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Review

Metabolic engineering for advanced biofuels production and recent advances towards commercialization[†]

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Abbreviations: ABE, acetone-ethanol-butanol fermentation; **MVA**, mevalonate; **DXP** 1-deoxy-D-xylulose 5-phosphate; **FAEE**, fatty acid ethyl esters; **FASE**, fatty acid short chain alcohol esters; **FAME**, fatty acid methyl esters; **FAS,** fatty acid synthase; **BCFA**, branched-chain fatty acids; **FABCE**, fatty acid branched chain alcohol esters; **GPP**, geranyl diphosphate; **FPP**, farnesyl diphosphate; **IPP**, isopentenyl diphosphate; **DMAPP**, dimethylallyl diphosphate.

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Abstract

Research on renewable biofuels produced by microorganisms has enjoyed considerable advances in academic and industrial settings. As the renewable ethanol market approaches maturity, the demand is rising for the commercialization of more energy-dense fuel targets. Many strategies implemented in recent years have considerably increased the diversity and number of fuel targets that can be produced by microorganisms. Moreover, strain optimization for some of these fuel targets has ultimately led to their production at industrial scale. In this review, we discuss recent metabolic engineering approaches for augmenting biofuel production derived from alcohols, isoprenoids, and fatty acids in several microorganisms. In addition, we discuss successful commercialization ventures for each class of biofuel targets.

1 Introduction

As evidenced by recent treaties such as the Paris Agreement and the renewal of the Kyoto Protocol, energy diversification and climate change are being addressed very broadly on a global scale. Over the past twelve years, approximately $2.3 trillion USD has been invested in all forms of renewable energy worldwide, with investment in 2015 reaching an all-time high of nearly $286 billion USD [1]. Policies, including the elimination of methyl *tert*-butyl ether as a gasoline additive, the Renewable Fuel Standard (RFS), and the Energy Independence and Security Act (EISA) prompted increased private investment in renewable ethanol production [2]. However, several difficulties render either first- or second-generation ethanol a poor candidate as a primary biofuel. Storage and distribution, engine design, energy content, crop depletion, and landmass shortages are contributing to the maturity of bioethanol technology [3-5].

While ethanol has proven to be a successful industrial venture as a renewable fuel blend, nearly 90% of all energy consumed for transportation in the United States is still derived from fossil fuels. Three primary petroleum-derived fuels consumed for transportation are gasoline (56%), diesel (21%), and jet fuel (11%), and the higher carbon content within these fuels (C_4-C_{20}) are more amenable for combustion engines than ethanol due to their higher energy density and lower hygroscopicity. In addition, viscosity, freezing point, octane or cetane number, vapor pressure, flash point, and toxicity are the properties that must be considered when selecting alternatives for fossil fuels [6].

Development of renewable fuels for commercial scale is of immediate concern, regardless of the temporary abundance of inexpensive crude oil. The most effective means for producing non-ethanol, petroleum-free alternatives is to engineer microorganisms which can synthesize advanced biofuels utilizing renewable carbon sources such as lignocellulosic biomass. Well studied organisms like *Escherichia coli* and *Saccharomyces cerevisiae* contain the metabolic

capabilities to produce a broad variety of compounds found in fossil fuels using sugars or glycerol as a carbon source.

As tools for synthetic biology such as gene cloning, bioinformatics algorithms, and gene sequencing and synthesis technologies become cheaper and faster, enzymes from other species becomes available and can be heterologously expressed within an engineered host organism to direct carbon flux towards producing the biofuel of interest. After significant efforts for optimization, the organism could be cultured to produce biofuels on an industrial scale. Commercial scale production of renewable biofuels other than ethanol, however, has proven to be a more difficult challenge than ethanol commercialization. Because the short-term economic pressures for industrial-scale production are challenging, the need for strategies to increase yield and productivity of biofuel production within microbial strains is paramount. In this review, we discuss recent strategies for increasing titers, yields, and production rates of various advanced biofuels in three representative hosts, *E. coli, S. cerevisiae*, and *Yarrowia lipolytica*. In the first three sections, we cover the biosynthesis of higher alcohols, isoprenoids, and fatty acid-derived fuels with a focus on recent progress; we summarize these recent advances in Table 1. Within each section's conclusion, we highlight the strategies employed in recent successful commercial ventures for renewable biofuel production and critique obstacles associated with targets whose production levels are still premature for commercial scale. In the final section, we address the role of industrial ventures that tangentially support strain engineering by focusing on high-throughput testing methodologies. Moreover, we address potential bottlenecks downstream of strain engineering that can affect the future of new commercial ventures in biofuel production.

2. Alcohol-based fuels via fermentative and keto acid-like pathways

An early example of microbial higher alcohol production was discovered in *Clostridium* strains via the acetyl-CoA dependent fermentation pathway, and the use of *Clostridium* has been studied and discussed extensively throughout the past century [7]. However, *Clostridium* strains have proven difficult to engineer solely for butanol, the primary value-added fuel byproduct of acetone-butanol-ethanol (ABE) fermentation. Moreover, slow culturing rates and

spore formation have rendered them unideal for commercial-scale alcohol production [8, 9]. A substantial number of studies have since been performed in *E. coli* and *S. cerevisiae* that enhanced the variety and productivity of various linear and branched-chain alcohols. Various optimization strategies in these organisms revolve around two core pathways, the acetyl-CoA dependent ABE pathway and the keto acid pathway derived from amino acid biosynthesis (Figure 1).

2.1 *n*-Butanol and isobutanol

Both isobutanol and *n*-butanol have recently become popular blending agents for gasoline as a primary fuel based on their low water solubility, anti-knock properties, and energy densities [10]. In the past several years, isobutanol and *n*-butanol have been arguably the most frequently studied fuel targets for microbial production.

In *E. coli*, several approaches have since been published that could be of interest for future commercial implementation. In one study, a previously engineered biosynthetic pathway for isobutanol was pushed to its theoretical yield under anaerobic conditions by engineering the cofactor preference for NADH over NADPH in *ilvC* and *adhA* [11, 12]. In another study, NADH accumulation was leveraged as a driving force to boost *n*-butanol production to 30 g/L [13]. An NADH-dependent CoA-reductase, *ter*, was heterologously introduced to an *E. coli* strain in which all fermentative NADH-consuming pathways were blocked. A similar study leveraged this *ter* reductase to reveal the nature of how bottlenecks upstream of crotonyl-CoA affected *n*-butanol production [14].

Approaches other than heterologous enzyme expression have been explored to improve titers and potential relevance for industrial application. One study focused on decreasing cytotoxicity by *in situ* removal of volatile products. Implementation of this technology with isobutanol overproducing strains has pushed titers to their highest reported at 50 g/L [15]. Another study utilized protein hydrolysates instead of sugar as a carbon source to produce isobutanol [16]. Three glutamate-dependent transamination/deamination cycles were introduced in *E. coli* to feed pyruvate through a previously engineered Erlich-like pathway [17]. Ammonia excretion,

serving as the main driving force for pyruvate production, was driven by deleting both *gdhA* and *glnA* genes used in nitrogen assimilation.

n-Butanol and isobutanol production have improved considerably in *S. cerevisiae*, where many noteworthy breakthroughs have been achieved in recent years. The initial proof of principle experiment in *S. cerevisiae* resulted in titers of only 2.5 mg/L of *n*-butanol [18]. Since then, strategies have generally focused on diverting carbon flux toward cytosolic acetyl-CoA to improve *n*-butanol biosynthesis. Titers improved roughly six-fold by implementing a "pyruvate dehydrogenase bypass" strategy, in which an acetaldehyde dehydrogenase (*ald6*) and an acetyl-CoA synthetase (*acs)* were overexpressed in tandem with the NADH-dependent CoA reductase (*ter*) [19]. In a more comprehensive study, heterologous expression of the cytosolic *pdh* gene was demonstrated; when introduced to a strain inhibiting ethanol and glycerol formation, titers of *n*-butanol were boosted to 120 mg/L in *S. cerevisiae* [20]. However, the highest *n*-butanol titers (242.8 mg/L) have been reported in the strain that utilizes a native keto acid-like pathway and contains a double deletion of *ilv2* and *adh1* to direct cytosolic pyruvate to mitochondrial threonine metabolism [21].

The first demonstration of microbial production of isobutanol in yeast was achieved by overexpressing genes involved in valine metabolism (*ilv2, ilv5,* and *ilv3*) [22]. Like *n*-butanol production studies, isobutanol production improved 13-fold by deleting *pdc1* and upregulating *kdc* and *adh* expression to divert carbon flux from α-ketoisovalerate [23]. In native *S. cerevisiae*, however, α-ketoisovalerate metabolism is localized in mitochondria, and is subsequently exported to the cytosol for downstream reduction to isobutanol. Substantial advances showed that localizing the entire Ehrlich pathway to either mitochondria or cytosol could obtain equally improved titers of approximately 630 mg/L of isobutanol [24, 25]. However, localization to mitochondria results in much greater yields relative to its cytosolic counterpart (14.2 vs. 6.4 mg/g glucose). More improved titers were reported for isobutanol production in *S. cerevisiae* (1.6 g/L) by a combination of multiple strategies including

overexpression of the fully localized cytosolic *ilv2, ilv5,* and *ilv3* genes, introduction of a transhydrogenase-like shunt to regenerate NADPH, and deletion of the *pdc* gene [26].

2.2 Extended linear- and branched-chain alcohols

Linear and branched alcohols with chain lengths ranging anywhere from five to eight carbons are now potential targets for biofuel production by *E. coli* or *S. cerevisiae.* Higher alcohols having chain lengths of C_5 and longer have mostly been produced by a non-native keto acid pathway in *E. coli*. Bacterial production of various linear and branched C_5-C_8 alcohols was demonstrated by overexpressing the promiscuous leucine biosynthesis enzymes (*LeuABCD*) in a hyperproducing threonine strain [27]. Rational enzyme mutagenesis was then performed on *leuA* and *kivD* to accommodate longer substrates. This work was expanded upon using additional modeling tools to accommodate elongated intermediates in the *EcLeuA** enzyme to form *n*-alcohols as large as 1-octanol [28]. Other approaches for *de novo* pathway design invokes modular retrobiosynthetic screening of enzymes tailored for specific products. Such an approach substantially increased the relative purity of products in *E. coli* strains harboring *de novo* pathways designed for 4-methyl-pentanol and *n*-pentanol [29, 30].

2.3 Commercialization successes of *n*-butanol and isobutanol

Among the higher alcohols discussed above, *n*-butanol and isobutanol have successfully achieved commercial scale production. The biological pathways utilized to produce these two biofuels have high stoichiometric efficiency, allowing achievement of relatively higher yields compared to other biofuel products [31]. In addition, various metabolic engineering strategies have pushed commercialization of these well-developed pathways further by increasing the availability of reducing cofactors by blocking other NADH-consuming fermentative pathways and by developing solvent removal technologies (i.e., gas stripping) to avoid implementing other energy-intensive purification processes like distillation [32].

With significant advances in titers and yields of n-butanol and isobutanol, the recent efforts for commercialization were more focused on process optimization, product recovery, and utilization of cheaper and alternative feedstocks. For example, the product toxicity of *n*-butanol

has limited conventional ABE fermentation for production, and can be overcome by implementing one or more separation technologies [32].

Solvent separation was one of the core issues that significantly impaired Gevo's isobutanol production in *S. cerevisiae* (Englewood, CO), and successful commercialization required switching to a continuous solvent removal process that required less energy input needed from distillation [32]. Furthermore, in October 2016, Gevo announced the successful deployment of cellulosic jet fuels derived from renewable isobutanol for commercial flights (www.gevo.com). In addition to improved product recovery technologies, they also implemented alternative feedstocks for their production process, using cellulosic sugars derived from wood waste for their isobutanol-based Alcohol-to-Jet (ATJ) fuels. These fuels have been approved for use in commercial transportation, and Alaska Airlines has contracted with Gevo to use ATJ fuels for their commercial flights.

Retrofitting and modification of existing ethanol production plants for isobutanol production has also been a critical strategy for commercializing isobutanol. Green Biologics (Milton, UK), a UK-based biotechnology company using engineered a variety of *Clostridia*, recently announced initiation of *n*-butanol shipments from its first commercial plant in Minnesota. A 21 million gallon/year ethanol-producing plant was retrofitted for *n*-butanol and acetone production (http://www.greenbiologics.com). Butamax (Wilmington, DE), a joint venture between BP and DuPont, has aimed to retrofit ethanol facilities suited for isobutanol commercialization, and announced its complete Phase 1 retrofit of Highwater Ethanol LLC's plant in Lamberton, MN in August 2014 (http://www.butamax.com). This plant, harboring a proven ethanol capacity of 50 million gallon/year, was utilized to produce isobutanol in recombinant yeast. Similarly, Butamax recently acquired Nesike Energy in 2017 (Scandia, KS) to produce introduce isobutanol production capabilities to its ethanol facility. In addition to this, Butamax has been in contact with several biofuel production companies called the "Early Adopters Group (EAG)", which could produce a total of about 900 million gallons of ethanol.

3 Isoprenoid-based fuels

Alcohols and hydrocarbons derived from isoprenoid biosynthesis provide a larger diversity of potential fuel targets. Two distinct pathways biosynthesize isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), the precursors for all isoprenoid-based fuels. One pathway is mevalonate (MVA)-dependent, which begins with acetyl-CoA; the other is the 1-deoxy-D-xylulose 5-phosphate (DXP) pathway, which begins from pyruvate and glyceraldehyde-3-phosphate (Figure 2). Unsaturated C_5 alcohols derived from either IPP or DMAPP are precursors for desirable gasoline additives based on their energy densities and octane numbers relative to gasoline [33, 34]. Downstream recursive condensation of the IPP/DMAPP building blocks form larger hydrocarbons, serving as more suitable fuel candidates for diesel and jet engines [35].

3.1 Hemiterpenoid-based isopentenol and prenol

Production of terpenols in *E. coli* has garnered substantial promise for commercial scale production based on recent yield and titer improvements. Isoprenoid-derived branched C_5 alcohol production in *E. coli* was first demonstrated using overexpression of the *nudF* gene from *Bacillus subtilis*, partially alleviating the rate-limiting step of diphosphate hydrolysis [36]. Introducing an *E. coli* ortholog, *nudB,* increased isopentenol production to 8% of its theoretical yield [34]. Metabolic engineering efforts for isopentenol production have undergone a series of improved metabolic engineering strategies to increase titers and pathway efficiency. Product purity and yield of isopentenol could be improved when the pathway was overexpressed in the absence of *idi*, which isomerizes IPP to DMAPP [37]. Further optimization of the MVA pathway enzyme expression based on multiple "-omics" data analysis, coupled with ribosome binding site optimization for *nudB*, pushed the yield of isopentenol significantly to 2.2 g/L from 1% glucose, 70% of its theoretical yield [38, 39]. Efforts to tailor microbial isopentenol for industrial implementation were leveraged by creating a more energetically efficient "IPP-bypass" pathway [40], and subsequently, the isopentenol titer was further improved by engineering a key enzyme, a phosphomevalonate decarboxylase (*pmd*), which enables bypass of

one phosphorylation step in the original MVA pathway [41]. Collectively, the overall efficiency of the revised pathway and the strain was increased, showing better performance under aeration-limited conditions [40].

3.2 Monoterpenoids

Most recently, rapid developments have been demonstrated in metabolic pathways for monoterpene biofuels derived from geranyl diphosphate (GPP, C_{10}). For example, geraniol titers increased by nearly 14-fold from initial demonstrations in an *E. coli yjgB* deletion strain containing a plant-based geraniol synthase from basil by minimizing endogenous geraniol dehydrogenation [42]. However, geraniol volatilization prompted heavy losses during fermentation, and implementation of a two-phase fermentation platform in *E. coli* improved titers to 2.0 g/L [43]. Efforts in *S. cerevisiae* were also underway, positing the IPP-DMAPP isomerase (*idi1*) is a rate-limiting enzyme for geraniol production by limiting the supply of precursors for GPP synthesis [37]. Overexpression of *idi1* in strains containing a geraniol synthase has pushed titers to its highest level reported in *S. cerevisiae* at 293 mg/L [44]. Substantial advances have been reported for biologically producing several potential jet fuel candidates. Introduction of a codon-optimized limonene synthase from *Mentha spicata* on an *E. coli* strain engineered with the heterologous MVA pathway improved limonene titers over 100-fold relative to initial demonstrations [45]. Further optimization aided by targeted proteomics data improved the highest known titers to 650 mg/L [46]. Pinene production was improved six-fold over initial studies by introducing nine geranyl diphosphate synthase/pinene synthase fusion enzymes in *E. coli* harboring the heterologous MVA pathway, notably concluding that substrate inhibition in both enzymes are rate-limiting in pinene biosynthesis [47]. Furthermore, a recent study utilized an evolved pinene synthase from *Pinus taeda*, improving titers to 140 mg/L in *E. coli* [48].

For myrcene, initial microbial production efforts (58 mg/L) were demonstrated in *E. coli* containing a heterologous MVA pathway with a myrcene synthase from *Quercus ilex* [46]. Recently, linalool and cineol were produced in *E. coli* at relatively high titers (505 and 653

mg/L, respectively) by mutating *E. coli*'s genomic copy of FPP synthase *(ispA)* and increasing the availability of GPP for monoterpene synthases [49]. Efforts to produce linalool in *S. cerevisiae* were initiated by incorporating a linalool synthase (*lis*) from *Clarkia breweri*, but the strain produced linalool at titers of less than 1 mg/L due to issues with *hmgr* overexpression upstream within the MVA pathway [50]. Further attempts to improve *S. cerevisiae* production by down regulating squalene synthase (*erg9*) also resulted in similarly low titers of linalool, which was ultimately attributed to its high toxicity in yeast [51]. Another new target, sabinene, has been produced at similar levels in *E. coli* (82 mg/L), with fed-batch fermentation levels reaching as high as 2.7 g/L at a yield of 3.5% using glycerol as a carbon source [52]. Analogous studies in *S. cerevisiae* have produced up to 17.5 mg/L of sabinene by altering a squalene synthase (*erg20p*) for GPP [53].

3.3 Sesquiterpenes

Finally, biofuel candidates produced from farnesyl diphosphate (FPP, C_{15}) have advanced significantly toward commercial scale applications. An *E. coli* strain facilitating increased FPP flux containing an overexpressed codon-optimized α-farnesene synthase boosted α-farnesene production over 300-fold relative to initial studies [54]. Further development of farnesene production was achieved using *in vitro* methods where individual enzymes involved in the MVA pathway and farnesene biosynthesis were subsequently titrated to a measured steady-state concentration of all relevant metabolites, pushing titers to 1.1 g/L in *E. coli* [55]. Industrial efforts for production of farnesene in *S. cerevisiae*, however, have outpaced efforts to produce farnesene in *E. coli*. Most noteworthy, Amyris recently engineered *S. cerevisiae* strains for production of farnesene at titers of 130 g/L in fed-batch fermentation by introducing novel pathways to increase cytosolic acetyl-CoA levels [56]. A monocyclic sesquiterpene bisabolene has also been discovered as a precursor for diesel fuel replacement, and its microbial production was demonstrated in both *E. coli* and in *S. cerevisiae* at a comparable level to farnesene [57]. In *E. coli*, further engineering aided by proteomics improved titers to 1.2 g/L by balancing

pathway enzyme levels *in vivo* [46]. Recently, the first demonstration of *E. coli* strains producing β-caryophyllene was achieved at 220 mg/L in flasks and 1.5 g/L in fed-batch fermentation [58].

3.4 Commercial successes with isoprenoid-based fuels

It is noteworthy that several companies have successfully commercialized isoprenoids produced from microbes utilizing either the MVA or the DXP pathways, namely via the Braskem/Michelin/Amyris (https://amyris.com/products) and DuPont/Goodyear partnerships (http://biosciences.dupont.com/about-us/collaborations/goodyear). However, commercial viability for utilizing these pathways for biofuel targets is still generally unrealized with a couple of notable exceptions.

Successful engineering of the MVA pathway in both *E. coli* and *S. cerevisiae* has suggested that its theoretical yield as well as redox balance for its enzymes are critical factors for determining commercial viability of producing larger molecules containing higher energy density. The MVA pathway has a relatively lower theoretical yield compared to the MEP pathway for producing one molecule of a C_5-diphosphate intermediate (IPP and/or DMAPP) [31], but there have been isoprenoid-based biofuels produced via the MVA pathway towards commercialization. Farnesane, a fully hydrogenated form of farnesene, is one of the promising isoprenoid-based alternative transportation substitutes for diesels and jet fuels. Cathay Pacific Airlines recently announced a two-year contract with Total and Amyris to use farnesane-blended jet fuel for flights from Toulouse to Hong Kong. In addition, farnesene is a good industrial solvent and polymer building block for cosmetics, detergents, and industrial lubricants. In 2015, Amyris announced commercial production of farnesene (Biofene®), achieving a production cost of $1.75 per liter. A recent study demonstrated the collective metabolic engineering efforts for more efficient farnesene production, which can be applicable to other isoprenoids. In this study, the overall stoichiometry was improved to 3.81 molecules of glucose consumed per molecule of

farnesene produced by rewiring yeast's central carbon metabolism with heterologous enzymes (theoretical limit: 3.5 glucose/farnesene) [56].

4 Fatty-acid based fuels

Conventional biodiesels consist of fatty acid derivatives such as fatty acid ethyl esters (FAEEs) and fatty acid methyl esters (FAMEs), which are produced via trans-esterification of lipids extracted from plant oils and animal fats with ethanol or methanol, respectively. Given that the production cost of biodiesel is determined by production costs of plant oils, year-round production capabilities of microbial fermentation is expected to lower overall production costs as well as reduce carbon debts and competitive land usage associated with biodiesel production [59]. Also, microbial fermentation enables production of structurally diverse biodiesel molecules with higher purities, improving the performance of biodiesels and oleochemicals.

A recent review article compares advantages and disadvantages of various biological routes to produce aliphatic molecules [59], including fatty acid biosynthesis. Fatty acid biosynthesis generates diverse intermediates with long aliphatic moieties (i.e. free fatty acids, fatty acyl thioesters and β-ketoacyl thioesters), which are good candidates as blending agents for diesels and jet fuels. Enzymatic reactions involved in fatty acid biosynthesis have been discussed in detail [59, 60]. Fatty acid biosynthesis is initiated by ATP-dependent activation of short acyl-CoAs such as acetyl-CoA. Acyl-thioesters (acyl-CoA or acyl-ACP) are iteratively condensed with other CoA- or ACP-activated moieties, followed by β-ketoacyl group reduction. Elongation proceeds until the desired chain length is achieved (C_8-C_{22}), and is most commonly terminated by thioesterases. Both major types of FASs (type I in yeast and type II in *E. coli*) produce hexadecanoyl-CoA (C_{16}) as a major product. However, type II FAS in *E. coli* can produce a wider range of acyl thioesters ranging from C_8 to C_{19} [59].

Fatty acid biosynthesis pathways have been exploited mainly in two model organisms (*E. coli* and *S. cerevisiae*) and oleaginous yeasts for the biological production of conventional biodiesels and petroleum-based diesel substitutes including fatty acid alkyl esters, fatty alcohols, methyl ketones, alkanes, and alkenes (Figure 3). In this section, we summarize engineering strategies

for producing fatty acid-derived biofuels in *E. coli*, *S. cerevisiae* and oleaginous yeasts such as *Yarrowia lipolytica*, and update the most recent titers.

4.1 Fatty acids

Various metabolic engineering approaches were used to increase titers and yields of free fatty acid production, including dynamic pathway regulation [61, 62], organismal growth control [63], and pathway modification [64, 65]. High yields of fatty acid production were reported in two studies. Dynamic regulation of FadR, a transcription factor that alters DNA-binding affinity in response to acyl-CoA, achieved yields of 0.26 g/g glucose [66]. A slightly higher yield was achieved by implementing a malonyl-CoA sensor, FapR [61]. A high titer (8.6 g/L) fatty acid production was reported in *E. coli*, where combinatorial optimization of pathway modules tuned the supply of acetyl-CoA and consumption of malonyl-CoA/ACP [67]. Most recently, 21.5 g/L fatty acids production was achieved in *E. coli* by linking upregulated *FadR* to growth performance, which subsequently selects high-performing non-genetic variants among the heterogeneous ensemble of cells [68].

In addition to the efforts to dynamically regulate carbon flux toward fatty acids, advances in pathway modification also improved efficiency of the fatty acid-based biofuel production. In one study, the β-oxidation pathway was reverse-engineered to effectively bypass the ATP-dependent activation of acetyl-CoA (C_2) to malonyl-CoA (C_3), resulting in improved yields of short chain fatty acids [64].

To decrease the freezing point of fatty acid fuels, there have been studies focused on reducing chain length [69-73] and increasing methyl branching within the linear hydrocarbon chains [74, 75]. One study showed that anteiso-branched chain fatty acids could comprise 20.4% of the total free fatty acid content by increasing the distribution of branched amino acids in *E. coli* [74]. Another study found that incomplete lipoylation of 2-oxoacid dehydrogenases was the bottleneck of branched chain fatty acid (BCFA) production [76]. When an optimized lipoylation pathway was expressed, *E. coli* produced 276 mg/L BCFA, resulting in 85% of the total free fatty acid content.

Fatty acid fuels production in yeast was first demonstrated using acyl-CoA as a primary precursor [77]. A higher titer of fatty acids (1.0 g/L) was reported in plasmid-free *S. cerevisiae* via accumulating fatty acids and blocking fatty acid activation by deleting two fatty acyl-CoA synthetases (FAA1 and FAA4) [78]. Shorter chain fatty acids were also produced in *S. cerevisiae* by facilitating premature cleavage of shorter fatty acid chains [79]. Pathway optimization and partial disruption of the β-oxidation pathway led to 119 mg/L of C_6-C_{10} fatty acids production in *S. cerevisiae* [80].

4.2 Fatty alcohols and alkyl esters

Fatty alcohols and alkyl esters are synthesized from fatty acyl-CoAs which are activated from free fatty acids by fatty acyl-CoA synthetase (encoded in *fadD*). The fatty acyl-CoAs are either subsequently reduced to fatty alcohols by fatty acyl-CoA reductase or react with alcohol to form fatty alkyl esters by acyltransferases (wax ester synthase/acyl-CoA:diacylglycerol acyltransferase, WS/DGAT) [35].

6.3 g/L of fatty alcohols were produced in *E. coli* where acyl-ACP thioesterase (*tesA*, *tesB* and *tesC*) and the competing pathways for acetyl-CoA supply (*ldhA*, *pta* and *ackA*) were removed [81]. High fatty alcohol yields (0.13 g fatty alcohol/g glucose) were achieved in *E. coli* by expression of heterologous FAS I from *Corynebacterium glutamicum*, which directly releases acyl-CoAs as a final product [82]. This heterologous FAS I could alleviate tight regulation of the native FAS II system in *E. coli* and decreased the impact of free fatty acid synthesis and CoA re-activation. In *S. cerevisiae*, 1.5 g/L fatty alcohol production was reported by directly converting free fatty acids into fatty aldehydes using a carboxylic acid reductase from *Mycobacterium marinum* (*MmCAR*) [78]. In addition, the cytoplasmic acetyl-CoA supply was increased by heterologous expression of chimeric citrate lyase pathway enzymes.

To improve fatty acid ethyl esters (FAEE) titers, dynamic regulation of FadR was used to control gene expression and resulted in a titer of 1.5 g/L in *E. coli* [62]. Recently there were also attempts to produce various alkyl esters such as short chain or branched chain esters in *E. coli*. One study demonstrated that fatty acid esters were formed with short chain alcohols such as

isobutanol and 3-methyl-1-butanol by expressing 2-keto acid decarboxylase (*aro10*) and alcohol dehydrogenase (*adh2*) from *S. cerevisiae* in addition to acyltransferases (*ws/dgat*) introduced from *Acinetobacter baylyi* [69].

Branched chain keto acids were directly used to produce branched chain biodiesels by co-expressing β-ketoacyl-ACP synthase III (FabHB) and branched-chain α-keto acid dehydrogenase complex from *B. subtilis* [75]. This study increased titers of branched chain fatty acid esters to 273 mg/L and achieved a high proportion of the branched chain esters (99.3 % of the total fatty acid ester content). Branched shorter chain alkyl ester production (230 mg/L) has been also achieved in *S. cerevisiae* by localizing isobutanol pathway enzymes to mitochondria, increasing flux to acyl-CoA, and performing high-cell density fermentation [83]. However, titers of fatty acid alkyl esters reported in *S. cerevisiae* were relatively lower than titers in *E. coli* [77, 84].

4.3 Alkanes, alkenes, and methyl ketones

Fatty acid-derived alkane production generally requires two steps from fatty acid biosynthesis pathways. The first step is the reduction of acyl-ACP to fatty aldehydes, followed by the deformylation of fatty aldehydes to alkanes. Heterologous expression of an acyl-ACP reductase (AAR) and an aldehyde-deformylating oxygenase (ADO) in a fatty acid overproducing host led to alkane production at a titer of 300 mg/L [85]. The highest titer of 1.3 g/L was recently achieved for long chain alkanes. Native *E. coli* aldehyde reductases were expressed to co-produce fatty alcohols, relieving the toxic effects of aldehyde accumulation [86]. The production of short chain alkanes, mainly consisting of nonane (327.8 mg/L) and dodecane (136.5 mg/L), was also reported by modifying specificity of thioesterase (TesA) for shorter acyl chains [71].

Two enzymes that catalyze production of α-olefins have been recently identified: fatty acid decarboxylase, $OleT_{JE}$ from *Jeotgalicoccus sp*. ATCC 8456 [87] and cyanobacterial elongase-decarboxylase from *Synechococcus sp*. strain PCC 7002 [88]. More recently, a non-heme iron

oxidase has been identified as an enzyme that synthesize terminal α-olefins from medium-chain (C_{10}-C_{14}) fatty acids [89].

Methyl ketone production was demonstrated by re-engineering β-oxidation to overproduce ketoacyl-CoAs, a precursor of methyl ketones. Methyl ketones are formed via hydrolysis of this precursor by *fadM* thioesterase, followed by spontaneous decarboxylation [65, 90]. Recently, short chain methyl ketones were also produced using modular polyketide synthases [91].

4.4 Lipids

Oleaginous yeasts can naturally accumulate large amounts of intracellular lipids up to 25-36% of their dry cell weight (DCW), and over 80% of the lipids are stored as the form of triglycerides in endoplasmic reticulum (ER) where lipid droplets are formed [59, 92]. When two enzymes, ACC1 and DGA1, were overexpressed in *Y. lipolytica*, lipids amassed up to 62% of DCW [93]. In addition, expression of a stearoyl-CoA desaturase (*scd*), a positive regulator to increase fatty acid flux, resulted in a productivity of 22 kg/m^3/day [94]. The most recent study demonstrated direct production of fatty acid-derived fuels such as FAEE by localizing the last tailoring enzymes where specific intermediates are available [95]. The highest yields and titers of lipids were subsequently achieved by decoupling acetyl-CoA flux and nitrogen starvation. With alternative cytosolic acetyl-CoA pathways, the best strain produced 66.4 g/L lipids with a yield of 0.229 g/g glucose and a productivity of 0.565 g/L/h.

4.5 Future ventures in fatty-acid based biofuels

Biodiesels have been traditionally produced from plant and animal oils in commercial scale. One biodiesel producing company, Renewable Energy Group (REG), recently acquired LS9 for their fatty acid-based chemicals and fuels technology (www.reglifesciences.com); however, commercialization of these products is still ongoing.

Improvements in metabolic engineering has significantly progressed production of fatty acid based biofuels in *E. coli* and *S. cerevisiae* towards commercialization. In addition, oleaginous yeast's capability to accumulate lipids at considerably high concentrations has warranted considerable attention as a host for producing fatty acid based biofuels from lipids. While *E.*

coli and *S. cerevisiae* produces free fatty acids (or its derivatives) extracellularly, oleaginous yeasts accumulate lipids intracellularly. This requires additional downstream processes to harvest biomass and extract lipids from the biomass. A recent study demonstrated production of these final products from *Yarrowia lipolytica*, but the titers of tailored products were significantly lower than that of lipids [95]. Novogy (Cambridge, MA), another notable startup company backed by the French energy company Total, aims to produce fatty acids and lipids on an industrial scale using oleaginous yeasts, yet its technology has yet to reach commercial-level production (http://novogyinc.com). Therefore, successful commercialization of fatty acid-based biofuels would be determined not only by high yields and productivity, but also by cost-effectiveness of the overall process that includes fermentations and downstream processes for recovery of precursors or final products.

Another apparent direction of recent metabolic engineering efforts involves improving fuel properties of fatty acid based biofuels by either decreasing the length of fatty acyl chains or by adding methyl branches. Production of shorter and/or more branched fatty acids based biofuels is important to improve the quality of fatty acid biofuels as transportation fuels and their proof-of-concept production has been demonstrated in many recent works. However, titers of these fuels still require significant improvement before commercial scale production occurs.

5 Conclusions – Future directions towards successful commercialization: more factors to consider

With the exponentially increasing potential of synthetic biology and metabolic engineering technologies, researchers and engineers are now capable of developing economically feasible high-yielding microorganism strains for industrial applications. The successful example of biofuel commercialization warrants that current hurdles are overcome by collective efforts in strain development and process optimization. Research during the past decade focused on identifying novel biological pathways for biofuels production and optimizing the pathway by identifying and addressing bottlenecks to improve titer, production rate, and yield. Now, more

deliberate selection of fuel targets with high yields, combined with more intensive and systematic engineering strategies for a specific target is necessary to accelerate commercialization of biofuels. For example, Zymergen (Emervyille, CA) and Gingko Bioworks (Boston, MA), which provide high-throughput organism and enzyme development for bioindustrial fermentation, demonstrate the emergence and fast growth of companies focused on metabolic engineering infrastructure. Moreover, selection and development of highly productive pathways and strains must be accompanied by efficient downstream process development such as biomass separation, product recovery, and economic utilization of existing fermentation facilities for commercial scale production of biofuels.

On the other hand, with continuing debates on "food vs fuel" and concerns on sustainability of fuel crops, utilization of more sustainable and cheaper feedstocks such as lignocellulosic materials is currently being addressed as another important factor for successful commercialization. Cellulosic materials are already utilized as feedstock for ethanol (POET-DSM) and isobutanol (Gevo, cellulosic ATJ fuels). POET-DSM announced successful production of cellulosic ethanol at the industrial scale for commercialization in 2016 (http://poet-dsm.com), and in 2015, DuPont Cellulosic Ethanol completed its 30 million gallon per year plant in Nevada, Iowa. In addition to the utilization of cellulosic sugars, researchers have been aiming for complete utilization of lignin-derived carbons to increase carbon yields for fuel producing processes. In the meantime, research on fuel crops such as switchgrass coupled with novel pre-treatment processes are critical steps for the successful biofuel commercialization.

Despite temporary low crude oil prices, the destructive global impact of petroleum-based fuels and chemicals emphasizes the increased importance of biofuel developments and commercialization. In addition, demand for more energy-dense biofuels are still unmet for heavy vehicles and jets, and cannot be fulfilled by electricity or low-energy density fuels such as ethanol.

Although the development of advanced biofuels is an important task for sustainability and energy security, commercialization of biofuels has encountered many hurdles. Its economic viability is determined by myriad factors including the price of commodities, productivity and yields from microbial fermentation, and the efficiencies associated with downstream recovery. Regardless of prior difficulties, we have presented several successful examples by which those hurdles were overcome, leading to large-scale production and commercialization of several types of renewable fuels. Therefore, developing advanced biofuels production should be continuously pursued in a multi-faceted approach as one of the core technologies for energy sustainability.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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1 **Table 1.** Summary of production levels for compounds featured in this review.
2

	Host	Compounds	Titers	Cultivation Method and Time	Key Engineering Strategies	Reference
Alcohols						
	E. coli	*n*-butanol	30 g/L	Batch, 100h	Removal of non-butanol forming fermentative pathways; introduction of *ter* reductase to produce butaryl-CoA	[13]
	S. cerevisiae	*n*-butanol	243 mg/L	Batch, 144h#	Deletion of adh1; overexpression of mitochondrial keto-acid pathway enzymes	[21]
	E. coli	Isobutanol	50 g/L	Fed-Batch, 72h	Gas stripping for *in situ* product removal	[15]
	S. cerevisiae	Isobutanol	1.6 g/L	Batch, 48h	Deletion of pyruvate decarboxylase; overexpression of enzymes responsible for NADH/NADPH cofactor balance	[26]
	E. coli	Extended *n*-alcohols	2.2 g/L	Batch, 48h	Overexpression *of LeuABCD* pathway; Rational enzyme mutagenesis on *leuA* and *kivD*	[28]
	E. coli	4-methyl-pentanol	192 mg/L	Batch, 48h	Retrosynthetic design for creation of *de novo* pathways	[29]
	E. coli	*n*-pentanol	109 mg/L	Batch, 48h	Retrosynthetic design for creation of *de novo* pathways	[30]
Isoprenoids						
	E. coli	Isopentenol	2.2 g/L	Batch, 48h	Optimization of ribosome binding site for *NudB*	[38]
	E. coli	Geraniol	2.0 g/L	Fed-batch, 68h	Two-phase fermentation platform	[43]
	S. cerevisiae	Geraniol	293 mg/L	Fed-batch, 48h	Overexpression of *idi1* and *tHMG1*	[44]
	E. coli	Limonene	650 mg/L	Batch, 72h	Principal Component Analysis of proteomics data to optimize MVA pathway protein expression levels	[46]
	E. coli	Myrcene	58 mg/L	Batch, 72h	Heterologous expression of myrcene synthase from *Quecrus ilex*	[96]
	E. coli	Cineol	653 mg/L	Batch, 48h	Chromosomal mutation of *ispA*; heterologous expression of cineol synthase from *Streptomyces clavuligerus*	[49]
	E. coli	Linalool	505 mg/L	Batch, 48h	Chromosomal mutation of *ispA*; heterologous expression of cineol synthase from *Streptomyces clavuligerus*	[49]
	E. coli	Pinene	140 mg/L	Batch, 24h	Evolved pinene synthase from *Pinus taeda* to decrease substrate inhibition	[48]
	E. coli	Sabinene	2.7 g/L	Fed-batch, 24h	Heterologous expression of *gpps2* from *Abies grandis* and sabinene synthase from *Salvia pomifera*	[52]
	S. cerevisiae	Sabinene	18 mg/L	Batch#	Altering a squalene synthase (*erg20p*) for GPP specificity	[53]
	E. coli	Farnesene	1.1 g/L	Batch, 96h	*In vitro* measurement of MVA enzyme activity; balanced expression based on *in vitro* activity of heterologous pathway	[55]
	S. cerevisiae	Farnesene	130 g/L	Fed-batch, 5-6d#	Rewiring central carbon metabolism to enhance cytosolic CoA availability	[56]
	E. coli	Bisabolene	1.2 g/L	Batch, 72h	Principal Component Analysis of proteomics data to optimize MVA	[46]

					pathway protein expression levels	
	E. coli	β-caryophyllene	1.5 g/L	Fed-batch, 72h	Balanced overexpression of MVA and DXP pathway enzymes	[58]
Fatty Acids						
	E. coli	Fatty acids	5.2 g/L	Batch, 72h	Dynamic regulation and control; tuning expression of *FadR*	[62, 66]
	E. coli	Fatty acids	8.6 g/L	Fed-batch, 70h	Optimization of transcription levels in three arbitrary modules within fatty-acid biosynthesis	[67]
	E. coli	Fatty acids	3.9 g/L	Fed-batch, 44h	Dynamic control using transcriptional regulator *FapR*	[61]
	E. coli	Fatty acids	7 g/L	Batch, 24h	Reversed β-oxidation cycle; overexpression of *FadBA* and select thioesterases in strain RB03 (RB02 Δ*yqhD* Δ*fucO* Δ*fadD*)	[64]
	E. coli	Branched fatty acids	276 mg/L	Batch, 48h	Incomplete lipoylation of 2-oxoacid dehydrogenases	[76]
	E. coli	Fatty acids with up to 20.4% anteiso-branched fatty acids	694 mg/L	Batch, 48h	Heterologous expression of Val, Leu, Ile biosynthetic pathways; overexpression of *bFabH2* and *'TesA*	[74]
	E. coli	Fatty acids	21.5 g/L	Fed-batch, 43h	Ensemble-based selection of bacterial strains upregulating *FadR*	[79]
	S. cerevisiae	Fatty acids	400 mg/L	Batch, 96h	Overexpression of fatty acid biosynthesis genes *ACC1, FAS1*, and *FAS2*	[77]
	S. cerevisiae	Fatty acids	140 mg/L	Batch, 72h[#]	*FAA1* and *ADH1* deletion; growth restoration by adaptation	[97]
	S. cerevisiae	Fatty acids	400 mg/L	Batch, 96h	Overexpression of all three fatty acid biosynthesis genes *ACC1, FAS1,* and *FAS2*	[77]
	S. cerevisiae	Short chain fatty acids < C_{12}	119 mg/L	Batch, 48h	Introduction of *hFAS* with heterologous phosphopantetheine transferases and heterologous thioesterases.	[80]
	Y. lipolytica	Fatty acids	9.7 g/L	Fed-batch, ca. 120h[#]	Targeted organelle-specific overexpression of acyl-CoA/acyl-ACP processing enzymes	[95]
Methyl Ketone						
	E. coli	Methyl Ketones	3.4 g/L	Fed-batch, 45h	Balanced overexpression of *fadR* and *fadD*; knockout of key acetate	[90]

					production pathways.	
Fatty alcohols						
	E. coli	Fatty alcohols	0.75g/L	Fed-batch, 15h#	Overexpression of *Synechococcus elongatus* fatty acyl-ACP reductase; introduction of *E. coli AdhP*	[98]
	E. coli	Odd-chain fatty alcohols	1.95 g/L	Fed-batch, 27.5h	Introduction of *αDOX* from *Oryza sativa* (rice) forming odd-chain aldehydes.	[70]
	E. coli	Fatty alcohols	1.65 g/L	Fed-batch, 24h	Overexpression of an acyl-ACP thioesterase *BTE*, an acyl-CoA ligase *FadD*, and an acyl-CoA/aldehyde reductase *MAACR* to produce 1-dodecanol and 1-tetradecanol intermediates	[99]
	E. coli	Fatty alcohols	3.8 g/L	Batch, 71h	Co-expression of various type I *FAS*	[82]
	E. coli	Fatty alcohols	6.3 g/L	Fed-batch, 55h	Deletions of acyl-ACP thioesterases to induce fatty acid starvation	[81]
	S. cerevisiae	Fatty alcohols	0.1 g/L	Batch, 96h	Overexpression of fatty acid biosynthesis genes *ACC1, FAS1*, and *FAS2*	[77]
	S. cerevisiae	Fatty alcohols	1.5 g/L	Fed-batch, 96h#	Screening of different *ALR/ADH* deletion strains	[78]
	Y. lipolytica	Fatty alcohols	2.2 g/L	Fed-batch, > 120h#	Introduction of *Marinobacter aquaeolei* fatty acyl-CoA reductase *Maqu2220* along with an *E. coli* fatty acyl-CoA synthetase *fadD*	[95]
FAEE						
	E. coli	FAEE	1.5 g/L	Batch, 72h	Dynamic regulation and control; tuning expression of *FadR*	[62]
	E. coli	Fatty acids short chain alcohol esters	1.0 g/L	Fed-batch, ~55h#	Co-expression of *ARO10* and *ADH2* from *S. cerevisiae* YPH499 for short-chain alcohol production; introduction of acyltransferase from *A. baylyi* strain ADP1 for catalytic esterification	[69]
	E. coli	Branched fatty acids/branched chain alcohol esters (BFABCEs)	273 mg/L	Batch, 28h	Combined expression of the branched fatty acid biosynthetic pathway and the branched-chain amino acid biosynthetic pathway.	[69]
	S. cerevisiae	FAEE, fatty acid short- and branched-chain alkyl esters	230 mg/L	Batch, 72h	Deletion of *Rpd3* and *Opi1* with targeted mitochondrial expression of five isobutanol pathway enzymes (*Ilv2, Ilv5, Ilv3, Aro10,* and *Adh7*)	[75]
	Y. lipolytica	FAEE	142.53 mg/L	Batch, 90h	ER-targeted expression of *Acinetobacter baylyi* wax-ester synthase, *AbAtfA*, and overexpression of a peroxisomal/mitochondrial	[83]

					carnitine acyltransferase, *perCat2*	
Alkanes	*E. coli*	Short chain alkanes	0.5 g/L	Fed-batch[#]	Deletion of *fadR*; expression of a modified thioesterase from *E. coli*	[71]
	E. coli	Long chain alkanes	1.3 g/L	Fed-batch, 40.5h	Modular transcriptional regulation of fatty acid biosynthesis, lipid degradation, and electron transport chain genes.	[86]
	S. cerevisiae	Alkanes	0.8 mg/L	Fed-batch, 72h	*Hfd1, pox1,* and *adh5* deletion with expression of *MmCAR*	[78]
	Y. lipolytica	Alkanes	23 mg/L	Batch[#]	Cytosolic expression of *Mycobacterium marinum* carboxylic acid reductase *Mm*CAR along with an ACP activation module, *Bsu*Sfp	[95]
Lipids						
	Y. lipolytica	Lipids; measured as FAME	55 g/L	Fed-batch, 78h	Expression of *SCD, ACC1*, and *DGA1*	[94]
	Y. lipolytica	Lipids	61.72% of Dry Cell Weight; 28.5g/L of biomass	Fed-batch, 120h	Co-expression of *ACC1* and *DGA1* with intron-enhanced *TEF* promoter, 0.143g/L/h	[93]
	Y. lipolytica	Triacyl glyceride (TAG)	66.4 g/L	Fed-batch, ~150h[#]	Five alternative cytosolic acetyl-CoA pathways; coupling of NADPH regeneration with acetyl-CoA formation	[95]

[#]Fermentation time was not clearly stated in the study and estimated from the figures.

Figure legends

Figure 1. Schematic of higher-alcohol producing pathways. Isobutanol and *n*-butanol are decarboxylated by α-ketoisovalerate decarboxylase (*kivd*) and reduced by alcohol dehydrogenase (*adh*) from native keto-acid intermediates 2-ketobutyrate and 2-ketoisovalerate. Extended chains are formed by diverting carbon flux from these intermediates through an engineered *EcLeuA** enzyme to form either *n*-alcohols (via leucine biosynthesis enzymes) or branched alcohols (via leucine and isoleucine biosynthesis enzymes).

Figure 2. Diagram showing pathways for microbial production of isoprenoid-based biofuels via the mevalonate (MVA) and the 1-deoxy-D-xylulose-5-phosphate (DXP) pathways. *atoB*: acetoacetyl-CoA thiolase; *hmgs*: HMG-CoA synthase; *hmgr*: HMG-CoA reductase; *mk*: mevalonate kinase; *pmk*: phosphomevalonate kinase; *pmd*: mevalonate diphosphate decarboxylase; *idi*: isopentenyl diphosphate isomerase; *dxs*: 1-deoxy-D-xylulose 5-phosphate (DXP) synthase; *dxr*: 1-deoxy-D-xylulose 5-phosphate reductoisomerase; MEP: 2-C-methyl-D-erythritol 4-phosphate; MEcPP: 2-C-methyl-D-erythritol-2,4-cyclopyrophosphate; HMBPP: hydroxymethylbutenyl diphosphate; IPP: isopentenyl diphosphate; DMAPP: dimethylallyl diphosphate; GPP: geranyl diphosphate; FPP: farnesyl diphosphate; *gpps*: GPP synthase; *fpps*: FPP synthase.

Figure 3. Fatty acid biosynthesis pathway derived fuels and relevant enzymes. Abbreviations: AAR, acyl-ACP reductase; ADO, aldehyde decarbonylase (or aldehyde deformylating oxygenase); CAR, carboxylic acid reductase; ACR, acyl-coenzyme A reductase; WS/DGAT, wax ester synthase/acyl coenzyme A: diacylglycerol acyltransferase; FAS, fatty acid synthase; FAEE, fatty acyl ethyl ester.

Figure 1.

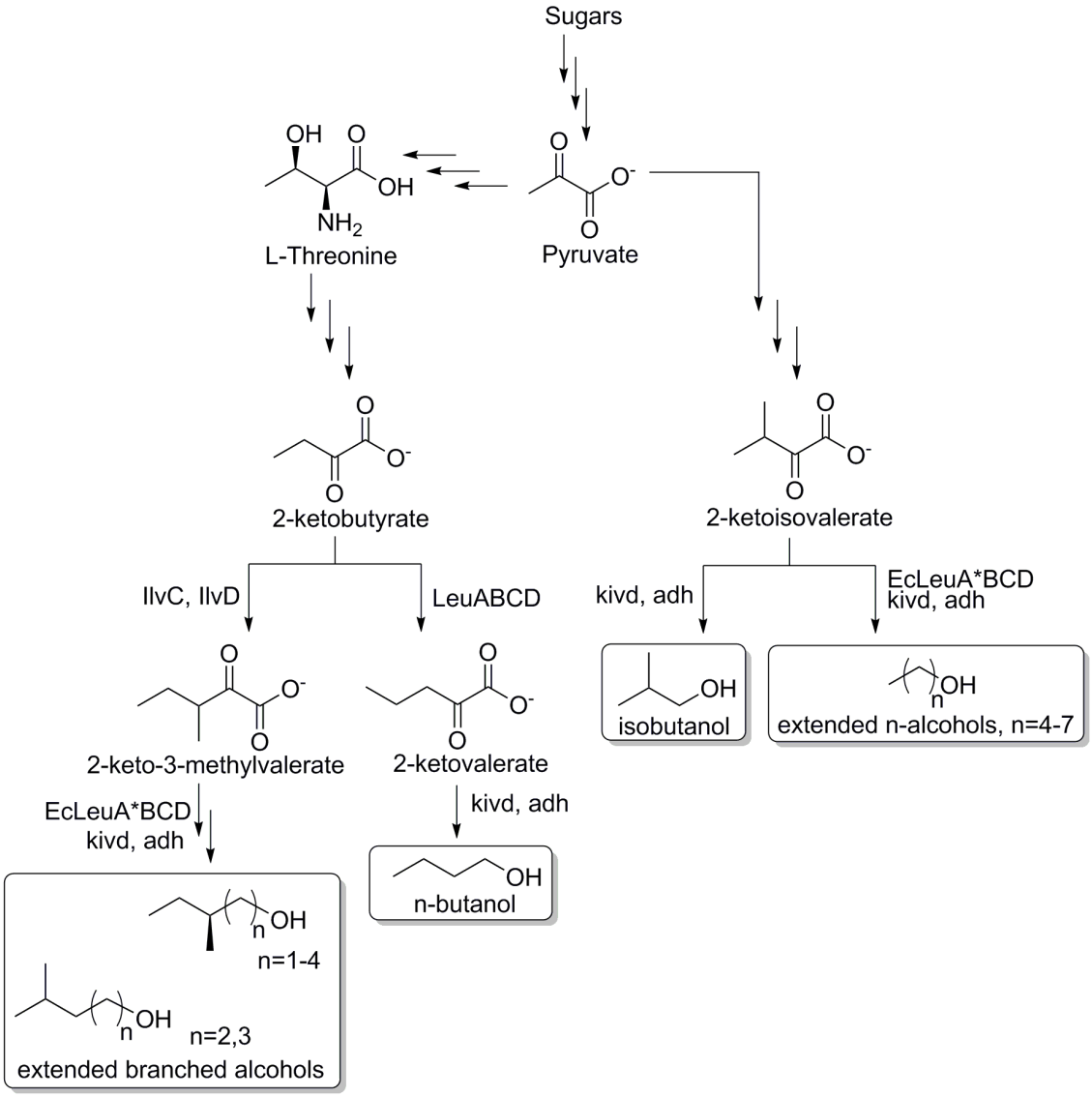

Figure 2

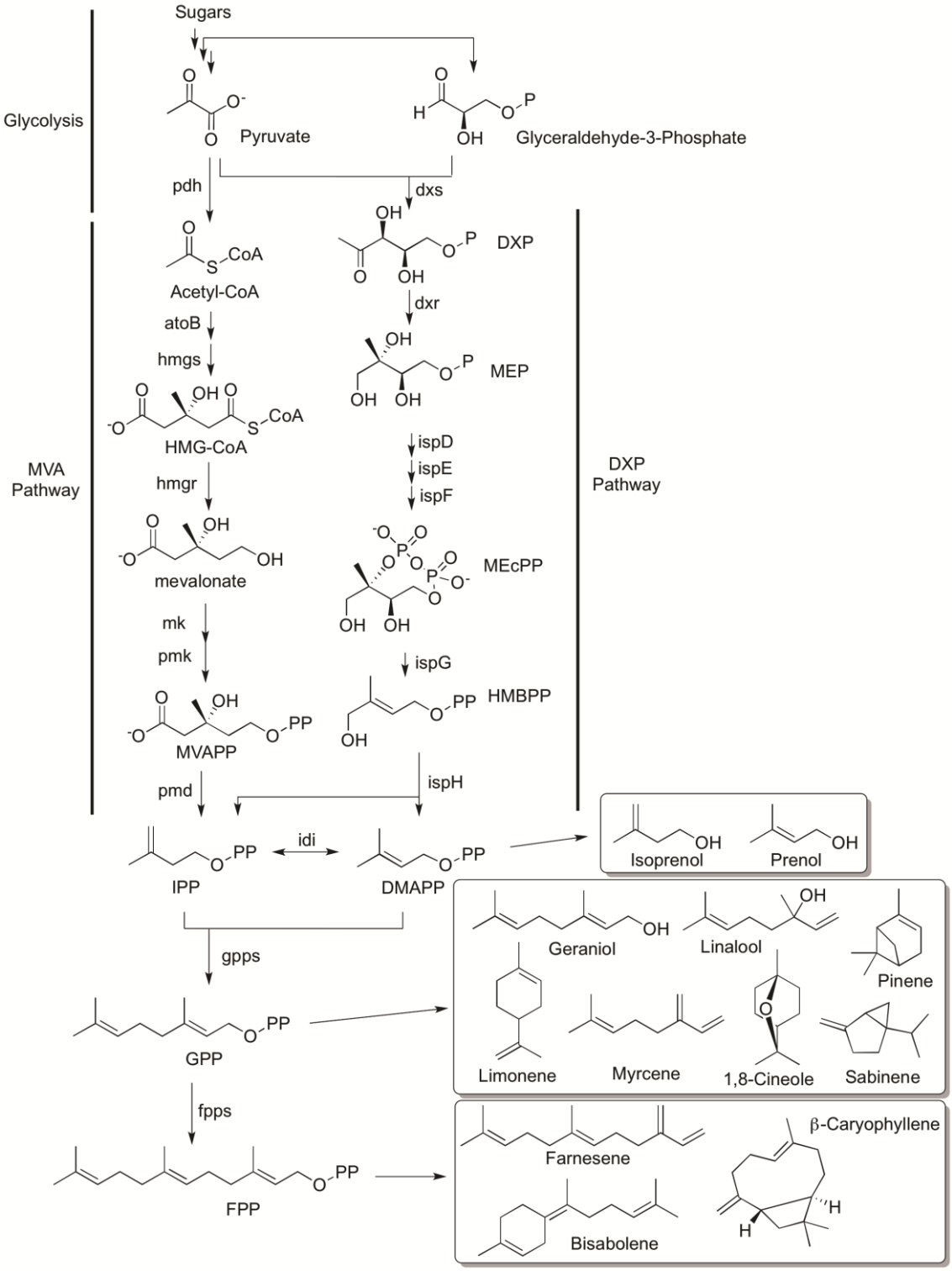

Figure 3