Biofuels: Biomolecular Engineering Fundamentals and Advances

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Abstract

The biological production of fuels from renewable sources has been regarded as a feasible solution to the energy and environmental problems in the foreseeable future. Recently, the biofuel product spectrum has expanded from ethanol and fatty acid methyl esters (biodiesel) to other molecules, such as higher alcohols and alkanes, with more desirable fuel properties. In general, biosynthesis of these fuel molecules can be divided into two phases: carbon chain elongation and functional modification. In addition to natural fatty acid and isoprenoid chain elongation pathways, keto acid-based chain elongation followed by decarboxylation and reduction has been explored for higher alcohol production. Other issues such as metabolic balance, strain robustness, and industrial production process efficiency have also been addressed. These successes may provide both scientific insights into and practical applications toward the ultimate goal of sustainable fuel production.

INTRODUCTION

The twenty-first century started with the resurrection of biofuels as a potential fossil fuel substitute. Petroleum, which powered the sustained economic growth of the past century, has begun to reach or has reached its peak. The rapid increase in demand has outpaced production in the past few decades. The energy shortage situation is further complicated by political uncertainty and the environmental impact associated with petroleum import and usage. In particular, CO_2 produced from fossil fuels has been implicated as a significant cause of climate change.

The current biofuel life cycle concept starts by recycling CO_2 with the help of solar energy and water to produce biomass via a well-known metabolic process, photosynthesis. Distinct from man-made solar energy–harvesting systems that mainly generate electrical power, biological systems utilize photosynthesis to capture and store solar energy in the form of chemical bonds in biomass. This naturally evolved process provides a unique opportunity to access and exploit solar energy via biological or thermochemical (1–3) conversion of biomass to produce liquid fuels. Biomass can be defined as the collection of all organic matter composing biological organisms, but the main components utilized for biofuel production are sugars (starch, simple sugars, and lignocelluloses) and lipids (4).

Sugars are the most abundant raw material for biofuel production. Bioethanol produced from plant starch and simple sugars has been the most successful biofuel to date. Traditionally, ethanol is produced in the yeast *Saccharomyces cerevisiae* or the proteobacteria *Zymomonas mobilis* from hexoses through a well-studied pathway known as glycolysis, which is followed by decarboxylation of pyruvate and further reduction (5). Lipids serve as another energy storage material in living organisms and can be readily extracted from oil plants, such as soybean and palm, and converted to biodiesel via transesterification. Because of the high C/O ratio in lipids, biodiesel generated from transesterification of biomass lipids enjoys the advantage of high energy density. In addition, the close resemblance between biodiesel and its counterpart derived from petroleum makes it compatible with existing petroleum-based infrastructure with only minor modifications (3).

Although ethanol can be produced by natural hosts with high yields, starch and simple sugars represent only a small fraction of the total plant biomass. Thus the utilization of corn or sugarcane as feedstock becomes economically challenging (4), in addition to presenting a food-versus-fuel issue. Utilization of nonfood lignocelluloses therefore presents a necessary direction for large-scale biofuel production. As for biomass lipids, biodiesel has yet to significantly substitute for petroleum-based diesel fuel due to the limited availability of oil plant feedstocks (6). Thus a complete solution to the biofuel problem requires integrative consideration of agricultural practice, land-use policy, water resource distribution, infrastructure of fuel distribution and usage, and environmental evaluation, in addition to the technical aspects of biological conversion. Nevertheless, this review focuses only on the biomolecular aspects of biofuel production, as improvement in the overall fuel production efficiency by biomolecular engineering for either the current or more desirable fuel molecules will certainly impact each step in the whole biofuel life cycle.

GENERAL ISSUES

The first steps in biofuel production are determining what kind of fuel to make and how to make it. Different types of fuel have different desirable qualities, and this impacts the choice of biocatalyst. These choices provide the backbone of production, for which there are several criteria for measuring performance. All of these issues must be considered in biofuel production.

Desirable Fuel Properties

Biofuels are designed to substitute for the liquid fuels currently used in internal combustion engines, diesel engines, and jet engines. Depending on the specific applications, each category has unique requirements to meet both performance and regulatory standards. All of these petroleum-based fuels consist primarily of alkanes of various lengths and branching patterns. The gasoline currently used for internal combustion engines consists of smaller alkanes containing six to nine carbons on average and must meet specifications for vapor pressure and octane number. In general, increasing carbon chain length lowers the octane number, whereas increasing chain branching increases octane number. Diesel fuels contain the largest alkanes, 12 to 20 carbons in length, and must meet cetane number requirements. Higher cetane number is desirable, but it increases with chain length and decreases with branching. The freezing point of diesel, and the related cloud point, is also an important consideration because long-chain alkanes can begin solidifying at temperatures as high as 10°C, which causes obvious complications for a liquid fuel engine. Jet fuels lie in between the other fuels, containing alkanes of 10 to 15 carbons in length, and the most important specification for jet fuel is low freezing point to maintain fuel liquidity at the low temperatures encountered at high altitudes.

Although ethanol represents an initial success as a biofuel because of its high production efficiency, it does not compare favorably to gasoline. It provides much less energy per volume, has a low vapor pressure, and is hygroscopic, which can lead to corrosion in pipelines and engine ducts (**Table 1**). Furthermore, when added to current gasoline blends, ethanol raises the vapor pressure of the mixture, ultimately increasing the price by forcing the extraction of other light components in the gasoline, although this is partially offset by an increase in octane number. Meanwhile, advanced biofuels such as n-butanol and other higher alcohols with longer carbon backbones have better properties as fuels than ethanol, including higher heating values and low hygroscopicity. Higher alcohols also lower the vapor pressure of current gasoline blends, and while the octane number of n-butanol is slightly less than standard gasoline, branched-chain isomers such as isobutanol have higher octane numbers, allowing for more flexibility in fuel design.

Common Choices

Regardless of the fuel molecules of interest, research and development in this area involve some common issues and choices that include the selection of the host organism, metabolic pathways,

Table 1 Comparison of chemical properties of automobile fuels

Fuel	Ethanol	n-Butanol	Isobutanol	Alkanes (gasoline)	Alkanes (diesel)	Fatty acid methyl esters (biodiesel)
Heating value (MJ/L)	21	29	29	32	39	37
Vapor pressure (psi)	1.1	0.077	0.17	0.1–30	<0.01	<0.01
Blended VP (psi)[a]	20[b]	6.4[b]	6.8[b]	7.8–15		
Avg octane number[c]	116	87	110	90		
Cetane number					45	49–58
Freezing point (°C)					−30–9.9	7.5–16
Hygroscopicity	High	Low	Low	Low	Very low	Very low
Fits current infrastruture?	No	Yes	Yes	Yes	Yes	No

[a]Represents vapor pressure of fuel mixture.
[b]Alcohol blended at 10% with gasoline.
[c]Average of research octane number (RON) and motor octane number (MON).

and enzyme origins. These steps are followed by system optimization to improve the metabolic process for the particular production condition of interest. Host organisms can be either native producers or user-friendly but non-native hosts. The advantages of native producers include higher production efficiency, at least initially, and higher tolerance of product toxicity. However, many native producers are not readily amenable to genetic engineering, and their physiological regulations are either poorly understood or not easily tractable. Therefore, non-native but well-characterized hosts such as *Escherichia coli* and *S. cerevisiae* may offer advantages for long-term success, provided that other shortcomings can be overcome. With the help of genetic tools, biosynthetic pathways can be transferred from one organism to the host of choice. The availability of genome sequencing and bioinformatic tools has greatly accelerated the discovery of candidate genes and pathways (7, 8), and the development of evolutionary techniques has enabled the rapid alteration and improvement of enzyme activities (9). Finally, optimization of whole-cell performance is carried out using a combination of biochemical, genetic, and modeling techniques.

Performance Criteria

A major challenge in biofuel production is the efficiency of the metabolic process. In addition to demonstrating scientific feasibility, the titer, yield, and productivity of the process need to be considered as the performance criteria for biofuel production. These quantities are somewhat related but not necessarily interdependent and present different challenges in research and development. Therefore, when considering the performance of a process, all three criteria need to be evaluated. Product titer is the concentration (e.g., grams per liter) of product accumulated in the bioreactor. It is perhaps the first performance index of interest in the early stage of research. In the industrial process, it determines the cost of product recovery. Yield is defined as the amount of product produced per unit of substrate consumed (grams product per grams substrate). This quantity is perhaps the most important performance index for industrial scale production because it directly determines the raw material cost, which is a dominating factor in biofuel production. For a given metabolic pathway, the theoretical maximum yield can be calculated based on the stoichiometry. The practical yield, however, depends on the physiology and regulation of the whole cell. Productivity (e.g., grams per liter per hour) refers to the rate of production per unit volume of reactor. It determines the cost of operation. Occasionally, productivity per cell (rate of production per cell mass) has been used to judge the performance of cells in the research stage.

Cell Growth versus Product Formation

It has been well recognized that product formation and cell growth are not necessarily correlated. According to mass balance, when cell growth increases, the product yield decreases when the same amount of materials is consumed. Thus, the ideal process minimizes the percentage of cell mass formation from the substrate while maximizing the percentage of product formation. One way to achieve this goal is to grow the cells before induction of product formation pathways. If the product formation pathways can be kept active for a long period of time without cell growth, the product yield can be increased. This type of operation is fed-batch in nature. Given the separation of product formation and growth phases, optimization of process with respect to growth does not necessarily lead to increased production, and tolerance to product toxicity evaluated based on cell growth is not necessarily informative. More sophisticated considerations are in order.

METABOLIC NETWORKS FOR FUEL PRODUCTION

Several biofuels that are naturally produced already have a long history of production, and this serves as the basis for the present state of the art. However, recent research has shown that a variety of potential new biofuels can be created by manipulating metabolic networks. Many of the pathways available for these new fuels are repeatable reaction schemes, which leads to the ability to create designer molecules for a variety of applications.

Ethanol

Ethanol production by fermentation has a long history dating back several thousand years. The natural pathways for ethanol production from sugars in *S. cerevisiae* and *Z. mobilis* have led to yields exceeding 95% of theoretical maximum, which is 0.51 g of ethanol per g of glucose. With such an efficient metabolic process, further improvement mainly resides in broadening the substrate range, enhancing resistance to product toxicity, and increasing robustness in various process conditions.

Natural ethanologenic hosts *S. cerevisiae* and *Z. mobilis* lack the ability to ferment pentoses, which are significant hydrolysis products of lignocellulosic biomass. To tackle this problem, one possibility is to introduce pentose-metabolizing pathways into *S. cerevisiae* (10–12) and *Z. mobilis* (13). However, one can express the ethanologenic pathways into *E. coli*, whose broad range of carbohydrate metabolizing capacity makes it a top candidate for biocatalyst engineering (14). Thus, the homoethanologenic pathway from *Z. mobilis* has been packed into a portable cassette (PET operon, which contains genes for *Z. mobilis* pyruvate decarboxylase and alcohol dehydrogenase B) and integrated into the *E. coli* chromosome at the *pfl* locus, while the *frd* gene was deleted to eliminate succinate production and thus prevent carbon loss. The resulting recombinant strain KO11 was capable of producing ethanol at a yield as high as 95% in a complex medium (14, 15). In addition, biomass-derived feedstock such as rice hulls or sugarcane bagasses have been tested for fermentation, and yields greater than 95% of theoretical yield were achieved (14). However, unlike natural ethanol producers, *E. coli* has a much lower inherent ethanol tolerance. To address this issue, metabolic evolution was used to generate better ethanol tolerance in these strains (16). More recently, to meet the need of lignocellulosic ethanol production, a strain with higher tolerance to toxic side products (e.g., furfural) generated in the acid hydrolysis of hemicellulose has been isolated (17). Interestingly, although current work has focused on introducing heterologous pathways to combine both pentose utilization and ethanologenesis traits in one biocatalyst organism, ethanol fermentation in *E. coli* has been achieved without foreign gene expression. The *E. coli* strain SE2378 has been reported to harbor mutations in the pyruvate dehydrogenase (PDH) operon that result in a mutant PDH that functions in anaerobic conditions and thus allows the balanced production of ethanol at a yield of 82% from glucose or xylose under anaerobic conditions (18). This discovery demonstrated the surprisingly high malleability of natural pathways as well as the potential power of evolutionary methods to generate novel metabolic networks not existing in nature (14).

Isopropanol and 1-Butanol

Both isopropanol and 1-butanol have long been known to be produced in various strains of *Clostridium* via the acetone-butanol-ethanol (ABE) fermentation pathway (**Figure 1**) (19–21). The recent call for longer-chain alcohols as renewable fuels has rekindled the enthusiasm to investigate and optimize these natural hosts. For example, the hyperamylolytic and

hyperbutanologenic strain *Clostridium beijerinckii* BA101 was isolated after chemical mutagenesis and selection and can produce total solvent titers as high as 33 g L^{-1} (22–24). Furthermore, global transcriptomic studies of physiological regulation in *Clostridium acetobutylicum* have provided significant guidance into additional strain improvement (25), such as widening the solvent production window by manipulating the sporulation program (24, 25). Besides upstream optimization of the *Clostridium* host, improvement of downstream fermentation techniques, such as utilization of a fibrous bed bioreactor that immobilizes cells during continuous production (26), represents another avenue toward economically competitive production. More recently, these natural pathways have also been transplanted into user-friendly hosts for further engineering. For example, acetone production pathways from *C. acetobutylicum* were introduced into *E. coli* in combination with a secondary alcohol dehydrogenase (SADH) to convert acetone to isopropanol. This led to a maximum production of 4.9 g L^{-1} isopropanol, which out-competed the production of these pathways in their natural host (27). Similarly, 1-butanol production pathways from *Clostridium* species have also been imported into *E. coli* (28) and yeast (29), resulting in the production of 550 mg L^{-1} and 2.5 mg L^{-1} of 1-butanol, respectively, which are much lower than the yields produced by *Clostridia*. Although these results demonstrated scientific feasibility, technical difficulty in engineering these platform hosts for the synthesis of higher alcohols remains.

Keto Acid Chain Elongation Pathways

As discussed above, although ethanol represents the predominant portion of biofuels produced currently, it suffers from nonideal physicochemical properties when used as a fuel (**Table 1**). Higher alcohols with more favorable fuel properties, such as 1-butanol, can only be produced naturally in some *Clostridium* species (30). Recombinant organisms (27, 31) expressing the Coenzyme A (CoA)-dependent pathway have only achieved relatively low titers. This CoA-dependent pathway is an extension of the oxidative decarboxylation of pyruvate to produce acetyl-CoA, which is also a precursor to ethanol (**Figure 2**). However, this CoA-dependent pathway for ethanol production does not produce ethanol efficiently. The natural ethanol producers *S. cerevisiae* and *Z. mobilis* use a nonoxidative decarboxylation pathway that is independent of CoA (**Figure 2**). This pathway starts by decarboxylation of pyruvate (a keto acid) to form acetyl aldehyde, which is then reduced to ethanol by an alcohol dehydrogenase. The above comparison implies that the keto acid decarboxylation pathway is a more efficient route for alcohol production.

This observation suggests that if a long-chain keto acid can be synthesized, decarboxylated, and reduced, the corresponding long-chain alcohol may be produced efficiently. Such pathways represent an extension of the efficient ethanol production pathway via nonoxidative decarboxylation of pyruvate. Luckily, nature provides metabolic engineers with toolkits for keto acid chain elongation and decarboxylation.

In amino acid biosynthesis, two types of keto acid chain elongation are involved (**Figure 3**): the 2-isopropylmalate synthase (IPMS or LeuA) chain elongation, which adds one net carbon while retaining the branching number, and the acetohydroxy acid synthase (AHAS) chain elongation, which increases the carbon number by two with a branch in the main chain. In these two pathways, acetyl-CoA and pyruvate, respectively, are utilized as elongation units. Reducing power is also applied to convert the added carbonyl carbon into an alkane carbon so that the functional nature of the carbon chain is reset after each cycle of elongation. Moreover, thanks to the promiscuity and potential evolvability of the key enzymes catalyzing the carbon condensation (32), these two types

of chain elongation modules can be applied repetitively in tandem or hybrid fashion to generate a broad panel of 2-keto acids with different carbon numbers and structures.

Isobutanol and 3-methyl-1-butanol. Under the keto acid elongation scheme, pyruvate (a three-carbon keto acid), which is a common central metabolite, can be converted to 2-ketoisovalerate (a five-carbon keto acid) via the AHAS chain elongation (**Figure 3**). 2-Ketoisovalerate is the precursor for valine and leucine biosynthesis, and this pathway is used in almost all microorganisms. Once the five-carbon keto acid is formed, it can be decarboxylated by a keto acid decarboxylase (KDC), such as 2-ketoisovalerate decarboxylase (KIVD) from *Lactococcus lactis* (33, 34). This enzyme is a homolog of pyruvate decarboxylase but was found to have a larger active site cavity to accommodate larger substrates (32). The decarboxylation of a keto acid generates an aldehyde, which can be reduced to the corresponding alcohol by various alcohol dehydrogenases (ADHs) such as Adh2 in *S. cerevisiae* (34, 35), AdhA in *L. lactis* (36), and YqhD in *E. coli* (36). Thus, 2-ketoisovalerate produced from the AHAS elongation pathway is converted to isobutanol (34). This reaction scheme is very efficient and produces more than 20 g L^{-1} of isobutanol from glucose, with a yield reaching 85% of the theoretical maximum (34). Note that the final concentration far exceeded the toxicity level that inhibits cell growth, and the cells continued to produce isobutanol in the nongrowing phase for a long period of time. This is an example of separation between cell growth and product formation resulting in high-yield production of isobutanol.

2-Ketoisovalerate produced from the AHAS elongation pathway can be further elongated via the IPMS elongation pathway to produce 2-keto-4-methylvalerate (a six-carbon keto acid) (**Figure 4**). This compound can then be decarboxylated by a KDC and reduced by an ADH to produce 3-methyl-1-butanol (34, 37).

1-propanol, 1-butanol, and 2-methyl-1-butanol. One round of IPMS chain elongation can also extend pyruvate to 2-ketobutyrate (a four-carbon keto acid) through an artificially evolved citramalate synthase from *Methanococcus jannaschii* (38). Alternatively, the threonine pathway can produce 2-ketobutyarate following the deamination of threonine (39). The resulting 2-ketobutyrate has several possible metabolic paths (**Figure 4**). KDC can directly decarboxylate it and reduce it to 1-propanol. It also can enter another round of IPMS chain elongation to generate 2-ketovalerate (a five-carbon keto acid) for 1-butanol production (34, 39). Alternatively, it can enter a round of AHAS chain elongation to generate 2-keto-3-methylvalerate (a six-carbon keto acid with one branch) for 2-methyl-1-butanol production (34, 40).

The AHAS elongation from 2-ketobutyrate to 2-keto-3-methylvalerate is used for isoleucine biosynthesis, whereas the IPMS chain elongation from 2-ketobutyrate to 2-ketovalerate is nonnative and only used accidentally by cells to make the toxic amino acid norvaline, which may result in its misincorporation into proteins. Thus, to make 1-butanol using this pathway, the *leuABCD* operon must be overexpressed and the endogenous AHAS chain elongation pathway needs to be inactivated (39). By contrast, to enhance 2-methyl-1-butanol production, AHASs from different microorganisms were introduced into *E. coli* and compared for best performance, whereas the *leuABCD* pathway was suppressed (40).

Six- to eight-carbon alcohols. To expand the substrate range of the IPMS chain elongation pathway to longer carbon chains for the production of alcohols ranging from C5 to C8 (**Figure 4**), IMPS was subjected to structure-guided protein engineering to tailor the keto acid binding pocket (32). These IPMS mutants, with various sizes of substrate docking cavity and thus different substrate preferences, were able to catalyze several sequential rounds of IPMS chain elongation from existing keto acid intermediates in amino acid biosynthesis toward production of

Table 2 Functional diversity of amino acid elongation pathways

Substrate	No. carbons	Pathway	Product	No. carbons
Pyruvate	3	KDC-ADH	Ethanol	2
		IPMS	2-ketobutyrate	4
		AHAS	2-ketoisovalerate	5
2-ketobutyrate	4	KDC-ADH	1-propanol	3
		IPMS	2-ketovalerate	5
		AHAS	2-keto-3-methylvalerate	6
2-ketovalerate	5	KDC-ADH	1-butanol	4
		IPMS	2-ketohexanoate	6
2-ketohexanoate	6	KDC-ADH	1-pentanol	5
		IPMS	2-ketoheptanoate	7
2-ketoheptanoate	7	KDC-ADH	1-hexanol	6
2-ketoisovalerate	5	KDC-ADH	Isobutanol	4
		IPMS	2-keto-4-methylvalerate	6
2-keto-4-methylvalerate	6	KDC-ADH	3-methyl-1-butanol	5
		IPMS	2-keto-5-methylhexanoate	7
2-keto-5-methylhexanoate	7	KDC-ADH	4-methyl-1-pentanol	6
2-keto-3-methylvalerate	6	KDC-ADH	2-methyl-1-butanol	5
		IPMS	2-keto-4-methylhexanoate	7
2-keto-4-methylhexanoate	7	KDC-ADH	3-methyl-1-pentanol	6
		IPMS	2-keto-5-methylheptanoate	8
2-keto-5-methylheptanoate	8	KDC-ADH	4-methyl-1-hexanol	7
		IPMS	2-keto-6-methyloctanoate	9
2-keto-6-methyloctanoate	9	KDC-ADH	5-methyl-1-heptanol	8

non-natural keto acids with the maximal chain length of nine (32), which are precursors for the corresponding eight-carbon alcohol (**Table 2**).

To optimize the flux to this non-natural chain elongation pathway, precursor supply was increased and competing pathways were eliminated to enhance the production rate (34, 37, 39, 40). Because these pathways are derived from amino acid biosynthesis, the robust and hyperproducer strains currently available for amino acid production provide a unique advantage as a platform for future application of these metabolic pathways for biofuel production.

Decarboxylase and dehydrogenase. As described above, after chain elongation the hydrocarbon chains with desired length and branching characteristics then undergo two sequential reactions involving decarboxylation and reduction to become alcohols of one carbon less than the original keto acids. KDC and ADH, respectively, carry out these reactions (**Figure 4**). In many organisms these two enzymes enjoy significant flexibility in substrate binding and thus are suitable to convert a diverse range of 2-keto acids into alcohols. Nevertheless, enzyme optimization for the specific product is still necessary. For example, KIVD from *L. lactis* has been engineered for multiple specific keto acid chain lengths (32). Interestingly, acetolactate synthase (ALS) from *Bacillus subtilis*, which is used in efficient isobutanol production as an AHAS homologue, also contains keto acid decarboxylase activity (41). Although the K_m for 2-ketoisovalerate is relatively high (approximately 300 mM), its activity is appreciable when the pathway flux is increased, presumably leading to a high intracellular concentration of 2-ketoisovalerate.

Various ADHs were characterized for isobutanol production. In particular, YqhD from *E. coli* and AdhA from *L. lactis* were shown to be the most active for isobutanol production (36). These

two enzymes are NADH and NADPH dependent, respectively, and can be used in systems where cofactor specificity is important.

Fatty Acid Chain Elongation Pathways

A significant amount of the energy captured by photosynthesis is stored in the form of lipids in plants and algae (42). The mechanisms for fatty acid biosynthesis have been well documented in biochemistry textbooks. The universal fatty acid biosynthetic pathway starts with the ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA, which is then charged with the acyl carrier protein (ACP) and serves as the repetitive unit to be added in fatty acid chain elongation through decarboxylative condensation (**Figure 3**). Within each cycle of chain elongation, two carbons in the form of an acetyl group are added to the growing fatty acyl-ACP carbon chain, followed by sequential input of reducing equivalents to remove the oxygen and saturate the carbon bond. This restores the original acyl-carbon chain structure, which is then poised for the next round of elongation. Although development in molecular biology and metabolic engineering in oil plants and algae have revealed the potential feasibility of enhancing fatty acid yield as well as tailoring this fatty acid chain elongation pathway (43–45), much work has been done recently in industry-friendly microorganisms to optimize fatty acid chain elongation toward production of carbon chains of specific length (46–48). For example, to increase fatty acid synthesis in microorganisms, the enzyme catalyzing the first committed step of fatty acid chain elongation, acetyl-CoA carboxylase (ACC), was overexpressed and fatty acid degradation genes (e.g., *fadD* in *E. coli*) were deleted (46). To alter final elongation products from the predominantly 16–18-carbon chains found in most organisms to the shorter carbon chains necessary for higher-quality fuel production (46), a thioesterase from plants has been heterologously expressed in *E. coli*. Because this enzyme releases fatty acids from ACP to terminate elongation, thus determining the carbon chain length, 12–14-carbon fatty acids were able to be produced in significant quantities (46–48).

Although the direct products of fatty acid biosynthesis have a high energy density, they make very poor fuels. Therefore, modifications are needed to transform those long-chain acids into liquid transportation fuels. The predominant portion of fatty acids in living organisms is esterified with glycerol, other polyols, and fatty alcohols. Current biodiesel production utilizes chemical transesterification reactions between triacylglycerols from oil plants and algal feedstock and short-chain alcohols to form fatty acid methyl esters (FAMEs) and fatty acid ethyl esters (FAEEs) (49). Intracellular fatty acid esterification for FAEE production has been demonstrated in *E. coli* by coupling ethanol production pathways from *Z. mobilis* with the broad substrate range wax ester synthase/acyl-CoA–diacylglycerol acyltransferase (WS/DGAT) from *Acinetobacter baylyi* (50). Similarly, production of FAEEs and fatty acid isoamyl esters (FAIEs) has also been achieved in recombinant *S. cerevisiae* (51). In addition, other naturally occurring mechanisms for fatty acid chain reduction and/or defunctionalization are under investigation, such as fatty alcohol formation (52) and decarboxylative alkane formation (53), although corresponding pathways have yet to be applied to biofuel production. Thanks to the exponentially increasing genomic sequencing data and ever-advancing DNA recombination technologies, more diverse and efficient fatty acid conversion systems may be discovered to provide knowledge and raw materials for future engineering purposes.

Isoprenoid Chain Elongation Pathways

Isoprenoids are another category of hydrocarbons synthesized in a broad range of organisms and are used as pigments, antioxidants, and organic solvents (54). The products, intermediates, and derivatives from isoprenoid biosynthetic pathways are also attractive targets as nutraceuticals

and pharmaceuticals (21), as well as for jet fuel alternatives and gasoline additives (8, 42). Despite their structural diversity, all isoprenoids are synthesized through a universal chain elongation pathway shared by numerous species. The isomers isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are the basic units to be added consecutively to the growing carbon chain; these are coupled by the release of a diphosphate (**Figure 3**). Thus, five carbons are added in each cycle of chain elongation. IPP or DMAPP can be synthesized either through the mevalonate pathway or the 1-deoxy-D-xylulose-5-phosphate (DXP) pathway. The former uses acetyl-CoA as the starting metabolite, whereas the latter starts from glyceraldehyde-3-phosphate (GAP) and pyruvate. In contrast to fatty acid chain elongation and keto acid chain elongation, no CO_2 is released and no reducing equivalents are input after carbon condensation in the mevalonate and DXP pathways. As a result, double bonds in the building blocks are preserved, thus causing the periodic occurrence of carbon double bonds throughout the hydrocarbon chain. Owing to the potential of isoprenoid compounds to deliver commercially attractive chemicals, much research has been published on engineering isoprenoid biosynthetic pathways, especially in microorganisms (55–61). One major strategy is to enhance carbon flux for building block supply by manipulating DXP pathways in *E. coli*. Specifically, the genes *dxs* and *idi*, encoding DXP synthase and IPP isomerase, respectively, were overexpressed to enhance carbon flux for IPP supply (55–58, 62–65). Moreover, both combinatorial and rational methods have been used to fine-tune gene expression within this pathway as well as to discover and engineer its regulatory mechanisms (59–61, 65). For example, in a recent study 24 endogenous genes shown to affect lycopene production in recombinant *E. coli* harboring the *crtEBI* operon were subjected to a recombination-based combinatorial genome engineering approach in search of the optimal expression pattern. The mutant that delivered the highest lycopene yield of 9000 ppm had four genes (*dxs*, *idi*, *dxr*, *rpoS*) overexpressed and one gene (*ytjc*) knocked out simultaneously (65). However, mevalonate pathways from yeast have also been transplanted into *E. coli*, providing an efficient precursor supply platform for further engineering of downstream pathways (62, 63). Similarly, combinatorial approaches have also been used to manipulate the activity of other genes (such as *atoB*, *HMGS*, *HMGR*) in these pathways by controlling their post-translational processing (64).

In addition, to determine the chain length in isoprenoid synthesis pathways, isoprenyl pyrophosphate synthases (IPPSs) that yield final products of different lengths have been identified and the mechanisms underlying chain length determination have been studied (66). This has provided knowledge and materials for protein engineers to tailor isoprenoid synthesis pathways. For example, by introducing and engineering geranyl-geranyl diphosphate (GGPP) synthase from *Archaeoglobus fulgidus*, the production of the desired 20-carbon product GGPP was enhanced (55, 56).

Isoprenoid biosynthesis pathways generate a large family of branched and cyclic hydrocarbons that may possess the properties favorable for jet fuels and diesel fuels (8, 42, 49). Although most efforts in isoprenoid biosynthesis engineering have targeted nutraceuticals and pharmaceuticals (such as lycopene) as final products, the IPP/DMAPP overproducing systems and elongation pathways mentioned above may be readily adaptable for potential biofuel production. For instance, isopentenol, a proposed gasoline additive or substitute, was produced by overexpressing *nudF* from *B. subtilis* in combination with the mevalonate pathway in *E. coli* and reached a titer of 112 mg L^{-1} (67).

THE CELL AS A SYSTEM

It is not enough to merely have all the pieces for biofuel production in one biocatalyst. Once the pathways have been designed for the desired compounds, further modifications are necessary to

exploit the full potential of these pathways. Balance must be achieved between precursors and cofactors, and wasteful side reactions must be eliminated where possible.

Strain Optimization

The above pathways explored for production of next-generation biofuels, including higher alcohols, fatty acid derivatives, and isoprenoid derivatives, can be divided into two parts: chain elongation and functional group modification. The first part synthesizes molecules of the desired chain length. In the second part these keto acids, fatty acids, or isoprenoids are turned into fuel-quality molecules such as alcohols, esters, and alkanes. Nonetheless, construction of desired biosynthesis pathways is only the first step toward economically viable biofuel production. Increasing the titer, yield, and productivity to the economically viable level is a major challenge and will ultimately determine the feasibility of each approach.

Once the target pathway is selected, maximum theoretical yield can be calculated based on the stoichiometry of the pathway (**Table 3**). This is done by either simple hand calculation or linear optimization to maximize the yield. Assumptions such as interconversion between NADH and NADPH and the existence of recycling pathways will affect the results of such calculations. Because the ideal production occurs after cell growth, the maximum theoretical yield calculation assumes no growth during the production phase.

However, the maximum theoretical yields are difficult to achieve in practice for several reasons: (*a*) Other endogenous pathways may compete with the target pathway for carbon metabolites, cofactors, or energy (8); (*b*) the desired pathway may cause an imbalance of cofactors such as NADH and NADPH (5, 21); and (*c*) final products or intermediates generated by the target pathway may disturb host metabolism in a specific or nonspecific way (5). To effectively incorporate synthetic pathways into host metabolic networks as well as optimize host physiology for efficient

Table 3 Energy yield of various fuels

Fuel	Mass energy density (MJ kg^{-1})	Volumetric energy density (MJ L^{-1})	Max biochemical yield (g g^{-1})[a]	Pathway stoichiometric yield (g g^{-1})	Energy yield from glucose (%)[b]
Gasoline	42.7	32.0	–	–	–
Jet fuel	43.8	34.8	–	–	–
Diesel	45.5	38.7	–	–	–
Ethanol	29.7	20.8	0.511	0.511	97.6
Propanol	33.6	27.0	0.444	0.444	95.9
1-butanol	36.1	29.2	0.411	0.411	95.4
Isobutanol	36.1	29.0	0.411	0.411	95.4
1-pentanol	37.7	30.8	0.391	0.326	79.0
3-methylbutanol	37.7	30.5	0.391	0.326	79.0
2-methylbutanol	37.7	30.5	0.391	0.391	94.8
Fatty acids (C12–C22)	37–41	33–35	0.35–0.39	0.34–0.37	89–90
Isoprene–Mev[c]	43.8	29.8	0.324	0.252	71.0
Isoprene–DXP[d]	43.8	29.8	0.324	0.300	84.5

[a]Theoretical yield based on best available cell metabolic pathways.
[b]Based on an energy density for glucose of 15.6 MJ kg^{-1}, using pathway yield.
[c]Using the mevalonate (Mev) pathway for isoprenoids.
[d]Using the 1-deoxy-D-xylulose-5-phosphate (DXP) pathway for isoprenoids.

production, both rational and combinatorial approaches have been applied in many different schemes (5).

Rational strategies take advantage of accumulated knowledge of genetic and biochemical metabolic regulations and propose focused and usually small-scale alterations to existing systems. Inspection of the pathways or stoichiometric models readily identifies competing pathways, and a remedy is usually straightforward. Similarly, pathway or stoichiometric model inspection relatively easily suggests potential NADH/NAD^+ or NADPH/$NADP^+$ limitations. However, proof or remedy of this problem is nontrivial. In general, various mathematical modeling techniques may help to identify potential targets for gene knockouts (68–71) and enzyme overexpression (72–75).

Some illustrative examples of rational metabolic engineering involve alleviation of a reduction/oxidation (redox) imbalance or cofactor imbalance caused by heterologous pathway importing. For instance, to enable the most efficient ethanologenic yeast, *S. cerevisiae*, to utilize xylose for bioethanol fermentation, xylose reductase (XR) and xylitol dehydrogenase (XDH) genes from *Pichia stipitis* were overexpressed (10). However, this two-enzyme pathway was not redox balanced by itself owing to NADPH dependence of XR and NAD^+ dependence of XDH, causing a significant accumulation of the intermediate xylitol (76–78). To transform this pathway into a closed redox loop, protein engineering has been carried out to switch the coenzyme specificity of either of the two enzymes, which resulted in significant yield improvement (9, 76–78). To solve similar problems from a different angle, some host genes from outside the production pathways, such as the ones encoding malate dehyrogenase, formate dehydrogenase, and pyridine nucleotide transhydrogenase, have also been manipulated to compensate for cofactor need for product synthesis (79–81).

By contrast, combinatorial strategies are especially suitable to solve metabolic engineering problems in which little information is known, such as those concerning product toxicity (5). Almost all biofuel products have solvent-like properties and may be harmful to microbial membranes. Although traditional mutagenesis and stress adaptation methods have enjoyed significant success in developing desirable phenotypes including product tolerance (5, 16, 82, 83), mechanisms involved in those traits may be complicated and elusive. In addition, classic mutagenesis methods such as chemical or UV treatment introduce multiple mutations that are not readily identifiable traditionally and may include both beneficial and detrimental mutations. However, thanks to the development of rapid genome sequencing technologies, mutations can now be detected and their individual phenotypes can be potentially characterized and transferred.

In contrast to traditional mutagenesis, transcription machinery (84–87) and transcription factors (88) can be targeted for mutagenesis to alter global transcriptome profiles and thus the phenotype. In combination with high-throughput screening methods, these combinatorial approaches have enjoyed promising success in improving complex cellular phenotypes such as glucose and ethanol tolerance in yeast (84) as well as butanol tolerance (87), heat-shock resistance (88), and production yield of chemicals in isoprenoid biosynthesis pathways in *E. coli* (64, 65, 89).

Synthetic Host

In addition to optimizing naturally existing hosts and pathways, researchers have proposed the possibility of creating production microorganisms from scratch with a more defined metabolism based on the concept that standardized and interchangeable gene network modules can be assembled into a larger system with predictable behavior (8, 90, 91). To advance toward this ultimate goal, advanced molecular tools are required. In particular, DNA assembly technologies capable of integrating large DNA fragments have enabled quick assembly of complex pathways and, more strikingly, the whole genome of a microorganism (92–94). Regulatable protein expression

platforms and devices have also been developed to sustain the proper functioning of heterologous proteins (especially those from organisms living in extreme environments) (8, 9, 95), as well as to fine-tune expression levels of multiple pathway components simultaneously to achieve global metabolic optimization (64, 89, 96).

LIGNOCELLULOSIC BIOMASS

Although starch and simple sugars from corn or sugarcane are currently used on a large scale as feedstock, it has been speculated that lignocellulosic biomass, which potentially enjoys a more abundant, widely distributed, and cost-effective resource base, can play an indispensable role in the paradigm transition from fossil fuel–based energy production to renewable energy production (4, 5, 7, 97). However, the recalcitrant nature of lignocellulosic biomass, as well as its complicated composition, places challenging obstacles to its efficient utilization as feedstock (7, 98). Current lignocellulosic biomass biofuel production starts from chemical and physical pretreatment of plant biomass to expose and partially hydrolyze cellulose (the most abundant component of plant cell walls composed of polymerized glucose), followed by hydrolysis of cellulose by the cellulase enzyme to liberate simple sugars for microbial fermentation (7, 97). Whereas recently emerged plant engineering (7, 97, 99) and pretreatment technologies (100) have promised significant opportunities to tackle the problem of biomass conversion, metagenomic sequence mining (101, 102) and protein engineering approaches based on an ever-expanding template bank have been proposed to play central roles in cellulase optimization (7, 9). Of particular interest, by manipulating protein interactions between individual cellulolytic enzymes and a common scaffold comprising a cellulosome, the multienzyme machinery for the degradation of cellulosic substrates, protein engineers sought to mix and match a divergent array of enzymes with different catalytic specificity and properties. A synergy effect allowed these "designer cellulosomes" to deliver higher enzymatic efficiency, providing better performance in the deconstruction of more complex lignocellulosic substrates due to an expanded substrate spectrum (9, 103, 104). Recently, to increase the robustness of cellulase during industrial processes, SCHEMA structure-guided protein recombination was performed to shuffle fragments from different parent fungal class II cellobiohydrolases (CBH II cellulases); this led to the identification of CBH II chimeras with better thermostablity and broader pH/activity profiles (105). This work revealed opportunities for future enzyme optimization by combinatorial protein engineering methods. Accordingly, high-throughput strategies have recently been developed for cellulase assessment and selection (106, 107), which has set the stage for further utilization of library-based protein engineering strategies.

CONCLUSION

Although biofuel production is intrinsically an engineering problem, new developments in molecular biology, metabolic engineering, and systems biology have broadly expanded the spectrum of possible fuel molecules and production platforms. These new developments address the shortcomings of existing biofuels and provide more flexible choices for industrial production.

Three chain elongation pathways, either naturally existing or synthetic, have shown potential to deliver suitable hydrocarbons for desirable fuels: keto acid chain elongation, fatty acid chain elongation, and isoprenoid elongation. To enhance the production capacity of these pathways, metabolic engineering and protein engineering have been applied to (*a*) seek the best combination of genes from a variety of organisms to compose pathways in user-friendly hosts, (*b*) fine-tune the activity of different genes within the synthetic pathways, and (*c*) tailor individual enzymes for higher efficiency or novel catalytic ability. Global engineering of host metabolic networks as

well as physiological regulation circuits is also necessary to boost carbon source, cofactor, and energy supply to support the synthetic pathways. In addition, to meet the needs of large-scale industrial production, the ideal hosts need to be robust under process conditions and tolerant to product toxicity. Advanced biomolecular approaches, both rational and combinatorial, coupled with traditional strain selection strategies, may also develop these traits. In summary, biofuel production with its interdisciplinary nature represents great challenges and opportunities for chemical and biomolecular engineers. The rapidly advancing tools will pave the way for biofuel to become a significant solution to energy and environmental problems. During production of this article, isobutanol production directly from CO_2 was achieved using photosynthetic bacteria (108).

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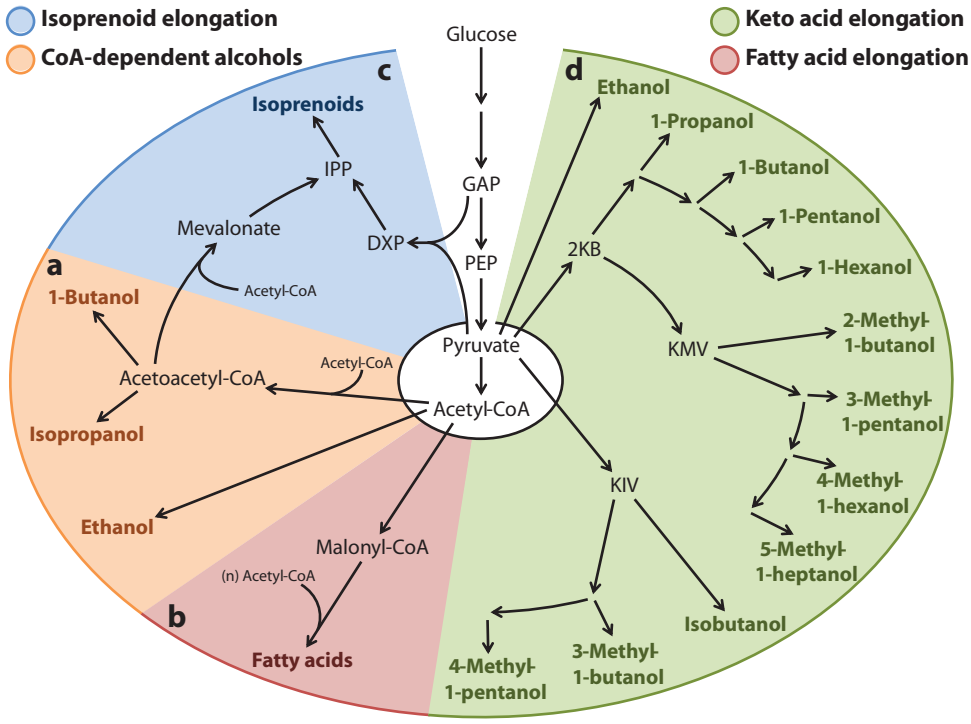

Figure 1

Schematic of biosynthetic pathways used in the production of biofuels. Metabolic pathways currently being explored for biofuel production can be grouped into four categories: (*a*) natural alcohol production pathways, which reside in natural hosts to produce ethanol, isopropanol, and 1-butanol (*orange*); (*b*) fatty acid elongation pathways for production of fatty acids that can then be converted to biodiesels (*red*); (*c*) isoprenoid elongation pathways for the production of isoprenoid-derived hydrocarbon fuels (*blue*); and (*d*) keto acid elongation pathways, which elongate the simple keto acid, pyruvate, to form keto acids with chain length ranging from 4–9 for the production of higher alcohols with 3–8 carbons (*green*). GAP, glyceraldehyde-3-phosphate; PEP, phosphoenol pyruvate; AceCoA, acetyl Coenzyme A; 2KB, 2-ketobutyrate; KMV, 2-keto-3-methylvalerate; KIV, 2-ketoisovalerate; DXP, 1-deoxy-D-xylulose-5-phosphate; IPP, isopentenyl diphosphate.

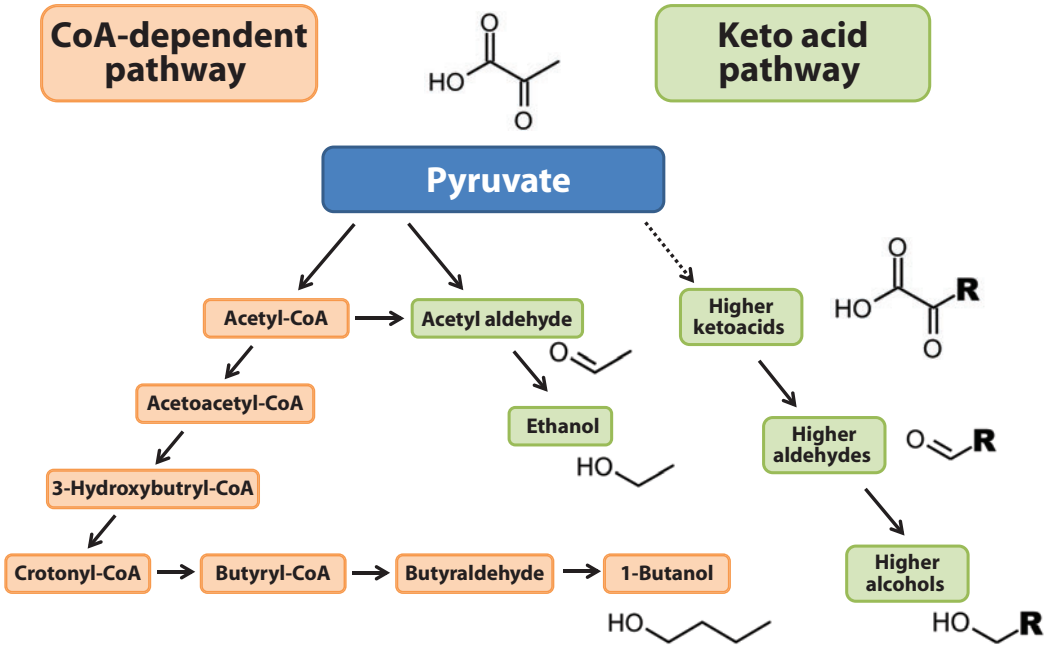

Figure 2

Pathway diversity for alcohol production from pyruvate. Higher alcohols such as 1-butanol can be produced in natural hosts from pyruvate via the Coenzyme A (CoA)-dependent pathway. Alternatively, the highly efficient ethanol-producing pathway in natural producers *S. cerevisiae* and *Z. mobilis* proceeds via decarboxylation of pyruvate (a keto acid) to form acetyl aldehyde, which is then reduced to ethanol by an alcohol dehydrogenase. Based on the comparison above, if higher keto acids with longer chains can be synthesized, decarboxylated, and reduced, higher alcohols may be produced efficiently.

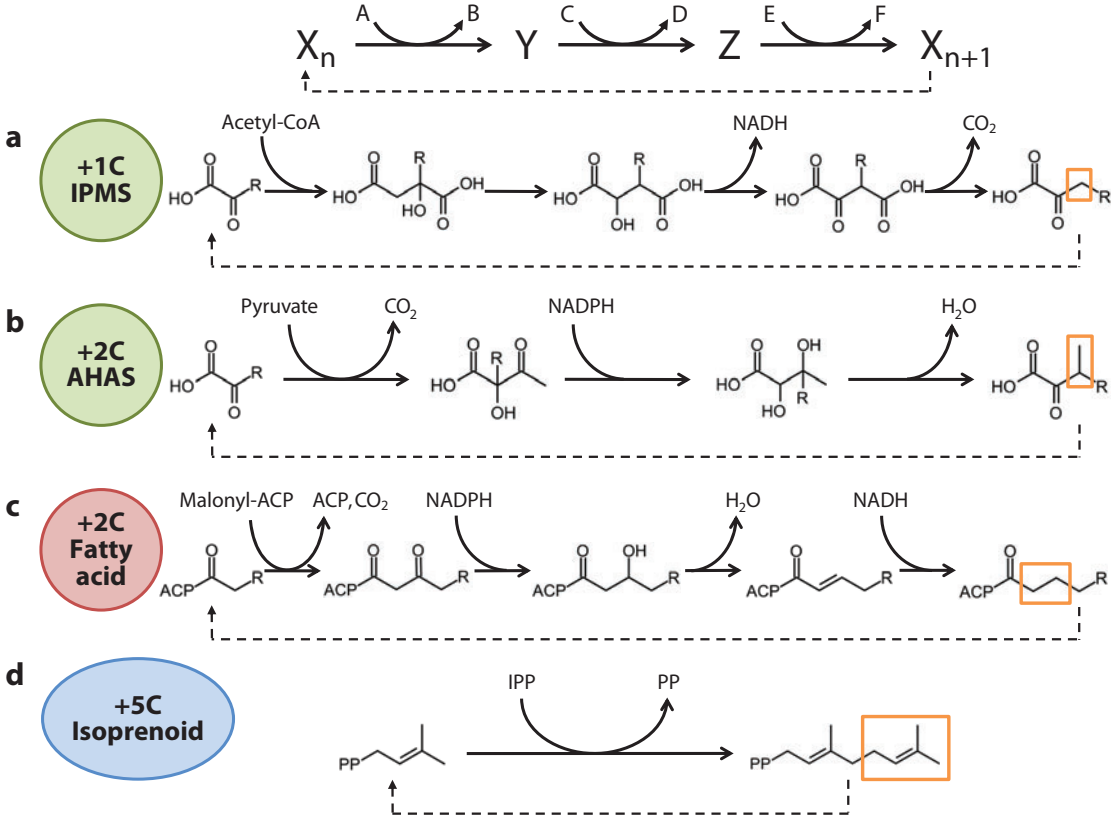

Figure 3

Biosynthetic pathways for carbon chain elongation. Four chain elongation pathways are currently used to compose desirable hydrocarbon chains for biofuel molecules: (*a*) isopropylmalate synthase (IPMS, leucine biosynthesis) chain elongation, which elongates a keto acid carbon chain by one carbon in each cycle; (*b*) acetohydroxy acid synthase (AHAS, valine/isoleucine biosynthesis) chain elongation, which increases the carbon number of a keto acid by two and generates a branch point; (*c*) fatty acid elongation, which adds two carbons linearly to a fatty acyl-acyl carrier protein (ACP); and (*d*) isoprenoid elongation, which adds isopentenyl diphosphate (IPP) monomers to elongate the carbon chain by five in each cycle. The larger subunits can also be used as monomers for additions of 10 carbons, 15 carbons, etc.

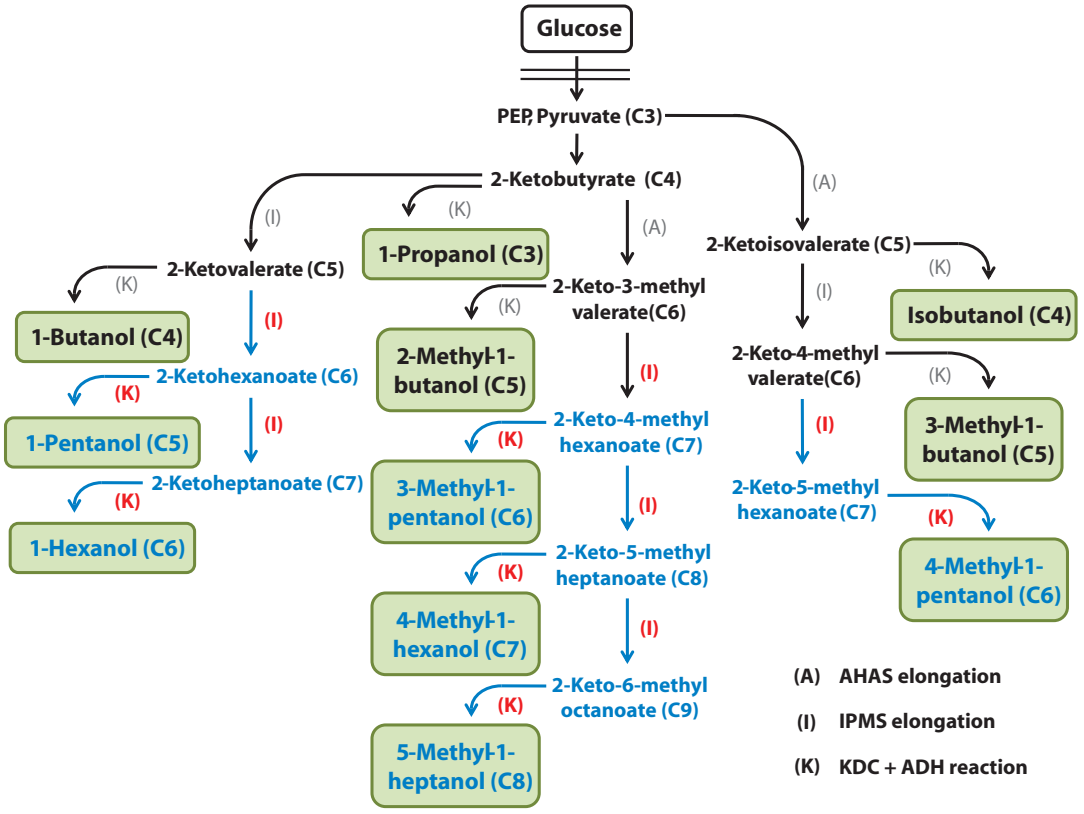

Figure 4

Non-natural synthetic pathway for longer-chain alcohol production. AHAS elongation (*A*) can produce natural keto acids such as 2-ketoisovalerate (valine) and 2-keto-3-methylvalerate (isoleucine) from pyruvate and 2-ketobutyrate, respectively. The engineered IPMS elongation pathway (*I*) can elongate some natural keto acids, such as 2-ketovalerate (norvaline) and 2-keto-4-methylvalerate (leucine), as well as 2-keto-3-methylvalerate. They are then decarboxylated by the redesigned keto acid decarboxylase (KDC) and reduced by alcohol dehydrogenase (ADH) (*K*) to become non-natural C5–C8 alcohols. All nonnatural reactions are represented with blue arrows and red letters.

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Contents

Errata

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