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Next generation biofuel engineering in prokaryotes

Luisa S. Gronenberg[1], **Ryan J. Marcheschi**[1], and **James C. Liao**[1,2,3,4]

[1]Department of Chemical and Biomolecular Engineering, University of California, Los Angeles, CA 90095, USA

[2]The Molecular Biology Institute, UCLA, Los Angeles, CA 90095, USA

[3]Department of Chemistry and Biochemistry, UCLA, Los Angeles, CA 90095, USA

[4]Institute of Genomics and Proteomics, UCLA, Los Angeles, CA 90095, USA

Abstract

Next-generation biofuels must be compatible with current transportation infrastructure and be derived from environmentally sustainable resources that do not compete with food crops. Many bacterial species have unique properties advantageous to the production of such next-generation fuels. However, no single species possesses all characteristics necessary to make high quantities of fuels from plant waste or CO_2. Species containing a subset of the desired characteristics are used as starting points for engineering organisms with all desired attributes. Metabolic engineering of model organisms has yielded high titer production of advanced fuels, including alcohols, isoprenoids and fatty acid derivatives. Technical developments now allow engineering of native fuel producers, as well as lignocellulolytic and autotrophic bacteria, for the production of biofuels. Continued research on multiple fronts is required to engineer organisms for truly sustainable and economical biofuel production.

Introduction

Geopolitical instability in petroleum-producing areas and concerns about global climate change are driving interest in biofuels. Although first-generation biofuels, like ethanol and biodiesel, have achieved significant milestones, next-generation fuels will need to have higher fuel density and be more compatible with current engines and infrastructure. Additionally, most biofuels are currently produced from sources grown on valuable agricultural land, leading to direct competition with food crops. For next-generation biofuels to be economically and environmentally sustainable, they must have high mitigation potential for greenhouse gas emissions and be produced from renewable resources that do

Corresponding author: James, C. Liao (liaoj@seas.ucla.edu).

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not compete with food, i.e. lignocellulose from plants grown on marginal land, agricultural waste (polysaccharides, lignin, triglycerides, and proteins)[1], or CO_2. Some bacterial species natively use these carbon sources, while others natively produce advanced fuels such as butanol; however, to date, no organism can efficiently achieve both. Efforts are underway to engineer these traits into one organism (Figure 1). Here we review recent progress in metabolic engineering of prokaryotes for this purpose. Work done in eukaryotes was covered in a recent comprehensive review of microbial fuel production [2].

Model organisms - *Escherichia coli, Bacillus subtilis*

Significant genetic manipulations are required for bacteria to produce biofuel at economically relevant yields and rates. Since many natively fuel-producing bacteria are difficult to engineer, one approach has been to engineer biofuel pathways into genetically-tractable model organisms. In this section we introduce important biofuel pathways and describe recent developments expressing them in model bacteria (*E. coli*, *B. subtilis*). We then discuss efforts to change the feedstock specificities of these bacteria.

Alcohols (Coenzyme A (CoA) dependent pathway)

The clostridial, CoA-dependent butanol pathway (Figure 2) has been heterologously expressed to produce isopropanol and 1-butanol. Under optimized conditions, isopropanol was produced by engineered *E. coli* at effective titers up to 143 g L^{-1} [3] with *in situ* product removal by gas-stripping. A key development was replacing the reversible, flavin-dependent butyryl-CoA dehydrogenase (Bcd) with an irreversible *trans*-enoyl-CoA reductase (Ter) for the reduction of crotonyl-CoA, driving the equilibrium towards 1-butanol [4,5]. Deletion of competing pathways increased NADH and acetyl-CoA driving forces and led to 30 g L^{-1} of 1-butanol [5]. This pathway was extended to produce 1-hexanol by adding β-ketothiolase (BktB) to elongate butyryl-CoA [6]. A selection requiring a functional pathway to recycle NADH [5], coupled with a long-chain-specific acyl-CoA-thioesterase, was used to increase activity of a key enzyme [7] to produce 469 mg L^{-1} 1-hexanol and 60 mg L^{-1} 1-octanol. Longer alcohols were produced by reversing β-oxidation through deregulation of *E. coli* enzymes to perform the CoA-dependent pathway (see fatty acids section)[8].

Alcohols (Keto-Acid pathway)

Alcohols can also be derived by decarboxylation of keto-acids and reduction of the resulting aldehyde [9]. This pathway uses intermediates of amino acid biosynthesis (Figure 2), ubiquitous in prokaryotic organisms. Keto-acids can also be derived from deamination of amino acids themselves, allowing protein waste to be used as a biofuel feedstock [10]. Isobutanol and other branched-chain alcohols are produced by commandeering the valine biosynthesis pathway (Figure 2). Combined with continuous gas-stripping, the effective titer of isobutanol in *E. coli* can exceed 50 g L^{-1} [11]; separately, co-factor alignment can lead to production of isobutanol at theoretical yield [12]. Linear-chain alcohols are derived from the threonine biosynthesis pathway (Figure 2). A range of alcohols, from 1-propanol to 1-octanol (1395 mg L^{-1} total alcohols), was produced in a threonine-overproducing *E. coli* strain by deamination of threonine, recursive carbon-chain elongation using engineered

leucine biosynthesis enzymes, and decarboxylation with an engineered broad-range decarboxylase [13]. Another example, using the citramalate synthesis pathway (Figure 2), with competing metabolic pathways deleted, produced more than 3.5 g L^{-1} 1-propanol and 524 mg L^{-1} 1-butanol from glucose [14]. *B. subtilis,* which has higher isobutanol tolerance than *E. coli*, has been engineered to produce 2.62 g L^{-1} isobutanol [15].

Isoprenoids

Isoprenoids have recently been explored as biofuels. Isoprenoid precursors are produced by the methyl-erythritol-4-phosphate (MEP) pathway, native to *E. coli*, or by the mevalonate pathway (Figure 2). Isopentanol can also be produced via the keto-acid pathway [16]. The smallest isoprenoid derivatives, isopentanol and isopentenol are potential gasoline substitutes. Larger isoprenoids are substitutes for diesel or jet fuel [17].

To minimize regulation, expression of the non-native mevalonate pathway in *E. coli* has been a focus. A farnesyl diphosphate (FPP)-overproducing strain, originally used to synthesize amorphadiene [18], was used in a screen that identified an active bisabolene synthase from *Abies grandis*. Balanced expression levels and codon optimization led to 900 mg L^{-1} of the biodiesel precursor bisabolene [19]. Farnesene is another diesel fuel precursor. In *E. coli,* metabolic engineering efforts, including protein fusion of FPP synthase and farnesene synthase, have led to production of 380 mg L^{-1} of farnesene [20]. Pinene, which can be modified to a jet-fuel alternative [17], has been produced from pretreated switchgrass in *E. coli* expressing cellulases and hemicellulases [21]. Expression of efflux pumps in limonene-producing *E. coli* increased not only biofuel tolerance but also production [22].

Overexpression of the native MEP pathway, which has a higher theoretical yield of IPP from glucose than the mevalonate pathway, can also increase terpene production in *E. coli*. Overexpression of isoprene synthase and key steps of the MEP pathway allowed for production of 314 mg L^{-1} of isoprene (a potential aviation fuel precursor) in *E. coli* [23]. In contrast, 6.3 g L^{-1} isoprene was produced via the mevalonate pathway [24].

Fatty Acids

Due to their ionic nature, fatty acids (FAs) must first be esterified to esters, reduced to fatty alcohols, or decarboxylated to alkanes and alkenes to yield good diesel alternatives. Though much focus has been on plants and algae, recent progress has been made in overproducing fatty acids or derivatives in prokaryotes. Engineering efforts to increase FA production in *E. coli* were recently reviewed [25].

During biosynthesis, growing FA chains are attached to acyl-carrier proteins (ACPs) (Figure 2). Fatty acyl-ACPs feedback-inhibit FA biosynthesis, therefore overexpression of thioesterases increases the production of FAs [26]. Additionally, eliminating FA breakdown via β-oxidation, while overexpressing the rate-limiting acetyl-CoA carboxylase (ACC), increased yields up to 4.5 g L^{-1} day^{-1} in fed batch culture [27]. Similar modifications also increase extracellular FAs, which are cheaper to extract [28]. An *in vitro* system for analyzing fatty acid biosynthesis showed that malonyl-CoA levels are limiting, while NADPH levels are not [27]. Mutations that force flux towards malonyl-CoA have been

identified computationally and validated experimentally [29]. A dynamic sensor-regulator system has been used to balance FA pathway intermediates, increasing the yield of fatty acid ethyl-esters (FAEEs) to 28% of theoretical maximum [30]. *In vitro* reconstitution allowed for kinetic analysis of FA biosynthesis and indicated that altering the ratio of FA synthase subunits may increase FA yields [31]. Deletion of the second gene of the β-oxidation pathway and overexpression of thioesterases produced up to 1.2 g L^{-1} of fatty acids in flask culture [32], which were directly converted to fatty alcohols (60 mg L^{-1}) by overexpression of fatty acyl-CoA reductases, or to FAEEs by overexpression of acyltransferases along with an ethanol production pathway (674 mg L^{-1})[32]. Varying the fatty acyl-CoA synthases and reductases affects the chain length of produced fatty alcohols [32,33]. Free FA overexpression in *E. coli* decreases membrane integrity by changing phospholipid composition. Balancing acyl-ACP pools by overexpressing a second thioesterase can improve viability, but does not lead to a greater production of FAs [34].

Methyl-ketones have received attention as potential biofuels. Modification of β-oxidation produces β-keto-fatty acids, which can be hydrolyzed to ketones by the native thioesterase FadM [35]. Alternatively, 500 mg L^{-1} methyl-ketones can be produced by codon-optimized heterologous β-ketoacyl-ACP thioesterases and β-ketoacid decarboxylases in *E. coli* strains lacking native fermentative pathways [36].

A novel approach to producing FAs is by reversing β-oxidation [8] (Figure 2). After deregulation of *E. coli* metabolism, overexpression of key genes, and knockouts of competing pathways, native *E. coli* enzymes achieve the same transformations as the clostridial CoA-dependent pathway. Because this pathway uses acetyl-CoA instead of malonyl-CoA it is more energy efficient than fatty acid biosynthesis. It allowed for production of 7 g L^{-1} of extracellular fatty acids. Recent *in vitro* characterization of the enzymes involved and *in vivo* reconstruction of the pathway allows for more deliberate modification of the genes involved [37].

Alkanes were previously made by chemically reducing fatty acids [38]. Recently an alkane biosynthetic pathway composed of an acyl-ACP-reductase and an aldehyde-deformylating oxygenase (ADO) was discovered in cyanobacteria [39,40]. ADO was initially incorrectly identified as aldehyde decarbonylase [41–44]. Heterologous expression led to production of alkanes and alkenes in *E. coli* [39]. Terminal alkenes can be produced by heterologous expression of a P450 enzyme from *Jeotgalicoccus* to decarboxylate fatty acids [45] (Figure 2).

Feedstock utilization

To ultimately convert biomass directly to fuel, bacteria must be able to break down lignocellulose and efficiently consume the resulting sugars: glucose, galactose, xylose and arabinose. While *E. coli* can consume all four, it shows a preference for certain sugars when fed a mixture due to carbon catabolite repression (CCR). Strategies to deactivate CCR have increased the amount of isobutanol produced from mixtures of glucose and xylose [46]. Inserting additional copies of the transcription factor XylR increased ethanol production from xylose/arabinose mixtures [47].

Cellulases are complex enzymes and difficult to express heterologously (Figure 3)[48]. Most work in this area has focused on *S. cerevisiae*, but some has been performed in prokaryotes as well: xylanases were engineered into *E. coli* to make FA-fuels directly from hemicellulose. FAEEs were produced at 11.6 mg L^{-1} from a glucose, hemicellulose mix [32]. *E. coli* strains have also been engineered to grow on either cellulose or hemicellulose and, separately, produce pinene, butanol or FAEEs [21]; co-cultures of two such strains produced 71 mg L^{-1} FAEEs from pretreated switchgrass [21]. *E. coli* expressing a β-glucosidase was able to produce isopropanol from cellobiose at titers up to 4 g L^{-1} [49]. Overexpression of an endoglucanase in *B. subtilis* allowed growth on cellulose and production of lactate [50]. *B. subtilis* has also been engineered to express minicellulosomes on its surface [51].

Other feedstocks currently of interest are glycerol, a waste product from the conversion of triacylglycerides to biodiesel [52,53], and protein waste, often a byproduct of biofuel refineries. Introduction of deamination capabilities into *E. coli* allows it to convert protein to alcohols, via the keto-acid pathway [10].

Native Biofuel Producers

Clostridium acetobutylicum and *Clostridium beijerinckii* have long been used for the industrial production of biofuels via the Acetone-Butanol-Ethanol (ABE) fermentation process. Modern genetic tools have recently been developed for solventogenic clostridial species [54]. The group II intron-based ClosTron system for knocking in and out genes [55] is also used in lignocellulolytic strains (see below).

A new spin on the ABE process was modifying *C. acetobutylicum* to synthesize isopropanol instead of acetone [56–58]. Overexpression of an alcohol dehydrogenase yielded 7.6 g L^{-1} of isopropanol and 23.9 g L^{-1} total alcohols in the butanol tolerant strain Rh8 [58]. Expression of the entire acetone operon [56,57] led to isopropanol titers up to 8.8 g L^{-1} [57] in *C. acetobutylicum* ATCC824. When an anhydrotetracycline-inducible expression system and gas-stripping were used, over 35.6 g L^{-1} of total alcohols were produced [56]. Similar conditions produced effective alcohol titers up to 143 g L^{-1} in *E. coli* [3]. Non-solventogenic *C. acetobutylicum* strains have been engineered to synthesize butanol, thus increasing butanol over acetone selectivity [59]. While not a native butanol producer, *C. tyrobutyricum* produces butyric acid and has high butanol tolerance. Overexpression of an alcohol dehydrogenase in *C. tyrobutyricum* and deletion of competing pathways led to production of 10 g L^{-1} butanol from glucose, 66% of the theoretical yield [60].

Although progress has been made towards engineering *C. acetobutylicum* to express cellulases [61], to date, solventogenic Clostridia have not been engineered to grow on cellulose alone. Deletion of a xylose repressor and overexpression of a xylose transporter in *C. beijerinckii* led to increased xylose consumption [62]. Similarly, disruption of the glucose phosphoenolpyruvate transport system of *C. acetobutylicum* improved co-utilization of xylose, arabinose and glucose [63].

Zymomonas mobilis has a long history as an ethanol producer. It does not produce butanol and is limited in the sugars it can consume, but progress has been made in engineering

strains to consume pentose sugars [64,65] and express cellulases [66]. *Corynebacterium glutamicum* is an amino acid producer, widely used in industry [67]. Due to high flux through its amino acid pathways and its high tolerance for isobutanol [68], titers up to 12.97 g L^{-1} of isobutanol have been reached [69]. *C. glutamicum* has recently been engineered to express minicellulosomes [70]

Lignocellulolytic organisms and thermophiles

Since heterologous expression of cellulases has proven difficult, engineering of natively lignocellulolytic organisms is of interest. Some cellulolytic species natively produce ethanol and metabolic engineering has increased ethanol yields from cellulose. There is only one example published to date of an advanced biofuel produced by a native cellulolytic bacterium [71]. Genetic manipulation is still challenging, though knockouts can now be made using intron-based technology [55]. A recent review provides a comprehensive list of biofuel production from cellulose and hemicellulose by strain [72].

One category of cellulolytic bacteria secretes monomeric cellulases into the medium. Of these, *Thermoanaerobacter* and *Thermoanaerobacterium sp*. can be engineered using methods developed for high titer ethanol production in non-cellulolytic *Thermoanaerobacterium saccharolyticum* [73]. Similar methods allow for knockouts in *Cellvibrio japonicas*, where ethanol production genes have also been expressed [74], and *Clostridium phytofermentans* [75], where acetate accumulation remains a drawback.

A second category of cellulolytic bacteria secretes large cellulosome organelles. A recently developed genetic system allows for engineering of one such species, *Clostridium thermocellum* [76]. Co-cultures of engineered *C. thermocellum* and *T. saccharolyticum* produce ethanol at titers up to 38 g L^{-1} [77]. Knocking out lactate dehydrogenase and malate dehydrogenase in *C. cellulolyticum* increased ethanol production and lowered the amount of organic acids produced [78]. To date, *C. cellulolyticum* is the only cellulolytic strain engineered to produce an advanced biofuel, isobutanol [71].

Geobacillus thermoglucosidasius is not cellulolytic, but is capable of cellobiose metabolism. Its thermophilic nature is desirable for biomass breakdown and fuel isolation. Knockouts of competing pathways in *G. thermoglucosidasius* have led to ethanol yields >90% theoretical [79].

Autotrophs

Autotrophic organisms have recently been used to produce biofuels directly from CO_2, altogether removing the requirement for solid feedstocks. Energy for the fixation of CO_2 is provided by either sunlight or electricity, which can be renewably sourced. Biofuel production in cyanobacteria has been reviewed in detail [80], and will be briefly discussed here.

Previously, *Synechococcuselongatus* sp. PCC7942 was engineered to produce 450 mg L^{-1} isobutanol by introduction of the Ehrlich pathway [81]. Optionally, isobutyraldehyde can be produced and continuously removed, extending production time. More recently, *S. elongatus*

was engineered to produce 14.5 mg L^{-1} 1-butanol [82] via the CoA-dependent pathway (Figure 2). Titers were increased to 29.9 mg L^{-1} by introduction of an ATP-driven step [83]. Another recent study described the production of 5.5 g L^{-1} ethanol in *Synechocystis* sp. 6803 overexpressing a native alcohol dehydrogenase and a *Z. mobilis* pyruvate decarboxylase [84].

Cyanobacteria naturally produce isoprene precursors via the MEP pathway. Addition of an isoprene synthase allowed *Synechocystis sp.* PCC 6803 to produce approximately 50 μg isoprene per gram of dry cell weight per day [85]. A polyketide synthase pathway for alkene production has also been described in cyanobacteria [86]. Production and secretion of fatty-acids (83.6 mg L^{-1}) has been attained in *Synechocystis sp.* PCC 6803 by the addition of a mutated *E. coli* thioesterase, and genomic knockout of the fatty-acid activation gene [87]. Further genomic knockouts designed to weaken polar cell wall layers, codon-optimization of the thioesterase, and addition of other codon-optimized heterologous thioesterases increased the fatty-acid yield to 197 mg L^{-1} [87]. Cellular biomass can be recovered by CO_2-limitation-induced lysis and lipolytic degradation of membrane lipids, producing a further 18.6 – 26.5 mg L^{-1} fatty acids over baseline [88]. Several cyanobacterial species, including *Synechocystis sp.* PCC 6803 and *S. elongatus* sp. PCC7942, are able to naturally produce small amounts of alkanes, primarily pentadecane, heptadecane, and methyl-heptadecane [39].

Instead of using photons, the facultative chemolithoautotrophic organism *Ralstonia eutropha* uses hydrogen as a source of reducing power to provide energy for carbon fixation. Recently, *R. eutropha* H16 has been shown to produce 270 mg L^{-1} isobutanol and 40 mg L^{-1} 3-methyl-1-butanol from fructose after removal of various carbon sinks and introduction of the isobutanol pathway [89]. *R. eutropha* H16 can also consume formic acid, and produced 846 mg L^{-1} isobutanol and 570 mg L^{-1} 3-methyl-1-butanol when engineered to disrupt polyhydroxybutyrate synthesis and express isobutanol biosynthesis genes [90]. Further, this same strain produced 536 mg L^{-1} isobutanol and 520 mg L^{-1} 3-methyl-1-butanol when grown solely on a mixture of H_2, O_2, and CO_2, and over 140 mg L^{-1} combined biofuels when provided with electricity (to produce formate electrochemically) and CO_2 as the sole sources of energy and carbon, respectively [90].

Conclusions

Studies of biofuel pathways in model organisms allowed for identification of bottelnecks; optimization of these has resulted in strains that produce high titers of advanced fuels. While it has proven difficult to heterologously express cellulases and re-engineer bacterial metabolism to allow for utilization of lignocellulose, progress is being made on both fronts. The use of co-cultures of multiple strains, not discussed here in depth, provides an interesting alternative to engineering all desired properties into one strain.

Bacteria that natively utilize carbon sources such as lignocellulose or CO_2 are harder to engineer than *E. coli*, and new genetic tools are required to fully take advantage of these organisms. Improvements in transformation techniques have allowed for metabolic engineering to increase ethanol production and recently produce isobutanol in

lignocellulolytic organisms. Further, successful biofuel production in cyanobacteria and in *R. eutropha* showed that it is feasible to make advanced fuels directly from CO_2; the next step will be to increase production in these strains.

While it remains unclear which of the approaches described here will yield the most effective biofuel-producing organism, improved genetic tools will be required to push forward the metabolic engineering of all these strains to make them industrially relevant.

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Highlights

- Next generation biofuels must be compatible with current infrastructure.
- Model organisms can be engineered for high-yield, sustainable fuel production.
- Genetic tools now allow engineering of native biofuel producers.
- Lignocellulolytic organisms and autotrophs can be engineered to make advanced fuels.

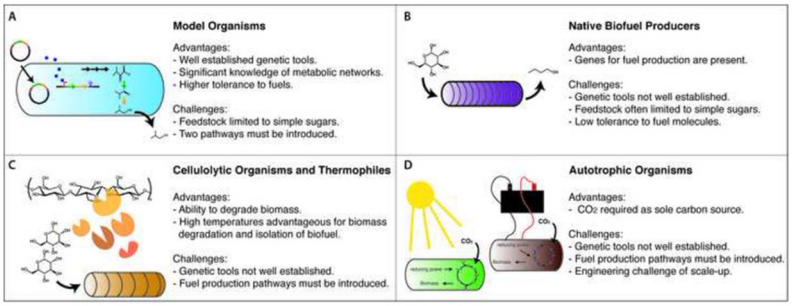

Figure 1. Four approaches to engineering next generation biofuel producers

Ideal biofuel producers should grow on cheap, renewable feedstock and, at the same time, produce high titers of advanced fuel. **(A)** One approach is to engineer both traits into model organisms, which have the major advantage of genetic tractability. A number of fuel pathways have been expressed in these organisms; less progress has been made in engineering them to utilize sustainable feedstocks. **(B)** A second approach involves using native fuel producers and re-engineering feedstock preferences. Solventogenic strains of *Clostridium* have long been used for industrial fuel production via the acetone–butanol–ethanol (ABE) fermentation process. However, fuel tolerance is low and genetic manipulations still remain challenging. **(C and D)** A third approach is to engineer biofuel production into organisms that are naturally capable of growth on renewable feedstocks. **(C)** Lignocellulolytic and some thermophilic organisms have the ability to use such feedstocks and grow at elevated temperatures, making biomass degradation and fuel extraction more efficient; however, genetic are sparse tools for these organisms and introduction of biofuel pathways is challenging. **(D)** Autotrophic organisms can naturally use CO_2 as a carbon source, but genetic tools are again limiting for the introduction of biofuel pathways.

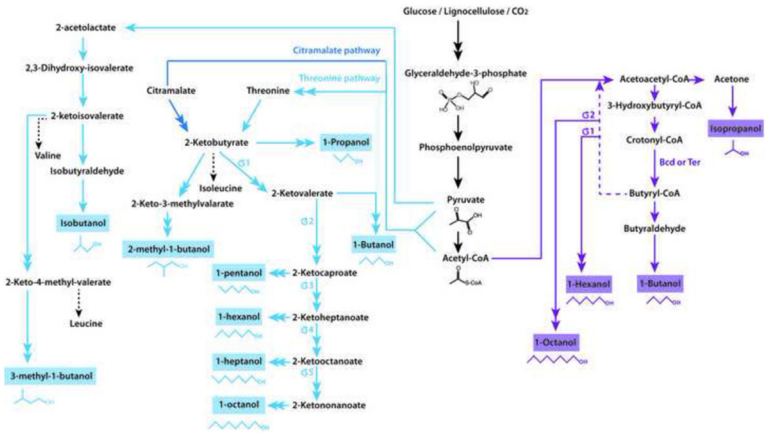

Figure 2. Biosynthesis of alcohol biofuels

Alcohols can be synthesized *via* the CoA-dependent pathway (purple) or the keto-acid pathway (blue). The latter takes advantage of native amino acid biosynthesis by decarboxylation and reduction (via the Ehrlich pathway) of the keto-acid intermediates of the Val, Leu or Ile pathways. 2-Ketobutyrate can be produced via the native threonine or the heterologous citramalate pathway. Elongation using engineered enzymes can recursively increase the product length by one carbon each round. The CoA-dependent mechanism is similar to ABE fermentation in *Clostridium*. Replacing the key enzyme butyryl-CoA dehydrogenase (Bcd) with an irreversible *trans*-enoyl-coA reductase (Ter) increases flux to butanol. Successive cycles of this pathway elongate the product by two carbons per cycle, producing hexanol from one turn, and octanol from two turns of the pathway. Double-headed arrows represent multiple steps.

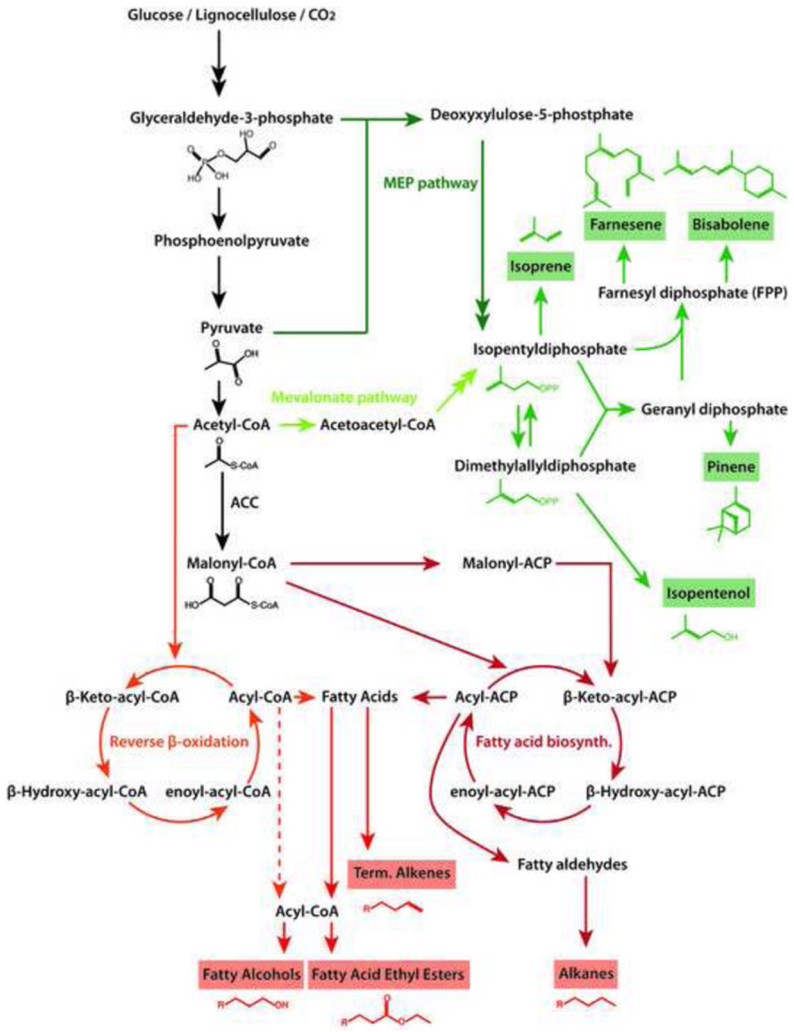

Figure 3. Biosynthesis of isoprenoid and fatty acid biofuels

Isoprenoids (green) are produced by successive condensation of the 5-carbon precursors isopentenyl-pyrophosphate (IPP) and dimethyl-allyl pyrophosphate (DMAP), which are isomers of each other. IPP and DMAP are synthesized by either the mevalonate pathway (found in the cytosol of plants) or the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway (also known as the non-mevalonate or 1-deoxy-D-xylulose-5-phosphate (DXP) pathway), which is native to *E. coli* and cyanobacteria. Fatty acids (FAs, red) are synthesized from malonyl-CoA by multi-enzyme fatty acid synthases. Malonyl-CoA is made by acetyl-CoA carboxylase (ACC), the rate-limiting and committed first step in FA biosynthesis. The growing FA chains are attached to acyl-carrier proteins (ACP). Thioesterases cleave fatty acids off the ACP. Reverse β-oxidation offers an alternative pathway that uses CoA as a carrier molecule and acetyl-CoA to elongate the growing chain instead of malonyl-CoA. Double-headed arrows represent multiple steps.

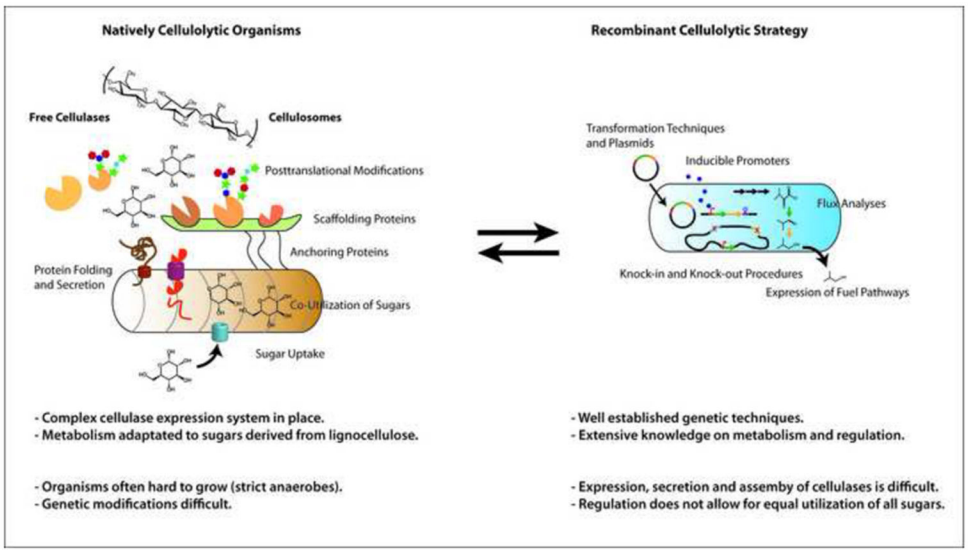

Figure 4. Strategies for Consolidated Bioprocessing

There are two strategies for engineering functionality for both lignocellulose degradation and fuel production into one strain. Natively cellulolytic organism can be engineered to express fuel pathways, or cellulolytic enzymes can be expressed recombinantly in model organisms (or native fuel producers, which is more difficult and not diagrammed in this figure). Key features of each organism are highlighted in the figure and advantages and challenges are mentioned.