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,

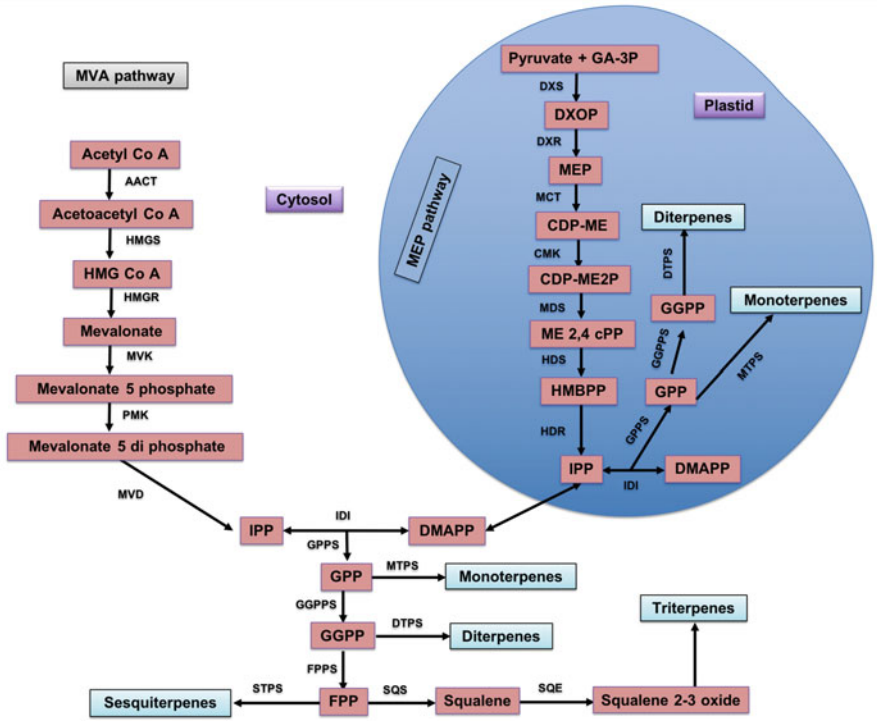

Fig. 14.1 Schematic representation of terpene biosynthetic pathway. Abbreviations: *AACT* acetoacetyl-CoA thiolase/acetyl-CoA acetyltransferase, *HMGS* hydroxymethylglutaryl-CoA synthase, *HMGR* hydroxymethylglutaryl-CoA reductase, *MVK* mevalonate kinase, *PMK* phosphomevalonate kinase, *MVD* mevalonate 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 anti-microbial 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.

Conflict of Interest: Authors declare no conflicts of interest.

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