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Alternative biofuel production in non-natural hosts

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Global energy and environmental concerns have stimulated increased efforts in synthesizing petroleum-derived products from renewable resources. Biological production of metabolites for fuel is increasingly becoming a feasible, renewable, environmentally sound alternative. However, many of these chemicals are not highly produced in any known native organism. Here we review the current progress of modifying microorganisms with heterogeneous elements for the production of biofuels. This strategy has been extensively employed in a variety of hosts for the development of production of various alcohols, fatty acids, alkenes and alkanes.

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Introduction

National interest toward mitigating dependence on foreign fossil fuel is growing in the face of challenging socioeconomic unrest, increasing price volatility, and expanding climate concerns. The US Department of Energy has identified alternative, renewable liquid fuels as a core solution to these challenges [1]. Desirable, renewable liquid fuels have qualities that are similar to current fuels in physical and energetic characteristics while minimizing green house gas emissions. One current strategy to develop cost-effective synthesis of such 'drop-in' fuels is engineering microorganisms to produce these fuels from a renewable chemical feedstock [2]. However, drop-in fuels are generally not highly produced by common industrial microorganisms. Here we review the current efforts of engineering microorganisms to highly produce metabolites for advanced biofuels.

Using this synthetic biological approach in conjunction with metabolic engineering tools requires some considerations. Synthetic biology is the artificial reassembling of individual chemical pieces of life into new systems for a useful purpose [3,4]. Common industrial microorganisms are desirable production platforms owing to an abundance of genetic manipulation tools and techniques, as well as commonly used production conditions. These platforms become the biological systems that are easily tuned for maximum production [5]. Lastly, a target fuel must be chosen on the basis of energy content, hygroscopicity, vapor pressure, as well as productivity.

Many promising fuels have been produced in encouraging yields from non-natural hosts. For these metabolites to be produced in non-natural organisms, synthetic metabolic pathways are introduced into the system, allowing the host to catalyze new chemical reactions. These reactions are engineered to divert carbon toward the production of new fuels. To enhance the fuel production in the host, competing reactions are removed from the system. Each process must be monitored to avoid buildup of toxic metabolites and maximize metabolic flux of the system toward higher production of the target fuel.

Ketoacid based fuels

Amino acid biosynthesis is a group of ubiquitous metabolic pathways essential to life. Amino acid production has been exploited for many years in industry [6,7]. Amino acid biosynthesis has been harnessed in *Escherichia coli* to convert 2-ketoacid precursors to numerous C3–C5 alcohols [8]. By mimicking the Ehrlich pathway [9], a single heterologous ketoacid decarboxylase (KDC) is added to the system, allowing for the production of C3–C5 alcohols diverting 2 ketoacids to aldehydes (Figure 1). Alcohol dehydrogenases will oxidize the aldehydes to the desired alcohol. These alcohols are desirable alternative fuels that may be mixed with conventional gasoline or used as a drop-in replacement (Figure 1).

The 2-ketoacid pathways for alcohols have been augmented in *E. coli* with additional heterologous genes to increase the production of these viable alternative fuels. Complementing this pathway with an additional, powerful alcohol dehydrogenase more fully catalyzes the conversion of aldehydes to alcohols [10]. Another heterologous gene, aceytlactate synthase (AlsS) from *Bacillus subtilis* improves the first reaction in the pathway for branched amino acids by having an increased affinity for pyruvate over 2 ketobuytrate [11]. This causes an increase in carbon flow from glycolysis toward branched alcohol production. Some metabolic engineering approaches used to enhance the production of these useful alcohols include deleting some non-essential enzymes [12–14]. Another interesting strategy using an

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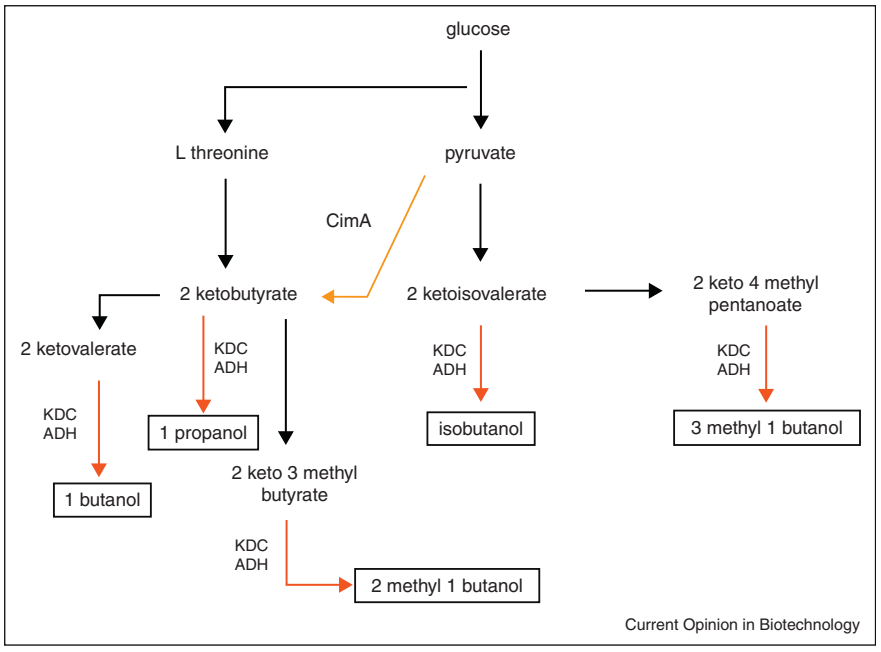
Figure 1



2 Ketoacid metabolism for biofuels. Synthetic Ehrlich mimicking pathway shown in red. Synthetic citramalate pathway for more direct production of 2 ketobutyrate shown in orange. KDC, ketoacid decarboxylase; ADH, alcohol dehydrogenase; CimA, citramalate synthase.

alternate, more direct pathway through amino acid biosynthesis by an additional heterologous gene has increased coproduction of 1-butanol and 1-propanol [15]. Additionally, random mutagenesis approaches have been utilized for further productivity gains [16,17•]. These approaches combine whole cell chemical mutagenesis with amino acid analogue growth based selection. After multiple rounds of selection, higher production for several different higher chained alcohols as well as increased strain tolerance to these alcohols have been shown [16,17•].

Examples of alcohols ranging from C3 to C8 have been produced and detected with the 2-ketoacid pathways in *E. coli* [8]. Exploiting valine biosynthesis, isobutanol has been produced at 100% theoretical yield of 0.41 g/g of glucose as a feedstock with titers as high as 50 g/l [18••,19•]. Utilizing threonine biosynthesis, 2-methyl 1-butanol has been produced at a yield of 0.17 g/g of glucose and a titer up to 3 g/l [20]. The same strategy for leucine biosynthesis produced a maximum yield of 0.11 g of 3-methyl 1-butanol/g of glucose with a maximum titer of 9.5 g/l [16]. 1-Butanol has been produced through the unnatural amino acid norvaline pathway with a maximum titer of 2 g/l [13].

This ubiquitous pathway for biofuel production has been transferred to new non-natural hosts including: *Corynebacterium glutamicum*, *Clostridium cellulolyticum*, and *Synechococcus elongatus* [21•,22,23•]. *C. glutamicum* is a Gram positive bacterium that is used extensively for industrial production of amino acids [7,24]. While *C. glutamicum* is naturally an aerobic organism, recently it has been shown to have increased production of industrially relevant organic molecules in anaerobic conditions [25]. This host has been engineered to produce isobutanol with titers as high as 4.9 g/l in aerobic conditions [21•].

S. elongatus is a photoautotrophic eubacterium that has been extensively studied owing to its unicellular circadian rhythm mechanism [26]. This organism has been genetically modified to recycle atmospheric carbon dioxide, through the Calvin–Benson–Bassham (CBB) cycle and convert it to isobutanol [22]. Carbon is fixed using energy obtained through the photosystems and shunted to the key intermediate, pyruvate [27]. Utilizing the same heterologous enzymes as *E. coli*, *S. elongatus* also was able to produce isobutanol at 450 mg/l. Tolerance to isobutanol is a concern with cyanobacteria production; therefore, *S. elongatus* also was engineered to produce isobutyraldehyde, a molecule that is easily removed from the culture with gas stripping methods [27]. This molecule may easily be catalyzed into isobutanol using chemical or biochemical methods. Isobutyraldehyde production was greater than isobutanol with a maximum titer of 1.1 g/l [22].

Clostridium is a quintessential chemical producing organism [28]. *C. cellulolyticum* is particularly enticing as an

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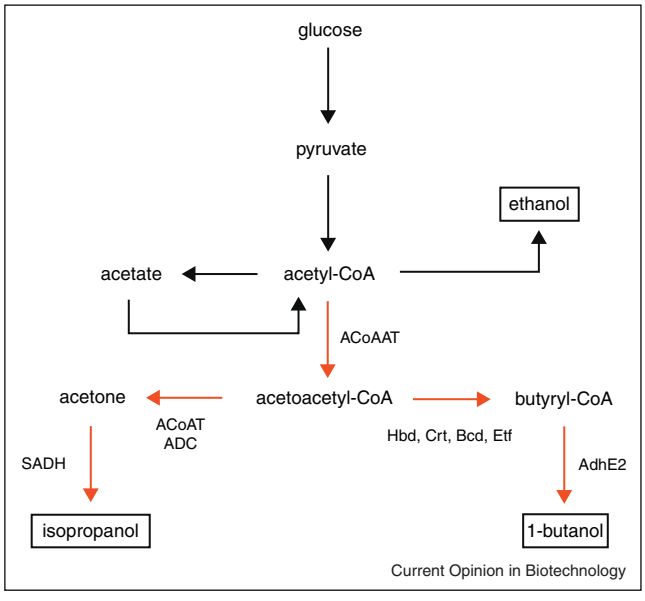
industrial platform owing to the rare ability to catabolize crystallized cellulose without the need to chemically or exoenzymatically breakdown the polysaccharide [29]. *C. cellulolyticum* was successfully engineered in the same method for production of isobutanol at titers up to 660 mg/l directly from crystallized cellulose [23•].

Traditional fermentation alcohols in non-native organisms

Native pathways for 1-butanol production exist, namely in *Clostridium's* ABE pathway (Figure 2) [28]. This pathway has been used for chemical production extensively in industry [30]. Metabolic engineering approaches allow the ABE pathway to be more fully directed toward the production of 1-butanol [31]. Five genes from the butanol producing part of this pathway of this system have been heterologously reconstructed within *E. coli*, allowing conversion of glucose to 1-butanol (Figure 2) [32]. The maximum titer for this biosynthetic pathway was shown to be 580 mg/l. This pathway has also been transferred into other microbial platforms including *Pseudomonas putida* and *B. subtilis* where titers were much lower [33]. To optimize this system, artificial driving forces have been engineered into *E. coli* bearing this pathway. Exploiting mechanistic differences in similar enzymes to create a kinetically trapped product increased titers up to 2.95 g/l [34•]. Knocking out competing NADH consuming pathways and irreversibly coupling NADH consumption to 1-butanol production, titers for 1-butanol have been raised to 30 g/l and 15 g/l, with and without continuous removal of the product, respectively [35•]. Cumulatively, this effort has led to the transferring of a natural biochemical pathway from *Clostridum*, allowing *E. coli* to ferment glucose to 1-butanol at reasonable biofuel production titers.

Figure 2



ABE pathway for 1-butanol and isopropanol production. Heterologous essential enzymatic steps in non-natural host shown in red. AcoAAT, acetyl-CoA acetyltransferase; Hdb, 3-hydroxybutyryl-CoA dehydrogenase; Crt, crotonase; Bcd, butyryl-CoA dehydrogenase; Etf, electron transfer flavoprotein; AdhE2, aldehyde/alcohol dehydrogenase; ACoAT, acetoacetyl-CoA transferase; ADC, acetoacetate decarboxylase; SADH, primary–secondary alcohol dehydrogenase.

Isopropanol is another natural byproduct in *Clostridium*. It is synthesized from the acetone branch of the ABE pathway (Figure 2) [36]. However, the total concentration of isopropanol is often very small in natural conditions [36]. One industrial natural producer of isopropanol, *Clostridium isopropylicum*, has been found to produce up to 4.6 g/l of isopropanol in 67 h [37]. Recent work has shown that the acetone production portion of the ABE pathway can be inserted into *E. coli* [38,39]. To this end, plasmid based expression of four heterologous genes (thiolase, CoA transferase, acetoacetate decarbonoxylase, and alcohol dehydrogenase) supplements natural glycolysis enzymes to produce isopropanol from glucose. In this approach, naturally competing butanol and ethanol pathways are absent in non-native organisms. The titers for this production have been shown up to 13.6 g/l with a maximum productivity of 0.727 g/h. However, the inherent toxicity of isopropanol has been shown to limit possible titers of production. This hurdle has been mitigated with gas stripping techniques, allowing concurrent production and removal of isopropanol to increase titer and yield [40]. After implementing these extraction methods, titer and productivity increased to a maximum of 140 g/l in 60 h and 0.775 g/h respectively. These titers and productivity are much higher than natural producers traditionally used in industry.

Fatty acids

Fatty acids are energy dense carboxylic acids featuring alkyl chains that typically range from 4 to 24 carbons in length. These molecules are one important way many organisms store energy among other vital cellular function [41]. Efforts are concentrating on exploiting the synthesis of this energy storage toward the production of fuels, particularly biodiesel. A traditional method for producing fuel from these molecules is chemical esterification of oils and fats from plants or animals to produce biodiesel [42]. The natural fatty acid biosynthetic pathway has been altered in *E. coli* to overproduce free fatty acids (FFA) (Figure 3) [43•]. In this alteration, a thioesterase (TesA) that normally associates with the cell membrane was modified to disassociate into the cytoplasmic matrix [44]. To further augment production, the first two genes in a competing pathway, β oxidation, were removed and titers of free fatty acids increased to about 1.2 g/l, about 14% of the maximum theoretical yield [45]. To increase the value of these biological products, an ethanol pathway including pyruvate decarboxylase and alcohol dehydrogenase was inserted and optimized in *E. coli* to produce

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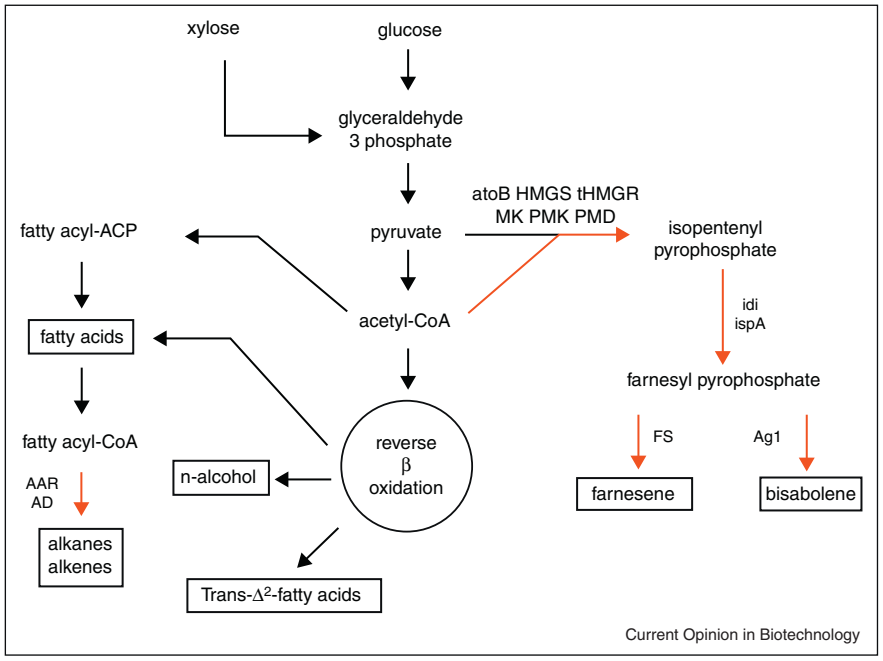
Figure 3



Biosynthesis of fatty acids, alkanes and alkenes. Heterologous pathways expressed in *E. coli* shown in red. AAR, acyl-ACP reductase; AD, aldehyde decarbonylase; atoB, acetyl-CoA acetyltransferase; HMGS, 3-hydroxy-3-methylglutaryl-CoA synthase; tHMGR, truncated 3-hydroxy-3-methylglutaryl-CoA reductase; MK, mevalonate kinase; PMK, phosphomevalonate kinase; PMD, mevalonate diphosphate decarboxylase; idi, isoprenyl diphosphate isomerase; ispA, farnesyl diphosphate synthase; Ag1, (E)-alpha-bisabolene synthase.

fatty acid ethyl ester, a more valuable and usable product than FFA alone. Lastly, this production platform was modified to consume hemicellulose directly, through the engineered excretion of xylanases, allowing for direct conversion of xylan to FFA. This approach shows the viability of building a microbial platform capable of consuming cellulosic sugar sources for advanced biofuel production.

Using a similar method, fatty acid production has also been achieved in cyanobacteria, *Synechocystis* sp. PCC6803, using only light and carbon dioxide as a feedstock [46]. This approach uses the same *E. coli* thioesterase mutant in conjugation with several similar carbon flux optimizations to allow photosynthetic production of FFA. Maximum titers of fatty acid production were reported as high as 0.2 g/l. Additional modifications to this system allowed for heterogeneous lipases to be expressed [47]. This serves as a possible way to increase FFA harvest at the expense of destroying the producing culture. This method increased the total amount of recovered FFA by as much as 0.025 g/l [48••].

One recent development is exploiting β oxidation to produce a multitude of different kinds of fuel molecules from glucose [49•]. This pathway shares ubiquity and some targets with ketoacid biosynthesis. In the presence of an excess of glucose and absence of fatty acids, the β oxidation cycle enzymes can catalyze the reverse reactions for the construction of various long chain alcohols and fatty acids (Figure 3). This method is only possible with extensive modification to the β oxidation cycle: limiting natural degradation of fatty acids, removing other fermentative carbon flux, and avoiding feedback inhibition. Owing to the cyclic nature of this pathway, the length and saturation of the target molecule can be adjusted by the specificity of the chosen terminal enzyme. Several different types of fuels have been produced using this production platform including: N-C4–10 alcohols and even-chained C10–18 fatty acids. Maximum reported titer from this system were 7 g/l of long chained fatty acids, with the most efficient reported production of alcohols greater than C5.

Alkanes and alkenes

Alkanes and alkenes are energy rich, hydrophobic molecules used for current industrial and transportation uses. Alkanes are produced in many biological specimens, for use as energy storage and structural formation in the form of fatty acids or waxes [50]. The biosynthetic pathway for these molecules has been isolated from cyanobacteria and engineered in *E. coli* for production [51•]. This pathway includes two recently characterized proteins, an acyl–acyl carrier protein reductase and aldehyde decarboxylase [51•] (Figure 3). The characterization of these proteins shows the ACP reductase preference toward acyl groups

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and the byproduct of this pathway to be formate [52,53]. The mechanism of this reaction seems to be unique among nonheme diiron enzymes [54]. This production method leads to a mixture of lengths of alkanes or alkenes that is valuable owing to the strong similarity to gasoline and the ease of separation of product from culture.

Another chemical pathway for developing alkanes and alkenes is the mevalonate pathway, traditionally studied in plants. This shunts carbon from acetyl CoA toward IPP and finally DMAPP (Figure 3). This pathway has previously been transplanted, in its entirety, to both *E. coli* and *S. cerevisiae* for the production of the immediate precursor of the antimalaria drug, artemisinin [55]. With slight changes to this pathway, it can be harnessed to make farnesene and subsequently transformed chemically to farnesane among other possible chemicals suitable for replacing diesel fuel [56]. Another possible chemical using this mevalonate pathway is bisabolene, a precursor to bisabolane that has been shown to have favorable attributes as an alternative diesel fuel [57].

Conclusions

Use of synthetic biology has continued to develop the nascent field of biological production of fuels. The environmental and economic advantages of renewable fuels are worth the considerable investment of strain development. Optimization for high production is one of the current limitations of the synthetic biology approach. This optimization must occur at three levels: the individual component level, the overall metabolic level, and at the complete organism level. At the most basic level, predicting non-native enzymes that will be active and efficient in the chosen host is difficult and inaccurate. Protein engineering will continue to be used to mitigate this difficulty as well as promote the discovery of new biologically based reactions. At the metabolism level, regulation remains one of the biggest advantages of natural systems over synthetic biological systems. Current synthetic metabolic implementations are largely digital—either completely overexpressed or knocked out. New advancements in supporting biological machinery such as scaffolds, regulatory elements and feedback mechanisms are necessary to regulate synthetic systems to the same degree as nature systems [58]. Lastly, ideal organism wide physiology suited for biofuel production varies with the target molecule. Further use of system wide biological analysis approaches with—omics data will lead to a better cellular environment for biofuel production. Using this data will lead to a better prediction of the metabolic flux and manipulation of system-wide regulation methods [59]. These sorts of additional breakthroughs will continue to increase yield, titers and productivities for the production of various biofuels. As the understanding of the complex intercellular interactions from heterologous elements increases, industrial synthetic biology will develop to an ever more mature field.

Acknowledgement

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An isobutanol-tolerant mutant was isolated with serial transfers. Using whole-genome sequencing followed by gene repair and knockout, five mutations (acrA, gatY, tnaA, yhbJ, and marCRAB) that were primarily responsible for the increased isobutanol tolerance were identified. However, improved isobutanol tolerance did not increase the final titer of isobutanol production.

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