Review

Advanced biofuel production in microbes

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The cost-effective production of biofuels from renewable materials will begin to address energy security and climate change concerns. Ethanol, naturally produced by microorganisms, is currently the major biofuel in the transportation sector. However, its low energy content and incompatibility with existing fuel distribution and storage infrastructure limits its economic use in the future. Advanced biofuels, such as long chain alcohols and isoprenoid- and fatty acid-based biofuels, have physical properties that more closely resemble petroleum-derived fuels, and as such are an attractive alternative for the future supplementation or replacement of petroleum-derived fuels. Here, we review recent developments in the engineering of metabolic pathways for the production of known and potential advanced biofuels by microorganisms. We concentrate on the metabolic engineering of genetically tractable organisms such as *Escherichia coli* and *Saccharomyces cerevisiae* for the production of these advanced biofuels.

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1 Introduction

To address energy security and climate change concerns, the US enacted the Energy Independence and Security Act of 2007, setting a goal to produce 36 billion gallons of renewable fuel by 2022, with 16 billion to be obtained from cellulosic ethanol (http://energy.senate.gov/public/_files/RL342941.pdf). Two years have seen great advances in the development of renewable biofuels. Ethanol produced from starch remains the most produced biofuel in the US, with nine billion gallons produced in 2008 (http://www.ethanolrfa.org/industry/outlook). Currently, there are 12 cellulosic ethanol plants either constructed or planned for construction across the US [1]. However, ethanol is not the ideal biofuel. Ethanol's corrosivity and hygroscopicity make it incompatible with existing fuel storage and distribution infrastructure [2], and the construction of novel infrastructure for an ethanol economy would cost hundreds of billions of dollars [3]. Further, despite its high octane number (116), ethanol contains only 70% of the energy content of gasoline [2]. The challenge, therefore, is to produce advanced biofuels that have high energy content and are compatible with storage and transportation infrastructures designed for petroleum based products, but which are also economically feasible to produce on an industrial scale.

In the near future, advanced biofuels need to have very similar properties to current transportation fuels. This will allow for maximum compatibility with existing engine design, distribution sys-

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Abbreviations: 2MB, 2-methyl-1-butanol; **3MB,** 3-methyl-1-butanol; **3MP,** 3-methyl-1-pentanol; **ACP,** acyl carrier protein; **AHAS,** acetohydroxy acid synthase; **DMAPP,** dimethylallyl pyrophosphate; **DXP,** deoxyxylulose; **FAEEs,** fatty acid ethyl esters; **FAMEs,** fatty acid methyl esters; **FPP,** farnesyl pyrophosphate; **GGPP,** geranylgeranyl pyrophosphate; **GPP,** geranyl pyrophosphate; **IPP,** isoprenyl pyrophosphate; **KMV,** 2-keto-3-methylvalerate; **MEV,** mevalonate; **PDH,** pyruvate dehydrogenase; **RBS,** ribosomal binding site

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tems, and storage infrastructure. Three transportation fuels in need of biosynthetic alternatives are gasoline, diesel, and jet fuel.

Gasoline, the fuel for spark ignition engines, is a mixture of C_4–C_{12} hydrocarbons. Linear, branched, and cyclic alkanes compose 40–60% of the fuel mixture, while aromatics compose the remaining 20–40%. With regard to fuel properties, biosynthetic alternatives to gasoline must achieve comparable energy content (32 MJ/L), transportability, and octane number (87–91) – a measurement of knocking resistance in a spark ignition engine. Potential advanced biofuels that could supplement or replace gasoline include short-chain alcohols and alkanes [2].

Diesel, the fuel for compression engines, is a mixture of C_9–C_{23} hydrocarbons with an average carbon length of 16. Linear, branched, and cyclic alkanes compose 75% of the fuel mixture, while aromatics compose the other 25%. Biosynthetic alternatives to diesel must achieve a comparable freezing temperature (–9.5°C), vapor pressure (0.009 psi at 21°C), and cetane number (50–60) – a measurement of combustion quality of diesel fuel during compression ignition. Potential advanced biofuels that could supplement or replace diesel include fatty acid methyl esters (FAMEs, biodiesel), fatty alcohols, alkanes, and linear or cyclic isoprenoids [2].

Finally, jet fuel, the fuel for gas turbines, is a mixture of C_8–C_{16} hydrocarbons. Jet fuel has a maximum of 25% aromatic compounds in the fuel mixture. Biosynthetic alternatives to jet fuel must achieve comparable net heat of combustion, low freezing temperature (–40°C), and high energy density (53.4 MJ/L). Potential advanced biofuels that could supplement or replace jet fuel include fatty acid- and isoprenoid-based biofuels [2].

Currently, the most convenient and cost-effective approach for large-scale production of advanced biofuels may be the engineering of microorganisms. First, recent advances in molecular, systems, and synthetic biology now allow for the rapid engineering of microbial biosynthetic pathways to produce a variety of advanced biofuel candidates such as alcohols, esters, alkanes, and alkenes from the isoprenoid and fatty acid pathways. Second, industrial fermentation knowledge can be readily applied to the microbial production of advanced biofuels. Third, as microbial advanced biofuels would be produced in bioreactors, production facilities could be placed wherever needed. Finally, once the breakdown of lignocellulosic biomass is economically feasible, the microbes could generate biofuel not from starchy agricultural products but rather from lignocellulosic biomass that cannot be used for food.

In the last 2 years, production of many potential advanced biofuels has been reported. In this review we concentrate on known and potential advanced biofuels produced by the genetically tractable organisms *Escherichia coli* and *Saccharomyces cerevisiae*. We divide this review into sections by the metabolic pathway exploited for the production of known and potential advanced biofuels (Fig. 1). We start by presenting the heterologous expression of the Clostridial C_3–C_4 alcohol biosynthetic pathway for the production of isopropanol and butanol. Then, we discuss the re-routing of the amino acid biosynthetic pathway for the production of medium- and long-chain alcohols. Next, we highlight advances in the metabolic engineering of the isoprenoid biosynthetic pathway for the generation of potential isoprenoid-based biofuels. We then touch on advances in the fatty acid metabolism for the generation of fatty acid-based biofuels. Finally, we note how synthetic and system biology tools have aided the development of advanced biofuel pathways and discuss how these tools may enable us to achieve titers of advanced biofuels large enough to ultimately replace petroleum-based transportation fuels.

2 Metabolic pathways

2.1 Heterologous expression of the Clostridial C_3–C_4 biosynthetic pathway for the production of isopropanol and 1-butanol in *E. coli* and *S. cerevisiae*

In nature, various species of *Clostridium* produce isopropanol and 1-butanol from acetyl-CoA [4] (Fig. 2). Isopropanol, a C_3 alcohol, has an energy density of 23.9 MJ/L, somewhat less than that of gasoline (32 MJ/L), a high octane number (108), and low water solubility when compared to ethanol. Isopropanol is currently used as a gasoline and diesel additive (http://www.epa.gov/otaq/regs/fuels/additive/web-dies.htm, http://www.epa.gov/otaq/regs/fuels/additive/web-gas.htm). The *Clostridium* isopropanol pathway requires 1 mol of glucose to produce 1 mol of isopropanol. The highest reported isopropanol concentration produced by *Clostridium acetobutylicum* is 1.8 g/L [5].

Butanol, a C_4 alcohol, is a biosynthetic alternative to gasoline. Butanol has an energy density of 29.2 MJ/L, which is comparable to that of gasoline (32 MJ/L), an octane number of 87, and can be mixed with gasoline at any percentage or even completely replace it [6]. Further, butanol's high

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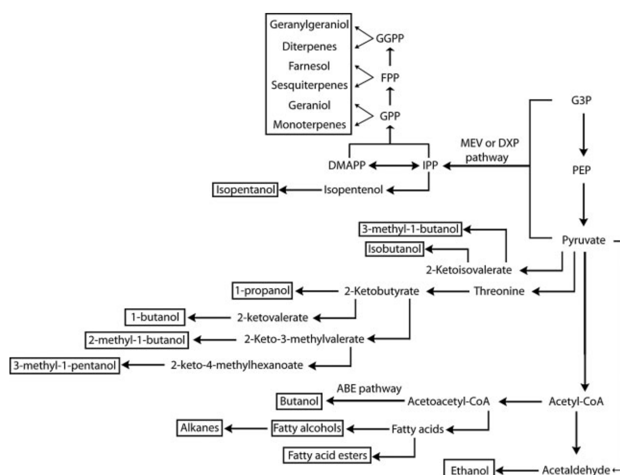

Figure 1. Overview of advanced biofuel biosynthetic pathways discussed in this review.

hydrophobicity may allow the use of existing fuel transportation and storage infrastructure without major modifications. The *Clostridium* butanol pathway requires 1 mol of glucose and 4 mol of NADH to produce 1 mol of butanol. The highest reported butanol concentration produced by *Clostridium beijerinckii* is 19.6 g/L [7].

Although native *Clostridium* produces butanol and isopropanol, the fact that it is a strict anaerobe with a slow growth rate means that it is necessary to tightly control fermentation conditions in order to maximize solvent production [8]. Further, the limited genetic tools available in *Clostridium* currently hinder the engineering of alcohol production in this organism to reach higher yields [8, 9]. To overcome these problems and reach higher alcohol titers, the *Clostridium* isopropanol pathway has been heterologously expressed in *E. coli*, while the butanol pathway has been expressed in both *E. coli* and *S. cerevisiae*.

Hanai *et al.* [9] reconstructed the *Clostridium* isopropanol biosynthetic pathway in *E. coli*. The authors expanded on a previous report that showed acetone production from acetyl-CoA in *E. coli* after introduction of three *C. acetobutylicum* genes: acetyl-CoA acyltransferase (*thl*), acetoacetyl-CoA transferase (*ctfAB*), and acetoacetate decarboxylase (*adc*) [10]. By overexpressing these three genes together with the *E. coli* codon optimized *C. beijerinckii* alcohol dehydrogenase (*adh*) to convert acetone to isopropanol, Hanai *et al.* elegantly reconstructed the isopropanol biosynthetic pathway in *E. coli*. Thinking that the low GC content of *C. acetobutylicum* genes may lead to poor protein expression, thus reducing overall isopropanol production, the authors tested native *E. coli* isozymes for acetyl-CoA acyltransferase (*atoB*) and acetoacetyl-CoA transferase (*atoAD*) to use in place of their counterparts from *C. acetobutylicum*. The authors also separately tested the *Thermoanaerobacter brockii* alcohol dehydrogenase (*adh*) in place of the *C. beijerinckii* alcohol dehydrogenase. The *E. coli* strain expressing *C. acetobutylicum thl* and *adc*, *E. coli atoAD*, and *C. beijerinckii adh* achieved the highest isopropanol production (4.9 g/L). Isopropanol was the major product of the *E. coli* isopropanol-producing strain followed by acetone and ethanol. The production achieved by Hanai *et al.* after 9.5 h of cultivation corresponds to 43.5% of the maximum theoretical yield. The 4.9 g/L isopropanol production by the engineered *E. coli* surpasses the highest reported production of isopropanol in *Clostridium* (1.8 g/L). The authors partially rationalized the higher isopropanol production achieved using the *E. coli atoAD* rather than the *C. acetobutylicum ctfAB* by the 22-fold lower K_m of AtoAD for acetate when compared to that of *C. beijerinckii* CtfAB. Further, the authors partially explain the higher isopropanol production in the presence of *C. beijerinckii* Adh by the fact that *C. beijerinckii* Adh showed higher *in vitro* alcohol

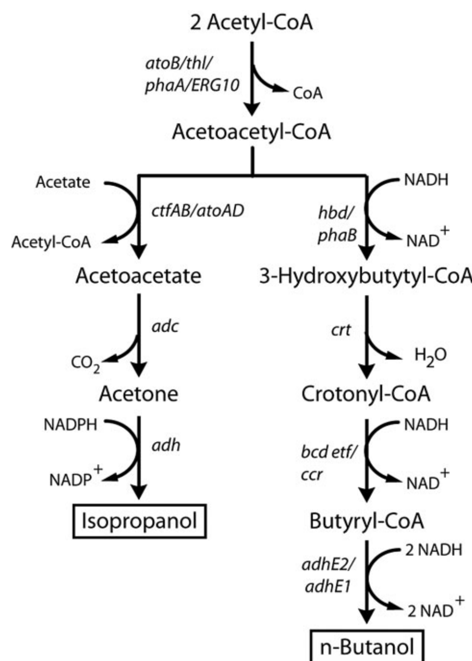

Figure 2. Adaptation of the Clostridia acetone–butanol–ethanol (ABE) fermentation pathway for the production of isopropanol and butanol in *E. coli* and *S. cerevisiae*. Gene symbols and the enzymes they encode: *atoB/thl/phaA/ERG10*, acetyl-CoA acetyltransferase; *hbd/PhaB*, 3-hydroxybutyryl-CoA dehydrogenase; *crt*, crotonase; *bcd etf/ccr*, butyryl-CoA dehydrogenase/electron transfer flavoprotein; *adhE2/adhE1*, aldehyde/alcohol dehydrogenase; *ctfAB/atoAD*, acetoacetyl-CoA transferase; *adc*, acetoacetate decarboxylase; *adh*, secondary alcohol dehydrogenase.

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dehydrogenase activity when compared to that of *T. brockii* Adh.

Jojima *et al*. [11] also reconstructed a *Clostridium* isopropanol pathway in *E. coli*. In contrast to Hanai *et al*., the *C. acetobutylicum thl*, *ctfAB*, *adc*, and the *C. beijerinckii adh* genes were each expressed from a dedicated promoter in a single vector. Expression of this construct resulted in an isopropanol production of 13.6 g/L, the major product, followed by acetone. The production achieved by Jojima *et al*. after 36 h of cultivation corresponds to 51% of the maximum theoretical yield. Jojima *et al*.'s isopropanol production (13.6 g/L) and theoretical yield (51%) were higher than those reported by Hanai *et al*. We speculate that the higher isopropanol production reached by Jojima *et al*. may be partially due to the separate expression of *thl*, *ctfAB*, and *adc* from dedicated promoters rather than polycistronic expression of the *thl–atoAD–adc* operon. The higher production reached by Jojima *et al*. may also be partially due to the use of a single vector isopropanol production system rather than the two vector system used by Hanai *et al*.

The *Clostridium* butanol biosynthetic pathway was reconstructed in *E. coli* by Atsumi *et al*. [8]. The authors introduced the *C. acetobutylicum* genes necessary to produce butanol from acetyl-CoA in two operons. The first operon encoded acetyl-CoA acetyltransferase (*thl*) and aldehyde/alcohol dehydrogenase (*adhE2*) while the second operon encoded 3-hydroxybutyryl-CoA dehydrogenase (*hbd*), crotonase (*crt*), and butyryl-CoA dehydrogenase/electron transfer flavoprotein (*bcd/etfAB*). Using this system, the engineered *E. coli* produced 13.9 mg/L of butanol. To increase butanol production, the authors replaced *C. acetobutylicum thl* with *E. coli atoB*, a move that increased butanol production more than three-fold. Next, the authors replaced *C. acetobutylicum bcd/etfAB* with *Megasphaera elsdenii bcd/etfAB* and *Streptomyces coelicor ccr*, but these replacements led to a decrease in butanol production. To further increase butanol production, the authors deleted host genes that would compete with the butanol pathway for the precursor acetyl-CoA or the cofactor NADH. The strain carrying the lactate dehydrogenase, ethanol dehydrogenase, and fumerate reductase deletions (Δ*ldhA*, Δ*adhE*, and Δ*frdBC*) and expressing the genes encoding the butanol biosynthetic pathway doubled butanol production over the strain with native copies of the chromosomal genes. However, deletion of phosphate acetyltransferase (Δ*ldhA*, Δ*frdBC*, Δ*adhE*, and Δ*pta*) together with expression of the butanol production pathway decreased butanol production. Deleting the gene encoding pyruvate formate lyase (Δ*ldhA*, Δ*frdBC*, Δ*adhE*, Δ*pta*, and Δ*pfl*) from the strain expressing the butanol production pathway nearly abolished butanol production, indicating that pyruvate formate lyase rather the pyruvate dehydrogenase (PDH) complex was the primary enzyme responsible for acetyl-CoA production. To activate PDHc and gain 1 mol of NADH *per* mole of acetyl-CoA produced, thus balancing the butanol production pathway, the authors deleted *fnr*, whose gene product regulates expression of the genes encoding PDH. The resulting strain (Δ*ldhA*, Δ*frdBC*, Δ*adhE*, Δ*pta*, and Δ*fnr*) had the highest butanol production (373 mg/L). Based on the glucose added to the minimal medium, butanol production corresponds to 11.7% of the maximum theoretical yield. Unfortunately, butanol was a minor product compared to ethanol (714 mg/L), formate (966 mg/L), and pyruvate (2.0 g/L), in part because the *fnr* deletion was not sufficient to fully activate PDH. Utilization of PDH rather than Pfl should balance redox in the butanol pathway and result in higher butanol production.

Inui *et al*.[16] [12] also reconstructed the butanol biosynthetic pathway in *E. coli*. Unlike Hanai *et al*., the authors expressed the complete butanol biosynthetic pathway from *C. acetobutylicum* in a single vector. Briefly, the first promoter drove expression of the *crt-bcd/etfAB-hbd* operon, while a second promoter drove expression of *thl*, and a third promoter drove expression of the alcohol dehydrogenase. In order to improve butanol production, Inui *et al*. also tested two *C. acetobutylicum* alcohol dehydrogenase isozymes (*adhE1* and *adhE2*). While expression of the butanol pathway with *adhE1* resulted in butanol production of 320 mg/L, expression of the same pathway with *adhE2* led to production of 540 mg/L. Increasing the production time to 60 h resulted in a maximal butanol production of 1.2 g/L. Butanol was not produced by the original not engineered *E. coli* strain. Taking into account the glucose added to the minimal medium, this production corresponds to a 7.2% maximum theoretical yield.

The third group to reconstruct the *Clostridium* butanol pathway in *E. coli* was Nielsen *et al*. [13]. Their work differentiates itself from its predecessors by studying the effects of polycistronic *versus* individual expression of butanol pathway genes on butanol production. The authors constructed the polycistronic version of the *C. acetobutylicum* butanol pathway in two vectors. The first vector expressed the *crt-bcd/etfAB-hbd* operon, while the second vector expressed *thl* and *adhE1* as an operon. The polycistronic butanol pathway produced 34.5 mg/L of butanol. Next, the authors construct-

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ed the butanol pathway with individual genes expressed from dedicated promoters in a total of four vectors. Individual expression of butanol biosynthetic genes resulted in 200 mg/L butanol production. It is notable that despite having an increased cellular burden from the four-vector system, the authors were still able to achieve a six-fold increase in butanol production when compared to the polycistronic two-vector system. Replacement of *C. acetobutylicum thl* for *E. coli atoB* improved butanol production marginally (220 mg/L). To increase the intracellular pool of NADH, the authors expressed the gene encoding *S. cerevisiae* formate dehydrogenase (*fdh1*), which catalyzes the conversion of formate to CO_2 while producing one molecule of NADH. Overexpression of *fdh1* increased butanol production 82%, to 400 mg/L. Separately, the authors tested butanol production by increasing the carbon flux through the glycolytic pathway by overexpressing the gene encoding glyceraldehyde-3-phosphate dehydrogenase (*gapA*), thus accelerating the metabolism of glycerol and achieving production of 580 mg/L butanol. Taking into account the glycerol added to the complex rich medium, this production corresponds to a 28.8% maximum theoretical yield from glycerol. Succinate (1.4 g/L), lactate (1.7 g/L), and ethanol (1.6 g/L) were the major products from this strain. Similar to Atsumi *et al*. [8], Nielsen *et al*. had a redox imbalance problem in its butanol-producing system as overexpressing formate dehydrogenase increased butanol production.

Steen *et al*. [14] constructed a butanol biosynthetic pathway in *S. cerevisiae*. The authors reasoned that *S. cerevisiae* may be a good host organism for butanol production because it is the current industrial host for ethanol production. Further, since butanol differs from ethanol by two carbons, *S. cerevisiae* may be able to tolerate butanol *via* the same mechanism it tolerates ethanol. First, Steen *et al*. tested expression of three acetyl-CoA acyltransferases (*Ralstonia eutropha* (*phaA*), *E. coli* (*atoB*), and *S. cerevisiae* (*ERG10*)) with *R. eutropha* 3-hydroxybutyryl-CoA dehydrogenase (*phaB*), *C. beijerinckii* crotonase (*crt*) and alcohol dehydrogenase (*adhE2*), and *Streptomyces collinus* butyryl-CoA dehydrogenase (*ccr*). The acetyl-CoA acyltransferase from *R. eutropha* (*phaA*) worked best with *phaB*, *crt*, *adhE2*, and *ccr*, producing 1 mg/L of butanol. To increase butanol production the authors tested the *C. beijerinckii* 3-hydroxybutyryl-CoA dehydrogenase (*hbd*) with all three acetyl-CoA acyltransferases (*phaA*, *atoB*, and *ERG10*). The authors hypothesized that the preference of *hbd* for NADH, rather than for NADPH as is the case of *phaB*, would be advantageous for butanol production under fermentative conditions when there is excess NADH. The biosynthetic pathway containing *Erg10*/*hbd* produced 2.5 mg/L of butanol, double the production of butanol when compared to the pathway containing *phaA*/*phaB*. The authors attributed this result to the fact that *Erg10* is the native acetyl-CoA acyltransferase and the fact that *hbd* uses NADH instead of NADPH. The authors also argue that PhaA/PhaB may have evolved to work together to maximize pathway flux in *R. eutropha*. This may be the reason why the pathway containing *phaA*/*phaB* produced more butanol (1 mg/L) than *phaA*/*hbd*, *atoB*/*phaB*, or *ERG10*/*phaB* (<0.5 mg/L). Finally, the authors substituted the butyryl-CoA dehydrogenase from *C. beijerinckii* (*bcd*/*etfAB*) for that from *S. collinus* (ccr), but it led to lower butanol production. The butanol yield obtained from the engineered *S. cerevisiae* (2.5 mg/L) was two orders-of-magnitude lower than that obtained from *E. coli* (550 mg/L). The authors suggest a potential bottleneck at the final reduction steps in the pathway due to the insolubility of AdhE2. Additionally, this may be explained by the fact that acetyl-CoA is the precursor for butanol production. In yeast, some of the acetyl-CoA pool is trapped in the mitochondrion, preventing it from being utilized in the cytosolic butanol pathway. We note, therefore, that solubilizing AdhE2 and increasing the cytosolic acetyl-CoA pool – for example, by creating a PDH bypass using pyruvate decarboxylase, acetaldehyde dehydrogenase, and acetyl-CoA synthase [15] – should increase the butanol yield.

On a final note, Atsumi *et al*. [8] mentioned that *E. coli* can tolerate butanol up to a concentration of 1.5%, meaning that without improving *E. coli*'s butanol tolerance, the current limit for butanol production will be in the vicinity of 10 g/L. Although none of the current butanol titers reach g/L levels, butanol toxicity may be of concern in further butanol production optimizations.

2.2 Re-routing of the amino acid biosynthetic pathway for production of higher alcohols

There are a number of alcohols that may have better fuel qualities than ethanol as gasoline replacements, and these alcohols can be produced from amino acid biosynthetic pathway intermediates (Fig. 3). Briefly, the amino acid biosynthetic pathway generates a number of keto acid intermediates. The yeast *S. cerevisiae* converts the keto acids in the leucine, valine, isoleucine, phenylalanine, tryptophan, and methionine pathways into "fusel" alcohols as a byproduct of fermentation [16]. Yeast produces these alcohols using the "Ehrlich pathway,"

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by sequential keto acid decarboxylation to the aldehyde followed by a reduction of the aldehyde to the alcohol [16]. *Via* the Ehrlich pathway it may be possible to overproduce: propanol and 2-methyl-1-butanol (2MB) from 2-ketobutyrate and 2-keto-3-methylvalerate (KMV), respectively, which are intermediates in the biosynthesis of isoleucine; isobutanol from 2-ketoisovalerate, an intermediate in biosynthesis of valine; 3-methyl-1-butanol (3MB) from 2-keto-4-methylpentanoate, an intermediate in the biosynthesis of leucine; 2-phenylethanol from phenylpyruvate, an intermediate in the biosynthesis of phenylalanine; and 1-butanol from 2-ketovalerate, a precursor in the norvaline biosynthetic pathway.

To access the potentially large array of higher alcohols from the amino acid biosynthetic pathway, Atsumi *et al*. reconstituted the Ehrlich pathway in *E. coli* by taking advantage of the promiscuity of 2-ketoacid decarboxylases (KDCs), to convert 2-keto acids to aldehydes, and alcohol dehydrogenases (ADHs), to convert the aldehydes into alcohols [17]. To test the capability of the endogenous pool of 2-ketoacids to be converted to alcohols, the authors tested five different KDCs with broad substrate specificity and coexpressed them with the *S. cerevisiae* alcohol dehydrogenase (*adh2*). *L. lactis kivd* led to the highest alcohol production. Further, due to the low levels of aldehydes in the metabolite analysis, the authors concluded that *adh2* was sufficiently active for the production of alcohols. Using *kivd* and *adh2*, six different 2-keto acid intermediates were converted into alcohols yielding: 1-propanol (31 mg/L), 2MB (68 mg/L), isobutanol (389 mg/L), 3MB (132 mg/L), 2-phenylethanol (40 mg/L), and 1-butanol (16 mg/L). Overall, production of alcohols *via* the keto acid pathway in *E. coli* requires the introduction of fewer heterologous enzymes (two) than the acetyl-CoA mediated production of isopropanol (three) and butanol (four) *via* the *Clostridium* pathway. A potential drawback of the use of this pathway for alcohol production stems from the broad substrate specificity of the decarboxylase and dehydrogenase steps, which results in the production of alcohol mixtures rather than single alcohols as all 2-keto acids can be converted to alcohols.

Atsumi *et al*. [17] targeted the isobutanol pathway for optimization due to isobutanol's high energy density (26.4 MJ/L), limited water solubility, and compatibility with existing fuel infrastructure. To increase isobutanol production, Atsumi *et al*. [17] overexpressed the *E. coli ilvHCD* genes – to increase the pool of available 2-ketoisovalerate – together with *kivd* and *adh2*. These changes led to a five-fold increase in isobutanol, to 1.7 g/L. Deletion of genes that divert the 2-ketoisovalerate pool from the isobutanol pathway (Δ*adhE*, Δ*ldhA*, Δ*frdAB*, Δ*fnr*, and Δ*pta*) resulted in a further increase in isobutanol production, to 2.2 g/L. Replacing *E. coli IlvIH* with the *B. subtilis alsS*, which has a higher affinity for pyruvate, increased isobutanol production to 3.7 g/L. The combination of deletions and overexpressions together with the deletion of *pflB*, which competes for pyruvate pools, resulted in 22 g/L isobutanol production. The 22 g/L production represents 86% theoretical yield conversion of glucose to isobutanol. This is a remarkable result, as the highest alcohol production previously reported, using the competing 1-butanol technology in *Clostridium*, was 19.6 g/L [7].

A strategy similar to the one used to optimize the isobutanol biosynthetic pathway was used to increase the production of 1-butanol from 2-ketovalerate [17]. As 2-ketovalerate is not naturally abundant in *E. coli*, Atsumi *et al*. increased the levels of 2-ketovalerate by taking advantage of the broad substrate specificity of LeuABCD to convert 2-ketobutyrate, which can be generated from threonine, to 2-ketovalerate. By overexpressing *ilvA* and *leuABCD* the authors obtained a three-fold increase in 1-butanol production (44.4 mg/L) over wild type. Since 2-ketobutyrate is also a 1-propanol intermediate, a mixture of 1-butanol/1-propanol was obtained. To further improve 1-butanol production, the authors deleted the *ilvD* gene. This deletion worked in two ways. First, *ilvD* deletion eliminates production of the native LeuABCD substrate, 2-ketoisovalerate, thus freeing up LeuABCD to convert more 2-ketobutyrate to 2-ketovalerate. Second, *ilvD* deletion eliminates production of 2-keto-3-methyl-valerate, a Kivd substrate, thus freeing up Kivd for 1-butanol production. Introduction of *ilvD* deletion led to a doubling of 1-butanol production when threonine was externally fed.

Via feeding experiments, Atsumi *et al*. [17] identified threonine as the limiting substrate in the production of 1-butanol/1-propanol. Deregulation of the threonine biosynthetic machinery enabled higher production of threonine and improved 1-butanol/1-propanol production [18]. By overexpressing the nonfeedback-resistant enzyme ThrA ($ThrA^{fbr}$) in the $ThrA^{fbr}BC$ operon, using a nonnative promoter, the authors achieved a three-fold increase of 1-butanol/1-propanol levels. Subsequent deletion of *metA* and *tdh* eliminated competing pathways for threonine consumption, bringing 1-butanol/1-propanol production to 1.2 g/L. To further reduce the diversion of 2-ketobutyrate away from 1-butanol/1-propanol, *ilvB* and *ilvl* were also deleted leading to a two-fold improvement in 1-butanol production and no change in 1-propanol

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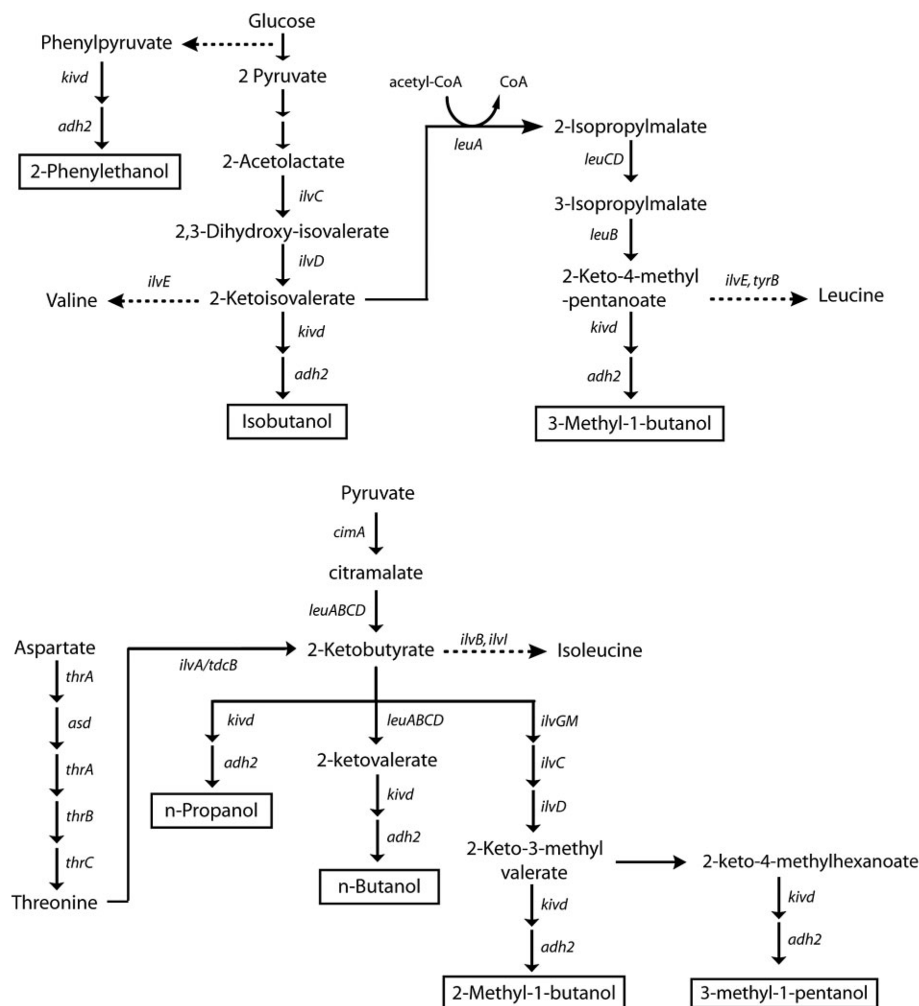

Figure 3. Re-routing of the amino acid biosynthetic pathway for production of higher alcohols. Gene symbols and the enzymes they encode: *ilvC*, acetohydroxy acid isomeroreductase; *ilvD*, dihydroxy acid dehydratase; *ilvE*, leucine transaminase; *kivd*, ketoisovalerate decarboxylase; *adh2*, alcohol dehydrogenase; *leuA*, 2-isopropylmalate synthase; *leuCD*, 2-isopropylmalate isomerase; *tyrB*, leucine aminotransferase; *thrA*, aspartate kinase/homoserine dehydrogenase; *asd*, aspartate semialdehyde dehydrogenase; *thrB*, homoserine kinase; *thrC*, threonine synthase; *tdh*, theonine dehydrogenase; ilvA, threonine deaminase; *tdcB*, threonine dehydratase; *ilvB*/*ilvI*, acetolactate synthase; *kivd*, ketoisovalerate decarboxylase; *adh2*, alcohol dehydrogenase; *ilvGM*, acetohydroxybutanoate synthase; *ilvC*, acetohydroxy acid isomeroreductase; *ilvD*, dihydroxy acid dehydratase; *cimA*, citramalate synthase; *leuB*, 3-isopropylmalate dehydrogenase.

production. Finally, to reduce the amount of acetyl-CoA being diverted from butanol to ethanol production, *adhE* was deleted. The combined effect of overexpressing *ilvA*, *leuA^{fbr}BCD*, *kivd*, and *adh2* overexpression and deleting *ilvB*, *ilvL*, *metA*, *tdh*, and *adhE* led to a highest combined production of 1-butanol/1-propanol of around 2 g/L. Further attempts at improving the mixed alcohol yield, by testing feedback resistant enzymes for two other steps of the pathway (TdcB for IlvA and LeuA^{fbr} for LeuA), did not increase alcohol production.

To reach a 1-butanol/1-propanol production higher than 2 g/L, Atsumi *et al*. introduced the *Methanococcus jannaschii* citramalate synthesis pathway in *E. coli*. Citramalate synthase (*cimA*) produces citramalate from pyruvate and acetyl-CoA, and the resulting citramalate can be directly converted to 2-ketobutyrate by LeuABCD [19]. To reconstruct the citramalate pathway, the authors overexpressed *M. jannaschii cimA* and the *E. coli leuABCD* genes in a strain unable to synthesize 2-ketobutyrate, a key intermediate in the synthesis of isoleucine. In such a strain, *cimA* overexpression allowed isoleucine production and accelerated the cell's growth rate. The authors observed partial rescue of the isoleucine auxotrophy by *cimA*, which they used as a platform to screen for *cimA* mutants with increased activity. After six rounds of mutagenesis and selection, the authors isolated CimA3.7. Overexpression of CimA3.7 in the 1-butanol/1-propanol biosynthetic pathway led to 3.5 g/L 1-propanol and 524 mg/L 1-butanol. The introduction

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of the citramalate synthase pathway in *E. coli* bypassed the threonine biosynthetic pathway for the generation of 2-ketobutyrate, thus creating a more direct route for the production 1-butanol/1-propanol.

Continuing with the theme of using amino acid biosynthetic pathways to produce higher alcohols, Connor and Liao [20] improved the production of 3MB, a C_5 alcohol with an energy density of 30.5 MJ/L close to that of gasoline (32 MJ/L). The production of 3MB builds onto previous work by Atsumi *et al*. [17] to produce isobutanol from 2-ketoisovalerate. For 3MB production, 2-ketoisovalerate must be re-routed through the leucine biosynthetic pathway to generate 2-ketoisocaproate, which can then be reduced to 3MB. To produce 3MB, Connor and Liao overexpressed *E. coli ilvIHCD* and *leuABCD*, *L. lactis kivd*, and *S. cerevisiae adh2* to yield 56 mg/L 3MB. To improve 3MB production, *ilvIH* was replaced with *B. subtilis alsS*. Introduction of the 3MB-producing genes into the strain previously optimized for isobutanol production (Δ*adhE*, Δ*frdBC*, Δ*ldhA*, Δ*pta*, Δ*fnr*, and Δ*pflB*) produced to 76 mg/L 3MB. Next, the authors addressed the issue of isobutanol by-product formation (about 2 g/L). To determine if 3MB production was low due to competition of Kivd for 2-ketoisovalerate or weak LeuABCD activity to generate 2-ketoisocaproate, the authors increased the intracellular concentration of LeuABCD using a synthetic ribosomal binding site (RBS). Increasing LeuABCD levels did not significantly increase 2-ketoisocaproate production, suggesting that LeuABCD activity rather than Kivd competition was the major bottleneck in the pathway. To increase the LeuABCD activity, the authors used the feedback inhibition mutant $LeuA^{fbr}$ and with LeuBCD, resulting in a 2-ketoisocaproate production of 1.61 g/L. Interestingly, a similar 2-ketoisocaproate production was obtained when *leuABCD* was expressed in a Δ*ilvE* and Δ*tyrB* strain. The overexpression of *ilvCD*, *alsS*, *kivd*, *adh2*, and *leuABCD* using a synthetic RBS in a Δ*ilvE*, Δ*tyrB*, Δ*adhE*, Δ*frdBC*, Δ*ldhA*, Δ*pta*, Δ*fnr*, and Δ*pflB* strain led to a production of 806 mg/L 3MB. Increasing the glucose concentration to 10 g/L and allowing longer fermentation time led to final 3MB titers of 1.28 g/L.

To improve the production of 2MB from 67 mg/L [17], Cann and Liao attempted to improve the production of the immediate precursor KMV [21]. The first committed step in the production of KMV is the condensation of 2-ketobutyrate and pyruvate to form 2-aceto-2-hydroxybutyrate. This step is catalyzed by acetohydroxy acid synthase (AHAS). To improve this step, the authors tested four different AHAS isozymes and determined that AHASII from *S. typhimurium* produced the highest titers of 2MB with the greatest selectivity. The next committed step in 2MB production is the transamination of threonine to 2-ketobutyrate. To improve this step, the authors tested three different threonine transaminase isozymes. With exogenously supplied threonine, overexpression of *C. glutamicum ilvA* led to the highest concentration of 2MB, converting 88% of the supplied threonine into 2MB. The overexpression of *S. typhimurium the* AHASII gene, *C. glutamicum IlvA*, *E. coli thrABC*, *L. lactis kivd*, and *S. cerevisiae adh2* produced 970 mg/L 2MB. Deletion of *metA* and *tdh* further boosted 2MB production (to 1.25 g/L) and reduced 1-propanol by-product formation. As 2MB is toxic to *E. coli* at concentrations as low as 1 g/L, improvement in *E. coli*'s tolerance to 2MB may need to accompany further improvements in 2MB production.

To access longer chain alcohols (>C_5), Zhang *et al*. [22] used LeuABCD to elongate KMV, a C_6 carboxylic acid intermediate in the synthesis of isoleucine, into 2-keto-4-methylhexanoate, which was converted into 3-methyl-1-pentanol (3MP) using a broad substrate specificity decarboxylase and dehydrogenase [22]. To construct the 3MP pathway, Zhang *et al*. overexpressed (i) the *E. coli* $thrA^{fbr}BC$ operon to increase 2-ketobutyrate pools; (ii) *E. coli tdcB* and *ilvGMCD* to convert 2-ketobutyrate into KMV; and (iii) *E. coli* $leuA^{fbr}BCD$, *K. lactis kivd*, and *S. cerevisiae adh6* to convert KMV into 3MP. The authors deleted *ilvE* and *tyrB* from *E. coli* to decrease flux away from the 3MP pathway. These optimizations resulted in the production of 40.8 mg/L of 3MP. To increase the production of 3MP, the authors used rational design to refine the substrate specificity of Kivd for 2-keto-4-methylhexanoate. Overexpression of Kivd:F381L/V461A led to a nine-fold improvement in 3MP production to 384.3 mg/L. To further increase 3MP production, the authors used rational design to engineer the gatekeeper enzyme of this pathway, LeuABCD, to more efficiently condense KMV and acetyl-CoA into 2-keto-4-methylhexanoate. Overexpression of LeuA:G462D/S139G led to the production of a mixture of nine different alcohols where 3-methyl-butanol had the highest production at 901 mg/L followed by 3MP with 793.5 mg/L, the highest production for this alcohol. The authors hypothesized that LeuA rational design mutants with larger binding pockets, in conjunction with Kivd:F381L/V461A, could produce alcohol mixtures containing novel long-chain alcohols. For example, expression of LeuA:G462D/S139G/N167A produced 4-methyl-1-hexanol, as part of a mixture of ten different alcohols, while expression of LeuA:G462D/S139G/N167A/H97A pro-

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duced 5-methyl-1-heptanol as part of a mixture of nine different alcohols. Further, expression of LeuA:G462D/S139G/H97A resulted in hexanol production as part of a mixture of nine alcohols. We believe the take away point of these findings is not necessarily the alcohol yields achieved, but the use of enzyme engineering to generate metabolic pathways able to produce novel, potential, advanced biofuels. Currently, the lack of specificity of the engineered enzymes, Kivd and LeuA, results in the production of alcohol mixtures rather than sole production of the desired alcohol. This problem can be potentially solved by further engineering of Kivd and LeuA for improved product specificity which may lead to the production of the desired alcohols as a single product.

2.3 Metabolic engineering of the isoprenoid biosynthetic pathway for the generation of potential isoprenoid-based biofuels

The different structures accessible *via* the isoprenoid biosynthetic pathway may allow the production of potential biosynthetic alternatives to gasoline, diesel, and jet fuel. For example, branched-chain, short terpenes or alcohols may be suitable gasoline substitutes, while longer cyclic or branched-chain terpenes may be appropriate as diesel and jet fuel substitutes. The feasibility of using the isoprenoid biosynthetic pathway for the production of potential advanced biofuels has been recently shown by the production of farnesene in *E. coli*, with yields of more than 14 g/L [23, 24]. Farnesene is a fuel precursor that can be hydrogenated to farnesane, which has a high cetane number (58) [24]. Terpene compounds currently being explored for jet fuel replacement include pinene, sabinene, and terpinene [24, 25].

Isoprenoids are assembled using the five-carbon monomer isoprenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP) (Fig 4). IPP and DMAP are condensed by IPP synthases into geranyl pyrophosphate (GPP, C_{10}), farnesyl pyrophosphate (FPP, C_{15}), or geranylgeranyl pyrophosphate (GGPP, C_{20}). Terpene synthases convert these long chain pyrophosphate molecules into branched chain or cyclic alkenes. Isoprenoid tailoring enzymes can then oxidize the alkenes into alcohols, or reduce them to alkanes. Alternatively, isoprenyl phosphatases or IPP synthase mutants can generate long-chain alcohols such as farnesol, geraniol, and geranylgeraniol from GPP, FPP, or GGPP, respectively.

Most of the work related to the isoprenoid pathway optimization has been done in relation to the production of amorphadiene, a C_{15} isoprenoid that is the key precursor in the semisynthesis of therapeutics and nutraceuticals. Therefore, in this section we emphasize the metabolic engineering of the isoprenoid biosynthetic pathway rather than the potential advanced biofuels generated by it.

One of the largest obstacles to the efficient microbial biosynthesis of isoprenoids is the production of the universal precursors IPP and DMAPP. In nature, *E. coli* produces IPP and DMAPP using the deoxyxylulose (DXP) pathway from pyruvate. Thus, previous metabolic engineering efforts have focused on optimization of the native DXP pathway [26–29]. However, Martin *et al*. [30] took an alternative approach to increase IPP and DMAPP production. The authors heterologously expressed the *S. cerevisiae* mevalonate (MEV) pathway in *E. coli* to generate IPP and DMAPP from acetyl-CoA. The MEV pathway was constructed in two vectors. The "upper" portion of the MEV pathway was composed of *E. coli* acetoacetyl-CoA thiolase (*atoB*) and *S. cerevisiae* HMG-CoA synthase (*HMGS*), a truncated soluble version of HMG-CoA reductase (*tHMGR*). The "lower" portion of the MEV pathway was composed of *S. cerevisiae* MEV kinase (*ERG12*), phosphomevalonate kinase (*ERG8*), and MEV pyrophosphate decarboxylase (*MVD1*), and *E. coli* IPP isomerase (*idi*) and FPP synthase (*ispA*). Expression of MEV pathway together with and *Artemisia annua* amorphadiene synthase produced 122 mg/L of amorphadiene. Production of amorphadiene was increased using a two-phased partitioning system which trapped over 97% of the amorphadiene produced in the organic phase resulting in a production of 480 mg/L of amorphadiene [31].

When transferring the *S. cerevisiae* MEV pathway into *E. coli*, the native pathway regulation was lost, thus potentially leading to an imbalance in gene expression and enzyme activity. Overexpression of a gene may deplete precursors or resources necessary for growth and production [32] or induce stress response from excessive heterologous protein expression [33], while imbalances in the total activity of an enzyme in the pathway can restrict carbon flux and/or lead to accumulation of a toxic intermediate in the pathway. To optimize the carbon flux through the MEV pathway, Pitera *et al*. [34] used feeding experiments to identify MEV as the limiting intermediate in the conversion of acetyl-CoA to amorphadiene. To address this imbalance, the authors overexpressed the "upper" part of the MEV pathway to overproduce MEV. However, this upregulation led to 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) accumulation, which was toxic and could be relieved by introducing extra copies of tHMGR. To balance the pathway flux gen-

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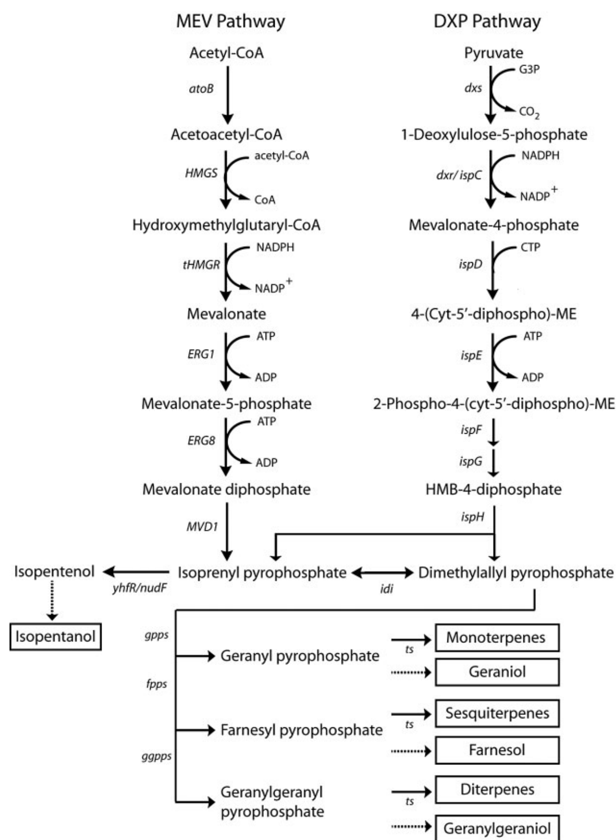

Figure 4. MEV and DXP pathway for the production of isoprenoids. Gene symbols and enzymes they encode: *atoB*, acetoacetyl-CoA thiolase; *HMGS*, hydroxymethylglutaryl-CoA synthase; *tHMGR*, truncated hydroxymethylglutaryl-CoA reductase; *Erg12*, MEV kinase; *Erg8*, phosphomevalonate kinase; *MVD1*, MEV pyrophosphate decarbonylase; *idi*, IPP isomerase; *dxs*, 1-deoxyxylulose-5-phosphate synthase; *dxr/ispC*, 1-deoxyxylulose-5-phosphaste reductoisomerase; *ispD*, methylerythritol-4-phosphate citidyltransferase; *ispE*, methylerythritol cyclodiphosphate synthase; *ispF*, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; *ispG*, 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase; *ispH*, IPP/DMAPP synthase; *yhfr/nudF*, isoprenylpyrophosphate phosphatase; *gpps*, geranyl pyrophosphate synthase; *ispA*, farnesyl pyrrophosphate synthase; *ggpps*, geranylgeranyl pyrophosphate synthase; *ts*, terpene synthase.

erated by the flux-limiting HMGR, Dueber *et al*. [35] scaffolded the three enzymes in the "upper" MEV pathway and obtained a 77-fold increase in MEV production over the unscaffolded pathway.

To increase amorphadiene production, Anthony *et al*. [36] focused on balancing the expression of the MEV pathway genes. The authors achieved a five-fold increase in the production of amorphadiene from the original three-plasmid system by (i) using the *E. coli* codon optimized genes for the "upper" portion of the pathway, (ii) placing the "upper" and "lower" portions of the pathway under control of a stronger promoter, and (iii) placing the complete MEV pathway in a single vector. Using the one vector system, the authors titrated genes in the "lower" part of the MEV pathway and identified MEV kinase as the rate-limiting enzyme. By expressing the single vector MEV pathway together with a vector harboring amorphadiene synthase and an extra copy of MEV kinase the authors reached their highest amorphadiene production of 293 mg/L/OD.

Tsuruta *et al*. [37] improved amorphadiene production by bioprospecting for enzymes in the "upper" portion of the MEV pathway. Knowing that accumulation of HMG-CoA limits flux through the MEV pathway [38], the authors tested *Staphylococcus aureus* and *Enterococcus faecalis* enzymes in place of the *E. coli*-codon optimized *S. cerevisiae HMGS* and *HMGR*. The construct carrying *E. coli atoB* and *S. aureus mvaS* and *mvaA* improved amorphadiene production 30%. Improvements in fermentation conditions increased amorphadiene titers to 27 g/L.

A eukaryotic platform for the production of isoprenoids could accelerate the bioprospecting of terpene synthases and terpene tailoring enzymes – most being of plant origin – which may be difficult to express in a prokaryote host due to the different codon usage. Ro *et al*. [39] engineered a *S. cerevisiae* platform for the overproduction IPP and DMAPP. By overexpressing *S. cerevisiae tHMGR*, downregulating squalene synthase (*ERG9*), which competes for FPP pools, replacing the sterol transcription regulator *upc2* with a semifunctional mutant, *upc2-1*, and overexpressing FPP synthase, *Erg20*, the engineered *S. cerevisiae* strain produced 153 mg/L amorphadiene. As with *E. coli*, this platform can be readily adapted to produce biofuel-relevant terpenes by changing the prenyl transferase and the terpene synthase.

The *S. cerevisiae* and *E. coli* platforms for IPP and DMAPP overproduction have been used to generate oxidized sesquiterpenes. Artemisinic acid, a closer intermediate to the antimalarial drug artemisinin than amorphadiene, has been produced at 115 mg/L in yeast by overexpressing amorphadiene synthase together with *A. annua* P450 CYP71AV1 and its native redox partner, CPR [39]. Production of artemisinic acid in *E. coli* at 105 mg/L was achieved by expressing amorphadiene synthase and the *E. coli* codon optimized CYP71AV1 with an engineered N-terminal transmembrane domain for better *E. coli* expression [40].

Demonstrating the wide range of structures that the isoprenoid biosynthetic pathway is able to access, Yoshikuni *et al*. [41] introduced terpene synthase mutants into the *E. coli* platform for IPP and DMAPP overproduction. The authors were able to produce eight different terpenes: α-farnesene, β-

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farnesene, sabinene, γ-humulene, sibirene, longifolene, α-longpinene, α-ylange, and β-bisabolene in different ratios. Some of these structures may be advanced biofuels or biofuel precursors, such as farnesene.

The high energy density of isopentanol was a motivation for the re-routing of the isoprenoid pathway for the production of isopentenols *via* dephosphorylation of IPP. Exploiting an observation that prenyl diphosphate accumulation is toxic to *E. coli* and leads to a slower cell growth phenotype, Withers *et al*. [42] screened a *B. subtilis* cDNA library for enzymes that were able to relieve the toxicity of accumulated prenyl diphosphates in *E. coli*. Although the intended purpose was to find an isoprene synthase able to convert DMAPP to isoprene, the authors discovered two genes, *yhfR* and *nudF*, able to relieve prenyl diphosphate toxicity. Expression of *nudF* in the IPP- and DMAPP-overproducing strain led to the production of isopentenol at a final titer of 112 mg/L, which has since been improved to 1.2 g/L [4].

2.4 Metabolic engineering of the fatty acid biosynthetic pathway for the generation of fatty acid based biofuels

Long-chain alkanes and esters are valuable molecules for diesel and jet fuel, and the fatty acid biosynthetic machinery could be utilized to produce these valuable fuels. Alcohols could be produced by sequential reduction of the fatty acid to the fatty alcohol, whereas alkanes could be produced by reduction of the fatty acid to the aldehyde followed by decarbonylation [43, 44], or from further reduction of the fatty aldehyde to alcohol and then to an alkane [45], although this route could not be later corroborated [46]. Finally, fatty acids could be converted to esters (biodiesel) *via* esterification with small alcohols [47].

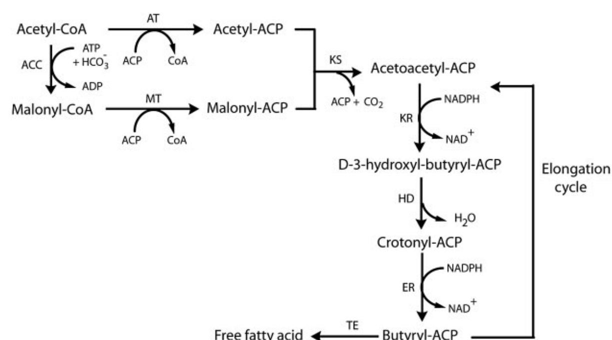

Figure 5. Fatty acid biosynthetic pathway. Symbols and enzymes they encode: ACP, acyl carrier protein; ACC, acetyl-CoA carboxylase; AT, acetyl transacylase; MT, malonyl transacylase; KS, β-ketoacyl-ACP synthase KR, β-ketoacyl-ACP reductase; HD, β-hydroxyacyl-ACP dehydratase; ER, enoyl-ACP reductase; TE, thioesterase

In fatty acid biosynthesis, two carbon units are sequentially condensed onto a growing fatty acyl chain (Fig. 5). First, acetyl-CoA carboxylase generates malonyl-CoA from acetyl-CoA and bicarbonate in the committed first step of the fatty acid biosynthesis pathway. Next, acetyl transacylase and malonyl transacylase convert acetyl-CoA and malonyl-CoA into acetyl-acyl carrier protein (acetyl-ACP) and malonyl-ACP, respectively. In the elongation step, β-ketoacyl-ACP synthase condenses acetyl-ACP and malonyl-ACP to form acetoacetyl-ACP. This molecule is then reduced (β-ketoacyl-ACP reductase), dehydrated (β-hydroxyacyl-ACP dehydratase), and reduced a second time (enoyl-ACP reductase), with the end result of generating butyryl-ACP. The elongation cycle will then repeat itself. In eukaryotes, the four enzymes necessary for fatty acid elongation are contained in a multidomain protein (type 1 fatty acid synthase), while in *E. coli* each enzymatic activity in the elongation step is performed by a monofunctional enzyme (type 2 fatty acid synthase). Once the fatty acid has reached a certain carbon length (*e.g.*, C_{16}), in eukaryotes a thioesterase hydrolyzes the fatty acid-ACP to generate free fatty acid. In prokaryotes, the fatty acid-ACP is directly transferred to glycerol-3-phosphate without releasing a free fatty acid.

The first step in generating biofuels from fatty acids is to overproduce free fatty acids. Michinaka *et al*. [48] evolved a *S. cerevisiae* strain with improved free fatty acid secretion. The authors began with a yeast strain carrying an acyl-CoA oxidase with reduced activity. When compared to wild type, this strain accumulated 2.6-fold more total intracellular fatty acids, but secreted the same amount of free fatty acids into the medium (0.24 mg/10^9 cells). *Via* mutagenesis and screening, the authors obtained a mutant strain that secreted 4 mg fatty acids *per* 10^9 cells, a 16-fold increase in free fatty acid secretion over wild type. *Via* complementation assays, fatty acyl-CoA synthase (*FAA1*), which is involved in fatty acid degradation, was identified as a key regulator in fatty acid secretion. Deletion of *FAA1* from the wild-type yeast strain led to fatty acid secretion, although it did not account for all the fatty acid secretion observed in the mutant strain. To determine the genotype responsible for fatty acid secretion in *S. cerevisiae*, Scharnewski *et al*. [49] deleted two of the four acyl-CoA synthases, *FAA1* and *FAA4*. After these deletions the yeast strain was able to secrete 220 µmol/L of free fatty acids. Deletion of all four acyl-CoA synthases did not improve fatty acid se-

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cretion. The mutants showed a tight correlation between cell growth and fatty acid secretion and reimported a portion of those fatty acids during the stationary phase.

To increase fatty acid production one can try to decrease the break down of fatty acids by targeting fatty acyl-CoA synthase genes involved in the aerobic (*e.g.*, *fadD*) or anaerobic (*e.g.*, *yfcY* and *yfcX* [50]) oxidation of fatty acids. Alternatively one can divert the acetyl-CoA pool toward fatty acid biosynthesis by overexpressing genes in the fatty acid biosynthetic pathway. For example, increased expression of acetyl-CoA carboxylase has led to a 6.5-fold increase in free fatty acid accumulation [51]. Overproduction of free fatty acids in *E. coli* was accomplished by Lu *et al*. [52], who showed that deletion of *E. coli fadD* leads to a three-fold improvement in total fatty acid production (255 nmol/mL) over wild type (81 nmol/mL). Using a Δ*fadD* strain, the authors improved total fatty acid production by overexpression of the *Cinnamonum camphorum* acyl-ACP thioesterase, doubling total fatty acid production to 511 nmol/mL. The greatest production increase was seen in medium-chain fatty acids (C_{14}–C_{16}). Additional overexpression of *E. coli* acetyl-CoA carboxylase (*acc*) led to slight improvement in fatty acid production (635 nmol/mL). To increase the production of long chain fatty acids (C_{16}–C_{18}), the authors overexpressed the *E. coli* acyl-ACP thioesterase and *acc* as well as *C. camphorum* acyl-ACP thioesterases to reach titers of 1.5 mM. Under fermentation conditions, the fatty acid strain produced about 2.5 g/L. The authors determined that 70% of the fatty acids were in the free form and less than 10% can be found in the media. Based on this observation, the authors speculated that the free fatty acids were reabsorbed by the cell, to be utilized as an energy and carbon source, and that most fatty acids should be sequestered in the inner membrane. It should be possible to convert the free fatty acids to FAMEs or fatty alcohols that can be used as biofuels or specialty chemicals. The esterases and reductases would not be in competition with the diffusion of free fatty acids across the cell membrane and thus would be able to modify them inside the cell, thereby producing the final product more efficiently.

Biodiesels are alkyl esters of long-chain fatty acids with short chain alcohols. FAMEs and fatty acid ethyl esters (FAEEs) are produced after the esterification of fatty acids with methanol and ethanol, respectively. Biodiesel can be mixed at any ratio with pure diesel to run diesel engines. The major problems of biodiesel production are the geographical and seasonal restrictions of producing plant oil, and the noncost-effective transesterification with bioethanol. To address this problem, Karlscheuer *et al*. [47] engineered *E. coli* to produce FAEEs. First, the authors introduced the pyruvate decarboxylase (*pdc*) and the alcohol dehydrogenase (*adh*) from *Zymomonas mobillis* to generate an ethanol-producing pathway in *E. coli*. Next, the gene encoding the wax ester synthase/acyl-coenzyme A: diacylglycerol acyltransferase from *Acinetobacter baylyi* (*ws/dgat*), which is able to esterify ethanol with a fatty acid, was overexpressed. The authors successfully produced 1.28 g/L of FAEES by overexpressing *pdc*, *adh* and *ws/dgat*, and supplementing the media with oleic acid and glucose. Using the same principle, overexpression of *Acinetobacter calcoaceticus ws/dgat* in a *S. cerevisiae* background lacking four key enzymes in the triacylglycerol and steryl ester biosynthetic pathways led to the production of FAEEs and fatty acid isoamyl ester [53].

In addition to the reported efforts in the literature, the patent literature is rife with reports of fatty acid production [54–57], indicative of the technology's increasing viability. Finally, the reduction of fatty acids to the aldehyde followed by decarbonylation, the sequential reduction of fatty acids, or the decarboxylation of fatty acids all may lead to the production of long-chain alkanes. These products would be good alternatives to petroleum-based fuels. Reports in the patent literature suggest that such conversions are not only possible [57, 58], but may also be commercially viable.

3 Systems and synthetic biology for the optimization of metabolic pathways

As described above, several potential advanced biofuels have been produced in microbes. However, to attain titers that enable the cost-effective commercial production of biofuels, further engineering of the production pathways and the microbes themselves will be needed. To date, the majority of improvements in the production of advanced biofuels have been achieved by fine tuning discrete steps within the production pathway, or by improving the reactions that regulate carbon flux to and from the pathway. Yet, it may be possible to achieve higher advanced biofuel titers by simultaneously modifying variables that, at first glance, appear further removed from the pathway. To identify these variables, we need a system-level understanding of microbial metabolism and the effects of fuel toxicity [59]. By taking advantage of functional genomic techniques to monitor thousands of parameters simultaneously, and by then integrating

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these system-wide data into models, we should be able to accurately represent a snapshot of the cell metabolism. We could use these representations to predict potential bottlenecks in biofuel production that can be addressed experimentally. Therefore, to achieve high biofuel titers, iterative rounds of metabolic engineering and systems analysis would be needed. First, the microorganism is engineered for biofuel production. Then, the engineered microorganism is profiled using functional genomics and metabolic flux analysis to identify potential bottlenecks in production and toxicities resulting from pathway expression. Finally, the systems biology predictions are incorporated into the microbes and fuel production is tested. The metabolic engineering/systems biology cycle has been successfully applied for the microbial production of 1,3-propanediol [60] and amorphadiene/artemisinic acid [61]. Though this cycle has not yet been applied to the improvement of advanced biofuel production, it should be only a matter of time before we see improvements in biofuel production using this cycle, as papers studying the systems biology of biofuel producing microbes have been recently published [62].

A limitation of the metabolic engineering/systems biology cycle is the time and resource allocation necessary to execute functional genomic studies, integrate the system-wide data into models, and predict potential bottlenecks in microbial biofuel production. Synthetic biology offers a less resource-intensive and more time-efficient alternative with which to achieve improved biofuel production. Synthetic biology conceptualizes the cell as an assembly of parts that leads to a specific phenotype. By standardizing part connections, standardizing part analysis methods, analyzing the behavior of these parts, and developing models that describe part behavior, synthetic biologists should be able to predictably assemble the parts into functional devices to accomplish a particular goal (*e.g.*, development of a biofuel production pathway). Because the engineered parts are standardized, they can be introduced into any pathway, thus providing general tools for improving metabolic pathways experimentally. Synthetic biology is not without its challenges, however, chief among them the fine tuning required for the assembly of engineered parts into devices that work as desired. The problem of fine tuning is further compounded by the length of time necessary to manually assemble metabolic pathways with numerous and varied promoters, ribosome binding sites, *etc*.

Synthetic biology has been most successful at the *in vivo* regulation of enzymes. Codon optimization of heterologous genes has increased the expression of heterologous proteins [63]. Transcriptional control has been achieved by engineering promoters with a wide range of strengths in *S. cerevisiae* and *E. coli* to achieve the desired mRNA levels [64, 65]. Post-transcriptional control has been attained using synthetic RBSs [66] and tunable intergenic regions, the latter consisting of mRNA secondary structures, RNAse cleavage sites, and RBS-sequestering sequences [67]. In a more global approach, transcription factors have been engineered to achieve differential expression of several genes and achieve improvement of several phenotypes at once [68]. Finally, enzymes with improved activity or changed substrate or product specificity have been engineered using rational design and directed evolution methods that can lead to higher biofuel product yields [69, 70]. Some of these synthetic biology tools have already been used in the production of advanced biofuels as highlighted in this review. In the future, application of synthetic biology tools, as well as systems biology tools, may help us engineer microorganisms able to reach production titers necessary to make the production of advanced biofuels commercially feasible.

4 Conclusion

In the last 2 years, there have been great advances in the production of advanced biofuels in microorganisms, highlighted by the fact that some of the technologies developed in this time period are in the process of commercialization (http://www.gevo.com).

Re-routing amino acid biosynthetic pathways for production of medium-chain alcohols has been highly successful. However, use of the amino acid biosynthetic pathway for alcohol production also has its drawbacks. First, amino acid biosynthesis is tightly regulated *via* feedback inhibition of its intermediates; deregulation of these feedback mechanisms leads to the greatest increases in alcohol production. Application of this approach is limited to steps known to be feedback inhibited, and further limited by the paucity of known mutants that are feedback resistant. Therefore, further application of this approach will require the discovery of novel-feedback resistant enzymes. Second, the adaptation of the amino acid biosynthetic pathway to generate alcohols relies on the promiscuity of the decarboxylase and dehydrogenase to transform 2-keto acids to alcohols. This promiscuity leads to the production of a mixture of alcohols, though this may not be unduly detrimental as gasoline is currently a mixture of alkanes. We speculate however

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that improving the substrate specificity of the promiscuous enzymes *via* protein engineering will overcome this obstacle, resulting in the production of single alcohols. The most exciting part of the alcohol work is probably the utilization of protein engineering in conjunction with metabolic engineering to generate these novel molecules.

With respect to isoprenoid-based biofuels, the *E. coli* and *S. cerevisiae* platforms for isoprenoid production are in place to generate a wide array of terpene structures. The pressing questions here are, first, what kind of terpene molecules will work as the best biosynthetic alternatives to gasoline, biodiesel, and jet fuel, and second, whether the production of isoprenoid-based biofuels will reach the yields and productivities needed for economically viable production. Clearly, the branched nature and the large number and diversity of products generated from three basic precursors (IPP, GPP, and FPP) is clearly an advantage.

Fatty acid production appears to be a lively biofuel technology research area in industry. Press releases have shown that at least one company is currently able to produce biodiesel from microorganisms (http://www.ls9.com/news). The literature shows that free fatty acids can be overproduced in *E. coli* [52], and that fatty acids can be esterified with small alcohols *in vivo* [47]. These accomplishments lead us to believe that we will be able to produce fatty acid-derived fuels directly using *E. coli* in the future.

At this point no advanced biofuel production technology has come out on top. However, given the diversity of molecules in petroleum-based fuels and the significant quantities of renewable fuels that will be needed to power transportation, there is room for all of the technologies to be used. Of upmost importance will be economic viability, net energy gain, and carbon neutrality. The development of advanced fuels that will work with the existing transportation infrastructure is certainly a step in the right direction toward usability and economic viability.

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JDK has financial interest in Amyris and LS9, both of which are involved in producing advanced biofuels.

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