Chem Soc Rev

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Journal:	*Chemical Society Reviews*
Manuscript ID:	CS-REV-02-2015-000159.R1
Article Type:	Review Article
Date Submitted by the Author:	10-Apr-2015
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Synthesis of Chemicals by Metabolic Engineering of Microbes

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Abstract

Metabolic engineering is a powerful tool for sustainable production of chemicals. Over the years, the exploration of microbial, animal and plant metabolism has generated a wealth of valuable genetic information. The prudent application of this knowledge on cellular metabolism and biochemistry has enabled the construction of novel metabolic pathways that don't exist in nature or enhance existing ones. The hand in hand development of computational technology, protein science and genetic manipulation tools has formed the basis of powerful emerging technologies that make the production of green chemicals and fuels a reality. Microbial production of chemicals is more feasible compared to plant and animal systems, due to simpler genetic make-up and amenable growth rates. Here, we summarize the recent progress in the synthesis of biofuels, value added chemicals, pharmaceuticals and nutraceuticals via metabolic engineering of microbes.

1. Introduction

Since the industrial revolution oil has become the blood of our economy, serving as the major source of energy, petrochemicals and influencing the market of a plethora of industries globally. Over-dependence on oil has on one hand led to rapid urbanization, and on the other hand has led to severe environmental damage and pollution. Immoderate exploitation and consumption has depleted oil reserves drastically over the past 150 years as majority of the fuels and chemicals are currently produced using petrochemical feedstocks. Generally, the production of chemicals via chemical approaches has advantages of being a well established production platform associated with low production costs. However, some severe drawbacks exist in petroleum-based manufacture approaches, such as: (a) use of toxic/ environmentally harmful or expensive catalysts (b) generation of toxic intermediates (c) requirement of high temperature/ pressure processes (d) high energy inputs (e) production of stereo-specific chemicals is difficult. The concerns of oil crisis and environment deterioration compel us to look for alternative ways to produce petroleum-based fuels and chemicals.

While the biological production of commodity chemicals has gained significant interest over the years by tapping into the petrochemicals market, the biosynthesis of natural products via metabolic engineering has tapped into the large pharmaceutical and food industry markets. Natural products are a rich source of food additives, pharmaceuticals and nutraceuticals, which are widely used in our daily life. The industrial scale production of these compounds by extraction is inefficient and uneconomical due to two reasons: (1) native producers are known to usually grow slowly; (2) the amount of the target compounds generated by native producers is not in significant quantities.

Metabolic engineering of microbes provides a promising alternative for the production of these petroleum-derived or natural compounds.

Metabolic engineering is a technique that first emerged in the early 1990s. Since then, this technology has been developing rapidly, which has been greatly dependent on the significant advances in its contiguous fields. DNA sequencing and bioinformatics analysis reveals new metabolic pathways and enzyme variants; enrichment in protein structure information provides foundation for rational protein engineering; advanced analytical tools identify pathway bottlenecks from RNA, protein or metabolite levels; the availability of a series of genetic tools facilitates pathway optimization; advancement in fermentation technology enables scale up for industrial scale production.[1]

Microbes (eukaryotic or prokaryotic) that have a simple genetic background and fast growth rate are usually used as hosts to produce various compounds. This strategy serves both metabolic logic as well as industrial scale process economics. Some successful examples include production of 1,3-propanediol in *Escherichia coli*[2], engineering *Saccharomyces cerevisiae* for the production of antimalarial drug precursor artemisinic acid[3]. While *E. coli* and *S. cerevisiae* are the two most commonly used hosts for metabolic engineering, other non-conventional hosts have also been explored for their distinctiveness. In this review, we summarize the recent progress in microbial production of chemicals including biofuels, value added chemicals, pharmaceuticals and nutraceuticals, by metabolic engineering (Table 1). We emphasize powerful metabolic engineering efforts with the goal of large scale manufacture. In this light, the metabolic engineering strategies to enable efficient production platforms such as evaluation of thermodynamic feasibility of pathway(s), protein engineering, carbon flux redirection,

manipulation of cellular energetics, use of alternate carbon sources, engineering substrate uptake mechanism, removal of final product from culture broth to alleviate end-product inhibition and cell toxicity, optimization of process parameters, etc. will be discussed.

2. Microbial production of biofuels

Globally, over 50% of crude oil is used for manufacturing transportation fuels.[4] Biofuels are compatible with internal combustion engines and can be used as fuel additives to reduce the consumption of oil. Among biofuels, bioethanol and biodiesel are currently the most widely used. In recent years, the microbial production of higher alcohols, also called advanced biofuels has drawn increasing attention. Compared with ethanol, higher alcohols have energy density closer to petroleum and are not corrosive to the existing infrastructure. Other alternative biofuels include biologically produced alkanes/alkenes and hydrogen. In this section, we highlight the recent advances in the production of these biofuels by metabolic engineering.

2.1 Alcohols

Ethanol

Currently, ethanol is the most widely used biofuel. In 2013, approximately 1040 million m^3 of ethanol was produced worldwide, over 80% of which was consumed for application as a fuel additive. The largest producer of bioethanol is USA with 518 million m^3, followed by Brazil with 277 million m^3. It is reported that annually over 98% of bioethanol is made from corn.[5] As the concerns of fuel and food competition increase, the production of biofuels from non-edible feedstocks has gained increasing attention. Here we summarize the recent progress on the metabolic engineering of microorganisms to use

non-edible feedstocks, such as lignocellulosics and brown macroalgae for ethanol production.

Lignocellulosics have been long considered as a good alternative carbon source for biofuel production. However, due to its recalcitrant nature, lignocellulosic biomass usually needs to be pretreated and hydrolyzed before it can be used by microorganisms for biofuel production. Lignocellulosic biomass contains several hexoses and pentoses, with glucose and xylose as the two most abundant components. *S. cerevisiae* can efficiently produce ethanol from glucose but is unable to natively use pentose sugars such as xylose as a carbon source.[6] To enable D-xylose fermentation the conversion of D-xylose to D-xylulose is necessary, for which heterologous enzymes such as xylose reductase (XR) and xylitol dehydrogenase (XDH) or just xylose isomerase (XI) are required. The expression XI has the advantage of overcoming redox imbalance, which is commonly an issue while expressing the XR-XDH system. However, bacterial XI genes are not well expressed in yeast. To surpass this hurdle, a fungal XI gene was identified and shown to have high activity in yeast. When it was over-expressed along with the genes of the non-oxidative pentose phosphate pathway, the resulting yeast strain grew anaerobically on D-xylose and produced ethanol with the yield comparable with that on glucose.[6] Transportation of D-xylose into the cell is another factor that limits xylose utilization. To further improve ethanol production from *S. cerevisiae*, a sugar transporter gene was expressed in a xylose-assimilating yeast strain. Xylose uptake ability and ethanol productivity were significantly improved via both xylose fermentation and xylose-glucose co-fermentation.[7] Another problem that hinders successful utilization of mixed sugars in cellulosic hydrolysates is the sequential consumption of xylose after

glucose depletion. To address this difficulty, an engineered *S. cerevisiae* strain was constructed to co-ferment xylose and cellobiose. Cellobiose was transported into yeast cells by a high-affinity cellodextrin transporter, and then hydrolyzed by an intracellular β-glucosidase. It was found that the intracellular hydrolysis of cellobiose minimizes glucose repression on xylose fermentation. The resulting yeast strains utilized xylose and cellobiose simultaneously, leading to an increase in ethanol titer of about 20% compared to that of glucose and xylose co-fermentation.[8]

The above-mentioned process requires pretreatment and lignocellulosic hydrolyzation to fermentable sugars before large scale production. Consolidated bioprocessing (CBP) combines cellulase secretary expression, cellulose hydrolysis, and biofuel production into a single step and represents a more effective technology for biofuel production.[9] To achieve CBP, ethanologenic microorganisms have been engineered to express either non-complexed or complexed cellulase (cellulosomes) systems. A non-complexed cellulase system was constructed by expressing an endoglucanase and β-glucosidase in *S. cerevisiae*. The resulting strain was able to grow on amorphous cellulose and produced up to 1.0 g/L of ethanol under anaerobic conditions.[10] Compared to non-complexed cellulase systems, the cellulosomes exhibit much higher degradation efficiency because of their highly ordered structural organization. Tsai and coworkers assembled a functional mini cellulosome on the yeast surface by using a synthetic yeast consortium.[11] One yeast strain displayed a trifunctional scaffoldin carrying three divergent cohesin domains. The others three strains secreted one of the three corresponding dockerin-tagged cellulases. The secreted cellulases were docked onto the displayed scaffoldin in a highly organized manner. By adjusting the

ratios of these four strains, 1.87 g/L ethanol was produced and the yield reached 93% of the theoretical maximum value.[11]

To simplify this system, researchers co-expressed scaffoldin and dockerin-tagged cellulases in one yeast cell. Wen and coworkers displayed a series of uni-, bi-, and trifunctional mini cellulosomes. Compared with the uni- and bifunctional mini cellulosomes, the trifunctional complexes showed better cellulose hydrolysis efficiency. The engineered yeast strain produced 1.8 g/L ethanol using phosphoric acid-swollen cellulose as the carbon source.[12] To further increase the display level, Fan and coworkers displayed cellulosomes using two individual mini scaffoldins. The engineered *S. cerevisiae* was able to hydrolyze microcrystalline cellulose efficiently, and produce ethanol with a titer of 1.4 g/L.[13] Sakamoto and coworkers constructed a *S. cerevisiae* strain that not only hydrolyzed hemicelluloses by co-displaying endoxylanase, β-xylosidase, and β-glucosidase but that also assimilated xylose by expressing XR, XDH and xylulokinase. The recombinant strain produced 8.2 g/L of ethanol directly from rice straw hydrolysate with the yield of 0.41 g/g.[14]

Besides lignocellulosics, brown macroalgae has also been investigated as feedstock for bioethanol production. Its cultivation requires no arable land, fresh water or fertilizer. Alginate, mannitol and glucan are the main sugars in brown macroalgae and these sugars can be easily separated by simple biorefinery processes such as milling, leaching and extraction. A yeast strain was engineered to assimilate major sugars in brown macroalgae for ethanol production. An alginate monomer (4-deoxy-L-erythro-5-hexoseulose uronate, or DEHU) transporter was discovered from *Asteromyces cruciatus*. The expression of this transporter gene together with other alginate and mannitol

174 catabolism genes enabled a *S. cerevisiae* strain to efficiently metabolize DEHU and
175 mannitol. Under anaerobic conditions, 36.2 g/L of ethanol was produced, which
176 corresponds to 83% of the maximum theoretical yield.[15] *E. coli* has also been engineered
177 to use brown macroalgae as the sustainable feedstock for ethanol production. First, a
178 secretable alginate lyase system was introduced into *E. coli* to enable efficient and rapid
179 degradation of alginate. Coupling this system with alginate uptake and metabolic
180 pathway generated a microbial platform that can produce ethanol directly from
181 macroalgal biomass with a titer of 4.7 % v/v and a yield of 0.281 g/g macroalgae (80% of
182 the theoretical maximum yield).[16]

183 **1-Propanol**

184 Compared to ethanol, 1-propanol has a higher octane number and is considered to be a
185 better biofuel. 1-Propanol is also an important feed stock in chemical industry and its
186 annual market is over 130,000 tonnes. It can be dehydrated to produce propylene, a
187 starting material for polypropylene plastics. It can also replace methanol during the
188 production of biodiesel fuel to avoid using organic solvents.[17]

189 1-Propanol can be biosynthesized via different precursors, such as 2-ketobutyrate,
190 1,2-propanediol and propionyl-coenzyme A (CoA). 1-Propanol production from 2-
191 ketobutyrate is an example of synthesis of higher alcohols using keto-acid pathways.[18]
192 Through these pathways, various alcohols can be synthesized from the corresponding
193 keto acids, which are catalyzed by promiscuous keto-acid decarboxylase (KDC) and
194 alcohol dehydrogenase (ADH) (Fig. 1). Shen and Liao reported the production of 2-
195 ketobutyrate by extending the *E. coli* threonine biosynthetic pathway.[19] The production of
196 1-propanol was systematically optimized through elimination of competing pathways and

deregulation of amino-acid biosynthesis, leading to a final titer of about 1 g/L. Atsumi and Liao designed a shorter route which bypasses threonine biosynthesis and directly converts pyruvate to 2-ketobutyrate.[17] This pathway relies on a foreign enzyme citramalate synthase (CimA). CimA variants with improved activity and resistance to feedback inhibition were obtained by directed evolution. These efforts led to 1-propanol production at 3.5 g/L after 92 hours.[17]

The production of 1-propanol via 1,2-propanediol commences from dihydroxyacetone- phosphate (DHAP), an intermediate in glycolysis pathway . First, the production of 1,2-propanediol was achieved via 3 steps at 0.8 g/L by screening for efficient enzymes. To further realize 1-propanol production, three diol dehydratases were tested for catalytic efficiency and the one from *Klebsiella oxytoca* (encoded by *ppdABC*) was identified to be optimal. Introduction of this enzyme into 1,2-propanediol producing strains resulted in the biosynthesis of 0.25 g/L of 1-propanol.[20] Based on this work, a fusion diol dehydratase with higher catalytic efficiency towards 1,2-propanediol was then constructed by linking its 3 subunits. Expression of this fusion dehydratase led to the production of 1-propanol at 0.62 g/L. Further enhancement in 1-propanol production was achieved by utilizing a dual strain strategy to express the 1,2-propanediol pathway and the 1-propanol pathway separately. These efforts led to 1-propanol production at 2.91 g/L.[21]

1-Propanol production via propionyl-CoA was first achieved in *Thermobifida fusca*.[22] Based upon computational analysis of the metabolic network, two distinct pathways leading to the synthesis of propionyl-CoA were identified by extending the threonine biosynthesis pathway or succinyl-CoA pathway (Fig. 2). The introduction of a

bifunctional butyraldehyde/alcohol dehydrogenase led to 1-propanol production from various carbon sources. The highest 1-propanol titer (0.48 g/L) was obtained using switchgrass as the carbon source. These two propionyl-CoA based pathways have been reconstituted in *E. coli*, resulting 1-propanol production at 10.8 g/L and 150 mg/L, respectively.[23, 24]

Isopropanol

Isopropanol is natively produced by several species of *Clostridium*. It can also be used as a biofuel. In chemical industry, isopropanol can be dehydrated to yield propylene, a monomer for manufacturing polypropylene. Isopropanol is also used as an alternative to methanol to produce biodiesel.[25] Currently, the only reported isopropanol pathway is the extension of *Clostridium* acetone pathway. Acetyl-CoA is converted to isopropanol by the sequential catalysis of acetyl-CoA acetyltransferase (AtoB), acetoacetyl-CoA transferase (AcoAT), acetoacetate decarboxylase (ADC), and secondary alcohol dehydrogenase (ADH) (Fig. 2). Hanai and coworkers screened these enzymes from different microorganisms to identify the most suitable candidates.[25] The expression of the most suitable set of enzymes resulted in the production of 81.6 mM isopropanol with a yield of 43.5% (mol/mol) in shake flask studies.[25] In a fed-batch process incorporated with a gas stripping recovery method, 143 g/L of isopropanol was produced after 240 hours with a yield of 67.4% (mol/mol).[26]

Soma and coworkers investigated isopropanol production from lignocellulosic biomass. In their work, a recombinant *E. coli* strain displaying β-glucosidase on the cell surface and expressing the isopropanol synthetic pathway produced 69 mM isopropanol after 21 hours of fermentation from cellobiose.[27] Kusakabe and coworkers constructed

the isopropanol pathway in cyanobacteria *Synechococcus elongatus*. The enzyme-coding genes were integrated into the genome. Under optimized conditions, the engineered cyanobacteria produced 26.5 mg/L of isopropanol directly from solar energy and carbon dioxide.[28] This pathway was also introduced into *Cupriavidus necator* strain Re2133. The synthetic production pathway was rationally designed through codon optimization, gene replacement, and manipulating gene expression levels in order to efficiently divert carbon flux toward isopropanol. These efforts led to 3.44 g/L isopropanol from fructose.[29]

1-Butanol

1-Butanol has been considered as an excellent biofuel due to hydrophobicity and similar energy density to gasoline. 1-Butanol can be mixed with gasoline at any ratio or completely replace gasoline. It is not corrosive and is compatible with existing pipeline infrastructure. In addition, the vapor pressure of 1-butanol (4mm Hg at 20ºC) is much lower than that of ethanol (45 mm Hg at 20ºC), which makes its separation more cost-effective.[30] 1-Butanol can be produced by two distinct biosynthetic pathways: the keto-acid pathway and the CoA-dependent pathway. In the keto-acid pathway, the precursor 2-ketovalerate is synthesized from 2-ketobutyrate by the action of LeuABCD (leucine biosynthesis operon) and then 2-ketovalerate is converted to 1-butanol by the two broad substrate enzymes KDC and ADH. Using this pathway, about 1 g/L of 1-butanol was produced from *E. coli*[31] and 242.8 mg/L from *S. cerevisiae*.[32]

1-Butanol can be produced natively via CoA-dependent pathway by various species of *Clostridium*. This pathway starts from acetyl-CoA, and the enzymes involved include acetyl-CoA acetyltransferase (Thl), acetoacetyl-CoA thiolase (Hbd), 3-hydroxybutyryl-CoA dehydrogenase (Crt), butyryl-CoA dehydrogenase (Bcd), electron

transfer flavoprotein (Etf), aldehyde/alcohol dehydrogenase (AdhE2) (Fig. 2). However, as *Clostridium* species are strict anaerobes and the growth rate is slower than *E. coli*, metabolic engineering strategies have been adopted to construct recombinant strains of aerobic microorganisms for 1-butanol production with high titer and productivity.[33, 34] Atsumi and coworkers reconstituted *Clostridium* 1-butanol pathway in *E. coli* and the initial strain produced only 13.9 mg/L of 1-butanol.[34] The pathway was further optimized by identification of alternative enzymes from other organisms, deletion of competing pathways and optimization of culture media. These efforts led to improving 1-butanol titer up to 552 mg/L in rich media supplemented with glycerol.[34]

Three major hurdles were identified with the *Clostridium* 1-butanol pathway: firstly, all the reactions are reversible; secondly, a total of four NADH molecules were consumed to produce one 1-butanol molecule; thirdly, the condensation reaction of acetyl-CoA to produce acetoacetyl-CoA was determined to be rate limiting. The first concern was addressed by introducing an irreversible *trans*-enoyl-CoA reductase (Ter). To address the second difficulty, (competing) NADH-consuming pathways were disrupted and a formate dehydrogenase was expressed to generate more NADH from formate. In order to overcome the third hurdle, the *Clostridium* Thl was replaced with *E. coli* native AtoB due to its higher specific activity. These modifications resulted in NADH and acetyl-CoA driving forces and enabled high-titer (30 g/L) and high-yield production of 1-butanol anaerobically.[35]

Isobutanol

Isobutanol is another potential biofuel with high octane value and energy density. It can mix with gasoline at any proportion and is also known to be compatible with existing combustion engines and fuel transportation infrastructure.[36] As an important building block, it is used as the feedstock for the production of p-xylene, isobutyl acetate and isobutyl esters.

Isobutanol production has been reported from 2-keto-isovalerate, the intermediate of valine biosynthesis. The by-product generating pathways that consume pyruvate and acetyl-CoA were disrupted. The resultant strain JCL260 achieved isobutanol production at 22 g/L from shake flasks under micro-aerobic conditions.[18] In a 1-L bioreactor this strain achieved over 50 g/L isobutanol within 72 hours with *in situ* isobutanol removal using gas stripping.[37]

Desai and coworkers engineered *E. coli* to produce isobutanol from cellobiose. A beta-glucosidase was expressed extracellularly by either excretion into the media or anchoring to the cell membrane. The excretion system allowed *E. coli* to grow with cellobiose as a sole carbon source at rates comparable to those with glucose. The genes expressing isobutanol production pathway enzymes were then introduced to this system. The most productive strain converted cellobiose to isobutanol with a titer of 7.64 g/L and a productivity of 0.16 g/L/h.[38]

Unlike carbohydrates and lipids, proteins are not commonly used as feedstocks for biofuel production. Recently, Huo and coworkers engineered *E. coli* to assimilate protein hydrolysates by introducing three heterologous transamination and deamination cycles for isobutanol production. Isobutanol was produced from different protein sources,

such as the biomass generated from *S. cerevisiae*, *E. coli* and *Bacillus subtilis*. Isobutanol titer of 4 g/L was achieved, which corresponds to about 56% of theoretical yield. [39]

Isobutanol production from *S. cerevisiae* has also been investigated. Overall, the titers are lower than those obtained in *E. coli*. Matsuda and coworkers improved isobutanol production from *S. cerevisiae* by eliminating competing pathways and resolving cofactor imbalance.[40] The precursor pyruvate pool was increased by disrupting genes related to pyruvate metabolism. NADPH supply was improved by introducing transhydrogenase-like shunts. This resulted in 1.62 g/L of isobutanol production with a yield of 0.016 g/g glucose via batch fermentation.[40] It was determined that in yeast, the upstream pathway is confined to mitochondria, whereas the downstream pathway is located in the cytoplasm. The transport of the intermediate 2-keto-isovalerate across membranes decreases productivity. To improve isobutanol production, the valine biosynthesis enzymes were relocated from the mitochondrial matrix into the cytosol.[41] To this end, the N-terminal mitochondrial targeting sequences of Ilv2, Ilv3 and Ilv5 were deleted. About 0.63 g/L of isobutanol was produced at a yield of 15 mg/g glucose. In another work, the isobutanol pathway was completely assembled in the mitochondria. Compared with the control, compartmentalization of the pathway into mitochondria improved isobutanol production by 260%, whereas over-expression of the same pathway in the cytoplasm only increased yields by 10%.[42]

Others alcohols

Besides the above mentioned alcohols, other alcohols with even longer chains have also drawn increasing attention. Both 2-methyl-1-butanol (2MB) and 3-methyl-1-butanol

(3MB) were produced via the keto-acid pathways. 2-Keto-3-methylvalerate (KMV), an intermediate in isoleucine biosynthetic pathway, is converted to 2MB by KDC and ADH. Over-expressing the key enzymes in the upper pathway and deleting competing pathways led to an *E. coli* strain that produces 1.25 g/L 2MB in 24 hours with yields of up to 0.17 g/g glucose.[43] Similarly, 3-methyl-1-butanol (3MB) was synthesized from 2-ketoisocaproate (KIC), the direct precursor to leucine. With pathway optimization, 1.3 g/L 3MB was produced.[44] Marcheschi and coworkers engineered *E. coli* LeuA by structure-based protein engineering to carry out recursive chain elongation reactions of keto-acids.[45] This enzyme has been successfully used for the production of a series of long chain alcohols, such as 1-pentanol, 1-hexanol, 1-heptanol, 1-octanol, directly from glucose (Fig. 1).[45]

Production of 1-hexanol and 1-pentanol was also achieved by extending the CoA-dependent 1-butanol pathway (Fig. 2). β-Ketothiolase (BktB) catalyzed the condensation of acetyl-CoA and butyryl-CoA to 3-ketohexanoyl-CoA. The subsequent reactions were catalyzed by Hbd, Crt, Ter, AdhE2, leading to 1-hexanol production. Under anaerobic conditions 1-hexanol was produced at a titer of 47 mg/L from glucose.[46] Similarly, to achieve 1-pentanol production, BktB was used to catalyze the condensation of acetyl-CoA and propionyl-CoA to form 3-ketovaleryl-CoA. Through several reduction and dehydration reactions, 3-ketovaleryl-CoA was converted to 1-pentanol. After systematic pathway optimization and strain engineering, the engineered *E. coli* strain achieved 19 mg/L of 1-pentanol production from glucose and 109 mg/L of 1-pentanol from glycerol.[47]

2.2 Fatty acids and biodiesel

As a renewable, biodegradable and non-toxic fuel, biodiesel is proposed as an environmentally friendly solution to energy dilemma. Fatty acids can be used as precursors for the production of biodiesel or chemicals.[48, 49] Among all the fuel molecules produced from living organisms, fatty acids and their derivatives have the highest volumetric energy density as most of the carbons in the long hydrocarbon chains are in the reduced state.[50]

As shown in Fig. 3, the first committed step of fatty acid elongation cycle is the conversion of acetyl-CoA to malonyl-CoA, which is catalyzed by acetyl-CoA carboxylase (AccABCD). Malonyl-CoA is converted to malonyl-acyl carrier protein (ACP) by malonyl-CoA-ACP transacylase (FabD), and further to acetoacetyl-ACP by the action of β-ketoacyl-ACP synthase III (FabH). Acetoacetyl-ACP undergoes reduction, dehydration and elongation, and subsequently enters another chain elongation cycle.[51-53] In the additional cycles FabH is replaced by β-ketoacyl-ACP synthase I (FabF) and β-ketoacyl-ACP synthase II (FabB). Acyl-ACP thioesterase (AAL) can break the elongation cycle and release fatty acids, so production of fatty acids can be achieved by introducing an AAL. Zhang and coworkers characterized several AALs from different sources and investigated their effect on fatty acid production.[51] These enzymes showed distinct degrees of chain length specificity, and the quantity and compositions of fatty acids produced depends on the AAL used.[51] To assess the limiting steps in fatty acid biosynthesis, *E. coli* fatty acid synthase (FAS) was reconstituted *in vitro* and steady-state analysis was carried out. The results showed that fatty acid production was strongly depended on the availability of malonyl-CoA[54] and compared to other components, higher concentrations of *holo*-ACP and AAL were required to give maximum FAS

activity.[55] Modular engineering strategies have been used to optimize fatty acid production in *E. coli*. Fatty acid pathway was split into three modules: the upstream acetyl-CoA formation module; the intermediary acetyl-CoA activation module; and the downstream fatty acid synthase module. These modules were optimized and balanced at both transcriptional and translational levels. The engineered strain produced 8.6 g/L of fatty acids via fed-batch cultivation.[56] Fatty acid production has been pursued usually under aerobic conditions. However, anaerobic conditions can conserve the carbon source for product synthesis and provide more reducing equivalents (NADH). To achieve anaerobic production of fatty acid, a homolog of *E. coli* FabG (β-ketoacyl-ACP reductase) was identified from *Cupriavidus taiwanensis*, with 35-fold higher preference for NADH than NADPH. Compared with the control, the titer of free fatty acid was improved by 60% under anaerobic conditions.[57] To achieve the production of fatty acid short-chain esters (FASEs) the fatty and 2-keto acid pathways were combined. The engineered *E. coli* strain produced 1 g/L of FASEs in fed-batch experiments.[58]

It has been shown that fatty acids can also be produced via reverse β-oxidation pathway. In previous section, we have reviewed alcohol production using this pathway (CoA-dependent 1-butanol pathway). *E. coli* strains with engineered reverse β-oxidation cycle, coupled with thioesterases can produce fatty acids with different chain lengths (C4-C12).[59] Steen and coworkers demonstrated the engineering of *E. coli* to produce structurally tailored fatty esters, fatty alcohols, and waxes directly from simple sugars.[49] They eliminated the first two competing enzymes associated with β-oxidation, FadD and FadE, resulting in 1.2 g/L of fatty acids.[49] Using a similar method, Dellomonaco and

coworkers engineered the reverse β-oxidation pathway and over-expressed endogenous dehydrogenases and thioesterases, to achieve 6.9 g/L fatty acids production in *E. coli*.[60]

2.3 Alkanes/alkenes

Many microorganisms have shown great capacity for the production of bioethanol and biodiesel. Other hydrocarbon products, like alkanes, alkenes and also hydrogen are also important biofuels and can be produced from biomass or even directly from sunlight and CO_2.[61]

Alkanes and alkenes are the most important components of natural gas, gasoline, diesel and aviation kerosene. Alkanes/alkenes containing carbon numbers more than four are present as liquids, implying that a majority of them can be produced via regular bioreactors. Usually, C3-C12 alkanes and alkenes are referred to as short-chain alkanes/ alkenes while the C13–C17 ones are referred to as long-chain alkanes/alkenes.[62]

Methane, the simplest alkane, is the major component of natural gas. Two distinct pathways for methane have been identified and characterized in methanogens.[63] This provides the foundation for constructing methane over-producing strains. However, two challenges are required to be addressed for methane production. Firstly, methane production requires strict anaerobic conditions, and secondly, capture and storage of methane is difficult.[63] In addition, the emission of methane into the atmosphere will intensify the green house effect. Considering these disadvantages, the production of alkanes and alkenes with longer chain lengths has gained more attention.

In 2010, an alkane biosynthesis pathway was identified from cyanobacteria.[64] In this pathway, fatty acyl-ACP, the intermediate of fatty acid metabolism, was converted to

fatty aldehyde by an acyl-ACP reductase (AAR), and subsequently into alkanes and alkenes by the action of an aldehyde decarbonylase (ADD) (Fig. 3). This pathway was introduced into *E. coli* , which led to the production of alkanes and alkenes with varying chain lengths (C13 to C17).[64] The activity of ADDs is a major impediment in efficient alkane biosynthesis. To improve the activity of ADDs, an ADD-AAR fusion protein was constructed and expressed in *E. coli*.[65] Compared with the strain expressing two enzymes separately, alkane production increased by 4.8-fold in the strains expressing the fusion protein. Meanwhile, the assembly of ADD and AAR on DNA scaffold resulted in 8.8-fold increase in alkane production.[65] In 2013, a novel pathway was designed to achieve the production of shorter chain alkanes (SCAs).[66] Short-chain fatty acyl-ACPs were converted to the corresponding free fatty acids (FFAs) by a mutated thioesterase. FFAs were further converted to SCAs, by the action of fatty acyl-CoA synthetase (FadD), acyl-CoA reductase (ACR) and ADD (Fig. 3). The genes *fadE* and *fadR* were disrupted to prevent the degradation of fatty acyl-CoAs and enhance fatty acid biosynthesis. The resultant strain produced 580.8 mg/L of SCAs.[66] Recently, a similar strategy has been used for propane production. This pathway relies on a thioesterase specific for butyryl-ACP, achieving 32 mg/L of propane from shake flask studies.[67]

2.4 Hydrogen

Hydrogen has high energy content (142 MJ/kg) and is regarded as an efficient and clean energy resource.[68] In nature, green algae can produce hydrogen by utilizing just water and sunlight. Compared with microalgal hydrogen production, fermentative hydrogen production doesn't require high density light and holds great potential. *E. coli* is the most common host used for hydrogen production. In *E. coli*, hydrogen is produced from

446 formate by formate hydrogen lyase (FHL) system (Fig. 3). Under anaerobic conditions,
447 formate is produced from pyruvate by pyruvate-formate lyase (PFL). Metabolic
448 engineering efforts have enhanced hydrogen production in *E. coli*.[68, 69] Gene *fhlA*
449 encoding an essential activator of FHL was over-expressed while several other genes
450 related to formate and hydrogen consumption were deleted. The best strain produced 141
451 fold more hydrogen than the control strain from formate and threefold more hydrogen
452 from glucose.[68] In another study *E. coli* strain with the best combination of gene
453 knockouts produced hydrogen from glycerol with the maximum theoretical yield (1 mol
454 H_2/mol glycerol).[69]
455

456 # 3. Microbial production of bulk chemicals

457 ## 3.1 Diols

458 The biological manufacture of attractive petrochemical derived diols has gained
459 significant commercial interest over the past decade due to their diverse applications and
460 increasing annual global demand. The commercial scale biological manufacture of 1,3-
461 propanediol (1,3-PDO) has been undertaken by DuPont[70], Tate & Lyle[71] and Genencor[72];
462 while the commercial scale bio-manufacturing of 1,4-butanediol (1,4-BDO) is established
463 by Genomatica[73]. In following section, the microbial production of C4 diols (2,3-
464 butanediol and 1,4-butanediol) and C3 diols (1,3-propanediol and 1,2-propanediol) will
465 be highlighted.

466 **2,3-Butanediol**

467 The three stereo isomers of 2,3-butanediol (2,3-BDO) find application in various
468 industries and their derivatives have an annual combined market of over 32 million

tons.[74] (R,R)-2,3-BDO in particular is used as an antifreeze; while 2,3-BDOs in general are used for the production of various chemicals like methyl ethyl ketone (MEK), 1,3-butadiene, acetoin, diacetyl, etc. Additionally, 2,3-BDOs find application in the manufacture of perfumes, printing ink, food supplements, pharmaceuticals, fumigants, etc.[74]

The microbial production of 2,3-butanediol has been pursued by both native producers and heterologous hosts.[75] The metabolism of native producers generally results in generating a mixture of 2,3-BDO stereoisomers. For instance, *Klebsiella* and *Enterobacter* species are known to produce both (S,S)- and meso-2,3-BDO, while *Bacillus* species are known to produce both (R,R)- and meso-2,3-BDO.[75] By engineering *Klebsiella pneumoniae*, 2,3-BDO titer of 150 g/L has already been achieved via fed-batch fermentation.[76] The production of enantio-pure 2,3-BDO has been largely achieved by introducing stereo-specific 2,3-BDO pathways in heterologous microbes such as *E. coli*. Due to the large market of 2,3-BDO and its derivatives, significant progress in its biological production from a variety of carbon sources has been achieved.[75] In this section, the general biochemical scheme of its microbial production, followed by metabolic engineering strategies implemented to achieve enhanced production in both native producers and in *E. coli* will be discussed.

The biosynthesis of meso/ (R,R)-2,3-BDO is a 3 step metabolic process from an endogenous precursor metabolite produced in nearly all microbes – pyruvate. The enzymes involved in its bio-catalysis include acetolactate synthase (ALS), acetolactate decarboxylase (ALDC) and a stereospecific secondary alcohol dehydrogenase (sADH) leading to the formation of acetolactate, acetoin and 2,3-BDO respectively (Fig. 4). It has

been determined that the expression of *K. pneumoniae* meso-dehydrogenase as the sADH leads to the formation of meso-2,3-BDO, while the expression of *B. subtilis*/*C. beijerinckii*/*T. brockii* sADH leads to the formation of (R,R)-2,3-BDO from acetoin.[77] The biosynthesis of (S,S)-2,3-BDO can be achieved from diacetyl as the target precursor, contrary to pyruvate. The expression of diacetyl reductase leads to the generation of (S)-acetoin from diacetyl, while the subsequent expression of (S)-2,3-butanediol dehydrogenase catalyzes the formation of (S,S)-2,3-BDO.[78]

In 1993, high titer production of 2,3-BDO was achieved at 118 g/L from *K. oxytoca* using molasses, where the biomass generated from a batch process was recycled for use in subsequent batch processes. This strategy overcame growth inhibition in the presence of high initial substrate concentrations and achieved a productivity of 2.4 g/L/h.[79] Recently, further engineering of *K. oxytoca* resulted in achieving a 2,3-BDO titer of 130 g/L with a yield of 0.48 g/g glucose in fed-batch studies. In their work, *K. oxytoca* was engineered to eliminate the production of a dominant by-product – ethanol via disruption of *aldA*. This resulted in diverting the carbon flux into the 2,3-BDO pathway.[80] In another metabolic engineering work, the production of 2,3-BDO by *K. pneumoniae* achieved a titer of 150 g/L with an impressive productivity of 4.21 g/L/h. In order to achieve this, first shake flask studies were performed to identify significant factors influencing nutritional requirements. Following the identification of corn steep liquor powder and $(NH_4)_2HPO_4$ as significant factors, an optimal media was designed to promote 2,3-BDO production. Finally, the parameters influencing fed-batch studies were optimized.[76] Recently, an engineered *Serratia marcescens* strain growing on sucrose reported the highest 2,3-BDO titer of 152 g/L via fed-batch studies. In their work, the

disruption of *swrW* encoding serrawettin W1 synthase significantly lowered the production of serrawettin W1 (a harmful exolipid that causes excessive foaming). Fed-batch culture of the engineered strain resulted in 2,3-BDO productivity of 2.67 g/L/h.[81] The production of enantio-pure 2,3-BDO has been demonstrated by engineering *E. coli*. The production enantio-pure meso-2,3-BDO from glucose was reported by expressing *K. pneumoniae* ALS, ALDC, and meso-2,3-BDO dehydrogenase in *E. coli* achieving a titer of 17.7 g/L.[82] In another work, the production of enantio-pure meso- or (R,R)-2,3-BDO was achieved from *E. coli* growing on glucose. In order to achieve stereo-specific production, the activity of sADH from *K. pneumoniae*, *B. subtilis*/ *C. beijerinckii*/ *T. brockii* were characterized. By expressing the complete pathway using the stereo-specific sADH, production of either meso-2,3-BDO or (R,R)-2,3-BDO was reported.[77] Based on this work, the production of enantio-pure (R,R)-2,3-BDO from glycerol was also reported by engineering *E. coli*. It was determined that the disruption of pathways leading to acetate accumulation (*ackA*/ *poxB*) prevented its accumulation and also the accumulation of acetoin. The engineered strain achieved (R,R)-2,3-BDO biosynthesis at 9.56 g/L from 10 mL shake flask studies.[74]

The production of enantio-pure (S,S)-2,3-BDO from *E. coli* was achieved at 73% conversion efficiency by feeding diacetyl to cultures.[78] In their work, the expression of *K. pneumoniae* diacetyl reductase (also known as meso-2,3-butanediol dehydrogenase) and *Brevibacterium saccharolyticum* (S)-2,3-butanediol dehydrogenase led to the production of (S,S)-2,3-BDO via the formation of (S)-acetoin.[78] Recently, highly pure (S,S)-2,3-BDO production at 26.8 g/L was achieved by feeding 40 g/L diacetyl. In their work, 2,3-butanediol dehydrogenase from an *Enterobacter cloacae* species was expressed in *E.*

538 *coli*. Using optimal fed-batch strategies to feed diacetyl at regular time intervals, (S,S)-
539 2,3-BDO production was achieved.[83]

540 The production of 2,3-BDO from carbon sources such as xylose, corn-corb
541 hydrolysate, molasses, acid hydrolysates of Jatropha hulls, Jerusalem artichoke stalk and
542 tuber, sucrose, even steel industry gas wastes and syngas has been reviewed elsewhere.[75]

543 **1,4-Butanediol**

544 Among butanediols, the biological manufacture of 1,4-butanediol (1,4-BDO) was
545 achieved most recently.[84] 1,4-BDO has an annual market of over 2.5 million tons
546 worldwide and finds applications in the manufacture of plastics, solvents, fibers,
547 polyesters, etc.[84] Until 2011, the production of 1,4-butanediol was solely dependent on
548 petrochemical derivatives due to the lack of any identified natural plant/ microbial routes.
549 In 2011, the *de novo* biosynthesis of 1,4-BDO via a biological platform was reported for
550 the first time.[84] In their work, two heterologous pathways led to the production of 1,4-
551 BDO in *E. coli* by channeling carbon from the tricarboxylic acid cycle (TCA cycle)
552 intermediates – succinate and α-ketoglutarate via 6 and 5 steps respectively (Fig. 4). In
553 both routes, the production of common precursor 4-hydroxybutyrate (4-HB) was first
554 achieved followed by establishment of downstream pathway leading to 1,4-BDO
555 production. The production of 4-HB from succinate required the expression of two
556 heterologous enzymes – succinate semialdehyde dehydrogenase and 4-hydroxybutyrate
557 dehydrogenase. Whereas, the production of 4-HB from α-ketoglutarate required the
558 expression of heterologous enzyme 2-oxoglutarate decarboxylase in addition to 4-
559 hydroxybutyrate dehydrogenase. With the successful production of key intermediate 4-
560 HB, an efficient bio-catalytic method for conversion of 4-HB to 1,4-BDO was then

pursued. 4-HB was catalyzed to 4-hydroxybutyrl-CoA, via the action of 4-hydroxybutyryl-CoA transferase. In order to catalyze the final two steps leading to 1,4-BDO, a bifunctional alcohol dehydrogenase was expressed. For *de novo* biosynthesis, the upstream and downstream pathways were co-expressed in *E. coli*. Furthermore, in order to establish an efficient production platform, a metabolic model based approach was utilized as a guide to engineer *E. coli*'s native metabolism, promoting a more thermodynamically favorable process. The metabolic routes leading to production of native fermentative products (lactate, succinate, ethanol, and formate) were disrupted; additionally, the carbon flux towards reductive TCA cycle was disrupted to promote oxidative TCA cycle metabolism. With this, more reducing power was endogenously available to drive desired biocatalysis leading to efficient 1,4-BDO biosynthesis from renewable feedstocks such as glucose, xylose. Their work demonstrates effective utilization of metabolic engineering strategies to optimize synthetic metabolic routes, exemplified by achieving over 18 g/L 1,4-BDO in 2 L bioreactors.[84]

Although 18 g/L of 1,4-BDO was achieved, the final two steps leading to 1,4-BDO production was determined to be inefficient. It was shown that the bifunctional alcohol dehydrogenase (AdhE2) catalyzing 4-hydroxybutyryl-CoA and 4-hydroxybutyrlaldehyde to 1,4-BDO displayed low specificity.[84, 85] Recently, another approach to efficiently catalyze these two reactions was reported with the use of two substrate specific dehydrogenases. In their work, *Clostridium saccharoperbutylacetonicum* butyraldehyde dehydrogenase (Bld) was engineered to enhance specificity towards 4-hydroxybutyryl-CoA, while *C. saccharoperbutylacetonicum* butanol dehydrogenase (Bdh) was used for the catalysis of

584 the final reaction.[85] In order to enhance activity of Bld towards 4-hydroxybutyryl- CoA,
585 random mutagenesis was performed, which led to the identification of a critical amino
586 acid site (L273) influencing the cofactor binding affinity and structural stability. A four-
587 fold increase in titer of 1,4-BDO biosynthesis was achieved with the use of Bld (L273T)-
588 Bdh module compared with the module utilizing AdhE2 for the final two catalytic
589 steps.[85]

590 **1,3-Propanediol**

591 Among the C3 diols, 1,3-propanediol (1,3-PDO) is regarded as an important platform
592 chemical with an annual demand of over one million tons; widely used for the
593 manufacture of various polymers with desirable properties, for production of drugs,
594 cosmetics, lubricants, etc.[70, 86] The first report of microbial production of 1,3-propanediol
595 was in 1881 by *Clostridium pasteurianum* growing on glycerol anaerobically.[87] The
596 biological production of 1,3-PDO has since been achieved from a wide range of
597 microorganisms using glucose or glycerol as the carbon source. Some of the microbes
598 known to natively produce 1,3-PDO include *Clostridium*, *Lactobacilli*, *Klebsiella*,
599 *Enterobacter*, and *Citrobacter*.[87, 88] Among these, the fermentation of glycerol led to
600 achieving 83.6 g/L of 1,3-PDO with a yield of 0.54 g/g glycerol using an engineered *C.*
601 *acetobutylicum* strain as reported by Metabolic Explorer researchers.[89] Furthermore, the
602 highest reported titer of 1,3-PDO at 135 g/L was achieved using glucose fermentation
603 from an engineered *E. coli* strain.[2] The most successful metabolic engineering strategies
604 to enhance the production of 1,3-PDO will be discussed in this section.

605 While utilizing glycerol as the carbon source, 1,3-PDO production is achieved by
606 engineering its reductive branch via two steps (Fig. 4). In the first step, 3-

hydroxypropionaldehyde (3-HPA) is generated by the action of glycerol dehydratase by dehydrating glycerol. Following subsequent reduction by a 1,3-PDO oxidoreductase, 1,3-PDO is produced. However, the oxidative branch of glycerol dissimilation pathway is known to compete for glycerol via the action of glycerol dehydrogenase and dihydroxyacetone kinase, leading to the formation of DHAP.[87] The major bottlenecks to improve 1,3-PDO include oxygen sensitive nature of dehydratase, NADH availability and harmful by-products accumulation.

It was observed that under aerobic conditions, when the substrate is absent, coenzyme B12-dependent glycerol dehydratase undergoes inactivation.[90] In order to overcome this limitation, strategies such as addition of ATP, Mg^{+2}, expression of B12-independent dehydratase, and reactivating factors have been used.[91, 92] The production of 1,3-PDO at high titers also results in toxic accumulation of 3-HPA, which is detrimental to achieving industrial scale production. In order to overcome this hurdle, a two stage fed-batch fermentation strategy was utilized by sequentially feeding glycerol to achieve 38.1 g/L 1,3-PDO without 3-HPA toxification by a *K. pneumoniae* strain.[93] Several studies have since been conducted to identify *Klebsiella* strains with improved tolerance to high 1,3-PDO titers. In 2009, *K. pneumoniae* strain HR526 enabled 1,3-PDO production at 102.06 g/L via fed-batch fermentation using glycerol as carbon source. In order to achieve this, *K. pneumoniae* strain was engineered to disrupt lactate dehydrogenase activity in order to prevent the accumulation of lactate.[94]

Metabolic engineering of *C. acetobutylicum* with the introduction of a B12-independent pathway of *C. butyricum* led to 1,3-PDO production at 1104 mM (83.6 g/L).

Fed-batch studies resulted in high volumetric productivity (3 g/L/h) of 1,3-PDO, emphasizing the advantage of an alternative dehydratase.[89, 95]

In order to establish a more efficient production platform, an *E. coli* strain was engineered to generate glycerol from glucose. The *S. cerevisiae* glycerol 3-phosphate dehydrogenase (*dar1*) and glycerol 3-phosphate phosphatase (*gpp2*) enzymes were expressed in *E. coli* in addition to the expression of *K. pneumoniae* glycerol dehydratase and its reactivating factors (*dhaB1*, *dhaB2*, *dhaB3, dhaBX* and *orfX*). *E. coli* strains natively expressing NADPH dependent YqhD for 1,3-PDO oxido-reductase activity was found to be more efficient in 1,3-PDO production, as compared to a strain expressing NADH dependent heterologous DhaT. Further metabolic engineering strategies include down-regulation of glyceraldehyde 3-phosphate dehydrogenase to limit the carbon flux towards TCA cycle; replacement of phosphoenolpyruvate (PEP)-dependent glucose uptake system with ATP-dependent glucose transport system to enhance glucose uptake efficiency. This was achieved via replacement of PTS with galactose permease and glucokinase. Additionally, in order to prevent the loss of glycerol to native metabolism, major competing pathways (*glpK*, *gldA*) were disrupted. These efforts resulted in achieving 1,3-PDO at 135 g/L from 10 L fed batch fermentations with a high yield of 0.62 mol/mol (at a rate of 3.5 g/L/h).[2, 70, 96] In another work, the improvement of YqhD activity towards 3-HPA was pursued in order to overcome the limitation of 1,3-PDO oxidoreductase activity via error-prone PCR. This led to the identification of a mutant (D99QN147H) with 4 four-fold higher activity towards 3-HPA. The expression of this mutant YqhD in *E. coli* led to an increase in 1,3-PDO conversion from 3-HPA.[97]

Detailed reviews encompassing metabolic engineering efforts to engineer the glycerol dissimilation pathways, use of two stage fermentations, improvement of strain tolerance, use of glucose to produce glycerol, and engineering *Clostridia*, *S. cerevisiae*, *Klebsiella* microbes for 1,3-PDO production can be found elsewhere.[2, 70, 87, 95, 96]

1,2-Propanediol

1,2-Propanediol (1,2-PDO) has a reported annual market of over 1 billion pounds and is primarily used as an antifreeze; while also finding applications in textile industry for the manufacture of ink, in the pharmaceutical industry, and also for the manufacture of detergents, cosmetics.[98, 99] The biosynthesis of 1,2-PDO was first reported in 1954, when a *Clostridium* species was identified to be capable of its production natively.[100] Since then several bacterial and yeast species have been identified with the ability of natively producing 1,2-PDO. Initially, the production of 1,2-PDO from *E. coli* was reported via the fermentation of uncommon sugars like fucose and rhamnose.[101] Additionally, it was reported that under phosphate limiting conditions, *C. sphenoides* was capable producing of 1,2-PDO from fructose, mannose and cellobiose as well. In their work, it was reported that the maximum amount of 1,2-PDO (72.6 mM) was produced with the fermentation of rhamnose.[102] The highest reported titer of 1,2-PDO till date was achieved at 9.1 g/L with a yield of 0.20 g/g glucose using *C. thermosaccharolyticum* via fermentation.[103] Recently, metabolic engineering efforts have enabled the anaerobic production of 1,2-PDO in *E. coli* using glucose.[98, 104, 105] It was also reported that utilization of more reduced carbon sources like sorbitol and gluconate did not improve 1,2-PDO production in *E. coli*.[99] Furthermore, the production of 1,2-PDO using *S. cerevisiae* has been

674 demonstrated by metabolic engineering using glucose and glycerol.[106, 107] Here, the
675 biochemical pathways leading to 1,2-propanediol using fucose/rhamnose or glucose/
676 glycerol will be highlighted with an emphasis on important metabolic engineering efforts
677 to enhance its production depending on the microorganism (*E. coli*/ *S. cerevisiae*/
678 cyanobacteria).

679 The two major biochemical pathways leading to 1,2-PDO production involves the
680 production of lactaldehyde as its immediate precursor. It was initially found that L-
681 rhamnose is first isomerized to L-rhamnulose followed by subsequent phosphorylation to
682 L-rhamnulose-1-phosphate. Similarly, L-fucolose-1-phosphate is produced via
683 isomerization and phosphorylation of L-fucose. Following cleavage of these two
684 phosphorylated sugars by aldolase, DHAP and L-lactaldehyde are generated
685 simultaneously. L-lactaldehyde is then reduced to L-1,2-PDO via the action of
686 propanediol oxidoreductase.[100] Although the metabolic routes from rhamnose and fucose
687 involve only 4 steps, the high cost of these sugars prevents commercial scale
688 manufacture.[100] Due to this, majority of metabolic engineering efforts for its production
689 have focused on utilization of cheaper carbon sources like glucose and glycerol.

690 The production of 1,2-PDO from glucose/glycerol involves the production of
691 methylglyoxal from the glycolytic intermediate DHAP via the action of methylglyoxal
692 synthase (Fig. 4). The production of 1,2-PDO from methylglyoxal can be achieved either
693 through the formation of lactaldehyde or through the formation of hydroxyacetone
694 (acetol) via the action of secondary alcohol dehydrogenase or methylglyoxal reductase
695 respectively. The subsequent action of alcohol dehydrogenase on lactaldehyde/
696 hydroxyacetone leads to the formation of 1,2-PDO.[20] It was initially determined that just

the over-expression of either methylglyoxal synthase or alcohol dehydrogenase led to the production of 1,2-PDO in *E. coli* using glucose.[98] Enhanced production of 1,2-PDO was then achieved using glucose, by expressing the complete pathway via lactaldehyde (*E. coli* methylglyoxal synthase, glycerol dehydrogenase and 1,2-propanediol oxidoreductase) in an engineered *E. coli* strain lacking lactate dehydrogenase activity. About 4.5 g/L of 1,2-PDO was produced with a yield of 0.19 g/g glucose in 2 L fed-batch anaerobic fermentations.[105] The highest reported titer of 1,2-PDO (5.6 g/L) achieved in *E. coli* was reported by engineering its glycerol dissimilation pathway. In their work, DHAP availability was increased by replacing *E. coli*'s native PEP-dependent dihydroxyacetone kinase with *C. freundii* ATP- dependent dihydroxyacetone kinase. By over-expressing *E. coli* methylglyoxal synthase, glycerol dehydrogenase and aldehyde oxidoreductase, 1,2-PDO production was achieved. Combining these strategies with the disruption of major fermentative by-product pathways (lactate and acetate) led to 1,2-propanediol production at 5.6 g/L in a fermenter with 400 mL media containing glycerol.[108]

The production of 1,2-PDO from *S. cerevisiae* was achieved by engineering the glycolysis pathway along with the expression of *E. coli* methylglyoxal synthase and glycerol dehydrogenase enzymes. With the disruption of the gene encoding triose phosphate isomerase, carbon flux to DHAP was increased in the engineered *S. cerevisiae* strain which led to 1,2-PDO production at 1.11 g/L.[106] In another report, *S. cerevisiae* was engineered to increase glycerol uptake rate as a strategy to enhance 1,2-PDO production. With the over-expression of the glycerol dissimilation pathways along with *E. coli* methylglyoxal synthase and glycerol dehydrogenase enzymes, 1,2-PDO was

produced at 2.19 g/L using a modified YEPD media containing 1% glycerol and 0.1% galactose.[107]

While the production of 1,2-PDO has been largely pursued utilizing a range of carbon sources and metabolic engineering of *E. coli* and *S. cerevisiae*, in 2013, its production was reported from CO_2, using an engineered cyanobacterial strain. In their work, the *E. coli* 1,2-PDO pathway was introduced in cyanobacteria, however, with the replacement of NADH-specific secondary alcohol dehydrogenase with a *C. beijerinckii* NADPH-specific secondary alcohol dehydrogenase to enhance its production. This strategy resulted in 0.15 g/L of 1,2-PDO production with the consumption of CO_2.[109] Recently, 1,2-propanediol production was achieved from *E. coli* at enhanced titer and yield simultaneously for the first time.[21] In their work, the expression of optimal minimal set of enzymes, carbon flux redirection and manipulation of cellular energetics (NADH availability) led to 1,2-propanediol production initially at 0.59 g/L (with a yield of 0.34 g/g glucose). Furthermore, adapting the engineered strain to the fermentation environment during the preparation of inoculum enhanced the titer of 1,2-propanediol (5.13 g/L) with 94% theoretical maximum yield (0.48 g/g glucose). This work represents the highest 1,2-propanediol yield and titers achieved till date from shake flask studies.[21]

3.2 Organic acids

Organic acids play important roles in various modern industries. They can be used as pharmaceuticals, polymer precursors, food additives, antibacterial agents. In this section, we summarized the production of important organic acids like lactic acid, succinic acid, fumaric acid, muconic acid, malic acid and 3-hydroxypropionic acid by metabolic engineering.

Lactic acid

Lactic acid is a widely used compound in the food and pharmaceutical industry. Lactic acid bacteria have the capability to produce abundant lactate under low pH conditions.[110] However, these bacteria require complex medium and produce a mixture of lactic acid stereoisomers. *E. coli* was used for lactic acid production with metabolic engineering that focused on disruption of competing pathways.[111] After deleting the genes encoding fumarate reductase (*frdABCD*), alcohol/aldehyde dehydrogenase (*adhE*), pyruvate formate lyase (*pflB*), and acetate kinase (*ackA*), the resulting strain could oxidize NADH only via D-lactate synthesis in a simple medium with glucose, and achieved 96% of the maximum theoretical yield. In mineral salts medium, an engineered *E .coli* W derivative strain efficiently consumed 120 g/L glucose and produced 110 g/L D-lactate.[111] After further optimization by deleting methylglyoxal synthase gene *mgsA* to eliminate L-lactate production, D-lactate producing strain, TG114 converted 120 g/L glucose to 118 g/L D-lactate with a high yield (98%) and impressive productivity (2.88 g/L/h).[112] To alleviate the inhibition effect of D-lactate production on cell growth in the early stage, the native promoter of D-lactate dehydrogenase gene (*ldhA*) was replaced with a thermo-controllable promoter. Expression of *ldhA* was turned off at 33ºC and the cell growth was improved by 10%. The temperature was then switched to 42ºC and 122.8 g/L of D-lactate was produced.[113] To achieve L-lactate production in *E. coli*, the native D-lactate specific dehydrogenase was replaced with L-lactate dehydrogenase from *Streptococcus bovis*, and the methylglyoxal bypass pathways and native aerobic L-lactate dehydrogenase were disrupted. The engineered strain produced 50 g/L of L-lactate from 56 g/L of crude glycerol at a yield of 93% of the theoretical maximum and with high optical (99.9%) and

chemical (97%) purity.[114] Recently, similar strains were constructed and using a temperature-shifting fermentation strategy, 142.2 g/l of L-lactate was produced.[115]

Succinic acid

Succinic acid, a dicarboxylic acid, is platform chemical that can be used for the manufacture of solvents, plastics, detergents, anti-bacterial and neutralizing agents. While the current global succinic acid production is 30,000 to 50,000 tons annually, with the market price of 2400-3000 US $/ton, the market is expected to reach 125,000 tons by 2020.[116, 117] Succinic acid can be produced by chemical approaches, however, these process produce succinic acid with low yields and purity.[118, 119]

Succinic acid is an intermediate metabolite of tricarboxylic acid (TCA) cycle and is known to be produced as a fermentative product. *E. coli* is one of the most studied microorganisms for succinate production. Metabolic engineering approaches have been used for high yield succinic acid production, which include knockout of by-product pathways, improvement of substrate transportation and utilization, enhancement of pathways directly involved in succinate production, and enabling redox balance.

The initial strategy for succinic acid production in *E. coli* focused on deletion of competitive pathways, such as alcohol/aldehyde dehydrogenase (AdhE), lactate dehydrogenase (LdhA), and acetate kinase (AckA). However, the resulting strains grew poorly in mineral salts medium under anaerobic conditions and accumulated only trace amounts of succinate. The same phenomenon was observed in pyruvate formate-lyase (PflB) deficient strain because these deletions lead to lack of cellular energy or insufficient levels of electron acceptors.[120] Metabolic evolution was used to improve both

cell growth and succinate production. After inactivation of *pflB* and *mgsA* to eliminate formate and lactate production, the final strain KJ073 produced near 670 mM succinate (80 g/L) in mineral salts medium with a high yield (1.2 mol/mol glucose) and high productivity (0.82 g/L/h).[120] Through inactivation of the *pflB*, *ldhA*, and *ptsG* genes and over-expression of the pyruvate carboxylase gene, strain AFP111/pTrc99A-pyc produced 99.2 g/L succinate with a yield of 1.1 g/g glucose by use of a dual-phase fermentation process.[121] Directed evolution strategy has been used to improve succinate production in *E. coli* without introduction of plasmids or foreign genes. Together with gene deletions, the result strains produced 622–733 mM of succinate with molar yields of 1.2-1.6 mol/mol glucose.[122] In evolved strains, PEP carboxykinase (*pck*) served as the major carboxylation pathway instead of phosphoenolpyruvate carboxylase (*ppc*). Meanwhile, the GalP permease (*galP*) and glucokinase (*glk*) replaced the native PEP-dependent phosphotransferase system as the major glucose transporter. These changes increased energy efficiency of succinate production.[123]

Fumaric acid

Fumaric acid is a four-carbon dicarboxylic acid and is known to be 1.5 times more acidic than citric acid. So, it is commonly used as an antibacterial agent in food and beverage industries. Fumaric acid can also be polymerized to produce synthetic paper resin, plasticizer, and environmentally friendly polymers.[124] As an important intermediate of TCA cycle, fumaric acid is a valuable feedstock in the preparation of food additives, such as L-malic acid and L-aspartic acid. [125]

Fermentation production of fumaric acid began since early last century. Among the fumaric acid producing microbes, *Rhizopus* species were shown to be the best

producers. Recently, 22.81 g/L of fumaric acid was produced using *R. arrhizus* in the co-fermentation of crude glycerol and glucose at 144 h.[126]

In most microbes, fumaric acid synthesis involves three enzymes commencing from pyruvate. Pyruvate carboxylase is the first enzyme that catalyzes the carboxylation of pyruvate to oxaloacetate with the consumption of ATP and CO_2. Oxaloacetate is then converted to malic acid by malate dehydrogenase and then to fumaric acid by fumarase. Recently, yeast and *E. coli* were engineered for fumaric acid production.[127] In fed-batch culture, the engineered *E. coli* strain produced 41.5 g/L fumaric acid from glycerol with 70% of theoretical maximum yield.[128] In *E. coli*, succinate is converted to fumaric acid by succinate dehydrogenase, and fumaric acid is then catalyzed to malate through fumarase. There are three known fumarases in *E. coli*[129], which are encoded by *fumA, fumB* and *fumC*, respectively. Based on previous research, fumarase A and C mainly function under aerobic and microaerobic conditions, while fumarase B was determined to be the dominant enzyme expressed under anaerobic conditions. It was reported that knockout of *fumAC* genes failed to improve fumaric acid accumulation, but the simultaneous deletion of *fumAC* and *fumB* resulted in increasing fumaric acid production.[130] Li and coworkers disrupted these three genes in a succinate over-producing *E. coli* strain and achieved 3.65 g/L fumaric acid production.[128]

Muconic acid

Muconic acid (MA), a six-carbon dicarboxylic acid, is an important platform chemical in plastic industry. It is a synthetic precursor to terephthalic acid, a chemical used for manufacturing polyethylene terephthalate (PET) and polyester.[131] MA can also be easily hydrogenated and converted to adipic acid, which is a raw material for nylon-6,6 and

polyurethane.[132] The global annual demand of terephthalate and adipic acid is about 71 million and 2.8 metric tons, respectively.[131] Currently, both compounds are synthesized from petroleum-derived chemicals, which is non-renewable and environmentally unfriendly.

In 1994, Draths and Frost first reported a *de novo* route for MA biosynthesis by an engineered *E. coli* strain.[133] This pathway was extended from the shikimic acid (SA) pathway. Three foreign enzymes, 3-dehydroshikimate dehydratase (AroZ), protocatechuate decarboxylase (AroY) and catechol 1,2-dioxygenase (CatA) were introduced in *E. coli* and the intermediate 3-dihydroshikimate (DHQ) was converted to muconic acid via protocatechuate and catechol (Fig. 5). *E. coli* SA pathway was strengthened by over-expressing *tktA*, *aroF* and *aroB* while shikimate dehydrogenase gene was inactivated to prevent DHQ consumption. The engineered strain was reported to produce 2.4 g/L and 38.6 g/L of muconic acid in shake flask studies and fed-batch fermentations, respectively.[133, 134] This pathway has also been constructed in *S. cerevisiae*, where new pathways enzymes were screened and MA production was achieved at a titer of 141 mg/L.[131]

Recently, three novel pathways have been developed for MA production in *E. coli* (Fig. 5). These pathways are all shunted from chorismate, the end product of SA pathway. In the first pathway, *E. coli* native anthranilate synthase was utilized to convert chorismate to anthranilate. Subsequently, two foreign enzymes, anthranilate 1,2-dioxygenase (ADO) and catechol 1,2-dioxygenase (CDO), catalyzed anthranilate to MA via catechol. Efficient ADO and CDO were screened from different microorganisms and enabled MA production from anthranilate. By expressing the key enzymes in SA

858 pathway, blocking tryptophan biosynthesis and introducing a glutamine recycling system,
859 *de novo* production of MA was achieved at a titer of 389.96 mg/L.[132] In the second
860 pathway, four enzymes: isochorismate synthase (ICS), isochorismate pyruvate lyase
861 (IPL), salicylate1-monoxygenase (SMO) and CDO were required to produce MA from
862 chorismate via salicylate and catechol. A phenylalanine over-producing *E. coli* strain was
863 engineered into a salicylate over-producer by expressing efficient ICS and IPL and
864 enhancement of shikimate pathway. Introducing SMO and CDO into the salicylate over-
865 producer led to *de novo* production of MA. After modular pathway optimization, 1.5 g/L
866 of MA was produced in shake flask studies.[135] Similarly, in the third pathway, two
867 foreign enzymes, 2,3-dihydroxybenzoic acid decarboxylase (BDC) and CDO catalyzed
868 *E.coli* native 2,3-dihydroxybenzoic acid (2,3-DHBA) into MA via catechol. Over-
869 expression of *entCBA* and the key enzymes in the SA pathway resulted in the production
870 of 900 mg/L of 2,3-DHBA. Assembly of the complete pathway led to the *de novo*
871 production of muconic acid up to 480 mg/L.[136]

872 **Other acids**

873 Malic acid is another important C4 dicarboxylic acid which is used by food and
874 pharmaceutical industries, as well as for the production of polymers.[137] The production of
875 malic acid from glucose was achieved at 9.25 g/L using an engineered *E. coli* strain
876 expressing a heterologous PEP carboxykinase from *Mannheimia succiniciproducens*.[137]
877 Another approach to engineer *E. coli* for the production of malic acid involved the
878 disruption of target genes followed by growth-based evolution, leading to its production
879 at 516 mM.[138] Further metabolic engineer efforts improved the production of malic acid
880 using *E. coli* to 34 g/L[139], and using *S. cerevisiae* to 59 g/L[140]. Recently, a new approach

was developed for the production of malic acid from its polymer polymalic acid (PMA). A PMA producing *Aureobasidium pullulans* strain was used for fermentation studies which led to the production of PMA at high titers from a fibrous-bed reactor. The resultant PMA was then recovered and purified at high efficiency following which malic acid was produced via acid hydrolysis at 142.2 g/L with a productivity of 0.74 g /L/ h.[141]

3-Hydroxypropionic acid (3-HP) is an important chemical recognized by the US Department of Energy (DOE) due to its wide applications. 3-HP is used for the production of chemicals (1,3-PDO, ethyl 3-HP, acrylic acid, acrylamide, malonic acid, poly 3-HP), and also in the manufacture of polyesters, metal lubricants, textiles, etc.[142] The initial efforts for biological production of 3-HP was pursued from glycerol using different *Lactobacillus* sp., from acrylic acid using *Byssochlamys* sp., *Geotrichum* sp. and *Trichoderma* sp. The microbial routes for the production of 3-HP has been extensively reviewed recently.[142] In order to pursue the commercial production of 3-HP, glucose and glycerol were used as carbon sources. The highest reported titer of 3-HP till date has been achieved from glucose (49 g/L), with a yield of 0.46 mol/ mol using an *E. coli* strain in 1 L fed-batch studies.[143] While the highest titer of 3-HP reported till date from glycerol (49.3 g/L), has been achieved using a *K. pneumoniaea* strain having a yield of 0.18 mol/ mol via 5 L fed-batch studies.[144] Recently, the highest yield of 3-HP production in *E. coli* from glycerol (54.1% mol/ mol) was reported by using dual synthetic pathways at a titer of 56.1 mM.[145]

4. Microbial production of pharmaceuticals and nutraceuticals

4.1 Amino acids

Amino acids, as the building blocks for life, are one of the most important and widely distributed natural chemicals in biological systems. Amino acids have been long used as feed additives to animals or food nutraceuticals to humans. Essential proteinogenic amino acids including L-valine, L-leucine, L-isoleucine, L-lysine, L-threonine, L-methionine, L-histidine, L-phenylalanine and L-tryptophan cannot be synthesized in animals and humans.[146] Some amino acids have shown to exhibit even pharmaceutical functions like L-glutamine against gastroenterological disorders and gastric ulcer, L-tryptophan against depression and L-tyrosine against Basedow's disease.[147, 148] Besides, amino acids of different scaffolds are key precursors for synthesis of enormous metabolites of critical biological importance like hormones, antibiotics and anti-cancer drugs.[149, 150] Nowadays, nutrition supply and health maintenance are the main impetus for large scale production of amino acids. Major food flavoring and feed additive amino acids L-glutamate and L-lysine has been produced at over 5 million tons in 2013 and the commercial market for amino acids is increasing with an annual growth rate of 6–8%.[151, 152] Compared with traditional processes to produce amino acids (extraction, chemical synthesis and enzymatic catalysis), microbial synthesis has accelerated industrial production of amino acids since the discovery of glutamate production by *Corynebacterium glutamicum* more than 50 years ago.[153] Microbial synthesis is becoming the dominant and optimal process for amino acid production because of its ease to produce enantiomerically pure amino acids, low costs and ecological acceptability.[147, 152] Particularly, recent progress in systematic metabolic engineering of *C. glutamicum* and *E. coli* enabled the feasible large scale production of nearly all amino acids.

L-Glutamate is one of the most largely produced amino acids and its sodium salt mono-sodium glutamate (MSG) is used worldwide as a commercial flavor enhancer. Metabolic engineering efforts for L-glutamate production has been largely pursued in *C. glutamicum*. To increase L-glutamate production, improving the availability or reducing the consumption of precursor 2-oxoglutarate by deleting native competing pathways were the main strategies.[152] 2-oxoglutarate production was also achieved by over-expression of pyruvate carboxylase or deletion of competing pyruvate kinase.[154, 155] Meanwhile, consumption of 2-oxoglutarate was reduced by deletion of oxoglutarate dehydrogenase (ODHC) or inhibition of ODHC either by antisense RNA or by use of an enzyme inhibitor (OdhI).[156-158] Over-expression of heterologous genes into *C. glutamicum* was another strategy to increase L-glutamate titer. By over-expressing *Vitreoscilla* hemoglobin (VHb) gene *vgb* into *C. glutamicum*, both cell growth and L-glutamate productivity were enhanced.[159] Over-expression of polyhydroxybutyrate (PHB) biosynthesis genes *phbCAB* from *R. eutropha* was found to regulate L-glutamate production in *C. glutamicum* and increased L-glutamate titer by 39–68%.[160] L-Glutamate production was also reported to be enhanced by reducing carbon dioxide emission levels via employing phosphoketolase (PKT) pathway from *Bifidobacterium animalis* into *C. glutamicum*.[161] With this, the carbon flux was directed to L-glutamate production via metabolic engineering of native PKT pathway.

L-Lysine is a preferred additive to animal feeds for pig breeding and poultry, with 1.5 million tons produced annually.[146, 152] Metabolic engineering for L-lysine production by *C. glutamicum* has been enhanced by use of synthetic biology tools. An outstanding example is using promoter engineering to redirect carbon flux to improve L-lysine

production. Oxaloacetate, the precursor for L-lysine production is generated from carboxylation of pyruvate. Exchange of the native promoter of pyruvate dehydrogenase complex (PDHC) with a reduced *dapA* promoter variant led to enhanced production of L-lysine.[162] The activity of isocitrate dehydrogenase (ICD) was reduced by mutating its start codon; this also resulted in reducing the carbon flux towards the TCA cycle. These efforts enhanced L-lysine production by 40%.[163] These two approaches addressed precursor supply from central metabolism, concomitant with improving cell growth. Other attempts encompassed increasing availability of NADPH via over-expression of fructose 1,6-bisphosphatase (encoded by *fbp*) or G6P dehydrogenase (encoded by *zwf*), which are required for L-lysine production.[164] Recently, by systems metabolic engineering of wild type *C. glutamicum*, an L-lysine over-producing strain was constructed, capable of achieving a titer of 120 g/L in fed-batch cultures.[165]

Aromatic amino acids, including L-tyrosine, L-phenylalanine and L-tryptophan, are known to be produced via SA pathway in bacteria, fungi, algae, parasites and plants. They are essential amino acids for human diet and are also important precursors for synthesis of a plethora of high value by-products (nitric oxide, polyamines, glutathione, taurine, thyroid hormones, serotonin, etc.). Aromatic amino acid biosynthesis is initiated by the condensation of phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P) to form 3-deoxy-d-arabino-heptulosonate 7-phosphate (DAHP), which is subsequently catalyzed into chorismate, the branching point of the three aromatic amino acid pathways.[166-168] Thus, metabolic engineering of aromatic amino acids have firstly focused on strengthening the pathway leading to chorismate. Over-expression of PEP synthase (encoded by *ppsA*) and transketolase (encoded by *tktA*) is a commonly used strategy to

improve the supply of intracellular PEP and E4P. Feedback inhibition-resistant (*fbr*) enzymes DAHP synthase (encoded by *aroG*^{fbr}) and chorismate mutase- prephenate dehydrogenase (encoded by *tyrA*^{fbr}) were constructed and used for chorismate accumulation and subsequent L-tyrosine over-production.[166, 167] Secondly, TyrR and TrpR-mediated repression were determined to alleviate the production of aromatic amino acid biosynthesis. Removal of transcriptional attenuation via deletion of *tyrR* resulted in improved production of L-phenylalanine or L-tyrosine.[167, 168] Additionally, disruption of a global regulatory gene *csrA* led to a two-fold increase in L-phenylalanine production.[169] Additionally, engineering the transport mechanism of aromatic amino acids was used as a strategy to increase yields. The *in vivo* accumulation or uptake of aromatic acids was found to inhibit production capacity, which was relieved by modifying transport systems.[170, 171] Applications of these approaches for metabolic engineering of *E. coli* and *Corynebacterium glutamicum* have achieved L-phenylalanine, L-tyrosine and L-tryptophan production with titers as high as 50 g/L, 55 g/L and 60 g/L respectively.[172-175]

Recently, metabolic engineering for production of branched chain amino acids including L-valine, L-leucine and L-isoleucine have gained significant interest for their potential applications in animal feed additive, cosmetics and pharmaceuticals.[176] Microbial production of L-threonine,[177] L-proline,[178] L-serine,[179] L-cysteine,[180] L-arginine[181] and L-alanine[182] has also been achieved by metabolic engineering. With understanding of genetic background and amenability of metabolic engineering in *C. glutamicum* or *E. coli*, microbial production of amino acids will be further elevated to meet the increasing demand for amino acids.[183] Metabolic engineering for amino acids production has been achieved by utilizing different carbon sources like glucose, starch,

arabinose, molasses, sucrose, fructose, xylose and even methanol in engineered microbes.[148, 184-187] In addition to metabolic engineering of biosynthetic pathways, other improvements have been made to achieve industrial scale production of amino acids including optimizing cultivating process, utilizing different carbon sources and dynamically controlling the pathway. Recent metabolic engineering of amino acids production have been successfully extended to their derivative chemicals like L-glutamate derived γ-amino-butyrate,[188, 189] L-tyrosine derived caffeic acid[190] and L-tryptophan derived 5-hydroxytryptophan[191, 192].

4.2 Phenylpropanoids

Phenylpropanoids are a diverse family of plant secondary metabolites derived from phenylalanine. According to their molecular structure, this family can be further classified as hydroxycinnamic acids, flavonoids, stilbenoids, coumains, etc.[193] Their broad use as supplements in foods and cosmetics and potentially even as pharmaceuticals is due to their superior antioxidant, anti-inflammatory, anti-virus and anti-cancer activities.[194, 195] Naturally-occurring phenylpropanoids are usually conjugated with one another or with other molecules such as sugars or organic acids but rarely exist in free forms. Therefore, it is laborious and inefficient to manufacture these compounds through conventional extraction methods. Alternatively, reconstitution of heterologous biosynthetic pathways in microbial hosts such as *E. coli* and *S. cerevisiae* with advantageous genetic and fermentation properties has led to facile production of various phenylpropanoids from inexpensive precursors or even simple carbon sources (Fig. 6).

Hydroxycinnamic acids

Hydroxycinnamic acids refer to the hydroxylated analogs of cinnamic acid with simple substituent groups, such as *p*-coumaric, caffeic and ferulic acid. *p*-Coumaric acid is a central intermediate for the biosynthesis of a majority of phenylpropanoids. In plants, it is produced through phenylalanine deamination by the action of phenylalanine ammonia lyase (PAL), followed by *para*-hydroxylation catalyzed by cinnamate 4-hydroxylase (C4H). This pathway was initially reconstituted in yeast, leading to the production of *p*-coumaric acid.[196] However, its efficiency is very low due to the employment of C4H, a cytochrome P450 hydroxylase. A more straightforward approach was established to produce *p*-coumaric acid directly from tyrosine deamination catalyzed by tyrosine ammonia lyase (TAL) or PALs with broad substrate tolerance.[197] Notably, this pathway is compatible with *E. coli* system, which enabled the efficient *de novo* production of *p*-coumaric acid from utilizing simple carbon sources. About 974 mg/L of *p*-coumaric acid was produced in shake flask studies by a tyrosine over-producing strain with the over-expression of *Saccharothrix espanaensis* TAL (encoded by *sam8*).[198]

The natural caffeic acid biosynthesis in plant involves another cytochrome P450 enzyme, *p*-coumarate 3-hydroxylase (C3H).[199, 200] Recently, the identification of C3H substitutes that can replace the plant P450 enzyme has enabled the microbial production of caffeic acid. The earliest identified C3H of bacterial origin was the Sam5 from *S. espanaensis*.[201] Interestingly, it was reported that bacterial species including *E. coli* and *Pseudomonas aeruginosa* natively possess 4-hydroxyphenylacetate 3-hydroxylases (4HPA3H) and are highly capable of hydroxylating *p*-coumaric acid.[190, 202] Furthermore, it was reported that 10.2 g/L of caffeic acid was produced by feeding *p*-coumaric acid from *E. coli* strains carrying 4HPA3H from *P. aeruginosa*.[202] In order to achieve *de novo*

biosynthesis of caffeic acid, both 4HPA3H and TAL were expressed in *E. coli*.[190] Engineering of a phenylalanine over-producer to increase tyrosine supply in combination with the heterologous pathway optimization allowed caffeic acid production up to 776 mg/L in shake flask studies from glucose and glycerol.[203] In the same period, Sam5 and Sam8-mediated caffeic acid biosynthesis was also established, leading to caffeic acid production from glucose, although the titer achieved was lower than that of the 4HPA3H-mediated pathway.[198] The additional expression of the *O*-methyltransferase from *Arabidopsis thaliana* with the above mentioned enzymes led to the extension of caffeic acid pathway and achieved the production of ferulic acid.[198] By utilizing the broad substrate specificity of 4HPA3H, production of salvianic acid A (or 3,4-dihydroxyphenyl lactic acid) was also achieved by shunting the tyrosine biosynthesis pathway via 4-hydroxyphenylpyruvate. A metabolically optimized strain was reported to produce this molecule up to 7.1 g/L by fed-batch fermentation, with a yield of 0.47 mol/mol glucose.[204] In addition to free acids, microbial production of hydroxycinnamic acid esters has been reported recently. For example, *E. coli* over-expressing 4-coumarate CoA: ligase (4CL) and hydroxycinnamoyl transferases (HCTs) can make use of endogenously produced quinate and shikimate, as well as supplemented *p*-coumaric acid to produce chlorogenic acid and *p*-coumaroyl shikimates, respectively.[205] Furthermore, *de novo* production of rosmarinic acid was also achieved in *E. coli* by simultaneous reconstitution of caffeic acid and 3,4-dihydroxyphenyl lactic acid biosynthesis pathways, in combination with the action of rosmarinic acid synthase.[206]

Flavonoids

Flavonoids consist of various phenylpropanoids such as flavones, flavanones, isoflavones, flavanols, flavonols and anthocynidins. So far, the majority of studies concerning flavonoid biosynthesis are focused on pinocembrin and naringenin, the simplest flavanones serving as gateway molecules to all other flavonoid compounds. Biosynthesis of pinocembrin and naringenin starts from the ligation of CoA with cinnamic acid and *p*-coumaric acid catalyzed by 4CL to form the starter molecules cinnamoyl-CoA and *p*-coumaroyl-CoA, respectively. Then chalcone synthase (CHS) catalyzes the repeated condensation of 3 molecules of malonyl-CoA with the starter molecules to form flavanone chalcones via Claisen cyclization. Finally, the chalcones can be converted to pinocembrin and naringenin either spontaneously or by the action of chalcone isomerase (CHI). An engineered *E. coli* strain expressing PAL/TAL, 4CL, CHS and CHI produced Naringenin and pinocembrin were produced 58 mg/L of pinocembrin from phenylalanine and 60 mg/L of naringenin from tyrosine.[207] Similarly, *S. cerevisiae* strains expressing 4CL, CHS and CHI produced 16.3 mg/L of pinocembrin and 0.2 mg/L of naringenin from the corresponding phenylpropanoid acids.[208]

Further increase in the production of flavanones was achieved by elevating the intracellular malonyl-CoA availability. On one hand, over-expression of acetyl-CoA carboxylase (ACC) and biotin ligase can increase the conversion of acetyl-CoA to malonyl-CoA, leading to significant improvement in the production of pinocembrin (429 mg/L), naringenin (119 mg/L) and eriodictyol (52 mg/L) from cinnamic acid, *p*-coumaric acid and caffeic acid, respectively.[207, 209] On the other hand, over-expression malonyl-CoA synthetase (MatB) and a putative malonate transporter (MatC) that promotes the conversion of exogenous malonate to malonyl-CoA resulted in similar improvement in

flavanones production.[210] Besides, minimization of genetic interventions assisted by a genome-sale metabolic model allowed the carbon flux to be redirected to malonyl-CoA and led to higher titers of naringenin (474 mg/L).[211] Recently, a strategy employing antisense RNAs was reported to enrich malonyl-CoA concentration, which shows the potential to replace the use of certain antibiotics that inhibit fatty acids synthesis.[212] Optimized modular expression by adjusting promoter strengths and plasmid copy numbers enabled the balance of metabolic pathways, capable of producing 100.64 mg/L (2S)-naringenin in *E. coli* from glucose.[213]

Production of more complicated flavanoids derived from flavonones, such as 5-deoxyflavanones, flavones, isoflavones, flavonols and anthocyanins has been reviewed elsewhere.[214]

Stilbenoids

Stilbenoids are the analogs of stilbene with simple substituent groups. A representative of this group is resveratrol which is well-known for its antioxidant, anti-aging, and cancer preventative effects. Similar to naringenin, resveratrol is also biosynthesized via decarboxylative condensation of 3 malonyl-CoA molecules with the *p*-coumaroyl-CoA (Fig. 6); whereas the final cyclization step follows the Adol rather than Claisen pattern.[215] Heterologous production of resveratrol was initially established in *S. cerevisiae* by co-expressing 4CL and STS utilizing supplemented *p*-coumaric as the substrate.[216, 217] An optimized industrial yeast strain was reported to produce 391 mg/L resveratrol when the 4CL1 from *A. thaliana* and STS from *Vitis vinifera* were introduced simultaneously.[218] Further efforts to enhance resveratrol production in *E. coli* included synthetic biology approaches to engineer different vectors, leading to its biosynthesis at

1.4 g/L. In their work, improving malonyl-CoA availability further enhanced resveratrol production to 2.3 g/L.[219] Recently, 4HPA3Hs from *E. coli* and *P. aeruginosa* were found to display high catalytic efficiency to hydroxylate resveratrol to piceatannol.[220, 221]

Coumarins

Compared with flanovoids and stibenoids, microbial production of plant-specific coumarins is less explored due to the limited knowledge on their biosynthesis pathways.[222] Recently, Yan group reported the first microbial synthesis of scopoletin and umbelliferone using an artificial pathway that circumvented the unknown steps and obviated the use of P450 hydroxylases.[223] On the basis of the 4HPA3H-mediated caffeic acid biosynthesis pathway, additional expression of caffeate 3-*O*-methyltransferase, 4CL and feruloyl-CoA 6'-hydroxylase led to the production of scopoletin. In addition, another different biosynthesis mechanism not derived from phenylpropanoids was explored for the production of 4-hydroxycoumarin (4HC), a precursor for the anticoagulant warfarin. By introducing isochorismate synthase (ICS), isochorismate pyruvate lyase, salicyol-CoA ligase and a promiscuous bacterial quinolone synthase (PqsD) into *E.coli*, endogenous metabolites chorismate and malonyl-CoA were converted to 4HC via salicylate.[224] Very recently, the *E. coli* 4HPA3H was reported to be capable of catalyzing the region-specific hydroxylation of umbelliferone to esculetin with high yield (2.7 g/L). Interestingly, this enzyme is also displayed the capability of hydroxylating resveratrol into piceatannol (1.2 g/L), which exhibited superior activity than all the identified and engineered CYP hydroxylases.[220]

4.3 Isoprenoids

Isoprenoids, one of the largest classes of plant natural products, have been identified with more than 40,000 different molecules.[225] Some pharmaceutically important compounds such as taxol (a potent anticancer drug), artemisinin (a widely used anti-malarial drug) and carotenoids (e.g. lycopene and β-carotene) belong to this class. Their biosynthesis usually starts from the generation of two common precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Carbon chain elongation is then achieved via the continuous condensation of IPP to DMAPP. In nature, IPP and DMAPP are generated from two distinct isoprenoid pathways: the mevalonate (MVA) pathway occurring in all eukaryotes and the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway in most bacteria (Fig. 7).

Artemisinin precursors

Artemisinin is a sesquiterpenoid lactone endoperoxide produced by *Artemisia annua*. So far, the direct microbial production of artemisinin has not been achieved yet. Alternatively, the biosynthesis its precursor artemisinic acid has been extensively studied, since it can be easily converted to artemisinin via chemical processes. The first step towards artemisinic acid production is the formation of the intermediate amorpha-4,11-diene by expressing the amorpha-4,11-diene synthase (ADS) from *A. annua* which catalyzes the cyclization of farnesyl diphosphate (FPP).[226] Engineering of the MVA pathway in *S. cerevisiae* to increase supply of FPP and the introduction of the amorphadiene synthase and a cytochrome P450 monooxygenase (AMO, along with its reductase) responsible for the final oxidation steps resulted in the production of significant amount of artemisinic acid up to 100 mg/L.[3] Further improvement was reported by optimizing the selection markers and media compositions. The use of a high-

density fed-batch fermentation process allowed the titer of artemisinic acid to be enhanced to 2.5 g/L when galactose was used as sole carbon source.[227] Other than yeast, biosynthesis of artemisinic acid was also explored in *E. coli*. Indeed, *E. coli* natively carries the MEP, as well as prenyldiphosphate pathway. The initial expression of heterologous enzymes in the downstream isoprenoid biosynthesis pathways led to the first *E. coli* strains producing plant sesquiterpenes. However, the titers achieved were very low due to the low availability of prenyldiphosphate which significantly limited isoprenoid production.[228] To overcome this, the MVA pathway from *S. cerevisiae* was partially or entirely reconstituted in *E. coli*.[229] Optimization of the expression level of the MVA pathway enzymes alleviated the growth inhibition caused by the accumulation of a toxic intermediate and further improved isoprenoid production.[230] In addition, employment of HMG-CoA synthase and HMG-CoA reductase from *Staphylococcus aureus* instead of the counterparts from *S. cerevisiae* achieved a two-fold increase in the production of amorpha-4,11-diene. This led to boosting the titer to 27.4 g/L in fermenter under carbon and nitrogen-restricted conditions.[231] On this basis, expression of the codon-optimized genes of AMO and CPR from *A. annua* allowed their functional expression in *E. coli*, leading to the conversion of amorpha-4,11-diene to artemisinic acid. Interestingly, replacement of the AMO membrane anchor with the sequences from other cytochrome P450s (CYPs) resulted in the accumulation of 100 mg/L artemisinic acid under optimized cultivation conditions.[232] Alternatively, a distinct semi-biosynthetic approach for artemisinin production was established via a non-natural intermediate artemisinic-11S,12-epoxide. This compound was produced up to 250 mg/L through the

selective oxidation of amorpha-4,11-diene catalyzed by a rationally engineered hydroxylase P450 BM3 from *Bacillus megaterium*.[233]

Taxol precursors

Taxol (paclitaxel) is a widely used cancer drug that was first isolated from the bark of the Pacific yew tree. Due to its extremely low abundance in nature, exploration of the microbial synthesis of taxol is highly desirable to achieve its inexpensive supply.[234] Compared with artemisinin, the reconstitution of taxol biosynthesis pathway is more challenging. This pathway involves 19 enzymatic steps, about half of which are catalyzed by cytochrome P450 related enzymes.[234] So far, direct production of taxol in microorganisms has not been realized yet. Taxa-11,4-diene is a major intermediate that has been targeted for microbial production. The earliest attempt to taxa-11,4-diene production was through a series of *in vitro* enzymatic reactions catalyzed by isopentenyl diphosphate isomerase from *Schizosaccharomyces pombe*, taxadiene synthase, and geranylgeranyl diphosphate synthase from *Erwinia herbicola*. On this basis, expression of the three enzymes in combination with the native deoxyxylulose-5-phosphate (DXP) synthase in *E. coli* led to the reconstitution of a hybrid biosynthetic pathway and the production of taxadiene (1.3 mg/L) *in vivo*.[235] Recently, a multivariate modular optimization approach was successfully employed, which improved taxadiene production dramatically.[236] In their work, the taxadiene biosynthesis pathway was divided into two modules: the native MEP upstream pathway forming IPP and the heterologous pathway yielding taxadiene. Systematic adjustment of their expression levels separately resulted in a balanced metabolism by which the accumulation of a toxic molecule indole was minimized. The optimized strain was capable of producing taxadiene up to 300 mg/L in

shake flask studies. This titer was finally boosted to about 1 g/L in fed-batch fermentation using glycerol as the carbon source. In the same study, one more step towards taxol biosynthesis was also explored to convert taxadiene to taxadien-5α-ol by a cytochrome P450-mediated oxidation.[236] In addition, taxadiene production has also been investigated in yeast.[237] Introduction of the geranylgeranyl pyrophosphate (GGPP) synthase and the taxadiene synthase from *Taxus chinensis* reconstituted a part of the pathway from IPP and DMAPP, leading to the taxa-4(5),11(12)-diene in *S. cerevisiae*. Similarly, taxadiene production was also improved by the elevation of precursor supply. Moreover, 50% increase in titer was achieved by the truncation of HMG-CoA reductase that alleviated feedback inhibition. Finally, 8.7 mg/L of taxadiene was produced by replacement of *T. chinensis* geranylgeranyl diphosphate synthase with its counterpart from *Sulfolobus acidocaldarius*, as well as the use of a codon-optimized *T. chinensis* taxadiene synthase gene.[237]

Carotenoids

Carotenoids are tetraterpenoids that are biosynthesized by condensing two molecules of GGPP. The initial metabolic engineering efforts for their production were in yeast. However, enhanced production has been reported in *E. coli*, notably for the production of *β*-carotene and lycopene. Since *E. coli* natively harbors the MEP and prenyldiphosphate pathway, the expression of phytoene synthase (PS) and phytoene desaturase (PD) from *Erwinia* enabled the production of lycopene in *E. coli*. Based on this, the production of *β*-carotene was achieved by expressing a lycopene *β*-cyclase.[238] Two approaches were utilized to improve carotenoids production in *E. coli* – enhancing prenyldiphosphate pathway to improve GGPP and DMAPP availability or enhancing MEP pathway to

improve IPP and DMAPP availability. The prenyldiphosphate pathway was engineered via over-expression of native GGPP synthase (GGPPS) and IPP isomerase. Alternatively, over-expression of heterologous GGPPS and IPP isomerase also resulted in enhancing carotenoids production.[239, 240] In another approach, IPP and DMAPP availability was increased via over-expression of the MEP pathway enzymes.[241] Other than these two strategies, a similar approach as used in artemisinic acid production was also used- to circumvent the undesirable native regulatory mechanisms. This was achieved via engineering of an exogenous MVA pathway in *E. coli* consisting of an upper pathway module and a lower pathway module from *Streptococcus pneumonia* and *Enterococcus faecalis*, respectively. These efforts boosted lycopene titer to 465 mg/L in *E. coli*.[242] Additionally, another critical insight to improving lycopene production in *E. coli* was elucidated by shifting the balance of intracellular pyruvate availability towards glyceraldehyde-3-phosphate (GA3P).[243]

In addition to the commonly used metabolic engineering strategies mentioned above, carotenoids production was also explored with intriguing synthetic biology approaches. For instance, an artificial dynamic regulatory circuit capable of responding to cellular metabolic states was used in order to direct the carbon flow into the heterologous carotenoid (lycopene) producing pathway. This strategy overcame metabolic imbalance and improved lycopene production from trace amounts to 150 mg/L.[244]

A computational analysis of lycopene production pathway led to the identification of critical mutagenesis targets and guided the construction of superior strains, producing lycopene at about 18 mg/g DCW.[245] Another approach to identify critical genes influencing lycopene production was by building a shotgun library and carefully

screening it via colorimetric methods.[246, 247] Apart from genetic manipulations, the effects of process optimization via feeding different carbon sources or amino acids have also been studied. This was shown to achieve β-carotene production at 2.47 g/L and lycopene production at 1.35 g/L.[248, 249] The lycopene/ β-carotene pathways have been further expanded to other valuable carotenoids such as zeaxanthin, astaxanthin, lutein.[250]

5. Challenges and perspectives

The synthesis of chemicals from microbes can be assigned to three categories. (1) The production of simple, naturally occurring chemicals which are achieved via traditional metabolic engineering efforts. (2) The production of natural chemicals involving complex reaction chemistry (polyketide synthases, CYPs, membrane bound proteins) and molecules that can be achieved via the efficient production of the target chemical's precursor, following which chemical methods can be used for its production. (3) The most challenging category of metabolic engineering involves the biological production of non-natural chemicals. The hurdle of achieving their biological production can be overcome by identifying enzymes capable of carrying out similar reaction chemistry as by the chemical routes. An example of this approach is well demonstrated for the production of 1,4-butanediol.[84]

The establishment of novel metabolic pathways often requires expansion and/ or manipulation of native metabolism. To this end, bottlenecks in enhancing their production can be overcome by use of synthetic biology and protein engineering tools. Regulation of metabolic pathways, fine tuning protein expression levels and improving biocatalysis have been demonstrated via recent development of enabling technologies such as antisense RNA[251], MAGE (multiplex automated genome engineering)[252],

compartmentalization of metabolic pathways[42], use of DNA/protein scaffolds[253], directed evolution[17].

Although a number of metabolic routes have been established for production of chemicals, there is a dearth in the number of successful industrial scale production processes. Various hurdles need to be overcome for commercialization of biological processes. First, the microbial host has to be systematically engineered, following which the target chemical has to be produced at high titer, high yield and high volumetric productivity. Furthermore, manufacturing costs need to be reduced via process optimization and scale up, and lastly the separation process needs to be economical and efficient. More detailed discussions on these approaches have been extensively reviewed recently.[254-256]

Acknowledgements

This work is supported by grants from The National High Technology Research and Development Program of China (2011AA02A207); National Natural Science Foundation of China (21176018, 21376017 and 21406010) and Program for Changjiang Scholars and Innovative Research Team (IRT13045). We also acknowledge the support from National Science Foundation, USA (grants 1335856 and 1349499); and the American Heart Association Scientist Development grant (11SDG6960001).

Figure Legends

Fig. 1. Microbial production of higher alcohols by keto acid pathways. ADH, alcohol dehydrogenase; KDC, keto acid decarboxylase; LeuABCD: leucine biosynthesis operon.

Fig. 2. Microbial production of alcohols using CoA-dependent pathways. AcoAT, acetoacetyl-CoA transferase; ADC, acetoacetate decarboxylase; ADH, alcohol dehydrogenase; AdhE2, aldehyde/alcohol dehydrogenase; ALDH, acetaldehyde dehydrogenase; AtoB, acetyl-CoA acetyltransferase; BktB, β-ketothiolase; Crt, crotonase; Hbd, acetoacetyl-CoA thiolase; Ter, *trans*-enoyl-coenzyme A reductase.

Fig. 3. Biosynthesis of fatty acids, biodiesel, alkanes/alkenes and hydrogen. AAR, fatty acyl-CoA reductase; AccABCD, ACR, fatty acyl-ACP reductase; acetyl-CoA carboxylase; ADD, aldehyde decarbonylase; FabA, 3-hydroxydecanoyl-ACP dehydratase; FabD, malonyl-CoA-ACP transacylase; FabH/B/F, β-ketoacyl-ACP synthase; FabG, 3-oxoacyl-ACP reductase; FabI, enoyl-ACP reductase; FadD, fatty acyl-CoA synthetase; FHL, formate hydrogen lyase; PFL, pyruvate-formate lyase; TesA, fatty acyl-ACP thioesterase.

Fig. 4. Microbial routes for the production of 2,3-butanediol, 1,4-butanediol, 1,3-propanediol and 1,2-propanediol. Intermediates: 4HB, 4-hydroxybutyrate; 4HB-CoA, 4-hydroxybutyrl-CoA; 4HBA, 4-hydroxybutyraldehyde; DHAP, dihydroxyacetone-phosphate; G3P, glycerol 3-phosphate; GA3P, glyceraldehyde 3-phosphate; OAA, oxaloacetate; SSA, succinyl semialdehyde. Enzymes: 4HBd, 4-hydroxybutyrate dehydrogenase; ALDC, acetolactate decarboxylase; ALS, acetolactate synthase, AdhE2, aldehyde/alcohol dehydrogenase; Cat1, succinate-CoA transferase; Cat2, 4-hydroxybutyryl-CoA transferase; Dar1, glycerol 3-phosphate dehydrogenase; DhaB123, glycerol dehydratase; FucO, 1,2-PDO oxidoreductase; GldA, glycerol dehydrogenase; Gpp2, glycerol 3-phosphate phosphatase; MgsA, methylglyoxal synthase; sADH, stereospecific secondary alcohol dehydrogenase; SucA, 2-oxoglutarate decarboxylase; SucD, succinate semialdehyde dehydrogenase; YdjG, methylglyoxal reductase; YqhD, 1,3-PDO oxidoreductase.

Fig. 5. Engineering and extending shikimate pathway for the production of muconic acid. Intermediates: PEP, phosphoenolpyruvate; E4P, erythrose 4-phosphate; DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate; 3-DHS, 3- dihydroxy shikimate; TYR, tyrosine; TRP, tryptophan; PHE, phenylalanine. Enzymes: AroF/G/H, 2-dehydro-3-deoxyphosphoheptonate aldolase; AroB, 3-dehydroquinate synthase; AroD, 3-dehydroquinate dehydratase; AroE, shikimate dehydrogenase; AroL/K, shikimate kinase; AroA, 3-phosphoshikimate-1-carboxyvinyltransferase; AroC, chorismate synthase; PDC, protocatechuate decaboxylase; CDO, catechol 1,2-dioxygenase; ADO, anthranilate 1,2 dioxygenase; $TrpE^{fbr}G$, anthranilate synthase (fed-back inhibition resistance mutant);

ICS, isochorismate synthase; IPL, isochorismate pyruvate lyase; SMO, salicylate1-monoxygenase; EntC, isochorismate synthase; EntB, isochorismatase; EntA, 2,3-dihydro-2,3-DHBA dehydrogenase; BDC, 2,3-DHBA decarboxylase.

Fig. 6. Biosynthesis of phenylpropanoids. 4CL, 4-coumaroyl-CoA ligase; C2'H, p-coumaroyl-CoA o-hydroxylase; C3H, p-coumarate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; CHI, chalcone isomerase; CHS, chalcone synthase; F6'H, feruloyl-CoA o-hydroxylase; ICS, isochorismate synthase; IPL, isochorismate pyruvate lyase; OMT, 3-O-methyltransferase; PAL, phenylalanine ammonia lyase; PQS, *Pseudomonas* quinolone synthase; SCL, salicoyl-CoA lyase; TAL, tyrosine ammonia lyase; STS, stilbene synthase.

Fig. 7. Biosynthesis of isoprenoids. Intermediates: ADS, amorphadiene synthase; AMO, amorphadiene oxidase; CPR, cytochrome P450 reductase; DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; GA3P, glyceraldehyde 3-phosphate; GGPP, geranylgeranyl diphosphate.MVA, mevalonate; IPP, isopentenyl diphosphate; LCY, lycopene β-cyclase; MEP, 2-C-methyl-D-erythritol 4-phosphate. Enzymes: FPPS, FPP synthase; GGPPS, GGPP synthase; IPPI, IPP isomerase; PD, phytoene desaturase; PS, phytoene synthase; TS, taxadiene synthase.

Legend for Table of Contents. This review provides a comprehensive outlook at the progress and developing trends in biosynthesis of chemicals.

Table 1. Synthesis of chemicals by metabolic engineering of microbes. This table represents the most significant metabolic engineering efforts and may not necessarily represent the highest titers achieved biologically.

Category	Subcategory	Chemical	Applications	Host/ Carbon source	Titer (g/L)	Ref.
Biofuels	Alcohols	Ethanol	Fuel additive, plastics, solvents, p-xylene, isobutyl acetate, isobutyl esters	*S. cerevisiae*/ DEHU and Mannitol	36.2	[15]
		1-Propanol		*E. coli*/ Glucose	10.8	[23]
		Isopropanol		*E. coli*/ Glucose	143	[26]
		1-Butnaol		*E. coli*/ Glucose	30	[35]
		Isobutanol		*E. coli*/ Glucose	50	[37]
	Fatty acids		Fuel additive, detergents, paints, food products, cosmetics, etc.	*E. coli*/ Glucose	6.9	[60]
	Alkanes/ Alkenes		Natural gas, gasoline, diesel, aviation kerosene	*E. coli*/ Glucose	0.58	[66]
	Other	Hydrogen	Fuel	*E. coli*/ Glycerol	1 mol/ mol	[69]
Bulk Chemicals	Diols	2,3-Butanediol	Polymers, antifreeze, perfumes, printing ink, food supplements, pharmaceuticals, fumigants, solvents, cosmetics, detergents	*Serratia marcescens*/ Sucrose	152	[81]
		1,4-Butanediol		*E. coli*/ Glucose	18	[84]
		1,3-Propanediol		*E. coli*/ Glucose	135	[2]
		1,2-Propanediol		*E. coli*/ Glucose	5.1	[21]
	Organic Acids	Lactic acid	Pharmaceuticals, polymer precursors, food additives,	*E. coli*/ Glucose	142.2	[115]
		Succinic acid		*E. coli*/ Glucose	99.2	[121]
		Fumaric acid		*E. coli*/ Glycerol	41.5	[128]

		Muconic acid	antibacterial agents, solvents, detergents	*E. coli*/ Glucose	38.6	[134]
		Malic acid		*S. cerevisiae*/ Glucose	59	[140]
		3-Hydroxypropionic acid		*K. pneumoniaea*/ Glycerol	49.3	[144]
Pharmaceuticals and Nutraceuticals	Amino acids	L-Glutamate	Feed additives, food nutraceuticals, hormones, antibiotics, anti-cancer drugs	*C. glutamicum*/ Glucose	37	[159]
		L-Lysine		*C. glutamicum*/ Glucose	120	[165]
		L-Phenylalanine		*E. coli*/ Glucose	50	[172]
		L-Tyrosine		*E. coli*/ Glucose	55	[175]
	Hydroxycinnamic acids	p-Coumaric acid	Pharmaceuticals, supplements in foods, cosmetics	*E. coli*/ Glucose	0.974	[198]
		Caffeic acid		*E. coli*/ Glucose and Glycerol	0.776	[203]
	Flavonoids	Naringenin		*E. coli*/ Glucose	0.474	[211]
		Pinocembrin		*E. coli*/ Glucose	0.429	[209]
	Stilbenoids	Resveratrol		*E. coli*/ p-Coumaric acid	2.3	[219]
	Coumarins	4-Hydroxycoumarin		*E. coli*/ Glycerol	0.5	[224]
	Isoprenoids	Amorpha-4,11diene		*E. coli*/ Glucose	27.4	[231]
		Taxadiene		*E. coli*/ Glycerol	1	[236]
		Lycopene		*E. coli*/ Glucose, Glycerol, L-Arabinose	1.35	[248]
		β-Carotene		*E. coli*/ Glycerol	2.47	[249]

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