H_2]_p - [H_2]_C}{d_{PC}} \tag{10.13}$$

where, $J =$ flux, $A =$ surface area of propionate degrading microorganism; $d =$ distance; $D =$ diffusion constant in water, [] stands for concentration and the subscripts H_2, subscripts P and C stand for hydrogen, producer, and consumer, respectively.

It follows from the above equation that the flux of hydrogen will be the maximum at the minimum distance between interspecies, i.e., syntrophic acetogen and hydrogenotrophic methanogen. Therefore, it is likely that in order to maximize the flux of hydrogen, the syntrophic partners would like to maintain the interspecies distance as small as possible. Such small distances between interspecies can be maintained through aggregate forms of microbes such as granules. It was

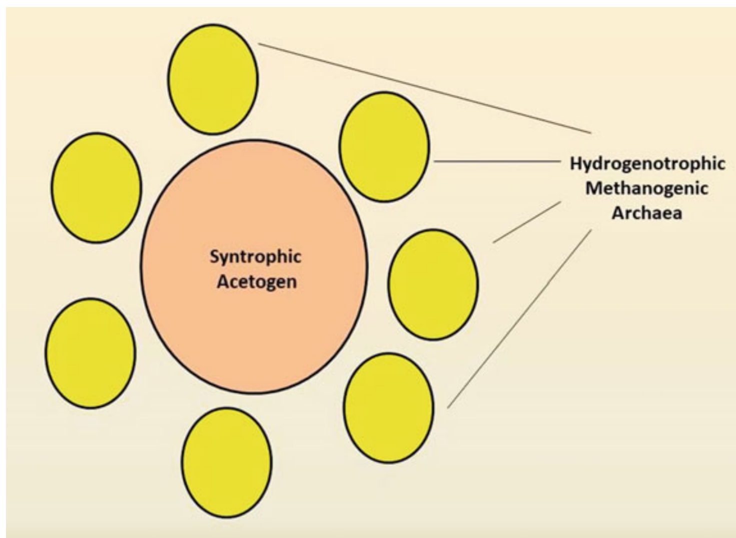


Fig. 10.3 Syntrophic acetogen surrounded by a number of hydrogenotrophic methanogenic archaea

demonstrated that bacterial cultures offered a higher rate of propionate degradation in aggregated form as compared to those in suspended form (Cobb and Hill 1991). A biofilm or granular anaerobic reactor could be found to degrade propionate efficiently (Singh et al. 2016; Tatara et al. 2008; Zheng et al. 2009; Zellner and Neudörfer 1995). In this configuration, abundance of syntrophs remains unexposed to high partial pressure of H_2 of the reactor indirectly. Kus and Wiesmann (1995) reported that in a mixed culture grown on a porous support and effectively degrading propionate could resist the inhibitory effect of high concentration of H_2 which was added from outside. Figure 10.3 is an evidence of close packing of syntrophic association within a granular structure which was collected from a reactor where syntrophic reactions were taking place (De Bok et al. 2005). The De Bok et al. (2005) showed in his study that one large syntrophic acetogen remains surrounded by a number of hydrogenotrophic methanogenic archaea. This is a direct proof that the syntrophic partners should form aggregates in such a way that there is maximization of hydrogen flux during interspecies hydrogen transfer between the participating organisms.

It is also possible that the granules formed in continuously stirred tank reactor may also help in the complete degradation of carbohydrate into the end products. Fig. 10.4 provides a conceptual structure of a granule for optimal complete anaerobic degradation of carbohydrates, where routes through propionic acid and butyric acid are also possible (Liu et al. 2003).

It is also possible that being small in size, formic acid can also at as carrier of electron. Further, it can be easily diffused, just like hydrogen. Literature also revealed that CO_2/formate couple has nearly similar value of redox potential as

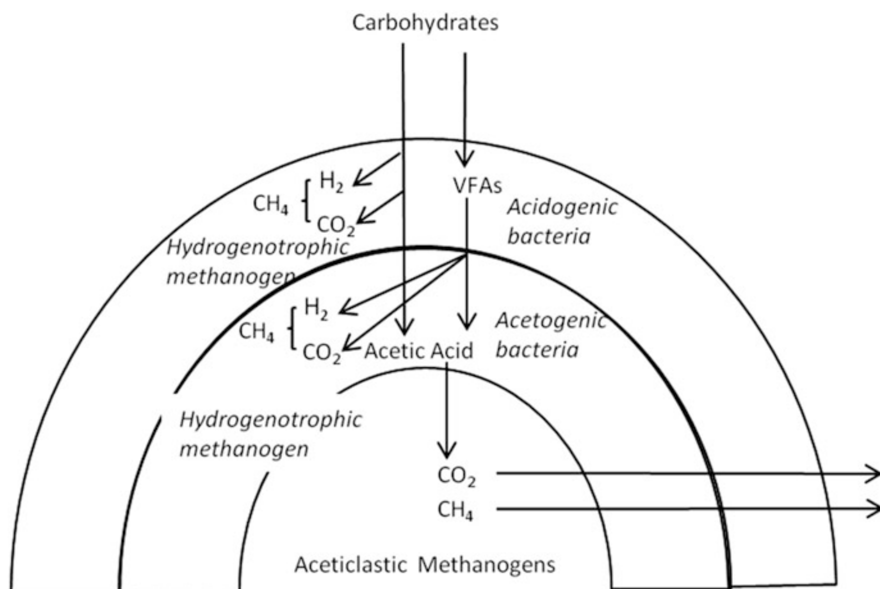


Fig. 10.4 A conceptual diagram of a possible granular structure of microbial aggregate responsible for the complete anaerobic conversion of carbohydrate to methane and carbon dioxide

H^+/H_2 combination. Because of similarities, it is difficult to predict whether formate or hydrogen acts as electron carrier and takes part in interspecies transfer. However, some recent experimental data have pointed out to the abundance of hydrogen as electron carrier (Schink 1997). The diffusion kinetics of anaerobic processes suggested that suspended growth systems are expected to offer formate/CO_2 couple as a preferred electron transfer system, where carrier has the tendency to diffuse a long distance in an aqueous medium. On the other hand, hydrogen is reported to be offering a better electron transfer system in attached/biofilm growth systems.

10.5 Microbial Diversity within an Anaerobic Reactor

The overall anaerobic process is governed by the functioning of the four trophic groups of bacteria, which are named as hydrolytic, acidogenic, acetogenic bacteria, and methanogenic archaea. Figure 10.5 illustrates the different groups of bacteria that are involved in different processes that an anaerobic treatment process generally consists of. During the hydrolysis stage of the anaerobic metabolism, low molecular weight soluble compounds (sugars, long-chain fatty acids, amino acids, glycerin, etc.) are formed from their high molecular weight precursors like carbohydrates, proteins, and lipids. These conversions are facilitated by the action of extracellular enzymes, excreted by the hydrolytic bacteria. On the basis of the nature of substrate, there may be diverse group of the hydrolytic bacteria. *Clostridium*, *Bacteroides,*

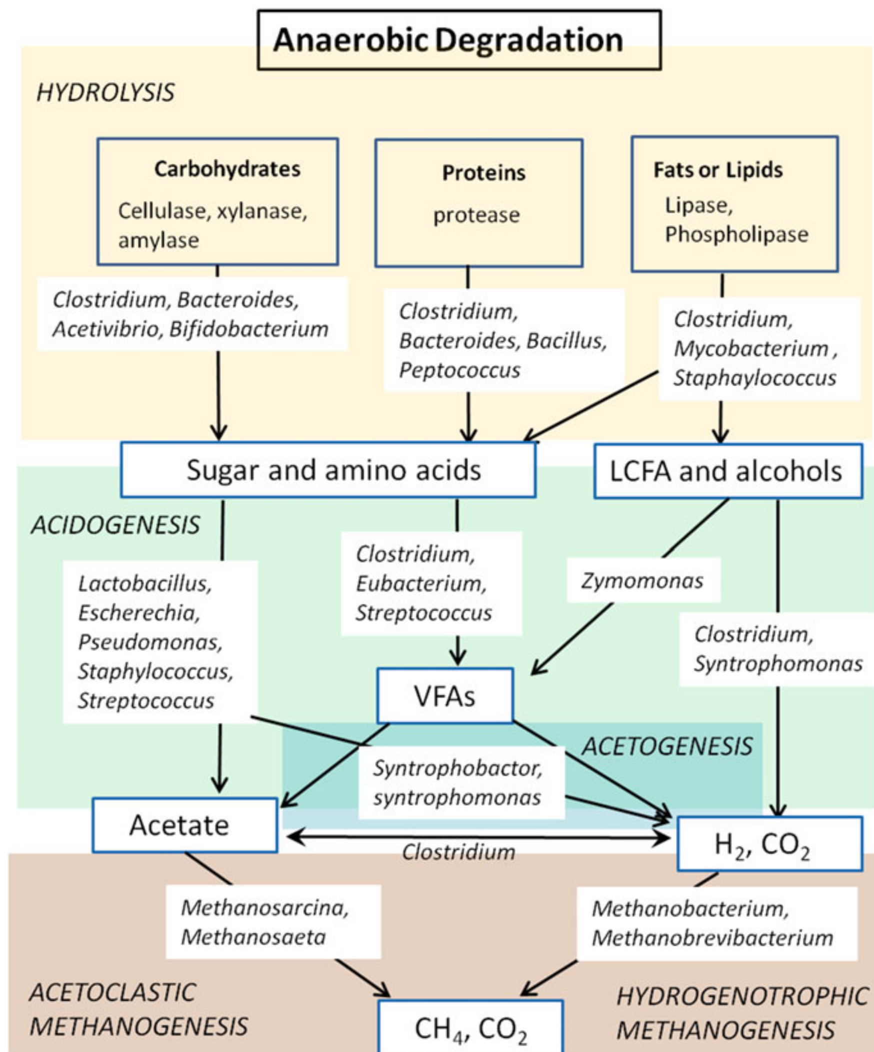


Fig. 10.5 Schematic showing the involvement of diverse group of bacteria during the process of anaerobic treatment of wastewater

Cellulomonas, Acetivibrio, etc. produce extracellular enzymes such as cellulase, amylase, and xylanase to convert the carbohydrate polymers into monomers such as simple sugar or glucose. *Clostridium*, *Bacillus*, *Peptococcus*, etc. are responsible for breaking down of protein into amino acids with or without concomitant generation of simple sugar. Species like *Clostridium*, *Mycobacterium*, and *Staphylococcus* are responsible for the release of enzymes like lipase and phospholipase to convert fats or lipids into simple sugar and long-chain fatty acids (LCFAs) with or without the concomitant formation of alcohols. In fermentation or acidogenesis process, the

simple monomers are converted into short-chain fatty acids or volatile fatty acids such as acetate, butyrate, propionate, and valerate along with the formation of other compounds such as lactate, ethanol, butanediol, formate, and succinate to a smaller extent. A vast diversity of bacteria is capable of such transformation. Major acetate-producing bacteria belong to the genera *Acetobacterium*, *Clostridium*, and *Sporomusa*. Although the main domain of ethanol fermentation is by yeast, such as *Saccharomyces*, other genera such as *Erwinia, Sarcina*, and *Zymomonas* can produce ethanol due to the fermentation. At low pH values, *Enterobacter* and *Serratia* are two genre that produce alcohol from glucose. Strictly anaerobic bacteria of the genera *Clostridium* and *Butyrivibrio* are known to ferment sugars into butyric acid. Under low pH, some clostridium bacteria are known for the small amount of production of *n*-butanol and acetone. The lactate-producing genera are generally *Bifidobacterium, Lactobacillus, Streptococcus, Leuconostoc*. Propionate and succinate are produced by the genera like *Bacteroides, Clostridium*, *Peptostreptococcus*, and *Selenomonas*. Various bacteria of the genera *Salmonella*, *Escherichia*, *Serratia*, *Enterobacter*, *Erwinia*, and *Shigella* are very well known to carry out mixed acid fermentation—acetate, formate, lactate, and succinate from sugar. Acidogenesis or fermentation is the most diverse phase of the whole anaerobic treatment process, where plethora of intermediate reduced end product can be formed by diverse group of microbes which become dominant under different specific environmental and concentration conditions.

In the acetogenesis process, the acetogenic bacteria oxidize the VFAs and other reduced products into acetates and hydrogen. Such class of bacteria also includes the genera *Syntrophobacter* and *Syntrophomonas*. The end products of this process, i.e., acetic acid and H_2, are then utilized by the methanogens, which helps in sustaining the low hydrogen concentration (essential for sustaining the otherwise thermodynamically unfavorable reactions) (Schink 1997). The primary alcohols such as ethanol are oxidized into acetate by the bacterial class of genera *Desulfovibrio*, *Thermoanaerobium*, and *Pelobacter*. The genera *Syntrophomonas* is predominantly responsible for the oxidation of propionic acid, butyric acid, and higher homologs. *Syntrophomonass apovorans, Syntrophomonas wolfei* and *Syntrophomonas bryanti* can convert butyrate and higher homologs into acetate, while *Syntrophobacter wolinii* is responsible for the conversion of propionate into acetate. Pentanoic acids are converted into propionic acid by the bacteria like *Methanobacterium suboxydans*, on the other hand *Methanobacterium propionicum* are mainly related to the conversion of propionic acid into acetic acid. The release of hydrogen occurs during acetogenesis, which exerts inhibitory effects on the responsible bacteria involved in the process. Therefore, a symbiotic relationship between the acetogenic bacteria with autotrophic methanogenic bacteria is necessary as they consume hydrogen. The acetogenic phase dominate in qualitative as well as quantitative production of biogas, as approximately 70% of methane is expected to produce during the acetate reduction. Consequently, methane digestion process produces acetates as a key intermediate product. During acetogenic phase, approximately 25% of the formation of acetic acid takes place and about 11% of hydrogen is produced in the decomposition of organic compounds.

Syntrophic bacteria produce hydrogen and thus these bacteria cannot form a pure culture. The metabolic processes of syntrophic bacteria are dependent upon the bacteria which can consume hydrogen. Methanogenic archaea is an example for consumption of hydrogen produced by syntrophic bacteria and subsequent methane production, these bacteria perform metabolic processes in association. During the oxidation process of propionic acid, many species of the *Syntrophobacter* genus of bacteria can utilize sulfate as terminal electron acceptor. *Desulfotomaculum* may use sulfate as electron acceptors. *Desulfo vibrio* may also use sulfate and lactate to form acetate and H_2, through syntrophic association with *Methanobacterium* genus. *Desulpho vibrio* may compete with methanogens by using the same substrate and producing H_2S and thereby hindering the methane formation.

It is well understood that acetate, H_2, and CO_2 are utilized mainly by the methanogenic bacteria for methane. Based on chemical oxygen demand (COD), decarboxylation of acetic acid is responsible for about 72% of production of methane, whereas the remaining 28% of methanogenesis takes places from carbon dioxide reduction (McCarty 1964). Aceticlastic methanogens are primarily responsible for the conversion of acetic acid into methane which consists of major part of the methanogenesis process. Remainder part of methanogenesis takes place by utilizing H_2 and CO_2 to form methane by the hydrogenotrophic methanogens.

Previously, methanogens were classified as bacteria; but with the increasing understanding of anaerobic processes, these microorganisms were classified as archaea which are bit different from the microbes. In particular, these comprise of membrane lipids and distinctive ribosomal RNA (Pandey and Sarkar 2019b; Boone et al. 1993), whereas the lack of basic cellular characteristics (e.g., peptidoglycan) are observed in such class of bacteria. Methanogens are obligate anaerobes. There are three major pathways known for the methanogenesis: acetotrophic or aceticlastic methanogenesis, CO_2 reducing or hydrogenotrophic methanogenesis, and methylotrophic pathways. The splitting of acetic acid to form methane according reaction 10.4 is known as aceticlastic pathway, and the methanogens taking part in such a pathway are called aceticlastic methanogens or aceticlastic archaea. Example of aceticlastic methanogens are *Methanosarcina* and *Methanosaeta*. The other most commonly encountered pathway of methane formation is anaerobic oxidation of hydrogen where carbon dioxide acts as the terminal electron acceptor. Reaction 10.3 is the representation of such pathway. In anaerobic treatment system ~28% of the methane generation is achieved through hydrogenotrophic pathways. Example of such hydrogenotrophic methanogens are *Methanobacterium*, *Methanobrevibacterium*, and *Methanospirillum*. Hydrogenotrophic methanogens are the hydrogen scavangers and help in maintaining a low partial pressure of H_2 which is a prerequisite for the acetogenesis process to proceed in the forward direction. There is a wide class of hydrogenotrophic methanogens which are known to use formate as electron source to reduce CO_2 to methane. Few numbers of methanogens are known to oxidize alcohols to reduce carbon dioxide to methane. Methanosarcina typically forms granules of spherical cell units and it is known to many other substrates like methylamines, methanol, and also H_2/CO_2 at several occasions. Utilizing acetate, *Methanosarcina* typically doubles its number in a time

of 1–2 days. On the other hand, the rod-shaped *Methanosaeta* can only utilize acetate for its growth and doubles its number in a time of 4–9 days (Lee and Zinder 1988). Therefore, for a short solid retention time (SRT) and a completely mixed anaerobic reactor, *Methanosaeta* would easily wash out from the system making *Methanosarcina* as the predominant genera. In the reports of Raskin et al. (1995), where he used anaerobic digesters in which SRT was set to 20 days or more, *Methanosaeta* were observed to be the dominant methanogens. As per the reports of Conklin et al. (2006), *Methanosaeta* is predominantly found in a majority of anaerobic reactors. As per the reports, *Methanosarcina* is the most dominant methanogen in anaerobic bioreactors in which acetate is the primary substrate for utilization with a concentration greater about 236 mg/L having HRTs of 10 days or less. Noike et al. (1985) predicted that in continuous stirred tank reactors, the most dominant methanogen at an SRT of 6.5 days or less would be *Methanosarcina*, whereas for an SRT of 9.6 days or more, the bioreactor would be dominated by *Methanosaeta*.

Compounds like methanol, mono-, di-, and trimethylamine, and dimethyl sulfide, which contain methyl groups, are catabolized by methylotrophic pathways. Through this pathway, the methyl carrier takes up the methyl group to reduce it to methane. For the reduction of methyl, the required electron can be gained by either using H_2 as an electron donor or by the oxidation of methyl groups (Boone et al. 1993).

Homoacetogens play a vital role during anaerobic digestion process as they produce acetate as the end product, which is an essential precursor for the aceticlastic methanogenesis. Either of the autotrophic or heterotrophic bacteria can mediate the process. The autotrophic homoacetogens are capable of utilizing CO_2 and H_2, with CO_2 being the only source of the carbon for cell synthesis. Few homoacetogens can also make use of CO as the sole source of carbon. On the other hand, heterotrophic homoacetogens produce acetate as the end by-product by using organic substrates such as methanol and formate. In the reports of Novaes 1986 the two isolates from the sewage sludge were identified as *Acetobacterium woodii* and *Clostridium aceticum* which are homoacetogenic bacteria thriving in mesophilic environment. These homoacetogenic bacteria are known to have a high thermodynamic efficiency; due to this attribute the accumulation of hydrogen and carbon dioxide does not take place even when multicarbon substrates are fed to the reactor for the bacterial growth. The Gibb's free-energy changes are analogous to hydrogenotrophic methanogenesis, which very closely competes for the available electron donor (H_2). During the stress conditions of low temperature or low pH, aceticlastic methanogens may compete very successfully with the hydrogenotrophic methanogens and can adapt better. However, extensive research is needed to explore more about the metabolism of aceticlastic and hydrogenotrophic methanogenesis.

10.6 Major External Factors Affecting Anaerobic Digestion

The anaerobic degradation of organic compound is affected by a variety of external factors. For enhancement of the activity of the microorganisms and increase in the overall efficiency of anaerobic treatment process, the process should be optimized by controlling the operating parameters so that the effect of the external factors can be minimized.

10.6.1 Nutrient

Just like any other biological process, the requirement for the macronutrients (nitrogen and phosphorus) and micronutrients (trace elements) is also inevitable for the anaerobic bacteria to support their growth activities. Compared to aerobic process, nutrient requirements in anaerobic process are significantly different. The major reason behind such difference is that fermentative and methane-forming bacteria have significantly lower cell yield as compared to aerobic bacteria (Uke and Stentiford 2013; Ortner et al. 2014; Long et al. 2012). In general, municipal wastewater has a balance of macronutrients so that there is no need of extraneous addition of macronutrients. Sometimes, industrial wastewaters may not possess sufficiently balanced nutrients, and it may be highly desirable to add nitrogen and phosphorus for the maintenance of C, N, P at sufficient ratios for the efficient biological treatment of wastewater. The suitable C:N:P ratios of about 100:5:1 and 100:1.8:0.28 have been reported for anaerobic microorganisms (Diez-Gonzalez et al. 1998; Yilmaz et al. 2008). Besides nitrogen and phosphorus, other trace elements are essential at low concentration stimulating the activity of anaerobic microorganisms. The micronutrients of importance are magnesium, molybdenum, iron, nickel, and cobalt. Out of these, cobalt was shown to be the most critical micronutrient (Florencio et al. 1993) for efficient anaerobic degradation of wastewater. Kayhanian and Rich (1995) reported that molybdenum, cobalt, and nickel are essential for the growth of *Methanobacterium*.

10.6.2 pH

Fundamentally, the anaerobic digestion process consists of two major processes: acid formation through breakdown of substrates and methane formation by conversion of acid generated in acid formation stage. Therefore, essentially acidogenesis process runs at acidic pH, while methanogenesis process should operate at near neutral pH. It is well known that the range of pH for the optimum activity of acidogenic bacteria is 5.5–7.2, while the optimum range of pH value for methanogenic archaea is 6.6–7.6 (Visvanathan and Abeynayaka 2012). A closer examination of the fermentation or acidogenesis reactions mentioned earlier in this review should reveal that the characteristics and loading of the substrate have immediate effect on the pH of the reactor during acidogenesis reaction. The acid-

forming bacteria are kinetically faster than the methanogenic bacteria. An increase in the loading of simple and easy-to-be-degraded substrate would allow for a spike in the production of VFAs, but the methanogens, having slower kinetics, might not be able to convert the acetate or hydrogen into methane. Thus, there will be accumulation of VFAs and excess production of CO_2 within the reactor. Consequently, for both the changes, there should be concomitant lowering of the pH of the system. The pH may fall below the threshold range of values required for the methanogens to work effectively. This is when the reactor is considered to have become sour along with the washout of methanogens from the reactor. In order to circumvent the problem of acidification of the reactor as a result of increased organic load, anaerobic treatment processes should require sufficient capacity of buffering or alkalinity to minimize the effects of pH variations caused by either inlet conditions or by the increased substrate loading. Typically, pH of anaerobic system is maintained by natural alkalinity or self-producing alkalinity. The use of sodium bicarbonate, sodium hydroxide, or lime can efficiently control the lowering pH. While lime can cause scaling problem in the reactor with a precipitation of $CaCO_3$, sodium bicarbonate is preferred due to its high buffering capability. Metcalf (2003) suggested that the alkalinity should be maintained within the range of 1–5 g/L as $CaCO_3$.

10.6.3 Temperature

Like all other biological processes, temperature variations affect the metabolic rate, bacterial growth, and the activity of the bacteria in anaerobic wastewater treatment. As the microorganisms involved in the anaerobic reactions have to thrive on the small energy budget as compared to other type of degradation, their activities are quite sensitive to small variations in temperature at which the reactions take place. Thus, in many cases it is possible to correctly predict the effect of the change in temperature on these reactions if the thermodynamic basis for the availability of free energy from the reactions is known. Hydrolysis process is favored at elevated temperature. Bouallagui et al. (2004) reported that thermophilic hydrolysis rate of cellulose is higher than mesophilic hydrolysis rate around 5–6 times. Acid formation reactions have highly negative ΔG^0 values which indicate the spontaneity of the reactions and also availability of high amount of free energy from these reactions for meeting the metabolic requirements of the fermenting microorganisms. Acetogenesis reactions have positive values of ΔG^0 which indicate that in order for the reactions to take place, one of the reaction products should be present in very low concentrations. The reaction schemes and discussions mentioned in previous sections clearly indicate that partial pressure of hydrogen has to be maintained to be minimum through mutual cooperation with other syntrophic partner who utilize hydrogen by oxidizing it through hydrogenotrophic methanogenesis to form methane in order to release the energy that will be required for carrying out of metabolic activities. It has been reported that under psychrophilic conditions (<15 °C), homoacetogenesis leading to the formation of acetate from CO_2 and H_2 dominates, and it helps in the removal of hydrogen. During this process, the aceticlastic

methanogenesis takes over as compared to hydrogenotrophic methanogenesis (Kotsyurbenko et al. 2001). Hydrogenotrophic methanogenesis reactions are thermodynamically favorable at low and high partial pressure of H_2. However, with the increase of temperature, the ΔG^0 value increases considerably, indicating that less free energy shall be available for metabolic activities at higher temperature. Alternatively, acetic acid can be converted to other forms in two possible ways: aceticlastic cleavage to form methane and acetic acid oxidation to form CO_2 and hydrogen. At a temperature of 35 °C or lower aceticlastic methanogenesis produces more energy than hydrogenotrophic methanogenesis.

Under standard conditions, hydrogenotrophic methanogenesis yields more energy than homoacetogenic hydrogen oxidation in which hydrogen and carbon dioxide react to form acetate. Thus at standard conditions, homoacetogens would not compete with hydrogenotrophic methanogens. However, the situation dramatically changes at slightly acidic situation under low temperature, low acetate concentration and low partial pressure of H_2. When the acetic acid concentration becomes 10 mM and H_2 partial pressure reaches lower than 10 Pa, the process of homoacetogenesis reaches the same gain of energy at 5 °C as hydrogenotrophic methanogenesis does at 35 °C. Therefore, at low temperature, the pathway of homoacetogenesis and aceticlastic methanogenesis would be predominant over the hydrogenotrophic methanogenesis. The opposite scenario takes place at high temperature under which aceticlastic methanogenesis becomes less significant, homoacetogenesis operates in the opposite direction making the electron to flow from acetate through either formate or carbon dioxide and driving hydrogen toward methane though hydrogenotrophic methanogenesis. Nielsen et al. (2004) indicated that the thermophilic hydrolytic and fermentation bacteria and hydrogen consuming methanogens work efficiently in the range of 55 °C–75 °C and 55 °C–70 °C, respectively. Ahring et al. (1995) reported that conversion of acetate, butyrate, and propionate to methane had an optimum temperature range at 55–60 °C. It has been reported that the anaerobic reactors operated at thermophilic condition have produced more methane than that at mesophilic condition (Ramakrishnan and Surampalli 2013). Prokaryotic microorganisms can better adapt to higher temperature than eukaryotes do. Madigan et al. (2003) showed that for eukaryotes the limiting temperature for growth is around 60 °C, which for prokaryotes are much higher: 70 °C (for bacteria) and 113 °C (for archaea).

10.6.4 Toxic Compounds

Anaerobic microorganisms and its activity can be inhibited by anaerobic inhibitors present in wastewater or by-products from metabolic activities of anaerobic microorganisms. Furthermore, anaerobic inhibitors largely depend on wastewater characteristics. Ammonia, heavy metals, phenol, and halogenated compounds are the examples for toxic materials of anaerobic microorganisms. Generally, varying concentrations of different toxic compounds are reported by many researchers. The probable reason is investigation on reactors of different configurations as well as

time and approach of seed sludge acclimatization. One of the interesting findings of anaerobic system-based research studies is that many anaerobic microorganisms have also the potential to degrade the refractory organics or recalcitrant compounds (Singh et al. 2018; Stronach et al. 2012). This toleration/degradation can be expected through the acclimation of microorganism to such toxic compounds. These findings can also be helpful in exploring the possibilities of anaerobic treatment of industrial wastewaters laden with varying concentrations of toxic/recalcitrant compounds (Pandey et al. 2016; Chen et al. 2008; Basri et al. 2010).

10.7 Conclusions

Being a complex system with a variety of bacteria involved in an anaerobic system, it needs a meticulous control over the processes for stable operation. Despite of all the advantages of anaerobic systems, lack of understanding about the processes makes it less used technology as compared to aerobic systems. The performance of the reactors under various adverse physicochemical conditions like pH, nutrient stress, toxic substances, detergents, and varying hydraulic loading rates affect the performance of the reactor heavily. These parameters should be meticulously taken care of in order to keep the bioprocess under control. A complete understanding should be a pre-condition before scaling up the process to industrial-level application. Accurate control of the anaerobic reactor may help in maximizing and recovering target intermediates which may prove to be commercially more attractive than methane production. Along with organic acids, hydrogen gas production is also one established and lucrative intermediate which can be economically beneficial and sustainable.

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Microbial Production of Polysaccharides 11

Urmi Halder, Raju Biswas, and Rajib Bandopadhyay

Abstract

A wide variety of bacterial species, and few algae, fungi, and yeasts have shown to produce a number of polymeric substances. The molecular weights of polysaccharides of different origins may differ widely. Different types of bacterial polysaccharides have been reported, and a few numbers was approved as commercial products. Polysaccharide production from pathogenic bacteria are appeared to be cost-effective, and maintenance of product quality is found quite difficult. Still several products, including xanthan and gellan from a small number of Gram-negative bacteria are acknowledged in chemical industry. Potent microbial polysaccharide has been commercialized due to versatile physical properties, which is appropriate for industrial usages. Since twentieth century, potential bioactivities of polysaccharides have been focused and finally it gets medical applicability by modification; it is used in drug delivery, bone and tissue engineering, surgery, artificial organs, and dental problems, as well as immunomodulator, antitumor, and antiviral.

Keywords

Polymeric substances · Bacterial polysaccharides · Cost-effective · Versatile physical properties · Medical applications

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11.1 Microbial Polysaccharides: An Introduction

11.1.1 Microbial Polysaccharides

In the middle of the nineteenth century, Louis Pasteur for the first time discovered microbial polymer dextran in wine, and later on Van Tieghem identified the bacterium *Leuconostoc mesenteriodes* which produced dextran. At the end of this century, microbial cellulose production was also reported. Discovery of microbial polymers had become a subject of extensive research from early to mid-twentieth century onwards due to the distinctive chemical structures and versatile functional properties (Rehm 2010). Bacteria, endophytes, microalgae, cyanobacteria, and microbial consortia have shown the capability to secrete polysaccharides (Freitas et al. 2017). In oceans, a major part of marine organic matter is composed of heterotrophic bacterioplankton polysaccharides (Sengupta et al. 2019; Piontek et al. 2010).

Usually there are three distinct classes of microbial polysaccharides based on localization, viz. intracellular, capsular, and extracellular polysaccharides (EPSs) (Mollet 1996). Intracellular cytosolic polysaccharides are energy sources for the microbial cells (Donot et al. 2012), whereas the capsular polysaccharides basically are the cell wall components like peptidoglycans, lipopolysaccharides, and teichoic acids. It remains bound to the cell wall and therefore it is not easy to harvest. Finally, the exopolysaccharides that are secreted into the extracellular environment in the form of homopolysaccharides and heteropolysaccharides to protect themselves from different environmental cues, and help to cell adherence and serve as carbon and water reservoir. Most microorganisms secrete EPS naturally and a very few exude high amount of EPS under stress (Papinutti 2010; Ravella et al. 2010; Lin and Casida 1984). EPSs exist in the form of either capsule or slime. Different classes of exopolysaccharides have been reported from a large group of bacterial populations: dextran, curdlan, xanthan, alginate, levan, cellulose, gellan, hyaluronic acid, fucoPol, fucoGel, and galactopol (Freitas et al. 2017).

Generally, microbial polysaccharides are modified to make useful properties as because the natural configuration does not match with the requirement of food hydrocolloids. It had been popularized in the food industry from the time when xanthan and gellan were acceptable as food preservatives in the United States and Europe (Sutherland 1998). The commercial productions of EPS from microbes are required few days compared to plant-based hydrocolloids, and no land is required for cultivations.

In this era, usages of eco-friendly and cost-effective microbial biopolymers have been gaining more attention rather than synthetic one. This chapter will focus on an overall scenario of the production and application of extracellular polysaccharides (EPSs) and capsular polysaccharides (CPSs).

11.1.2 Capsular Polysaccharides (CPS)

Cell surface of most microbial pathogen has negatively charged and highly hydrated long-chain polysaccharide having high molecular weight known as capsular polysaccharides (CPSs) (Willis and Whitfield 2013; Liston et al. 2018). CPSs are responsible for the determination of virulence in pathogens which influence host innate immune responses and serve as a receptor for bacteriophages (Liston et al. 2018; Williams et al. 2018; Alvarez and Cobb 2019; Vasiliu et al. 2016). Selective pressures are responsible for the diverse structures of CPSs by variation of sugar units, non-sugar part, and linkage. *Escherichia coli* alone secrets more than 80 different structures of capsular polysaccharides: glycan, hyaluronan, heparosan, chondroitin, polysialic acid, etc. (Williams et al. 2018; Whitfield 2006) (Table 11.1). Other than bacteria, CPSs were rarely investigated in microalgae and diatoms (Rossi and De Philippis 2016). *S. pneumoniae* type 3 capsule is basically covalently linked with the phospholipid molecules on the cell surface and often serves as endogenous receptor (Cartee et al. 2005). Bacterial polysaccharides have high molecular weight, biocompatibility, and viscoelastic properties (Rehm 2010). It becomes catchy targets for production in genetically engineered heterologous host which resulted in improved and increased CPS production compared to wild-type strains (Schmid and Sieber 2015).

11.1.3 Exopolysaccharides (EPS)

Microbial cells secret exopolysaccharides into the extracellular environment in the form of homopolymer and heteropolymer (Donot et al. 2012). Both prokaryotes (eubacteria, archaebacteria) as well as eukaryotes (phytoplankton, fungi, algae) have the ability to secrete exopolysaccharides (Freitas et al. 2017) (Table 11.1). Homopolysaccharides are basically neutral sugars, but polyanionic heteropolysaccharides are decorated with the inorganic or organic substitutes like uronic acid or pyruvate, phosphate, sulfate, acetyl groups (Schmid and Sieber 2015). Bacterial homopolymers include cellulose and dextran. Different classes of exopolysaccharides such as dextran, curdlan, xanthan, alginate, levan, cellulose, gellan, hyaluronic acid, fucoPol, fucoGel, and galactopol have been reported from a large group of bacterial populations (Freitas et al. 2017). Polysaccharides in the different organisms involve in a number of tasks: cellulose mediates structural stability of the cells in plants and algae, and glycogen or starch stores as intracellular components. Microbial exopolysaccharides (EPS) work as natural glues and protect cells from stresses such as extreme pH, desiccation, and antibiotics (indiscriminate and high doges). Microbial EPS also associates in host–pathogen interactions (Corbett and Roberts 2009; Limoli et al. 2015). EPS produced by food-associated microbes has been widely used in food and beverage productions (Brandt et al. 2016).

Despite having natural function, the microbial EPSs are important hydrocolloids which are mainly exploited in industries such as food, pharmaceutical, brewing,

Table 11.1 Types of microbial polysaccharides

Microorganisms	Polysaccharides	Monosaccharides	Primary structure	References
Bacteria:				
Lactobacillus spp. *Leuconostoc* spp. *Streptococcus* spp.	Dextran (extracellular)	Glucose	α-(1,2)/α-(1,3)/α-(1,4)-branched α-(1,6)-linked homopolymer	Rehm (2010); Freitas et al. (2017)
Xanthomonas spp.	Xanthan (extracellular)	Glucose, mannose, and glucuronate	β-(1,4)-linked repeating heteropolymer consisting of pentasaccharide units	Rehm (2010); Freitas et al. (2017)
Agrobacterium spp. *Alcaligenes* spp. *Cellulomonas* spp. *Rhizobium* spp.	Curdlan (extracellular)	Glucose	β-(1,3)-linked homopolymer	Rehm (2010); Freitas et al. (2017)
Azotobacter spp. *Pseudomonas* spp.	Alginate (extracellular)	Mannuronic acid and guluronic acid	β-(1,4)-linked non-repeating heteropolymer	Rehm (2010); Freitas et al. (2017)
Bacillus sp. *Halomonas* sp. *Zymomonas* sp.	Levan (extracellular)	Fructose	-	Rehm (2010); Freitas et al. (2017)
Acetobacter sp. *Glucanoacetobacter* sp. *Rhizobium* sp. *Sarcina* sp.	Cellulose (extracellular)	Glucose	β-(1,4)-linked homopolymer	Rehm (2010); Freitas et al. (2017)
Sphingomonas spp.	Gellan (extracellular)	Glucose, rhamnose and glucuronate	β-(1,3)-linked repeating heteropolymer consisting of tetrasaccharide units	Rehm (2010); Freitas et al. (2017)
Streptococcus spp. *Pasteurella* spp.	Hyaluronic acid (extracellular)	Glucuronate and *N*-acetyl glucosamine	β-(1,4)-linked repeating heteropolymer consisting of disaccharide units	Rehm (2010); Freitas et al. (2017)
Enterobacter sp.	FucoPol (extracellular)	-	-	Rehm (2010); Freitas et al. (2017)
Klebsiella sp.	FucoGel (extracellular)	-	-	Rehm (2010); Freitas et al. (2017)
Pseudomonas sp.	Galactopol (extracellular)	-	-	Rehm (2010); Freitas et al. (2017)

Escherichia coli, *Shigella* spp. *Salmonella* spp. *Enterobacter* spp.	Colanic acid (extracellular)	Fucose, glucose, glucuronate and galactose	β-(1,4)-linked repeating heteropolymer consisting of hexasaccharide units	Rehm (2010)
Bacteria and archaea	Glycogen (intracellular)	Glucose	α-(1,6)-branched α-(1,4)-linked homopolymer	Rehm (2010)
Escherichia coli	K30 antigen (capsular)	Mannose, galactose, and glucuronate	β-(1,2)-linked repeating heteropolymer consisting of tetrasaccharide units	Rehm (2010)
Fungi:				
Aureobasidium sp.	Pullulan	Glucose	-	Freitas et al. (2017)
Sclerotium sp.	Scleroglucan	Glucose	-	Freitas et al. (2017)
Schizophyllum sp.	Schizophyllan	Glucose	-	Freitas et al. (2017)
Antrodia sp.	–	Fuc, GlcN, Gal, Glc, Man	-	Freitas et al. (2017)
Aspergillus sp.	–	Man, Gal	-	Freitas et al. (2017)
Diaporthe sp.	–	Glc, Gal, Man	-	Freitas et al. (2017)
Fusarium sp.	–	Rha, Gal	-	Freitas et al. (2017)
Ganoderma sp.	–	Gal, Man, Glc, Ara, Rha	-	Freitas et al. (2017)
Microalgae:				
Anabaena sp.	–	Glc, Gal, Man, Xyl, Fuc, Rha, GalN, GlcN, GalA, GlcA	-	Freitas et al. (2017)
Dunaliella	–	Glc	-	Freitas et al. (2017)
Gyrodinium	–	Gal	-	Freitas et al. (2017)
Phormidium	–	Rha, Rib, Man, Glc, Fuc, Gal, Ara, GalA, GlcA	-	Freitas et al. (2017)
Porphyridium	–	Xyl, Gal, Glc, GlcA	-	Freitas et al. (2017)
Rhodella	–	Xyl, Gal, Glc, Rha, Ara, GlcA	-	Freitas et al. (2017)
Synechocystis	–	Fuc, Glc, Rha, Xyl, Man, GlcN, GalA, GlcA	-	Freitas et al. (2017)

– Not available

textiles, detergents, adhesives, downstream processing, cosmetology, wastewater treatment, dredging (Ahmad et al. 2015; Ruhmann et al. 2015). Microbial polysaccharides like xanthan, dextran, and pullulan have shown substantial market values due to their useful properties: physicochemical and rheological. However, plant and algae still provide a major part of the hydrocolloid in form of starch, pectin, galactomannans, alginate, and carrageenan in the markets. It had a market value of 4 and 3.9 million US dollars in 2008 and 2012, respectively, and this worth would be expected to get in touch with around 7 billion USD by 2019 (Patel and Prajapat 2013). Under controlled environment, microbial EPSs are economically competitive to polysaccharides of plant and algae (Kaur et al. 2014). Although a very few bacterial EPSs, xanthan, gellan, and dextran, have been commercialized due to unusual production prices (Freitas et al. 2011; Llamas et al. 2012). Therefore, it becomes crucial to understand bacterial EPSs biosynthesis mechanism and pathways in order to improve EPSs productivity.

11.2 Biosynthesis of Microbial Polysaccharides

Microbes adopted various pathways to produce complex extracellular and capsular polymers (Fig. 11.1). A set of genes are involved for its biosynthesis and export (Reid and Szymanski 2010). Polysaccharide precursors like dTDP-rhamnose, UDP-*N*-acetyl glucosamine (diphosphate sugar) or GDP-mannuronic acid (sugar derivatives) are formed by the enzymatic transformations that are controlled by a set of gene cluster. Primary glycosyltransferase (GT) plays the key role in the polymerization of sugar units to form the backbone. In case of heteropolysaccharides, non-sugar substitutes are incorporated by different types of linkage, whereas, extracellular sucrase-based biosynthesis of homopolysaccharides follows the substrate cleavage and polymerization (Schmid 2018; Halder et al. 2017; Becker 2015; Ates 2015). An enzyme family copolymerase finally shapes up the polymer structure by controlling the chain length (Schmid and Sieber 2015; Wu et al. 2017).

Secretion of heteropolymeric EPSs is facilitated by the flippase (Wzx)/polymerase (Wzy)-dependent pathway or ABC transporter and synthase-based pathway. Protein machineries are conserved for the assembly and secretion of CPSs except structurally diverse glycan type (Liston et al. 2018). Secretion of CPSs across the inner membrane is mainly facilitated by multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) export flippase (Wzx) pathway or through the widespread ABC transporter (ATP-binding cassette) (Reid and Szymanski 2010; Islam and Lam 2013; Liston et al. 2018; Williams et al. 2018). Nucleotide-binding domain is responsible for the conformational changes of the CPSs and secretion across the membrane enabled by another domain called transmembrane of glycol-ABC transporter protein. Finally, translocation from periplasm to outer membrane facilitated by polysaccharide copolymerase and outer membrane polysaccharide export protein complex. The inner membrane flippase or ABC transporter proteins interact with the interfaces of outer membrane protein through adopter protein complex in periplasm

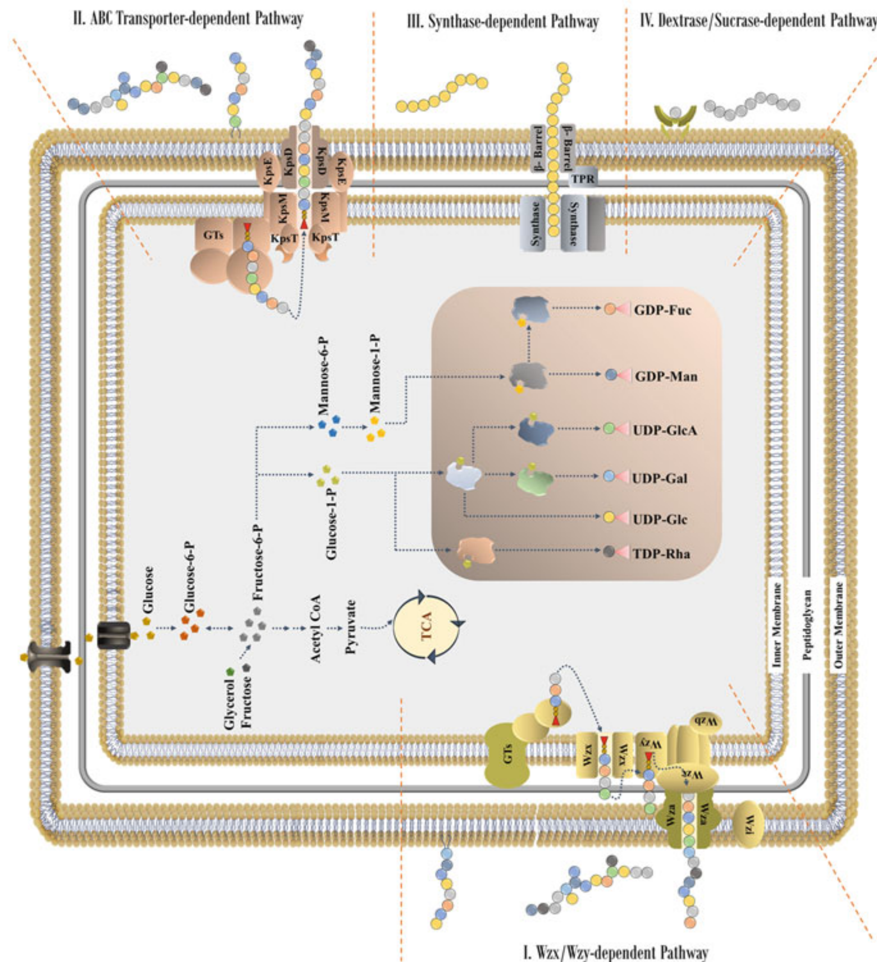

Fig. 11.1 Generalized pathway of microbial EPS and CPS biosynthesis and their secretion (Developed from Reid and Szymanski 2010; Ates 2015; Schmid 2018)

and formed a "tripartite drug efflux pump" (Willis and Whitfield 2013; Liston et al. 2018).

11.3 Metabolic Engineering of Polysaccharide

Nowadays researcher is interested to enhance the declining resources of the microbial polysaccharides having biomedical and commercial applications. Genetic manipulations of microbes were experimented to enhance the productivity by the implementation of synthetic biological methods like promoter engineering, gene

knockout, and control gene expression (Williams et al. 2018). Development of genetically engineered heterologous host using "platform cell factories" was quite predictable by introducing de novo or manipulating existing pathway to deal with potential contamination risk and other obstacles of "difficult to handle" microbes (Nielsen and Keasling 2016; Niu et al. 2016; Chae et al. 2017). Some trials have been made on the overexpression of glycosyltransferases to increase the EPS productivity. But the domain swapping strategy for both the donor and acceptor sites of glycosyltransferases could introduce a potent variant to produce diverse polysaccharide structures (Schmid and Sieber 2015).

11.4 Commercial Microbial Polysaccharides

Very few bacterial polysaccharides have been commercialized: xanthan gum, gellan gum, dextran, and succinoglycan (Sanderson 1982; Gonçalves et al. 2009; Falconer et al. 2011; Halder et al. 2017; Banerjee et al. 2017). Xanthan gum from *Xanthomonas campestris* has been used as food grade for a long time. It consists of glucan main chain [β (1 → 4) linkage] and having alternating side chain of glucuronic acid and two mannose residues (Sanderson 1982). Besides food industry, xanthan is used in medical applications. In food industry, xanthan is utilized as thickener, stabilizer, and emulsifier of having high viscosity, solubility, and stability (in highly acidic condition). Modification of xanthan could be possible at the initial and terminal mannose residues of that trisaccharide side chain and the helical backbone by insertion of galactomannans.

Gellan gum, an anionic extracellular polysaccharide, is extracted from *Sphingomonas elodea*, which consists of linear repeating tetrasaccharides: β-D glucose, L-rhamnose, and D-glucuronic acid with L-glyceric (Omoto et al. 1999). It is used expansively in food products like jams, jellies, fabricated foods, ice cream, milkshakes, yogurt, and cheese (Banik and Santhiagu 2006). Gellan exhibits an extensive range of gelatine characteristics: soft, elastic, and thermo-reversible gels (acylated gellan), but de-acylated form shows reverse phenomenon. This hydrocolloid also replace agar in several Japanese foods, hard bean and misumame jelly, tokoroten noodles, etc. (Kampf and Nussinovitch 2000).

Dextran, an exopolysaccharide made up of α-D-glucopyranose residues [(1 → 6) linkage], which is familiarly excreted by *Leuconostoc mesenteroides* (Banerjee and Bandopadhyay 2016; Qader et al. 2005). Different genera of *Streptococcus* and *Acetobacter* have also been reported to produce dextran (Niven et al. 1941; Hehre and Hamilton 1951). Mostly, dextran and its derivatives are applied in biomedicine. In food products, it is also used as thickener by de-crystallization of sugar and retains moisture, and maintains flavor (Qader et al. 2005; Naessens et al. 2005; Purama and Goyal 2005).

Alginate which is isolated from seaweed and bacteria, *Pseudomonas aeruginosa* and *Azotobacter vinelandii*, consists of linear polymers of repeated mannuronic and guluronic acid residues forming poly-mannuronic, poly-guluronic acid, and mixed sequences (GMGMGM) (Rehm and Valla 1997; Tonnesen and Karlsen 2002).

Though algal alginates are typically cost-effective, but the environment becomes polluted during its production. Generally, algal alginate differs from microbial source in terms of length of each building block, i.e., G and M proportion except *A. vinelandii* having similar structural blocks of copolymer to the algal alginate (Tonnesen and Karlsen 2002). Alginate are extensively used in food, textile, paper making, and biomedicine industries as thickeners, stabilizers, gelling agents, and emulsifiers due to its biocompatibility and low toxicity (Gombotz and Wee 2012).

11.5 Medical Applications of Microbial Polysaccharide

Cancer is a serious disease that destroys body tissue. Recent studies have demonstrated that polysaccharides from biological resources have shown an effective anticancer activity (Fan et al. 2017; Xie et al. 2013; Yu et al. 2015; Zong et al. 2012). Microbial EPS shows exclusive applications especially medical usages: pharmaceutical science and therapy. It is obtained from renewable, biocompatible, and non-toxic resources.

Dextran, a microbial EPS, was discovered in nineteenth century and used remarkable for the first in pharmaceutical applications in United States and Europe: controlling wounds shock since 1953 (Nwodo et al. 2012; Amspacher and Curreri 1953). Xanthan was discovered in 1950 (Born et al. 2002) and permitted as a food preservative in the USA (1969) and Europe (1982). Subsequently, it was also included in 2012 and 2014 in United States Pharmacopeia [USP] and European Pharmacopeia [EP], respectively. Bacterial alginate was discovered in 1964 and differentiated from product extracted from seaweeds by the presence of acetyl groups in the linear structure of mannuronic and guluronic acid. It is a good disintegrating agent in tablets: thickening and stabilizing agent in pharmaceutical and cell microencapsulation for drug delivery (Mukherjee and Atala 2005; Nwodo et al. 2012) and used as sodium salt in capsules protecting stomach (Mc Hugh 1987). FDA-approved alginate fibers to be used as wound dressings and bandages (Mc Hugh 1987; Mukherjee and Atala 2005; Nwodo et al. 2012).

11.6 Polysaccharide-Based Vaccine

Exopolysaccharide can serve as antigens for preparing vaccine. Alginate-tetanus toxoid microparticles could induce an effective immune response in rabbits. Hyaluronic acid attached in hemagglutinin influenza H1N1 was found to be useful for immunization in rabbits, pigs, and mice (Sharma et al. 2009). Curdlan sulfate boosted vaccine adjuvant in mice administered by human recombinant hepatitis B protein (Li et al. 2014). Type B capsular polysaccharide (*Haemophilus influenza*) conjugated to a protein may act as a constituent of polyvalent vaccines for kids (Albani et al. 2015; De Oliveira Cintra and Takagi 2015). In an adjuvant condition, EPS could enhance immune response through vaccination targeting challenging pathogens, influenza pandemic strains H1N1, malarial parasites, viruses (hepatitis

C and AIDS), and resistant mycobacteria (tuberculosis). It could also eliminate cancer cells by stimulating an immune response to target.

Capsular polysaccharides (CPSs) of bacteria, a large-sized surface carbohydrate, were coupled to carrier proteins to produce glycoconjugate vaccines in the 1980s. Capsules are required to evoke the host immune system as the capsular polysaccharides basically masked pathogen associated molecular patterns on the cell surface. So, CPSs have an extraordinary efficiency to develop vaccines (Liston et al. 2018; Willis and Whitfield 2013). Conjugate polysaccharide vaccines had a remarkable effect to reduce meningitis caused by *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Neisseria meningitidis* (McIntyre et al. 2012; Bottomley et al. 2012). Several glycoconjugate vaccines were developed to prevent infectious diseases caused by *Haemophilus influenzae* type b, *Streptococcus pneumoniae*, and *Neisseria meningitides* (Avci and Kasper 2009; Kelly et al. 2005).

11.7 Gene Delivery

EPS and its derivatives have confirmed a huge potential as vectors for gene delivery. Besides viral vectors, polymers are favoured for genetic material delivery to avoid lysosomal degradation. Plasmids were successfully internalized by encapsulation with pullulan nanoparticles (Gupta and Gupta 2004). A successful transfection of gene silencing siRNA into human lung, colon cancer cell lines, macrophages (derivatives of monocyte leukemia cell line), and stem cells was achieved by 6-amino-deoxy-curdlan and this technique is also carried out into hepatoma Huh-7 cells by dextran derivative nanoparticles along with a photosensitizer (Han et al. 2015; Raemdonck et al. 2010). Availability of the biopolymer and reasonable prizes are expected to determine the achievement of BNC as drug delivery system.

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Microbial Production of Industrial Proteins and Enzymes Using Metabolic Engineering 12

Pooja Doshi, Manju Shri, Poonam Bhargava, Chaitanya G. Joshi, and Madhvi Joshi

Abstract

Metabolic engineering is a field of science, which takes advantage of previously gathered information about a particular pathway in a living organism and utilizes this for the improvement of product that could be either metabolite, enzyme, or any protein. Advances in various field of science specifically r-DNA technology, bioinformatics, synthetic biology, molecular genetics as well as other protein engineering technologies had given wings to metabolic engineering. Metabolic engineering has the capacity to mold the flux of a completely enzymatic pathway to a very newly designed pathway. It allows the modulation and production of either previously working metabolite or the production of a new novel enzyme in a different microbial strain. In the present era, there is huge demand of microbial enzymes and proteins for various purposes such as medication, oil and gas industry, dairy industry, baking industry, etc. Microbial strains are utilized as micro factories for the production of microbial enzymes and proteins via metabolic engineering. Therefore, in this book chapter we are dealing with the various criteria that are utilized for the selection of the strains, various approaches that are routinely utilized for the higher expression of genes, as well as various metabolic engineering strategies.

Keywords

Metabolic engineering · Strain selection · Mutagenesis · Microbial host

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12.1 Introduction

Microbes have been traditionally used in fermentation processes to obtain desired products. Scientific studies of the fermentation processes have led to the great advancements in this area. Many significant products, which were previously synthesized by chemical processes, are now being produced by fermentation using microbial cell factories and biotransformation. Microbes have various features such as to the ease of their mass cultivation, fast growth, use of cheap substrates and the diversity of the potential products produced by them, which makes them suitable for the production of value-added proteins. In the recent years, the application of microbial proteins has been increased enormously. The industries involving the use of microbial proteins include detergents, starch processing, animal feed, paper and pulp, fruit/vegetable processing, oil and gas, dairy, brewery, textile, baking, tanning, and so on. There could be further increase in the production as well as supply of the microbial products by improving our understanding of the recombination in microbes, metagenome mining, various fermentation processes, and recovery methods.

Metabolic engineering is a new emerging field with huge impact on human socioeconomic level, for example, its applications in the production of fuels, materials, chemicals, medicine, and pharmaceuticals where modifications are made at the genetic level. It can be defined as enhancement of formation of desired product and/or cellular properties via editing-specific biochemical reaction(s) involved in its formation or the complete designing and introduction of new reactions with the use of r-DNA technology. The targeted biochemical reaction needs to be very specific to obtain maximum yield of the desired product. The procedure involves identification of the target reactions followed by the use of r-DNA techniques to augment, knockdown or delete, transfer, or deregulate the corresponding genes or their protein products. In this book chapter, we are dealing with the various criteria that are utilized for the selection of the strains, various approaches that are routinely utilized for the higher expression of genes, as well as various metabolic engineering strategies.

12.2 Strain Selection Criteria

There is a vast diversity of microbes available in nature. Many microbes especially those isolated from extreme environments are being explored as a source for production of industrially important enzymes since their optimum growth conditions help avoid contamination and produce more stable enzymes. The microbial strain to be used for industrial production of desired protein varies greatly and is usually selected on the basis of the following:

1. The input of efforts required in engineering and the toolsets as well as the availability of resources.
2. The nature and compatibility of the product in the selected host.

3. Metabolic requirements of the selected microbe for the production of the desired protein, viz. synthesis pathways, substrates/precursors, enzymes, and cofactors.

There are various factors which must be considered, such as metabolic resources, secretion of products, proper folding of proteins when selecting a microbe as a host for metabolic engineering. In the coming section, these factors will be discussed briefly.

12.2.1 Metabolic Resources

For the production of protein of interest by a microbe, it requires the precursors and various organic and inorganic cofactors. Cofactors may either act as redox carriers for the biosynthetic reactions or mediate energy transfer for the cell. Manipulation of cofactors include change in culture conditions, modification of pathway for increased availability or introduction of novel cofactors for enhancement of biochemical processes and overproduction of protein of interest (Wang et al. 2013). The advancements in the tools of bioinformatics and the availability of whole genome sequences of microbes helped a lot in exploring their biodiversity and evaluating the diverse potential of the microbial hosts thus enabling to identify the best potential host that can accumulate the pathway for production of desired protein. The availability of databases and computerized methods of model building like ModelSEED (Henry et al. 2010) and Path2models (Buchel et al. 2013) aid in the development of models for potential hosts for production of desired protein. There are various other tools also available like MetaNetX that can facilitate the direct assimilation of the new synthetic pathways available in other genome-scale models, where in their interactions and effects on the host metabolic network can be understood.

12.2.2 Minimum Metabolic Adjustment

Metabolic engineering of bacteria many a times forces it to pass through the evolutionary pressure to overcome its wild-type form and function and attain optimality to produce protein of interest (Fong and Palsson 2004). Therefore, it might be always advantageous to use a microbial host requiring marginal metabolic adaptations and minimum adjustment through progression (Fisher et al. 2014). Advancements in synthetic biology is making use of different bioinformatic tools for selection of microbial host for different de novo biosynthetic pathways for the production of protein of interest. Toolsets used for modeling and substrate-based analysis make use of global sensitivity analysis and agent-based modeling for screening significant components. Along with components, various other parameters such as genome-scale metabolic flux modeling have been done for identification of metabolic networks and medium formulations so that the expression of a biosynthetic pathway can be maximized (Apte et al. 2014).

12.2.3 Secretion of Proteins

The kinetics of protein production and purification vary greatly from lab scale to industrial scale. When going for industrial production, it is an added advantage if the protein of interest is secreted extracellularly. It aids in down streaming as well as the yield could be higher in comparison to the intracellular expression. *Bacillus* spp. are favored as the host for secretion of protein in comparison to *E. coli*. However, research is still ongoing for the identification of other protein secretion hosts (Ferrer-Miralles and Villaverde 2013a), such as Streptomyces (Okesli et al. 2011) halophiles that enhance solubility (Tokunaga et al. 2010) and yeast (Mattanovich et al. 2012). Yeast has some other advantages also such as posttranslational modifications. The final protein of interest can influence the microbial host as well.

12.2.4 Genomic Toolsets

Although *E. coli* is a common and preferred host for expression and production of recombinant protein due to its wide exploration both genetically and physiologically, other microbes are also being investigated in the same direction for protein production by metabolic engineering on an industrial scale. Toolsets like exonucleotide-based Gibson assemblies (Zhang et al. 2012) are of great help that increase the reliability of the assembly to be used for the protein synthesis. This assembly method is useful for the construction of natural and synthetic genes, pathways, and entire genomes (Fisher et al. 2014).

12.2.5 Proper Folding and Functionality of Protein of Interest

A critical step in recombinant protein production is to obtain properly folded and functional protein in the host used for expression. In case of use of metabolic engineering especially when expression of eukaryotic proteins is required, various factors like availability of the necessary chaperons and chapronins as well as transcript reading for synthesis and folding and posttranslational modifications to yield a functional protein need to be taken into consideration (Hartl et al. 2011; Bernal et al. 2014; Osterlehner et al. 2011). Eukaryotic protein expression in microbes where posttranslational modifications are required usually made use of yeasts like *Pichia pastoris* as a host besides the human and baculovirus-based insect cell lines. There is also significant interest being shown in transferring the posttranslational capabilities of eukaryotic cells to other microbes as well (Fisher et al. 2014). Heterologous expression, proper folding and N-linked glycosylation of eukaryotic proteins AcrA and IgG have been achieved in *E. coli* using combinatorial libraries, codon optimization, and shotgun proteomics (Pandhal et al. 2013). Earlier metabolic engineering experiments used to fail at the stage of translation because of lack of tools to check if the heterologous protein will be expressed properly or not. A translational coupling cassette is now available to quickly determine if the

heterologous mRNA will be translated in the chosen host along with its level of expression, even when it expresses large multidomain enzymes. It also helps to isolate the translation problems to the C-terminal domains, and to optimize conditions for expressing a codon-optimized sequence variant (Mendez-Perez et al. 2012).

12.3 Approaches to Attain Higher Expression of Industrial Enzymes

12.3.1 E. coli as a Host

E. coli is still one of the favorite organisms for the expression of heterologous proteins. Its usage as a microbial cell chassis is very highly acknowledged and well explored. Multiple studies carried out on its expression and regulation have made it the most popularly used expression platform. For *E. coli* system, various molecular biology tools and protocols are available which give higher expression and production of heterologous proteins. There is a huge number of expression plasmids, and many engineered strains and diverse cultivation strategies are also available for *E. coli* system (Rosano and Ceccarelli 2014). The different levels at which the E. coli expression system can be regulated are described in the following.

12.3.1.1 Transcriptional Regulation

A strong and controlled promoter as well as tight regulation of expression of protein is a requisite for efficient and high-level recombinant protein expression in E. coli chassis. In E. coli, large-scale production of protein makes use of chemical or thermal inducers for expression (Chao et al. 2004). Promoters that are tightly regulated help in designing many novel and highly repressible or inducible expression systems. Studies on regulation of promoters help in providing vital tools as information on regulation for gene expression.

Besides transcription promoters, transcription terminator elements are also crucial in controlling the expression and stability of heterologous gene expression, by enhancing the mRNA stability, which further increases protein expression (Newbury et al. 1987).

12.3.1.2 Translational Regulation

Translational regulation involves controlling and optimizing the factors involved in translation. Regulation can be done at the level of initiation codon, secondary structures at the site of initiation, or even stability of translated mRNA (Sprengart et al. 1996). The initiation codon AUG has been shown to be much more efficient as compared to GUG or UUG. The residues at 3′ end of initiation codon especially the second one have been shown to influence the translation rate. The Shine-Dalgarno sequence upstream to the initiation codon that initiates translation is also shown to be more proficient than other translational initiators. The efficacy also varies directly to the distance of the translation initiation sequence (Ringquist et al. 1992). The bases

458–466 of the 16S rRNA of *E. coli* when placed upstream of the ribosomal binding site (RBS) has also shown to increase the translation efficiency by 110-fold (Olins and Rangwala 1989). All these factors can be regulated and can be taken into consideration during strain development.

12.3.1.3 Enhancement by Formation of Additional Proteins

Attempts for overexpression of proteins many times lead to accumulation of inclusion bodies within the cell. Purification and refolding to obtain active proteins from inclusion bodies is many a times a tedious job and leads to losses in downstreaming. Methods like use of molecular chaperones and low temperatures has been shown to be efficient in reducing the inclusion body formation and proper refolding of recombinant proteins. Proper folding of proteins is influenced by the oxidative environment of the periplasm and proper signal peptide cleavage during translocation. Various strategies have been used for efficient transport and protein folding. These include overproduction of signal peptidase I (Zhang et al. 1997), simultaneous expression of the secEand prlA4 genes (Pérez-Pérez et al. 1994), addition of Golgi retention or endoplasmic reticulum sequences (Zhan et al. 1998), and mutations in secY (Brinkworth et al. 2011). Uthandi et al. 2012 carried out deletion of the twin-arginine translocation motif, whereas Brinkworth et al. 2011 has described use of type III secretion chaperone to facilitate translocation.

The extracellular secretion of proteins is always favored over the intracellular expression due to several advantages like high expression levels and simplified downstream purification and protein folding. Since E. coli secretes very few proteins, use of signal peptide mutations compatible to E. coli membrane (Ismail et al. 2011) and limited leakage of outer membrane by synergistic use of EDTA and lysozyme (Liu et al. 2012) have also been employed for extracellular protein secretion.

12.3.1.4 Use of Fusion Proteins or Molecular Chaperones

A fusion protein is the product of two or more genes that are translated together with no stop codon in between them. In protein overexpression systems, fusion proteins serve to increase protein yield due to various modes of action like increased solubility of expressed protein, improved folding, efficient mRNA translation (Rosano and Ceccarelli 2014). Translational fusion of trpE gene fragments has positive effect of the expression heterologous genes in E. coli (Makoff et al. 1989). Molecular chaperones are very well known for their assistance in protein folding under normal and various stress conditions like heat or temperature stress (Hartl 1996) as well as they are also capable of providing correctly folded, biologically active proteins that were found to be difficult to be produced in E. coli. It is not necessary to use target protein as well as chaperones from the same organisms. In a two-vector system in *E. coli*, soluble gp37 protein was reported to be effectively produced by co-expression of two bacteriophage T4 chaperones (Bartual et al. 2010).

12.3.1.5 Codon Optimization

The presence of biased codons or codons requiring the rare tRNAs for their expression results in alteration of the proficiency of expression of heterologous protein in *E. coli*. Expression of such genes (without codon optimization) in *E. coli* displays a nonrandom utilization of identical codons which further affects the expression of host genes, or it may lead to an adversely rigorous response (Burgess-Brown et al. 2008). For example, AGA and AGG the arginine codons are mainly infrequent in *E. coli*, and they lead to lower protein expression and mistranslational errors (Calderone et al. 1996). In such cases, there is a need for codon optimization strategies to improve the fidelity of transformation as well as expression of enzymes (Hutterer et al. 2012). The issue can be overcome by conversion of rare codons to commonly used codons by site-directed mutagenesis. Another approach for rescue involves the co-expression of the genes encoding rare tRNA along with the protein of interest (Kleber-Janke and Becker 2000). Kim and Lee (2006) and Gustafsson et al. (2004) have reported use of synthetic DNA with optimized and commonly used codons for successful expression of desired enzymes.

12.3.2 Other Bacteria as a Host

The exploration of microbes, their physiology, and metabolism is attracting other bacterial hosts as microbial chassis. The diversity and biosynthetic potential which is the result of the adaptation due to exposure to varied environments makes other bacteria as a useful host (Ferrer-Miralles and Villaverde 2013b). Besides E. coli, *Bacillus* species are also regularly used as a host for production of recombinant protein due to their high capacity of secretion of protein and ability to export proteins directly into extracellular media. Different *Bacilli* systems that have been explored for protein production include *B. licheniformis,* B. subtilis, B. megaterium, B. brevis, and B. amyloliquefaciens. Another group of potential and promising microbes used for recombinant protein expression is lactic acid bacteria. They are safer expression hosts since they do not produce endotoxins like *Bacillus* spp. Besides these, proteobacteria and actinobacteria have also been used as host for protein expression. Table 12.1 highlights significant bacterial groups used as cell factories for recombinant protein production (Ferrer-Miralles and Villaverde 2013b).

12.3.3 Yeasts as a Host

Yeasts are also exceptional microbial hosts used for recombinant proteins expression since they have a dual advantage of being unicellular, fast growth and easy genetic manipulation like prokaryotes as well as secretory pathway, protein processing, and ability of posttranslational modifications as in eukaryotes. Hence they are of significant interest as microbial chassis especially when the source of protein to be produced is of eukaryotic origin. Yeasts hosts mainly used as expression systems

Table 12.1 Significant bacterial groups used as cell factories for recombinant protein production

S. no	Group	Advantage	Host	Protein product
1	Caulobacteria	Easy purification of secreted RSaA fusions	*Caulobacter crescentus*	Hematopoietic necrosis virus capsid proteins
2	Phototrophic bacteria	High production of membrane proteins	*Rodhobacter sphaeroides*	Membrane proteins
3	Cold adapted bacteria	Improved protein folding	*Pseudoalteromonas haloplanktis*	3H6 Fab, human nerve growth factor
			Shewanella sp. *strain Ac10*	β-Lactamase, peptidases, glucosidase
4	Pseudomonads	Efficient secretion	*Pseudomonas fluorescens*	Human granulocyte colony-stimulating factor
			Pseudomonas putida	Single-chain Fv fragments
			Pseudomonas aeruginosa	Penicillin G acylase
5	Halophilic bacteria	Solubility favored	*Halomonas elongate*	β-Lactamase
6	Streptomycetes	Efficient secretion	*Streptomyces lividans*	M. tuberculosis antigens
			Streptomyces griseus	Trypsin
7	Nocardia	Efficient secretion	*Nocardia lactamdurans*	Lysine-6-aminotransferase
8	Mycobacteria	Posttranslational modifications	*Mycobacterium smegmatis*	Hsp65-hIL-2 fusion protein
9	Coryneform bacteria	High-level production and secretion; GRAS	*Corynebacterium glutamicum*	Protein-glutaminase
			Corynebacterium ammoniagenes	Pro-transglutaminase
			Brevibacterium lactofermentum	Cellulases
10	Bacilli	High-level production and secretion	*Bacillus subtilis*	β-Galactosidase
			Bacillus brevis	Disulfide isomerase
			Bacillus megaterium	Antibodies
			Bacillus licheniformis	Subtilisin
			Bacillus amyloliquefaciens	Amylases

(continued)

Table 12.1 (continued)

S. no	Group	Advantage	Host	Protein product
11	Lactic acid bacteria	Secretion; GRAS	*Lactococcus lactis*	Fibronectin-binding protein A, internalin A, GroEL
			Lactobacillus plantarum	β-Galactosidase
			Lactobacillus casei	VP2-VP3 fusion protein of infectious pancreatic necrosis virus
			Lactobacillus reuteri	Pediocin PA-1
			Lactobacillus gasseri	CC chemokines

include *Pichia pastoris, Arxula adeninivorans,* Saccharomyces cerevisiae*, Schizosaccharomyces pombe, Hansenula polymorpha*, and *Yarrowia lipolytica*.

Details of the different yeasts that have been used as microbial host for recombinant proteins production, their advantages, and shortcomings are mentioned in Table 12.2. Besides use of yeast vectors, integration of gene encoding the protein product of interest into yeast genome has also been done for more expression and added stability of the insert.

12.3.4 Filamentous Fungi as a Host

Besides bacteria and yeasts, the research efforts for the production of industrially important proteins have also focused on filamentous fungi as a host. For production from these fungi, their already known best regulatory, expression, and secretory machinery is employed in conjunction with heterologous gene producing recombinant protein for commercial production. Table 12.3 highlights the fungal strains employed for recombinant protein production. The use of fungi as industrial hosts for protein production is still under exploration phase since fungi encompasses a very large and diverse group of microbes, and many of them are yet to be well characterized for their biosynthetic potential. Filamentous fungi also have disadvantages like their ability to produce and secrete homologous proteases, which might degrade the recombinant product. Also, the process of glycosylation of proteins is different in mammalian and fungal cells. This can also affect the final product synthesized using fungal machinery (Ward 2012).

Table 12.2 Yeast strains used as microbial host for recombinant proteins production, their advantages, and shortcomings

S. no.	Name	Advantages	Shortcomings	Products	Strains used
1	*Saccharomyces cerevisiae*	Well-known expression system, generally recognized as safe, tolerance to wide pH range, resistance to elevated osmotic pressures, intra- and extracellular expression of proteins	The hyperglycosylation of proteins, low protein yield, and plasmid instability (26)	Hepatitis B surface antigen, hirudin, insulin, glucagon, urate oxidase, macrophage colony-stimulating factor, and platelet-derived growth factor	BY4716, W303, S288c, A634A, CEN.PK, BJ5464, ∑1278b,SK1, BY4741, BY4742, BY4743
2	Yarrowia lipolytica	Produce high levels of heterologous proteins and metabolites, grow on n-paraffin, utilize acetate, alcohols, and hydrophobic substrates including oils, alkanes, and fatty acids	Can use only limited range of C6 sugars such as glucose, fructose, and mannose	Single cell protein (SCP), citric acid, single cell oil (SCO)	W29, E150, E129, YB423, CX161-1B
3	Pichia pastoris	Powerful methanol-regulated alcohol oxidase promoter (PAOX1), highly efficient secretion mechanism, posttranslational modification capabilities, and high cell density growing on defined medium	The high concentration of proteases, difficulties in the systematic study due to the product-specific effects and risks related to storage of large amounts of methanol	Human erythropoietin, phospholipase C, phytase, human superoxide dismutase, trypsin, human serum albumin, collagen, and human monoclonal antibody 3H6 Fab fragment	Y-11430, X-33, Mut+ (AOX1+, AOX2+), Muts (AOX1−, AOX2+), Mut− (AOX1−, AOX2) SMD1163 strain (his4pep4prb1), SMD1165 strain (his4prb1), SMD1168 strain (his4pep4), GS115 (his4), KM71 (Δaox1::SARG4 his4 arg4), SMD1168 (His4, pep4)
4	Hansenula polymorpha	Use methanol as the only carbon and energy source, multicopy integration system and the powerful inducible promoters, thermotolerant (48–50 °C), resistant to heat, oxidative stress, and heavy metals	Accessibility of the multicopy integration system, powerful inducible promoters, thermotolerant (48–50 °C), resistanceto heat, oxidative stress and heavy metals, showed reduced hypermannosylation	Hepatitis B vaccine, insulin, interferon alpha-2a, hirudin, phytase, hexose oxidase, and lipase.	CBS4732 (CCY38–22-2; ATCC34438, NRRL-Y-5445), DL-1 (NRRL-Y-7560; ATCC26012) and NCYC495 (CBS1976; ATAA14754, NRLLY-1798)

Table 12.3 Fungal strains used as microbial host for recombinant proteins production, their advantages, and shortcomings

S. no.	Genus	Proposed hosts	Advantages	Shortcomings	Some potential products
1	*Aspergillus*	*A. niger, A. nidulans, A. oryzae, A. awamori, A. terreus*	Generally recognized as safe (GRAS) list of the Food and Drug Administration (FDA), genetic characterization	Produces aflatoxins (*A. niger*)	Alpha1-proteinase inhibitor, antigen-binding (Fab′) fragment, corticosteroid binding globulin, epithelial growth factor, granulocyte macrophage colony-stimulating factor, growth hormone, humanized IgG1 (kappa) antibodies, interferon-alpha-2, interleukin-6, lactoferrin, lysozyme, mucus proteinase inhibitor, parathyroid hormone, single-chain variable region fragment (scFv) anti-lysozyme construct, superoxide dismutase, and tissue plasminogen activator.
2	*Trichoderma*	*T. reesei, T. altoviride, T. vireus*	Genome sequence available, extraordinary ability to secrete proteins	–	Cellulase, hemicellulose, endoglucanase I, endoglucanase II and cellobiohydrolase I, cinnanoyl esterase from an unsuitable host, the anaerobic fungus *Piromyces equi*
3	*Penicillium*	*P. purpurogenum, P. funiculosum, P. emersonii, P. chrysogenum, P. Camemberti, P. roqueforti*	Efficient promoters	Limited genomic characterization	Arabinofuranosidases, beta-glucosidase, xylanases, acetyl esterases and ferulyl esterases, pectin lyase
4	*Rhizopus*	*Rhizopus oryzae, Rhizomucor miehei*, and *Rhizomucor pusillus*	Characterized annotated expanded gene families of secreted proteases, source of important extracellular industrial and medical enzymes	Pathogenic	Aspartic proteases and subtilases
5	*Neurospora*	*N. crassa*	Biochemically and genetically characterized, grown in simple media with high growth rates producing high amounts of proteins	Not yet recognized as an important industrial host	Influenza hemagglutinin (HA) and neuraminidase antigens (NA)

12.4 Metabolic Engineering Strategies

12.4.1 Directed Evolution

The use of enzymes on an industrial scale many times needs properties like thermostability and resistance to osmotic pressure under actual process conditions that are not found in naturally occurring enzymes. Directed evolution can be defined as the tuning of the natural enzyme in a lab by a process similar to natural evolution by random mutagenesis and recombination followed by efficient screening and selection of the mutants so obtained for desired activity.

Random mutagenesis for evolution makes use of the DNA libraries generated by techniques like error-prone PCR, combinatorial oligonucleotide mutagenesis, DNA shuffling, and staggered extension process (StEP recombination) for strain development followed by screening to obtain a better host with enhanced efficacy. Recombination target modifications make use of nonhomologous recombination, exon shuffling, or alternative splicing to develop and select folded proteins from the obtained secondary structure elements (Urvoas et al. 2012). Although the method is feasible and proficient, a limitation of this method many times involves a compromise between the targeted gene and other essential properties of host required for proper growth and survival.

12.4.2 Site-Directed Mutagenesis (SDM)

It is a highly versatile and precise molecular biology technique to confer tailored mutation in double-stranded DNA. It is also a widely used technique for protein engineering. The approach can be used in traditional cloning, in mapping and control of regulatory as well as in functional analysis of proteins. It also facilitates genome editing in a defined manner through homology-directed repair. Both single-site-directed mutagenesis (point mutation, insertion, deletion, multiple nearby substitutions) as well as multiple site mutations (100 bp apart) have been reported for modification and regulation of the desired gene/s for protein expression (Hsieh and Vaisvila 2013). Irfan et al. 2018 have reported to improve the thermostability of xylanase from *Geobacillus thermodenitrificans* by using SDM. Thermostability of bacterial chitinase was also shown to improve by 15% by using site-directed mutagenesis to alter the enzyme structure (Emruzi et al. 2018).

12.4.3 Site Saturation Mutagenesis (SSM)

SSM is used for substitution of targeted residue (of a protein) with some other naturally occurring amino acid to improve the expression and efficacy the desired significant proteins. The protocol makes use of single amino acid substitutions achieved by using different sets of primers having degenerate mixture of the four nucleotide bases at the three positions of the codon at the site linked to the

functionality of the protein. The amplified mutated PCR products transformed to competent cells and the activities of all substitutions are checked to determine the substitution having the maximum efficiency (Steffens and Williams 2007). When multisites are iteratively subjected for enzyme optimization by saturation mutagenesis, the procedure is called iterative saturation mutagenesis. SSM has led to the evolution of enzymes with stability, enhanced activity, manipulation of binding properties of antibodies and transcription factors (TFs) as well as stereoselectivity. Besides modifying the gene encoding functional protein, SSM has some applications in the engineering of promoters, transcriptional enhancers, RBS, *trans*-acting factors and *cis*-regulatory (Guazzaroni et al. 2015). ISM for evolution experiments has proved to be the most efficient technique even when compared to error-prone PCR (Yang et al. 2017).

12.4.4 Protein Truncation

Truncation is the random or directed deletion of the protein domains that are not necessary for its activity. It has been reported for enhancement of the desirable properties of the enzymes in some cases and may also lead to reduction of specific activity in others. Truncated versions of endo-dextranases from *Streptococcus mutans* were rendered resistant to degradation by proteases during long-term storage by truncation to remove domains not involved in catalytic activity (Kim et al. 2011). Amylases that are active at a high pH are in demand in textile and detergent industries. The stability and specific activity of the alkaline α-amylase of *Bacillus pseudofirmus* were found to improve to almost 35-fold due to N-terminal truncated mutant (Lu et al. 2016).

12.4.5 Fusion to Generate Chimeric Enzymes

Chimeric proteins are proteins prepared by fusion of the structural genes of the two different proteins/polypeptides having different functions or physico-chemical properties to produce a single protein with higher efficiency. Fusion proteins may be a product of two end-to-end fused sub-units linked by a linker or a product of gene where the amino acids from both sources are interspersed with each other. The product of the end fusion genes usually shows the activity of both the parent genes, whereas the product of the later type often shows a novel activity (Irfan et al. 2018). The fused molecules range from short, synthetic oligonucleotides to full-length structural genes. The increasing amount of publicly available sequenced gene databases provides endless number of fusion partners, thus making this technique a valuable and versatile tool for expression of desired proteins.

12.5 Conclusions

Proteins are present and are synthesized by all living forms. They are a part of cytoskeleton as well as help body functions to be carried out smoothly by serving as biocatalysts. Use of microbes as cell factories for production of proteins and enzymes is increasing due to their higher efficiency as compared to chemical catalysts and they are ecofriendly and renewable. They usually have simple nutritional requirements and are easy for handling and manipulation as well. Microbes as chassis for production make use of bacteria, fungi, and yeasts. The use of advanced metabolic engineering strategies has led to assimilation of various microorganisms that can be used in microbial cell factories. Metabolic orthogonality is a main objective that is to be achieved in metabolic engineering for the production of different products by microbes. It is preferred because microbes when engineered have to compromise on their natural pathways. The availability of resources in the form of genetic exploration of microbes will help in achieving this goal, and even researchers will have to input less efforts for bioproduction. Amongst bacteria, a major area is still occupied by E. coli. *Bacillus* spp. are employed as microbial chassis due to their secretory properties that help increase the expression level of proteins. Lactic acid bacteria are still under the process of exploration as a host. Yeasts and fungi are preferred hosts due to posttranslational modifications as well as their high secretion capacity. The choice of best microbial skeleton for industrial protein production is a diverse area as still under exploration phase as the information on genetic and metabolic resources is continuously being added. The addition of the more knowledge in terms of databases will also help for selection of metabolic engineering strategy from the above-discussed methods for the production of desired proteins.

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13 Microbial Production of Antibiotics Using Metabolic Engineering

Sonam Gupta, Payal Gupta, and Vikas Pruthi

Abstract

Metabolic engineering is an advanced production technology to produce novel antibiotic factories by enabling development of high-performance engineered microbial strains. Since, antibiotic yield and productivity are the keen design parameters therefore in metabolic engineering main focus is directed on the carbon flux to increase the antibiotic yield. To achieve this goal, various approaches like genetic modifications, heterologous production, and metabolic alterations are introduced which resulted in substantial elevation of the existing conventional production processes of antibiotics. This book chapter deals with current state of art in metabolic engineering for higher yield of antibiotics.

Keywords

Metabolic engineering · Microbial strains · Antibiotics · Genetic modifications · Yield

13.1 Introduction

Antibiotics are microbial secondary metabolites and are proved crucial therapeutic agents for the treatment of broad-spectrum pathogens including Gram-positive, Gram-negative bacteria, and virulent fungi. However, low productivity and poor activity are the most important parameters always remained a great concern for the commercial production of the antibiotics. Hence, to overcome the problems

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associated with conventional antibiotic production techniques, designing of novel strategies of strain improvement and higher yield are in great demand. These strategies basically involve the optimization of production process with a main aim of having increased product flux. Specially for low value-added products such as antibiotics metabolic engineering provide has become a very important tool to increase the antibiotic production rate along with decreasing the by-products production. Conventional method although leads to the discovery of potent therapeutics; however, it is a high time-consuming process. The traditional approach to discover potent antibiotics involves growth of native producers on different culture media, extraction, and analysis of antimicrobial property using different activity assays (Weber 2014). Metabolic engineering of microbial strain is an alternative strategy to modulate the antibiotic production which specifically target the specific parts of biosynthetic pathway involved in the antibiotic production. Furthermore, metabolic engineering is valuable in numerous aspects as compared to the simple approaches like genetic engineering or random mutagenesis. Earlier, fermentation engineering and random mutagenesis were used for the increased antibiotic production; however, the results were not satisfactory in terms of microbe engineering and genetic modifications. Sometimes conventionally modified microbes were not even able to cultivate in laboratory. To counter these problems, advanced metabolic engineering techniques were evolved which account for purposeful genetic modifications including gene deletion, gene overexpression, or heterologous expression of new genes which distinguish itself from simple genetic engineering and lead to the high-titer rate of antibiotic production (Shomar et al. 2018; Lee et al. 2009; Stephanopoulos et al. 1998; Weber 2014). Numerous types of engineered microbes were developed using metabolic engineering to produce antibiotics such as amidated polyketide from *Streptomyces coelicolor* (Zhang et al. 2016), daptomycin from *Streptomyces lividans* (Penn et al. 2006), clavulanic acid from *Streptomyces clavuligerus* (Li and Townsend 2006), and Erythromycin A from *Saccharopolyspora erythraea* (Chen et al. 2008). For example, actinomycetes showed ten-fold more antibiotic production after genome sequencing (Bachmann et al. 2014).

Present book chapter deals with the advanced metabolic engineering approaches and associated challenges and risk factors involved in the microbial production of antibiotics.

13.2 Metabolic Engineering of Microbes for Antibiotic Production

Chemical synthesis involves complex structures while natural production process account for very minimum synthesis. In this context, metabolic engineering has emerged as a useful tool which offers several advantages including consistent and controlled production rate over chemical and natural antibiotic production. Engineering of microbial flora provides rapid growth and can be easily accomplished as compared to the higher plants and mammalian systems (Nguyen et al. 2006). Genetic modifications of microorganisms cause alterations in the biosynthetic pathways.

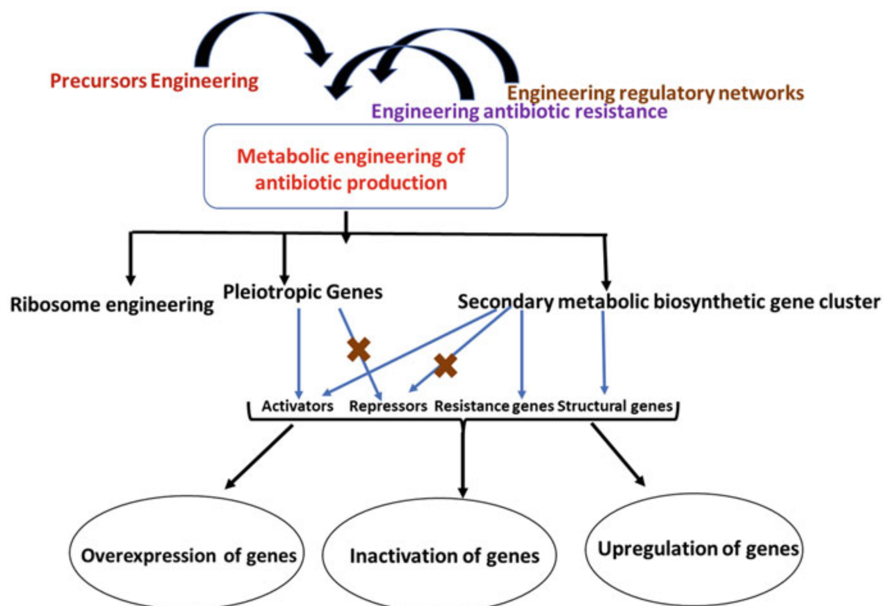

Fig. 13.1 Different approaches of metabolic engineering to produce antibiotics

Microbes contain antibiotic resistance machinery containing gene clusters which provide self-protection against one or more than one antibiotic toxicity. These biosynthetic genes encode different sets of enzymes with several activities such as antibiotic degradation, inactivation, and modification of antibiotic binding site (Cundliffe 1989; Méndez and Salas 2001). However, through metabolic engineering functions of these enzymes and their encoding genes can be altered, and produced mutants can provide high rate for antibiotics for which earlier they showed resistance (Olano et al. 1995; Menéndez et al. 2007; Yanai et al. 2006). Metabolic engineering for antibiotic production involved several approaches including engineering antibiotic resistance, engineering pathway-specific and other regulatory genes for the increased rate of antibiotic production as shown in Fig. 13.1. Presence of antibiotic biosynthetic genes also contain a different gene involved in self-protection from the toxic effects of synthesized antibiotics. Antibiotic resistance also helps in the mutant selection with increased antibiotic production rate. Some antibiotics are synthesized by pathway-specific genes, and mutation in these genes causes increased antibiotic production.

13.3 Ribosome Engineering

Ribosome engineering is one of the advanced metabolic engineering approaches used for the antibiotic production. Studies revealed that mutation in ribosomal synthetic genes could lead to the improved production of unusual antibiotics in the microbes which normally would not have been produced or produced in a very low concentration. Due to mutation, aberrant ribosome is produced which contains modified guanosine nucleotide, i.e., hyperphosphorylated guanosine nucleotide (ppGpp) (Okamoto-Hosoya et al. 2003; Wohlert et al. 1999). Induction of point mutation such as str-6 point mutation leads to the production of actinorhodin and undecylprodigiosin antibiotics by *S. lividans* and *S. coelicolor* (Shima et al. 1996). This mutation involves expression of rpsL mutant in single-copy-number plasmid. Thus, it can be conferred that ribosome engineering has been emerged as an advanced molecular technique to enhance antibiotic production in several *Streptomyces* spp. such as *S. chattanoogensis*, *S. antibioticus*, *S. lavendulae*, and *S. albus* enabling the high rate fredericamycin, actinomycin, formycin, and salinomycin antibiotic production, respectively (Hosoya et al. 1998; Tamehiro et al. 2003). Overexpression in *S. coelicolor* of genes such as frr encoding a ribosome-recycling factor whose overproduction has been detected in streptomycin-resistant rpsL mutants also leads to actinorhodin overproduction up to tenfold (Hosaka et al. 2006). Nishimura et al. (2007) reported that deletion mutation of rsmG gene encoding SAM-dependent 16S rRNA methyltransferase confers the actinorhodin production in *S. coelicolor* along with low level of resistance for streptomycin. Besides, *S. coelicolor*, *S. albus*, and *Planobispora rosea* strains, respectively, can also be engineered to produce high titer of antibiotics such as rifampin, paromomycin, geneticin, fusidic acid, thiostrepton, gentamicin, and lincomycin other than streptomycin (Hu and Ochi 2001; Tamehiro et al. 2003; Beltrametti et al. 2006). Wang et al. (2008) reported that metabolic engineering of *S. coelicolor* could be used for the 180-fold increased actinorhodin production, which is known for its resistance ability against rifampin, gentamicin, geneticin, paromomycin, thiostrepton, fusidic acid, and lincomycin in normal conditions. Additionally, mutant strains having mtmR and complement act II-orf4 also cause amplified synthesis of actinorhodin production in *S. coelicolor* JF1 (Lombó et al. 1999; Blanco et al. 2000). Apart from mutations, upregulation of secondary metabolic pathways can also be used for improvement in antibiotics production such as pimM encoding PAS/LuxR regulator enhance the pimaricin synthesis in *Streptomyces nataliensis* (Antón et al. 2007). rapH and rapG encoding proteins with LuxR and AraC-like HTH motifs, respectively, increase rapamycin production in *Streptomyces hygroscopicus* (Kuscer et al. 2007). SARP family proteins possess pleotropic functions which include regulation of different secondary metabolic pathways. For example, afsR gene induces overexpression of actinorhodin and undecylprodigiosin in *S. coelicolor* for its increased production (Horinouchi et al. 1983). Other homologous pleotropic activators such as SsmA and afsR-p are involved in the increased biosynthesis of actinorhodin, doxorubicin, nystatin clavulanic acid, streptomycin, and pikromycin, in *S. clavuligerus*, *S. lividans*, *S. griseus*, and *S. Venezuelae*

(Sekurova et al. 1999; Parajuli et al. 2005; Maharjan et al. 2008). pptA encodes a phosphotyrosine protein phosphatase, a member of signal transduction network basically involved in the production of actinorhodin and undecylprodigiosin (Umeyama et al. 1996).

13.4 Inactivation of Pathway-Specific Genes

The inactivation of pathway-specific proteins can also lead to overproduction of secondary metabolites which act like pleiotropic repressors. For example, overproduction of chromomycinis conferred by inactivation of specific transcriptional repressor cmmRII in *S. griseus* subsp. *griseus* (Menéndez et al. 2007). Inactivation of actVBorf10 encoding LysR-otype transcriptional regulator leads to the overproduction of actinorhodin in *S. lividans* (Martínez-Costa et al. 1999). Deletion or disruption of two-component system phoR-phoP has been found involved in the higher pimaricin production in *S. nataliensis* (Mendes et al. 2007). Sola-Landa et al. (2003) reported the 5- and 12-fold increased production of actinorhodin and undecylprodigiosin after deleting the same system in *S. lividans* (Sola-Landa et al. 2003). Disruption of nsdA, a pleiotropic gene of *S. coelicolor*, leads to the improved actinorhodin, calcium-dependent antibiotic and methylenomycin biosynthesis. Overexpression of antibiotic-producing genes is attained by activators or by repressors (Olano et al. 2008). Actinorhodin pathway can also be activated by different other gene alternations such as the inactivation of polyphosphate kinase gene ppk (Chouayekh and Virolle 2002).

13.5 Engineering of Structural Genes

Engineering of biosynthetic structural genes like inactivation, activation, or increased dose of biosynthetic structural genes is another important approach which can be used to increase the production of antibiotics. For example, overexpression of stcmM gene can lead to high titter of tetracenomycin D3. Actually, stcmM encodes acyl carrier protein (ACP) which is a type of polyketide synthase (PKS). Overexpression of stcmM causes the 30-fold increased expression of this enzyme leading to high production of teracenomycin D3 (Decker et al. 1994). Similarly, modifications of 6DOH pathway genes lead to increased synthesis of 6DOH 4-deoxy-4-(dimethylamino)-5,5-dimethyl-D-ribopyranose which is actually involved in the twofold increase of an antitumor enediyne antibiotic C-1027 by *S. globisporus.* An important gene of 6DOH pathway gene group is sgcA1. Therefore, in order to get further increased fold yield, this gene can be targeted for modification by several means including introduction of another gene caller cagA (Murrell et al. 2004). cagA encodes an apoprotein which helps in the increasing antibiotic production fold (Salas and Méndez 2005). Antibiotics like spinosyn can be obtained in higher titter by targeting gtt and gdh genes of L-rhamnose biosynthetic pathway which is interrelated to the 6DOH pathway. These genes encode most

important enzymes NDP-glucose synthase and NDP-glucose dehydratase, respectively, which are keen enzymes of L-rhamnose and L-dimethyl-forosamine biosynthesis (Madduri et al. 2001). Clavulamic acid is produced by *S. clavuligerus* at fivefold rate by engineering of cas2 encoding clavaminate synthase. Further, integration of an activator coding gene ccaR to cas2 leads to 23-fold increase in the earlier production of clavulamic acid (Hung et al. 2007). Modification in a different gene pah2 which encodes proclavaminate amidino hydrolase has also been reported to improve the clavulanic acid production (Song et al. 2008).

Li et al. 2005 reported that the nikkomycin X produced by *S. ansochromogenes* was obtained at higher titter after modifying the sanU and sanV genes. These biosynthetic genes encode glutamate mutase, a responsible factor for increased antibiotic synthesis. This enzyme basically converts glutamate to 3-methylaspartate, a precursor of nikkomycin X. Similarly, overexpression of lat gene in the high copy number plasmid helps in the increased cephamycin C production in the *S. clavuligerus* (Malmberg et al. 1993, 1995).Wang and Tan 2004 showed that the duplication of sanO which encodes non-ribosomal peptide synthetase (NRPS) also gives the higher titer of nikkomycin X. Gene duplication causes the enhancement of the 4-formyl-4-imidazolin-2-one leading to antibiotic increased production. Other approaches apart from gene modifications to get higher yield include engineering of genes to transform antibiotic production. Engineering of dnrX and dnrH genes involved in the production of daunorubicin and doxorubicin in *S. peucetius* (Lomovskaya et al. 1998; Scotti and Hutchinson 1996).

13.6 Genome Shuffling

Genome shuffling is a new and rapid method to enhance secondary metabolite production. Zhang et al. (2002) reported the six- to eightfold increase in tylosin production in *S. fradiae* using genome shuffling over a population of classically improved strains. In contrast to this, conventional methods require about 20 rounds of mutagenesis and screening methods. Genome shuffling of *Streptomyces* spp. U121 induced the overproduction of (2S,3R)- hydroxycitric acid within one round of nitrosoguanidine mutagenesis (Hida et al. 2007).

13.7 Conclusion and Future Studies

Antibiotics-resistant microbial infections have been emerged to be the most prevalent and serious threat to the society today in which we are living. This could be possible because of several causes such as lack of awareness, limited effective antimicrobials of natural origin, and low rate of new antibiotic discovery. Different metabolic approaches of which we have discussed in this chapter would be helpful to provide new dimension for the new and effective antibiotics production. Engineered microbes or engineering of their metabolic pathways in either way boost up the rate of antibiotic production which is an urgent requirement in the current scenario to

deal with different diseases including those caused by multi-drug-resistant pathogens.

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14 Metabolic Engineering Opening New Avenues for Therapeutics

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Abstract

Metabolic engineering involves advantageous manipulations in the metabolic pathways of suitable organisms for better yield of valuable metabolites like pharmaceuticals, fuels, dairy products, and cosmetics. In classical approach, cellular metabolism is altered by changing enzyme kinetics or regulatory proteins by employing genetic engineering tools, thus enhancing the yield of a metabolite or producing a novel bio-product. Several strategies like upregulation/ downregulation of key enzymes, elimination of toxic by-products, removing feedback inhibition, reducing competition, and engineering transporters/co-factors may be employed to optimize an engineered process in suitable host like bacteria. In this chapter, a broad overview of metabolic engineering is presented, describing various strategies of metabolic engineering, plant metabolic engineering, microbial metabolic engineering with evident examples and challenges of metabolic engineering.

Keywords

Metabolic engineering · Enzymes · Regulatory proteins · Feedback inhibition · Bio-product

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14.1 Introduction

Metabolic engineering is a tool to produce enhanced levels of desired metabolite (s) in an organism through genetic manipulations in a biosynthetic pathway of the metabolite of interest (Woolston et al. 2013). Metabolites are generally categorized into two groups, primary and secondary metabolites. Primary metabolites are essential for normal growth and development of an organism. For several decades, secondary metabolites have been speculated to be a by-product with no economic value. Though secondary metabolites do not play crucial role in growth and development but definitely play an important role in survival, cell signaling, and regulation of primary metabolite biosynthetic pathway (Carmichael 1992; Makkar et al. 2007). For example, in plant secondary metabolites play an important role in defensive mechanisms against pathogens as well as herbivores (Zaynab et al. 2018).

Plants are the major sources of secondary metabolites and others are bacteria, fungus, and animals. Plants are the unlimited source of structurally and chemically diverse secondary metabolites. These secondary metabolites have commercial and clinical implications and are widely utilized in pharmacology and therapeutics for treatment of various human diseases, e.g., taxol, vinblastine, and vincristine for cancer treatment (Seca and Pinto 2018). In 2015, Tu Youyou shared the Nobel Prize in Physiology or Medicine for the discovery of antimalarial drug artemisinin, a compound which is extracted from *Artemisia annua*, which highlights the importance of secondary metabolites (Youyou 2016). Although a large number of secondary metabolites of varied origins have been investigated for pharmacological activity, a major fraction of secondary metabolites remains unexplored till date. In addition, the biosynthetic pathway of several known metabolites is also not understood well until recently. The advent of powerful new technologies such as next-generation sequencing, mass spectrometry, genome editing techniques along with parallel development of sophisticated bioinformatics algorithms have revolutionized the understating of plant secondary metabolite biosynthetic pathways, which in turn permitted the fine-tuning of secondary metabolite production using genetic manipulations.

Depending on the complexity of the pathway, the metabolite production can be altered by overexpressing a gene or silencing a gene or introducing a complete pathway with multiple genes. In corn seeds, tocopherol production was increased by overexpressing geranylgeranyl transferase gene, but conversely zeaxanthin accumulation was increased by silencing zeaxanthin epoxidase gene (Giuliano 2014). In rice endosperm, several genes have been introduced in rice genome in order to synthesize β-carotene, famously known as golden rice, from the precursor isopentenyl diphosphate (Giuliano et al. 2008).

Undoubtedly, it is significant to understand the complexities of biosynthetic pathways with the available recent technologies in the plant biotechnology in order to modulate the levels of metabolite of interest. In this context, we will discuss in detail about various strategies of metabolic engineering, plant and microbial metabolic engineering approaches along with recent examples and various challenges in the engineering process.

14.2 Strategies of Metabolic Engineering

Metabolic flux analysis (MFA) and metabolic control analysis (MCA) are two most important aspects of metabolic engineering. Metabolic flux analysis is a stoichiometric method that quantifies the consumption and production in a biological system (Ando and Martin 2018). Likewise, metabolic control analysis helps to determine the control coefficients of various enzymes as flux control is distributed among different enzymes instead of one rate-limiting enzyme. Both MFA and MCA help to manipulate the metabolic flux distribution to optimize the desired cellular parameter and/or enhance the yield of metabolites (Yang et al. 2007). The primary requirements for metabolic engineering are understanding of the metabolic pathway of interest, genes coding the relevant enzymes, regulatory elements responsible for suppression, and/or overexpression of target genes, in vitro and in vivo mutation analysis, assembly of gene arrays in suitable host system (Lee et al. 2009). Several hosts like bacteria, yeast, fungi, plants, and animals are nowadays available for such studies.

Stephanopoulos and Vallino (1991) introduced the concept of network rigidity, flexible and rigid nodes which explain the mechanism of resistance to variations in metabolic pathways. For a successful metabolic engineering strategy, a thorough understanding of the host system is indispensable to choose the appropriate modification (Stephanopoulos and Vallino 1991). For example, it is important to examine metabolic burden of any potential strategy on the host system, e.g., the effect it causes on the growth of host system and possible effects on "unrelated" pathways.

There are several approaches of metabolic engineering available to achieve the desired goal. Lee et al. (2012) proposed that metabolic strategies could be classified into two groups—the rational intuitive approach and the systematic and rational-random approach. The former approach includes the conventional variations in metabolic engineering process like carbon source utilization engineering, precursor enrichment and by-product elimination, transport engineering, and cofactor engineering, while the latter approach includes omics-based engineering techniques and evolution-based strategies (Lee et al. 2012). Some of these approaches are discussed in the following.

14.2.1 Gene Overexpression

The overexpression of a gene or a gene family is the most basic approach to enhance the synthesis of desired bio-product and has been practiced widely in various sectors. The disease resistance in apple was demonstrated by overexpressing *MdMyb10* transcription factor in flavonoid pathway. The production of flavanols was elevated to 1.6 and 1.7 times in *MdMyb10* "HC" and "Gala" transgenic plants as compared to their non-transgenic counterparts (Rihani et al. 2017). Likewise, enhanced protein expression in engineered yeast can be achieved by manipulating the unfolded protein response (UPR) pathway wherein several targets are identified in several studies. For instance, a heterologous overexpression of *Hac1* increased protein secretion of

endogenous invertase by twofold, *Bacillus* amylase by 2.4-fold and recombinant α-amylase by 70% (Valkonen et al. 2003).

14.2.2 Heterologous Pathways

Another approach could be an introduction of exogenous or heterologous pathway in the host plant. In this method, a gene or set of genes naturally produced in a host organism can be introduced in a non-native host which is relatively easier to cultivate (Bock 2013). Park et al. (2018) constructed astaxanthin pathway in *Escherichia coli* by introducing heterologous *crt* genes and truncated *BKT* gene from *Pantoea ananatis* and *Chlamydomonas reinhardtii*, resulting in high productivity of astaxanthin (Park et al. 2018). Other notable examples include production of biofuel molecules, fatty acid ethyl esters in *E. coli* by introducing non-native enzymes from native plants and various bacteria (Lennen and Pfleger 2013).

14.2.3 By-Product Elimination

An alternative method of improving the production of desired metabolite is removal of an inhibiting enzyme or competing metabolic reactions involving same substrate, thus channeling the substrate toward a desired chemical reaction. For instance, the production of L-threonine and L-isoleucine in *Corynebacterium glutamicum* was significantly increased by eliminating L-lysine after deletion of chromosomal *ddh* and *lysE* (Dong et al. 2016).

14.2.4 Transporter and/or Co-factor Engineering

The overproduction of metabolites like amino acids is usually not the sufficient criteria to obtain the improved titer, several factors like transporters and co-factors also contribute toward the final titer. The overexpression of global regulator Lrp and BrnFE (two-component export system) increases the production of branched-chain amino acids significantly (Chen et al. 2015; Vogt et al. 2014). Similarly, co-factor regeneration also plays role in channeling the overproduction of metabolites. Bommaredyy et al. fabricated a de novo pathway by altering the coenzyme specificity of NAD-dependent GADPH to NADP which resulted in supplementary supply of NADPH through glycolysis (Bommareddy et al. 2014).

14.2.5 Systems Biology Models

System-based approaches are employed alternatively to eliminate the random and untargeted approaches of metabolic engineering. This approach aims to encode comprehensive information of an organism into a computational skeleton for

predicting physiological behavior from cellular genotype (Lee and Kim 2015). Constraint-based reconstruction and analysis (COBRA) schemes are widely utilized for in silico modeling of metabolic networks of an organism to optimize the genetic modifications (King et al. 2015; Oberhardt et al. 2013). A web server "FMM-From Metabolite to Metabolite" mimics the biological system and leads to the virtual synthesis of the targeted product by a redesigned pathway based on KEGG (Kyoto Encyclopedia of Genes and Genomes) and other databases (Chou et al. 2009).

14.3 Plant Metabolic Engineering

Plants possess two types of metabolites, primary and secondary. Primary metabolites such as lipid, protein, nucleic acids, and carbohydrates are needed for the growth and development of the plant, while secondary metabolites although do not play any role in plant growth and development but are required for their survival in unfavorable environment (Sangwan et al. 2018). Plant secondary metabolites are directly extracted from natural plant sources, and therefore they are also known as natural products. In addition to providing protection to the plant from herbivore attack, pathogens, and environmental stresses, secondary metabolites also provide specific odors, tastes, and colors to the plant parts like leaves, fruits, and flowers. Researchers have been using these plant secondary metabolites as medicinal drugs, food, fragrance, juice, cosmetics, etc. owing to their commercial and therapeutic significance (Narnoliya et al. 2018a, 2019; Narnoliya and Jadaun 2019; Sangwan et al. 2018).

Nowadays, the application of plant-derived bioactive molecules in medicine has increased significantly because of their role in curing various types of diseases including cancer, diabetes, malaria, tuberculosis, and infectious disease. Thus, natural bioactive products hold a substantial share in pharmaceutical industries, which can be estimated from the fact that 61% of anticancer and 49% of anti-infective medicines contains plant-bioactive compounds (Luo et al. 2015; Narnoliya et al. 2018a). Also, the alternate sources of medicines are expensive and prone to side effects. Hence, the demand of natural products is increasing day by day, but their production is limited. Generally, the chemical synthesis of secondary metabolites is not preferable due to their structural complexities, thereby increasing our dependence on their biological sources. Thus, it is important to enhance the production of natural products by altering their biosynthetic pathways. Metabolic engineering by using synthetic biology, genetic engineering, and CRISPR-Cas is the most successful approach to obtain elevated level of secondary metabolites (Sangwan et al. 2018; Maurya et al. 2019; Kumar et al. 2019).

A number of plants have been reported which have significant levels of secondary metabolites such as Neem, Geranium, Centella, Withania, Artemisia, Mentha, Catharanthus, and Cymbopogon (Narnoliya et al. 2014, 2018b, Narnoliya and Jadaun 2019; Sangwan et al. 2013; Sangwan and Sangwan 2014). Several types of secondary metabolites are produced by using metabolic engineering such as phenylylpropanoids, terpenoids, tropanes, carotenoids, flavonoids, alkaloids, sterols, saponins, terpenoid indole alkaloids, lignin, and benzenoid (Sangwan et al. 2018).

Metabolic engineering can also be used for the enhanced production of functional sugars, oligosaccharides, food products, dairy products, cosmetics, etc. (Jadaun et al. 2019). Recently, attempts have been made for the enhanced production of therapeutic compounds like artemisinin and taxol through metabolic engineering.

Metabolic engineering technology is able to provide enhanced yield of desired secondary metabolites for their application at commercial scale for therapeutics. Plant metabolic engineering provides several new opportunities in agriculture, food, environment, chemicals production, health and medical fields (Narnoliya et al. 2018a, Narnoliya and Jadaun 2019; Sangwan et al. 2018). Generally, three systems are used for metabolic engineering, whole plant, tissue-cultured plant cell, and plant gene(s) in microorganism (Sangwan et al. 2018). Here, we will discuss about secondary metabolites, their biosynthetic pathways, and metabolic engineering to obtain enhanced level of desired product.

14.3.1 Types of Secondary Metabolites

Generally, secondary metabolites are categorized into three main groups, terpenoids, nitrogen-containing compounds, and phenylpropanoids, and at present there are more than 50,000 metabolites from these three groups.

14.3.1.1 Terpenes

Terpenes are the most abundant secondary metabolites (>4000) in plants. They are biosynthesized through terpenoid biosynthetic pathways. The basic unit of the entire terpenoid group is five-carbon isoprene unit; therefore, terpenes are also known as isoprenoids. Further, assembly, cyclization, and group modification of isoprenes produce array of compounds (Nagegowda 2010; Narnoliya et al. 2018a). They are classified in several groups based on isoprene units like hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), triterpenes (C30), and higher terpenes (>C30) (Narnoliya et al. 2018a, 2019; Sangwan et al. 2018). The mevalonate (MVA) and non-mevalonate or 2-*C*-methyl-D-erythritol 4-phosphate (MEP) are the basic pathways for isoprene biosynthesis. These pathways are present in different parts of the cell like MVA pathway operated in cytosol, whereas MEP pathway operated in plastid (Fig. 14.1). Generally, monoterpenes and diterpene are biosynthesized by MEP/DOXP pathway, whereas sesquiterpenes and triterpenes are produced through MVA pathway produces (Narnoliya et al. 2017; Nagegowda 2010). However, incidences of cross-talks have also been reported. Some well-known terpenes compound having therapeutic applications are isovaleric acid, geraniol, terpineol, limonene, linalool, artemisinin, abscisic acid, farnesol, germacrene, paclitaxel, ginsenosides, steroids, etc. (Nagegowda 2010; Narnoliya et al. 2018a; Sangwan et al. 2018).

14.3.1.2 Nitrogen-Containing Compounds

The compounds of this class possess nitrogen atom within their structure. Alkaloids consist a major part of these compounds and others are cyanogenic glucosides,

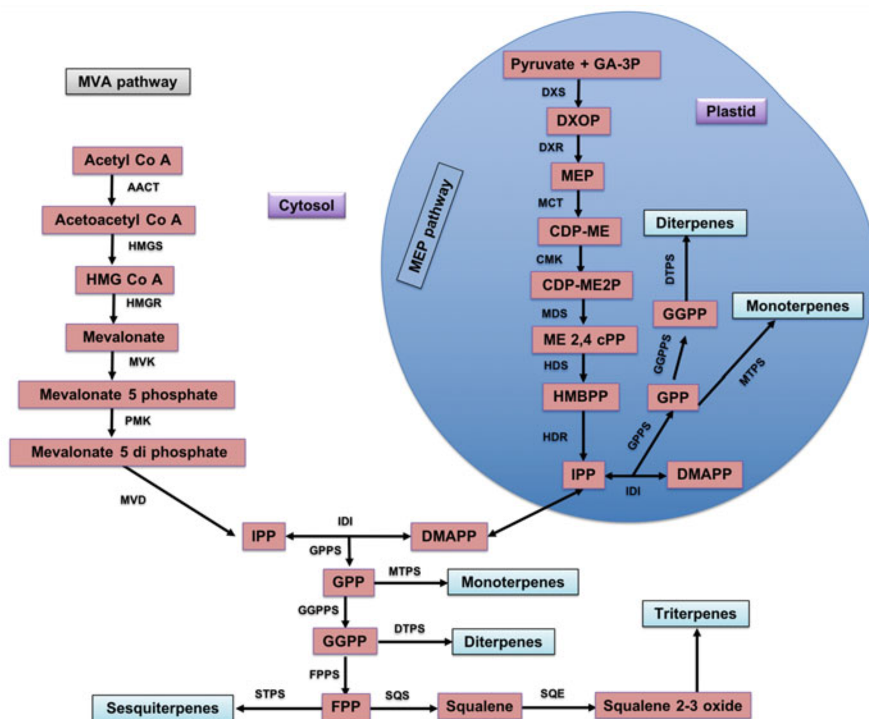

Fig. 14.1 Schematic representation of terpene biosynthetic pathway. Abbreviations: *AACT* acetoacetyl-CoA thiolase/acetyl-CoA acetyltransferase, *HMGS* hydroxymethylglutaryl-CoA synthase, *HMGR* hydroxymethylglutaryl-CoA reductase, *MVK* mevalonate kinase, *PMK* phosphomevalonate kinase, *MVD* mevalonate diphosphate decarboxylase, *DXS* 1-deoxy-D-xylulose 5-phosphate synthase, *DXR* 1-deoxy-D-xylulose 5-phosphate reductoisomerase, *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, *HDS* (E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase, *HDR* (E)-4-hydroxy-3-methylbut-2-enyl diphosphatereductase, *IDI* isopentenyl diphosphate delta isomerase, *GPPS* geranyl diphosphate synthase, *FPPS* farnesyl pyrophosphate synthase, *GGPPS* geranylgeranyl diphosphate synthase, *MTPS* monoterpene synthase, *STPS* sesqui-terpene synthase, *DTPS* diterpene synthase, *SQS* squalene synthase, *SQE* squalene epoxidase, *HMG* CoA, hydroxymethylglutaryl-CoA, *IPP* isopentenyl pyrophosphate, *DMAPP* dimethylallyl pyrophosphate, *GA-3P* glyceraldehyde 3-phosphate, *DXOP* 1-deoxy-D-xylulose-5-phosphate, *MEP* 2-*C*-methyl-D-erythritol-phosphate, *CDP-ME* 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol, *CDP-ME2P* 2-phospho 4-(cytidine 5′-diphospho)2-*C*-methyl-D-erythritol, *ME 2,4 cPP* *C*-methyl-D-erythritol 2,4-cyclodiphosphate, *HMBPP* 1-hydroxy-2-methyl-2-butenyl 4-diphosphate, *GPP* geranyl pyrophosphate, *FPP* farnesyl pyrophosphate, *GGPP* geranylgeranyl pyrophosphate, *MVA* mevalonic acid

non-protein amino acids, etc. Generally, they are originated from amino acids like tryptophan, tyrosine, lysine, histidine, and ornithine (Luo et al. 2015; Sangwan et al. 2018). In plants, Gramineae, Rosaceae, and Leguminosae are the main cyanogenic glucosides-producing families (Narnoliya et al. 2018a). Naturally, alkaloids are toxic and involved in the defense system of plants. Most commonly three types of

alkaloids are present in nature, monoterpene indole alkaloids (MIAs), benzylisoquinoline alkaloids (BIAs), and tropane-type alkaloids. Strictosidine, (S)-reticuline, and tropane are the key precursor molecules of the MIA, BIA, and tropane-type alkaloid compounds, respectively (Narnoliya et al. 2018a; Sangwan et al. 2018; Kushwaha et al. 2013).

14.3.1.3 Phenylpropanoids

Phenylalanine-derived secondary metabolites are known as phenylpropanoids. The plant phenylpropanoids are involved in the growth, development, and defense mechanism of plants as well as have several therapeutic applications for human kind. Generally, phenylpropanoids are classified into two main groups, flavonoids (flavones, flavonols, flavanones, anthocyanidins, and isoflavones) and non-flavonoids (hydroxycinnamates and stilbenes) (Narnoliya et al. 2018a; Baenas et al. 2014).

14.3.2 Modulation of Metabolic Flux for Enhanced Production of Therapeutic Products

Secondary metabolites are biosynthesized by their specific biosynthetic pathways, and to obtain enhanced level of desired metabolites, their metabolic flux needs to be altered. The first requirement for altering metabolic flux is the complete understanding of pathway enzymes and their regulation. Further, the key regulatory genes and regulatory factors (promoters/transcription factor/enhancers) can be modified to obtain a significant level of desired products. Generally product modulation can be performed via three processes: gene overexpression/silencing, cis-regulatory elements (promoters), and trans-regulatory elements (transcription factors). Nowadays, long noncoding RNA and small regulatory RNAs like micro-RNA (miRNA), short-interfering RNA (siRNA), piwi-protein-interacting RNA (piRNA), etc., are also identified as new key regulatory elements which can play a critical role in product modulation as well as production (Narnoliya et al. 2019).

14.3.2.1 Modulation Through Key Genes Overexpression and Silencing

This is the basic approach of the modulation of metabolite production. In this approach, key regulatory genes can be overexpressed or silenced, in in vivo as well as in vitro system to divert metabolic flux of desired product toward its maximal production. First of all, the key regulatory genes have to be identified through traditional methods and modern approaches like EST, transcriptome, and genome analysis. A number of secondary-metabolites-producing plants, genome, or transcriptomic data are available in public domain, which can be utilized for gene identification such as Withania, Centella, Artemisia, Neem, and Rose-scented geranium (Sangwan et al. 2013; Sangwan and Sangwan 2014; Narnoliya et al. 2014, 2018b; Narnoliya and Jadaun 2019).

Overexpression of key gene or silencing of gene, which catabolize the desired product, can efficiently enhance the productivity of desired product (Sangwan et al.

2018; Narnoliya et al. 2018a). Efficient genes from other sources can also be transferred into desired system, which plays similar role in metabolite production. The salicylic acid was produced in plant system by transferring microbial gene responsible for its biosynthesis (Verberne et al. 2000; Sangwan et al. 2018). For overexpression, single or multiple selected gene(s) from same source or another source with significant catalytic activity are introduced in in vitro or in vivo systems to produce elevated level of the desired product. In silencing approach, selected gene (s) are knockout through advance techniques like RNA interference (RNAi), CRISPR-Cas, and specific antibody (Tang and Galili 2004; Kumar et al. 2019). Modulation of single gene is an easy process, but multiple genes are quite complicated, therefore alternative techniques like modulation through cis- or trans-regulatory elements are used, which can alter whole pathways.

14.3.2.2 Modulation Through Cis-regulatory Elements

The flux of any metabolite can be modulated through highly expressive constitutive promoter. Promoter is a DNA element, which start the transcription of gene, consist by three regions, core, proximal, and distal. Generally, transcription start site and RNA polymerase binding site are present in core region, and proximal region contains most of the regulatory elements, which make promoter specific. The end distal part possesses some additional cis-regulatory elements. Preferably, constitutive promoters are used for any transgene expression for modulation of metabolite flux of desired products. A number of constitutive promoters are reported, which have remarkable potential such as 35S, ubiquitin, actin, Opaque-2, β-conglycinin, and APase (Sangwan et al. 2018; Narnoliya et al. 2018a). A constitutive promoter is required for the efficient expression of a transgene to modulate metabolic flux, which should express in each and every tissue as well as conditions.

Docosa-hexaenoic acid (DHA) is a long-chain polyunsaturated fatty acid used in the maintenance of human health and development. Deficiency of DHA in human body can cause cardiovascular and inflammatory diseases, therefore its proper requirement for human body is necessary. Generally, the source of DHA for humans is fish and algal oils, but due to its high demand their higher production or alternative source is required. Therefore, recently DHA biosynthesis genes from yeast/algae were introduced in several crops such as Arabidopsis, Brassica with a seed-specific strong promoter FAE1 (Arabidopsis origin). These crops are able to produce significant DHA to fulfill the marker demand. The transgenic *Brassica napus* produces as much as oil in 1 ha which is equal to the oil produced from 1000 fishes and it contains ~12% DHA in oil (Petrie et al. 2012; Sangwan et al. 2018).

14.3.2.3 Modulation Through Transcription Factors

Transcription factors (TFs) are the DNA-binding domains, which bind to the upstream region of the genes (promoters or enhancers) and alter the expression of responsible gene and pathway. TFs could enhance or repress the transcription of gene. TF interacts with cis-regulatory elements of promoter, and they can affect multiple genes or whole pathway at a time. Generally, metabolic flux is controlled by multiple gene, therefore modulation in TF is quite easy process rather than many

genes of that pathway (Mitsuda et al. 2007; Sangwan et al. 2018). The C1 and R transcription factor of maize, which regulates the level of anthocyanins in aleuronic layers, was successfully expressed in Arabidopsis with strong constitutive promoter, which modified the flavonoid biosynthetic pathway, and anthocyanin regulation was manipulated accordingly (Stracke et al. 2007). A number of TFs were successfully applied for the enhanced production of desired product through altering metabolic flux such as MYB, WRKY, DOF4, NAC, and P1 (Sangwan et al. 2018). Nowadays, beside natural TFs, artificial TFs were also used for modulating metabolic flux toward desired product (Jantz et al. 2004).

14.4 Metabolic Engineering in Microorganisms

Microorganisms are ubiquitous and include bacteria, viruses, fungi, and algae of both marine and terrestrial origin. These microorganisms have significant contribution in all life forms on the planet including human, plant, and veterinary life (Zengler and Zaramela 2018). Like plants, microorganisms also produce primary metabolites and secondary metabolites. These small-molecular-weight compounds are utilized to regulate their own growth, encourage the mutually beneficial organisms, and suppress their predators and/or competitors (Firáková et al. 2007). Microbial primary metabolites include amino acids, vitamins, enzymes, nucleotides, alcohol and organic acids, and secondary metabolites with a diverse array of functions including antibiotics, anti-tumor agents, plant growth stimulators, nutraceuticals, anabolics, anesthetics, herbicides, and insecticides. Since the serendipitous discovery of penicillin in 1929, scientists have been investigating the therapeutic potential of microbial metabolites (Vining 1990). This led to the mass production of bioactive products that have become inevitable in the cure and control of several infectious diseases (Mehra et al. 2019).

Although, the microbes are being extensively explored for industrially important materials like biofuel, plastics, and polymers, the scope of this chapter covers only the therapeutic use of the microbes. Microbial secondary metabolites having diverse range of bioactivities are profoundly utilized as antibiotics. It is estimated that around 45% of bioactive metabolites are produced by Streptomyces and actinomycetes; 17% are produced from *Bacillus*, *Pseudomonas*, *Myxo-,* and *Cyanobacteria* species, and the remaining 38% are produced from eukaryotic fungal species (Berdy 2005). These metabolites possess low molecular weight and unique structural features. It is estimated that around 40% of these microbial metabolites cannot be chemically synthesized (Feher and Schmidt 2003). Despite the dimmed interest of big pharmaceutical companies in the last decade, microorganisms still continue to be an interesting and productive source of bioactive metabolites (Marinelli et al. 2015). However, the microbial resistance toward the currently used anti-microbial drugs is a global issue, and this life-threatening issue calls for an urgent effort to find better alternatives and hunt novel as well as effective antimicrobial drugs (Gould and Bal 2013). Additionally, some of the anti-microbial drugs or drugs of non-microbial origin having very good efficacy are posing

challenge because of their very low yield (Ehrenworth and Peralta-Yahya 2017). The cultivation of conventional microbial producers with unnatural precursors, molecular alterations including random mutagenesis, or use of genetically engineered strains are better alternatives to enhance the yields and expand the chemical diversity. For example, the synthesis of penicillin V is a standard example of metabolic engineering (Demain and Elander 1999).

S. cerevisiae is extensively used for food and alcoholic beverages since centuries and has fetched enormous attention from pharmaceutical industries from past few decades. Due to its non-pathogenic nature, well-known fermentation as well as process technology, availability of genomic data, and prolonged history of usage in edible products, *S. cerevisiae* is a safe and preferred choice for metabolic engineering (Glick et al. 2010). Earlier genetic manipulation strategies involved random mutagenesis or classical breeding and genetic crossing of two strains followed by desired mutant screening. However, several advances in the field of genetic engineering have now enabled researchers to modulate specific pathways and directed improvements in the cellular metabolism to achieve a better product. Major products of therapeutic importance produced from microorganisms are discussed here.

14.4.1 Neutraceuticals

PUFA (Polyunsaturated fatty acids) are essential fatty acids required for normal growth of the human. Main focus of the microbial metabolic engineering is on Omega-3 fatty acids which play a pivotal role in reducing the risk of inflammatory and neurodegenerative diseases. The production of α-linolenic acid, eicosapentaenoic acid, and other such PUFAs has been successful in *Y. lipolytica* (Yuan and Alper 2019). Similarly, engineered micro-organisms like *E. coli* is able to produce 100 mg/L of naringenin, a polyphenol, from glucose. Also, the co-expression of naringenin biosynthesis genes and other modifications led to the production of naringenin in *S. cerevisiae*. Some other examples of metabolic engineering of microbes that lead to the production of nutraceuticals are carotenoids in *E. coli* and amino acids such as β-alanine in *S. cerevisiae* (Yuan and Alper 2019).

14.4.2 Antibiotics

Corynebacterium glutamicum and *E. coli* have been widely exploited in antibiotic synthesis by synthesizing precursor molecules like valine, methionine, lysine, and threonine. *E. coli* is generally the first choice amongst microorganisms for protein engineering, and most of the conventional knowledge of microbial physiology and microbial genetics is derived from this species. The initial developments in the field included steady-state accumulation, recombinant phages, plasmids, and GECs (gene expression cassettes).The biosynthesis of antibiotics by certain microbes has been observed to increase rapidly even by small twitching of the metabolic pathway like

overexpressing a single gene associated with it. Most of the times, all the genes associated with the antibiotic-biosynthetic pathway are present on the same chromosome. So, it becomes easier to overexpress the gene clusters and induce higher production of antibiotics. The production of penicillin by *P. chrysogenum* increased when increased copy number of *pcbC* and *penDE* genes were introduced (Yang et al. 2007).

Apart from antibiotics, many other drugs like artemisinin (for malaria) and taxol (anticancer), which are originally derived from plants, are now being engineered to be produced from microbes. For example, *B. subtilis*, a safe bacterium, has been engineered to ease the production of taxol (Abdallah et al. 2019). Microbes like bacteria and yeast which are relatively easier to grow and modulate provide a suitable framework for controlled and scalable production of various pharmaceutically important metabolites like proteins and secondary metabolites via metabolic engineering. In early 80s, the FDA approval for clinical use of recombinant human insulin from recombinant *E. coli* became a revolutionary move for pharmaceutical industry (Ferrer-Miralles et al. 2009). Metabolic engineering takes advantage of the occurrence of majority of the genes involved in the biosynthesis of secondary metabolites in the form of gene clusters. Combinatorial biosynthesis approach is opted for the generation of novel bioactive compounds in host microbes. In principle, prior knowledge of the chemical characteristics of the target compound, followed by identification of gene cluster proceeded by calculated and controlled modulations of the biosynthesis pathway are the basic prerequisites (Zhao 2011). There are many ways in which the metabolic pathways are being engineered in microorganisms to get therapeutically significant products.

14.4.3 Semi-Synthetic Drugs

The industrial production of plant secondary metabolites which form the precursors of many drugs pose many hurdles. These include low-level accumulation of these precursors in plants, slow doubling time, complexity in modulating the metabolic pathways, and difficulty in isolating the natural products. To overcome these hurdles, scientists are evolving a new approach in which the semi-synthesis of the precursor molecules is done in engineered microbes, thereby circumventing most of the technical challenges discussed earlier (Ehrenworth and Peralta-Yahya 2017).

14.5 Challenges of Metabolic Engineering

Increasing global burden of diseases and high cost of therapeutics derives the rewiring of metabolites for better yield and production. However, customizing biological systems into efficient factories is challenging because of extensive cross-talk of pathways and strict regulatory mechanisms. Any alteration in the complex cellular system does not imply a direct change in the targeted pathway, rather it is interconnected with several unrelated pathways (Yu et al. 2019).

Additionally, genome-wide regulation of pathways generally results in multiple transcriptional or translational responses which are otherwise unpredictable in nature.

Gene silencing or knockout mutants may behave in entirely different manner than the wild types by activating alternate metabolic pathways. Thus, what may look like a response to gene knockout might be because of any secondary mutation in the organism. Therefore, modulations in genetic makeup of the organism in or around their normal physiological range may be more effective. Sometimes fine-tuning of the gene expression might also be required to optimize the flux distribution (Jin et al. 2019; Zhang et al. 2018). The fine-tuning of D-lactate pathway and 2,3-butanediol pathway was demonstrated by engineered *nar* promoter which is dependent on dissolved oxygen (Hwang et al. 2018).

Omics is an effective approach for expanding knowledge and predictive analysis at molecular level in understanding the complex biological system. However, it has its own limitations, for example, majority of the genomes of the sequenced organisms are junk and have no known function. Besides, microarrays and genomic mapping techniques are error-prone, further complicating the analysis. The data analysis and interpretations are a big challenge in bioinformatics (García-Granados et al. 2019).

Overall, metabolic engineering is a useful tool to facilitate a well-planned hypothesis of metabolic flux and metabolic control on a suitable host to develop value-added products especially therapeutic compounds. The driving force behind these strategies is the sustainable development, cost factor, and increasing demands. Combined with these driving forces and scientific advancements, a series of remarkable achievements are available in literature. The limiting factor to this approach is our understanding of fundamental biology and complexity of interconnected metabolic pathways. Thus, it becomes inevitable to fill the knowledge gaps and address the bottlenecks in the developed processes. It is strongly believed that as the knowledge pool in the fundamental and applied biology expands and more powerful tools are developed, metabolic engineering will provide a truly robust framework for commercial applications.

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15 Biofuels Production Using Metabolic Engineering

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Abstract

Rapidly increasing environmental problems and limited fossil resources are motivating the development of sustainable energy options. In terms of this, microbial fuel always fascinated world community, but their implementation has number of hurdles. However, evolvement of metabolic engineering converts these microbes into efficient cell factories for biofuel production. It incorporates the techniques that work in a frame or synchronized manner to modify the existing enzyme and pathway, related to desired product. This chapter gives brief insight into different strategies and techniques conferring metabolic engineering and highlights the challenges on more advanced level.

Keywords

Sustainable · Metabolic engineering · Implementation · Evolvement · Microbes

15.1 Introduction

Continuous growth of any developed or developmental country depends on the production of goods that require constant supply of energy. Therefore, with increasing industrialization and modernization, consumption of energy sources increased with high rate. Currently, coal, petroleum, and natural gas are the prominent sources of energy which are non-renewable in nature, thus their exploitation to an extreme level is not appropriate worldwide, and it is expected that these sources of energy would be depleted very soon in near future. According to Shahid and Jamal (Shahid

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and Jamal 2011) and International Energy Agency (IEA) (World energy outlook 2007), demand of fossil fuels will increase up to 50% more in 2030 than today out of which 45% will be requisite for only China and India. In addition to this, negative impact on environment like increasing pollution, global warming, and degrading air quality are the major demerits of fossil fuel utilization. So, all these facts are prompting world scientific community to focus on the alternative fuels that would be renewable, sustainable, cost-effective, friendlier to the environment, and also could fulfill the demand of ever-increasing population. Which is not only crucial to protect the environment but also essential for economic sustainability (Mishra and Goswami 2018). Consequently, pioneer research in this arena results in the introduction of biofuels that have been considered as viable alternative for fossil fuels. Laterally several market forces also favored transition of petro-fuel to biofuels. Therefore, biofuel is gaining more attention of many countries day by day (Patel et al. 2016).

Biofuels that come under the category of liquid fuels are utilized for transportation or heating purposes. Its positive impact on rural development and reduced carbon emission are some advantageous properties accountable for its market value. Moreover, several countries already introduced, whereas many are trying to introduce policies that could increase use of biofuel in their energy portfolio. While the following progression will also lead to rapidly rise in the global demand of biofuel, but due to its major domestic consumption very little amount is entering in international market (Dufey 2006). So, government agencies are promoting existing research that focuses more toward biofuel production via utilizing different sources. Currently biomass-based biofuel production is in trend which takes advantage of diverse microorganisms as a factory for biofuel production using different biomass as feedstocks (substrates). These substrates can be variety of herbaceous and woody plants, lignocellulosic biomass, sugarcane bagasse, waste products like botchery waste, agricultural waste, and dairy waste. On the other hand, production of biofuel via utilizing these microorganisms has some hurdles related to narrow range of substrate utilization, slow generation time, dependency on physiological factors such as temperature, pH, and salinity. However, modification in their genome and metabolism is much easier so that these can be modified according to the demand of the system. Metabolic engineering is one of the fields among genetic engineering techniques which work on modulation and designing of multiple pathways according to the need. Nowadays, this technique is becoming more popular among researchers for enhanced biofuel production by changing, deleting, or fixing the route of different biological compounds so that the overall pathway can emphasize to produce interested product, biofuels, etc.

15.2 Biofuels

Progressive research results in the introduction of several energy alternatives like natural gas, biofuels syngas (synthesis gas), and hydrogen. Among all of these, only biofuel emerges as an important and sustainable fuel in foreseeable future. Liquid fuels from natural and renewable sources (plant biomass) are known as biofuels, and

these are categorized into primary and secondary biofuels on the basis of unprocessed form (wood ships, pellets, etc.) and production by processing of biomass (bioethanol, DME, biodiesel, etc.), respectively. According to the type of raw material with different technologies used for their production, secondary biofuels are categorized into four different classes: first-, second-, third-, and fourth-generation biofuels (Nigam and Singh 2011). Employment of biofuels in place of diesel fuels or its blending with petro-diesel is a favorable choice for improving air quality and reducing greenhouse gas emissions (Delfort et al. 2008; Nigam and Singh 2011). Moreover, biofuels are also being explored for their diverse properties such as biodegradability, renewability, non-toxicity, positive economy impact, reduced foreign oil dependence, and no net carbon addition to the atmosphere (Granda et al. 2007).

15.2.1 Types of Biofuels

Biofuels can be solid like wood pellets and charcoal or liquid like biodiesel, alcohol, and bioethanol, or gaseous such as biogas (methane) (Nigam and Singh 2011). This chapter provides a brief overview of biofuels that are currently in use with their economic viabilities and various approaches that are presently utilized for their production.

15.2.1.1 Biodiesel

Biodiesel is considered as a better alternative for petroleum-based fuel and chemically comprised of mono-alkyl esters of long-chain fatty acids derived from animal fats and vegetable oils. Pyrolysis, microemulsions, and transesterification are three primary ways of biodiesel production in which transesterification is the widely used method because of economic point of view and simple, easiest reaction conditions with high conversion (98%) that follows direct conversion to ester without any intermediate step. Basically oil and fats in the presence of catalyst (either acid or base) and alcohol (ethanol or methanol) undergo a chemical process and produce fatty esters along with glycerin by-product. The overhead reaction is known as transesterification due to formation of new esters by the exchange of organic group from esters to alcohol. Along with this type of reagent, feedstocks and operational conditions like catalyst, temperature, reaction time, water content, and free acid are some critical factors that affect biodiesel yield (Ma and Hanna 1999). Moreover, biodiesel can be used neat (B 100), but due to some issues including cold start problems, material compatibility, and loss of engine power, blending is the most preferred method. Biodiesel owns high oxygen content with low aromatics, water, and low sulfur that is primarily responsible for the lower GHG emissions (Shaine 2001). Although biodiesel is safe for environment, high cost of production process is the major demerit that can be overcome by increasing feedstock yields which accounts 60–70% of the total cost and introduction of novel technologies with increasing economic return from by-products produced during biodiesel production like glycerin (Granda et al. 2007).

15.2.1.2 Bioethanol

Unlike biodiesel, bioethanol is ethyl alcohol or chemically known as C_2H_5OH or EtOH. It is produced via microbial fermentation primarily from starch-bearing plants like sugarcane, sweet sorghum, corn, or lignocellulosic biomass and carbohydrate produced in sugar (Anyanwu et al. 2018). Apart from this, waste products like potato peel are also providing good source of feedstock, therefore reducing the cost of overall process. The production of this organic biofuel completes in three major units: pretreatment, enzymatic saccharification, and fermentation (Rocha-Meneses et al. 2017). Microorganisms also play an essential role in bioethanol production especially yeasts (*Saccharomyces cerevisiae*) *utilized by most of industrial plants due to its valuable properties such as* wide range of substrate utilization, high ethanol tolerance, high ethanol productivity, growth in inexpensive media, resistant to inhibitors, and contaminants (Azhar et al. 2017). Blending of bioethanol with gasoline not only provides substitute for fossil fuels but also enhances octane number which further reduces the emission of exhaust gases. Broader flammability limits, increased heat of vaporization, higher flame speed, less toxicity, biodegradability, and lesser production of air-borne pollutants are the main advantages of bioethanol over gasoline (Balat and Balat 2009; John et al. 2011). However, high cost involved in enzyme, feedstock, detoxification, recovery of ethanol, demand of high energy are the major obstacles in pilot-scale production of bioethanol. Thus, several efforts have been made in cost reduction like using engineered microbes and enzyme cocktails for improved yield and better saccharification, respectively, along with the production of high-value-added products to improve the economics.

15.2.1.3 Biobutanol

Currently utmost automobile industries are utilizing biodiesel and bioethanol for reduced emission of harmful gases and lower consumption fossil fuels. However, biobutanol is also a commendable option for safe environment. It is colorless, renewable, flammable, and clean burning fuel with four carbons (C_4H_9OH) (Anyanwu et al. 2018). Production of biobutanol is totally an anaerobic process which utilized common agricultural waste as feedstocks like straw, grass, leaves, spoiled grain, fruits, and other sources of plant biomass (algae culture) (Dürre 2008; Chisti 2008; Chisti 2007). The complete process of fermentation is accomplished in the presence of starch-degrading bacteria belonging to the genus Clostridium especially *Clostridium acetobutylicum* and popularly known as ABE (acetone-butanol-ethanol) on the basis of three main products of this process with a ratio of 3:6:1 (Kamiński et al. 2011). Irrespective of bioethanol, biobutanol is an attractive energy source because of its remarkable characteristics such as high calorific value (29.2 MJ/dm^3), easy transport (can be transported in pipelines unlike bioethanol transported by trucks, trains), non-hydroscopic, high energy density, noncorrosive, lower vapor pressure, relatively low latent heat of vaporization, and higher miscibility with diesel fuel (Anyanwu et al. 2018). Likewise, as opposed to bioethanol that can only be blend with gasoline up to 85%, biobutanol can be blend at any concentration without any engine modifications. These features enhance

its usefulness toward an additive to gasoline as well as biofuels. However, further research is carried out in this direction (Anyanwu et al. 2018).

15.2.1.4 Biomethane

Biogas-derived biomethane comes under the category of clean, green, flexible, easily storable burning fuel, and considered as a substitute for natural gas. Nearly all kinds of biomass and a wide range of waste products like food, distillery, landfill, sewage, oil residues, manure, livestock waste, and harvest surplus provide substrates for biogas production which are further converted into biomethane and utilized for domestic uses, transportation, and electricity generation (Molino et al. 2016; Anyanwu et al. 2018). The conversion process of biogas to biomethane is a biochemical reaction that is processed under oxygen-free environment with bacterial consortium known as anaerobic digestion (AD). Hydrolysis, acidogenesis, acetogenesis, and methanogenesis are four stages of AD which occurred in the presence of four different types of microbes: (1) hydrolytic bacteria that convert organic waste into sugars and amino acids, (2) fermentative bacteria that form organic acids, (3) acidogenic microorganisms that help in the conversion of acids into carbon dioxide, acetate, and hydrogen, and (4) methanogenic bacteria that produce biogas (Molino et al. 2016). Pretreatment of material is the first and very crucial step for biomethane production because it enhances biomass solubilization and biodegradability that directly increase the yield of biomethane. Mechanical (sonication, grinding, milling), biological (enzymatic), chemical, thermal, electrical, and irradiation (gamma-ray, microwave) are the well-established pretreatment methods (Anyanwu et al. 2018).

15.2.1.5 Biohydrogen

Hydrogen is the most abundant molecule on earth; therefore, biohydrogen H_2 produced by various kinds of biomass is considered as safer than other fuels due to zero emission. Furthermore, its production is sustainable, low cost, and clean with easy processing techniques: photofermentation, dark fermentation, biophotolysis, and indirect biophotolysis. Along with this, demand of less energy and formation of water as by-product are the major merits that make biohydrogen an attractive and promising alternative of energy sources (Anyanwu et al. 2018; Boboescu et al. 2016). As compared to traditional energy sources, biohydrogen provides high energy, and after combustion production of water vapors in place of toxic gases is the only thing that ascertains it as a better substitute for gasoline. In addition to this, utilization of biohydrogen as a power fuel is major advantage in those areas where combustion is hazardous or undesirable.

15.3 Microbial Commodities for Biofuel Production

The following discussion clears that all the approaches for biofuel production involve biological sources (plant materials) which mandate the utilization of microorganisms in the production process (Elshahed 2010). Moreover, it is

necessary for environmental point of view to concentrate on the biological way to generate alternatives of energy. Therefore, production of biofuels using microorganisms is in high demand. Unique metabolic diversity and easy genetic manipulation are the most important features of microorganisms that allow these to grow on various substrates. For example, where algae and cyanobacteria have the potential to convert atmospheric CO_2 into biofuel, methanotrophs produce methanol from methane. Unique molecular machinery of *Shewanella oneidensis* and *Geobacter sulfurreducens* bacterium can be used in biochemical devices for bioelectricity and biohydrogen generation (Kumar and Kumar 2017). Table 15.1 gives concrete knowledge about the role of different microbes in energy sector.

15.4 Role of Metabolic Engineering

Production of medicines, chemicals, pharmaceuticals, fuels, and several other value-added products are the multiple applications of microorganisms (Yadav et al. 2016). Although production of these compounds is presently on their peak, but it seems impossible to complete the sky-rocketing demand of global population because of several limitations related to low yield, feedstock availability, low enzyme secretion via microbes, etc. Though in the era of science, these limitations can be shorted out by applying a new and advantageous field of genetic engineering prevalently known as "metabolic engineering." Which can be defined by a process of modulating metabolism and specific pathway of cell in a way to produce desired product with greater yield. The process led by downregulation and overexpression of concerned protein or gene related to pathway so that all the machinery of cell could emphasize to accrue the desired compound in good amount (Koppolu and Vasigala 2016). As per the current scenario, humans are using splendid properties of microbes tremendously for green, safe, ecofriendly, and an alternative source of energy production. Moreover, application of genetic engineering in the field of biofuel production has great potential toward resolving the problems coming from mixed substrate utilization, harmful by-products, downstream processing, and problems associated with purity of products, etc. So, the process of fermentation is easier than before, and world can successfully reach at that level where an average percentage of transportation could run on microbial biofuel. However, more research is needed to explore this field in a way to produce advanced microbial fuel.

15.5 Strategies of Metabolic Engineering for Biofuel Production

Microorganisms are also well known for their potential in biofuel production; however, traditional methods for obtaining these were not appropriate because in some cases the amount of desired product was very less in cell. Since metabolism of native microbes are not evolved in such a way to meet the expected outcomes desired by humankind, thus naturally isolated microbes show less efficiency toward interested component. To address the following problems, microbiologists introduced a

Table 15.1 Summary of different microbes involved in energy sector

Microorganism	Strain description	Biofuel	Yield (g/L)	References
Escherichia coli	Bacteria	Butanol	30	Shen et al. (2011)
Escherichia coli	Bacteria	Ethanol	25	Romero-García et al. (2016)
Saccharomyces cerevisiae	Non-oleaginous yeast	Fatty acids	0.38	Yu et al. (2016)
Pseudomonas putida	Bacteria	Butanol	0.05	Nielsen et al. (2009)
Cryptococcus vishniaccii	Oleaginous yeast	Lipids	7.8	Deeba et al. (2016)
Zymomonas mobilis	Bacteria	Ethanol	–	Kremer et al. (2015)
Yarrowia lipolytica	Oleaginous yeast	Fatty acids	55	Beopoulos et al. (2009)
Rhodosporidiumkratochvilovae HIMPA1	Oleaginous yeast	Lipids	8.39	Patel et al. (2014)
Chlorella minutissima	Microalgae	Lipids	49.2 mg/L/d	Arora et al. (2016)
S. cerevisiae DA24-16BT3	Known for fermentation of glucose, cellobiose, and xylose	Ethanol	60	Ha et al. (2011)
Lipomycesstarkeyi	Oleaginous yeast having the ability to consume glucose as well as xylose simultaneously	Lipids	4.6	Yu et al. (2011)
Cryptococcus curvatus	Native oleaginous yeast (adaptive to glucose and xylose consumption)	Lipids	5.8	Yu et al. (2011)

brilliant technique of metabolic engineering and came up with a great idea which transforms these hesitant biofactories into highly efficient focused machines proficient in huge production (Lee et al. 2012). Now it is possible to produce advanced biofuel having similar characteristics with petro-fuels (Peralta-Yahya et al. 2012). Additionally it becomes easier to balance redox reactions and optimization of metabolic pathways in engineered microbes (Lee et al. 2008). While to transform a microbe metabolically, some basic fundamentals should be very clear; mechanism

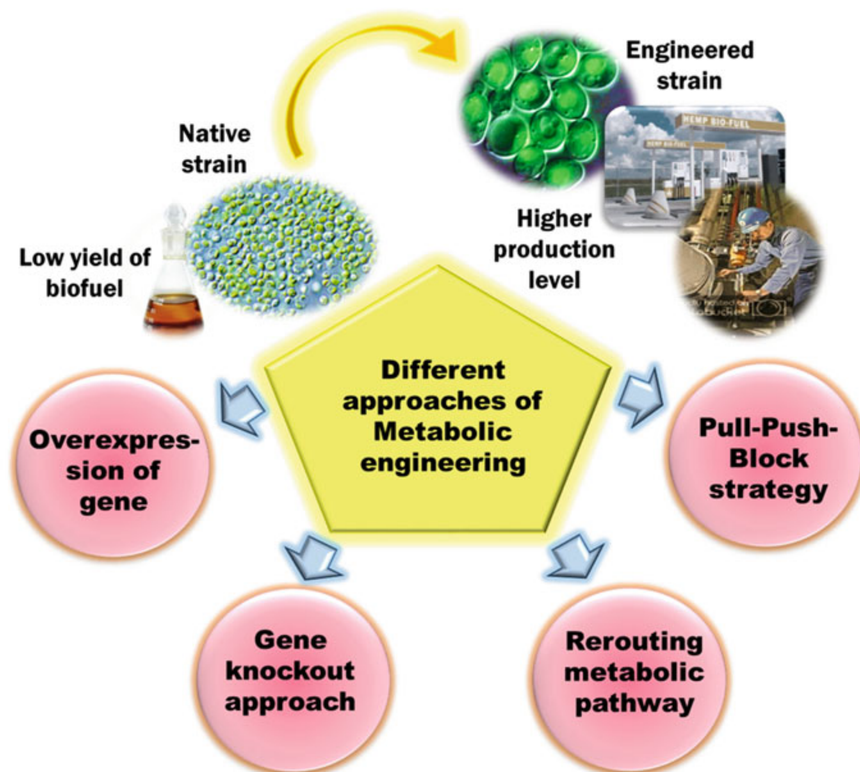

Fig. 15.1 Overview of different approaches in metabolic engineering

of host metabolism for desired compound, information of genes encoding related enzymes, knowledge about biosynthetic pathway, expression and suppression of required genes, in vivo and in vitro mutation, etc. Bacteria, fungi, yeast, animals, and plants are the most pioneering host for metabolic engineering. This section will brief about different approaches of metabolic engineering for efficient productivity of biofuels as shown in Fig. 15.1.

15.5.1 Rational and Intuitive Approaches

These approaches are basically applied to pursue multiple objectives at the same time. This is related to diversify substrate utilization by organisms, transporter modifications which restrict intracellular product accumulation, eradication of by-product formation, rerouting of metabolic pathways, etc. (Lee et al. 2012).

15.5.1.1 In Silico Gene Insertion Strategy

This advanced strategy allows microbes to utilize a wide range of substrates and therefore reduces the cost involved in upstream processing with maximum conversion of biomass to biofuel. Metabolic manipulation in *S. cerevisiae* for ethanol production is the best suited example in which two different genes encoding xylose reductase (Xyl1p) and xylitol dehydrogenase (Xyl2p) from *Pichia stipitis* are inserted into *cerevisiae* genome which enable it to degrade xylose, the most available form of carbohydrate in nature (Chu and Lee 2007). However, higher affinity of Xyl1p and Xyl2p for NADPH, NAD^+ leads to redox imbalance due to accumulation of NADH and shortage of NADPH (Jeffries 2006). This major roadblock was cleared by deletion and overexpression of genes, namely $NADP^+$-dependent glutamate dehydrogenase (GDH1) and NAD^+-dependent GDH2, respectively. The results are increased activity of Xyl1p for NADH due to which ethanol production is increased to a significant level along with decreased synthesis of by-product and increased tolerance toward lignocellulosic hydrolysate (Almeida et al. 2008; Lee et al. 2008).

15.5.1.2 Transporter Engineering

This is a new approach that improves microbial resistance capacity against toxic components as well as target products. A recent example of this is insertion of a gene encoding acyl-acyl carrier protein (ACP) thioesterase (TE) I in *Synechocystis* sp. PCC6803 weakens peptidoglycan layer and secretes FFAs approximately 197 mg/L of culture (Hegde et al. 2015). These techniques not only enhanced the level of production but also helps in balancing and monitoring intermittent metabolites.

15.5.1.3 Elimination of By-Product

Insertion of gene for glyceraldehyde-3-phosphate dehydrogenase showed remarkable changes in the reduction of glycerol 58% and xylitol 33% with 24% increase in ethanol productivity (Bro et al. 2006). In another example, fermentation efficiency of *E. coli* is improved by minimizing by-product formation, and this could be achieved by the disruption of tricarboxylic acid cycle which further eliminates the pathways for NADH oxidation (Causey et al. 2003).

15.5.1.4 Rerouting Metabolic Pathways

Rerouting metabolic pathway is a better option in those conditions when native pathway of microorganisms could not provide optimum concentration of the desired product. For example, n-butanol production from *E. coli* can be efficiently improved by using trans-2-enoyl CoA reductase enzyme (obtained by *Treponema denticola*; a gram-negative bacterium) in place of originally present butyryl-CoA dehydrogenase (Bcd) (produced by *Clostridium acetobutylicum*) (Shen et al. 2011). Gonzalez and his colleagues engineered an *E. coli* strain in a way so that it can activate β-oxidation pathway when glucose is present in the medium. This could be achieved by manipulation of several transcriptional regulators in *E. coli*. This novel reverse β-oxidation pathway directed to synthesize various fatty acids and alcohols by

introducing initiation and termination enzymes to catalyze the Claisen condensation of acyl-CoA and removal of the CoA moiety from acyl-CoA, respectively. Approximately, 14 g/L of n-butanol is produced via this approach that is far better than the traditional n-butanol producer, *C. acetobutylicum* (Dellomonaco et al. 2011; Lee et al. 2012).

15.5.1.5 Classical Metabolic Engineering (Overexpression of Gene)

Currently microalgae are front runner for biodiesel production on large scale due to its growth in severe environmental conditions, high lipid yields, and fast growth rate. Microalgae lipid can be improved by biochemical and transcriptional factor engineering by regulating different physiological conditions required for algal growth. However, admirable results could be possible by metabolic engineering. For example, a strain of microalgae has been modified by National Renewable Energy Laboratory in the USA (NREL) by overexpressing acetyl-coA carboxylase gene that controls the process of lipid accumulation. The result represented drastic change in lipid content of outdoor (40%) and indoor (60%) cultivation of microalgae as compared to native strain 5–20% (Hegde et al. 2015). Studies done by Davis and Cronan ascertain that thioesterase overexpression allows free fatty acids synthesis in *E. coli* by avoiding inhibition of fatty acids via acyl-ACP (acyl carrier protein) (Davis and Cronan 2001).

15.5.1.6 Gene Knockout Approach

In a study, Trentacoste et al. (2013) improved lipid productivity in a diatom *Thalassiosira pseudonana* under nutrient-deprived and -replete conditions by knockdown of multiple activities of Thaps3_264297 protein linked with multifunctional lipase, phospholipase, or acyltransferase. As a result, lipid was improved significantly from 3.3- to 4.1-fold under exponential and nutrient-limited conditions, respectively (Trentacoste et al. 2013). Another example in this category includes inhibition of phosphoenolpyruvate carboxylase (PEPC) enzyme required for phosphoenolpyruvate to oxaloacetate conversion which has shown elevation of fatty acid content in plant and microalgae (Hegde et al. 2015).

15.5.1.7 Construction of Synthetic Metabolic Pathways

This branch of metabolic engineering is very challenging and demand advanced and effective tools that could control mRNA and protein required for proper functioning of synthetic pathway. For example, a non-biofuel-producing microorganism can be transformed into biofuel-producing microbes by implanting imperative genes or enzymes from biofuel-producing organisms such as *Hansenula polymorpha*, a non-oleaginous yeast attained 40% increment in total fatty acid content after heterologous expression of ACC_1 from *Mucorrouxii* fungi (Ruenwai et al. 2009; Kumar and Kumar 2017).

15.5.1.8 Pull–Push–Block Strategy

In pull–push–block strategy, multiple genes play critical role where every gene works in a synchronized manner so that the expected outcomes could be achieved.

For example, overexpression of TesA ("pulling" carbon flux to fatty acid production), FadR ("push" fatty acid production along with blocking of fatty acid degradation), and simultaneously knocking out of FadE ("blocking" of β-oxidation pathway) improved fatty acid titer with 48% in batch cultivation (Hegde et al. 2015; Xiao et al. 2013).

15.5.1.9 Tolerance Engineering (Inverse Metabolic Engineering)

Lignocellulosic biomass is the easily available and furthermost form of carbon source; however, the presence of HMF, furfural, acetate, and phenol-like inhibitors restricts microbial growth. On the other hand, ethanol tolerance in *S. cerevisiae* is a big issue. However, few examples of current metabolic manipulations represent that this is not an impossible task. For example, overexpression of one of the three genes ADE17, PIR3, and HTA2 conferring furfural tolerance led to shorter lag phase without interruption in ethanol productivity (Dai and Nielsen 2015; Almario et al. 2013). While overexpression of another gene encoding oxidoreductase in *S. cerevisiae* provides resistance against HMF and furfural (Pinel et al. 2011; Almeida et al. 2008).

15.5.2 Systematic and Rational-Random Approaches

As the name suggests, these approaches are based on clear rationale. This approach includes in silico and omics-based techniques and applied to study various hidden bottleneck for strain development. Metabolic revolution, resequencing, reengineering, and enzyme evolution are some subcategories interrelated to this approach (Lee et al. 2012).

15.6 Future Avenues

In the past few years, microbiologists are working efficiently on different aspects of metabolic engineering like large-scale production of cellulase, tolerance engineering, and genetic manipulation of several organisms like yeast, algae, and *E. coli*. Although these processes working satisfactory on lab scale, commercialization of these innovations in an economical way is a challenging task (Kumar and Kumar 2017; Hegde et al., 2015). Therefore, it is necessary to explore new tools together with metabolic manipulations. In addition to this, data-driven, synthetic biology and protein engineering are some excellent approaches for optimizing and troubleshooting of engineered metabolic pathways. Further improvement in efficiency of microbial biofuels or to establish it as an alternative for indigenous techniques, intervention of more potent tools is required like those could control mRNA and protein in a precise manner so that the process run according to its own metabolic flux. The evolution of new pathways to tune the pathway flux, biofuel properties, expanded source of feedstock, optimization of hosts, and boosted production of advanced biofuel are demands of future market (Peralta-Yahya et al.

2012). Several other issues allied with secretion of oil, direct FAME synthesis by microbes, waste to biofuel production, and conversion of by-product to value-added products are some essential issues need to be addressed by world scientific society.

15.7 Conclusion

Metabolic engineering has great impact on microbial biofuels' production; as per the discussion, different tools and techniques play a significant role in the commercialization of bioethanol and other biofuels as well. This makes organisms more robust in nature compared to native strain and also provides resistance to toxic by-products with a more tuned lipid profile. The microorganisms manipulated by the abovementioned processes are more focused toward the generation of desired products along with maintaining optimal flux of pathway and a wide range of substrate utilization including different wastes.

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Microbial Utilization of Glycerol for Biomanufacturing

16

Valeriane Malika Keita, Miriam Gonzalez-Villanueva, Tuck Seng Wong, and Kang Lan Tee

Abstract

Glycerol is a by-product of transesterification, fat splitting, and saponification processes. The rise in biofuels led to excess glycerol and price volatility. Crude glycerol from the biodiesel and the oleochemical industries contains methanol or other impurities, and constitutes a disposal problem. This waste glycerol has invigorated the development of new processes, both chemical and biological, for crude and refined glycerol valorization. Nature has evolved sophisticated microbial pathways for glycerol uptake and dissimilation. The advancement of genetic engineering has allowed us to capitalize on this opportunity and developed microbial production hosts to manufacture a wide array of value-added chemicals from glycerol, ranging from bulk to fine chemicals. Although there are technical challenges associated with microbial glycerol utilization (feedstock composition, substrate and product inhibition, downstream processing, and process economics, to name a few), strain engineering and bioprocess optimization have empowered us to address some, if not all, of these issues. Therefore, the future for utilizing glycerol as a renewable raw material for biomanufacturing holds great promises. This book chapter seeks to consolidate the advances made in the sector of biological glycerol use, by putting equal weight on our discussions of feedstock (i.e., glycerol) and of microbial cell factory.

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Keywords

Glycerol · Crude glycerol · Sweetwater · Biodiesel · Transesterification · Fat splitting · Saponification

Abbreviations

μ_{max}	Maximum specific growth rate
1,2-PDO	1,2-propanediol
1,3-PDO	1,3-propanediol
2,3-BDO	2,3-butanediol
3-HPA	3-hydroxypropanal
ABC	ATP-binding cassette
ADP	Adenosine diphosphate
agmR	Gene encoding a transcription regulator
aqy3	Gene encoding a protein similar to fps1
ATP	Adenosine triphosphate
Btu	British thermal unit
c/lb	Cents per pounds
CAB	Columbia blood agar base
ccp	Gene encoding a bacterial complement control protein
ccpA	Catabolite control protein A
CCR	Carbon catabolite repression
Cg	*Corynebacterium glutamicum*
cre	Catabolite response element
Crh	Homologue of the phosphoryl carrier protein of PTS
crr	Gene encoding PTS glucose transporter subunit IIa
DDH	Diol dehydratase (encoded by the *dhaB* gene in bacteria)
DG	Diglyceride or diacylglycerol
DHA	Dihydroxyacetone
dhaB	Gene encoding bacterial glycerol dehydratase
DhaD	Bacterial glycerol dehydrogenase
DhaK	Bacterial ATP-dependent dihydroxyacetone kinase (encoded by *dak* genes)
DhaKLM	Bacterial PEP-dependent dihydroxyacetone kinase (trimeric: subunits DhaK, DhaL, DhaM)
DHAP	Dihydroxyacetone phosphate
DhaR	Dehydrogenation pathway regulator
dhaT	Gene encoding bacterial 1,3-propanediol-oxydoreductase
Ec	Escherichia coli
EMS	Ethyl methanesulfonate
EU	European Union
FAD	Flavin adenine dinucleotide

FAME	Fatty methyl esters (biodiesel)
FCC	Food chemical codex
FDA	Food and drug administration
FFA	Free fatty acid
FHL	Formate hydrogen lyase
FMN	Flavin mononucleotide
Fps1	Glycerol uptake/efflux facilitator protein
G3P	Glycerol-3-phosphate
G3PDH	Glycerol-3-phosphate dehydrogenase
gcy1	Gene encoding a yeast glycerol dehydrogenase
GDHt	B_{12}-dependent glycerol dehydratase
GldA	Bacterial glycerol dehydrogenase
GlpABC	Subunits of the heterotrimeric anaerobic bacterial G3PDH (encoded by the *glpABC* operon)
GlpD	Subunit of the homodimeric aerobic bacterial G3PDH (encoded by the *glpD* gene)
GlpF	Glycerol uptake facilitator protein (encoded by the *glpFK* operon)
GlpK	Bacterial glycerol kinase (encoded by the *glpFK* operon)
GlpP	Bacterial antiterminator protein
GlpR	Bacterial glycerol/G3P-responsive transcriptional regulator
gpd1/2	Gene encoding a yeast G3PDH
gpsA	Gene encoding bacterial NAD-dependent G3PDH
GTE	Glycerin-to-epichlorohydrin
gut1	Gene encoding a yeast glycerol kinase
gut2	Gene encoding a mitochondrial G3PDH in yeast
HOG	High-osmolarity glycerol
hog1	Gene encoding a yeast mitogen-activated protein kinase (MAP)
HPr or enzyme I	Phosphoryl carrier protein of PTS
IUPAC	International Union of Pure and Applied Chemistry
KEGG	Kyoto Encyclopedia of Genes and Genomes
MAP	Mitogen-activated protein
MES	2-(*N*-morpholino)ethanesulfonic acid
MG	Monoglyceride or monoacylglycerol
MIP	Major intrinsic protein
mmHg	Millimeter of mercury
MONG	Matter organic non-glycerol
mRNA	Messenger ribonucleic acid
n.r.	Not reported
NAD	Nicotinamide adenine dinucleotide
NPA	Asparagine-proline-alanine sequence
NTG	*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine
P(3HB)	Poly-3-hydroxybutyrate
pbs2	Gene encoding a MAP kinase of the HOG pathway

PDO	Propanediol
PDOR	1,3-propanediol-oxidoreductase (encoded by the *dhaT* gene in bacteria)
pduC-E	Gene encoding subunits of a propanediol dehydratase
pdxK	Gene encoding a bacterial pyridoxine kinase
PEP	Phosphoenolpyruvate
PEP:PTS	Phosphoenolpyruvate:sugar phosphotransferase system
pH	Potential hydrogen
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
Poly(3HP)	Poly(3-hydroxypropionate)
Rex	Redox-sensing regulator
RNA	Ribonucleic acid
RNAP	Ribonucleic acid polymerase
rpoB	Gene encoding the β subunit of bacterial RNA polymerase
rpoC	Gene encoding the β′ subunit of bacterial RNA polymerase
rpoS	Gene encoding a bacterial sigma factor S (σ^S)
Spo0A	Master transcriptional regulator of sporulation
spp	Several species
STL	Sugar transporter like
TFA	Trifluoroacetic
TG	triglyceride or triacylglycerol
UQ	Ubiquinone
USP	United States *Pharmacopeia*
UV	Ultraviolet

16.1 Introduction

Glycerol is a versatile molecule with over 2000 known applications, in sectors as diverse as transport, defense, personal care, pharmaceutical, nutraceutical, food, antifreeze, animal feed, etc. (Bauer and Hulteberg 2013). Found by Swedish chemist Carl Wilhelm Scheele in 1779 as the "sweet principle of fat," glycerol is one of the earliest organic molecules isolated by man (Pagliaro and Rossi 2010). Because of its sweet taste, the term *glycerine* was coined in 1823 by French chemist Michel Eugene Chevreul after the word *glukeros* that implies sweet in Greek (Nda-Umar et al. 2019). Glycerol was the raw material for manufacturing nitroglycerin, the base component for dynamite, discovered by Swedish industrialist Alfred Nobel in 1863 (Lichtman 2017). Nitroglycerin turned into a significant application during World War I and led to the establishment of the first glycerol-manufacturing plants in Europe, Russia, and the United States (Ciriminna et al. 2014). It was produced via microbial fermentation of sugar until 1943 when the high-temperature chlorination of propene to allyl chloride developed at IG Farben plants in Germany became the

predominant process for manufacturing synthetic glycerol from petroleum feedstock (Ciriminna et al. 2014).

The rapid growth of the biodiesel industry has prompted a huge and expanding excess of its glycerol by-product. This by-product is of little economic value due to the presence of impurities. In 2007, the price of refined glycerol was approximately $0.30 per pound in the United States (contrasted with $0.70 per pound before the expansion of biodiesel production), and crude glycerol price was $0.05 per pound (Kerr et al. 2007). Waste management and disposal of a large volume of waste glycerol became a severe financial burden and environmental liability for the biodiesel industry. A considerable amount of research has been devoted to developing chemical and biological processes for value-added conversion of glycerol to address the aforementioned challenge. Process development is affected by the characteristics of glycerol by-product, which are reliant on numerous factors including the feedstock, the manufacturing, and the purification processes. In this chapter, we first provide an overview of the manufacturing processes that produce glycerol as a by-product, which then leads to a discussion on crude glycerol composition and the contaminants present in this industrial waste stream. This is followed by a survey of microorganisms equipped for utilizing glycerol by-product as carbon/energy feedstock for biomanufacturing, the strategies applied to increase glycerol metabolism and therefore productivity, as well as bioproducts derived from glycerol conversion. The chapter concludes with a summary of technical challenges in the biological utilization of glycerol by-product and prospects.

16.2 Glycerol: A Potential Biomanufacturing Feedstock

Glycerol exhibits a unique set of chemical and physical characteristics: water solubility, hygroscopicity, almost colorless, biodegradability, absence of toxicity, viscosity, and high boiling point (Nda-Umar et al. 2019). With a chemical formula of $C_3H_8O_3$, pure glycerol has a specific gravity of 1.261 g/cm^3, a melting point of 18.2 °C and a boiling point of 290 °C under normal atmospheric pressure. Glycerol concentration is usually expressed in weight percentage and is obtained by conversion from specific gravity measurements made at either 20/20 °C or 25/25 °C (Ayoub and Abdullah 2012; Tan et al. 2013). It is stable under normal storage conditions (Pagliaro and Rossi 2008; Ayoub and Abdullah 2012), even when in contact with air (Tan et al. 2013). In spite of its apparent stability, glycerol is polyhydric alcohol that displays extraordinary reactivity due to the presence of primary and secondary hydroxyl groups (Nda-Umar et al. 2019), making it an indispensible oleochemical (Gervajio 2005). It is also ubiquitous, playing critical roles in biological processes as an essential intermediate in lipid metabolism and sometimes as a source of carbon and energy (Klein et al. 2017). Glycerol is involved in the osmoregulation of organisms (Klein et al. 2017). However, its role as a backbone of phospholipids and triacylglycerols is most universal (Klein et al. 2017).

16.2.1 Glycerol Nomenclature and Classification

The International Union of Pure and Applied Chemistry (IUPAC) name of glycerol is propane-1,2,3-triol although it is also commonly alluded to as glycerin(e), glyceritol, glycyl alcohol, and trihydroxypropane (Ayoub and Abdullah 2012; Tan et al. 2013). There is no clear distinction between the two most common names "glycerol" and "glycerin(e)," aside from in the United States where "glycerine" (sometimes spelled as "glycerin") is the most popular commercial name, and "glycerol" is used to refer to its chemical structure. In Europe, "glycerol" is a generic term used to refer to any propane-1,2,3-triol from crude products to commercial forms. In this chapter, we use "glycerol" as a generic term covering propane-1,2,3-triol of all purity and grades.

The designations of various types of glycerol are often confusing due to the lack of standardization. The terms used in this chapter are defined in Fig. 16.1. Glycerol of biotic origin is called natural glycerol or bio-glycerol, as opposed to synthetic glycerol chemically derived from C3 derivatives such as epichlorohydrin, allyl chloride, or allyl alcohol synthesized from propene (Bauer and Hulteberg 2013). Glycerol found in the waste streams of industrial processes is referred to as raw glycerol. Pretreatment is required to transform raw glycerol into semi-crude glycerol. This process removes impurities that either corrode pipes and storage equipment or do not comply with commercial specifications. Semi-crude glycerol, produced at the end of pretreatment, typically contains 60–80% glycerol and is pure enough to be used as animal feed (Mota et al. 2017). The impurities in semi-crude glycerol, however, avert sensitive applications such as human nutrition, pharmaceuticals, or cosmetics (Nda-Umar et al. 2019). The semi-crude glycerol can further be refined to give pure glycerol. Pure glycerol, from both bio-glycerol and synthetic glycerol routes, have a glycerol content of $\geq$95.5% (Ayoub and Abdullah 2012) and can be classified into three primary grades depending on their purity (Pagliaro and Rossi 2010):

- Grade I or technical-grade glycerol is purified up to 95.5–99.5%. It is suitable for synthetic chemistry but not for sensitive applications.
- Grade II or US Pharmacopeia (USP) grade contains glycerol purified to $\geq$99.5%, in compliance with the guidelines set forth by the US Food and Drug Administration (FDA). It can be used in delicate applications like human nutrition, pharmaceuticals, or cosmetics.
- Grade III is the Kosher equivalent of grade II. It has the same purity and applications as grade II, but the product has been prepared and maintained in compliance with Jewish religious dietary law.

Grades II and III glycerol for food applications must also conform to the requirements of Food Chemical Codex (FCC). Glycerol is often commercialized in both semi-crude and pure forms, and the term "commercial glycerol" is used generically for all glycerol products. The term "crude glycerol" is used loosely in

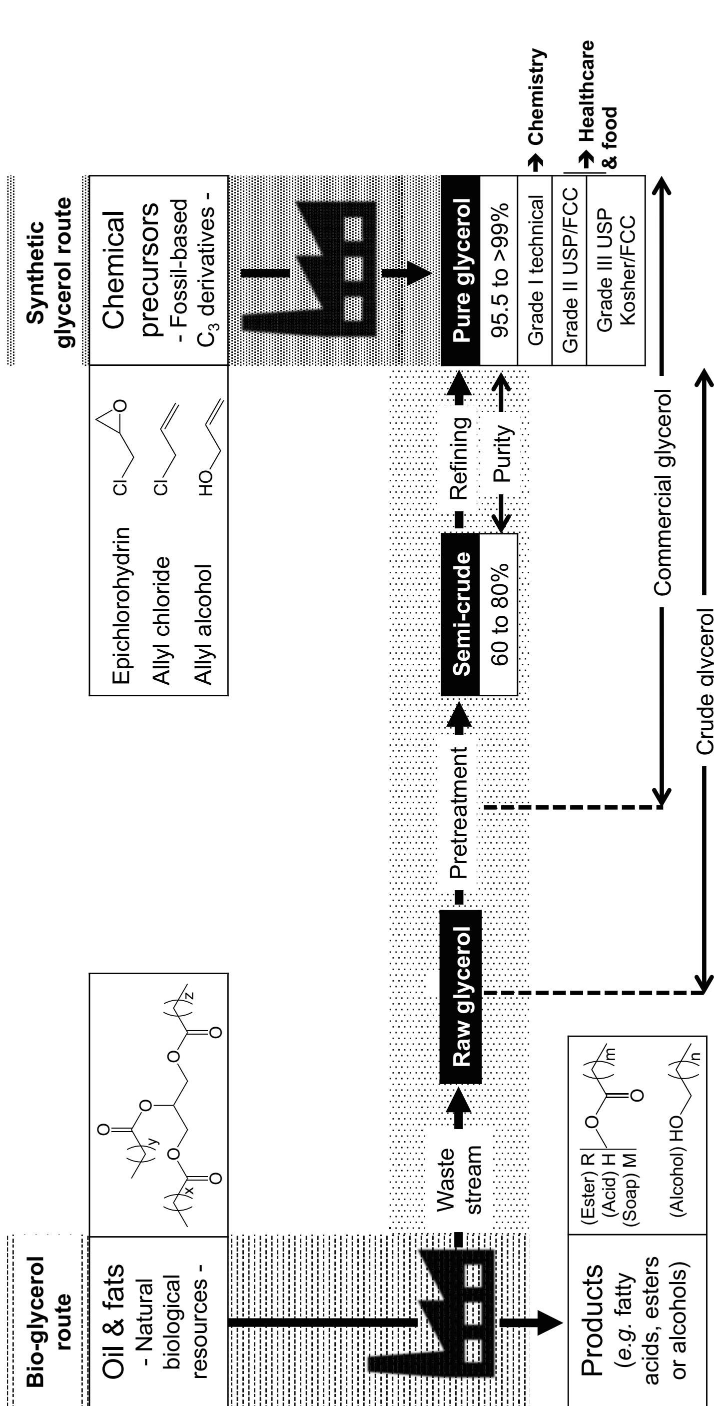

Fig. 16.1 Nomenclature and classification for glycerol of biotic (i.e., oil and fats) and synthetic (i.e., fossil-based C_3 derivatives) origins. Designations of bio-glycerol during processing from raw glycerol to pure glycerol are shown in black boxes

the literature, often without a clear description of its composition or prior treatment. This term is used in this chapter to cover all glycerol forms before "pure glycerol" when pretreatment or refining is ambiguous. All three forms of glycerol (raw, semi-crude, and pure) are a potential feedstock for biomanufacturing although raw glycerol requires the least processing postproduction and is, therefore, the most economical option.

16.2.2 Industrial Bio-glycerol: Processes and Impurities

The bulk of the glycerol accessible today is bio-glycerol originates from vegetable oil and animal fat. Bio-glycerol is derived from biodiesel production (60–70%), followed by fatty acids and fatty alcohols production (30%), with a minor fraction from soap making (Ciriminna et al. 2014; Pagliaro 2017). The chemical reactions in these three main processes are as follows: (1) transesterification utilizing methanol to produce fatty acid methyl ester (otherwise called FAME or biodiesel), (2) fat splitting or hydrolysis into fatty acids, and (3) saponification or hydrolysis under alkaline conditions to produce salts of fatty acid (also known as soap) as appeared in Fig. 16.2.

The composition and quality of the resultant crude glycerol from these industrial processes depend on their feedstock and process conditions. Therefore, the impurities found in crude glycerol are categorized into feedstock-specific and process-specific impurities. This section focuses mainly on transesterification and fat splitting as these two processes contribute ~90% of glycerol in the market.

16.2.2.1 Feedstock-Specific Impurities

Although oil and fat are both composed of triglycerides (TGs), they differ in their physical forms as liquid (oil) or solid (fat) at ambient temperature. The oleochemical industry mainly uses refined soybean oil, palm oil, rapeseed oil, sunflower oil, coconut oil, colza oil, palm kernel oil, tallow, and lard (Hill 2001). Among these, colza, soybean, and palm oils are most suitable for biodiesel production (Pagliaro and Rossi 2008). In any case, the use of edible oil for biodiesel production has been under scrutiny due to its competition with food security. To improve sustainability and cost-efficiency and reduce environmental impact, these edible oils (i.e., refined oils), also known as first-generation feedstocks for biodiesel production, are being supplanted by second-generation feedstocks including some unrefined oils (e.g., jatropha oil, waste cooking oil, and tallow oil) (Tan et al. 2013).

Food-grade oil refining includes steps such as degumming (lecithin and phosphorus removal), deacidification [free fatty acid (FFA) removal], neutralization, bleaching, and deodorization (Pagliaro and Rossi 2008; Patel et al. 2016). Consequently, impurities like colloidal matters, phospholipids, FFAs, and coloring agents are removed from food-grade edible oils, resulting in crude glycerol with a low level of contaminants (Jungermann and Sonntag 1991). These steps increase the shelf lives of refined oils and are especially suitable for fat splitting (Angers et al. 2003). On the contrary, unrefined oils used in biodiesel production (including soap stock

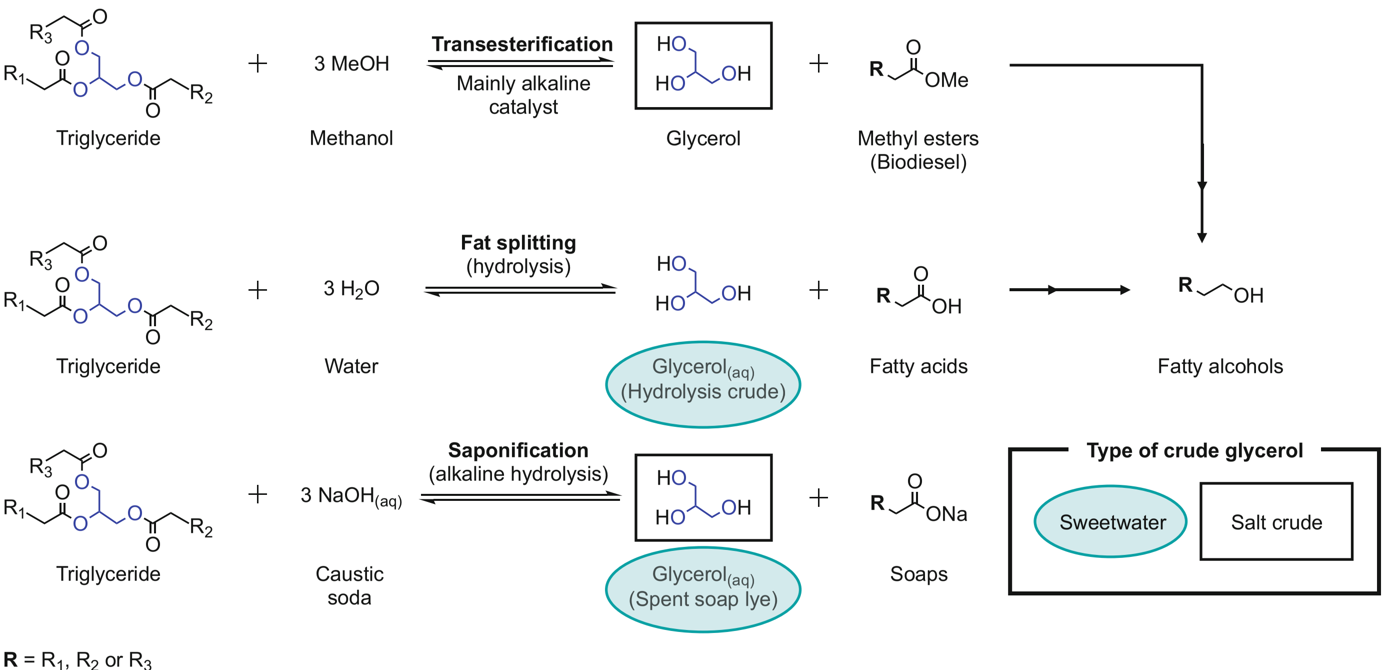

Fig. 16.2 Manufacturing processes leading to bio-glycerol as a by-product. The chemical reactions for industrial processes involving transesterification (top), fat splitting (middle), and saponification (bottom) of triglyceride (i.e., oils and fats) are shown. All three processes produce glycerol as a side product. Derivatives of fatty acids and methyl esters, like fatty alcohols, can be obtained via further processing

and rendered oils) contain all the above impurities, which eventually end up in the discharged crude glycerol. Depending on the type and origin of feedstock, additional contaminants may include sulfur compounds, proteins, nitrogenous compounds, aldehydes, ketones, oxidized fatty matters, fermentation side-products (Jungermann and Sonntag 1991), sodium chloride especially in waste cooking oil (Gao et al. 2016), carbohydrates (Ayoub and Abdullah 2012), and polyphenols (Pagliaro 2017). An examination of the total fatty acid composition revealed that the fatty acid profile of crude glycerol is consistent with that found in the underlying feedstock (Hu et al. 2012).

The source of feedstocks and the oil refining steps they undergo are the key determinants of feedstock-specific impurities. That said, the subsequent processing steps (e.g., chemical reaction and separation process) also influence the amount and the nature of impurities present in crude glycerol. For instance, during fat splitting, fatty acids are cleaved from TGs sequentially to form successively diglycerides (DGs), monoglycerides (MGs), and finally FFAs. Reaction by-products such as unprocessed oil, DGs, and MGs are found in crude glycerol due to an incomplete reaction. Entrained fatty matter (e.g., FAME, FFA) in Matter Organic Non-Glycerol (MONG) is also a consequence of inefficient phase separation, which in turn depends on the length of the alkyl chain of the fatty derivative (i.e., feedstock-specific) and the ionic form of FFA. The ionic form of FFA varies with the pH of the system, which is often dictated by the catalyst used. Therefore, impurities composition is an interplay of feedstock and process.

16.2.2.2 Process-Specific Impurities

The oil and fat processed via fat splitting or transesterification contain theoretically 9.0–13.5% of glycerol (D'Souza 1979). Both processes yield two phases, an oily phase (light phase) containing the target fatty acids or fatty esters and a glycerol phase (heavy phase) containing 8–20% glycerol (after fat splitting) (Jungermann and Sonntag 1991) or 50–60% glycerol (after transesterification) (Bauer and Hulteberg 2013) and impurities. Light and heavy phases are separated by gravity (settling) or centrifugation. The light phase can also be washed with water to recoup entrained glycerol to boost yield.

Crude Glycerol from the Fat Splitting Process

There are four ways to achieve fat splitting, of which only three have been industrially exploited: Twitchell process (batch process), batch autoclave, and the continuous countercurrent high-pressure Colgate-Emery process (Angers et al. 2003). The fourth enzymatic route is yet to be commercialized but has shown promises in reducing process energy consumption and cost. Hydrolysis in fat splitting has three characteristic stages: a slow initial reaction rate limited by the miscibility of water in oil, an increase in reaction rate as fatty acids released improve water/oil mixing and finally a reaction equilibrium when rates of hydrolysis and reesterification are the same. Continuous glycerol withdrawal to prevent the equilibrium stage enables a higher degree of splitting (Gervajio 2005). In all processes, water and fats are introduced at a ratio of around 1:1, thus the resulting crude

glycerol composed mainly of water and its solutes. The discharge glycerol/water mixture from fat splitting is also commonly known as "sweetwater." Main characteristics of the four fat-splitting processes are summarized in Table 16.1.

Twitchell process, taking place at atmospheric pressure and 100–105 °C, is catalyzed by sulfuric acid and a mixture of sulfonated fatty acids with naphthalene (Twitchell reagent). The process takes up to 48 h to achieve 80–85% split, which is the lowest among the four processes. The catalytic sulfuric acid is found in the crude glycerol released, lowering its pH to a value below 2 (Jungermann and Sonntag 1991). While neutralization of excess mineral acid is possible, it generates a large amount of salts as impurities in the crude glycerol. Batch autoclave process,

Table 16.1 Main characteristics of the four fat-splitting processes and common impurities found in their respective crude glycerol

Process	Twitchell process	Batch autoclave	Colgate-emery	Enzymatic
Reactor	Lead-lined or acid-resistant wooden vat, open kettle	Corrosion-resistant, insulated autoclave	Corrosion-resistant splitting tower	Not commercialized
Operation mode	Batch	Batch	Continuous	Batch or continuous
Pressure	Atmospheric	1135 kPa	6000 kPa	Atmospheric
Temperature and time	100–105 °C, 36–48 h (2–4 cycles)	150–175 °C, 6–10 h	250–260 °C, 2–3 h	26–46 °C, 48–72 h
Catalyst used	Twitchell reagent (sulfonated mixture of oleic acid and naphthalene) and sulfuric acid	2–4% oxides of Zn, Mg, or Ca	Not required	Free or immobilized lipases
Degree of splitting	80–85%	95%	99%	Up to 98%
Advantages	Low cost, simple installation	High yield	Fast, high yield, no catalyst required	High yield, substrate/product specific, low energy consumption
Disadvantages	Oxidative discoloration of fatty acid, darkening due to sulfonated products, high labor cost	Catalyst handling, high labor cost	Expensive equipment, restricted to relatively clean feedstock, energy-intensive	Poor enzyme solubility, high enzyme cost, limited substrate range, large volumes
Characteristic impurities in crude glycerol	Presence of sulfuric acid (pH 2)	Presence of metal oxides	Products from thermal degradation and polymerization of glycerol	Presence of buffer salt and protein residue

conducted at 1135 kPa and 150–175 °C, has a reduced processing time (6–10 h) and a higher degree of splitting (95%) compared to the Twitchell process. The crude glycerol from batch autoclave has a characteristic signature of the catalytic metal oxides used.

Unlike Twitchell and batch autoclave processes, the use of a catalyst is optional in the Colgate-Emery process as high temperature (250–260 °C) and pressure (6000 kPa) improve water/oil miscibility. It does, however, require a splitting tower uniquely designed to allow countercurrent circulation of the two reagents, leveraging the difference in density of oil and water by introducing oil from the bottom of the splitting tower and water from the top (Angers et al. 2003; Gervajio 2005). While Colgate-Emery is the most efficient (2–3 h process time) large-scale fat splitting process, it is energy-intensive (340 Btu of energy per pound of oil split; Piazza and Haas 1999). The glycerol produced also decomposes and polymerizes at temperatures above 204 °C (Gervajio 2005). Thermal degradation of glycerol produces acetaldehyde, acrolein, allyl alcohol, and other products (Qadariyah et al. 2011), while glycerol polymerization results in polyglycerol formation (Ardi et al. 2015). However, the high degree of splitting accomplished (99%) compensates for this glycerol loss.

Enzymatic processes are yet to be industrially viable. One of the fundamental restricting factors is the high enzyme cost. Research efforts are looking at enzyme immobilization on solid supports for enzyme reuse to circumvent this problem. Foreseeable impurities in crude glycerol from an enzymatic process are salts from the buffering system used, protein residues, amino acids, and traces of degraded immobilization support when enzyme immobilization is applied.

Crude Glycerol from Biodiesel Production

Biodiesel production is achieved via transesterification of TGs, otherwise called alcoholysis, to form alkyl fatty esters and glycerol. This reaction is similar to fat splitting (Fig. 16.2), except that an alcohol containing 1–4 carbons is used rather than water (Knothe et al. 2010). It can take place in batch reactors, but a continuous process using plug flow reactors or stirred tanks is favored in large facilities (Bart et al. 2010). As in fat splitting, the reaction shows three characteristic stages, where the initial reaction rate is limited by poor alcohol/oil miscibility, which improves as alkyl fatty ester and glycerol are discharged before finally reaching a reaction equilibrium (Knothe et al. 2010). The reaction equilibrium is shifted toward product formation by using overabundance of alcohol compared to the 3:1 (alcohol to oil) stoichiometric molar ratio. This can range from 30 to 100% excess alcohol (Knothe and Razon 2017), with the 6:1 alcohol to oil molar ratio being the most commonly used for methanol (Tan et al. 2013). Methanol is the preferred alcohol for transesterification because it is most economical in most countries except in Brazil, where it is more advantageous to use ethanol (Knothe et al. 2010). As reaction proceeds, the glycerol and oily phases are not miscible, leading to phase separation that mimics product withdrawal (Knothe et al. 2010), consequently favoring reaction progress. The main characteristics of different biodiesel production processes and

Table 16.2 Main characteristics of different biodiesel production processes with methanol and common impurities found in their respective crude glycerols

Process	Homogeneous alkaline-catalyzed	Homogeneous acid-catalyzed	Enzymatic
Catalyst used	NaOH/KOH	H_2SO_4/HCl	Lipases
Temperature and time	60 °C, 0.5–2 h	80–100 °C, 2–10 h	45 °C, 4–8 h
Yield	>96%	90–95% (for two-step process of acid esterification followed by base transesterification)	>95%
Advantages	Cheap, fast, efficient	Use for feedstock with high FFA	Little requirement on the initial feedstock, less energy-intensive
Disadvantages	Not compatible with high FFA content, soap formation	Slower reaction rate, higher temperature required	Slowest reaction rate, high enzyme cost, enzyme inactivation by methanol
Characteristic impurities in crude glycerol	High pH, soap, residual methanol	Low pH, water, residual methanol	Buffer salt, protein residue, residual methanol

the impurities found in their respective crude glycerols are summarized in Table 16.2.

Acid, base, and enzyme catalysts are used in the transesterification of oil and fat. Base-catalyzed (e.g., sodium hydroxide, potassium hydroxide, and sodium alkoxide) transesterification is faster than acid-catalyzed reaction and is most commonly used in commercial applications. The typical reaction temperature for transesterification with methanol (60 °C) is just below the boiling point of methanol (Knothe et al. 2010), but higher temperatures might be required for other alcohols. At 60 °C and atmospheric pressure, the reaction time is 1–2 h (Gervajio 2005). The reaction time can be as short as 30 min (Thanh et al. 2012), contingent upon the amount and type of catalyst, reaction temperature, alcohol to oil ratio, and mixing during the reaction. The alkaline catalyst is most suitable for high-quality raw materials [FFA <1% (w/w) and moisture <0.5% (w/w)] (Thanh et al. 2012). This is also one of the biggest drawbacks for the base catalyst as samples with high FFA react with the base catalyst to form soap as an impurity in crude glycerol. This side reaction neutralizes the catalyst, and the resultant soap inhibits the separation of glycerol from the reaction mixture. Removal of the saponified catalysts is also technically tricky and adds to the production cost of biodiesel. A small quantity of elemental impurities such as Na, Ca, K, Mg, P, S, and N are found in crude glycerol derived from biodiesel production using base catalysts (Thompson and He 2006). Metal impurities, except Na, are present at concentrations between 4 and 163 ppm (Ayoub and Abdullah 2012). The Na content can surpass 1% (Ayoub and Abdullah 2012), which likely originates from the base catalyst used. A catalyst-free process is hypothetically conceivable at

9000 kPa and 240 °C (Gervajio 2005). Unlike the Colgate-Emery process, it is not sought after because of the staggering expense (Knothe et al. 2010).

Acid catalysts (e.g., sulfuric, hydrochloric, or phosphoric acid) are favorable when the beginning raw materials contain a high measure of FFAs. For initial acid values >1, which is regularly the situation for unrefined oils, an acid catalyst like trifluoroacetic (TFA) acid is used (Tan et al. 2013) to prevent soap formation when alkaline catalyst reacts with FFA. However, acid-catalyzed transesterification is very sensitive to a water content of the raw materials, and an amount as small as 0.1% (w/w) water in the reaction mixture is known to influence the FAME yield of vegetable oil transesterification with methanol (Thanh et al. 2012). Compared to base catalysts, the process requires a higher molar ratio of methanol to oil of 12:1, higher temperatures of 80–100 °C and longer reaction times of 2–10 h (Thanh et al. 2012). Consequently, acid-catalyzed transesterification has higher alcohol content in the crude glycerol formed. Practically, acid catalysts are more commonly used for pretreatment of oil and fat to convert FFA to esters when necessary, before conventional base catalysts are used for the transesterification reaction. It is worth noting that this pretreated mixture likely has a higher water content as water is a product of esterification (Knothe et al. 2010).

The homogeneous acid and base catalysts used in transesterification are troublesome and exorbitant to separate from the reaction mixture. Heterogeneous solid catalysts such as alkaline metal oxides (e.g., CaO and MgO), mixed metal oxides (ZrO_2, SnO_2, TiO_2), and metal compounds supported on solid matrix (zeolites, hydrotalcites, and γ-alumina) have recently been developed to facilitate separation from the reaction mixture and reuse (Thanh et al. 2012). Similar to homogeneous catalysts, solid base catalysts are more active than solid acid catalysts. These heterogeneous catalysts are sometimes supported on materials with a large surface area like silica or zirconia. Impurities from these heterogeneous catalysts can result from leaching of the metal ions into the crude glycerol.

Lipases are used as enzyme catalysts for biodiesel production. They have high reaction specificity and are frequently employed in immobilized forms to address the issues of catalyst recovery or by-products removal. The principle downside is the high cost of lipases, the low solubility of the enzyme in methanol, and the enzyme inactivation prompting a diminishing yield. Although lipase-catalyzed transesterification is an attractive alternative, it has not been well received.

16.2.3 Composition of Crude Glycerol

Defining the composition of crude glycerol has proven difficult for various reasons: (1) the oil and fat feedstocks used in industrial processes are highly diverse, (2) these feedstocks are processed via different chemical reactions (transesterification, fat splitting, saponification), where different catalysts and reaction conditions are applied, (3) crude glycerol characterization is not standardized and data reported is often incomplete, (4) oil refining steps (e.g., degumming) prior to its transformation are usually not specified, and (5) crude glycerol concentrating and/or refining steps is

not reported in most cases. Some studies conclude that crude glycerol composition is consistent for a given source (Hu et al. 2012), while others found significant differences between crude glycerols generated by similar processes (Hansen et al. 2009).

A survey of crude glycerol compositions reported in the literature (Table 16.3) reveals a substantial compositional variation. In this table, MONG includes entrained fatty matters (e.g., FFAs, FAMEs, DGs, MGs, and unprocessed oil) and other organic matters (e.g., proteins) but excludes methanol and soap. Using transesterification as an example, the pH of crude glycerol varies between 2.0 and 10.8, while the glycerol concentrations range from 20.0% to 96.5%.

16.2.4 Glycerol Refining

Crude glycerol purification strategy is impacted by the nature of impurities present and the targeted use of the purified glycerol. This section centers around crude glycerol from biodiesel production. This is the largest source of crude glycerol, which tends to have a higher level of impurities due to the use of unrefined feedstock compared to fat splitting. Most purification schemes follow the typical process illustrated in Fig. 16.3. Variations between the schemes include the number of unit operations used which depend on the starting and the target purity and the addition of newer technology such as membrane separation.

16.2.4.1 Pretreatment

Pretreatment aims to produce semi-crude glycerol that can be either commercialized or further refined. The process relies on proper pH and temperature control to influence the physical form and solubility of MONG and salts to facilitate their removal. The first step is generally the use of a strong acid to neutralize the catalyst and remove soap. The acid converts soap to FFA and base catalyst to salt and water, giving three product layers of FFA, glycerol-enriched solution, and inorganic salt from top to bottom (Kongjao et al. 2010). The FFA can be decanted or skimmed off, and the precipitated salt can be removed by filtration. Unreacted methanol in crude glycerol is toxic, and a contaminant for the environment (Wan Isahak et al. 2015). It is removed from the middle glycerol-rich layer in a second step using vacuum evaporation in an evaporator or flash unit. This process takes place under vacuum at reduced temperature to avoid glycerol decomposition at high temperature. Vacuum evaporation also removes water from the glycerol solution. The semi-crude glycerol at this stage usually has a concentration of ~85% and is suitable for refining. Depending on the chosen method for refining, the pH of semi-crude may be adjusted. Semi-crude glycerol used for distillation should have water content $<15\%$ to avoid vacuum system overload and low fatty matter content to avoid co-distillation. On the contrary, refining by ion exchange (or deionization) requires semi-crude with at least 60% water. If necessary, the water content may be reduced by one or several evaporation/concentration step(s) before refining.

Table 16.3 Crude glycerol compositions reported in the literature

Sources of crude glycerol										
Feedstock	Catalyst (w/w)	Chemical reaction	pH	Glycerol (w/w)	Methanol (w/w)	Soap (w/w)	MONG (w/w)	Ash (w/w)	Moisture (w/w)	Reference
Soybean oil	KOH	Transesterification	n.r.	62.0%	12.8%	25.2%	n.r.	n.r.	n.r.	Pyle et al. (2008)
Chicken fat/soy bean oil (50/50, w/w)	KOH	Transesterification	n.r.	62.3%	14.4%	23.2%	n.r.	n.r.	n.r.	Pyle et al. (2008)
Canola oil	NaOH	Transesterification	n.r.	56.5%	28.3%	15.3%	n.r.	n.r.	n.r.	Pyle et al. (2008)
Sunflower oil	0.5% NaOH	Transesterification	n.r.	75.0%	1.0%	9.3%	n.r.	n.r.	n.r.	Rehman et al. (2008)
Sunflower oil	0.5% NaOH	Transesterification	n.r.	90.0%	<1.0%	n.r.	n.r.	n.r.	n.r.	Rehman et al. (2008)
Jatropha oil	n.r.	Transesterification	n.r.	18.0–22.0%	14.5%	29.0%	11.0–21.0%	n.r.	n.r.	Hiremath et al. (2011)
Palm oil	n.r.	Transesterification	n.r.	80.5%	0.5%	n.r.	<2.0%	n.r.	n.r.	Liu et al. (2013)
IdaGold oil	$NaOCH_3$	Transesterification	n.r.	62.9%	n.r.	n.r.	n.r.	n.r.	n.r.	Thompson and He (2006)
PacGold oil	$NaOCH_3$	Transesterification	n.r.	62.9%	n.r.	n.r.	n.r.	n.r.	n.r.	Thompson and He (2006)
Rapeseed oil	$NaOCH_3$	Transesterification	n.r.	65.7%	n.r.	n.r.	n.r.	n.r.	n.r.	Thompson and He (2006)
Canola oil	$NaOCH_3$	Transesterification	n.r.	67.8%	n.r.	n.r.	n.r.	n.r.	n.r.	Thompson and He (2006)
Soybean oil	$NaOCH_3$	Transesterification	n.r.	67.8%	n.r.	n.r.	n.r.	n.r.	n.r.	Thompson and He (2006)
Crambe oil	$NaOCH_3$	Transesterification	n.r.	62.5%	n.r.	n.r.	n.r.	n.r.	n.r.	Thompson and He (2006)

Waste vegetable oil	$NaOCH_3$	Transesterification	n.r.	76.6%	n.r.	n.r.	n.r.	n.r.	n.r.	Thompson and He (2006)
Waste vegetable oil	n.r.	Transesterification	n.r.	63.4%	4.4%	n.r.	9.6%	7.4%	13.9%	Chanjula et al. (2016)
Soybean oil	n.r.	Transesterification	n.r.	87.0%	0.0%	n.r.	5.7%	3.2%	9.2%	Lammers et al. (2008)
Vegetable oil	n.r.	Transesterification	n.r.	87.4%	0.1%	n.r.	n.r.	5.9%	8.0%	Orengo et al. (2014)
Palm oil	n.r.	Transesterification	n.r.	88.9%	0.5%	n.r.	0.0%	3.5%	5.6%	Chanjula et al. (2016)
Rapeseed oil	n.r.	Transesterification	n.r.	80.0%	0.5%	n.r.	n.r.	8.0%	n.r.	Bartoň et al. (2013)
Soybean oil	n.r.	Transesterification	n.r.	80.3%	n.r.	n.r.	1.6%	5.0%	12.0%	Lage et al. (2014a)
Soybean oil	n.r.	Transesterification	n.r.	80.3%	0.4%	n.r.	n.r.	n.r.	12.4%	Shin et al. (2012)
Pork fat	n.r.	Transesterification	n.r.	74.7%	0.0%	n.r.	0.9%	n.r.	10.3%	Silveira et al. (2015)
Castor/soybean/ sunflower/cotton seed oils	n.r.	Transesterification	n.r.	36.2%	8.7%	n.r.	0.4%	2.0%	6.2%	Lage et al. (2014b)
Soybean/sunflower oils	n.r.	Transesterification	n.r.	83.0%	0.0%	n.r.	n.r.	6.0%	11.0%	Carvalho et al. (2015)
n.r.	n.r.	Transesterification	n.r.	82.6%	0.4%	n.r.	0.6%	9.3%	7.1%	Kass et al. (2013)
Soybean oil	n.r.	Transesterification	n.r.	80.3%	0.0%	n.r.	3.0%	5.7%	12.0%	San Vito et al. (2015)
n.r.	n.r.	Transesterification	n.r.	86.6%	0.0%	n.r.	n.r.	5.9%	7.5%	Egea et al. (2016)

(continued)

Table 16.3 (continued)

Sources of crude glycerol										
Feedstock	Catalyst (w/w)	Chemical reaction	pH	Glycerol (w/w)	Methanol (w/w)	Soap (w/w)	MONG (w/w)	Ash (w/w)	Moisture (w/w)	Reference
n.r.	n.r.	Transesterification	n.r.	86.6%	0.0%	n.r.	0.5%	3.2%	9.2%	Lammers et al. (2008)
n.r.	n.r.	Transesterification	n.r.	84.4%	0.0%	n.r.	1.0%	3.2%	10.7%	Kim et al. (2013)
n.r.	n.r.	Transesterification	3.3	77.0%	0.0%	n.r.	4.6%	2.3%	16.1%	Hansen et al. (2009)
n.r.	n.r.	Transesterification	5.4	94.8%	0.0%	n.r.	3.2%	0.0%	2.0%	Hansen et al. (2009)
n.r.	n.r.	Transesterification	7.6	96.5%	0.0%	n.r.	1.0%	0.0%	1.3%	Hansen et al. (2009)
n.r.	n.r.	Transesterification	9.0	38.4%	0.1%	n.r.	57.0%	4.2%	0.3%	Hansen et al. (2009)
n.r.	n.r.	Transesterification	2.3	61.1%	0.2%	n.r.	5.6%	29.4%	2.5%	Hansen et al. (2009)
n.r.	n.r.	Transesterification	10.6	66.7%	11.4%	n.r.	18.8%	2.9%	0.2%	Hansen et al. (2009)
n.r.	n.r.	Transesterification	10.8	64.5%	13.9%	n.r.	18.1%	3.4%	0.0%	Hansen et al. (2009)
n.r.	n.r.	Transesterification	2.7	83.4%	0.2%	n.r.	4.2%	1.5%	10.7%	Hansen et al. (2009)
n.r.	n.r.	Transesterification	2.0	76.1%	1.8%	n.r.	6.9%	3.5%	11.7%	Hansen et al. (2009)
n.r.	n.r.	Transesterification	2.4	74.5%	0.6%	n.r.	6.7%	4.6%	14.3%	Hansen et al. (2009)
n.r.	n.r.	Transesterification	8.6	63.4%	4.7%	n.r.	25.3%	5.6%	1.0%	Hansen et al. (2009)

Soybean oil	n.r.	Transesterification	6.9	63.0%	6.2%	0.0%	0.0%	2.7%	28.7%	Hu et al. (2012)
Soybean oil	n.r.	Transesterification	9.7	22.9%	10.9%	26.2%	23.5%	3.0%	18.2%	Hu et al. (2012)
Soybean oil	n.r.	Transesterification	9.5	33.3%	12.6%	26.1%	22.3%	2.8%	6.5%	Hu et al. (2012)
Waste vegetable oil	n.r.	Transesterification	9.4	27.8%	8.6%	20.5%	38.8%	2.7%	4.1%	Hu et al. (2012)
Soybean/waste vegetable oils	n.r.	Transesterification	10.0	57.1%	11.3%	31.4%	0.9%	5.7%	1.0%	Hu et al. (2012)
n.r.	n.r.	Transesterification	n.r.	75.0%	n.r.	n.r.	6.0%	10.0%	10.0%	Tan et al. (2013)
n.r.	n.r.	Hydrolysis	n.r.	88.0–90.0%	n.r.	n.r.	3.1–4.1%	0.7–1.0%	8.0–9.0%	Tan et al. (2013)
n.r.	n.r.	Saponification	n.r.	83.0–84.0%	n.r.	n.r.	0.9–1.2%	8.5–9.5%	6.0–7.0%	Tan et al. (2013)

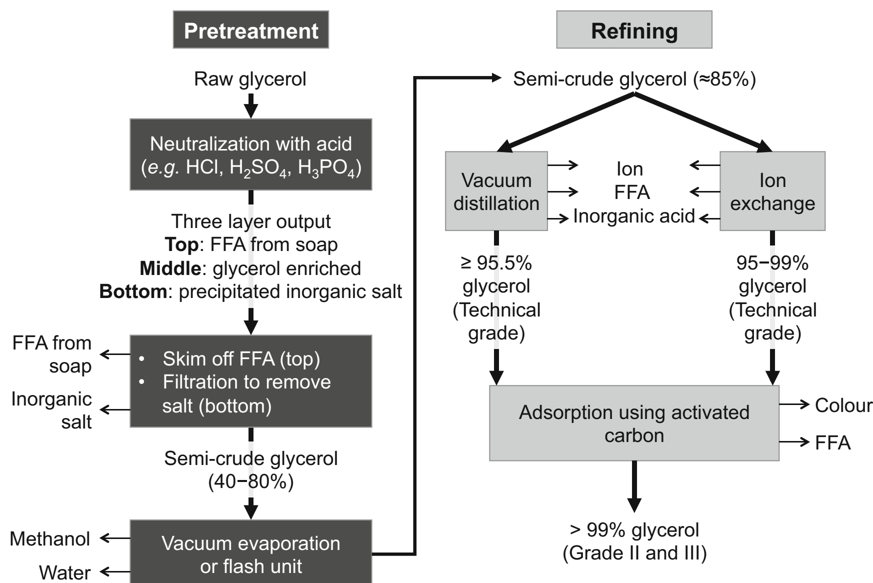

Fig. 16.3 Typical biodiesel crude glycerol purification process. Pretreatment steps shown in dark gray boxes convert raw glycerol to semi-crude. FFA from soap, precipitated inorganic salt, methanol, and water are removed in pretreatment. Extracted methanol can be reused as a reactant for transesterification. Refining steps are shown in light gray boxes where ion, FFA, and inorganic acid are removed via vacuum distillation or ion exchange before adsorption using activated carbon to remove color and traces of FFA further to produce Grade II and III pure glycerol

16.2.4.2 Refining

There are two main methods for refining glycerol, i.e., vacuum distillation and ion exchange. Distillation relies on the lower volatility of all the contaminants compared to glycerol, which has a boiling point of 290 °C at an atmospheric pressure. This process is however operated under vacuum (10–30 mmHg) to allow an operating temperature between 160 and 180 °C (Mota et al. 2017) to prevent glycerol decomposition at a temperature above 200 °C (Jungermann and Sonntag 1991). The distillation process is well-established, feasible for small- to large-scale continuous operation and has high process adaptability for purifying varying quality of semi-crude. However, it is energy-intensive as the glycerol is vaporized and condensed in multiple condensers operating at different temperatures to collect glycerol fractions of different purities (Jungermann and Sonntag 1991) and may lead to thermal decomposition with extended processing. Lower purity fractions can be reprocessed to increase the yield or sold as technical grade.

Ion exchange requires lower capital investment and gives a high-quality product. It is more suitable for small volume refining (<25 tons/day) (Jungermann and Sonntag 1991) and for semi-crude with low salt concentrations. It is primarily used to remove ionic contaminants by the passage of pretreated semi-crude through successive beds containing strong cation, weak anion, and mixed strong cation–

anion resins (Gervajio 2005). It also removes traces of FFAs, color, odor bodies, and mineral impurities. Following ion exchange, the water content may be reduced by evaporation and concentration, but concentration sometimes generates new color bodies.

The glycerol produced from vacuum distillation and ion exchange is usually polished to meet grade II or III specifications through adsorption on activated carbon to reduce color and sometimes to deodorize via vacuum extraction procedures (Gervajio 2005).

16.2.4.3 Cost

The various purification strategies used to convert raw glycerol to pure glycerol usually combine several methods and are resource-intensive (Tan et al. 2013). New developments involving membrane-based technology (ultrafiltration and reverse osmosis) are currently under evaluation as less energy-consuming alternatives to deionization, evaporation, and distillation (Jungermann and Sonntag 1991; Pagliaro and Rossi 2008). However, the cost and the lifetime of membranes are also limiting factors (Mota et al. 2017). In 2011, the lowest cost of glycerol purification achievable for purity of up to 98% (by the combination of neutralization, centrifugation, evaporation, and column distillation) was estimated at $0.15 per kg (Posada et al. 2011). Distillation generates a solid waste called glycerol pitch (Hazimah et al. 2003) or glycerol residue (Yong et al. 2001) that contains glycerides, salts, soaps, fatty acids, polyglycerols, ashes, and glycerol (Jungermann and Sonntag 1991; Mota et al. 2017). This glycerol pitch is classified as hazardous and poisonous (Irvan et al. 2018) and must be carefully disposed of. The cost of which increases the purification cost beyond $0.15 per kg. On the other hand, some isolated contaminants are co-products and are recycled to reduce the process cost. For instance, methanol recycling is well established in the biodiesel industry (Wan Isahak et al. 2015) and the unprocessed oils and some catalytic salts can be recovered (Jungermann and Sonntag 1991). Given the low price of refined glycerol [50c/lb. (USA) and €0.76 per kg (EU) in 2018] and the expense of raw glycerol purification, the economic viability of the biodiesel industry is firmly connected to its capacity to valorize glycerol (Tan et al. 2013).

16.2.5 The Glycerol Market

Before the expansion of the biodiesel market in 2004, glycerol production remained relatively stable at 0.8 million tonnes per annum, with prices of refined glycerol at around €1000 per tonnes in EU (Gunstone and Heming 2004) and 70 c/lb. in the USA (Heming 2005). The glycerol market has since become volatile due to the increasing production of glycerol in the fast-growing biodiesel industry. As a by-product, glycerol supply depends on the performance of the biodiesel and the oleochemical market rather than its demand. This independence of glycerol supply from its demand caused a significant drop and volatility in glycerol prices. The prices of glycerol reached a critical low in 2006 such that synthetic glycerol plants were

either shut down or retrofitted. The Solvay group in France was the first to initiate plant retrofitting in 2007 for their process of Epicerol to produce epichlorohydrin from glycerol (Pagliaro and Rossi 2008). Oleon retrofitted its propylene glycol production plant in 2015 to refine crude glycerol (Pagliaro 2017). The supply of crude glycerol was forecasted to be 3.8 million tonnes in 2018 (Vantage Oleochemicals) with prices of 10–15 c/lb. in the USA and €180–390 per tonne in EU.

16.2.5.1 Glycerol Supply

In 2005, the top three global glycerol suppliers were Procter & Gamble, Cognis, and Uniqema (presently Croda), with more than 33% combined market share. By 2010, the leading glycerol suppliers were biodiesel and oleochemical companies, mostly situated in Southeast Asia (Malaysia, Philippines, Thailand, and Indonesia) (Pagliaro 2017). Today Malaysia, Colombia, Argentina, and Brazil are among the biggest biodiesel producers, and the market has consolidated with four major companies (IOI Group, Wilmar International, KL Kepong and Emery Oleochemicals) representing >65% of the overall market ($2.47 billion) in 2015 (Global Market Insights). The glycerol market size is anticipated to reach $3.04 billion by 2022 (Global Market Insights).

16.2.5.2 Glycerol Demand and Applications

Three conventional applications dominate the demand of refined glycerol globally: personal and oral care products (30%), food and beverage (>13%), and pharmaceuticals (>10%) (IHS Markit 2018). New applications include the production of propylene glycol, epichlorohydrin, and polyether polyol used in polyurethane foam production. The applications of glycerol are however subjected to regional variation. For instance, in 2006, 40% of US refined glycerol was dedicated to personal and oral care products while this application only represented 15% of glycerol used in Japan. The proportion of glycerol used for pharmaceuticals was 25% and 8% for Japan and Western Europe, respectively (Greenea).

As a result of the impurities present, crude glycerol is often used in low value-added applications such as animal feed, feedstock in biotechnological applications, de-icing agent, concrete additive and road anti-dust (Ayoub and Abdullah 2012; Pagliaro 2017). The longevity of crude glycerol use for these applications strongly depends on glycerol price (Pagliaro 2017). Low prices favor the development of new applications for glycerol (e.g., epichlorohydrin) and created new markets, especially in Asia. For example, DowChemical designed a process to produce epichlorohydrin from crude glycerol [GTE (glycerine-to-epichlorohydrin) process] to capitalize on the low crude glycerol price (Bell et al. 2008). Refined glycerol is sometimes perceived as an application of crude glycerol. In 2014, China imported 795,000 tonnes of crude glycerol for refining (Greenea). China is currently the largest consumer of glycerol, driven by its glycerol-to-epichlorohydrin production (IHS Markit 2018).

16.2.5.3 The Future of Glycerol Valorization

While the demand and supply for glycerol remain volatile, it is expected that glycerol supply is 6 times higher than its demand by 2020 (Nda-Umar et al. 2019). This trend poses significant pressure on glycerol disposal, resulting in additional waste management costs and environmental impact. There is a need for innovations in glycerol purification and valorization. Profitability remains a priority when developing new applications for glycerol, but sustainability (e.g., feedstock and energy requirement) has gained increasing importance. Currently, chemical conversion to epichlorohydrin and propylene glycol is among the successful valorization strategies. Other processes leading to a wide range of valuable products include glycerol reforming (Bauer and Hulteberg 2013), gasification, pyrolysis, and bioconversion (Wan Isahak et al. 2015). Chemical conversions often require high-purity glycerol, whereas microorganisms can uptake glycerol from its crude form potentially saving on glycerol purification cost and time. Microbial utilization of glycerol is therefore a promising course to sustainable glycerol valorization.

16.3 Glycerol-Utilizing Microorganisms

The natural capacity to use glycerol as a carbon source for growth and energy and chemical production can be found in bacteria, archaea, fungi (including yeast and filamentous fungi), and microalgae. Although extremophiles have been shown to utilize glycerol (Sherwood et al. 2009), their industrial application remains low and is not discussed here. The efficiency of glycerol utilization varies between species and strains. This was clearly illustrated in a study where only 8 out of 126 species tested in the *Enterobacteriaceae* family were able to ferment glycerol (Holm 2013). The maximum specific growth rates (μ_{max}) in glycerol as a sole carbon source of various commonly used industrial hosts are summarized in Table 16.4. Worthy of a mention, μ_{max} is dependent on glycerol concentration and purity, other nutrients available, and cultivation conditions (e.g., temperature, stirring, and cultivation mode). Many other bacteria also utilize glycerol, for example, Staphylococcus aureus, Mycobacterium tuberculosis, *Nocardia asteroids, Salmonella typhimurium*, *Mycoplasma mycoides*, *Mycoplasma pneumoniae*, and Listeria monocytogenes (Lin 1976; Blotz and Stulke 2017). Their mechanisms for glycerol transport and dissimilation are well studied, but they show low potential for biomanufacturing due to pathogenicity, and thus are excluded from this chapter.

16.3.1 Glycerol Transport Across the Cell Membrane

Before it can be metabolized, glycerol molecules must cross the cellular membrane to enter the cytosol. This can be achieved via three general routes: passive diffusion, facilitated diffusion, and active transport (Fig. 16.4).

Table 16.4 Maximum specific growth rate (μ_{max}) of different microorganisms in glycerol

	Strain	Glycerol (type/ concentration)	Product	μ_{max} (h^{-1})	References
Bacteria	*Klebsiella pneumoniae* M5al	Pure/20 g.L^{-1}	1,3-PDO	0.19 (anaerobic) 0.17 (aerobic)	Cheng et al. (2005)
	Klebsiella pneumoniae BLh^{-1}	Crude (82.97%)/15 & 65 g.L^{-1}	1,3-PDO	0.35 (anaerobic)	Morcelli et al. (2018)
	Citrobacter werkmanii DSM17579	Pure/163 mM	1,3-PDO	0.33	Maervoet et al. (2016)
	Clostridium butyricum JKT37	Pure/40 g.L^{-1}	1,3-PDO	0.53	Tee et al. (2017)
	R. eutropha H16 *(engineered)*	Pure/0.5% (w/v)	PHB	0.15	Fukui et al. (2014)
	Bacillus subtilis subsp. Niger	Pure/5 g.L^{-1}	–	0.65 (continuous culture)	Kruyssen et al. (1980)
	Escherichia coli JM101	Pure/4 g.L^{-1}	–	0.49	Martinez-Gomez et al. (2012)
	Escherichia coli K12	Pure/20%	–	0.26	Chaudhary et al. (2011)
Fungi	*Saccharomyces cerevisiae CBS6412*	Pure/6% (v/v)	–	0.1	Swinnen et al. (2013)
	Pichia jadinii	Pure/2% (w/v)	–	0.32	Lages et al. (1999)
	Yarrowia lipolytica IBT 446	Pure/6% (w/v)	–	0.50	Klein et al. (2016)
	Pachysolen tannophilus CBS 4044	Pure/6% (w/v)	–	0.27	Klein et al. (2016)
	Aspergillus niger PJR1	[a]Crude (67%)/ 160 g.L^{-1}	Malic acid	0.1444	Iyyappan et al. (2019)
Microalgae	*Chlorella protothecoides* UTEX 256	[a]Pure/30 g.L^{-1}	TG	0.03	Chen and Walker (2011)
	Chlorella vulgaris	Pure/10 g.L^{-1}		0.012	da Silva and Fonseca (2018)

[a]Media contain 4 g.L^{-1} of yeast extract
[b]Media contain 1–1.5 g.L^{-1} of yeast extract

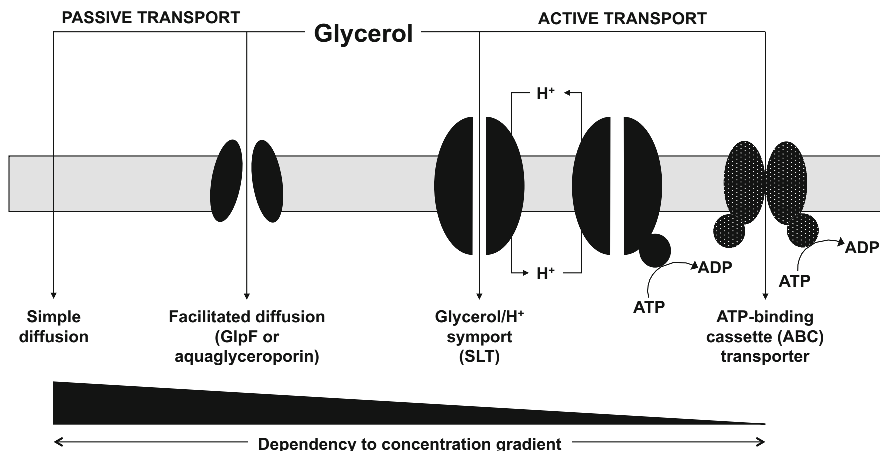

Fig. 16.4 Glycerol transport across the cell membrane. Passive transport of glycerol includes simple and facilitated diffusions, which are highly dependent on glycerol concentration across the cell membrane. Active transport occurs via glycerol/H+ symport and ATP-binding cassette transporters and can take place against a concentration gradient

16.3.1.1 Simple Diffusion

Simple or passive diffusion across a cellular membrane is driven by the concentration gradient of the solute, without requiring energetic input. Key parameters governing transmembrane diffusion are polarity and size of the solute. Being a small and uncharged molecule, glycerol can cross the microbial cell membrane by passive diffusion, driven by its higher concentration in the medium (Sutherland et al. 1997; Blotz and Stulke 2017; Klein et al. 2017; Westbrook et al. 2019). The permeability of a given molecule across the membrane is quantitatively represented by its permeability coefficient (typically expressed in a unit of cm/s), which is a measure of how quickly it crosses a membrane. Compared to small nonpolar molecule with very high permeability coefficient like O_2 (2.3×10^1 cm/s), small polar molecule like H_2O (3.4×10^{-3} cm/s), and charged molecule like Na^+ (5.0×10^{-14} cm/s), glycerol has a permeability coefficient of 5.4×10^{-6} cm/s (Yang and Hinner 2015).

It is worth mentioning that, while most proposed models of microbial glycerol transport across membrane include passive diffusion, the membrane lipid composition also influences its membrane permeability for solutes. It is thus unsurprising that the role and significance of passive diffusion in glycerol uptake remain widely debatable. For instance, Escherichia coli showed >99% decrease in glycerol uptake when the protein responsible for glycerol-facilitated transport (GlpF) was removed (Truniger and Boos 1993). Passive diffusion of glycerol in Saccharomyces cerevisiae has been highly contentious, partly due to very poor permeability of glycerol across its plasma membrane and the role of its Fps1 channel as glycerol facilitator (Oliveira et al. 2003). However, it is now widely accepted that the uptake

of glycerol is mainly driven by active transport in S. cerevisiae during growth in glycerol (Ferreira et al. 2005).

16.3.1.2 Facilitated Diffusion

Facilitated glycerol diffusion is mediated by members of the major intrinsic protein (MIP) family. MIP is a large family of transmembrane proteins, which includes the aquaporins and aquaglyceroporins (also known as glycerol facilitator proteins) subfamilies. While the aquaporins have a highly conserved amino acid sequence that forms 2 NPA boxes (asparagine-proline-alanine), the second NPA box of aquaglyceroporins has an aspartic acid instead of an asparagine. The smaller aspartic acid residue is believed to result in an expanded pore to accept the larger glycerol molecule (Benga 2012). The direction of glycerol flux (influx or efflux) is dependent on its concentration gradient as in passive diffusion. However, this process can be controlled by channel closure resulting in glycerol accumulation inside the cells (Tamas et al. 1999; Fakas et al. 2009).

The E. coli glycerol facilitator (GlpF) is a widely investigated protein. It is a highly selective channel with GlpF-mediated glycerol influx 100- to 1000-folds higher than expected for a transporter and is non-saturable to a glycerol concentration of >200 mM (Fu et al. 2000). Bacterial glycerol transport largely relies on facilitated diffusion involving homologs of GlpF (Lin 1976). Another well-studied glycerol transport facilitator is the Fps1 located in the S. cerevisiae plasma membrane. Fps1 is homologous to the GlpF in E. coli and is involved in osmoregulation. It is closed under hyperosmotic stress to allow intracellular accumulation of glycerol and opened under low-osmolarity conditions to enable glycerol efflux. Overexpression of Fps1 prompted a slight increment in the specific growth rate of S. cerevisiae in glycerol (Klein et al. 2016).

16.3.1.3 Active Transport

Among the glycerol transport mechanisms, active transport is the only one that can take place against a concentration gradient, supported by energy expenditure often in the form of an ATP molecule. The most common channels are proton/glycerol symports coupled to proton gradient creation via plasma membrane proton ATPase (Duskova et al. 2015). These symports are part of the Sugar Transporter Like family (STL) encoded by *stl* genes. Structurally, STL transporters resemble that of the hexose transporters with 12 transmembrane domains and conserved motifs that govern channel specificity and regulation (Duskova et al. 2015). Further to H^+/glycerol symports, sodium/glycerol symports in halotolerant yeast (Lages et al. 1999) and ATP-binding cassette (ABC) transporters in some species of *Mycoplasma* genus (Blotz and Stulke 2017) were also identified as active glycerol transport systems.

In response to high osmotic pressures, cells tend to accumulate or internalize solutes to balance the external pressure (Roberts 2005). In bacteria, the primary solutes are trehalose and glycine, whereas glycerol is used in fungi (Brisson et al. 2001). Active glycerol transport in bacteria is uncommon, but few cases have been described (Blotz and Stulke 2017). Given the critical role of glycerol in fungi

osmoregulation (Brisson et al. 2001; Klein et al. 2017), active glycerol transport systems are common to guarantee solute retention and accumulation within the cells (Lages et al. 1999). Active glycerol transport is well studied in yeast, and both H^+/glycerol and Na^+/glycerol symports have been described (Lages et al. 1999). Most species express only one type of symport, but species like Debaryomyces hansenii and *Pichia sorbitophila* express both H^+ and Na^+ symports (Lages et al. 1999). STL1 transporters mediate the active uptake of glycerol with protons and are characterized in detail in S. cerevisiae (Ferreira et al. 2005), pathogenic Candida albicans (Kayingo et al. 2009) and osmotolerant *Zygosaccharomyces rouxii* (Duskova et al. 2015). Na^+/glycerol symports are usually found in halotolerant yeast species and studied in connection to their capacity in osmoregulation (Klein et al. 2016).

Though the osmoregulation function of active glycerol transport is well studied, it is worth noting that glycerol accumulation does not necessarily lead to glycerol metabolism. For instance, Z. rouxii (yeast) and *Dunaliella parva* (microalgae) can internalize glycerol but are not equipped to catabolize it (Hard and Gilmour 1996; Duskova et al. 2015). The glycerol uptake in microalgae is less studied compared to bacteria and yeast, though active transport (Hard and Gilmour 1996; Lin et al. 2013) and facilitated diffusion (Anderca et al. 2004) have both been identified in different species.

16.3.2 Overview of Glycerol Metabolism in Microbes

There are two dissimilation pathways for glycerol metabolism: the phosphorylation pathway and the dehydrogenation pathway (Fig. 16.5, Table 16.5) (Doi 2019; Poblete-Castro et al. 2020; Westbrook et al. 2019).

The phosphorylation pathway exists in all the commonly used biomanufacturing hosts (Fig. 16.5). The pathway begins with glycerol phosphorylation by a glycerol kinase (GlpK, EC 2.7.1.30) at the expense of ATP. The resulting glycerol-3-phosphate (G3P) is subsequently oxidized by a G3P dehydrogenase (GlpD or GlpABC, EC 1.1.5.3). GlpD is an aerobic G3P dehydrogenase (G3PDH). It catalyzes the oxidation of G3P to dihydroxyacetone phosphate (DHAP), with concomitant reduction of flavin adenine dinucleotide (FAD) to $FADH_2$, and passes electrons onto ubiquinone (UQ) and ultimately to oxygen or nitrate. GlpABC, on the other hand, is an anaerobic G3PDH complex. GlpA and GlpB are flavoenzymes that bind FAD and flavin mononucleotide (FMN), respectively. GlpA is believed to be the subunit that performs G3P oxidation, and both GlpB and GlpC form a complex with GlpA to establish an electron transfer pathway to the inner membrane.

In the dehydrogenation pathway (Fig. 16.5), glycerol is first oxidized to dihydroxyacetone (DHA) by a glycerol dehydrogenase (GldA or DhaD, EC 1.1.1.6), and after that phosphorylated by a DHA kinase (DhaK, EC 2.7.1.29 or DhaKLM, EC 2.7.1.121). Two forms of DHA kinase have appeared to exist, using either an ATP or a phosphoprotein of the phosphoenolpyruvate:sugar phosphotransferase system (PEP:PTS) as the phosphate donor. The ATP-dependent kinases (DhaK) are single-subunit enzymes comprising two domains (K-domain and L-domain). The

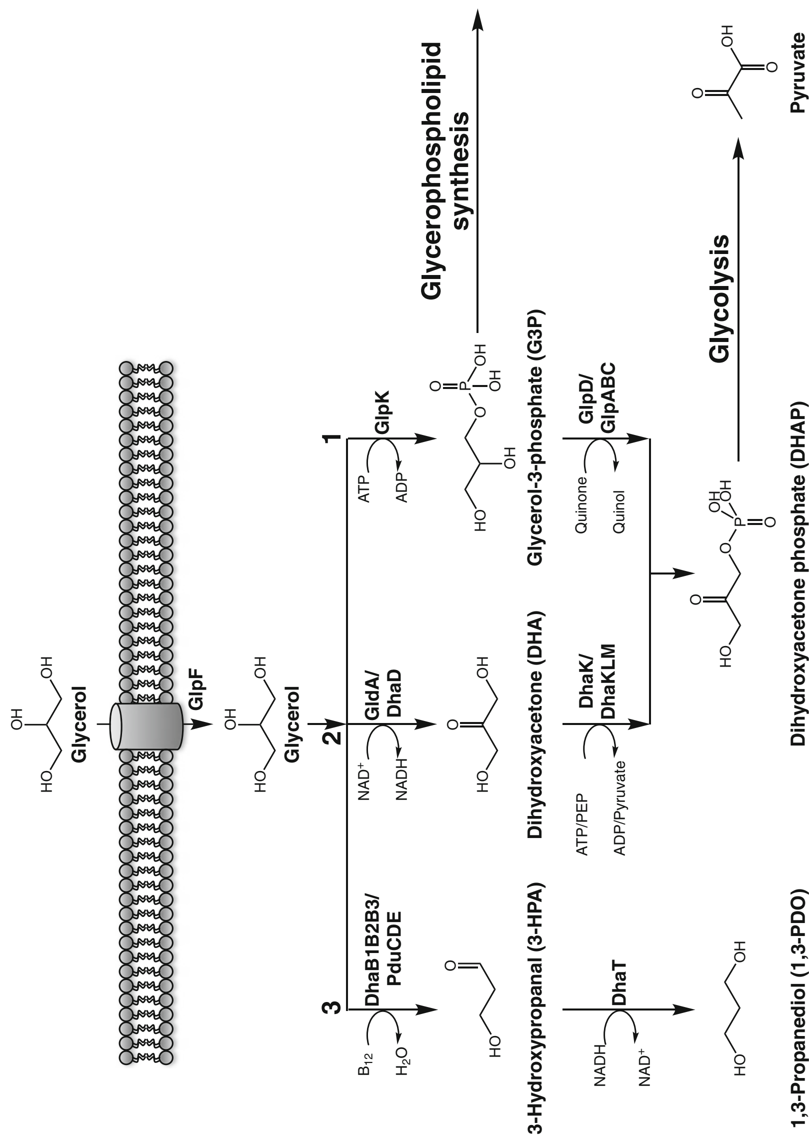

Fig. 16.5 Major glycerol catabolism pathways in microbes. The two oxidative pathways occur via phosphorylation (pathway **1**) and dehydrogenation (pathway **2**) to produce DHAP, which is then metabolized in glycolysis. Some microbes can convert glycerol via a reductive pathway (pathway **3**) to produce 1,3-PDO

Table 16.5 Genes encoding the enzymes involved in microbial glycerol metabolism

					Glycerol dissimilation				1,3-PDO synthesis pathway		
					Phosphorylation pathway		Dehydrogenation pathway				
Microorganism	Strain		KEGG entry	Glycerol uptake	Glycerol phosphorylation	G3P oxidation	Glycerol oxidation	DHA phosphorylation	Glycerol dehydration	3-HPA reduction	Regulation
Citrobacter freundii	CFNIH1	Gram-negative bacterium	T03077	*glpF*	*glpK*	*glpA, glpB, glpC, glpD, gpsA*	*gldA*	*dhaK, dhaL, dhaM*	*pduC, pduD, pduE*	*dhaT*	*glpR, glpP, dhaR*
Cupriavidus necator	H16	Gram-negative bacterium	T00416	*glpF*	*glpK*	*glpD, gpsA*	–	–	–	–	*glpR, glpP*
Escherichia coli	MG1655	Gram-negative bacterium	T00007	*glpF*	*glpK*	*glpA, glpB, glpC, glpD, gpsA*	*gldA*	*dhaK, dhaL, dhaM*	–	–	*dhaR*
Klebsiella aerogenes	KCTC 2190	Gram-negative bacterium	T01529	*glpF*	*glpK*	*glpA, glpB, glpC, glpD, gpsA*	*gldA*	*dhaK, dhaL, dhaM*	–	–	*glpR, dhaR*
Klebsiella pneumoniae	MGH 78578	Gram-negative bacterium	T00566	*glpF*	*glpK*	*glpA, glpB, glpC, glpD, gpsA*	*gldA, dhaD*	*dhaK, dhaL, dhaM*	*dhaB1, dhaB2, dhaB3, pduC, pduD, pduE*	*dhaT*	*glpR, dhaR*
Pseudomonas putida	KT2440	Gram-negative bacterium	T00114	*glpF*	*glpK*	*glpD, gpsA*	–	–	–	–	*glpR, agmR*
Bacillus megaterium	QM B1551	Gram-positive bacterium	T01208	*glpF*	*glpK*	*glpD, gpsA*	*dhaD*	*dhaK*	–	–	*glpP, ccpA, rex*

(continued)

Table 16.5 (continued)

					Glycerol dissimilation						
					Phosphorylation pathway		Dehydrogenation pathway		1,3-PDO synthesis pathway		
Microorganism	Strain		KEGG entry	Glycerol uptake	Glycerol phosphorylation	G3P oxidation	Glycerol oxidation	DHA phosphorylation	Glycerol dehydration	3-HPA reduction	Regulation
Bacillus subtilis	168	Gram-positive bacterium	T00010	*glpF*	*glpK*	*glpD*, *gpsA*	–	–	–	–	*glpP*, *ccpA*, *rex*
Clostridium acetobutylicum	ATCC 824	Gram-positive bacterium	T00056	*glpF*	*glpK*	*glpA*, *gpsA*	–	–	–	–	*glpP*, *ccpA*, *rex*
Lactobacillus plantarum	WCFS1	Gram-positive bacterium	T00115	*glpF1*, *glpF3*, *glpF4*, *glpF5*, *glpF6*	*glpK*, *gykA*	*glpD*, *gspA*	–	*dak1A*, *dak1B*, *dak2*, *dak3*	–	*dhaT*	*glpR*, *ccpA*
Saccharomyces cerevisiae	S228c	Yeast	T00005	*fps1*, *stl1*, *aqy3*	*gut1*	*gut2*, *gpd1*, *gpd2*	*gcy1*	*dak1*, *dak2*	–	–	–
Yarrowia lipolytica	CLIB122	Yeast	T01033	*glpF*	*glpK*	*glpD*, *gpd1*	*gcy1*	*dak*	–	–	–

phosphoprotein-dependent kinases (DhaKLM) are composed of three subunits: DhaK and DhaL, which are homologous to the domains from the ATP-dependent enzyme; and DhaM, which is a component of the PTS system. DhaK contains a binding site for DHA, and DhaL contains an ADP-binding site. DhaM is a phosphohistidine protein that transfers phosphoryl groups from a phosphoryl carrier protein of PTS (HPr or enzyme I) to the DhaL–ADP complex. Regardless of the phosphorylation or the dehydrogenation pathway, glycerol is converted into DHAP and then metabolized in glycolysis.

As opposed to the two oxidative pathways discussed above, some bacteria (e.g., microbes within the genera of *Citrobacter* and *Klebsiella*) convert glycerol into 1,3-propanediol (1,3-PDO) via 3-hydroxypropanal (3-HPA) in a reductive pathway (Fig. 16.5). The reductive pathway is regulated by a coenzyme B_{12}-dependent glycerol dehydratase (GDHt) (EC 4.2.1.30) correlated diol dehydratases (DDHs) (EC 4.2.1.28), converting glycerol to 3-HPA, and by a coenzyme NADH-dependent enzyme, 1,3-propanediol-oxidoreductase (PDOR) (EC 1.1.1.202), reducing 3-HPA to 1,3-PDO and regenerating NAD^+. GDHt and PDOR are encoded by *dhaB* and *dhaT*, respectively.

16.3.3 Regulation of Glycerol Metabolism

16.3.3.1 Regulation of the Phosphorylation Pathway

Metabolic substrates induce the gene expressions for glycerol metabolism. The glycerol/G3P-responsive transcriptional regulator GlpR represses the transcription of the *glpFK* operon, *glpD* and *glpABC* operon of Gram-negative bacteria. Either glycerol or G3P relieves this repression. In Gram-positive bacteria, the gene encoding the antiterminator protein GlpP is located upstream of the *glpFKD* gene cluster (Fig. 16.6). In the presence of G3P, GlpP upregulates the translation efficiency by binding to the 5′-untranslated region and stabilizing the secondary structure of mRNA transcribed from the gene cluster.

Other than the metabolic substrate-responsive regulators, the expression levels of genes encoding glycerol metabolic enzymes are controlled by other regulation factors. GlpK is the key regulatory and rate-limiting step in glycerol utilization. GlpK is allosterically regulated by fructose 1,6-bisphosphate. Rapidly metabolizable sugars repress *glpFK* operon. Carbon catabolite repression (CCR) of *glpFK* is partly mediated through a catabolite response element *cre* preceding *glpFK*. This operator site is recognized by the catabolite control protein A (CcpA) in complex with one of its co-repressors, phosphorylated HPr, or phosphorylated Crh. HPr is a component of the PTS, and Crh is an HPr homolog.

16.3.3.2 Regulation of the Dehydrogenation Pathway

The redox-sensing regulator Rex is implicated in the transcriptional regulation of bacterial central carbon and energy metabolism. The Rex-binding motif was found upstream of the glycerol-utilizing gene (e.g., *gldA*).

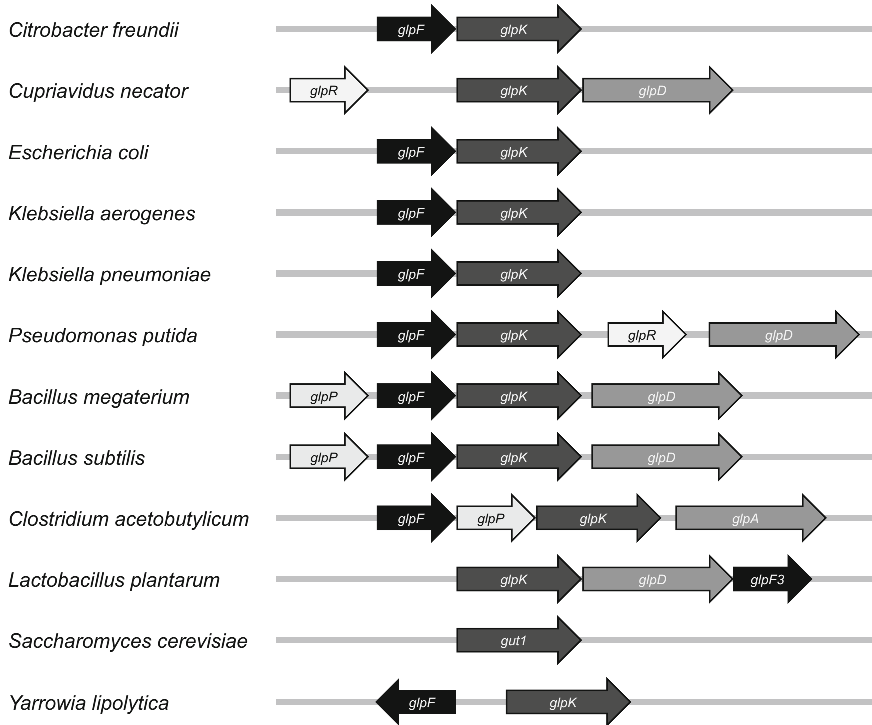

Fig. 16.6 Genetic organization of microbial glycerol metabolism via the phosphorylation pathway. Arrows and arrowheads represent the length, location, and orientation of genes

In E. coli, genetic and biochemical studies have demonstrated that the *dha* operon is regulated by DhaR and the two kinase subunits DhaK and DhaL (Fig. 16.7). DhaR is a regulator with two domains: transmitter domain and receiver domain. DhaK and DhaL act antagonistically; DhaK functions as a corepressor and DhaL as a coactivator of DhaR. In the presence of DHA, when the phosphoryl group is transferred from DhaL::ATP to DHA, the now-dephosphorylated DhaL::ADP binds to the DhaR receiver domain and activates the expression of the *dha* operon. In the absence of DHA, DhaL::ADP is rephosphorylated by DhaM to DhaL::ATP, which does not bind to DhaR.

16.4 Enhanced Glycerol Utilization via Strain Engineering

As discussed in Sect. 16.3, many microorganisms can naturally utilize glycerol, but at rates lower than desired for industrial applications (Table 16.4). Strain engineering is a widely used technique to increase microbial productivity in transforming glycerol (crude and pure) into desired products. It has also been used to engineer

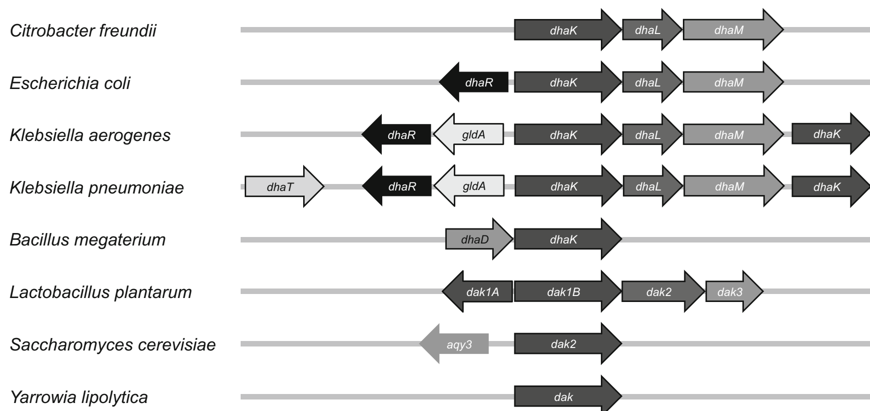

Fig. 16.7 Genetic organization of microbial glycerol metabolism via the dehydrogenation pathway. Arrows and arrowheads represent the length, location, and orientation of genes

industrially relevant microorganisms that cannot use glycerol naturally, such as *Corynebacterium glutamicum* (Rittmann et al. 2008).

The two key strategies to improve glycerol utilization are rational pathway design and evolutionary engineering. In rational pathway design, engineering targets include (1) improving glycerol influx to the cell, (2) improving glycerol catabolism to central metabolites (e.g., DHAP and 3-HPA), and (3) improving downstream metabolic pathway leading to the desired product. Targeting downstream metabolic pathways has been the subject of numerous reviews and is not discussed here. Improving glycerol influx and the production of central metabolite mainly revolves around the set of genes responsible for glycerol transport (Sect. 16.3.1), catabolism (Sect. 16.3.2), and regulation (Sect. 16.3.3). As a complementary approach, evolutionary engineering such as adaptive laboratory evolution is invaluable, mainly when there is a limited understanding of the glycerol metabolism of the target microorganism or a lack of molecular biology tools to introduce foreign genes or edit its genome. Knowledge of the specific proteins responsible for glycerol transport, catabolism, or regulation, however, does not preclude the use of an evolutionary approach. By nature, cellular metabolism of microorganisms is complex. It is therefore difficult for biochemical engineers to predict or account for all possible effects (beneficial or detrimental) resulting from rational pathway design.

16.4.1 Rational Pathway Design

In rational design, improving glycerol metabolism is often achieved by overexpression of proteins to facilitate glycerol transport and glycerol catabolism or remove catabolite repression. Based on known mechanisms of glycerol transport, catabolism, and regulation in microorganisms (Sects. 16.3.1–16.3.3), one of the

most common rational design strategies is to overexpress native or heterologous proteins for the glycerol catabolic pathways (i.e., phosphorylation, dehydrogenation, or 1,3-PDO synthesis). Alternatively, one can activate glycerol induction or remove regulation that limits glycerol metabolism. Table 16.6 summarizes the genes in glycerol dissimilation pathways targeted by various strain engineering works to enhance chemical production from glycerol.

The protein targeted for engineering is often product-dependent. In the production of 1,3-PDO, the enzyme PDOR (DhaT) is overexpressed to increase the conversion of 3-HPA to 1,3-PDO, sometimes in combination with the preceding GDHt (DhaB). On the other hand, PDOR (DhaT) is deleted when the product 3-HPA is desired, and GDHt (DhaB) responsible for converting glycerol to 3-HPA is overexpressed. Mazumdar et al. produced D-lactic acid from glycerol in recombinant strains of E. coli (Mazumdar et al. 2010). As D-lactic acid is derived from the central metabolite pyruvate, the authors tested two pathways (GlpK-GlpD and GldA-DhaK) to increase the production of glycolytic intermediate DHA. Expression of GldA-DHA kinase (*gldA* and *dhaKLM*) fermentative pathway caused a small increase in D-lactate production, but the best outcomes were seen in the heterologous expression of the respiratory G3P pathway (*glpK* and *glpD*) that led to ~fivefold increase in D-lactate concentration and yield. In the production of 1,2-propanediol (1,2-PDO), Clomburg and Gonzalez overexpressed the genes for the fermentative pathway from glycerol to 1,2-PDO (i.e., *gldA*) (Clomburg and Gonzalez 2011). Moreover, they removed the native E. coli PEP-dependent DhaK and replaced it with the ATP-dependent DhaK from *C. freundii* to decouple DHAP formation from PEP synthesis, which they postulated was constraining the yield. Results demonstrated higher glycerol consumption and 1,2-PDO yield.

Besides products, the microbial host also dictates the enzyme targets for rational engineering. The amino acid-producing microbial host *C. glutamicum* is a non-pathogenic, Gram-positive soil bacterium that cannot utilize glycerol as sole carbon and energy source (Rittmann et al. 2008). Engineered strains of *C. glutamicum* required the heterologous proteins GlpF, GlpK, and GlpD to assimilate glycerol (Rittmann et al. 2008; Meiswinkel et al. 2013). Rittmann et al. reported that expression of *glpKD* from E. coli ($glpKD_{Ec}$) was sufficient for growth on glycerol, but the addition of E. coli *glpF* ($glpF_{Ec}$) helped to increase growth rate and biomass formation (Rittmann et al. 2008). It was revealed in this study that no growth on glycerol was observed when $glpFK_{Ec}$ was expressed without $glpD_{Ec}$ due to the accumulation of sub-toxic G3P within the cells (Rittmann et al. 2008).

The inability to consume glycerol is not always due to the absence of glycerol utilization proteins but can be a lack of gene expression. Meiswinkel et al. identified putative homologs of E. coli GlpK (with 49% identity to $glpK_{Ec}$) and GlpD (with 32% identity to $glpD_{Ec}$) in *C. glutamicum* and investigated their effects on *C. glutamicum* glycerol utilization (Meiswinkel et al. 2013). The expression of the native $glpK_{Cg}$ or $glpD_{Cg}$ gene did not enable *C. glutamicum* to grow on glycerol. Growth in glycerol was observed when $glpKD_{Cg}$ was expressed in *C. glutamicum*, eventually reaching the same biomass as that achieved by the expression of $glpFKD_{Ec}$ in the strain. However, the growth rate when $glpKD_{Cg}$ ($\mu_{max} = 0.115\ h^{-1}$)

Table 16.6 Engineered strains for bioconversion of glycerol to a product

Strain	Product	Glycerol uptake	Phosphorylation pathway	Dehydrogenation pathway	1,3-PDO synthesis pathway	Regulation	References
Bacillus amyloliquefaciens	2,3-BDO			(+)*dhaD*			Yang et al. (2015)
Bacillus subtilis	3-HPA				(+)*dhaB123*	(+)*gdrAB*	Kalantari et al. (2017)
Citrobacter werkmanii	1,3-PDO			Δ*dhaD*			Maervoet et al. (2016)
Clostridium acetobutylicum	1,3-PDO				(+)*dhaB1B2*		Gonzalez-Pajuelo et al. (2005)
Clostridium pasteurianum	Butanol				ΔdhaT		Pyne et al. (2016)
Corynebacterium glutamicum	Succinate	(+)*glpF*	(+)*glpK*, (+)*glpD*				Litsanov et al. (2013)
Corynebacterium glutamicum	Glutamate/lysine/arginine/putrescine	(+)*glpF*	(+)*glpK*, (+)*glpD*				Rittmann et al. (2008); Meiswinkel et al. (2013)
Cupriavidus necator H16	Poly(3HB)	(+)*glpF*	(+)*glpK*				Fukui et al. (2014)
Escherichia coli	1,3-PDO				(+)*dhaB1B2*, (+)*dhaT*		Tang et al. (2009)
Escherichia coli	1,2-PDO			(+)*gldA*, Δ*dhaK*, (+)*dhaKL*			Clomburg and Gonzalez (2011)
Escherichia coli	D-lactate		(+)*glpK*, (+)*glpD*				Mazumdar et al. (2010)
Escherichia coli	L-lactate		(+)*glpK*, (+)*glpD*				Mazumdar et al. (2013)
Escherichia coli	3-HPA				(+)*dhaB123*	Δ*glpR*, (+)*gdrAB*	Chu et al. (2015)
Escherichia coli	Ethanol		(+)*glpK*	(+)*gldA*, (+)*dhaKLM*			Wong et al. (2014)
Escherichia coli	D-lactate		(+)*glpK*, (+)*glpD*				Wang et al. (2015)

(continued)

Table 16.6 (continued)

Strain	Product	Glycerol uptake	Phosphorylation pathway	Dehydrogenation pathway	1,3-PDO synthesis pathway	Regulation	References
Escherichia coli	Ethanol and formate			(+)*gldA*, (+) *dhaKLM*			Yazdani and Gonzalez (2008)
Escherichia coli	DHA			(+)*gldA*			Yang et al. (2013)
Klebsiella pneumoniae	D-lactate				Δ*dhaT*		Feng et al. (2014)
Klebsiella pneumoniae	1,3-PDO				(+)*dhaT*		Hao et al. (2008)
Klebsiella pneumoniae	1,3-PDO			(+)*dhaD*	(+)*dhaT*		Chen et al. (2009); Zhao et al. (2009)
Klebsiella pneumoniae	1,3-PDO	(+)*glpF*			(+)*dhaT*[a]	Δ*crr*	Wang et al. (2017a)
Klebsiella pneumoniae	1,3-PDO					Δ*crr*	Lu et al. (2018)
Klebsiella pneumoniae	1,3-PDO		Δ*glpK*	Δ*dhaD*	(+)*dhaT*		Lee et al. (2018)
Klebsiella pneumoniae	3-HPA				Δ*dhaT*		Ko et al. (2012)
Klebsiella pneumoniae	3-HPA		Δ*glpK*		Δ*dhaT*		Ashok et al. (2013)
Klebsiella pneumoniae	Poly(3HP)				(+)*dhaB123*, Δ*dhaT*	(+)*gdrAB*	Feng et al. (2015)
Pseudomonas denitrificans	3-HPA				(+)*dhaB123*	(+)*gdrAB*	Zhou et al. (2013)
Pseudomonas putida	*N*-methyl-glutamate					Δ*glpR*	Mindt et al. (2018)
Saccharomyces cerevisiae	1,2-PDO	(+)*gup1*	(+)*gut1*, (+)*gut2*	(+)*gldA*			Jung et al. (2011)
Yarrowia lipolytica	Erythritol and citric acid		(+)*gut1*, (+)*gut2*				Mironczuk et al. (2016)

[a]A protein variant was used

was expressed was more than twofold slower than with $glpKD_{Ec}$ ($\mu_{max} = 0.2790\ h^{-1}$). The engineered strains of *C. glutamicum* were used for the production of amino acids and diamine putrescine from crude and pure glycerol. This phenomenon of insufficient or dormant gene expression was also observed in *Cupriavidus necator* H16, where overexpression of the native GlpK improved growth in glycerol to $\mu_{max} = 0.15\ h^{-1}$(Fukui et al. 2014).

The removal of metabolic regulation, such as catabolite repression in mixed carbon cultivation, is also a viable strategy in rational engineering. Klebsiella pneumoniae assimilates glycerol naturally and is a natural producer of 1,3-PDO. To improve the economic viability of the process, Wang et al. enhanced the K. pneumoniae 1,3-PDO biosynthesis in a mixed carbon source with glucose and crude glycerol (Wang et al. 2017a). The work focused on achieving a higher glycerol consumption rate and eliminating glucose inhibition (i.e., catabolite inhibition) to increase the productivity and yield of 1,3-PDO from glycerol. The authors expressed different heterologous GlpF proteins (from E. coli, L. rhamnosus, and K. pneumoniae), aquaporin Z, and glycerol diffusion proteins from K. pneumoniae to improve glycerol uptake. GlpF from K. pneumonia*e* resulted in the most improved glycerol consumption of about twofolds. Interestingly, the investigation demonstrated that strains with a high expression level of the channel proteins had an even lower glycerol consumption rate. Glucose added to the K. pneumoniae fermentation process was beneficial to cell growth and 1,3-PDO production, but high glucose concentration resulted in catabolite repression of glycerol metabolism. The authors eliminated glucose repression by deleting the *crr* gene responsible for glucose transport in the PEP:PTS catabolite repression system. The engineered strains of K. pneumoniae delivered higher amount of 1,3-PDO and consumed glycerol faster in a glucose-glycerol fermentation process.

When considering the implementation of rational pathway design, the availability of plasmids, promoter systems, and genome engineering tools is a prerequisite. Different promoter systems (inducible or constitutive, strong or weak) are necessary for the adjustment of heterologous protein expression. Genome editing techniques are required for deleting unwanted pathways or inactivating regulatory proteins. This is one of the critical challenges of rational pathway design, as many microorganisms do not have well-established plasmid systems, promoters with varying strength and genome editing tools.

16.4.2 Evolutionary Engineering

Evolutionary engineering under a controlled laboratory setting is a versatile method for generating robust microbial hosts for varied conditions, for instance, to use crude glycerol. Microbial hosts may be adapted to grow in carbon sources through a temporary or a permanent change in the strain phenotype (Dragosits and Mattanovich 2013). The process hinges on the use of target-oriented selection pressure, in this case glycerol utilization, to create and select for improved mutant strains. Phenotypic changes can be hastened through the use of mutagens like UV,

ethyl methanesulfonate (EMS), or *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG). New technologies have also emerged to enable the creation of genome-level gene libraries (Choi et al. 2019). Once the desired phenotype has been found, the whole-genome sequencing of the mutant helps to identify beneficial mutations. This information is often used subsequently in combination with rational engineering to improve a strain further.

Evolutionary engineering has been used for the enhancement of glycerol utilization in E. coli for more than 40 years. Despite a complete pathway for glycerol catabolism, E. coli cells cultivated in a synthetic medium supplemented with glycerol displayed significant variations in growth rates (Lin 1976). Based on this premise, Herring et al. determined the genetic basis of adaptation to glycerol by whole-genome resequencing and identified 13 de novo mutations in five different E. coli strains (Herring et al. 2006). The introduction of each of these 13 mutations by site-directed mutagenesis showed the genes encoding the two major subunits (*rpo*B and *rpoC*) of RNA polymerase (RNAP) conferred the most substantial change in growth rate (48–65%) in glycerol. Mutations in *glpK* and *pdxK* (pyridoxine kinase) also had significant effects. The mutations identified in *glpK* resulted in reaction rates 51–130% higher than the wild type. While *pdxK* has no direct function in glycerol metabolism, it is interesting to note that it is adjacent to *crr*, a regulator of GlpK and a critical component in catabolite repression (PEP:PTS). Other beneficial mutations were located in global regulatory elements such as sigma factor S (σ^S, *rpoS*). This investigation showed that marked changes in phenotype could be observed with as few as two mutations. The mutations identified have no direct relation to the glycerol dissimilation pathways discussed in Sect. 16.3.2. This highlights the complexity of metabolic networks and regulations as well as the relevance of evolutionary methods in strain optimization.

A study by Conrad et al. consistently found specific small deletions within the *rpoC* gene, which encodes the β′-subunit of RNAP, after adaptation of E. coli K-12 MG1655 in minimal media with glycerol (Conrad et al. 2010). Mutants of E. coli grew 60% faster in glycerol compared to the parental strain and converted carbon source 15–35% more efficiently to biomass. However, the mutant strains were also capable of growing significantly faster in glucose and lactate in minimal media, which indicated that the growth improvement was not specific to glycerol but extended to growth in minimal media with other carbon sources. These findings suggested that these RNAP mutants reflect a general adaptation to minimal media. Adaptive evolution by serial cultivation has also been applied to improve the glycerol tolerance of *Xanthomonas campestris* (Wang et al. 2017b) and *Rhizopus oryzae* (Huang et al. 2015).

Other evolutionary engineering strategies for enhancing glycerol utilization include the use of chemical mutagens alongside adaptive laboratory evolution. Lee et al. continuously exposed E. coli cells to a low level of the chemical mutagen NTG to accelerate the timescale of adaptive evolution in glycerol (Lee et al. 2011). Perhaps unsurprisingly, they identified a superior growth rate phenotype with a larger than the usual number of mutations as verified through whole-genome sequencing of the endpoint strains. The authors concluded that, for adaptation of

E. coli to glycerol utilization, the paths leading to increased fitness are *glpK* and *rpoB*. Jensen et al. isolated a *Clostridium pasteurianum* mutant with increased tolerance toward crude glycerol after mutagenesis with EMS and cultivation in increasing concentration of crude glycerol (Jensen et al. 2012). The mutant *C. pasteurianum* can produce six times more cell mass compared to the wild type at 25 g/L of crude glycerol and had detectable growth at 105 g/L of crude glycerol. In a separate study, NTG-mutagenized *C. pasteurianum* with 91% increase in butanol production in 100 g/L of crude glycerol compared to the wild-type strain was obtained. The deletion of the master transcriptional regulator of sporulation, Spo0A, was identified as the reason for increased crude glycerol tolerance (Sandoval et al. 2015). Rahman et al. obtained a K. pneumonia*e* mutant that can be cultivated at 300 g/L of glycerol following UV and EMS mutagenesis (Rahman et al. 2017).

S. cerevisiae has the genetic capability to utilize an assortment of nutrient sources. Despite possessing all necessary pathways, it grows very poorly or not at all in a minimal glycerol medium, although it can assimilate glycerol when grown in a complex growth medium. Through adaptive evolution, Strucko et al. obtained mutant strains capable of growing efficiently in glycerol (mean $\mu_{max} \approx 0.22\ h^{-1}$) and showing a fourfold faster growth rate in glycerol compared to the parental strain in a rich medium containing amino acids (Strucko et al. 2018). After sequencing, mutations were found in genes encoding proteins associated with a range of functions from metabolism to regulation and signaling; three genes were frequently found to be mutated: *gut1* (encoding glycerol kinase), *hog1* (encoding mitogen-activated protein kinase), and *pbs2* (encoding MAP kinase of the HOG signaling pathway). These findings were similar to those previously observed in other microbial hosts, where the mutation was commonly found in *glpK* gene in E. coli (Herring et al. 2006) and other studies with S. cerevisiae (Ho et al. 2017). While the mutants have higher glycerol utilization efficiency, they demonstrated fitness trade-offs with regard to osmosensitivity or ethanol utilization, possibly explaining the suboptimal or no capacity for glycerol metabolism in S. cerevisiae.

16.5 Enhanced Glycerol Utilization via Physical Methods

The use of genetically modified organisms for biomanufacturing is constrained by the legal framework and social perceptions in some countries. Except for adaptive evolution (Satyanarayana and Kunze 2009), many strain engineering techniques described in Sect. 16.4 are less applicable when it comes to food and pharmaceutical applications. Besides strain engineering, it is possible to enhance cell growth on crude glycerol through optimizing process parameters such as media composition, aeration, temperature, mixing, and organism(s) used. Currently, the most challenging aspect of crude glycerol valorization is its varying composition (Table 16.3), which depends on feedstocks, production processes, and treatments (Hu et al. 2012).

Crude glycerol composition is often omitted or not precisely described in studies focusing on microbial crude glycerol utilization. This leads to ambiguities and variations in the product yield reported by different authors (Samul et al. 2014).

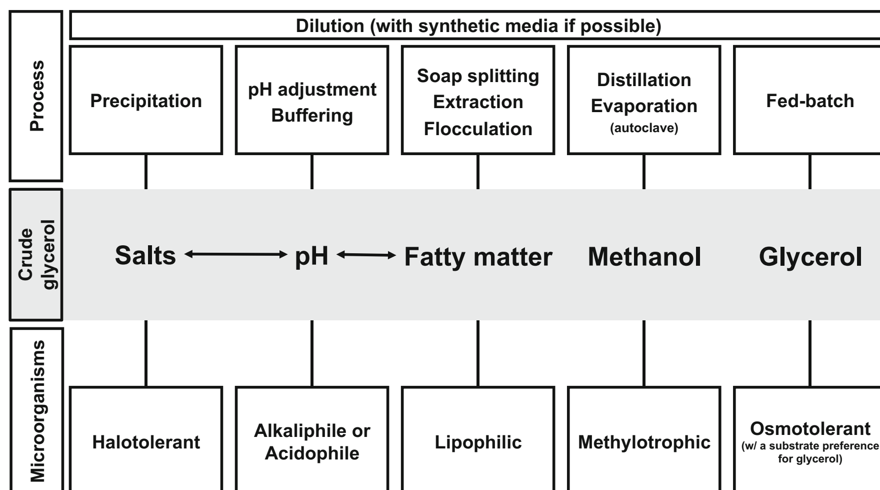

Fig. 16.8 Overview of strategies for crude glycerol pretreatment. The gray section identifies crude glycerol properties that potentially affect microbial growth. The top part of the diagram shows physical modifications that can improve the compatibility of crude glycerol to microbial growth. The bottom part of the diagram shows the class of microorganisms with specific tolerance against the identified property

Many components in crude glycerol are growth inhibitory, even the glycerol itself. That said, the impurities present do not necessarily harm the bioprocesses. Some might serve as co-substrates or exert no effects at all on the microbial host.

As untreated raw glycerol is the cheapest form of crude glycerol, it is the most economical feedstock. When necessary, raw glycerol concentration or composition could be modulated by concentrating or diluting step(s), minor additional treatments or supplementation of vitamins, essential amino acids, minerals, and other nutrients vital for microbial growth. An overview of treatment processes targeting various impurities in crude glycerol is presented in Fig. 16.8. The following section looks specifically at the impacts of common impurities in crude glycerol on bioprocesses and strategies to alleviate the problem. Even though crude glycerol components are discussed individually here, microbial growth behavior and productivity in crude glycerol are likely manifestations of combined effects from all components present. Dilution is the only treatment that modulates all components of crude glycerol. From the perspective of process development, the selected strategy should also take advantage of existing pretreatment and purification facilities.

Besides modifying crude glycerol to make it more compatible with microbial growth, one can also choose a microorganism that thrives in a given crude glycerol condition (Fig. 16.8). For instance, osmotolerant microorganisms grow in high glycerol concentrations. Microalgae are known for their osmotolerance (Day et al. 2012). Acidophiles and alkaliphiles survive in acidic and alkaline raw glycerol, respectively. Halotolerant species, for example, microalgae, maintain lipid production under high salt concentrations (Xu et al. 2012). Use of naturally occurring

microorganism is, however, limited by the requirement that the product of interest must be a natural product of the chosen microorganism.

16.5.1 Glycerol

Raw glycerol from fat splitting has 8–20% glycerol and that from transesterification contains 50–60% glycerol. These high concentrations result in substrate inhibition during microbial growth (Muniraj et al. 2015), which is often attributed to osmotic shock (Raimondi et al. 2014) and redox imbalance in cells (Mota et al. 2017). Due to its high reductance ($\kappa = 4.67$), glycerol dissimilation generates more reducing equivalents (i.e., NADH) compared to glucose ($\kappa = 4$) (Westbrook et al. 2019). Therefore, balancing excess NADH is vital during glycerol fermentation.

A common strategy is to dilute the crude glycerol with synthetic medium (Ito et al. 2005). Not only this strategy eliminates glycerol substrate inhibition but also it provides additional nutrients to support microbial growth or pH adjustment of the feedstock. In the same vein, the use of fed-batch cultivation has been reported as an efficient solution to circumvent glycerol substrate inhibition as it allows better control of glycerol and nutrient concentrations (Samul et al. 2014; Muniraj et al. 2015) to achieve higher cell density and metabolite production than in batch cultivation (Beopoulos et al. 2009).

In place of a synthetic medium, overcoming substrate inhibition by adding another carbon source or waste stream is also frequently adopted. There have been reports in the literature of crude glycerol being successfully blended with glucose (Mantzouridou et al. 2008; Chen et al. 2012) and other wastes such as whey (Ilić et al. 2013), tomato waste (Fakas et al. 2008), corn stover hydrolysate (Xin et al. 2016), and dairy cattle manure (Simm et al. 2017). The two carbon sources can be consumed either sequentially (diauxie) or at the same time (co-utilization). However, the selection of a second carbon source is crucial as some carbon sources can repress glycerol assimilation (e.g., glucose repression described in Sect. 16.3.3). Carbon sources are often assimilated in a hierarchy, but the molecular understanding of diauxie and co-utilization is limited. Wang et al. recently proposed a model explaining microbial growth on mixed carbon sources (Wang et al. 2019). The usual carbon sources (xylose, ribose, glucose, maltose, lactose, galactose, fructose, sorbitol, mannose, arabinose, and glycerol) can be sorted according to the growth rates they enable when used as sole carbon source, and the microbe naturally utilizes the most efficient carbon source first. Glycerol, fructose, maltose, and galactose are often the less preferred sources and are consumed later when mixed with other carbon sources. Furthermore, glycerol does not elicit catabolite repression of another carbon source, so its assimilation is repressed by the catabolite repression exerted by PEP:PTS sugars (all except maltose). The proposed model by Wang et al. relies on the fact that glycerol utilization is inefficient, but as described earlier, some organisms have developed sophisticated means to utilize glycerol. For example, Phaeodactylum tricornutum prefers glycerol over glucose (Perez-Garcia et al. 2011).

A further advantage of combining crude glycerol with other wastes is that the latter provides additional nutrients. Nutrients like vitamin B_{12} stimulate central metabolism and glycerol utilization as some enzymes, for example, GDHt involved in 3-HPA formation are B_{12}-dependent (Westbrook et al. 2019). That being said, nutrient supplementation is not always the best strategy. These are cases whereby nutrient starvation was purposely adopted to stimulate or accumulate a metabolite of interest (Mozejko-Ciesielska and Pokoj 2018). Further, adjustment of the glycerol/co-substrate ratio with an uncharacterized waste stream is also challenging compared to using pure sugars.

16.5.2 Methanol

High methanol concentration affects the fluidity of plasma membranes (Venkataramanan et al. 2012). It was described as an inhibitor of algal growth (Pyle et al. 2008). Nonetheless, the positive effects of methanol have also been documented for microalgae, but it is concentration-dependent and strain-specific (Miazek et al. 2017). Fortunately, methanol is absent in the crude glycerol from hydrolysis. Despite an initial concentration of up to 30% in crude glycerol from biodiesel production (Table 16.3), it is often recovered in the early purification stages for reuse. Xu et al. showed that a small amount of methanol found in the crude glycerol tested did not severely impact glycerol fermentation using R. toruloides (Xu et al. 2012). If crude glycerol did contain methanol, media sterilization via autoclaving before cultivation would evaporate the bulk of the methanol present (Athalye et al. 2009).

16.5.3 pH

The pH of crude glycerol has an impact on biological processes. pH influences aquaporin assembly (Sabir et al. 2017) and modulates the activation of hexose/proton symports (Perez-Garcia et al. 2011). Both aquaporin and hexose/proton symports share similarities with aquaglyceroporins (e.g., GlpF). Therefore, pH likely affects glycerol transport. Metabolic pathways can also be affected by pH. Lowering media pH has been shown to activate the transcription of formate hydrogenlyase (FHL) and the formate pathway in *E.coli* (Westbrook et al. 2019). In the worst-case scenario, an inappropriate pH prevents cell growth and results in cell death.

The pH of crude glycerol is dictated by the catalyst used during fat splitting or biodiesel production. Transesterification using NaOH/KOH catalyst produces crude glycerol of pH ~10, and fatty acids tend to form soap (i.e., metallic salt of fatty acids) at alkaline pH; the absence of a catalyst leads to an equilibrium between acidic and basic forms of fatty acids. Crude glycerol from the Colgate-Emery process with no catalyst exhibits a pH between 4 and 5, owing to the presence of fatty acids (Jungermann and Sonntag 1991). While pH adjustment may seem straightforward, it can lead to other undesirable outcomes. For example, lowering pH is one way to

split soap and extract fatty acids from the glycerol phase, but this pH adjustment also increases total salt content.

Moon et al. adjusted the pH of crude glycerol from biodiesel production to reduce its MONG concentration (Moon et al. 2010). The authors first diluted crude glycerol before adjusting its pH to 3–4 with HCl to neutralize fatty acids as well as the remaining catalyst and separating the two phases (oily and glycerol phases) by gravity followed by centrifugation. The initial dilution decreased the concentration of all contaminants (especially the salt concentration), and the addition of HCl was controlled such that the final salt level was similar to or below the initial level in undiluted crude glycerol. This pretreated crude glycerol with concentration between 15 and 25 g/L was then mixed with a modified CAB medium for *Clostridium* strains or a DMSZ n°427 medium for *Klebsiella* strains. Both media contain K_2HPO_4/KH_2PO_4, and the CAB medium has 2-(*N*-morpholino)ethanesulfonic acid (MES) to buffer the media with crude glycerol at a pH suitable for bacterial growth.

16.5.4 Fatty Matters

The main fatty matters found in crude glycerol include FFA, soaps (metallic salt of fatty acids), glycerides (mono-, di- and tri-), and FAME. The pH influence on fatty matters was briefly described in the previous section. In addition to pH adjustment, fatty matters in MONG can be reduced via solvent extraction. The solvent choice is crucial. For instance, the use of hexane was reported to turn crude glycerol into a gel (Moon et al. 2010).

Fatty matter exerts both positive and negative effects on bioprocess, depending mainly on the nature of the fatty matter and the microorganism used. The fatty matter in crude glycerol may serve as a carbon source, act as surfactants, and be incorporated into *ex novo* lipid synthesis pathway or into the lipids of the plasma membrane. Used as a carbon source, the fatty matter can promote growth and metabolite accumulation as described in fungi (Grewal and Kalra 1995) and yeast (Papanikolaou et al. 2006; Soccol et al. 2006). The outcome depends on the nature of the fatty matter. Some strains can consume FFAs but cannot process glycerides because they lack the suitable lipase(s) while others can utilize both FFAs and glycerides (Solaiman et al. 2006). Non-assimilated fatty matter can positively or negatively impact on cell membranes. Fatty acids interfere with the membrane structure by tail/tail interactions with the membrane fatty acids (Muranushi et al. 1981). In *C. pasteurianum*, the presence of oleic acid (a saturated fatty acid) did not affect growth and just slightly decreased metabolite production, while linoleic acid (an unsaturated fatty acid) inhibited growth from a concentration of 0.125 g/L (Venkataramanan et al. 2012). It was hypothesized that unsaturated fatty acids hindered the diffusion of nutrients (Venkataramanan et al. 2012).

The influence of fatty matter on microbial growth can be a combination of several effects. For instance, *Y. lipolytica* can use fatty matter as sole carbon source (Papanikolaou et al. 2006; Soccol et al. 2006), and its glycerol assimilation was also improved by surfactants (Amaral et al. 2009). FAME, soap, MG, and DG can

function as surfactants to emulsify the medium, improving nutrient intake by increasing cell membrane permeability (Gao et al. 2016). Given the effect observed with a surfactant, this strategy was adopted in process design to improve glycerol assimilation using commercial surfactant such as Tween 80 (Pachapur et al. 2016). Liu et al. tested nine surfactants on *Trichosporon fermentans* and T. cutaneum (Liu et al. 2017). Only potassium oleate was able to stimulate growth slightly.

16.5.5 Salt and Mineral

The effects of salts and minerals on microorganisms are diverse. Heavy metals can inhibit cell division (Moon et al. 2010). Iron activates an enzyme that consumes citric acid, and thus reduces the yield of this specific metabolite (Cavallo et al. 2017). Metal oxides have been shown to have antimicrobial activities through the generation of reactive oxygen species (Siddiqi and Husen 2016; Hao et al. 2017; Gold et al. 2018; Ko et al. 2018). Fortunately, most minerals are found only as traces in biodiesel crude glycerol (Ayoub and Abdullah 2012). Of these, the main mineral is sodium ion, which exists as NaOH (remaining catalyst) or NaCl (neutralized catalyst or present in initial oil feedstock). It should be noted that KOH can replace NaOH. Compared to potassium ion, the sodium ion is a stronger inhibitor for *Paracoccus denitrificans* and *Cupriavidus necator* (Mothes et al. 2007). As with high glycerol concentration, high salt concentration creates osmotic pressure (Xu et al. 2012). While the thresholds are strain-dependent, Gao et al. concluded that osmotic pressure from concentrations of NaCl below 16 g/L (300 mM) was not detrimental to cell growth but stimulated growth and enhanced lipid content of certain microorganisms (Gao et al. 2016).

If the salt content is detrimental to a bioprocess, dilution can be used to reduce its concentration. Salt precipitation is also a feasible alternative.

16.6 Biomanufacturing from Glycerol

Microbial biomanufacturing from glycerol is widely reported. Value-added chemicals synthesized by microbes from glycerol include polyhydroxyalkanoates (PHA), 1,3-PDO, alcohols (e.g., butanol and ethanol), organic acids (e.g., citric acid, succinic acid, and propionic acid), and other fine chemicals (e.g., DHA, lipids, pigments, and biosurfactants) (Table 16.7). These microbial hosts use either pure or crude glycerol as carbon feedstock.

16.6.1 Polyhydroxyalkanoate (PHA)

Widely known as bioplastic, PHA is microbial carbon and energy reserve, accumulated intracellularly in the form of granules when the bacterial cells experience stress. PHA shares similar properties to petroleum-derived plastics. They are

Table 16.7 Microbial conversion of glycerol into value-added products

Microorganism	Product	Pure glycerol (titer)	Crude glycerol (titer)	References
C. necator H16	[P(3HB)]	1.44 g/L	n. r.	Fukui et al. (2014)
C. necator JMP 134, *and P. denitrificans*	[P(3HB)]	n. r.	48% CDW	Mothes et al. (2007)
C. necator DSM 545	[P(3HB)]	n. r.	50% CDW	Cavalheiro et al. (2009)
P. oleovorans NRRL B-14682	[P(3HB)]	n. r.	30% CDW	Ashby et al. (2011)
Z. denitrificans MW1	[P(3HB)]	n. r.	67% CDW	Ibrahim and Steinbuchel (2009)
C. Acetobutylicum	1,3-PDO	1104 mM	1104 mM	Gonzalez-Pajuelo et al. (2005)
C. Butyricum	1,3-PDO	n. r.	63.40 g/L	Saxena et al. (2009)
K. pneumoniae KCTC2242	1,3-PDO	n. r.	86 g/L	Wang et al. (2017a)
K. pneumoniae DSM 2026	1,3-PDO	61.90 g/L	51.30 g/L	Mu et al. (2006)
K. pneumoniae DSM 4799	1,3-PDO	51.86 g/L	80 g/L	Jun et al. (2010)
C. pasteurianum DSM 525	Butanol and 1,3-PDO	n. r.	Butanol: 0.19–0.28 g/g 1,3-PDO: 0.06–0.21 g/g	Gallardo et al. (2014)
C. pasteurianum MTCC 116	Butanol and 1,3-PDO	n. r.	Butanol: 0.23 g/g crude glycerol 1,3-PDO: 0.61 g/g crude glycerol	Khanna et al. (2014)
C. pasteurianum DSM 525	Butanol and 1,3-PDO	n. r.	Butanol: 0.39 mol/mol crude glycerol	Johnson and Rehmann (2016)
C. pasteurianum CH4	Butanol and 1,3-PDO	n. r.	Butanol: 0.29 g/g crude glycerol	Lin et al. (2015)
E. aerogenes SUM I014	Ethanol	n. r.	34.54 g/L	Thapa et al. (2015)
K. pneumoniae GEM 167	Ethanol	21.50 g/L	24.60 g/L	Oh et al. (2011)
K. cryocrescens S26	Ethanol	n. r.	27 g/L	Choi et al. (2011)
P. tannophilus CBS 4044	Ethanol	n. r.	28.10 g/L	Liu et al. (2012)
Y. lipolytica Wratislavia K1	Citric acid	110 g/L	86 g/L	Rymowicz et al. (2008)
Y. lipolytica Wratislavia AWG7	Citric acid	139 g/L	131.50 g/L	Rywinska et al. (2009); Rywinska and Rymowicz (2010)
Y. lipolytica 1.31	Citric acid	124.50 g/L	124.50 g/L	Rymowicz et al. (2006)
B. succiniciproducens DD1	Succinic acid	n. r.	5.21 g/L	Scholten et al. (2009)

biodegradable and can be produced from renewable and cheap feedstocks such as crude glycerol (Zhu et al. 2010). Ashby et al. used *P. oleovorans* and P. corrugata as microbial hosts for PHB and medium-chain-length PHA production, respectively, utilizing crude glycerol from biodiesel production and pure glycerol (Ashby et al. 2004, 2005).

The impact of NaCl in crude glycerol on PHB production was investigated in *Paracoccus denitrificans* and C. necator JMP 134. The result demonstrated that polymers produced were very similar to the one obtained from glucose, but PHB production was reduced drastically (Mothes et al. 2007). On the other hand, *Zobellela denitrificans* MW1 was proven a strain with good tolerance to NaCl-contaminated crude glycerol, producing PHB at the high quantity (Ibrahim and Steinbuchel 2009).

16.6.2 1,3-Propanediol (1,3-PDO)

One of the most studied value-added chemicals that can be manufactured from crude glycerol is 1,3-PDO. This chemical has wide commercial applications in the polymer and cosmetic industries (Saxena et al. 2009). In the plastic industry, it is used as a monomer to synthesize polyesters, polyethers, and polyurethanes. Plastics synthesized from 1,3-PDO have a high biodegradability factor (Gonzalez-Pajuelo et al. 2004). A variety of microbial hosts have been used for 1,3-PDO production, but K. pneumoniae and *C. butyricum* are the most investigated microorganisms for this purpose (Table 16.7). *Clostridia* spp. have the advantage that their nutritional and vitamin supplementation requirement is less stringent than other 1,3-PDO-producing microorganisms (Willke and Vorlop 2008). It was observed that the yield of 1,3-PDO was dependent on the source of crude glycerol (Willke and Vorlop 2008). Interestingly, *C. butyricum* VPI 3266 showed similar productivity and yield, regardless of whether crude or pure glycerol was used (Gonzalez-Pajuelo et al. 2005). This same tolerance toward both pure and crude glycerol was also observed for K. pneumoniae DSM 2026 (Mu et al. 2006).

16.6.3 Alcohol: Butanol and Ethanol

Butanol offers better physical and chemical properties as a biofuel compared to ethanol due to its lower volatility and higher energy density (Gallardo et al. 2014). *C. acetobutylicum*, *C. beijerinckii*, and *C. pasteurianum* all produce butanol from crude glycerol. Taconi et al. demonstrated that the maximum butanol yield from crude glycerol (0.3 g/g) was the same as that from glucose for *C. acetobutylicum* (Taconi et al. 2009). Not only is crude glycerol a cheaper feedstock but it also does not produce acetone as a by-product during fermentation. Johnson et al. also converted crude glycerol to butanol obtaining a maximum yield of 0.29 g/g glycerol with *C. pasteurianum* (Johnson and Rehmann 2016).

Ethanol production from crude glycerol has also been described. The four microbial strains listed in Table 16.7 showed titers of ~25–35 g/L ethanol from crude glycerol. These titers were achieved through strain engineering. Thapa et al. blocked lactic acid formation to enhance ethanol production in E. aerogenes SUMI014 (Thapa et al. 2015), while Loaces et al. screened heterologous genes from a purge sludge metagenomic library in E. coli to improve glycerol conversion and ethanol production (Loaces et al. 2016).

16.6.4 Organic Acids: Citrate, Succinate, and Propionate

Citric acid is applied in diverse sectors from food, beverage, and pharmaceutical industries to additive in detergents, cosmetics, and toiletries (Rywinska and Rymowicz 2010). Citric acid can be produced from both pure and crude glycerol to similarly high levels using acetate-negative mutants of *Y. lipolytica* Wratislavia AWG7 and *Y. lipolytica* Wratislavia K1 (Rywinska et al. 2009).

Succinic acid is widely used in the food and pharmaceutical industries, and also for the production of surfactant and detergent, green solvents, and biodegradable plastics. It is an intermediate for the production of 1,4-butanediol, tetrahydrofuran, and adipic acid (Zeikus et al. 1999). *A. succiniproducens* has been used for efficient conversion of glycerol to succinate, where a maximum of 19 g/L succinate was achieved. An advantage of using glycerol as a carbon substrate instead of glucose is that higher succinic acid yield can be achieved with a reduced acetic acid formation (Lee et al. 2001).

Propionic acid is used as an antifungal in food and feed. It is also a primary chemical to produce cellulose-based plastics, solvents, flavors, and thermoplastics (Barbirato et al. 1997; Himmi et al. 2000). *Propionibacterium acidipropionici*, *P. acnes*, and *Clostridium propionicum* have been used for propionate production using glycerol as a carbon source, where the best strain for propionic acid production was *P. acidipropionici* with a productivity of up to 0.36 g/L/h of propionate using 80 g/L glycerol in the medium (Barbirato et al. 1997). The authors concluded that propionate production from glycerol had conversion yield and productivity similar or even superior to other carbon sources such as lactic acid or glucose.

16.6.5 Other Chemicals: Dihydroxyacetone (DHA) and Lipids

DHA is used in the cosmetic industry as a building block for organic synthesis of fine chemicals (Bauer et al. 2005). Microbial DHA production is more economical due to the high safety requirements of the corresponding chemical process (Hekmat et al. 2003). Production of DHA from glycerol was accomplished using G. oxydans (Bauer et al. 2005).

Crude glycerol has also been used as a feedstock for lipid production with Chlorella protothecoides, achieving a yield of 0.31 g lipid/g substrate (O'Grady

and Morgan 2011). The high production of single-cell oil from biodiesel crude glycerol was also achieved with *Thamnidium elegans* (Chatzifragkou et al. 2011).

16.7 Challenges, Prospects, and Conclusion

Although glycerol-utilizing microbes are ubiquitous, developing a sustainable and economical large-scale bioprocess for glycerol utilization remains a challenging pursuit. First, feedstock variability is a crucial concern because the physical and chemical characteristics of crude glycerol are dependent on its source, manufacturing process, and pretreatment/refining steps. Feedstock quality often affects product yield and quality. When mixed with other waste streams (i.e., mixed carbon sources), optimization is unavoidable to find the most optimal ratio, concentration, and pH. In this regard, the use of computational or statistical tools (e.g., design of experiments) coupled to high-throughput experimentation (e.g., high-throughput cultivation using miniature bioreactors) would simplify the optimization process. Second, the microbial host chosen must show high tolerance to the feedstock used (pH, presence of inhibitors, high glycerol concentration) and maintain high productivity. Potential product inhibition is another critical consideration in production host selection. During strain engineering to improve glycerol utilization, the actual crude glycerol should preferably be used as there is a considerable difference between crude glycerol and laboratory-grade glycerol. More often than not, carbon hierarchy is not well-understood or studied in commonly used microbial hosts. This understanding is fundamental to developing an integrated biorefinery for utilizing glycerol and other renewable carbon sources. Third, the recovery, isolation, and purification of microbial products from the often-diluted cultivation broth should not be overlooked. Downstream processing costs can be as high as 80% of the total manufacturing costs (Ramakrishnan and Sadana 2000). Last but not least, process economics often determines the viability of a bioprocess, bearing in mind that biological processes are continually facing fierce competition with chemical processes.

Despite the challenges described above, glycerol is a renewable carbon source whose supply is predicted to grow. The field of biological glycerol utilization has made a giant leap, supported by our ever-increasing comprehension of biomanufacturing hosts, advancement in synthetic biology, and process optimization. In summary, the future is bright for microbial glycerol utilization.

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17 Revealing Function of Amino Acids in Nitrifying and Anammox Systems Through Chromatography and Metagenomic Analyses

Yuepeng Sun, Yuntao Guan, Kai He, Shinya Echigo, and Guangxue Wu

Abstract

Extracellular polymeric substances (EPS) in nitrifying batch biofilm reactors (SBBRs) and anammox reactors are important for aggregation of autotrophic nitrifying and anammox bacteria. In addition, soluble microbial products (SMP) also play very important roles in biological degradation in these processes. Therefore, more comprehensive analytical methods are required for better characterization of EPS and SMP. Chromatography and metagenomic analyses can help to accelerate the EPS/SMP characterization. Amino acids, esters, dipeptides, and fatty acids are the widespread compounds in both EPS and SMP of all nitrifying and anammox reactors. Heterotrophs of *Chlorobi bacterium*_OLB7 proliferated in SBBRs and *Lautropia* sp. SCN_69–89 in the anammox reactors. The metagenomic-based analysis showed that these heterotrophs might grow by assimilating amino acids or peptides in EPS and SMP by possessing amino acids degradation genes. The organics utilization pattern was affected by the addition of organics in SBBRs. Heterotrophs in the SBBR with the addition of organics merely possessed amino acids degradation genes, whereas those in the SBBR without the addition of organics had both amino acids synthesizing and degrading genes. Heterotrophs in the anammox reactors could utilize amino acids or peptides produced by anammox bacteria and other heterotrophs. The clarification of amino acids function can be useful for accelerating the application of

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autotrophic nitrogen removal systems to better understand microbial aggregation and interactions.

Keywords

Anammox · Extracellular polymeric substances · Microbial interaction · Nitrification · Soluble microbial products

17.1 Introduction

Completely autotrophic nitrogen removal via nitrification and anammox has the high potential to realize the energy-neutral or -positive wastewater treatment (Kartal et al. 2010). During autotrophic partial nitrification, ammonium nitrogen (NH_4^+-N) is partially nitrified to nitrite nitrogen (NO_2^--N) by activities of the ammonium-oxidizing bacteria (AOB) under aerobic conditions. While during autotrophic anaerobic ammonia oxidation (anammox), NH_4^+-N and NO_2^--N are converted to dinitrogen under anaerobic conditions through activities of anammox bacteria. Compared to the conventional nitrification and denitrification process, the application of the nitrification/anammox process reduces the operating cost, waste sludge yield, and greenhouse gas emissions, and eliminates the need for external organic carbon for nitrogen removal (Kartal et al. 2010).

One major characteristic of these autotrophic processes is the slow growth rate of autotrophs. Aggregation in the form of granule or biofilm is an effective strategy to solve the above problem in these processes (Cirpus et al. 2006). In the aggregation system, extracellular polymeric substances (EPS) provide a matrix for the microbial aggregate especially for anammox bacteria (Cirpus et al. 2006), and EPS typically include proteins, humic substances, carbohydrates, lipids, and nucleic acids (Sheng et al. 2010). However, studies on the EPS are limited to the content analysis of protein, polysaccharides, humic acids, uronic acids, and DNA by using the colorimetric method; the element compositions and functional groups in the EPS have also been examined by using the 3-dimensional excitation-emission matrix fluorescence spectroscopy, X-ray photoelectron spectroscopy, nuclear magnetic resonance, and Fourier transform infrared spectroscopy (Sheng et al. 2010; Adav and Lee 2011). Some researchers attempted to reveal aggregation ability of anammox sludge by analyzing amino acids contents in the EPS by using high-performance liquid chromatography combined with tandem mass spectrometry (Tang et al. 2018). In the EPS, the amino acids of protein could contribute to the hydrophobic property of biomass, resulting in the high aggregation ability of microorganisms (Hou et al. 2015). Also, amino acids and peptides in the EPS could serve as carbon and/or energy sources for the growth of heterotrophs (Lawson et al. 2017). Therefore, characterization of EPS by using mass spectrometry may be important for understanding the interaction between heterotrophs and autotrophic nitrifiers/anammox bacteria. Likewise, as the source of organic compounds from microbial metabolism, soluble microbial products (SMP) are commonly found in the effluent of autotrophic

nitrogen removal process. The SMP in the anaerobic effluent was compromised of long chain esters and carbohydrates, accounting for 55–65% of the total organics (Zhou et al. 2009). However, the effects or roles of these components detected in SMP have not yet been evaluated. Hence, characterization of the composition and control of the effluent SMP are also required to better understand microbial function and improve the autotrophic performance.

This chapter aimed to underline the application of chromatography and metagenomic techniques for characterizing EPS and SMP in nitrification and anammox systems. By using high resolution mass spectrometry (quadrupole time-of-flight mass spectrometer combined with liquid chromatography system (LC-QTOF-MS)), main metabolites in EPS and SMP were clarified. Finally, microbial communities related to these metabolites were analyzed based on the metagenomics for better understanding of microbial interactions.

17.2 Putative Metabolites in EPS and SMP

Two 2 L nitrifying sequencing batch biofilm reactors (SBBRs) (SBBR_CN with the addition of organics and SBBR_N without the addition of organics) and two plug-flow anammox reactors (influent concentrations of NH_4^+-N and NO_2^--N in anammox-H were 110 mg/L, whereas 50 mg/L in anammox-L) containing suspended carriers were operated and described in Sun et al. (2018a, b). The EPS, SMP as well as the influent of SBBRs and anammox systems were analyzed by LC-QTOF-MS, and their metabolites are visualized in Fig. 17.1a, b. Metabolites of 4170 in EPS and 3730 in SMP were detected in anammox-H, whereas 3926 in EPS and 3390 in SMP in anammox-L. More types of metabolites were detected in EPS and SMP of anammox-H in comparison with anammox-L, suggesting that influent ammonium concentrations affected the microbial metabolic patterns. Zhou et al. (2009) also demonstrated that metabolites might be altered by chemical oxygen demand to nitrogen ratios and organic loading rates.

Components of EPS and SMP in nitrification and anammox systems were detected. Most of them were fatty acids, esters, dipeptides, free amino acids, amides, alcohols, and glucosides. Particularly, phthalate esters were ubiquitously found in EPS of SBBRs. Limited works have been conducted using mass spectrometry technique for characterizing EPS and SMP. Esters, which were reported to be distributed in the anaerobic effluent, were mainly from the microbial metabolism rather than related to the raw pollutants directly in all the four bioreactors (Zhou et al. 2009). Fatty acids found in all the four reactors were reported as one of the important contributors to the hydrophobicity of EPS (Conrad et al. 2003). Campbell et al. (1983) reported that oleic acid could induce bacteria to produce slime surrounding the cells, indicating its positive effect on the biofilm formation. As common plasticizers, phthalates, which were reported to present in sewage sludge samples, sediment, sewage water, and surface water (Fromme et al. 2002), were likely to come from the plastic reactor itself (Trzcinski and Stuckey 2009). Alcohols accounted for 74% in the SMP of anaerobic sludge blanket reactor for treating

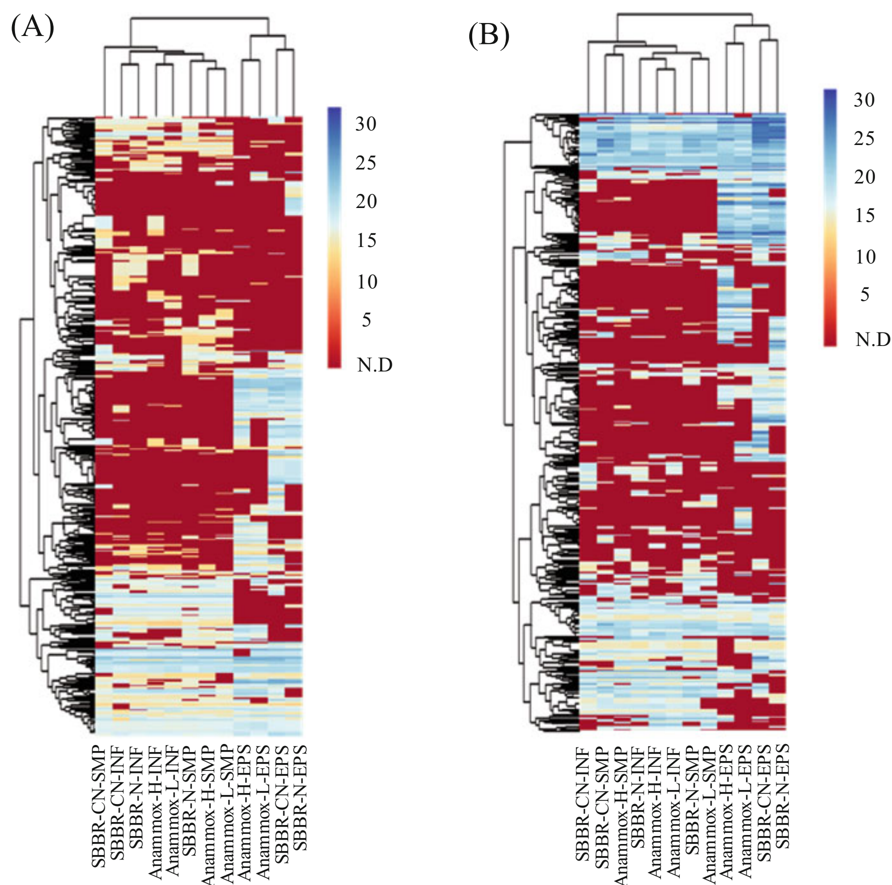

Fig. 17.1 Heatmap showing the profiles of metabolites of EPS and SMP detected by the negative (**a**) and positive (**b**) mode, respectively. Heatmap scale represents the abundance of genus normalized by log 2 transformation. Items with "EPS," "SMP," and "INF" represent samples of the extracted EPS solution, the effluent SMP, and influent wastewater

distillery wastewater were found by Wu and Zhou (2010). Other components including palmitic acid, ubiquinones, homoserine lactone, and adenosine were detected. Palmitic acid was the basic cell lipid composition in the anammox bacteria (Boumann et al. 2006), and was exclusively found in EPS of anammox systems. Homoserine lactones, as signal substances used for bacterial communication, were identified as *N*-hexanoyl-L-homoserine lactone and *N*-octanoyl-L-homoserine lactone (Sun et al. 2018b).

17.3 Amino Acids Analysis in Nitrification and Anammox Systems

The extracted EPS solution from both systems was analyzed by the LC-QTOF system. Free amino acids were mostly found in SBBRs or anammox systems. From Fig. 17.2a, phenylalanine was found in SBBRs and anammox reactors. Besides, tryptophan dominated in anammox-L and SBBRs, especially in SBBR-CN. Valine, arginine, lysine, and tyrosine were detected in both anammox systems. In SBBRs, tyrosine was detected in SBBR-N, whereas arginine in SBBR-CN. Other amino acids including isoleucine/leucine, threonine, histidine, and methionine were also detected in SBBRs. The detected amino acids suggested that amino acids could play a very important role in biofilm systems, especially for proteins of EPS and further for biofilm formation (Zhao et al. 2018). The different constituents in SBBRs and anammox systems operated under different conditions might result in diverse biofilm characteristics. Glycine, valine, phenylalanine, and leucine could be used to synthesize phospholipids, therefore benefiting the bacterial aggregation capacity (Zhao et al. 2018). The characteristics of hydrophobicity and hydrophilia might be also an indicator for the EPS production and therefore biofilm formation (Hou et al.

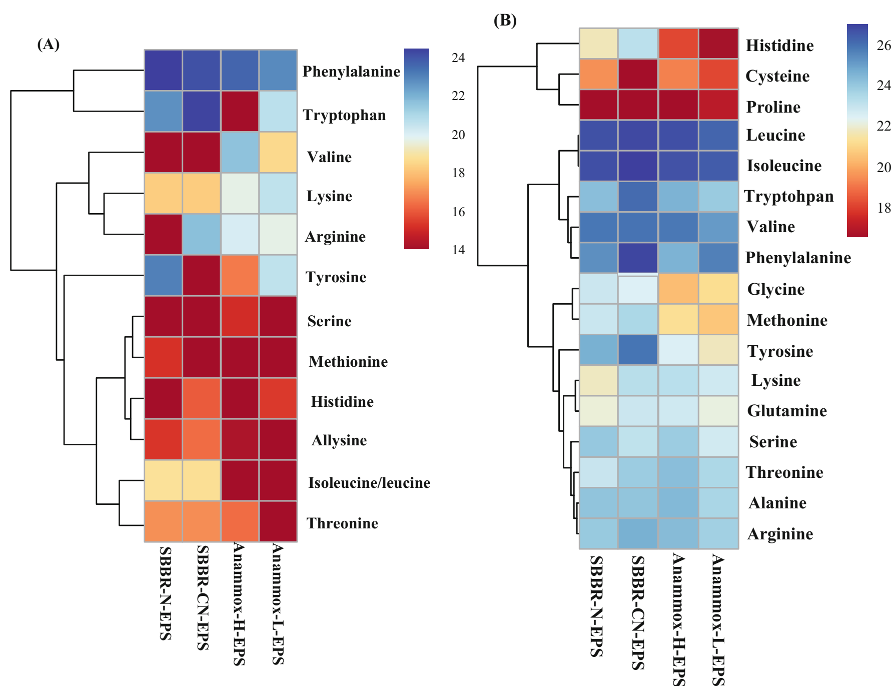

Fig. 17.2 Heatmap showing the profiles of main amino acids (**a**) and dipeptides (**b**) in SBBRs and anammox reactors. Partial figure content reproduced with permission from *Chemical Engineering Journal* (Sun et al. 2018b)©(2018) Elsevier

2015). Also, amino acids might serve as the carbon source for the growth of heterotrophic bacteria in the anammox system (Lawson et al. 2017).

The amino acids detected might not a reflection of the whole situation because amino acids could be utilized by heterotrophs for growth or existed in the form of peptides (Fig. 17.2b). Various dipeptides could be detected in EPS (Fig. 17.2b). Leucine and isoleucine with high abundances were detected in all the four reactors, followed by tryptophan, valine, and phenylalanine dipeptides. Other dipeptides including serine, alanine, and arginine were also identified. Furthermore, the types of amino acids (L-, D-, and DL-type) could not be distinguished based on LC-QTOF-MS results. L-amino acids are the necessary constituents of proteins and peptides, while D-amino acids are important components of microbial cell wall (Fujii 2002). As the most dominant amino acid, D-phenylalanine inhibited biofilm formation but did not affect cell growth of *Pseudoalteromonas* sp. SC2014 (Kolodkin-Gal et al. 2010). On the other hand, as carbon and energy sources, dipeptides could support the proliferation of heterotrophs originating from anammox cells (Lawson et al. 2017). Their metabolic pathways in autotrophic nitrogen removal processes need to be investigated by analyzing microbial communities related to peptides metabolism.

17.4 Amino Acids Metabolism in Nitrification and Anammox Systems

For further clarifying the amino acids function, genes involved in amino acids and peptides metabolism were analyzed according to metagenomic sequencing results (Fig. 17.3). The Kyoto Encyclopedia of Genes and Genomes database and the Evolutionary Genealogy of Genes Non-supervised Orthologous Groups database were applied for the amino acids and peptides metabolism genes annotation (Yin et al. 2018). For peptides and amino acids metabolism in SBBRs, more active peptides metabolism could be observed in SBBR-N (Fig. 17.3a, b). For amino acids metabolism in SBBR-N, nitrifiers of *Nitrospira defluvii* and *Nitrosomonas* sp. Is79A3 contained all selected genes for amino acids synthesis. *Nitrospira* sp. SCN_59–13 possessed genes for threonine and tryptophan synthase, serine-pyruvate transaminase, and branched-chain amino acid aminotransferase. In terms of amino acids degradation, *Nitrospira defluvii*, *Nitrospira* sp. SCN_59–13, and *Nitrosomonas* sp. Is79A3 contained genes for glutamine aminotransferase, glycine dehydrogenase, and threonine dehydratase, etc. Heterotrophs of *Dongia* sp. URHE0060, *Haliscomenobacter hydrossis* and *Gemmatimonadetes bacterium* SCN_70–22 harbored genes for amino acids production and degradation, indicating that these heterotrophs might survive in a self-sufficiency pattern. In contrast, although *Haliangium ochraceum* and *Chlorobi bacterium*_OLB7 contained less genes for amino acids synthesis, they could also participate in amino acid degradation, especially for glutamine, arginine, and alanine, suggesting that these heterotrophs might survive by assimilating amino acids or peptidase produced by autotrophs or other heterotrophs.

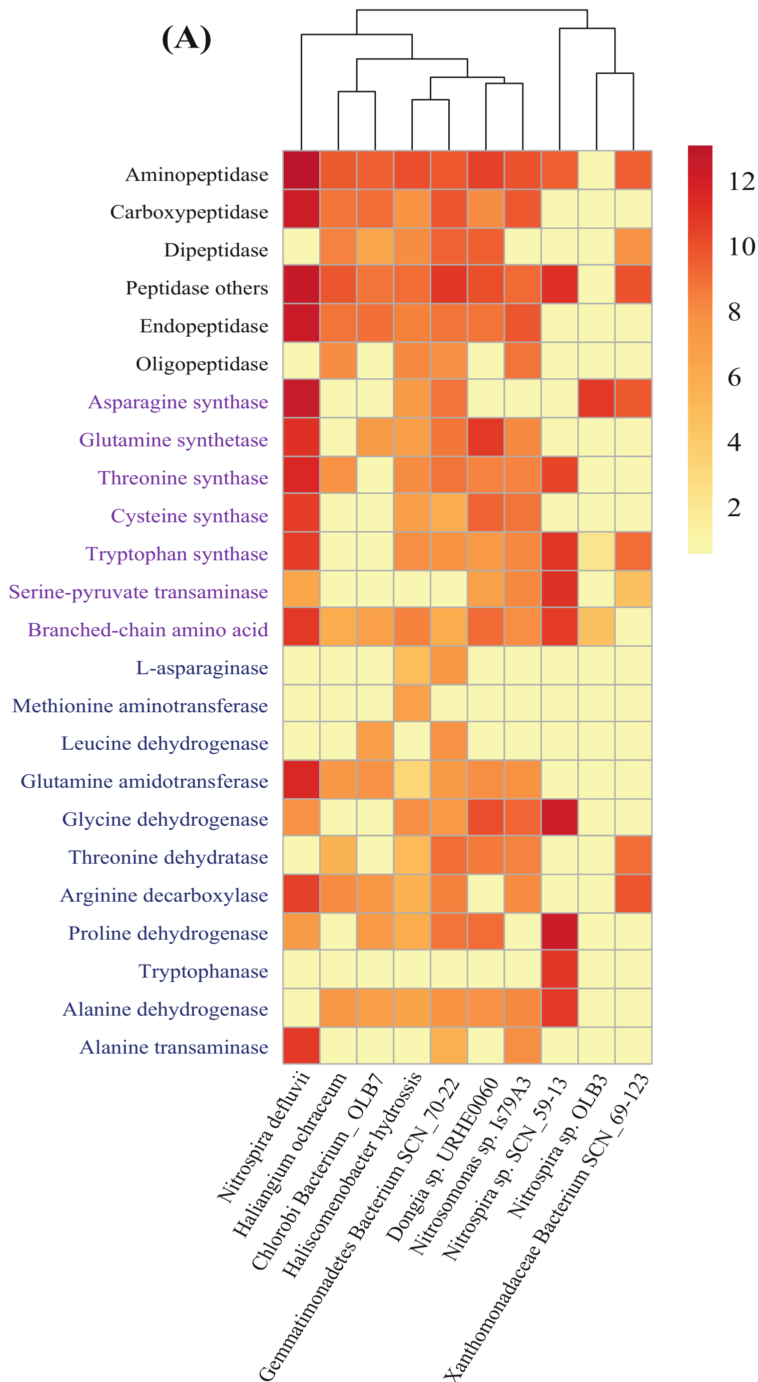

Fig. 17.3 Heatmap showing the abundance of genes involving in amino acids and peptides metabolism in SBBR-N (**a**), SBBR-CN (**b**), anammox-H (**c**), and anammox-L (**d**)

(B)

Aminopeptidase
Carboxypeptidase
Dipeptidase
Peptidase others
Endopeptidase
Oligopeptidase
Asparagine synthase
Glutamine synthetase
Threonine synthase
Cysteine synthase
Tryptophan synthase
Serine-pyruvate transaminase
Branched-chain amino acid aminotransferase
L-asparaginase
Leucine dehydrogenase
Glutamine amidotransferase
Glycine dehydrogenase
Threonine dehydratase
Arginine decarboxylase
Proline dehydrogenase
Tryptophanase
Alanine dehydrogenase
Alanine transaminase

12
10
8
6
4

Nitrospira defluvii
Gemmatimonadetes Bacterium SCN_ 70-22
Pyrinomonas methylaliphatogenes
Sorangium cellulosum
Haliangium ochraceum
Chloroflexi Bacterium_OLB14
Pedosphaera parvula
Chlorobi Bacterium_ OLB7
Nitrospira sp. SCN_59-13
Nitrospira sp. OLB3

Fig. 17.3 (continued)

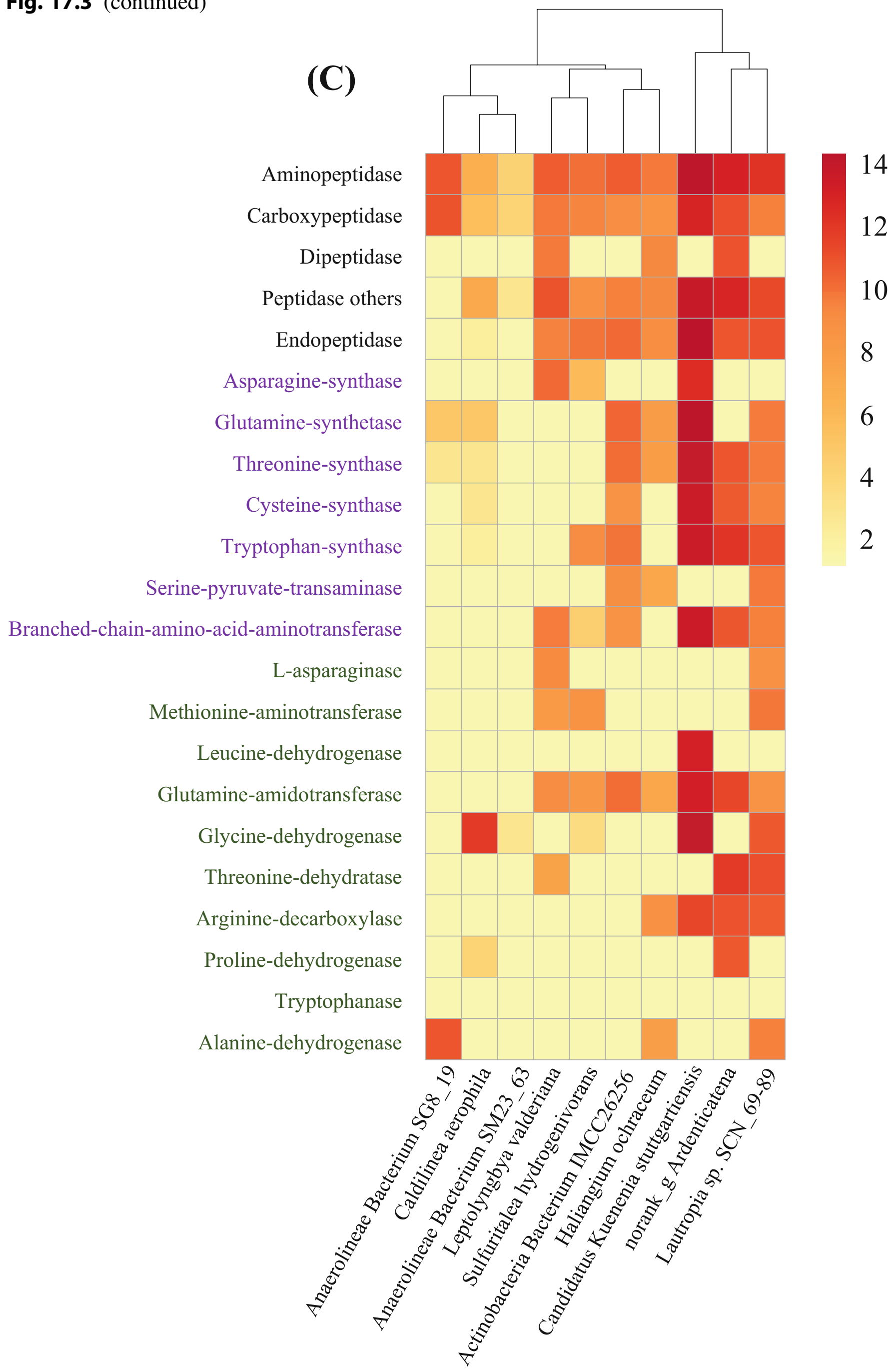

Fig. 17.3 (continued)

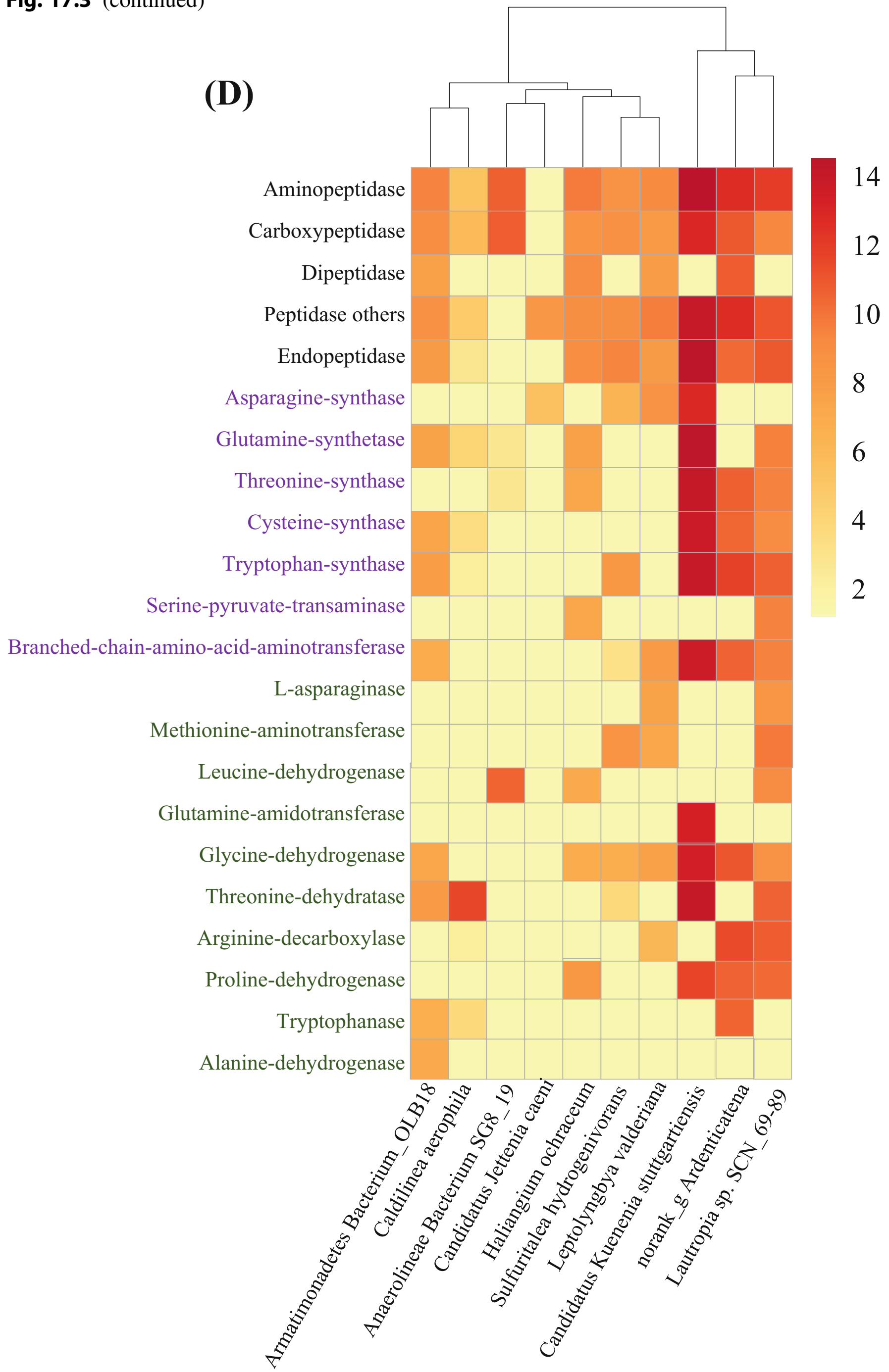

Fig. 17.3 (continued)

A totally different amino acid metabolism pattern was observed in SBBR-CN. Nitrifiers of *Nitrospira defluvii* possessed all genes for amino acid synthase. *Nitrospira* sp. SCN_59–13 merely possessed genes for threonine and tryptophan synthase, serine-pyruvate transaminase, and branched-chain amino acid aminotransferase. For amino acids degradation, all selected heterotrophs could involve in the amino acids degradation, suggesting that these heterotrophs could capture the amino acids produced by nitrifiers or supplied from organics. Different from heterotrophs in SBBR-N-containing genes for amino acid synthesis, most of heterotrophs from SBBR-CN were lack of genes for amino acids biosynthesis. Differences in the utilization of various types of organic matters might be correlated with the distribution and diversity of heterotrophic bacteria within the biofilm (Kindaichi et al. 2004). Liu et al. (2012) found that *Ignavibacterium album* within the phylum *Chlorobi* lacked key genes involving in the biosynthesis of several amino acids and might obtain amino acids from their living environment through other communities within the microbial mat. The organics supplied in SBBR-CN might affect the organics utilization pattern or microbial community of heterotrophs, contributing to diverse biofilm formation. Therefore, heterotrophs could possess the ability to synthesize amino acids after long-term acclimation under autotrophic circumstances.

For the peptides metabolism in anammox (Fig. 17.3c, d), most of selected species in anammox-H and anammox-L harbored the genes encoding aminopeptidase and carboxypeptidase, indicating their potential roles in free amino acids production from peptides. Genes for dipeptidase (responsible for hydrolyzing dipeptides to free amino acids) were specifically harbored by *Leptolyngbya valderiana*, *Haliangium ochraceum*, *norank_g Ardenticatena*, etc. In addition, endopeptidase and others peptidase genes were mainly possessed by *Leptolyngbya valderiana*, *Haliangium ochraceum*, *Lautropia* sp. SCN_69–89, etc.

For amino acids metabolism in anammox systems, *Lautropia* sp. SCN_69–89 and *Candidatus* Kuenenia stuttgartiensis owned all the selected genes for various amino acids synthase or aminotransferase, suggesting that these species were major amino acid producers. In contrast, *Candidatus* Kuenenia stuttgartiensis merely contained genes for enzymes related to leucine, glutamine, glycine, and arginine degradation. *norank_g Ardenticatena*, *Lautropia* sp. SCN_69–89, and some other species including *Leptolyngbya valderiana*, *Haliangium ochraceum*, and *Sulfuritalea hydrogenivorans* were the major species possibly responsible for amino acids utilization due to their possessing genes for amino acids degradation. Therefore, this result revealed that heterotrophs could utilize amino acids or peptides produced by anammox bacteria and some other heterotrophs (*Lautropia sp.* SCN_69–89). van de Vossenberg et al. (2013) found that *Candidatus* Scalindua contained genes involving in oligopeptide and dipeptide metabolism and transport. *Lautropia* sp. SCN_69–89 also harbored nitrogen metabolism-related genes. This bacterium might also contribute to nitrogen removal using amino acids and peptides as carbon and energy sources. Heterotrophs of *Chlorobi* in anammox granules could be capable of degrading and catabolizing extracellular peptides and amino acids bounded in the EPS matrix, while reducing nitrate to nitrite for the growth of anammox bacteria (Lawson et al. 2017; Speth et al. 2016).

17.5 Microbial Interaction in Nitrification and Anammox Systems

The biofilm biomass from SBBRs and anammox reactors was collected for the DNA extraction. After extraction, the DNA was amplified by polymerase chain reaction, and further analyzed through the high-throughput sequencing method. Microbial community was analyzed based on high-throughput sequencing results. Nitrifiers of *Nitrospira defluvii*, *Nitrospira* sp. SCN_59–13, and *Nitrospira* sp. OLB3 were enriched in both SBBRs, whereas *Nitrosomonas* sp. Is79A3 (1.3%) was detected in SBBR-N (Fig. 17.4a, b). A different heterotrophic community was observed. For heterotrophs in SBBR-N, *Dongia* sp. URHE0060, *Haliangium ochraceum*, *Chlorobi bacterium*_OLB7, etc., were identified. In contrast, SBBR-CN supported the growth of *Sorangium cellulosum*, *Gemmatimonadetes bacterium* SCN_70–22, *Haliangium_ochraceum*, etc. The discrepancy of community in two SBBRs might be due to the availability of organic carbons. Heterotrophs of *Chlorobi bacterium* OLB7 within the phylum *Chlorobi* and *Chloroflexi bacterium*_OLB14 within the phylum *Chloroflexi* might participate in the nitrogen conversion (with genes for nitrate reductase and nitrite reductase) based on Speth et al. (2016) and metagenomics-based results.

Anammox bacteria of *Candidatus* Kuenenia stuttgartiensis were the major species in both anammox systems (Fig. 17.4c, d). Besides, heterotrophs of *norank_g Ardenticatena* (6.6 and 5.1%), *Lautropia* sp. SCN_69–89 (3.5 and 3.0%), and *Anaerolineae bacterium*_SG8–19 (2.8 and 2.2%) proliferated in anammox systems, albeit no organics was supplied. *Lautropia* sp. SCN_69–89 could not only involve in the nitrogen conversion but also in the TCA cycle, pyruvate metabolism, glycolysis, and fatty acid metabolism from the metagenomics analysis result. *norank_g Ardenticatena* and *Anaerolineae bacterium*_SG8–19 within the phylum *Chloroflexi* were the other dominant heterotrophs, which were reported as ubiquitous phylum in anammox or nitrification biofilm systems (Kindaichi et al. 2012; Okabe et al. 2005).

The observation of heterotrophs and their potential ability to use amino acids and peptides indicated that heterotrophs might be supported by amino acids or dipeptides of SMP and EPS excreted by nitrifiers or anammox bacteria, or substrates from the decay of biomass (such as fatty acid and esters identified in SMP and EPS). These amino acids and dipeptides might be derived from autotrophic nitrifiers or anammox bacteria. The addition of organics altered the metabolic pattern of heterotrophs, thus regulating the microbial community. In nitrification systems, heterotrophs in SBBR-N could simultaneously synthesize and assimilate amino acids or peptides. While heterotrophs in SBBR-CN could only assimilate amino acids or peptides. Heterotrophs have positive effects. Some heterotrophs appeared to be denitrifiers (supply NO_2^--N to autotrophs) by using amino acids as carbon source. Autotrophic nitrifiers of *Nitrospira* could be the primary amino acid producers, which supplied heterotrophs with organics as carbon and energy sources (Dolinšek et al. 2013). In anammox systems, coexisting *Chloroflexi* could scavenge organic matters through activities of anammox bacteria (Kindaichi et al. 2012).

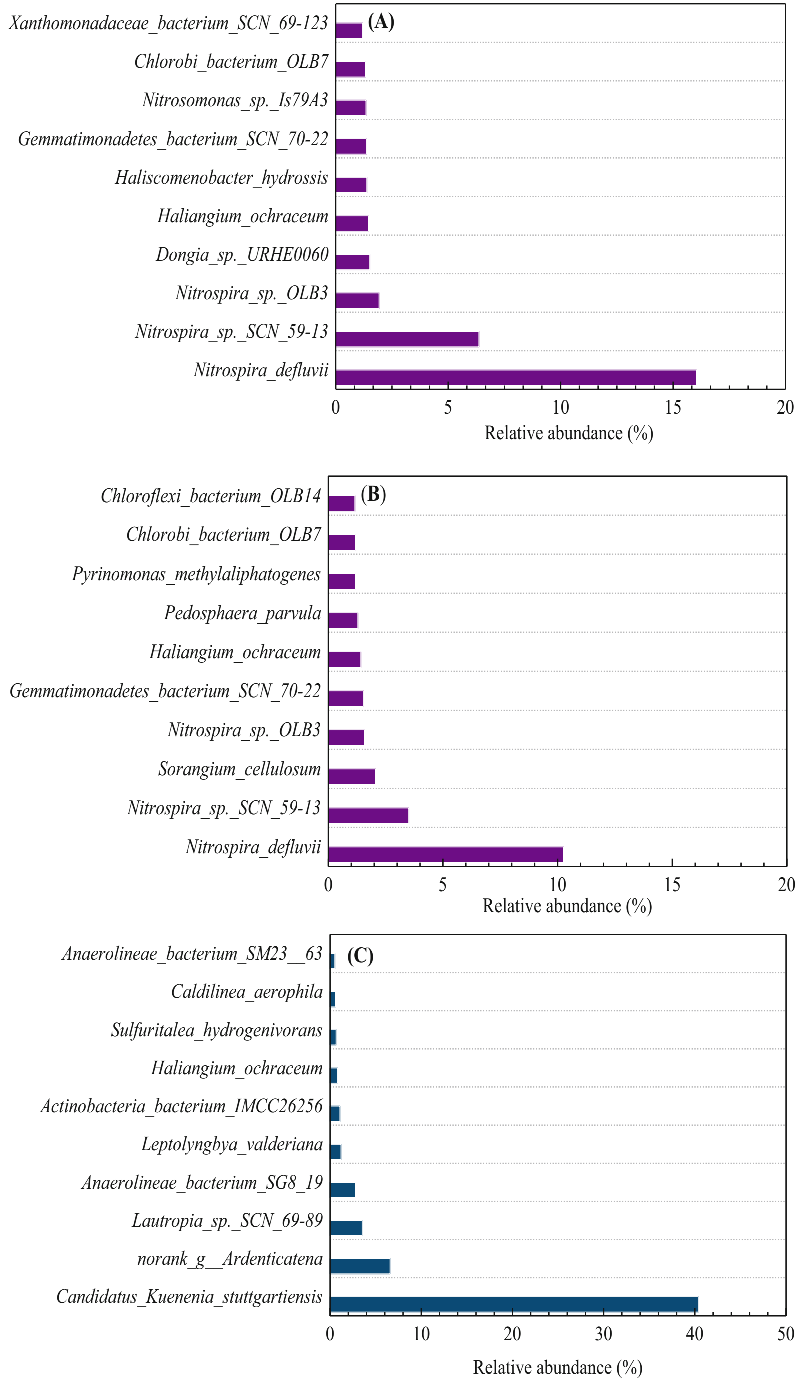

Fig. 17.4 Microbial community (top 10) in SBBR-N (**a**), SBBR-CN (**b**), anammox-H (**c**), and anammox-L (**d**)

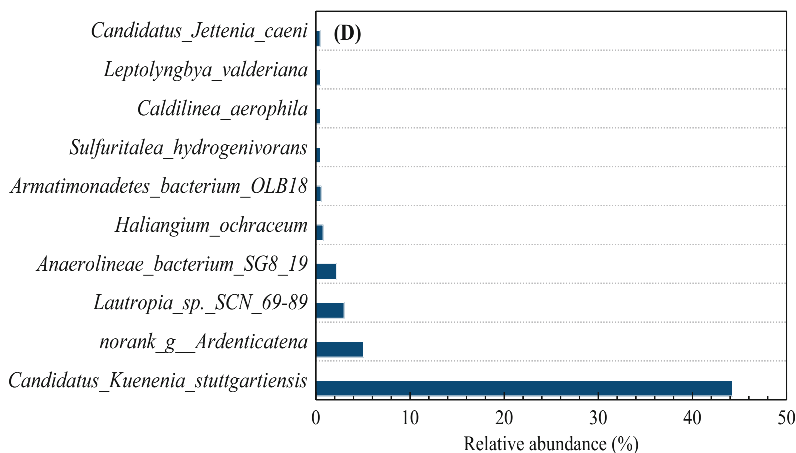

Fig. 17.4 (continued)

According to Lawson et al. (2017), amino acid and peptides might provoke the proliferation of heterotrophs in anammox systems.

The interaction between heterotrophs and nitrifiers or anammox bacteria might exert important role for system performance and biofilm formation. The growth of heterotrophs might induce more active quorum quenching process as the trigger to disperse biofilm (Sun et al. 2018a). Besides, microbial interaction might be regarded as a strategy to regulate the biofilms formation and microbial diversity, and resist environmental stress. More researches are needed to decipher the microbial interaction from quorum sensing or other aspects to promote the practical application.

17.6 Conclusions and Future Perspective

Combination of chromatography and metagenomic analysis allows better characterization of EPS and SMP in nitrifying and anammox systems. Esters, amino acids, and fatty acids were the main components in EPS and SMP of SBBRs and anammox systems. Various dipeptides and amino acids were detected in SMP and EPS of all the four reactors. Heterotrophic bacteria of *Lautropia* sp. SCN_69–89 and *Chlorobi bacterium*_OLB7 proliferated in SBBRs and anammox, respectively, and might be supported by amino acids and peptides produced by autotrophic nitrifiers, anammox bacteria, and other heterotrophs. These results suggested that heterotrophic community within autotrophic nitrifying and anammox enrichments may exert important roles. Furthermore, more advanced analytical techniques including transcriptomic, metabolomics, and proteomics should be adopted in future studies so as to provide more deep insights for microbial interactions or the metabolic roles played by heterotrophs. In addition, all these information may help to find hints for

accelerating the growth rates of nitrifying and anammox bacteria and advancing the comprehensive control and widespread application of this promising technology.

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