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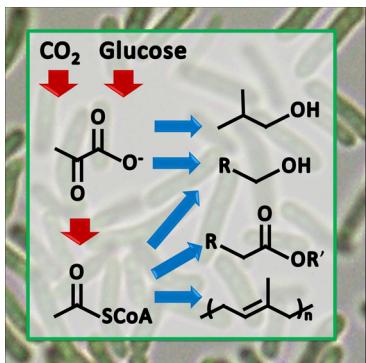
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Synthetic Biology and Metabolic Engineering Approaches To Produce Biofuels

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1. INTRODUCTION

Fossil fuels (crude oil, coal, and natural gas) represent the current primary source of the world's energy supply, accounting for 87% of global energy.¹ World energy consumption is projected to increase more than 50% by 2035, from 505 quadrillion British thermal units (Btu) in 2008 to 770 quadrillion Btu in 2035.² Liquid fuels account for the largest proportion (34%, approximately 170 quadrillion Btu) of total world energy consumption, and the demand is steadily increasing. The majority of liquid fuels are utilized in transportation sectors (54%), with another 30% consumed in industrial sectors.² Of the liquid fuels used in the US, 86% are petroleum-based fuels, with 60% of those from imports.³ World oil prices are expected to continue to steadily increase due to dwindling supply and increasing demand, both of which are affected by economic and political factors that can be difficult to predict.^{4,5} While total petroleum imports are forecasted to decrease over the next several decades, the U.S. is still expected to be dependent on imported petroleum for more than 40% of liquid fuel demand.³ The specific time frame in which finite energy sources dwindle and the rate of production decrease after peak are the subject of some debate. Rational analysis has predicted that crude oil supply will peak around 2040 and decrease sharply thereafter.^{4,6} What is not under debate is that fossil fuel resources are finite and will eventually need to be replaced with renewable substitutes.

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Another serious consequence of global long-term use of fossil fuels has been its contribution to accumulating greenhouse gases. Combustion of fossil fuels is the single greatest anthropogenic contribution to the increase of greenhouse gases and global warming.² The climate changes associated with global warming include rising sea level and acidity, average global temperature, and increasingly intense weather events. These changes negatively impact most ecosystems on Earth and threaten the survival of countless forms of life.⁷ Thus, political, economic, and environmental reasons drive the development of suitable replacements for liquid petroleum-based fuels that are renewable.

Bioethanol is currently the most widely produced and utilized biofuel and combined with biodiesel accounts for 90% of the biofuel market.^{2,8} It has been traditionally generated via yeast fermentation of easily utilized sugars and starches from sugar beet or corn biomass and, more recently, also from nonfood (and thus sustainable, but more recalcitrant to degradation) lignocellulosic plants.⁹ Production of bioethanol, using plant biomass as substrate for microbial biosynthesis, has been well-studied, developed, and optimized for a wide range of aspects, including enzyme activity and substrate range, reaction chemistry, and genetics.^{8,10} Ethanol has an energy content approximately two-thirds that of gasoline (19.6 vs 32 MJ/L, respectively), is hygroscopic, volatile, and has a higher octane rating than gasoline (129 vs 91–99).¹¹ Because of these chemical characteristics, bioethanol is used as a fuel additive to gasoline or in high concentration in newly developed engines and infrastructure.^{12,13}

In the interest of developing bioethanol alternatives and, by doing so, broadening the array of available renewable liquid transportation fuels, investigations of bioethanol have inspired an explosive expansion in the field of “advanced” biofuels. A great deal of recent biofuel research has focused on development of advanced biofuels, especially longer-chain alcohols ($\geq C_4$), alka(e)nes, and fatty acid-based fuel molecules.^{14,15}

With the exceptions of 1-butanol and 2-propanol,¹⁶ and in contrast to ethanol, advanced biofuels are generally produced naturally only in trace amounts. However, with synthetic biology, organisms can be constructed that express pathways specifically engineered to generate advanced biofuels (and many other compounds of interest) (Table 1). It is the aim of the field of synthetic biology to design, build, and optimize metabolically engineered biosynthetic pathways, or to redesign natural pathways, to achieve high yields of advanced biofuels from the producing organism.^{17–19} This is made possible first by evaluation of a gene, regulatory element (such as a promoter), cofactor, or protein for thermodynamic, regulatory, catalytic, and chemical properties. That component (or module) of a biosynthetic pathway is then manipulated to optimize module characteristics, such as expression efficacy, substrate identity, cofactors, regulation, driving forces, and thermodynamics. A library of such modules enables researchers to piece together an engineered metabolic pathway with desired characteristics and increased overall efficiency and product yield.^{15,20} The field of synthetic biology has been key in recent developments in advanced biofuel production from microorganisms.²¹

Due to comparatively faster growth rates, higher potential product yield per cell, genetic tractability, and depth of knowledge of the organism, well-studied bacteria or yeast are the ideal hosts for advanced biofuel production.²² The Gram-

Table 1. Highest Reported Titers for Various Biofuels and Chemicals

biofuel	host	titer (g/L)	ref
2-propanol	<i>E. coli</i>	143	140
1-butanol	<i>E. coli</i>	30	116
isobutanol	<i>E. coli</i>	50	79
3-methyl-1-butanol	<i>E. coli</i>	9.5	239
2-methyl-1-butanol	<i>E. coli</i>	1.25	240
isoprene	<i>E. coli</i>	0.32	209
bisabolene	<i>S. cerevisiae</i>	0.994	223
farnesene	<i>E. coli</i>	0.38	218
limonene	<i>E. coli</i>	0.005	233
(S)-3-methyl-1-pentanol	<i>E. coli</i>	0.79	84
1-hexanol	<i>E. coli</i>	0.21	145
1-heptanol	<i>E. coli</i>	0.08	85
1-octanol	<i>E. coli</i>	0.02	85
free fatty acids (FFA)	<i>E. coli</i>	7	145
fatty acid ethyl esters (FAEEs)	<i>E. coli</i>	0.922	173
heptadecane	<i>E. coli</i>	0.042	189

negative bacterium *Escherichia coli* and the yeast *Saccharomyces cerevisiae* are examples of user-friendly hosts that have been genetically engineered to generate a range of biofuels from the simple sugar glucose, from pathways based around the central metabolism of fermentation or respiration.^{19,20} In this way, renewable advanced biofuels can be generated from simple sugars.

A recent development in advanced biofuel production is the use of cyanobacteria as production hosts.²³ Cyanobacteria are photosynthetic bacteria able to utilize CO₂ and light as sole carbon and energy sources, respectively.²⁴ (Please see refs 25, 26 for reviews on carbon fixation and photosynthesis in cyanobacteria.) Utilization of a CO₂-fixing organism to produce biofuels would have the strong advantage that no exogenous carbon source (such as glucose) would be required, saving production cost.²⁷ This is especially useful due the recent shift in production of glucose substrate from renewable nonfood lignocellulosic plants. This source, while sustainable, is difficult to glean glucose from, largely due the recalcitrance of cellulose to biological degradation.^{9,28} Natural catabolic cellulose pathways have been identified and studied in detail,^{9,29,30} but efficient conversion of cellulose into simple sugars remains a bottleneck in renewable lignocellulosic-based biofuels.^{9,28} Another advantage to cyanobacteria hosts for biofuel production is that sequestration of CO₂ would utilize the greenhouse gas for beneficial purposes³¹ and close an energy cycle between fuel combustion (CO₂ emission) and subsequent CO₂ fixation.²¹

The goal of this review is to provide a comprehensive understanding of advances (but not an exhaustive list of all studies) in microbial production of prominent advanced biofuels [longer-chain alcohols, fatty acids, and alka(e)nes] from sugars and CO₂, with a special focus on the use of metabolic engineering and the synthetic biology involved.

2. ADVANCED BIOFUELS: LONG-CHAIN ALCOHOLS

Biosynthetic production of long-chain ($\geq C_4$) alcohols has been achieved primarily by manipulation of two natural biosynthetic pathways: a keto acid-based pathway based on amino acid biosynthesis³² and a CoA-dependent pathway naturally found in some *Clostridia* species.¹⁶ Recent advances in development

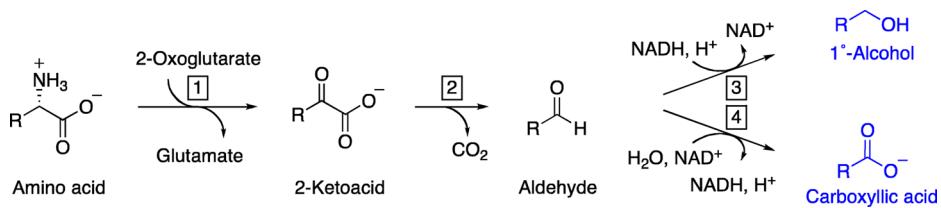


Figure 1. Ehrlich pathway: (1) transamination, (2) decarboxylation, (3) reduction, and (4) oxidation.

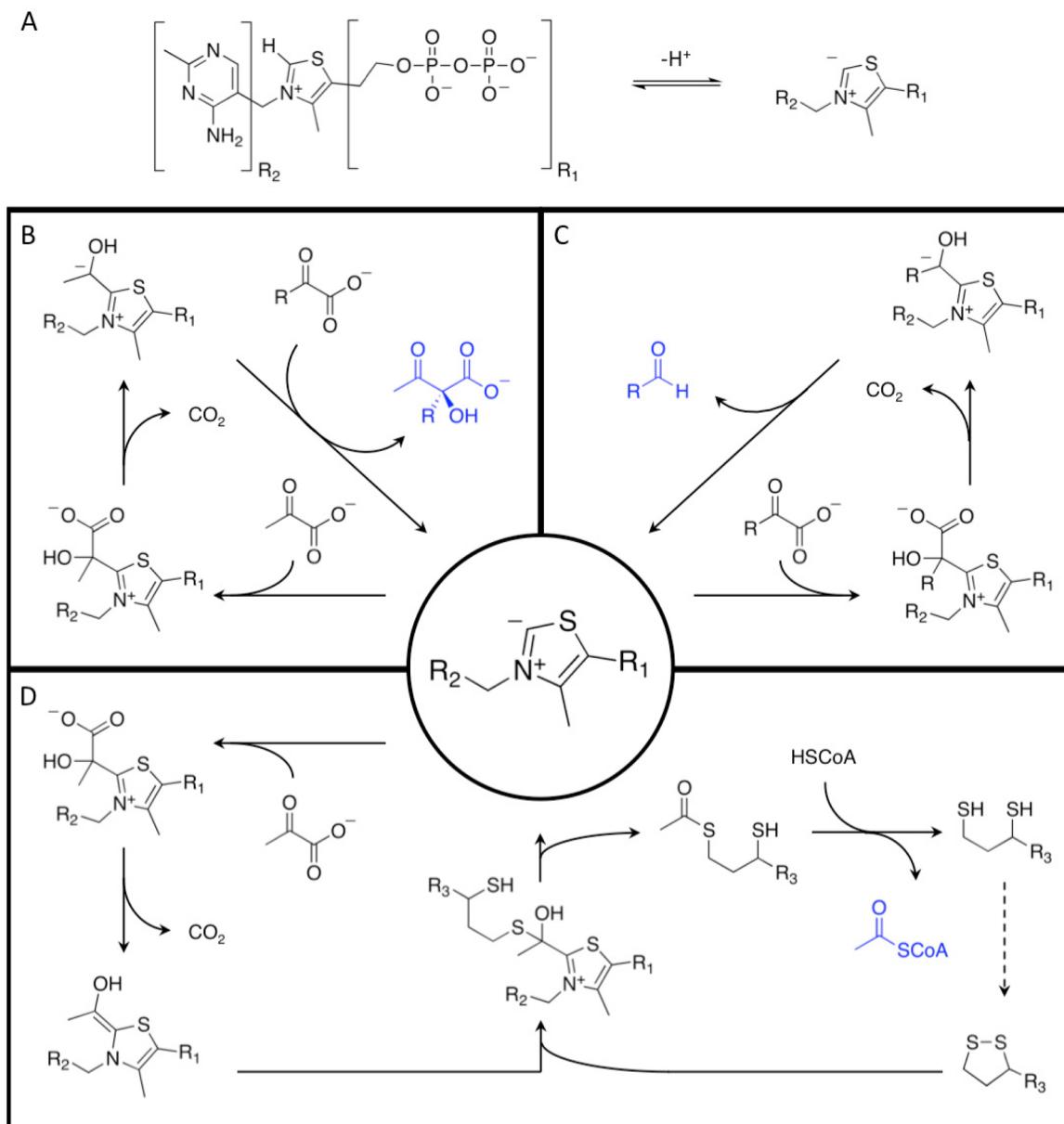


Figure 2. The general mechanism for TPP-dependent enzymes: (A) generation of an active C-2 carbanion on the thiazolium ring, (B) formation of acyl-lactate derivatives by attack of decarboxylated pyruvate on 2-keto acids, (C) formation of aldehydes by decarboxylation of 2-keto acids, and (D) formation of acetyl-CoA through acetylation of a lipoyl group.

of these two strategies for production of long-chain alcohols will be discussed herein.

2.1. Keto Acid-Based Alcohols

Manipulation of innate nonfermentative biosynthetic pathways has been used to generate valuable product(s) such as advanced biofuels and chemical feedstocks.³² In brief, amino acid biosynthesis keto acid intermediates are diverted from their

respective pathways to formation of biofuels through synthetic reactions similar to the Ehrlich pathway (Figure 1).^{33–35} The synthetic pathway catalyzes a transamination and decarboxylation, the latter by a thiamine pyrophosphate (TPP)-dependent enzyme, and lastly a dehydration. The details of these processes are discussed below.

2.1.1. Natural Production of Substrates: Amino Acid Biosynthesis Intermediates. 2.1.1.1. *Leucine, Valine, Isoleucine, Norvaline.* Synthesis of L-valine, L-leucine, and L-isoleucine differs primarily in the first step of the biosynthesis.³⁶ These three pathways result in 2-keto acids that are converted to their respective amino acids by amination with glutamate and an aminotransferase (except for 2-ketobutyrate). 2-Ketoisovalerate (KIV, which is subsequently aminated to L-valine) is generated from pyruvate by the catalysis of three enzymes, acetohydroxyacid synthase (AHAS), 2-ketol-acid reductoisomerase (IlvC), and dihydroxyacid dehydratase (IlvD). These enzymes catalyze the condensation of two pyruvates, followed by reduction and then dehydration to form KIV.³⁷ 2-Ketoisocaproate (precursor for L-leucine) is formed from the action of LeuA, LeuB, and LeuCD with KIV as substrate. These enzymes are a 2-isopropylmalate synthase (LeuA), 3-isopropylmalate dehydrogenase (LeuB), and the isopropylmalate isomerase (LeuCD), respectively. These enzymes catalyze the transfer of an acetate group to KIV, followed by isomerization, oxidation, and decarboxylation to form 2-ketoisocaproate.³⁸ 2-Keto-3-methylvalerate (precursor for L-isoleucine) is generated from catalysis of 2-ketobutyrate with the same chemistry as with L-valine biosynthesis.³⁶ L-Norvaline, an isomer of L-valine, is a nonproteinogenic, modified branched-chain amino acid found across the bacterial kingdom.^{34,39} It is generated by a side-pathway of threonine/isoleucine biosynthesis, which modifies 2-ketobutyrate via LeuABCD.³²

2.1.1.2. *Phenylalanine.* An intermediate in phenylalanine recycling pathways is phenylpyruvate. Phenylpyruvate is generated during phenylalanine catabolism by a glutamine aminotransferase, for the purpose of discarding toxic amounts of phenylalanine or for utilization of the fixed nitrogen.^{40,41} Glutamine aminotransferases have been identified in a range of yeast including *Kluyveromyces marxianus* CBS 600,⁴² *S. cerevisiae*,⁴¹ animals,⁴³ and one bacterium, *Thermus thermophilus* HB8.⁴⁴ In these innate pathways, phenylalanine is catabolized through phenylpyruvate via the Ehrlich pathway, often to 2-phenylethanol. However, generation of this valuable compound occurs naturally only at very low titers.⁴⁰ Heterologous expression of genes and optimization of the pathway and conditions to allow generation of high titers of 2-phenylethanol would be highly valuable.

2.1.2. Ehrlich Pathway. In order to artificially generate valuable keto acid-based alcohols, the keto acid intermediates from amino acid metabolism and catabolism can be diverted to a reconstructed heterologous Ehrlich pathway. The Ehrlich pathway is a series of three reactions with a primary function in amino acid recycling (Figure 1).³³ Enzymes shown to be involved in the Ehrlich pathway have been demonstrated in yeast and lactic acid bacteria (Figure 1).^{45,46} The first reaction in the pathway is a transamination, in which an aminotransferase catalyzes the exchange of a keto group from 2-oxoglutarate for the amino group on the to-be-catabolized amino acid. This reaction results in a 2-keto acid and glutamate.³⁶ The second reaction is an irreversible decarboxylation catalyzed by a TPP-dependent decarboxylase. This reaction forms a fusel (aliphatic or aromatic) aldehyde with the release of CO₂. The third reaction either oxidizes the aldehyde to an acid by an NAD(P)⁺-dependent aldehyde dehydrogenase or reduces the aldehyde to an alcohol by an NAD(P)H-dependent aldehyde reductase/alcohol dehydrogenase. The path the aldehyde takes, either oxidation or reduction, depends

largely on culture conditions.⁴⁶ The acid or alcohol is often then exported from the cell, either actively⁴⁷ or likely by passive diffusion.⁴⁶

Since 2-keto acids are naturally generated by bacterial amino acid metabolic and catabolic pathways, production of valuable alcohol compounds (in this case, biofuels) required a mere two enzymatic steps, which can be lifted from the Ehrlich pathway: decarboxylation and reduction. There are several enzymes known that are capable of catalyzing nonoxidative keto acid decarboxylation: Pyruvate decarboxylases from *Zymomonas mobilis*⁴⁸ and from *S. cerevisiae*,⁴⁹ benzoylformate decarboxylase from *Pseudomonas putida*,⁵⁰ indolepyruvate decarboxylase from *Enterobacter cloacae*,⁵¹ phenylpyruvate decarboxylase from *S. cerevisiae*,⁴¹ and keto acid decarboxylases from *Lactococcus lactis* IFPL730 (Kivd)⁵² and from *L. lactis* B1157 (KdcA).⁵³ In general, these keto acid decarboxylases share little amino acid identity except for those residues involved in TPP (cofactor) binding.⁵⁴ These enzymes do, however, seem to generally share an overall homotetrameric structure⁵⁵ (with the exception of KdcA⁵⁶) and the position in the active site of key functional groups.⁵⁴

2.1.3. Making Use of Ehrlich Pathway Keto Acid Intermediates. 2.1.3.1. *Thiamine Diphosphate (TPP) Dependent Enzymes.* TPP-dependent enzymes are ubiquitous in nature and are involved in various major metabolic processes.^{57–59} It is generally accepted that the initial step in catalysis by these enzymes is the deprotonation of the thiazolium ring of thiamine at the C-2 atom, producing a nucleophilic anion (Figure 2A).^{57–59} Thiamine alone, under physiological conditions, is a very poor catalyst due to the relatively high pK_a (17–19) of the C-2 atom. However, once bound to Mg²⁺ inside the tailored environment of its protein, conformational and electrostatic interactions allow the C-2 proton to be readily extracted.⁶⁰ Common of all TPP enzymes is the nucleophilic C-2 anion, which undergoes nucleophilic attack on carbonyl compounds. Then via a biological umpolung, the carbonyl carbon is converted into an “activated aldehyde”, which attacks a second substrate. The general mechanism for TPP-dependent enzymes is illustrated in Figure 2B–D.

2.1.3.1.1. *Kivd.* Decarboxylases are the most abundant type of TPP enzymes. The best-characterized Kdc's are Kivd and KdcA from *L. lactis*. Kivd was isolated from *L. lactis* IFPL730,⁵² and the gene *kivd* has been cloned and sequenced. The protein was purified, 20 amino acids of its N-terminus were sequenced, and the mass of recombinant Kivd was measured by gel filtration, revealing that it was likely a TPP-dependent 60 kDa enzyme that functions as a homotetramer. The enzyme activity was assayed with respect to pH, temperature, metal cofactors, and inhibitors. Kivd was reported to have a *K_m* and *V_{max}* of 1.9 mM and 118 μmol min⁻¹, respectively, with an optimum pH of 6.5 and temperature of 45 °C. Kivd shows its highest activity with 2-ketoisovalerate (KIV) as substrate and also shows activity toward other branched-chain 2-keto acids.⁵²

2.1.3.1.2. *KdcA.* A second TPP-dependent keto acid decarboxylase, KdcA, was reported from *L. lactis*.⁵³ KdcA also shows a strikingly broad substrate range, and the highest reported decarboxylase activity is with branched-chain 2-keto acids as substrate. Like all TPP enzymes, KdcA undergoes the same general mechanism, except that the reaction stops after decarboxylation (Figure 2C). Decarboxylation of KIV, the preferred substrate of KdcA (of those tested), results in formation of 2-methylpropanal (isobutyraldehyde) and

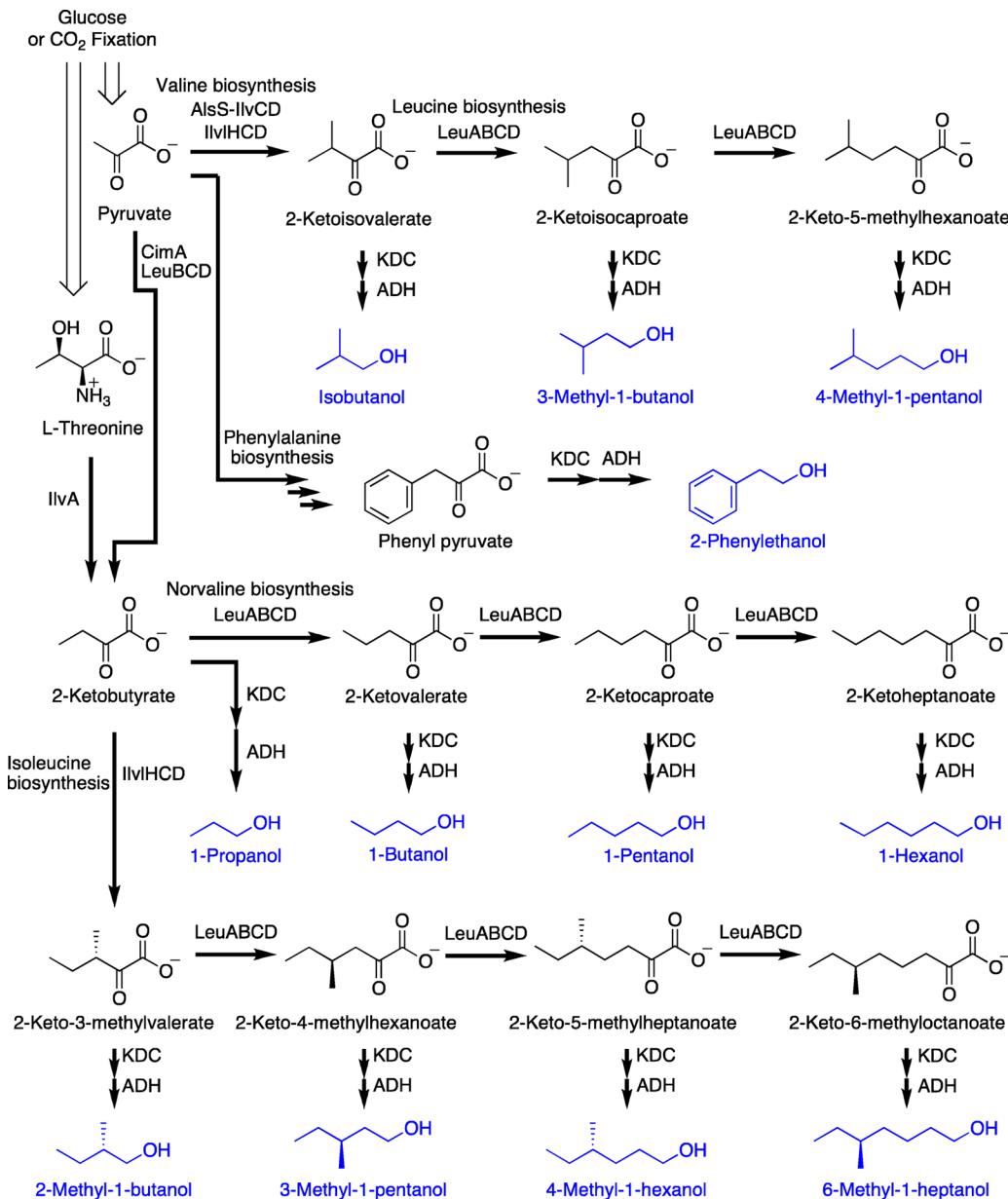


Figure 3. Higher alcohol synthesis via 2-keto acid intermediates. AlsS, acetolactate synthase; KDC, keto acid decarboxylase; ADH, alcohol dehydrogenase; IlvIH, acetolactate synthase large and small subunit (*E. coli*); IlvC, 2-hydroxy-3-ketol-acid reductoisomerase (*E. coli*); IlvD, dihydroxy-acid dehydratase (*E. coli*); LeuA, 2-isopropylmalate synthase; LeuB, 3-isopropylmalate dehydrogenase; LeuCD, isopropylmalate isomerase.

CO_2 .^{53,55,61} KdcA has been successfully expressed in *E. coli*, and its kinetic parameters have been determined with respect to substrate specificity.⁵⁵ KdcA has been reported to have a K_m and V_{max} of 5.2 mM and 182 $\mu\text{mol min}^{-1}$, respectively, and is functional in a pH range of 5–8, with an optimum pH 6.3 and temperature of 50 °C.⁶¹ Homology modeling and site-directed mutagenesis identified four residues (S286, F381, V461, M538) that contribute to substrate specificity. Three of them (S286, F381, M538) are located in the substrate binding pocket.⁵⁵ KdcA also shows a broad substrate range for the side carboligation reaction common to some TPP-dependent decarboxylases.^{61–63} Carboligation reactions form asymmetric carbon–carbon bonds and yield a range of 2-hydroxy ketones.^{62,63} While most other TPP-dependent enzymes function as homotetramers, including Kivd, KdcA functions as a homodimer. Interestingly, Kivd is nearly identical in amino

acid sequence to KdcA.^{56,61} The crystal structures of holo-KdcA and of KdcA complexed with an inhibitory TPP analog have been reported and show that the unusually broad substrate profile for carboligation was due to an unusually large binding pocket.⁵⁶

2.1.3.1.3. AlsS. AlsS is a potential alternative to Kdc and to AHAS (such as IlvIH) in keto acid-based isobutanol production pathways.⁶⁴ alsS encodes an acetolactate synthase (ALS) from *Bacillus subtilis*⁶⁵ that can perform dual function in the isobutanol production pathway. It catalyzes the aldo condensation of two pyruvate molecules to form 2-acetylacetate⁶⁶ and is useful in this step of isobutanol synthesis due to its higher specific activity for pyruvate than IlvIH of *E. coli* (which prefers 2-ketobutyrate).^{32,67} AlsS also catalyzes the decarboxylation of 2-ketoisovalerate (KIV) to isobutyraldehyde and CO_2 .⁶⁴ This second reaction is irreversible due to the release of

CO_2 . Through structural modeling and enzyme assays, the residue Q487 was found to be important for ketodecarboxylase activity by AlsS (*B. subtilis*), but not for acetolactate synthase activity.⁶⁴

ALS is a TPP-dependent enzyme. Notably, the similar subgroup AHAS (a group that includes IlvIH) also catalyzes the condensation of two molecules of pyruvate. Despite the structural similarities of these two enzymes, they are separated by one distinct feature: AHAS contains the cofactor FAD⁺, while ALS does not.⁶⁸ This FAD⁺ is noncatalytic, however, and was previously suggested to play a structural role. With the discovery of the non-FAD⁺ containing ALS form, such as AlsS, the reasoning for FAD within the enzyme is now thought to be simply a remnant of an earlier form of TPP-dependent enzymes that evolved into AHAS.⁶⁸ This hypothesis is supported by differences in the catalytic activity of the two enzyme forms. The ALS from *Klebsiella pneumoniae*, a homologue of AlsS (*B. subtilis*), has high specific activity that corresponds to a k_{cat} of $\sim 533 \text{ s}^{-1}$. This ALS activity is 10-fold higher than even the most active AHAS enzymes.⁶⁹ Also, the fact that AlsS catalyzes a decarboxylation reaction supports the hypothesis that TPP-dependent enzymes have diverged from a common ancestor during their evolution.

2.1.3.2. Adh. The last enzymatic step required to generate higher alcohols from 2-keto acids is the reduction of the aldehyde (produced from a keto acid decarboxylase) to an alcohol. Alcohol dehydrogenases (Adhs) commonly perform such a reduction and are found across all kingdoms of life.^{70,71} They have roles in many functions, from degradation of toxins to biosynthesis of metabolites, and are utilized for many applications, including production of industrial solvents and alcoholic beverages. Adhs have a wide range of substrate specificities and cofactor requirements. Adhs catalyze the oxidation of alcohols to either aldehydes or ketones, many are NAD(P)⁺-dependent, and most catalyze a reversible reaction.^{70,71}

The *E. coli* genome contains seven genes that code for Adhs: *adhE*, *adhP*, *eutG*, *yiaY*, *yqhD*, *fucO*, and *yjgB*. Of these, *yqhD* is of the most interest with respect to production of higher alcohols because it codes for an enzyme with preferred activity toward longer ($\geq \text{C}4$) alcohols, with no activity detected for short-chain alcohols.⁷² YqhD has also been shown to reduce aldehydes in an NADPH- and Zn²⁺-dependent reaction that is the reverse of those typical of Adhs.⁷³ Due to the relatively high K_m for longer-chain alcohols (5–30 mM)⁷² and low K_m for aldehydes (0.6–4 mM), it is likely that the biologically relevant, preferred substrate for YqhD is aldehydes.⁷³ A later study on the other Adhs encoded on the genome of *E. coli* revealed that all except YiaY are active toward acetaldehyde, and AdhP, EutG, FucO, and YjgB were shown to have similar activity toward isobutyraldehyde.⁷⁴ AdhP, EutG, and FucO prefer to utilize NADH as a cofactor, while YjgB prefers NADPH.⁷⁴

adhA was identified in *L. lactis* as an Adh-encoding gene by nucleotide sequence similarity to other Adh-encoding genes.⁷⁵ AdhA has been characterized with respect to enzyme kinetics and was found to have high NADH-dependent activity toward acetaldehyde and isobutyraldehyde.⁷⁶

Since more than 100 Adh crystal structures have been solved,⁷⁷ it is possible that other dehydrogenases will yet be identified and deemed useful candidates for advanced biofuel production.

2.1.3.3. Production of Keto Acid-Based C3–C6 Alcohols. Since their identification and initial characterization, these

enzymes (acetolactate synthase, keto acid decarboxylase, and alcohol dehydrogenase) have been combined in various genetic backgrounds and, in some cases, altered to produce a wide range of higher alcohols suitable for use as transportation biofuels (Figure 3).

The pioneering nonfermentative production of six advanced biofuels was achieved through recombinant expression of *alsS*, *kivd*, and *adh2* in *E. coli* (Figure 3).³² The keto acid intermediates from the biosynthesis of L-valine, L-norvaline, L-leucine, L-isoleucine, and L-phenylalanine were diverted toward the production of several valuable aldehydes by Kivd from *L. lactis*. The aldehyde intermediates were then reduced to a range of higher alcohols³² by an alcohol dehydrogenase (Adh2) from *S. cerevisiae* (Figure 3).⁷⁸ Higher alcohols produced were isobutanol, 1-butanol, 3-methyl-1-butanol, 2-methyl-1-butanol, 1-propanol, and 2-phenylethanol. These products were initially generated in a range from 2 to 10 mM. The *E. coli* genome was optimized for biofuel production by deletion of genes for competing carbon flow pathways, which increased the pool of desired keto acid substrate. Isobutanol production was further optimized by expression of *alsS* in place of the native *ilvIH*. AlsS has higher affinity for pyruvate than IlvIH, which further focuses carbon flow toward production of isobutanol. With these genome modifications, the highest titer of isobutanol achieved was approximately 22 g/L (300 mM) from glucose (86% of theoretical yield).³² Gas-stripping harvest methods allowed isobutanol yield as high as 50 g/L.⁷⁹ Adh2 was later found to be essentially irrelevant in *E. coli* isobutanol production, likely due to native enzymes such as YqhD with aldehyde dehydrogenase activity.⁷⁶

2.1.4. Pathway Improvement. The keto acid based 1-propanol and 1-butanol production was improved by evolving a citramalate synthase (CimA) from *Methanococcus jannaschii*⁸⁰ in *E. coli*.⁸¹ CimA catalyzes the addition of an acetate group from acetyl-CoA to pyruvate to synthesize citramalate, which is then converted to 2-ketobutyrate by LeuCD and LeuB. Because 2-ketobutyrate is an essential precursor in the biosynthesis of L-isoleucine, an *E. coli* strain deficient in *ilvA* and *tdcB* cannot synthesize 2-ketobutyrate and becomes auxotrophic for L-isoleucine. By leveraging a growth selection based on a requirement for 2-ketobutyrate, the directed evolution of CimA was able to enhance the activity of this pathway and increase the production of 1-propanol and 1-butanol by 9- and 22-fold, respectively.⁸¹

In order to identify mutations that allow improved isobutanol production, an *E. coli* strain was randomly mutagenized with N'-nitro-N-nitrosoguanidine (NTG), and isolates better able to grow with the toxic valine analog norvaline were selected for by growth with L-norvaline.⁸² This selection strategy theoretically results in a strain better able to survive by upregulating biosynthesis of natural amino acids like L-valine, to overcome the toxic effects of nonproteinogenic L-norvaline. The result is an increase in carbon flux to the desired amino acid precursor keto acids, which in turn increases biofuel production. This strategy successfully increased short-term (over 24 h) isobutanol production from 5.3 g/L in the parent strain to 8.0 g/L.⁸² A similar strategy was used to increase 3-methyl-1-butanol synthesis in *E. coli* by growth with the L-leucine analog 4-aza-D,L-leucine.⁸³

A non-native metabolic pathway was constructed in *E. coli* to generate longer-chain (C5–C8) alcohols from natural pathway intermediates (Figure 3).⁸⁴ Simple overexpression of *leuABCD*, *kivd*, and *adh6* in a threonine-hyperproducing strain of *E. coli*

allowed for production of 1-hexanol (17.4 mg/L) and (S)-3-methyl-1-pentanol (299.2 mg/L). Neither of these compounds were detected in wild-type fermentations. Further, mutagenesis of Kivd (V461A/F381L) to widen the binding pockets increased preference for a larger substrate (2-keto-4-methylhexanoate, a C7 compound) instead of the natural substrate (2-ketoisovalerate, C5). Mutagenesis of LeuA (the first enzyme in the recursive chain elongation pathway, G462D/S139G) allowed for generation of longer-chain keto acid substrates. The combination of overexpression of *leuA*^{*}*BCD*, *kivd*^{*}, and *adh6*, increased the yield of 1-hexanol to 37.4 mg/L and (S)-3-methyl-1-pentanol (793.5 mg/L).⁸⁴ The cumulative result was an altered keto acid pathway able to generate longer-chain alcohols.

The work of Zhang et al. has been expanded to optimize LeuA for generation of longer-chain keto acids.⁸⁵ Quantum mechanical and protein–substrate modeling^{86,87} were used to predict how to mutagenize 2-isopropyl malate synthase (LeuA) to allow repetitive condensation of acetyl-CoA with substrates larger than the natural substrate, KIV.⁸⁵ Hypothetically (in silico) mutated LeuA with a larger binding pocket was analyzed for substrate–enzyme interactions and for likely water exclusion and was found to accommodate substrates up to C8 for catalysis. Enzyme assays verified in silico predictions that a mutant LeuA with smaller amino acids in the binding pocket preferred C6 and C7 substrates and also accommodated C8 and aromatic substrates. The mutated *leuA* was expressed in the *E. coli* strain previously developed⁸⁴ for increased threonine production (the precursor for 2-ketobutyrate and L-isoleucine) and included the optimized 2-keto acid decarboxylase (Kivd*) originally from *L. lactis*⁸⁴ and an alcohol dehydrogenase (Adh6) from *S. cerevisiae*. The yield of 1-hexanol was improved to 146 mg/L. 1-Heptanol (80 mg/L) and 1-octanol (2 mg/L) were produced biologically for the first time from the resulting strain.⁸⁵ Similar efforts to expand the substrate range and substrate size of downstream enzymes (LeuBCD) may increase yields of longer-chain alcohols and are underway.

The key to efficient microbially generated biofuels is the balance of energy in that spent versus that produced. An energy balance maintained during production ensures that the cell is not energetically overtaxed and also that the cell has a regenerated/working sink for accumulated reducing power (i.e., NAD[P]H). Anaerobic microbial production of biofuels such as isobutanol has much higher potential yield and lower operating costs than aerobic production.⁸⁸ However, only aerobic or microaerobic production has been feasible due to imbalanced production energetics.³² It was hypothesized that anaerobic isobutanol yield in *E. coli* is low because the required enzyme, 2-keto acid reductoisomerase (IlvC, *E. coli*), requires an NADPH, which is generated only in aerobic conditions from the pentose phosphate pathway or the TCA cycle. Only NADH (two per one glucose) is generated in anaerobic conditions from glycolysis.⁸⁸ To overcome this limitation, two strategies were tested: (1) expression of a transhydrogenase (PntAB, *E. coli*) to convert NADH to NADPH and (2) generation of mutated IlvC* and AdhA* variants with a preference for NADH as a cofactor and high catalytic activities.⁸⁸

E. coli contains two transhydrogenases: the transmembrane PntAB^{89,90} and the soluble SthA.⁹¹ These enzymes have been used previously to increase productivity of useful chemicals;^{92,93} however, the endogenous activity of these enzymes was not sufficient to allow significant isobutanol production in anoxic conditions from NADPH-dependent enzymes.⁸⁸ To increase

cellular PntAB content, an extra copy of *pntAB* was expressed in cis with an inducible promoter. Also, an IlvC variant with preference for NADH⁹⁴ was mutated to increase acetolactate reductase activity. A mutant enzyme with four amino acid changes (A71S;R76D;S78D;Q110V) showed a K_m for acetolactate similar to that for the wild-type enzyme, 40 mM, and a 54 000-fold increase in specificity for NADH cofactor over NADPH.⁸⁸ AdhA, an NADH-dependent dehydrogenase,⁷⁶ was engineered with three mutations (Y50F, I212T, and L264 V) to reduce K_m from 11.7 to 1.7 mM and increase k_{cat} from 30 to 140 s⁻¹. The *E. coli* strain with the NADH-dependent pathway produced isobutanol anaerobically at 100% theoretical yield.⁸⁸

2.1.5. Other Organisms. A bacterial strain naturally able to degrade cellulose, *Clostridium cellulolyticum*, was utilized in the first example of consolidated bioprocessing (CBP) to generate isobutanol.⁹⁵ CBP is a method used to combine the steps of lignocellulosic (plant) biomass degradation and biofuel production in a single microorganism (reviewed recently by Olson et al.⁹⁶). The genes, *alsS* (*B. subtilis*), *ilvC* (*E. coli*), *ilvD* (*E. coli*), *kivd* (*L. lactis*), and *yqhD* (*E. coli*) were integrated into the *C. cellulolyticum* chromosome.⁹⁵ To reduce the toxic effects of AlsS, optimal gene order was determined. Isobutanol was generated at 660 mg/L from the complex polysaccharide cellulose by engineered *C. cellulolyticum*. It was not clear whether the heterologous IlvCD or the native valine biosynthetic IlvCD homologues were the primary players in isobutanol production.

S. cerevisiae also has a relatively high tolerance for alcohols,⁹⁷ and due to the ease of genetic tractability and the fact that it naturally produces isobutanol⁹⁸ from L-valine,⁹⁹ it was explored as a host for augmented isobutanol production by increased flux through the valine biosynthetic pathway.¹⁰⁰ The native genes *ILV2* (encoding an acetolactate synthase), *ILV5* (acetohydroxyacid reductoisomerase), and *ILV3* (dihydroxyacid dehydratase) were constitutively overexpressed in cis in *S. cerevisiae* to increase production of KIV from pyruvate. The native gene *BAT2* (branched-chain amino-acid aminotransferase) was also constitutively overexpressed from the chromosome. Bat2 catalyzes the first step in cytosolic conversion of valine to isobutanol,¹⁰¹ and overexpression of *BAT2* in rich media has been shown to increase isobutanol production.¹⁰² A maximum titer of 4.12 mg isobutanol/g glucose was generated from engineered cells grown aerobically in rich media. A maximum of 3.86 mg isobutanol/g glucose was produced aerobically in defined media.¹⁰⁰ In a strategy similar to that used for *E. coli* engineering, a further increase in isobutanol production [6.6 mg/g glucose (143 mg/L)] was achieved by microaerobic growth of a strain with overexpressed *ILV2* (*S. cerevisiae*), *kivd* (*L. lactis*), and *ADH6* (*S. cerevisiae*) and deletion of one of six pyruvate decarboxylase genes (that draw pyruvate to a competing ethanol-producing pathway).¹⁰³

Bacillus subtilis was also studied as a host for keto acid-based alcohol production due to the strain's relatively high solvent tolerance.^{104–106} The genes *alsS* (*B. subtilis*), *ilvC*, and *ilvD*, (both from *Corynebacterium glutamicum*) were overexpressed to generate KIV for isobutanol production. Kivd (*L. lactis*) and Adh2 (*S. cerevisiae*) were utilized to complete the biofuel production pathway.¹⁰⁶ Isobutanol, ethanol, 2-phenylethanol, 2-methyl-1-butanol, and 3-methyl-1-butanol (in order of yield, highest to lowest) were generated from both fermentative and microaerobic conditions. The highest isobutanol titer from

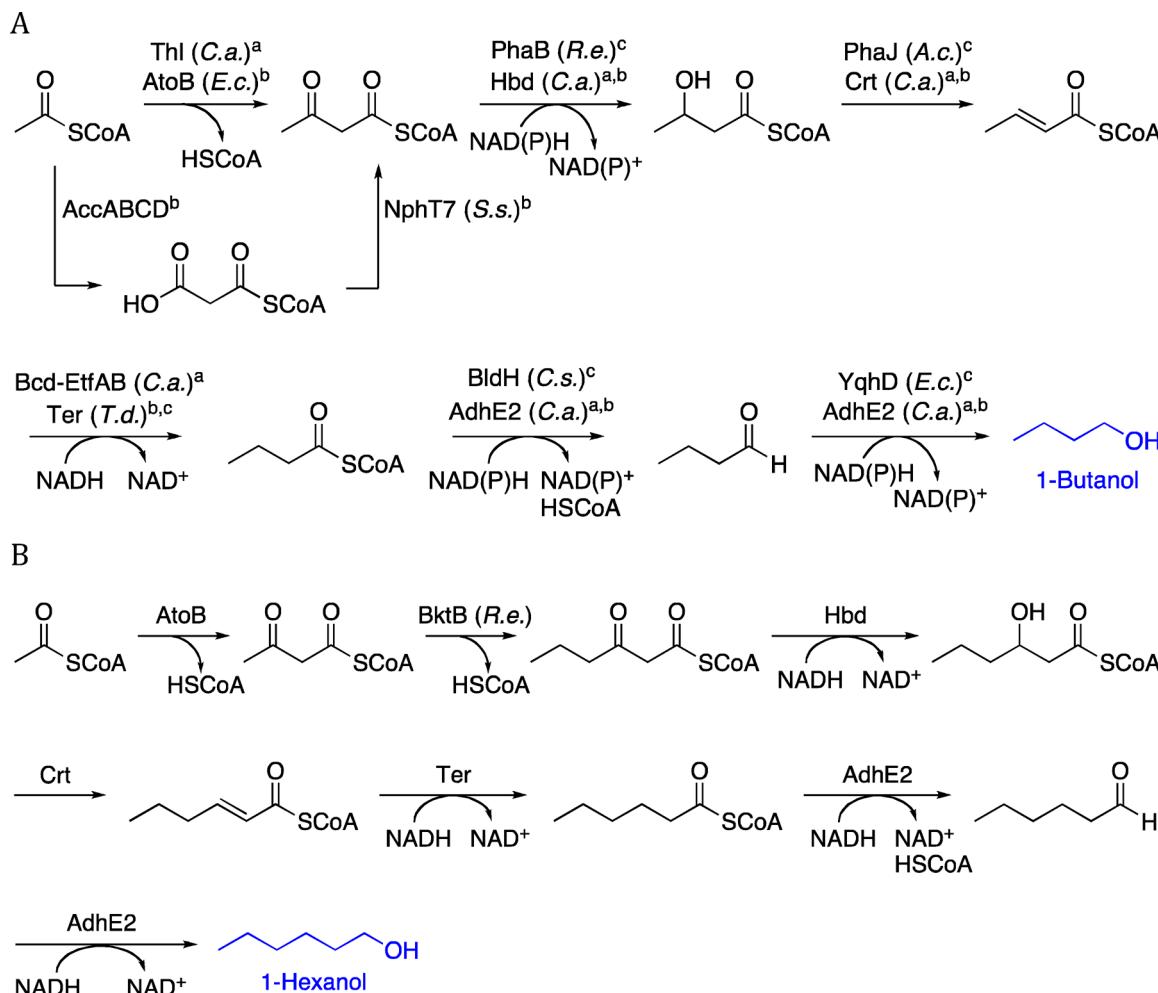


Figure 4. CoA-dependent 1-butanol and 1-hexanol pathways: (A) synthetic 1-butanol pathways [AtoB, acetyl-CoA acetyltransferase; PhaB, acetoacetyl-CoA reductase; PhaJ, enoyl-CoA hydratase; Hbd, 3-hydroxybutyryl-CoA dehydrogenase; Crt, crotonase; Ter, *trans*-2-enoyl-CoA reductase; and AdhE2, aldehyde/alcohol dehydrogenase; (E.c.), *E. coli*; (R.e.), *R. eutropha*; (C.a.), *C. acetobutylicum*; (A.c.), *A. caviae*; (T.d.), *T. denticola*; (C.s.), *C. saccharoperbutylacetonicum* N1–4; (S.s.), *Streptomyces* sp. strain CL190. ^aAtsumi et al.¹²³ ^bShen et al.¹¹⁶ ^cLan et al.¹³⁵] and (B) synthetic 1-hexanol pathway (BktB, β -ketothiolase).

engineered *B. subtilis* was 2.62 g/L, from cells grown microaerobically in shake-flask fed-batch fermentation.¹⁰⁶

C. glutamicum is a Gram-positive soil bacterium utilized for high industrial productivity of amino acids¹⁰⁷ and has relatively high tolerance for isobutanol.¹⁰⁸ Overexpression of *alsS* (*B. subtilis*), *ilvCD* (*C. glutamicum*), *kivd* (*L. lactis*), and *adhA* (*C. glutamicum*) resulted in fermentative production of 2.6 g/L isobutanol.¹⁰⁸ Other higher alcohols were also detected: 3-methyl-1-butanol, 2-methyl-1-butanol, 1-butanol, and 2-phenylethanol. Long-term (96 h) production from a strain with additional genetic changes to decrease carbon flow to competing pathways (deletion of *ldh* that codes for lactate dehydrogenase and *pyc* that codes for pyruvate carboxylase) increased isobutanol production to 4.9 g/L.¹⁰⁸

2.1.6. Keto Acid-Based Alcohols from CO₂. Isobutanol and its precursor isobutyraldehyde have been produced from direct recycling of CO₂ by expression of *alsS*, *kivd*, and *yqhD* in the cyanobacterium *Synechococcus elongatus* PCC7942.¹⁰⁹ Isobutyraldehyde, in addition to being a valuable feedstock for a range of useful chemicals, has a lower boiling point and higher vapor pressure than isobutanol. These characteristics allow isobutyraldehyde to be stripped from cultures during production much more easily than isobutanol, resulting in

higher product recovery due to decreased culture accumulation of toxic products.¹⁰⁹ Increased production of isobutyraldehyde was achieved by overexpression of non-native copies of *rbcLS*.¹⁰⁹ RbcLS are the large and small subunits, respectively, of the key enzyme in photosynthetic carbon-fixation, ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco).¹¹⁰ Rubisco is a known bottleneck in carbon fixation in cyanobacteria,¹¹¹ and increasing available RbcLS enhanced isobutyraldehyde production, likely by overcoming the limitation of poor turnover rate.^{109,111}

S. elongatus PCC7942 has recently been engineered to generate 200 mg/L 2-methyl-1-butanol by expression of genes encoding the citramalate pathway, Kivd, and YqhD.¹¹² The citramalate pathway is an alternative pathway to threonine biosynthesis for generation of the required precursor 2-ketobutyrate (2KB) and is primarily found in methanogens,⁸⁰ spirochaetes,^{113,114} and some cyanobacteria.¹¹⁵ The citramalate pathway requires only one NADH (versus three NADPH and two ATP for the threonine pathway) and involves a decarboxylation step that may drive 2KB synthesis forward.¹¹⁶

The metabolically flexible lithoautotrophic bacterium *Ralstonia eutropha* H16 was utilized to generate longer-chain alcohols, specifically isobutanol and 3-methyl-1-butanol,

through the keto acid-based pathway directly from CO₂ and without light.¹¹⁷ The genes *alsS* and *ilvCD* were expressed in cis, and *kivd* and *yqhd* were expressed in trans. The polyhydroxybutyrate (PHB) synthesis genes were deleted from the genome so that the biofuel pathways could act as the primary carbon and electron sink. The resulting strain LH74D was grown in a fermentor with formic acid as the sole carbon and energy source and with culture pH controlled by addition of formic acid. LH74D generated a combination of 846 mg/L isobutanol and 570 mg/L 3-methyl-1-butanol.¹¹⁷ Electrically charged cultures were bubbled with CO₂ and, with cells protected with a physical barrier (ceramic cup) from anode-generated reactive compounds (such as hydrogen peroxide and superoxide free radicals), produced approximately 100 mg/L isobutanol and 50 mg/L 3-methyl-1-butanol.¹¹⁷ Independency from light as an energy source may greatly increase the potential biofuel yield per cost due to fewer constraints on culture size, reactor surface area, available production time, and land area required.²¹

2.2. Advanced Biofuels: CoA-Dependent Alcohols

2.2.1. Natural CoA-Dependent Butanol Production in *Clostridia*.

Biological fermentative production of the advanced biofuel 1-butanol (along with acetone and ethanol) was first discovered by Pasteur in 1861 and later attributed to the Gram-positive strict anaerobic bacterium *Clostridium acetobutylicum*.¹¹⁸ Several other *Clostridia* species were also found capable of the acetone–butanol–ethanol (ABE) pathway. Some strains produce isopropanol in addition to acetone, butanol, and ethanol, while some produce isobutanol in place of acetone. Jones and Woods provide an excellent review on the history and early biological knowledge of the ABE pathway and use in industrial production.¹⁶

The ABE pathway in *Clostridia* species generates 1-butanol¹¹⁹ from a series of reactions that build on two molecules of acetyl-CoA substrate^{16,120} (Figure 4A). It is thus known as the CoA-dependent pathway of butanol production. In this pathway, two molecules of acetyl-CoA are condensed into acetoacetyl-CoA by an acetoacetyl-CoA thiolase (Thl). The acetoacetyl-CoA is reduced to 3-hydroxybutyryl-CoA by an NADH-dependent 3-hydroxybutyryl-CoA dehydrogenase (Hbd). A crotonase (Crt) then catalyzes a dehydration to yield crotonyl-CoA.¹²¹ An NADH-dependent butyryl-CoA dehydrogenase and its 2-subunit electron transfer flavoprotein (Bcd and EtfAB, respectively) reduce crotonyl-CoA to butyryl-CoA. The action of Bcd/EtfAB is a key step in the butanol pathway, due to the high oxygen-sensitivity of EtfAB,¹²² although it has been demonstrated to function in aerobic conditions.¹⁰⁵ Lastly, butyryl-CoA is sequentially reduced by a butyraldehyde dehydrogenase (AdhE) and a 2-subunit butanol dehydrogenase (BdhAB) to 1-butanol. These last two reactions are also NADH-dependent^{16,120} and can alternately be catalyzed sequentially by a single NADH-dependent aldehyde/alcohol dehydrogenase (AdhE2).¹²³ All of the reactions of the *Clostridia* butanol pathway are reversible and are driven toward formation of butanol likely by high acetyl-CoA and NADH pools.¹⁶

2.1.2. *E. coli* as Host for the CoA-Dependent Pathway.

Due to the requirement for anaerobic growth (resulting in slow growth), underdeveloped genetic tools, and difficult strain characteristics such as spore formation, the five key reactions of the *Clostridia* 1-butanol pathway were heterologously expressed in *E. coli* to develop a high-yield system.^{123,124}

The *Clostridia* 1-butanol pathway was expressed in *E. coli* and produced 13.9 mg/L of 1-butanol. This was not an improvement over the *Clostridia* pathway but demonstrated that the pathway could successfully generate 1-butanol in a non-native host. To improve 1-butanol generation in *E. coli*, the enzyme acetyl-CoA acetyltransferase (AtoB), which is native to *E. coli* and able to catalyze the same reaction as Thl but with higher activity,¹²⁵ was overexpressed in place of Thl. This change improved 1-butanol yield more than 4.5-fold to approximately 64 mg/L. Lastly, several host genes were deleted in order to siphon carbon (acetyl-CoA) and NADH away from competing fermentation pathways, such as that for ethanol and acetate. Deletion of genes from the host [*ldhA* (which codes for a lactate dehydrogenase), *adhE* (alcohol dehydrogenase), *frdBC* (fumarate reductase), *fnr* (DNA-binding transcriptional dual regulator), and *pta* (phosphotransacetylase)] resulted in excess NADH and inhibited cell growth. This was circumvented by growth in microaerobic conditions, which allowed NADH recycling to occur. These changes resulted in another sharp increase in 1-butanol production to 552 mg/L.¹²³

2.2.3. Pathway Improvement.

The type of biosynthetic strategy in which a pathway is driven toward completion by enzyme characteristics such as required cofactors, generation of particular side products, and irreversibility, as opposed to substrate surplus or removal of final product, has been referred to as driving a reaction by an “enzyme chemical mechanism”.¹²⁶ In this strategy, physical sequestration of an enzyme’s product by subsequent utilization as another enzyme’s substrate is the driving force for the forward reaction. By trial-and-error, different combinations of enzymes were tested to find the optimal synthetic pathway with increased 1-butanol productivity. Enzymes from the original *Clostridia* pathway and from a hand-chosen pool of enzymes with characteristics hypothesized to increase rate of the forward reaction toward generation of 1-butanol were tested for production. Most combinations were assayed, and great lengths were taken to identify the biochemical causes of the observed enzyme differences for any single catalytic step. The optimal pathway yielded 4650 mg/L 1-butanol and consisted of PhaA, Hbd, Crt, Ter, and AdhE2. This represents replacement of the first (Thl) and fourth/fifth (Bcd/Etf) enzymes of the original *Clostridia* pathway with PhaA and Ter, respectively. PhaA is a biosynthetic enzyme that catalyzes the condensation of two molecules of acetyl-CoA in polyhydroxyalkanoate (PHA) synthesis in *Ralstonia eutrophus*¹²⁷ (formerly *Alcaligenes eutrophus*) and is capable of high forward flux under in vivo conditions of product removal by a downstream enzyme (PhaC).¹²⁸ Ter is an NADH-dependent enzyme that catalyzes a direct and irreversible reduction of a C–C double bond (transforming crotonyl-CoA to butyryl-CoA) and acts as the primary driving force for this synthetic 1-butanol pathway.^{126,129} Use of PhaA and Ter in place of the *Clostridia* enzymes, combined with optimization of promoter strength for trans expression of all five genes, allowed more than 10-fold increase in 1-butanol yield from the previous reports.^{123,126}

Another heterologous version of the *Clostridia* 1-butanol pathway replaced Thl with AtoB (from *E. coli*) and replaced Bcd/Etf with Ter (from *Treponema denticola*) (Figure 4A).¹¹⁶ Use of AtoB for condensation of two molecules of acetyl-CoA drove formation of 1-butanol due to its higher specific activity for acetyl-CoA than Thl (or PhaA, both of which favor the reverse reaction).^{116,126} Competing dehydrogenases and other enzymes that catalyze NADH-consuming reactions were also

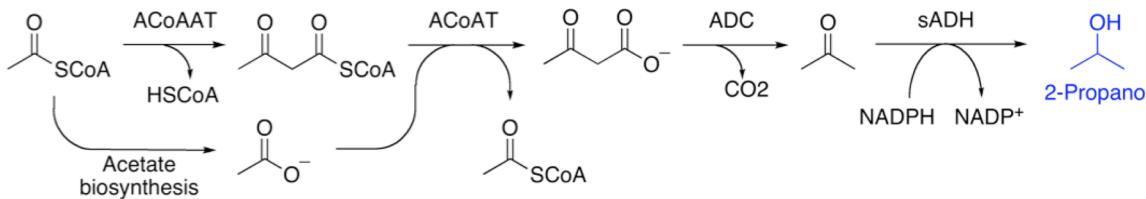


Figure 5. CoA-dependent 2-propanol pathway (ACoAAT, acetyl-CoA acetyltransferase; ACoAT, acetoacetyl-CoA transferase; ADC, acetoacetate decarboxylase; sADH, secondary alcohol dehydrogenase).

deleted,¹²³ which, as expected, affected the cell negatively due to loss of the ability to recycle NADH and thus use it as an electron sink. In theory, increasing NADH consumption in the 1-butanol pathway by use of the NADH-dependent Ter would drive the formation of 1-butanol by providing the cell an outlet for NADH recycling and alleviate the need for semiaerobic growth conditions.¹²³ Formate dehydrogenase (encoded by *fdh* from *Candida boidinii*) catalyzes the oxidation of formate to CO₂ with release of NADH.¹³⁰ Expression of *fdh* may increase the cellular NADH pool and add to the driving force for consumption of NADH via 1-butanol production. However, this correlation was not clearly demonstrated.¹¹⁶ Modifications in the driving forces for formation of 1-butanol, combined with anaerobic growth of the resulting *E. coli* strain, increased production to 15 g/L. Gas stripping to remove 1-butanol from anaerobic fermenters additionally drove production and increased product yield to 30 g/L (88% theoretical yield).¹¹⁶

2.2.4. Other Organisms. A synthetic pathway for 1-butanol has been constructed in *S. cerevisiae*.¹³¹ *S. cerevisiae* is relatively solvent-tolerant (up to 3%),^{97,104} and this makes it an attractive production host. However, initial 1-butanol yield was very low (2.5 mg/L),¹³¹ and the pathway in yeast has not since been optimized.

The CoA-dependent pathway for 1-butanol has also been expressed in *Pseudomonas putida* and *B. subtilis*.¹⁰⁵ Similarly to *S. cerevisiae*, these strains have higher solvent tolerance than *E. coli*^{132,133} and so would also be useful production hosts. Expression of genes *thiL*, *crt*, *bcd/etfAB*, *hbd*, and *adhE1* in *P. putida* S12 achieved 1-butanol production of 44 mg/L from glucose and 122 mg/L from the more reduced substrate glycerol. This represents the first example of aerobically grown bacteria generating 1-butanol from the *Clostridia* CoA-dependent pathway.¹⁰⁵ It was previously thought that enzymes of the pathway, EtffAB in particular, were inactivated in the presence of oxygen.¹²² The same genes were expressed in *B. subtilis*, and generated 23 mg/L 1-butanol from glucose and 24 mg/L from glycerol in anaerobic conditions. No 1-butanol was detected from *B. subtilis* in aerobic conditions.¹⁰⁵ 1-Butanol has also been generated from a recombinant strain of *Lactobacillus brevis*, which expressed *Clostridia thiL*, *crt*, *bcd*, *etfAB*, and *hbd* in trans, while utilizing its native thiolase and aldehyde/alcohol dehydrogenase. A maximum 300 mg/L 1-butanol was produced from glucose during *L. brevis* growth in semiaerobic conditions.¹³⁴

Although production in various organisms may be attractive for their toxin tolerance or growth conditions, *E. coli* will likely always be the optimal expression strain due to its metabolic versatility, genetic tractability, and very high growth rate under many useful conditions.

2.2.5. 1-Butanol from CO₂. The CoA-dependent pathway for 1-butanol production was subsequently moved into the cyanobacterium *S. elongatus* PCC7942 and would represent direct fixation of CO₂ into a valuable biofuel. 1-Butanol

production was low, 14.5 mg/L in 7 days, but the concept of direct CO₂ sequestration into a useful biofuel was proven.¹³⁵ 1-Butanol yield in *S. elongatus* was improved by substituting three of the four NADH-dependent enzymes for NADPH-dependent enzymes (Ter was the exception) (Figure 4A).¹³⁵ Since photosynthesis produces NADPH, but not NADH,¹³⁶ the switch allowed cells to pull from a much more abundant pool of reducing power instead of depleting a relatively small supply of NADH. Also, although the condensation of two molecules of acetyl-CoA by AtoB is relatively favored over that by Thl, the reaction is still thermodynamically unfavorable.¹³⁵ Thus, AtoB was replaced with two sets of enzymes: AccABCD, a native acetyl-CoA carboxylase complex that catalyzes the ATP-dependent formation of malonyl-CoA by carboxylative condensation of acetyl-CoA, and NphT7, a ketoacyl-ACP synthase III enzyme (from *Streptomyces* sp. strain CL190) that catalyzes the decarboxylation of malonyl-CoA to acetoacetyl-CoA.¹³⁵ These changes provide a thermodynamically favorable and irreversible pathway for 1-butanol production that does not require high concentrations of substrate (acetyl-CoA) to drive the reactions forward. The engineered pathway is also functional in the oxic conditions required for photosynthesis. From an ATP- and NADPH-dependent pathway with two irreversible steps, 30 mg/L 1-butanol was produced from *S. elongatus*.¹³⁵

2.2.6. 1-Hexanol. The high-yield CoA-dependent pathway for synthetic 1-butanol production in *E. coli*¹¹⁶ was subsequently modified to generate the longer-chain alcohol 1-hexanol for use as a biofuel.¹³⁷ The synthetic pathway generates butyryl-CoA from two molecules of acetyl-CoA, by the catalytic actions of AtoB, Hbd, Crt, and Ter (Figure 4B). However, instead of immediate reduction to 1-butanol, the butyryl-CoA is first lengthened by two carbons to 3-ketohexanoyl-CoA by condensation with acetyl-CoA in a reaction catalyzed by β -ketothiolase (BktB) from *R. eutropha*. 3-Ketohexanoyl-CoA is then transformed through the reductive pathway of Hbd, Crt, and Ter again to form hexanoyl-CoA. The six-carbon product is then reduced twice in NADH-dependent reactions by AdhE2 to 1-hexanol. Thus, 1-hexanol was generated by addition of a single enzyme to the CoA-dependent 1-butanol pathway. Genes encoding AtoB, BktB, Hbd, Crt, and Ter were expressed in trans in an *E. coli* strain with deletion of competing fermentative pathway genes.¹²³ This pathway produced 27 mg/L 1-hexanol.¹³⁷ However, 1-butanol was the dominant product detected, indicating that pathway enzymes could be altered or replaced to improve activity toward six-carbon substrates. It had been suggested that expression of *fdh* (which codes for formate dehydrogenase and would increase cellular NADH levels) might increase biofuel yield.^{105,116,138} Upon addition of *fdh* to the 1-hexanol-producing *E. coli* strain and a switch to growth in anaerobic conditions, the 1-hexanol yield increased to 47 mg/L. This study showed proof-of-concept that even-numbered, longer-chain alcohols valuable as transportation biofuels can

be generated from synthetic pathways and suggests that optimization may yield more concentrated and longer-chain alcohols.

2.2.7. 2-Propanol. Similarly to 1-butanol production, a trial-and-error method was used to find an ideal combination of enzymes for biosynthesis of 2-propanol in a synthetic heterologous pathway constructed in *E. coli* (Figure 5).¹³⁹ The synthetic pathway was based on the 2-propanol pathway from *Clostridium beijerinckii*, which produces 2-propanol from acetyl-CoA through acetone. The pathway involves condensation of 2 molecules of acetyl-CoA by an acetyl-CoA acetyltransferase, removal of CoA by an acetoacetyl-CoA transferase, formation of acetone by acetoacetate decarboxylase, and finally a reduction to 2-propanol by an alcohol dehydrogenase (Figure 5) (ref 139 and references therein). Enzymes tested include those from *C. acetobutylicum*, *C. beijerinckii*, *E. coli*, and *Thermoanaerobacter brockii*. The combination of a *C. acetobutylicum* Thl (an acetyl-CoA acetyltransferase), *E. coli* AtoAD (an acetoacetyl-CoA transferase), *C. acetobutylicum* Adc (an acetoacetate decarboxylase), and *C. beijerinckii* Adh (a secondary alcohol dehydrogenase) achieved the highest yield of 2-propanol: 4.9 g/L (81.6 mM).¹³⁹ Periodic addition of concentrated media to supplement spent nutrients and application of gas stripping to remove 2-propanol and circumvent toxic accumulation resulted in a drastic increase in 2-propanol titer to 143 g/L.¹⁴⁰

2-Propanol has also been produced from *E. coli* by CBP using cellobiose as substrate.¹⁴¹ Cellobiose is a disaccharide of glucose often used to represent partially degrade cellulosic biomass.⁹ A β -glucosidase from *Thermobifida fusca* (Tfu0937) was fused to an anchor protein for presentation at the exterior of an *E. coli* cell¹⁴¹ also expressing the 2-propanol production pathway.¹³⁹ The resulting strain generated 5.1 g/L 2-propanol from cellobiose in fed-batch fermentation experiments.¹⁴¹

2.2.8. Long-Chain Alcohols from Fatty Acids via Acetyl-CoA. The study and development of CoA-dependent synthesis of biofuels has focused almost exclusively on introduction of a combination of *Clostridia* genes and overexpression of native genes to achieve production of 1-butanol. However, the *Clostridia* 1-butanol pathway can be thought of as a direct reversal of the β -oxidation pathway of fatty acid degradation (Figure 6). There have been recent developments in the generation of long-chain alcohols (1-butanol, 1-hexanol, 1-octanol, 1-decanol) from glucose by reversal of the native fatty acid β -oxidation pathway. Fatty acids are synthesized by all forms of life. The degradative β -oxidation pathway has been especially well-studied in bacteria and normally functions in fatty acid catabolism through acetyl-CoA for harvest of carbon and energy stored in those high-energy molecules.¹⁴² A single turn of the β -oxidation cycle removes two carbons of the fatty acid and can release more than 12 ATP and add one acetyl-CoA into the TCA cycle.³⁶

In initial studies, the native genes of *E. coli* MG1655 were manipulated to effectively reverse the reactions of fatty acid degradation via β -oxidation to generate butyrate (which can be utilized as a butanol precursor) and 1-butanol.¹⁴³ No exogenous genes were introduced into the *E. coli*. The strategy was to first generate an *E. coli* strain able to grow with butyrate as the sole carbon source, which would indicate that reactions transforming butyrate into acetyl-CoA are functional, and then force the cell to reverse the direction of those reactions and generate butyrate from glucose through acetyl-CoA.¹⁴³ It is possible for an *E. coli* strain with modifications only in enzymes

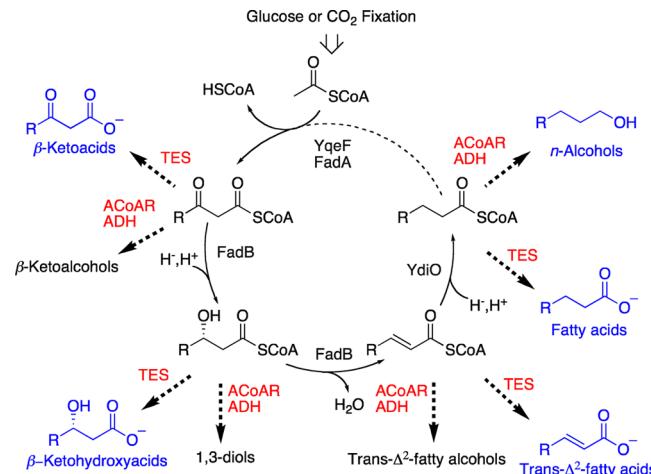


Figure 6. Reverse β -oxidation pathway [YqeF, acetyl-CoA acetyltransferase (*E. coli*); FadA, ketoacyl-CoA thiolase (*E. coli*); FadB, hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase (*E. coli*); YdiO, enoyl-CoA reductase (*E. coli*); TES, thioesterase; ACoAR, acylCoA reductase; ADH, alcohol dehydrogenase].

involved in fatty acid β -oxidation to generate very low levels (0.3–1 mg/L) of 1-butanol.¹⁴⁴ But a strain with a significant butanol titer requires manipulation of genes in other pathways as well. An *E. coli* strain with the genotype $\Delta fadR$ (the gene encodes a repressor for fatty acid β -oxidation), *adhE** (an alcohol dehydrogenase mutated with nitrosoguanidine), *atoC** (a transcriptional activator for the *atoDAB* operon), *P_{tet}-atoB* (a functional thiolase expressed constitutively), and Δldh (a lactate dehydrogenase for a competing pathway) produced up to 1.3 g/L butyrate and 75 mg/L 1-butanol.¹⁴³

Subsequent studies manipulated *E. coli* in a slightly different way to achieve reversal of the β -oxidation pathway¹⁴⁵ (Figure 6). The proteins FadR, AtoC, ArcA, and cyclic AMP (cAMP) receptor protein (Crp) control expression of the regulons of the β -oxidation pathway. Mutations were introduced into *fadR* and *atoC*, *arcA* was deleted, and the native *crp* was replaced with a cAMP-independent version to allow constitutive expression of the pathway genes.^{146,147} Genes that encode enzymes for competing pathways were deleted: *pta*, *adhE*, and *frdA*. *yqeF* and *fucO* were overexpressed in trans to supplement the low activity of likely useful enzymes (YqeF is an acetyltransferase with high sequence similarity to AtoB, and would supplement the activity of that thiolase). FucO is a 1,2-propanediol oxidoreductase and has high sequence and structural similarity to the aldehyde/alcohol dehydrogenases in the 1-butanol pathway of *Clostridia* species. Finally, expression of any one of several genes coding for alcohol dehydrogenases (*yiaY*, *eutG*, and *betA*) allowed generation of C5–C10 alcohols. The highest titer was 1-hexanol at approximately 210 mg/L.¹⁴⁵ This represents the highest titer of long-chain (C6–C10) alcohols generated biosynthetically to date and represents the longest-chain (C10) alcohols yet produced biosynthetically.

3. ADVANCED BIOFUELS: FATTY ACIDS

3.1. Natural Biosynthesis of Fatty Acids

Fatty acids are high-energy molecules utilized naturally for storage of carbon and energy. Fatty acid biosynthesis by both prokaryotes and eukaryotes is a well-studied field. The genes involved in fatty acid biosynthetic pathways, the enzymes they

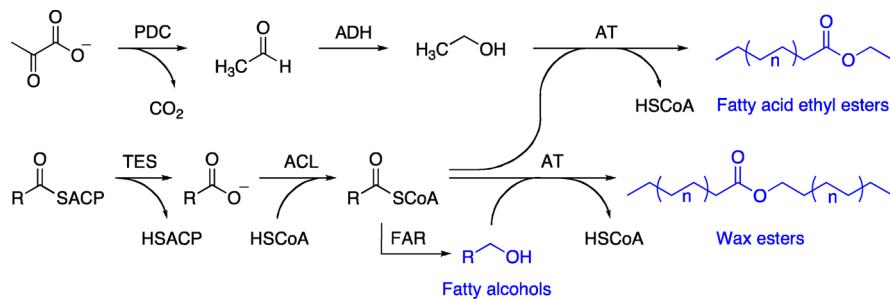


Figure 7. Fatty acid and fatty acid ethyl ester production (PDC, pyruvate decarboxylase; ADH, alcohol dehydrogenase; AT, acyl transferase; TES, thioesterase; ACL, acylCoA ligase; FAR, fatty acylCoA reductase).

encode, the reactions the enzymes catalyze, and the mechanisms of regulation are all well-understood (reviewed in refs 142, 148, 149). Briefly, in *E. coli*, an ATP-dependent acetyl-CoA carboxylase (Acc) converts acetyl-CoA into malonyl-CoA in the first dedicated step in fatty acid synthesis. Malonyl-CoA is then reduced in an NADPH-dependent reaction catalyzed by a multicomponent fatty acid synthase to a fatty acid compound bound to an acyl carrier protein (ACP) (Figure 7). These fatty acyl-ACPs are then used in structures or as carbon or energy storage molecules.¹⁵⁰

Petroleum-based diesel is composed primarily of very-long-chain or aromatic hydrocarbon molecules. Biodiesel fuel is typically generated from the transesterification of acylglycerols, usually produced from plant or animal oils, to form fatty acid methyl esters (FAMEs) or fatty acid ethyl esters (FAEEs).⁸ Wax esters are the esters of fatty acids and long-chain alcohols and do not have favorable fuel properties. It should also be noted that synthetic conversion of free fatty acids (FFAs) to biodiesel with current technology requires high levels of heat or catalysis for esterification.¹⁵¹

Due to similar chemical properties, petrodiesel can be replaced directly with biodiesel, without manufacture of new engines or infrastructure.⁸ Eukaryotic algae are famous for naturally producing significant amounts of acylglycerols, which can be converted to biodiesel, and sparked the initial interest in culturing photosynthetic microorganisms for biodiesel production.¹⁵² These precursor acylglycerols are not produced in appreciable amounts by bacteria. Despite this, the fast growth rate of bacteria, ease of genetic manipulation, and much smaller land area requirement make bacteria very attractive hosts for biodiesel generation.¹⁵³ FAMEs and FAEEs are not known to be generated naturally by any microorganism.

3.2. Biosynthetic Fatty Acid Production

Bacterial genomes can be manipulated to generate increased FFAs from glucose by at least two methods. A gene coding for a cytoplasmic (normally periplasmic) thiolase was expressed to hydrolyze fatty acyl ACPs,¹⁵⁴ and the fatty acid transport and degradation genes *fadDE*, respectively, were deleted from *E. coli* to achieve approximately 1.2 g/L FFA.¹⁵⁵ In another study, fatty acid metabolic regulation was similarly circumvented by deletion of *fadD* and overexpression of a plant thioesterase, a native thioesterase, and the native AccABCD. In addition to decreasing fatty acid degradation, these modifications increased malonyl-CoA substrate and flux through a regulated bottleneck of fatty acid synthesis, to generate 2.5 g/L fatty acids (Figure 7).¹⁵⁶

3.3. System Improvement

After several unsuccessful attempts to improve fatty acid yield over previous work using directed genetic manipulations,¹⁵⁶ a cell-free system was designed to convert acetyl-CoA into fatty acids in order to define limiting aspects and thus possible points for improvement.¹⁵⁷ Varying amounts of substrate (acetyl-CoA or malonyl-CoA), purified Acc enzyme complex (from several sources), reducing equivalents (NADPH), purified thioesterase (from several sources), or acyl carrier protein (ACP) were added to cell-free extracts of *E. coli* *ΔfadD*, and formation of fatty acids was monitored. Results demonstrated that malonyl-CoA and Acc complex are likely limiting in vivo, as supported by previous studies.¹⁵⁸ NADPH is likely not limiting, but if it were, it could be supplemented by conversion from NADH, a reaction catalyzed by a phosphite dehydrogenase (PtdH) from *Pseudomonas stutzeri*¹⁵⁹ modified for increased activity toward NADP⁺.¹⁶⁰ Thioesterase is limiting at low enzyme concentrations (0.66–4 μM, depending on the specific gene expressed) and severely decreases fatty acid yield at higher concentrations. ACP generally inhibited fatty acid synthesis (also supported by previous results¹⁵⁸), indicating that expression must be carefully regulated. An *E. coli* strain was constructed based on the optimal parameters elucidated from the cell-free system. The engineered strain contained a deleted *fadD*, overexpressed *accBCDA* and *tesA* (both from *E. coli*), and an overexpressed plant thioesterase. The strain generated 4.5 g/L fatty acids.¹⁵⁷

Concurrent with previous work,¹⁵⁷ it was found that medium-chain-length fatty acid production in *E. coli* was increased 7-fold over a negative control strain with three modifications similar to those previously found.¹⁶¹ Fatty acid production was increased by deletion of *fadD*, overexpression of *accDABC*, and heterologous expression of a plant ACP thioesterase (encoded by *bte* from *Umbellularia californica*). As in the previous studies,¹⁵⁷ it was found through expression of *bte* from plasmids of different copy number that lowest expression levels (from the lowest copy number plasmid) yielded the highest titer of fatty acids (as well as the most dense cultures by OD₆₀₀). The optimized engineered *E. coli* strain generated 0.81 mg/L medium (C12) chain length fatty acids from glycerol.¹⁶¹

A strain of *E. coli* engineered with a reversed β-oxidation pathway was able to generate a range of biofuels, depending on the identity of the terminal enzymes in the biosynthetic pathways expressed.¹⁴⁵ (See above in “CoA-dependent alcohol production”). The strain contained constitutively expressed genes of the β-oxidation pathway (*fad* and *ato*) and deletions of competing pathways for carbon flux. Heterologous expression of an acyl-CoA thioesterase yielded a range of extracellular fatty

acids, the identity and distribution of which varied depending on the specific thioesterase expressed. A range of fatty acids, in length (C10 to C18) and saturation, were produced in concentrations up to approximately 660 mg/L from engineered *E. coli* grown aerobically with glucose. When cells were grown in fermentors with minimal oxygen (2% saturation) and glucose, 7 g/L fatty acid was produced.¹⁴⁵

3.4. Fatty Acids from CO₂

Bacteria have also been engineered to produce fatty acids from CO₂ and light. While cyanobacterial fatty acid synthesis is not as thoroughly studied as that in *E. coli*, it is similar in that acyl-ACP inhibits fatty acid synthesis at many points in the pathway, including the activity of acetyl-CoA carboxylase (Acc). Also, high levels of thioesterase (i.e., TesA) reduce acyl-ACP levels and thus reduce feedback inhibition of the pathway.^{142,148,162} The genome of the cyanobacterium *Synechocystis* sp. PCC6803 was manipulated in order to explore fatty acid synthesis in cyanobacteria and to explore the possibility of utilizing cyanobacteria as factories for high-titer fatty acid synthesis.¹⁶²

Extensive genome modification was undertaken to ensure that high thioesterase activity (of multiple enzymes) reduced cellular acyl-ACP levels to produce a range of fatty acids, to decrease carbon flux to competing pathways, and to weaken cellular membranes.¹⁶² Five thioesterases were expressed in cis: two copies of *tesA* from *E. coli* and three plant thioesterase genes (from *U. californica*, *Cuphea hookeriana*, and *Cinnamomum camphora*). The *accBCDA* genes were expressed in cis downstream of highly active promoters. Six genes were deleted from the *Synechocystis* genome: The gene *slr1609*, which codes for an acyl-ACP synthetase (homologous to *fadd* in *E. coli*); *sll1951*, which codes for a hemolysin-like surface layer protein with function in cell membrane stability;¹⁶³ *slr1710*, a gene with a role in peptidoglycan assembly;¹⁶⁴ *pta*, which codes for a phosphotransacetylase;¹⁶⁵ and two cyanophycin synthetase genes to avoid formation of the chemical storage molecule cyanophycin.¹⁶⁶ Modifications were maintained in various combinations in a total of 18 strains. The strain with highest fatty acid productivity contained all of the modifications and produced 197 mg/L FFA.¹⁶² Studies are ongoing to further improve FFA yield from CO₂ by cyanobacteria. It was also found that thioesterases could yield fatty acids with different chain lengths or saturation when expressed in different hosts.¹⁶²

Bacteria can also be used to generate FFAs from their cell lipids. Lipids from cell membranes can be released from spent cell cultures by the recently developed Green Recovery method.¹⁶⁷ In *Synechocystis* sp. PCC6803 cells engineered for Green Recovery, lethal genes encoding the lipolytic enzymes from guinea pig (Gpl), fungus (*Fusarium oxysporum*, Fol), or bacteria (*Staphylococcus hyicus*, Shl) are induced for expression under CO₂-limiting conditions. Native lipase (LipA) also contributes to hydrolysis of carboxylic ester bonds of membrane diacylglycerols to release FFAs. This method has been used to generate up to 26.5 mg/L released FFAs.¹⁶⁷

3.5. Generating FAEEs

Petroleum-based diesel fuel is in high demand around the world.⁵ However, FFAs and acylglycerols cannot be directly utilized as a diesel and must first be converted into fatty acid ethyl esters (FAEEs) or fatty acid methyl esters (FAMEs), which have more favorable fuel properties. Thus, there has been exciting work to express essential FAEE and FAME biosynthetic genes from eukaryotic organisms in bacterial

hosts in order to generate compounds directly usable as biodiesel.

The first studies of bacterially generated biodiesel-like compounds were performed by Steinbuchel's group. It was demonstrated that recombinant *S. cerevisiae* were capable of FAEE and fatty acid isoamyl ester (FAIE) production¹⁶⁸ and that engineered *E. coli* were capable of fatty acid butyl ester (FABE) production.¹⁵³ These were accomplished by recombinant expression of the nonspecific wax ester synthase/acyl-coenzyme A:diacylglycerol acyltransferase (WS/DGAT), encoded by *atfA* (Figure 7). WS/DGAT is an enzyme complex with an extremely broad substrate range.^{169,170} It is capable of utilizing linear, cyclic, and aromatic substrates of short-chain to very long-chain-length primary and secondary alkyl alcohols, mono- and di- acyl glycerols, and sterols.¹⁵³ It is also capable of generating a range of substrates including wax diesters, thioesters, and triacyl glycerols,¹⁶⁹ the identity of which depend on factors such as the substrate utilized, the expression host, and growth conditions.¹⁵³

The study of FAEE generation from *E. coli* has involved expressing the gene encoding WS/DGAT from *Acinetobacter baylyi* strain ADP1, along with the ethanol-producing genes of *Zymomonas mobilis*.¹⁵³ Pyruvate decarboxylase (Pdc) and alcohol dehydrogenase (AdhB) from *Z. mobilis* were installed in *E. coli* to catalyze the reductive decarboxylation and subsequent reduction of pyruvate, respectively, to generate ethanol in high productivities. The ethanol produced was combined with oleic acid (exogenously added) by the acetyl transferase activity of WS/DGAT to generate a small range of FAEEs, including primarily ethyl oleate, but also ethyl palmitate and ethyl palmitoleate (Figure 7). The highest titer of FAEEs, 0.26 g/L [6.1% (w/w) of cell content], was obtained with aerobic growth of the recombinant *E. coli* with glucose as the carbon source and exogenous oleic acid.¹⁵³

The next step in engineering bacteria for generation of biodiesel was to achieve FAEE production without exogenous addition of fatty acids (Figure 7). This was achieved by installing several different sets of genes into *E. coli*. As in the previous study, the genes *pdc* and *adhB* (from *Z. mobilis*) were installed to encode the enzymes to produce ethanol.¹⁵³ The genes *fadD* and '*tesA*' (both from *E. coli*) and two copies of *atfA* (from *A. baylyi* strain ADP1) were overexpressed to generate and activate FFAs. FadD is an acyl-CoA synthetase¹⁷¹ with a role in transport of long-chain fatty acids across the inner cellular membrane into the cytoplasm while converting them to acyl-CoA thioesters (ref 142 and references therein). 'TesA, a thioesterase modified to be maintained in the cytoplasm instead of in the periplasm, along with the wax-ester synthase AtfA, constitutes the minimum FAEE biosynthetic pathway.¹⁵⁵ Lastly, the gene *fadE* was deleted to avoid degradation of synthesized acyl-CoA. *fadE* codes for an acetyl-CoA dehydrogenase, which catalyzes the first step of fatty acid β-oxidation by reducing an acyl-CoA group to enoyl-CoA.^{142,172} These genetic modifications allowed generation of 427 mg/L FAEEs from aerobic growth on glucose without exogenously added fatty acids. An organic phase dodecane overlay was utilized to avoid FAEE evaporation and increased FAEE yield to 674 mg/L.¹⁵⁵ A range of products including FAEEs (biodiesel), fatty alcohols, and waxes were generated from this study, depending on the identity of the biosynthetic enzymes. To generate fatty alcohols, *atfA* was replaced with *acr1* (from *Acinetobacter calcoaceticus* BD413), which codes for an acyl-CoA reductase, without expression of *pdc* and *adhB*. Approximately 57 mg/L fatty

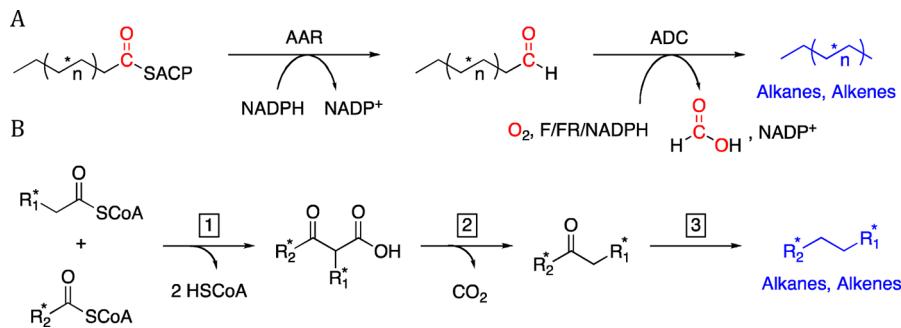


Figure 8. Alka(e)ne production pathways: (A) elongation–decarboxylation (red atoms have been traced to formation of formate by radiolabeling experiments; AAR, acylACP reductase; ADC, aldehyde decarboxylase) and (B) head-to-head condensation (1, aldol condensation; 2, decarboxylation; 3, decarbonylation; an asterisk (*) represents a carbon chain with unknown saturation).

alcohols were produced. To generate fatty esters, *mfar1* [which codes for an acyl-CoA reductase from *Mus musculus* (house mouse)] was expressed in addition to the FAEE synthesis genes, without the ethanol production pathway. Also, by expressing different thioesterase genes, fatty esters and fatty alcohols of products with varied chain length profiles (from C2 to C18) were generated. This study also demonstrated the possibility of generating FAEEs by CBP. The expression of two genes, *xyn10B* (encoding the catalytic domain of an endoxylanase from *Clostridium stercorarium*) and *xsa-osmY* (encoding an extracellular xylanase from *Bacteroides ovatus*), allowed the engineered *E. coli* to degrade hemicellulose (xylan) into xylose. The native metabolic pathways allowed production of 11.6 mg/L FAEEs from growth on xylan.¹⁵⁵

A more recent study¹⁷³ also expressed the *accBACD* operon in combination with other previously studied modifications in *E. coli*,¹⁵⁵ to overcome the proposed limiting step of fatty acid biosynthesis catalyzed by the encoded acyl-CoA carboxylase.¹⁷⁴ The addition of heterologous *accBACD* increased FAEE yield to 922 mg/L from engineered *E. coli* grown anaerobically with glucose.¹⁷³

4. ADVANCED BIOFUELS: ALKANES/ALKENES

4.1. Natural Biosynthesis

Alkanes and alkenes [alka(e)nes] make up a significant portion of petroleum-based gasoline, diesel, and jet fuel. They are traditionally generated via refining of alkane-containing oil deposits. Alkanes are produced naturally in plant pollen and cuticular waxes,¹⁷⁵ as components of insect pheromones,¹⁷⁶ and in a wide range of organisms such as fungus, algae, and cyanobacteria, in which the function is not well-understood but likely has a role in cell protection or normal membrane dynamics.^{27,177,178}

Natural alkane production pathways have been studied in depth biochemically and occur likely through one of two ways. In “elongation–decarboxylation”, an acyl-CoA is cyclically lengthened by a two-carbon unit derived from malonyl-CoA to a fatty acyl-CoA and then decarboxylated to form an odd-chain hydrocarbon. In “head-to-head condensation” two acyl-CoA are joined by aldol condensation, followed by decarboxylation of the resulting carboxylic acid, and decarbonylation to form an odd-chain hydrocarbon (Figure 8).²⁷ Variations in these pathways have been hypothesized on the basis of biochemical studies of alkane intermediates and final products.¹⁷⁹

4.2. In Eukaryotes

Natural alkane biosynthesis in plants, yeast, and fungi has been studied intensely.¹⁸⁰ Very long chain alkanes in plants have a role in protection from water loss,¹⁸¹ and in yeast and fungi, alkanes may regulate cell membrane function.²⁷ Eukaryotic, especially algal, alka(e)nes have been suggested as a possible biofuel.¹⁸² However, very few genes or enzymes required for biosynthesis have been identified.¹⁸³ The eukaryotic synthesis of terpenoids (branched hydrocarbon chains generated specifically from one or more isoprene units) has also been well-studied^{179,184–186} and is a quickly growing topic for application to the field of biofuels (see section 5 of this review).

4.3. In Bacteria

4.3.1. Discovery of the Alka(e)ne Pathway. Although many bacterial alka(e)ne pathways have been shown to be biochemically similar to those in eukaryotes,^{27,188} until recently almost no genetic information was available for the genes involved in natural bacterial alka(e)ne production. The pathway and genes for natural alka(e)ne production (primarily heptadecane, but also pentadecane) in the cyanobacterium *S. elongatus* PCC7942 were recently identified.¹⁸⁹ Subtractive genomic comparison was used to find 17 genes common to 10 sequenced cyanobacterial strains shown to produce alka(e)nes and absent from the nonalka(e)ne producing strain *Synechococcus* sp. PCC7002. Those 17 genes were narrowed down to 10 after excluding those with likely functions unrelated to alka(e)ne production, and study focused on those two with predicted homology to proteins capable of chemical reactions likely involved in alkane biosynthesis. The analysis yielded two genes, *orf1594* and *orf1593*: a putative dehydrogenase or reductase and a putative ferritin-like ribonucleotide reductase, respectively. Ribonucleotide reductases catalyze radical-based removal of hydroxyl groups and may have a similar function in decarbonylation of the hypothesized aldehyde substrate. These two genes are arranged in an operon on the *S. elongatus* PCC7942 genome, and homologues (percent amino acid identity range from 57%–100%) were identified in 57 of 59 sequenced cyanobacteria genomes.¹⁸⁹

4.3.2. In Vivo Experiments with *orf1593* and *orf1594*. Researchers investigated the function of *orf1594* and *orf1593* through “knock out” and “knock in” approaches.¹⁸⁹ These genes were deleted from their bicistronic position in the genome of *Synechocystis* sp. PCC6803, whereafter production of alkanes ceased. The two candidate genes were also expressed in *E. coli* MG1655, and the resulting strain produced detectable amounts of fatty aldehydes and fatty alcohols [alka(e)ne biosynthesis intermediates] and alka(e)nes in culture extracts.

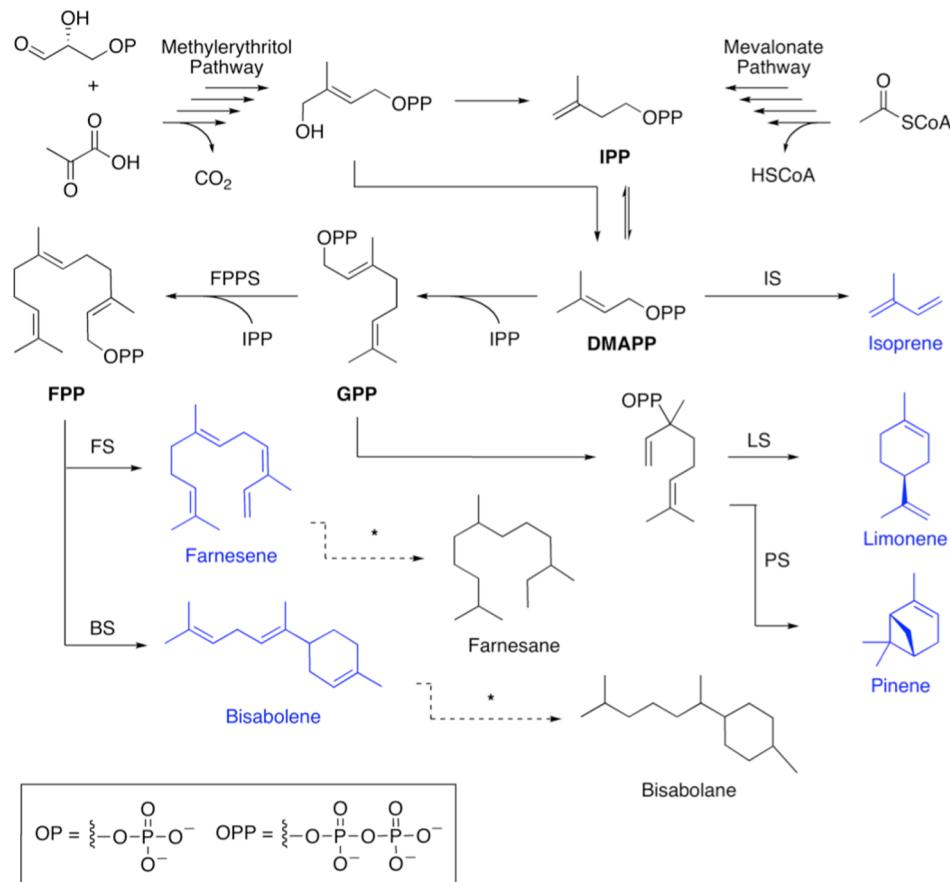


Figure 9. Isoprenoid production pathways (IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; FPPS, farnesyl diphosphate synthase; FS, farnesene synthase; BS, bisabolene synthase; IS, isoprene synthase; LS, limonene synthase; PS, pinene synthase; an asterisk (*) represents a proposed nonbiological synthetic step).

Expression of only *orf1594* generated fatty aldehydes and fatty alcohols [no alka(e)nes], and expression of *orf1593* alone generated compounds not different from the negative control strain (*E. coli* MC1655). Orthologs of *orf1594* from 15 other cyanobacteria were coexpressed with *S. elongatus* PCC7942 *orf1593* in MG1655, and alkanes and alkenes were detected from all culture extracts.¹⁸⁹

4.3.3. Acyl-ACP Reductase (AAR). In vitro characterization of purified recombinant enzymes showed that the product of *orf1594* preferred acyl-acyl carrier protein (acyl-ACP) as substrate to generate the corresponding fatty aldehyde in an NADPH- and divalent cation-dependent reaction. That enzyme was named acyl-ACP reductase (AAR).¹⁸⁹ The preference for acyl-ACP as a substrate differentiates the enzyme from previously characterized acyl-CoA reductases, which ultimately form fatty alcohols as part of wax ester formation and have been shown to be unreactive with acyl-ACP.¹⁹⁰ Specificity for acyl-ACP substrate is preferential for metabolic engineering given that acyl-ACP is predominantly produced during lipid biosynthesis and could be used directly in a constructive production pathway. Acyl-CoA is less desirable as it is predominantly produced through the degradation of fully synthesized fatty acids.^{150,189} To date no crystal structure information is known for acyl-ACP(CoA) reductases, and no detailed mechanism has been proposed. The reaction is likely analogous to acyl-ACP(CoA) hydrolysis. If this were true, attack of the hydride from NAD(P)H, instead of hydroxide, on the thioester carbonyl would drive cleavage of the sulphydryl

leaving group, generating unbound coenzyme A (after protonation) and an aldehyde.

4.3.4. Aldehyde Decarbonylase (ADC). Due to the production of alka(e)nes via decarbonylation of aldehyde substrates generated from AAR, the enzyme encoded by *orf1593* was named aldehyde decarbonylase (ADC).¹⁸⁹ The crystal structure of the ADC ortholog from *Prochlorococcus marinus* MIT9313 (PMT1231) showed a ribonuclease reductase (RNR)-like nonheme carboxylate-bridged dimetal enzyme. The vast majority of these enzymes utilize diiron reaction centers. In vitro activity assays showed that the ADC ortholog from *Nostoc punctiforme* PCC73102 (NpnR1711) required ferredoxin, ferredoxin reductase, and reducing equivalents (NADPH) (F/FR/NADPH) for alka(e)ne production. These components are common requirements for diiron enzymes.¹⁸⁹ Diiron RNR enzymes commonly utilize a diiron(III)-tyrosyl cofactor, which can stabilize a radical intermediate. In ADC, however, the coordinating position is a phenylalanine, which does not offer this activity. The nearest tyrosine residue (6 Å from the dimetal center) is not required for alkane production.¹⁸⁹ Thus, the mechanism for alkane formation is fundamentally different from the reduction of ribonucleotides catalyzed by RNRs.

4.3.5. Discussion of the ADC Reaction. ADC from *N. punctiforme* PCC73102 requires reducing cofactors (F/FR/NADPH)¹⁸⁹ and dioxygen for production of alka(e)nes.¹⁹¹ These are typical requirements for carboxylate-bridged dimetal-cluster-containing enzymes, which catalyze reactions through

activation of reduced dimetal clusters by oxidation with O₂ and subsequent formation of a substrate radical.¹⁹² In the case of ADC decarbonylation of a fatty aldehyde, the substrate radical would decompose into an alka(e)ne and formate,¹⁹² not CO as suggested previously.¹⁸⁹ Stoichiometric production of formate and alkane has since been demonstrated by expression of the ADC ortholog from *Nostoc punctiforme* PCC73102 (NpunR1711) in *E. coli*.¹⁹² Although specific knowledge of the radical intermediate is pending identification of the metal ions in the ADC reaction center, it is proposed that oxygen forms a bridged peroxide intermediate capable of acting as a radical nucleophile to attack the aldehyde. This is supported by radiolabeled oxygen being detected in the formate produced during *in vitro* assays.¹⁹¹ In this mechanism, ADC “oxygenates, without oxidizing, its substrate”,¹⁹¹ which is novel in enzymatic catalysis. Demonstration of this type of chemistry performed by a ferritin-like carboxylate dimetal protein represents a dramatic expansion of the chemistry catalyzed by diiron carboxylate proteins.¹⁹³

4.3.6. Bacterial Alka(e)ne Pathway. In summary, the two-step pathway reduces an acyl-ACP to an aldehyde molecule, which is then decarbonylated to yield an alkane (or alkene, depending on the saturation of the initial fatty ester substrate) and formate.^{189,192,194} The side product formate is formed from atoms including the substrate aldehydic hydrogen and an oxygen atom from O₂ or water. The terminal hydrogen of the alka(e)ne product is from solvent (H₂O).¹⁹² Using recombinant enzymes, approximately 42 mg/L of the alkane heptadecane was detected in *E. coli* culture extract (Figure 8).¹⁸⁹

5. ISOPRENOID-BASED BIOFUELS

Isoprenoids (also called terpenoids) describe a highly diverse and considerable set of compounds that vary in size, structure, and function, but are all built from at least one five-carbon isoprene unit via head-to-tail addition of the key intermediate isopentenyl diphosphate (IPP) (Figure 9).¹⁹⁵ *E. coli* have been engineered to produce IPP at levels that support a 20-fold increase in isoprenoid output.¹⁹⁶ Increased IPP production has also been engineered in *S. cerevisiae*.¹⁹⁷ Isoprenoid compounds are involved in a wide array of natural functions, including signal transduction and chemical storage,¹⁹⁸ cell membrane structures,¹⁹⁹ photoprotection,²⁰⁰ and insect pheromone precursors.¹⁷⁶ Isoprenoids are utilized in a wide range of applications, including biofuels, pharmaceuticals, pesticides, chemical feedstocks, disinfectants, flavors, and perfumes.^{195,201} Isoprenoids are naturally made by most eukaryotes, archaea, and in the cytosols and mitochondria of plant cells by the mevalonate (MVA) pathway, which begins with acetyl-CoA (Figure 9).^{202,203} Microbial engineering to generate isoprenoids was initially inspired by application in medicine, especially production of the antimalarial drug precursor artemisinic acid from yeast.²⁰⁴ Since then it has become apparent that isoprenoids can also be utilized as precursors for a range of “drop in” fuel replacements for diesel and jet fuel.²⁰⁵ They are made naturally in plant chloroplasts and bacteria (including cyanobacteria) by the methylerythritol phosphate (MEP, also called the mevalonate-independent or 1-deoxy-D-xylulose-5-phosphate) pathway, which begins with condensation of pyruvate and glyceraldehyde-3-phosphate (Figure 9).^{184,206} Isoprenoid biosynthesis and regulation in plants have been exceptionally well-studied. Examples of isoprenoids include carotenoids, ubiquinone, and plasquinone.²⁰⁷ Classes of

isoprenoids are based on the number of isoprene units in the compound.¹⁹⁵ Isoprenoid biosynthesis and engineering applications have been reviewed extensively.^{195,207,208} This section will serve as a general overview with key highlights in the field of isoprenoid biofuels.

5.1. Isoprene

The simplest isoprenoid is isoprene (C₅H₈). Isoprene is a hydrophobic, highly volatile compound typically generated from petroleum. It is used to produce millions of tons of rubber annually¹⁹⁵ and has been suggested as a liquid fuel.²⁰⁹ *E. coli* has been recently engineered to express both its native MEP pathway and heterologous MVA pathway in order to generate up to 320 mg/L isoprene (Figure 9).²⁰⁹ It was determined that the majority of isoprene product was a result of the heterologous MVA pathway.²⁰⁹ *Synechocystis* PCC6803 naturally generates IPP but not isoprene. The cyanobacterium has been engineered to generate isoprene by expression of a single codon-optimized plant gene [from kudzu vine (*Pueraria montana*)] encoding an isoprene synthase (IspS).^{187,210} Approximately 50 µg/L/g dry cell weight per day was produced²¹⁰ and can be harvested continuously for 8 days.²¹¹

5.2. Farnesene

The term farnesene represents a group of six isomer sesquiterpene compounds (three isoprene units, 15 carbons), half of which are generated naturally at low levels by insects and plants.¹⁹⁵ The biosynthesis of farnesene occurs through the well-studied MVA pathway to generate IPP and continues with reversible isomerization to the 5-carbon dimethylallyl pyrophosphate (DMAPP) (Figure 9). Sequential head-to-tail condensation catalyzed by farnesyl diphosphate (FPP) isomerase then generates FPP from two molecules of IPP and one molecule of DMAPP.^{212,213} FPP is subsequently converted by one of several single reactions to the oleic farnesene, useful as an industrial lubricant and a biodiesel precursor, or the alcohol farnesol, useful as a fragrance and medicinal compound.²¹⁴ Conversion of FPP to farnesene is catalyzed by farnesene synthases.^{215,216} The double bonds in farnesene can be hydrogenated to generate the biodiesel fuel farnesane by chemical reduction.¹⁹⁵ Farnesane is useful as a drop-in biodiesel or biojetfuel replacement for petrofuels.²⁰⁵

Farnesene is famously the target compound for large-scale industrial production of a petrofuel replacement.²¹⁷ It has been artificially generated from engineered *S. cerevisiae*¹⁹⁷ and *E. coli*²¹⁸ using one of the few α-farnesene synthases that has been characterized in detail (that from apple peel, *Malus x domestica*).^{219–221} A new sesquiterpene synthase has recently been identified from castor beans (*Ricinus communis*) that generates α-farnesene²²² and may be useful in future microbial engineering. The key to higher-yield farnesene production has typically been to increase the precursor (IPP and FPP) pool by expression of either the MVA or MEP pathways. The pathway chosen for heterologous expression is usually the non-native type (i.e., MVA pathway expression in *E. coli*²¹⁸), since that bypasses potentially complex regulatory limitations. Yields up to 380 mg/L have been achieved in *E. coli* by construction and expression of a fusion FPP synthase and codon-optimized α-farnesene synthase, which likely reduced inefficient diffusion of pathway intermediates.²¹⁸

5.3. Bisabolene

Bisabolene is a group of three (α, β, and γ) monocyclic sesquiterpene compounds naturally generated by plants.

Bisabolene is produced from cyclization of FPP by the enzyme bisabolene synthase (Figure 9). It can then be chemically hydrogenated to generate bisabolane, which has been demonstrated to be a candidate renewable replacement for the (petrol) D2 diesel based on similarity of chemical properties.²²³

Bisabolene has been generated in *E. coli* to 912 mg/L, which is 4% of the theoretical maximum from glucose. The five sesquiterpene synthases known (at the time) that generate bisabolene were screened for high titers. That from codon-optimized *A. grana* (AgBIS) demonstrated the highest titer (389 mg/L) and was used in subsequent production optimization. Genes from the heterologous *S. cerevisiae* MVA pathway were inserted^{196,224} and codon-optimized. Lastly, strong promoters were inserted upstream of key pathway genes.²²³ *S. cerevisiae* optimized for sesquiterpene production²⁰⁴ were additionally modified to express AgBIS in order to generate 994 mg/L bisabolene.²²³

5.4. Pinene

Pinenes are a group of four highly volatile bicyclic monoterpenes (two isoprene units, 10 carbons) generated naturally by plants and insects. Pinenes are toxic to yeast cells by inhibition of natural mitochondrial and plasma membrane functions. The very low minimum inhibitory concentration (MIC = 1.52 mM) can be alleviated somewhat by a liquid two-phase solvent extraction from yeast²²⁵ and by an engineered export system in *E. coli*.²²⁶ Head-to-tail condensation of one IPP and one DMAPP by geranyl diphosphate (GPP) synthase generates the direct pinene precursor GPP (Figure 9). Cyclization of GPP to form pinene is catalyzed by pinene synthase. Monoterpenes such as pinene may be useful as flavors, perfumes, and next-generation bio-jet fuel precursors.²²⁷ The energy density of pinenes can be increased by dimerization under moderate conditions (100 °C and atmospheric pressure) with the heterogeneous solid acid catalysts Montmorillonite K-10 (MMK-10) and Nafion.²²⁷

As with sesquiterpene production, monoterpene generation has been achieved in *S. cerevisiae*²²⁸ and *E. coli*²²⁹ and is limited by available precursors. GPP production in *S. cerevisiae* was increased by the cre-lox recombination system that allowed chromosomal insertion of removable (recyclable) strong promoters directly upstream of key native MVA enzymes and insertion of extra copies of key enzymes expressed from strong promoters.²²⁸ *S. cerevisiae* expression of an in trans cineole synthase encoding gene from Greek sage (*Salvia fruticosa*, SfCinS1) resulted in generation of β-pinene (31.59% of total products). Coexpression of a gene encoding HSP90, a protein found by yeast two-hybrid screening to directly interact with SfCinS1, further increased monoterpene production by 60%.²²⁸

By in trans expression of key (limiting) enzymes of the MEP pathway in *E. coli*, the intracellular pinene precursor (GPP) pool was increased. A GPP synthase was constructed by directed mutation (S81F) of IspA, the native *E. coli* FPP synthase. Combined with expression of the monterpene synthase 3-carene cyclase from Norway spruce (*Picea abies*),²³⁰ these modifications generated a range of monterpenes including very low levels of α-pinene (0.9% of total products, approximately 0.027 μg/L/OD₆₀₀) and limonene (0.8%, also see below).²²⁹

5.5. Limonene

Limonene is a pair of cyclic monoterpene compounds (two isoprene units, 10 carbons) found in citrus. Limonene is utilized

as a renewably generated solvent, flavor, fragrance, insecticide,²³¹ and has been verified as a viable biodiesel additive.²³² It is a highly toxic compound to yeast and bacteria (MIC = 0.44 mM in yeast), likely due to inhibition of cell membrane function. Toxicity can be partially alleviated by a liquid two-phase solvent extraction from yeast.²²⁵ It is naturally generated by cyclization of GPP via limonene synthase, and as with other isoprenoids, efficient synthesis depends on the precursor (GPP) pool (Figure 9).²³³

Limonene has been produced from engineered *E. coli*²²⁹ to low levels in two studies. Four genes that encode the pathway for synthesis of the monterpene ketone carvone were expressed in trans,²³³ including the GPP synthase from grand fir (*Abies grandis*) and the limonene synthase from spearmint (*Mentha spicata*).²³⁴ The carvone intermediate limonene was produced to nearly 5 mg/L (4% of theoretical maximum) and was naturally primarily excreted from the cell.²³³ Limonene has also been generated from *S. cerevisiae* engineered for a more efficient MVA pathway and for expression of exogenous SfCinS1. It represented 13.39% of total products.²²⁸

6. SUMMARY/CONCLUSIONS

Synthetic biology has been utilized to metabolically engineer a range of microbial hosts including yeast and photoautotrophic and heterotrophic bacteria, in order to produce many types of biofuels, including long-chain alcohols, fatty acids, alka(e)nes, and isoprenoids. The general method employed in the studies cited herein to develop microbial biofuels is to characterize the enzymes, regulation, and thermodynamics of relevant native metabolic pathways and then to adjust or redesign those pathways around catalytic capabilities, cofactor pools, and other driving forces toward production of the desired compound. In some cases, overlap of production of several types of biofuels is possible from a single strategy with minor adjustments.^{145,155} The majority of maximal titers have been produced from engineered *E. coli* (Table 1), likely due to the thoroughly studied metabolism, genetic tools, and fast growth rate of the bacterium. Yields for microbial production of some advanced biofuels have achieved the levels of industrial relevance, while most are not yet encouraging for industrial scale production (Table 1). However, the proof-of-concept studies and incremental improvements in production titers indicate great possibilities.

Advances in technology allowing for microbial genetic manipulation have increased exponentially over the past decade, along with understanding of enzymatic mechanisms, the driving forces behind a catalytic reaction, and especially the energetic balance required for efficient growth and production. The collective application of that information has presented a great opportunity for biologically synthesizing a range of compounds for use as chemical feedstocks^{74,235–237} and a wide array of biofuels. Microbes, especially bacteria, provide an ideal metabolic “factory” due to their incredible malleability and resiliency and their toolbox of evolutionarily honed central and peripheral metabolic pathways. Although more fastidious than other bacteria, photosynthetic cyanobacteria in particular offer an opportunity to generate valuable and useful products from some of the simplest and most abundant starting molecules usable by a living organism, at the same time decreasing the amount of a detrimental compound (CO₂). Some impressive accomplishments in microbial synthetic biology to generate biofuels have already been achieved. However, recent studies of driving forces,¹³⁵ bottlenecks in metabolic pathways,¹⁵⁷ catalytic

activity of key enzymes,²³⁸ and energetic balance⁸⁸ suggest that the full capacity for microbial biofuel production is yet to be realized.

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Notes

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John W. K. Oliver grew up in Rotorua, New Zealand. In 2006, he obtained his Bachelor's Degree in Chemistry from the University of California—Irvine, where he worked under Dr. Zhibin Guan. During this time he was awarded the Allergan SURP grant for undergraduate research to investigate synthetic polymers that mimic muscle fiber. He is currently a Ph.D. candidate in Chemistry with an Emphasis in Biotechnology, at the University of California—Davis. Under the

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