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Metabolic engineering of microbial pathways for advanced biofuels production

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Production of biofuels from renewable resources such as cellulosic biomass provides a source of liquid transportation fuel to replace petroleum-based fuels. This endeavor requires the conversion of cellulosic biomass into simple sugars, and the conversion of simple sugars into biofuels. Recently, microorganisms have been engineered to convert simple sugars into several types of biofuels, such as alcohols, fatty acid alkyl esters, alkanes, and terpenes, with high titers and yields. Here, we review recently engineered biosynthetic pathways from the well-characterized microorganisms *Escherichia coli* and *Saccharomyces cerevisiae* for the production of several advanced biofuels.

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Introduction

First generation biofuels such as bioethanol fermented from corn and biodiesel esterified from edible vegetable oils or animal fats capture 90% of the current biofuel market [1]. Replacing petroleum fuel with the first generation biofuels would require diverting farmland and crops for biofuel production, competing with world food supply and causing economic and ethical problems. Also, cultivating food crops for biofuel production consumes large amounts of water, fertilizers, and pesticides, which burden the environment [2]. Ethanol has the additional problems of containing about 70% of the energy content of gasoline and is miscible with water, making it difficult to distill from fermentation broth and corrosive to storage and distribution infrastructures [3]. Advanced biofuels are produced from nonfood cellulosic biomass, including but not limited to wheat straw, forest waste, and energy crops such as switchgrass. These feedstocks are either low cost agricultural byproducts or fast-growing, easily cultivated crops that provide more abundant cellulosic biomass for fuel production and do not compete with food supply while reducing water and fertilizer usage. Ideally, advanced fuels would have very similar energy content, storage and transportation properties, and combustion properties to current transportation fuels which would allow them to be used in existing gasoline, diesel, and jet engines [4]. Proposed advanced fuels include butanol, isopentanol, terpenes, fatty acid ethyl esters, and alkanes (Table 1).

Recent advances in microbial engineering offer the possibility to convert renewable resources into biofuels [5•]. After chemical treatment of feedstocks, cellulosic biomass is decomposed into simple sugars, which are metabolized by microorganisms and converted into biofuels. Although some microorganisms can produce certain biofuels naturally [6,7], native microorganisms often suffer from low growth rates, intolerance of toxic biofuel products, and incomplete carbon source usage (i.e. many microorganisms cannot metabolize xylose, which comprises approximately 30% of plant cellulosic biomass). Advanced techniques in synthetic biology and metabolic engineering enable the development of heterologous metabolic pathways in well-studied microbial hosts — such as *Escherichia coli* and *Saccharomyces cerevisiae* — for production of hydrocarbons from the entire sugar complement of biomass. These microorganisms have been used for industrial-scale production for many years, can be engineered to tolerate toxic biofuels [8] and metabolize a range of carbon sources [9,10]. The extensive characterization of these hosts and the plentiful genetic manipulation tools enable the engineering of heterologous pathways not only to improve production titers and yields but also to extend the choices of biofuels. Here we focus on the engineering of metabolic pathways within the microbial hosts *E. coli* and *S. cerevisiae* for the production of advanced biofuels. All the engineered metabolic pathways for biofuel production covered in this review start from three central metabolites: phosphoenolpyruvate, pyruvate, and acetyl-CoA (Figure 1), which are produced from simple sugars. Biofuels produced from green algae or photosynthetic cyanobacteria have been reviewed previously [11–13] and will not be discussed.

Table 1

Summary of advanced biofuels and their production.

Target fuel	Type of fuel	Strain	Key enzymes expressed	Titer (g/L)	Yield (%)	Fermentation time	Reference
Isopropanol	Gasoline additive	*E. coli*	Thl, AtoAD, Adc, Adh	143	67	240 h	[18]
Butanol	Gasoline additive	*E. coli*	AtoB, Hbd, Crt, Ter, AdhE2	30	70–88	7 days	[23]
Isobutanol	Gasoline additive	*E. coli*	AlsS, IlvCD, KDC, Adh	22	86	110 h	[24••]
Farnesol	Diesel/jet fuel	*S. cerevisiae/E. coli*	Mevalonate pathway, IspA	0.135[a]	nd[b]	48 h	[39,41]
FAEE	Diesel	*E. coli*	TesA, FadD, AtfA, Pdc, AdhB	0.43	9.4	72 h	[56••]
Fatty alcohols	Diesel	*E. coli*	TesA, FadD, Acr1	0.06	0.7	72 h	[56••]
Terminal alkenes	Diesel	*E. coli*	TesA, OleTJE	nd	nd	nd	[63]
Alkanes	Diesel	*E. coli*	AAR, ADC	0.3	3.5	40 h	[64••]

[a] The titer was measured in *E. coli*, Ref. [39].
[b] Not determined.

Short-chain alcohols from fermentative pathways

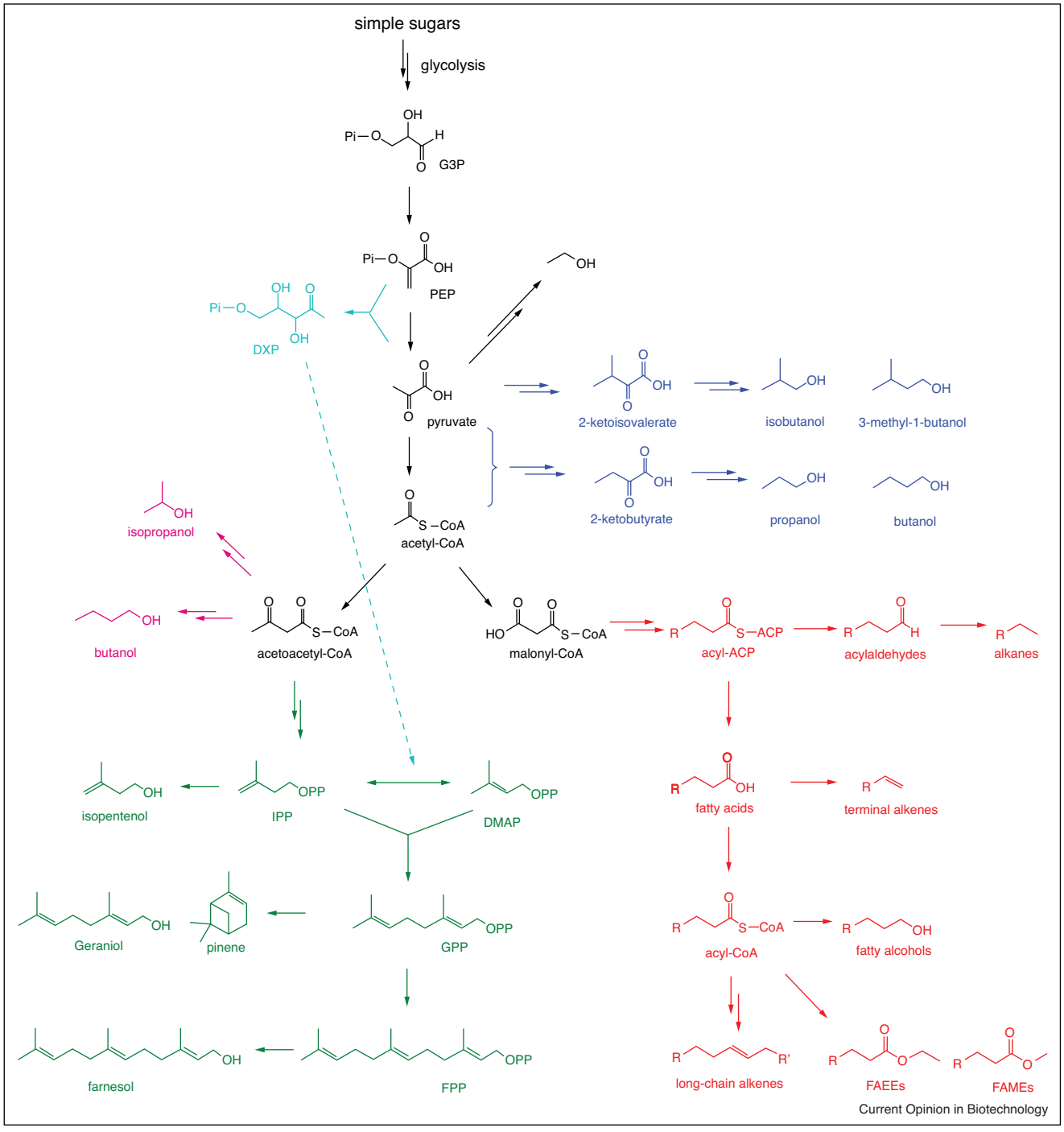
Isopropanol and butanol are considerably better alcohol fuels than ethanol because of their high energy density and low hygroscopicity, which make them less corrosive to pipelines during transportation [14,15]. *E. coli* production of isopropanol has been reported by two groups using similar biosynthetic schemes with genes from either *Clostridium acetobutylicum* (*thl*, *ctfAB*, and *adc*) or *E. coli* (*atoAD*) to convert acetyl-CoA into acetone through acetoacetyl-CoA and acetoacetate (Figure 2) [16,17]. Meanwhile, an alcohol dehydrogenase gene (*adh*) from *Clostridium beijerinckii* was expressed to convert acetone into isopropanol. These strains produced 4.9 g/L and 13.6 g/L of isopropanol from media with different glucose concentrations. In addition, gas trapping was used to remove the produced isopropanol from production medium to alleviate isopropanol toxicity to *E. coli*. Titer was increased to 143 g/L after 240 h fermentation with a yield of 67% (mol of isopropanol/mol of glucose) [18], much higher than the native clostridial strain (2 g/L).

Unlike isopropanol, when the clostridial butanol biosynthetic pathway was introduced into *E. coli* (*thl*, *hbd*, *crt*, *bcd*, *etfAB*, and *adhE2*, Figure 2), only 13.9 mg/L was produced [19]. Even after the deletion of competing pathways for carbon and reducing cofactor usage (*ldhA*, *adhE*, *frdBC*, *pta*, and *fnr*), the best butanol-production strain only yielded 1.2 g/L over 60 h period [20], much lower than the 19.6 g/L in a *Clostridia* strain [21]. Recently, two genes in the butanol biosynthetic pathway were replaced, including one gene whose enzyme product catalyzes an irreversible step, driving the pathway toward butanol production [22•]. In this study, the *thl* gene was replaced by the *pdaA* gene from *Ralstonia eutrophus* (Figure 2), which supports high efficiency acetyl-CoA condensation as demonstrated in the polyhydroxyalkanoates biosynthetic pathway, and the *bcd-etfAB* gene was replaced by the *ter* gene from *Treponema denticola*, a butyryl-CoA dehydrogenase that catalyzes the irreversible conversion of crotonyl-CoA into butyryl-CoA. This strain produced 2.95 g/L butanol after three days of fermentation. With the over-expression of the *E. coli* pyruvate dehydrogenase complex (*aceEF–lpd*) to provide both NADH and acetyl-CoA for butanol biosynthesis, production titer was further increased to 4.65 g/L with a yield of 28% from glucose. Similarly, three genes (*frd*, *ldhA*, and *adhE*) involved in pathways that consume NADH were deleted. In combination with the expression of a formate dehydrogenase (*fdh*) and the irreversible butyryl-CoA dehydrogenase Ter, 30 g/L of butanol was produced, reaching 70–88% of the theoretical limit [23].

Short-chain and medium-chain alcohols from 2-keto acid pathways

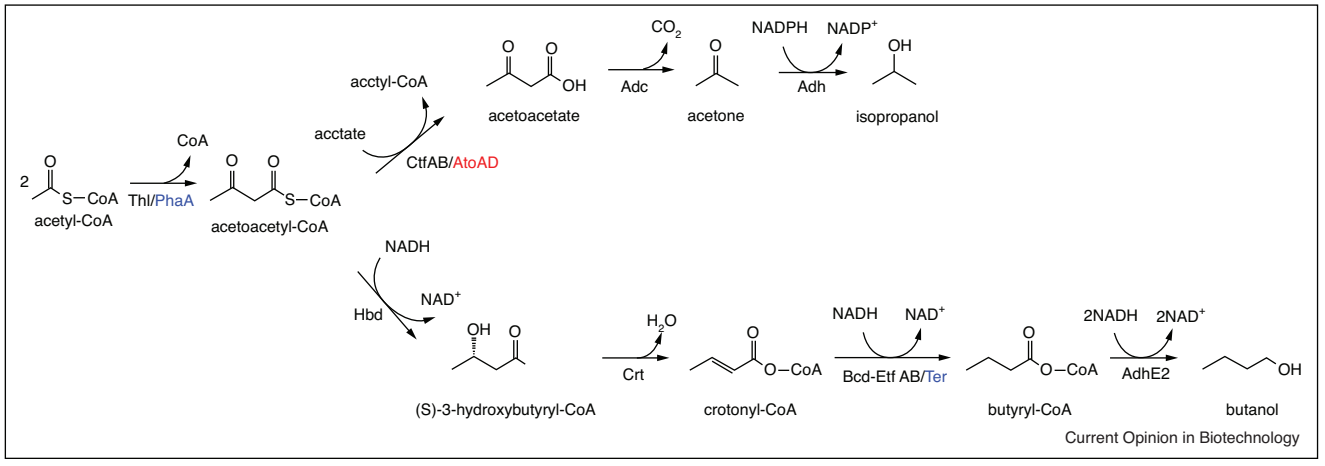
Liao and coworkers developed a series of nonfermentative metabolic pathways for short-chain alcohol biosynthesis from 2-keto acids, common precursors of *E. coli* amino acid biosynthesis. Taking advantage of the native, high flux, amino acid biosynthetic pathway [24••], 2-keto acids were converted into aldehydes by a broad substrate range 2-keto acid decarboxylase KDC (encoded by the *kivd* gene from *Lactococcus lactis*), and the aldehydes were then reduced to alcohols by a nonspecific alcohol dehydrogenase Adh (encoded by the *adh2* gene from *S. cerevisiae*) [24••]. The composition of alcohol product depends on the 2-keto acids pool of the engineered *E. coli*. When pathway genes were overexpressed to increase the flux toward a specific 2-keto acid — for example, *alsS* and *ilvCD* were overexpressed to enhance 2-ketoisovalerate biosynthesis — the corresponding alcohol, isobutanol, was produced at 22 g/L after 110 h at 86% of the theoretical yield. Using a similar approach, several short-chain alcohols including 1-propanol, 1-butanol [25], 2-methyl-1-butanol [26], and 3-methyl-1-butanol [27] have been synthesized at high yields and high specificity. In addition, structural-based protein engineering was used to enlarge the binding pocket of LeuA, a 2-isopropylmalate synthase that catalyzes the chain elongation reaction in leucine biosynthesis, to accommodate larger substrates. Using LeuA mutants, a series of medium-chain alcohols (C6–C8 alcohols) were produced [28]. Details about alcohol production from the 2-keto acid pathways have been reviewed elsewhere [29,30].

Figure 1



Engineered metabolic pathways for the production of advanced biofuels. The central metabolism is colored black. Short-chain alcohols produced by fermentative pathways are colored purple. 2-Keto acid pathways and the corresponding alcohol fuels are colored blue. Isoprenoid pathways and terpene-based fuels are colored green. Fatty acid pathway and corresponding fuels are colored red. Single arrows represent conversions catalyzed by one enzyme. Double arrows and dashed arrow represent conversions catalyzed by several enzymes. R and R′ represent for alkyl chains. OP represents a phosphate group and OPP represents a pyrophosphate group. G3P, glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate; DXP, 2-C-methy-D-erythritol-4-phosphate; ACP, acyl carrier protein; IPP, isopentenyl-diphosphate; DMAP, dimethyl-allylphosphate; GPP, geranyl-diphosphate; FPP, farnesyl-pyrophosphate; GGPP, geranylgeranyl-pyrophosphate; FAME, fatty acid methyl ester; FAEE, fatty acid ethyl ester.

Figure 2



Fermentative pathways for the production of isopropanol and butanol. *C. acetobutylicum* enzymes are colored black. *E. coli* enzyme is colored red. Special enzymes used in the irreversible butanol pathway are colored blue. Thl/PhaA, acetyl-CoA acetyltransferases; CtfAB/AtoAD, acetoacetyl-CoA transferases; Adc, acetoacetate decarboxylase; Adh, alcohol dehydrogenase; Hbd, 3-hydroxybutyryl-CoA dehydrogenase; Crt, crotonase; Bcd-EtfAB/Ter, butyryl-CoA dehydrogenases; AdhE2, aldehyde/alcohol dehydrogenase.

Fuels from isoprenoid pathways

Terpenes, otherwise known as isoprenoids, are derived from an isomeric five-carbon unit (C_5) called IPP (isopentenyl pyrophosphate) or DMAP (dimethyl-allyl pyrophosphate). IPP and DMAP can be formed from either the mevalonate (MEV) pathway or the 1-deoxy-D-xylulose 5-phosphate (DXP) pathway (Figure 3). After IPP or DMAP is biosynthesized, the 5 carbon units can be condensed via prenyltransferases to form geranyl pyrophosphate (GPP, C_{10}), farnesyl-pyrophosphate (FPP, C_{15}), and geranylgeranyl-pyrophosphate (GGPP, C_{20}). These prenyl-pyrophosphates can then be converted into monoterpenes (C_{10}), sesquiterpenes (C_{15}), and diterpenes (C_{20}), respectively, by terpene synthases.

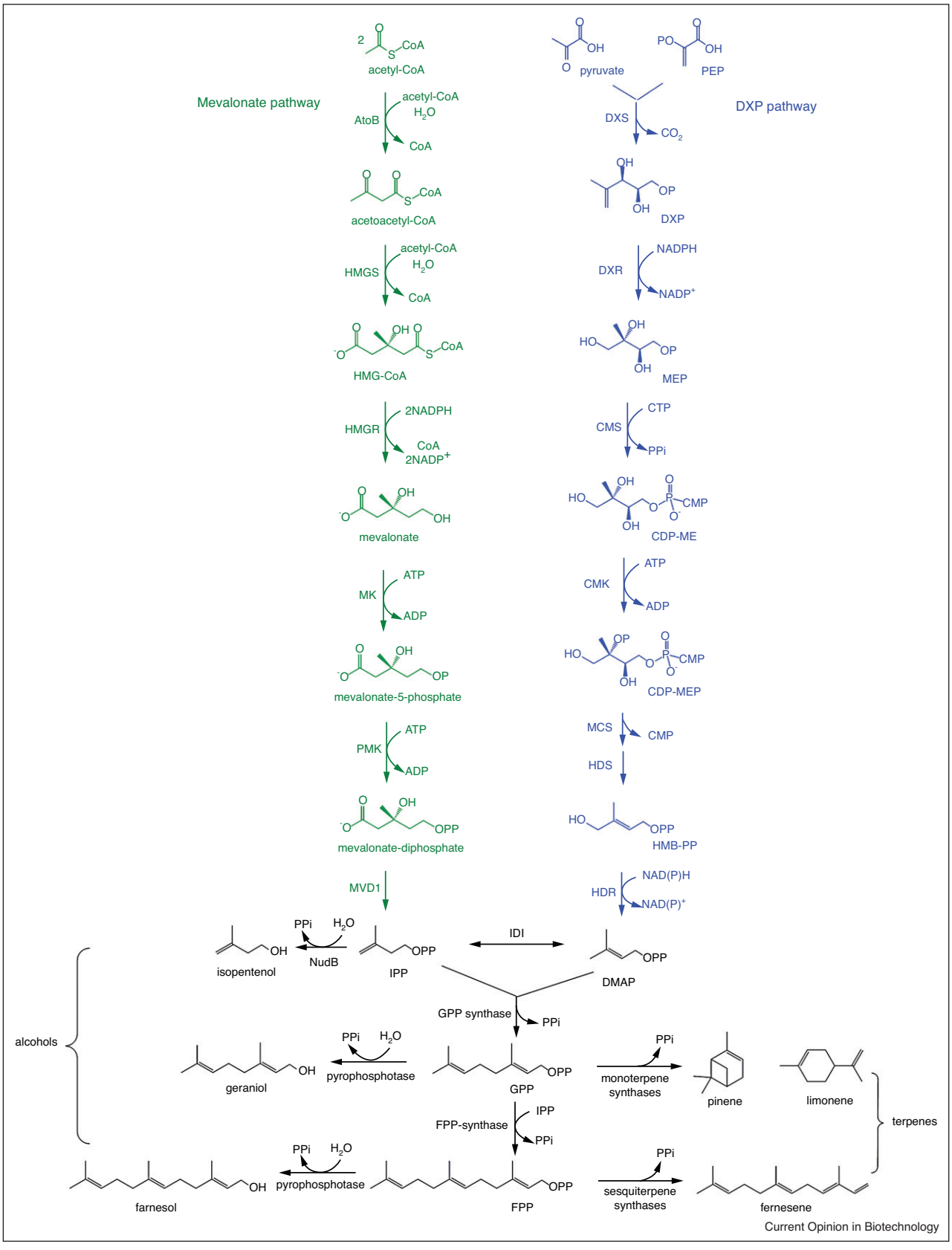
Several studies have recently reported to optimize carbon flux toward the production of terpenes. Using combinatorial approaches either by screening for enzyme mutations [31] or gene expression levels [32], the diterpenes levopimaradiene and taxadiene were produced at 700 mg/L and 1 g/L, respectively. Alternatively, using a rational design approach, Redding Johanson *et al.* employed targeted proteomics to determine potential bottlenecks of a pathway previously developed by Martin *et al.* [33], which led to production of the sesquiterpene amorphadiene at >500 mg/L [34]. Furthermore, sesquiterpene production in *S. cerevisiae* has been improved by down-regulation of key competing pathways, such as the native FPP utilizing squalene biosynthetic pathway [35].

The terpene biosynthetic pathway intermediates IPP, GPP, and FPP can be hydrolyzed by pyrophosphatases to form fuel-like alcohols. Specifically, isopentanol and isoamylacetate have been proposed as gasoline additives [36]. Isopentanol has been produced in *E. coli* using the pyrophosphatase *nudB* isolated from *B. subtilis*, by the hydrolysis of IPP or DMAP [37]. Farnesol and farnesene, both generated from FPP, have been proposed as diesel fuels [38]. Recently, *E. coli* was engineered to produce 135 mg/L farnesol by increasing FPP biosynthesis through the use of a heterologous MEV pathway and *ispA* (FPP synthase) over-expression [39]. It is believed that high intracellular FPP levels force endogenous phosphatases to nonspecifically hydrolyze FPP to farnesol. Farnesol has also been produced in *S. cerevisiae*, either by expression of a soluble phosphatase [40] or by down-regulation of the squalene synthase, Erg9 [41]. Erg9 catalyzes the first committed reaction to ergosterol biosynthesis and down-regulation of this enzyme results in accumulation of FPP [42•,43].

Cyclized monoterpene olefins such as limonene, pinene, sabinene, and terpinene have been identified as precursors to potential jet fuels [44]. For example, properties of pinene dimer mixtures, such as heats of combustion and densities, mimic those of the current jet fuel JP-10 [45]. While examples of heterologous production of pinene [46,47] and limonene [48] exist, high titer production of monoterpenes has not been reported.

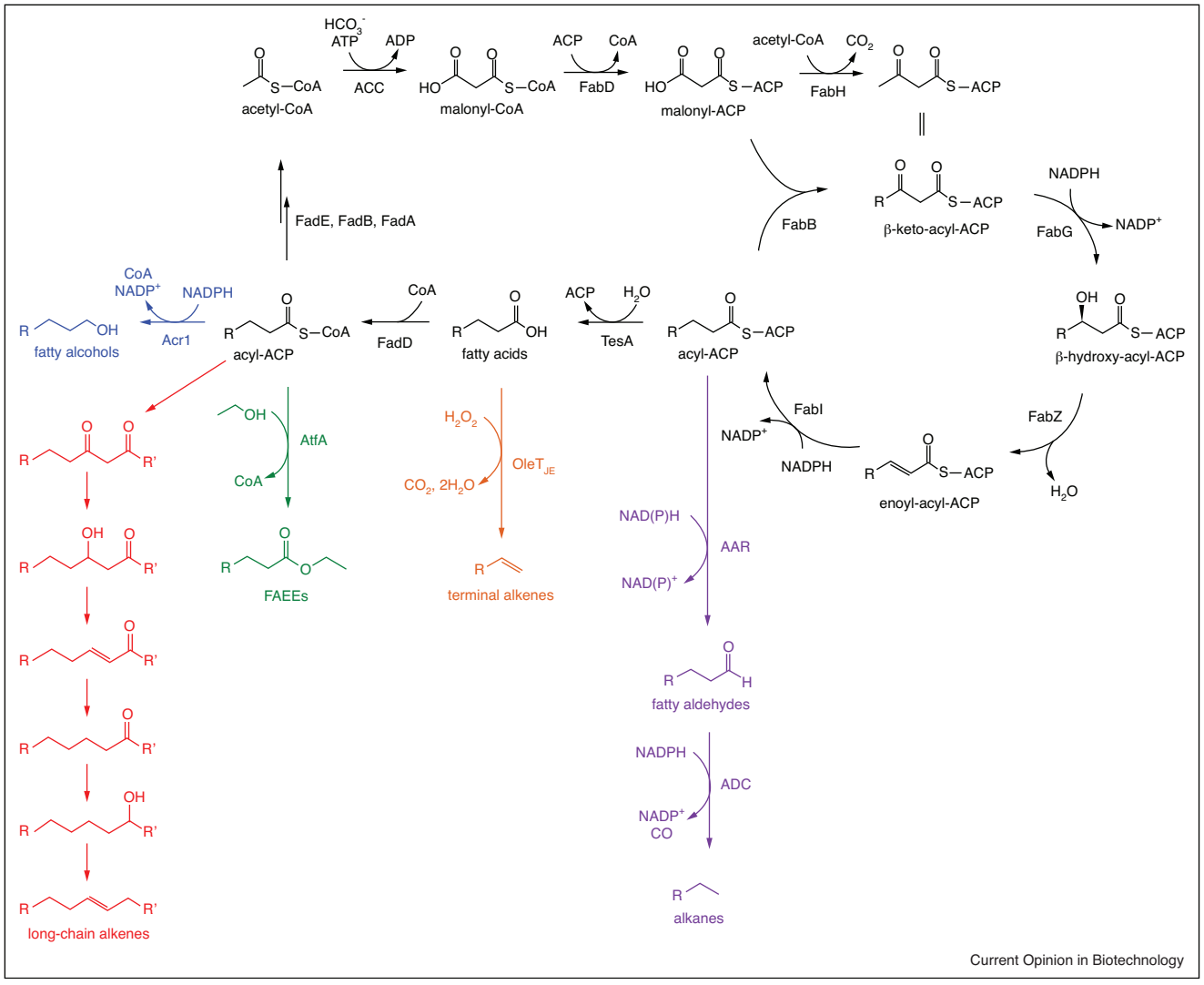
The vast diversity and abundance of terpene synthases [49] encourage us to discover new terpene-based biofuels and their corresponding synthetic enzymes. *Arabidopsis thaliana* alone has at least 32 putatively functional terpene synthase genes [50]. Moreover, organisms that generate fuel-like compounds provide us with the opportunity to

Figure 3



Biofuels from isoprenoid pathways. Mevalonate pathway is colored green. DXP pathway is colored blue. AtoB, thiolase; HMGS, HMG-synthase; HMGR, HMG-reductase; MK, mevalonate kinase; PMK, phosphor-mevalonate kinase; MVD1, mevalonate pyrophosphate decarboxylase; IDI,

Figure 4



Pathways for the production of fatty acid-based biofuels. Native *E. coli* fatty acid pathway is colored black. The proposed pathway for long-chain alkene biosynthesis is colored red [60]. Engineered pathways for the production of other derivatives are in different colors. ACC, acetyl-CoA carboxylase; FabD, malonyl-CoA:ACP transacylase; FabH, β-keto-acyl-ACP synthase III; FabB, β-keto-acyl-ACP synthase I; FabG, β-keto-acyl-ACP reductase; FabZ, β-hydroxyacyl-ACP dehydratase; FabI, enoyl-acyl-ACP reductase; TesA, acyl-ACP thioesterase; FadD, acyl-CoA synthase; FadE, acyl-CoA dehydrogenase; FadB, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase; FadA, 3-keto-acyl-CoA thiolase; Acr1, acyl-CoA reductase; AtfA, wax-ester synthase; AAR, acyl-ACP reductase; ADC, aldehyde decarboxylase; $OleT_{JE}$, a cytochrome P450 enzyme that reduces fatty acids to alkenes.

discover novel biosynthetic pathways. Recently, the endophytic fungus *Phomopsis* sp. has been found to generate the monoterpene sabinene along with other fuel-like compounds such as 1-butanol [51]. In addition, the promiscuous nature of terpene synthases lends to its use in enzyme engineering for new terpene fuels, because generally few mutations are needed to alter their product spectrum [52,53].

(Figure 3 Legend Continued) isopentenyl pyrophosphate isomerase; NudB, pyrophosphatase; DXS, deoxy-xylulose-phosphate synthase; DXR, deoxy-xylulose-phosphate reductoisomerase; MEP, 2-C-methylerythritol-4-phosphate; CMS, C-methyl-erythritol cyclodiphosphate synthase; CDP-ME, 4-diphosphocytidyl-2-C-methylerythritol; CDP-MEP, 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate; CMK, C-methyl-erythritol kinase; HDS, hydroxy-methylbutenyl diphosphate synthase; HMB-PP, (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate; HDR, hydroxy-methylbutenyl diphosphate reductase.

Fuels from fatty acid pathways

As a major component of cell membrane, fatty acids are synthesized in high flux and converted into phospholipids in all organisms. The long hydrocarbon, fatty acyl chain is energy rich, making it an ideal precursor for biofuels. Although the free fatty acids cannot be used as fuel directly, their derivatives including fatty alcohols, fatty acid alkyl esters, fatty acid-derived alkanes, and alkenes are good biofuel targets because of their low water solubility, high energy density, and low toxicity to the production hosts [4]. Furthermore, fatty acid biosynthesis and regulation have been extensively characterized [54,55], providing rich information for metabolic engineering.

The native fatty acid pathway starts from acetyl-CoA, which is converted into malonyl-CoA and malonyl-ACP (acyl carrier protein) by acetyl-CoA carboxylase (ACC) and malonyl-CoA:ACP transacylase (FabD), respectively (Figure 4) [54]. Initiation of fatty acyl elongation is catalyzed by FabH, which condenses malonyl-ACP and acetyl-CoA to generate acetoacetyl-ACP. The acetoacetyl-ACP (a β-keto-acyl-ACP) is then transformed into acyl-ACP by a series of β-keto-reduction, dehydration, and enoyl-reduction catalyzed by FabG, FabZ, and FabI, respectively. This same cycle can be repeated several times to elongate the growing acyl chain after addition of two carbon atoms from malonyl-ACP as catalyzed by FabB. On the other hand, excess fatty acid is activated to acyl-CoA by FadD for degradation through the β-oxidation pathway [55].

Several groups have reported the engineering of *E. coli* metabolic pathways to produce fatty acids in high yields. One study deleted the *fadE* gene, whose enzyme product catalyzes the first step of β-oxidation, and overexpressed a truncated version of endogenous thioesterase gene *tesA* (membrane insertion domain deleted) [56••]. The thioesterase catalyzes the hydrolysis of acyl-ACP, releasing free fatty acids from the endogenous fatty acid biosynthetic cycles (Figure 4). The engineered *E. coli* strain produced 1.2 g/L fatty acids after 72 h incubation, reaching 14% of the theoretical limit. In the other two studies, the *fadD* gene responsible for fatty acid activation to acyl-CoA was deleted; ACC was overexpressed to increase the supply of malonyl-CoA; and a plant thioesterase from either *Umbellularia californica* [57] or *Cinnamomum camphorum* [58] was expressed. The latter strain had a fatty acid production efficiency of 4.5 g/L day with 20% of theoretical yield. All the fatty acids produced contain 12–18 carbon acyl chains.

Biodiesel — including fatty acid methyl, ethyl or propyl esters (FAME, FAEE, and FAPE, respectively) — is currently used in diesel engines at greater than two billion gallons per year. A FAEE-producing *E. coli* strain was engineered based on the above-mentioned fatty acid production strain [56]. In this strain, an ethanol biosynthetic pathway was introduced by expressing a pyruvate decarboxylase (*pdc*) and an alcohol dehydrogenase (*adhB*) to convert pyruvate into ethanol. Meanwhile, the endogenous *fadD* gene was overexpressed together with a wax-ester synthase gene (*atfA*) [59] to activate free fatty acids to acyl-CoAs and esterify them to FAEEs. The engineered strain produced 427 mg/L FAEE in 72 h with a 9.4% theoretical yield [56]. Similarly, fatty alcohols were produced by overexpressing *fadD* and an acyl-CoA reductase from *Acinetobacter calcoaceticus* (*acr1*) [56].

Alkanes and alkenes (C_8–C_{21}) are the predominant components of diesel fuel [15]. Recently, a long-chain alkene biosynthetic pathway was constructed by the expression of a three-gene cluster from *Micrococcus luteus* in a fatty acid-producing *E. coli* [60]. The heterologous enzymes catalyzed head-to-head condensation of two acyl-CoAs [61,62] and a series of reduction and dehydration reactions to form internal alkenes, predominantly $C_{27:3}$ and $C_{29:3}$. Furthermore, terminal alkenes (mostly C_{18}–C_{20}) were synthesized in *E. coli* by the expression of a cytochrome P450 enzyme $OleT_{JE}$ from *Jeotgalicoccus* spp., which catalyzed the decarboxylation of free fatty acids to alkenes [63]. In addition, alkane biosynthetic genes were discovered in cyanobacteria [64••]. An acyl-ACP reductase (AAR) was able to reduce the acyl-ACPs to aldehydes, which were converted into alkanes by an aldehyde decarboxylase (ADC). When expressing these two genes in *E. coli*, a mixture of alkanes (C_{13}–C_{17}) was produced with a yield of 300 mg/L after 40 h [64••].

Conclusions

The recently engineered biosynthetic pathways in *E. coli* or *S. cerevisiae* have greatly expanded the fuel candidate repertoire. Of particular interest is the microbial production of alkanes and alkenes, whose biosynthetic pathways have been demonstrated in a few very recent studies [63,64••]. Each engineering endeavor, a given target molecule in a given selected host, comes with its own set of advantages and challenges, and guides us in the construction of biosynthetic pathways in the future.

When building a pathway in a highly engineerable host to generate a target compound of interest, it is advantageous to consider the use of native high flux pathways to provide key precursors. Heterologous enzymes, either found in nature or designed by protein engineering, can be expressed to catalyze the conversion of these precursors into the target compound. Preferably, the heterologous pathway enzymes chosen will be orthogonal to the host native pathways, meaning few heterologous intermediates will be used in native cellular metabolism. Furthermore, limiting the use of precursors by competing pathways and creating irreversible steps in the engineered pathway can increase flux toward product. Finally, other key determinants of the overall yield and productivity such as redox

balance, ATP usage, and cofactor requirements must be considered.

To date, neither a single microbial host nor a fuel candidate has completely supplanted the need for development of any other biofuel. The efficiency of converting a feedstock into the desired fuel can have considerable impact on the economic feasibility of fuel production. Fortunately, the era of genomics, transcriptomics, proteomics, metabolomics, and fluxomics has given us the ability to mine genomes for potential pathways; design, construct and characterize strains; then iterate through the process again quickly. In recent years, researchers have made steady progress toward engineering microbially derived fuels that will reduce our dependence on petroleum and emissions of carbon dioxide.

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