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Title: Synthetic biology for microbial production of lipid-based biofuels

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Abstract: The risks of maintaining current CO₂ emission trends have led to interest in producing biofuels using engineered microbes. Microbial biofuels reduce emissions because CO₂ produced by fuel combustion is offset by CO₂ captured by growing biomass, which is later used as feedstock for biofuel fermentation. Hydrocarbons found in petroleum fuels share striking similarity with biological lipids. Here we review synthetic metabolic pathways based on fatty acid and isoprenoid metabolism to produce alkanes and other molecules suitable as biofuels. We further discuss engineering strategies to optimize engineered biosynthetic routes, as well as the potential of synthetic biology for sustainable manufacturing.

Dear Editor and Reviewers,

Please find below responses to reviewer's comments, and later highlighted changes between the original and the resubmitted drafts.

Best,
Leo

Reviewers' comments:

Reviewer #1: The review paper is well-written. However, it did not have both page and line numbers so that it was difficult to review the manuscript. The authors summarized recent progress in microbial engineering efforts for producing advanced biofuels.

>>> Thank you for your comments. Page numbers have been added.

These are suggested minor changes:

- As the manuscript focused on advanced biofuels, the title can be changed into "Synthetic biology for microbial production of advanced biofuels"

>>> We have changed the title to "Synthetic biology for microbial production of lipid-based biofuels"

- All highlighted reference will need one sentence summary.

>>> Summaries are provided in the "Highlights" section at the beginning of the pdf

- Redundant paragraphs, the following two paragraph are too similar:
(omitted)

>>>Apologies for this oversight, which has been corrected.

- A well-known and genetically tractable host, such as Escherichia coli or Saccharomyces cerevisiae, can be engineered TO convert a simple feedstock

>>> This sentence has been changed.

Reviewer #2: The authors have provide a succinct-yet-thorough review of microbial biosynthesis of lipid-derived fuel molecules. The manuscript will undoubtedly provide a valuable resource for the biofuels community. I recommend the following minor revisions to strengthen the manuscript and enhance its value to the scientific community:

1. The subject material is primarily focused on classical metabolic engineering strategies ("push-pull-block"). There is an array of novel synbio tools currently being leveraged for microbial engineering that is overlooked here (such as functional optimization of gene clusters/synthetic gene networks, logic circuits, design-build-test strategies, in vivo editing, etc). The authors mention these in passing in the Conclusions section, but the manuscript would be strengthened significantly through addition of a short section focused on application of these tools for microbial biosynthesis of fuel molecules. Additionally, there are an array of non-lipid-derived fuel molecules and precursors currently under examination by the biofuels community (e.g. higher alcohols). These certainly fall outside the scope of this review, but it may be more accurate to entitle this work "Metabolic Engineering Strategies Targeting Lipid Biofuels" to more accurately reflect the subject matter.

>>> We appreciate these thoughtful suggestions. We have changed the title to "Synthetic biology for microbial production of lipid-based biofuels" to reflect our scope. We have also added and consolidated material into a section on next-generation synthetic biology tools.

2. The paragraph starting with "Alkanes and alkenes - the major constituents of petroleum diesel" - appears in the manuscript twice.

>>> Apologies, we have removed the second copy.

3. If available, the productivity (g/l/hr) associated with the lipid fuels in Table 1 would be a nice addition.

>>> Unfortunately, this information is not provided in most referenced reports.

4. Related to point 3 above, a brief discussion of the technoeconomic state of technology for the molecules in Table 1 would be welcome. For example, is a 7g/L titer at 28% yield for FFA technoeconomically viable? If not, how far from viability and how can synbio address the

limitations? A single sentence or two commenting on this would suffice to orient the reader within the context of real-world deployment.

>>> We felt that a thorough discussion would need to include considerations such as subsidies (both to biofuel and petrofuel producers, as well as to farmers, etc.) which would not fit due to space constraints. However we have added some thoughts on the viability of bioprocesses in relation to feedstock costs and other factors in the conclusion section.

5. General: overall, the manuscript reads as more of a review than a "current opinion." Space permitting, the authors are encouraged to explicitly share their opinion on the best path forward for synbio-guided biofuels.

>>> We have significantly changed several parts of the manuscript to allow for greater commentary and prognostication.

6. Final paragraph has minor grammatical issues that need to be addressed.

>>> We have changed the final section and removed much of the problematic language.

Highlights

- Biological lipids are naturally energy-dense and several make good biofuels.
- Metabolic pathways to produce these biofuels have been engineered in microbial hosts.
- A wide range of tools and methodologies are available for improving biofuel production.
- Synthetic biology offers great potential for sustainable manufacturing.

Reference Highlights

** Choi YJ, Lee SY. 2013. Microbial production of short-chain alkanes. *Nature* 502:571–4.

This paper shows production of short chain hydrocarbons for the first time using a fatty acyl-CoA reductase from *Clostridium acetobutylicum* and a fatty aldehyde decarbonylase from *Arabidopsis thaliana*.

** Haushalter RW, Groff D, Deutsch S, The L, Chavkin T a., Brunner SF, Katz L, Keasling JD. 2015. Fatty acid synthase produces acyl thioester intermediates as acyl-CoA Development of an orthogonal fatty acid biosynthesis system in *E. coli* for oleochemical production. *Metab Eng* 30:1–6.

Type I fatty acid synthases were expressed for the first time in parallel with native *E. coli* fatty acid synthase for the production of fatty alcohols and methyl ketones.

** Liu Q, Wu K, Cheng Y, Lu L, Xiao E, Zhang Y, Deng Z, Liu T. 2015. Engineering an iterative polyketide pathway in *Escherichia coli* results in single-form alkene and alkane overproduction. *Metab Eng* 28:82–90.

An iterative polyketide pathway was engineered for the production of alkenes.

* Alonso-Gutierrez J, Kim E-M, Batth TS, Cho N, Hu Q, Chan LJG, Petzold CJ, Hillson NJ, Adams PD, Keasling JD, Garcia-Martin H, Soon Lee T. 2014. Principal component analysis of proteomics (PCAP) as a tool to direct metabolic engineering. *Metab Eng* 28:123–133.

* Leber C, Polson B, Fernandez-Moya R, Da Silva N a. 2015. Overproduction and secretion of free fatty acids through disrupted neutral lipid recycle in *Saccharomyces cerevisiae*. *Metab Eng* 28:54–62.

* Shi S, Chen Y, Siewers V: Improving Production of Malonyl Coenzyme A-Derived Metabolites. *MBio* 2014, doi:10.1128/mBio.01130-14.Editor.

* Kozak BU, van Rossum HM, Luttik MAH, Akeroyd M, Benjamin KR, Wu L, de Vries S, Daran J-M, Pronk JT, van Maris AJA: Engineering acetyl coenzyme A supply: functional expression of a bacterial pyruvate dehydrogenase complex in the cytosol of *Saccharomyces cerevisiae*. [Internet]. *MBio* 2014, 5:e01696–14.

**Chowdhury A, Zomorodi AR, Maranas CD: k-OptForce: integrating kinetics with flux balance analysis for strain design. [Internet]. *PLoS Comput. Biol.* 2014, 10:e1003487.

A whole-genome scale computational model including flux balance analysis and kinetic data is used to predict beneficial strain improvements

** DiCarlo JE, Norville JE, Mali P, Rios X, Aach J, Church GM: Genome engineering in *Saccharomyces cerevisiae* using CRISPR-Cas systems. [Internet]. *Nucleic Acids Res.* 2013, 41:4336–43.

Cas9-mediated gene editing is demonstrated for the first time in *S. cerevisiae*.

Synthetic biology for microbial production of lipid-based biofuels

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Abstract

The risks of maintaining current CO₂ emission trends have led to interest in producing biofuels using engineered microbes. Microbial biofuels reduce emissions because CO₂ produced by fuel combustion is offset by CO₂ captured by growing biomass, which is later used as feedstock for biofuel fermentation. Hydrocarbons found in petroleum fuels share striking similarity with biological lipids. Here we review synthetic metabolic pathways based on fatty acid and isoprenoid metabolism to produce alkanes and other molecules suitable as biofuels. We further discuss engineering strategies to optimize engineered biosynthetic routes, as well as the potential of synthetic biology for sustainable manufacturing.

Introduction

Over the last century, human use of fossil fuels has raised atmospheric CO₂ to levels 40% higher than at any other time in the 800,000-year record [1,2]. The rising CO₂ can be linked to global climate change, including more frequent and intense extreme weather events, and rising mean global temperatures. The already-observed temperature increase of 0.7°C is projected to reach 2–8 °C by the end of the century, with potentially catastrophic consequences for our biosphere [1,3].

Of all anthropogenic CO₂ emissions, one quarter originate from the combustion of liquid transportation fuels[4]. Curbing these emissions will require a multi-faceted approach, including improved standards for vehicle fuel economy and emissions, alternative-powered vehicles, and biofuels. Biofuels reduce emissions because CO₂ produced by fuel combustion is offset by CO₂ captured by growing biomass, which is in turn used to produce more fuel(Figure 1). “Drop-in” biofuels that can be used with existing vehicles—especially trucks and planes that are impractical to power using current fuel cell technology—are especially desirable[5]. Stemming from these concerns, several governments including those of the United States, China, and the European Union have instituted mandates for biofuels to constitute an increasing percentage of total transportation fuel usage in the coming years [6].

Liquid transportation fuels

Today, only 2% of all transportation fuel is bio-based. By far the most prevalent biofuel is ethanol produced by microbial fermentation of sugars blended into gasoline as a volume booster and oxygenate [7]. However, ethanol can only be used as 10% of the blend due to its low energy density (and other factors). In the United States, most gasoline is already blended at this 10% ethanol limit.

Besides ethanol, the other predominant biofuel today is fatty acid alkyl ester (FAAE, marketed as biodiesel). FAAE is produced by thermochemical esterification of plant oils with alcohol—typically methanol or ethanol—and used as a diesel substitute. FAAE is chemically different from petroleum diesel, and from gasoline. Gasoline is primarily composed of linear and ringed C4-C9 hydrocarbons, diesel and jet fuel C8-21. FAAE, on the other hand, is composed of methyl- or ethyl-esters of linear C16-C22 alkyl chains (Figure 2). The increased oxygen content of FAAE leads to more complete fuel combustion, decreasing particulate and CO emissions [8]. However, oil crops used as feedstocks for FAAE production have low yields and divert agricultural resources from food crops.

The limitations of first-generation biofuels have generated interest in genetically engineering microorganisms to perform the bioconversion of an abundant and inexpensive feedstock into a biofuel. Here we discuss how various enzymes can be combined to biologically produce molecules suitable as transportation fuels, focusing on lipid-based replacements for diesel and jet fuel produced in the two most well-known microbial hosts, *Escherichia coli* and *Saccharomyces*

cerevisiae. We also provide an overview of the tools and methodologies of synthetic biology for creating and optimizing biological designs, and outlooks on its potential for future biomanufacturing.

Figure 1. (high-def color image in separate file)

Carbon cycle for a microbial biofuel. Biofuels reduce emissions because CO₂ produced by fuel combustion is offset by CO₂ captured by growing biomass, which is in turn used to produce more fuel. With synthetic biology, it may be possible to produce fuel from various sources of carbon and energy. It may also be possible to produce fuels, or other molecules, with improved properties using the diverse bioconversions observed in living organisms.

Hydrocarbons and lipids

The hydrocarbons we use as fuels today share striking similarity with some of the lipids most organisms use to store energy (Figure 2). Lipids are naturally energy-dense, and many exhibit other properties desirable in a biofuel. In fact, early demonstrations of the internal combustion (Diesel) engine used peanut oil as fuel [9]. The triacylglycerides (TAGs) predominant in oils, and other molecules structurally similar to fuels, are produced through the fatty acid biosynthetic pathway. This nearly universally conserved pathway produces some of the major cellular components—*e.g.*, phospholipids and TAGs—and also a great variety of other molecules.

Figure 2 (high-def image in separate file)

Hydrocarbons and lipids. Compounds present in liquid transportation fuels are chemically similar to compounds produced through fatty acid biosynthesis. (A) Iso-octane is a major component of gasoline, hexadecane of diesel fuel, and FAAE of biodiesel. ¹FAAE is typically produced using methanol and producing fatty acid methyl ester (FAME). (B) TAGs are produced in many organisms as energy storage molecules, and phospholipids as the main structural components of cellular membranes. Fatty acid biosynthesis produces acyl thioester intermediates that give rise to phospholipids, TAGs, and other molecules. ²The acyl chains in these thioesters can be bound to acyl carrier protein (ACP) or CoA, depending on the organism. Generally bacteria employ acyl-ACPs and eukaryotes acyl-CoAs. (C) Lesser-known microorganisms contain intriguing lipids. Annamox bacteria produce fatty acids containing linearly concatenated cyclobutane rings termed “ladderanes”. Archaeal membranes are composed of phospholipids containing isoprene-chains linked through ether (rather than ester) linkages.

Fatty acid metabolism

Fatty acid metabolism begins with the carboxylation of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase (ACC). Fatty acid synthase (FAS) then condenses one acetyl-CoA starter unit and several malonyl-CoA extender units iteratively to produce a linear acyl chain typically 12-22 carbons long, depending on the organism. Fatty acids are released as acyl thioesters, bound to either coenzyme A (CoA, in type I FAS) or to acyl carrier protein (ACP, in type II FAS). Most fungi and mammals employ type I FAS, most bacteria type II. Type II FAS is organized as discrete multifunctional polypeptides. By contrast type I FAS is organized as multimeric complexes of one or more polypeptides, each containing multiple enzyme activities.

After synthesis, acyl thioesters are mainly routed biologically toward membrane phospholipids (containing two acyl chains bound to a glycerol backbone) or energy storage TAGs (containing three). Most organisms can degrade TAGs or other fatty acids through β -oxidation. Some organisms perform additional bioconversions, such as consuming alkanes [10], or producing wax esters, polyhydroxyalkanoates, fatty alcohols, or other compounds through variations of this versatile pathway.

Engineered microbial production of fatty-acid derived biofuels

Genes encoding enzymes that perform desired chemical conversions can be introduced into an easy-to-culture and genetically tractable microbial host, allowing the engineered strain to convert a simple feedstock—*e.g.*, glucose—into a target molecule. A number of enzymes have been identified that catalyze the conversion of fatty acids or their intermediates into different products with good fuel properties.

FAAE—molecularly identical to oil-crop biodiesel—has been produced by heterologously expressing a wax ester synthase (WS) catalyzing the esterification of an acyl thioester with ethanol [11,12]. Several WS enzymes have been shown to catalyze this reaction [13].

Alkanes and alkenes—the major constituents of petroleum diesel—have been produced through various bioengineered routes, such as the reduction of acyl-thioesters (or free fatty acids) into fatty aldehydes followed by decarbonylation [14–16]. Other routes include the decarboxylation of free fatty acids directly into α -alkenes by a bacterial cytochrome P450 [16], or polyketide synthase (PKS)-mediated extension-decarboxylation [17].

Similarly, different pathways can be assembled to produce molecules not currently used as fuels, but with likely suitable properties, including fatty alcohols [12,18], methyl ketones [19,20], ω -hydroxy and dicarboxylic acids [21], and other fatty acid-derived products (Figure 3). It should be noted that different tailoring enzymes have different preferences for substrate chain length and terminal moiety, *e.g.*, acyl-ACP, acyl-CoA, or free fatty acids. These can mirror the nature of their host's central FAS pathway—type I produces acyl-CoA, type II acyl-ACP. However, it is possible to introduce a type I FAS pathway into a host that natively employs type II FAS [22], or vice-versa [23]. Additionally, the FAS pathway can be modified to incorporate branched amino acids and produce branched-chain fatty acids[24]. Chain branching lowers freezing point, which is important for fuel performance in high altitudes or cold weather.

Isoprenoid metabolism

Branching is a defining feature of isoprenoids, another main class of lipids that produces molecules similar to those found in fuels. Also called terpenoids, these molecules are defined by being formed from 5-carbon isoprene building blocks into thousands of molecules, encompassing 60% of known natural products[25]. Isoprenoids are better known as plant secondary metabolites. However they perform diverse functions in all kingdoms of life. In archaea, for example, isoprenoids are part of primary metabolism and comprise the hydrophobic chains of cell membranes (Figure 2).

Isoprenoids are synthesized through one of two pathways: the 1-deoxy-D-xylulose 5-phosphate (DXP) pathway (native to most bacteria) or the mevalonate (MVA) pathway (native to most eukaryotes and archaea). The MVA pathway begins with three acetyl-CoA molecules, which combine to form mevalonate through six enzyme-catalyzed steps. The DXP pathway begins with pyruvate and glyceraldehyde-3-phosphate, which form DXP through seven steps. Both pathways proceed further to form the five-carbon building blocks isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). IPP and DMAPP can be condensed and modified in various ways by various kinds of terpene synthases (TPSs) to form thousands of products. C₁₀ and C₁₅ isoprenoids, called respectively monoterpenes and sesquiterpenes, have appropriate carbon numbers for a liquid biofuel.

Engineered microbial production of isoprenoid derived biofuels

Monoterpenes (C₁₀) are produced by the condensation of IPP and DMAPP into geranyl pyrophosphate (GPP) catalyzed by GPP synthase, and further modified by any number of

monoterpene synthases. Synthetic pathways to monoterpenes such as α -pinene [26], sabinene [27], limonene [28], and geraniol [29], have been successfully constructed in microbial hosts.

Sesquiterpenes (C₁₅) are produced by the condensation of two IPP molecules and one DMAPP into farnesyl pyrophosphate (FPP) by FPP synthase, and subsequent modification by sesquiterpene synthases. Combining pathways to FPP with various sesquiterpene synthases in microbial hosts has produced bisabolene [30] and α -farnesene [31].

Figure 3(high-def color image in separate file)

Biosynthetic routes for the production of natural and synthetic fuels from glucose. Fatty acid biosynthesis (pink) naturally produces phospholipids for membrane composition, and TAGs for energy storage. Isoprenoid biosynthesis (green) naturally produces sterols and other compounds. These pathways can be coopted using heterologous genes to produce a number of biofuel molecules. From acyl thioesters: 1, Esterification with ethanol by wax synthase [11] to produce FAEE; 2, Reduction followed by decarbonylation [14–16], or PKS-

mediated extension-decarboxylation [32] to produce alkanes/enes; 3, Reduction either directly [22] or through fatty aldehydes intermediates [18] to produce fatty alcohols; 4, Other routes to other products [12,19]; 5, Heterologous FAS pathways [22,23]; 6, Monoterpene synthases can modify C10 geranyl-PP to produce pinene, limonene, or other monoterpenes [28,33]; 7, Sesquiterpene synthases can modify C15 Farnesyl-PP to form farnesene, bisabolene, or other sesquiterpenes [30,31]. Unsaturated lipids can be chemically hydrogenated for biofuel production (*e.g.*, farnesene to farnesane). ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase. The isoprenoid pathway shown is the mevalonate (MEV) pathway. Bacteria employ an alternative route, the DXP pathway, not shown for simplicity.

Metabolic engineering strategies for lipid-based biofuels

Having chosen a set of enzyme activities as a biosynthetic route to a biofuel, achieving good production levels is often challenging and time-consuming. Traditional metabolic engineering often employs a “pull-push-block” approach. “Pulling” on a pathway by overexpressing terminal enzymes or providing an irreversible sink—such as partitioning into a separate phase [12]—can create a thermodynamic driving force for product formation. “Blocking” consumption of products or intermediates can be achieved by deleting genes catalyzing undesirable reactions [12,34,35]. “Pushing” flux involves overcoming bottlenecks that may form along the pathway. The problems, and solutions, are often specific to the pathway and host organism.

In fatty acid biosynthesis, for example, a common bottleneck is the carboxylation of acetyl-CoA into malonyl-CoA by ACC [36]. In *E. coli*, ACC activity is inhibited by the product acyl-ACP. This inhibition can be minimized by converting acyl-ACP to other products that do not inhibit ACC—*e.g.*, by expressing a thioesterase [12]. In *S. cerevisiae*, ACC activity is inhibited post-translationally by phosphorylation in response to signals—such as glucose depletion. Mutating phosphorylation sites in ACC has resulted in increased titers of fatty acid products [37]. Another layer of regulation in yeast is transcriptional inhibition of ACC in response to elevated fatty acid levels. Replacing the native ACC promoter with one that is constitutively active has led to improved fatty product titers [34].

The availability of the central metabolite acetyl-CoA is important not only for fatty acid-derived products, but also isoprenoids produced through the mevalonate pathway, and many other targets. In *S. cerevisiae*, acetyl-CoA is provided to different subcellular pools through various

biosynthetic routes [38]. The route to cytoplasmic acetyl-CoA is energetically draining and suppressed in the high-glucose conditions typical of laboratory and industrial cultivation (for reasons that are still debated [39,40]). Strategies to overcome this bottleneck have included expressing heterologous pathways for the provision of this central building block [41–44].

A number of other strategies (reviewed elsewhere [45–48]) have been used to improve production levels of many biofuel molecules (Table 1). While many improvements in production levels have been realized, there is much room for further optimization to approach maximum theoretical yields.

TABLE 1. Yields of lipid fuels produced in engineered *E. coli* and *S. cerevisiae*

<i>Biofuel</i>	<i>Max Theoretical Yield^A</i> (g/g glucose)	<i>Host</i>	<i>Titer</i> (g/L)	<i>Yield</i> (g/g glucose)	<i>Percent of Max Theoretical Yield^F</i>	<i>Reference</i>
<i>FFA</i> (<i>C16</i>)	0.37	<i>E. coli</i>	7.0	0.28	76%	[49]
		<i>S. cerevisiae</i>	2.2	0.11	30%	[35]
<i>FAEE</i> (<i>C18</i>)	0.36	<i>E. coli</i>	1.5	0.075	21%	[50]
		<i>S. cerevisiae</i>	0.034	0.0017	<1%	[13]
<i>Fatty alcohol</i> (<i>C16</i>)	0.34	<i>E. coli</i>	3.8 ^B	0.13	38%	[22]
		<i>S. cerevisiae</i>	1.1	0.055	16%	[18]
<i>Alkanes</i> (<i>C15</i>)	0.30	<i>E. coli</i>	0.58	0.029	8% ^C	[15]
		<i>S. cerevisiae</i>	0.0037	0.00019	<1%	[51]
<i>Bisabolene</i> (<i>C15</i>)	0.27	<i>E. coli</i>	1.1	0.055	20%	[52]
		<i>S. cerevisiae</i>	1.0	0.050	19%	[30]

Titers and yields of current laboratory-scale demonstrations of selected microbial biofuels produced in *E. coli* and *S. cerevisiae*. In general, experiments were performed using 2% glucose in shake flask fermentations.

^A Maximum theoretical yields are calculated using an *in silico* optimization algorithm employing a whole genome-scale reconstruction [53,54].

^B This example used 3% glucose rather than 2%.

^C This example produced C8 alkanes, with a different maximum theoretical yield, which is nevertheless accounted for in the percent yield calculation.

Synthetic biology tools and methodologies

Synthetic biology today encompasses an increasing number of tools and methodologies to facilitate strain construction and optimization. Synthesizing, sequencing, and introducing DNA sequences into living cells [55] is cheaper and easier than ever. Codon-optimization, directed evolution [56], screening enzyme libraries, and incorporating non-natural amino acids[57] all provide ways of improving or generating novel enzymatic activities.

Moving beyond which genes to express (or delete), how to express them—when, where, and at what level—can have significant effects on the growth rate and product titer of engineered strains. It is a defining feature of biological systems that their constituent parts are interconnected—as compared to electrical circuits or chemical engineering unit operations. More fully characterizing and modeling biological systems will reveal principles and design rules for synthetic biology [52,58]. These can be implemented using an increasing number of tools to program gene expression: ribosome binding sites (RBSs)[59], promoters[60], trans-acting activators[61], sensors and switches[50,62], enzyme fusions [63], scaffolds [64], localization tags [65], and other genetic “parts”. Employing these in ways approaching the complex natural orchestration of metabolism [66] will be necessary for more sophisticated and better performing biological designs.

While we have constrained our discussion to *S. cerevisiae* and *E. coli*, several other microorganisms are already used as hosts for engineered bioproduction [67]. A different host organism may have a significantly different base metabolism, availability of substrates and cofactors, or compatibility with heterologous genes, which can lead to better pathways. Exploring and developing genetic tools for non-traditional hosts will open possibilities for novel metabolic pathways.

Conclusions

With more powerful synthetic biology tools, the concept of “host” gives way to a fundamentally different synthetic organism. It’s worth considering how the design objectives differ between a natural organism that evolved to maximize fitness in a given ecological niche, and one synthetically constructed to convert a feedstock into a product. How to realize these radically different objectives is a current aim in synthetic biology. The propensity of organisms to grow—rather than to necessarily produce a target molecule—can lead to lower product yields. The propensity to mutate—perhaps necessary to adapt to changing environmental conditions—may

not be desirable in constant fermentation conditions, and in fact yield to strain instability. Many of these concerns have significant impacts on the viability of microbial bioproduction.

For biofuels, strain performance is paramount. A gallon of petroleum gasoline sells for less than a gallon of water [68]. Techno-economic analysis shows that microbial biofuels provide for significant reductions in CO₂ emissions over using petroleum fuels [69]. However, these savings, and the economic viability of such bioprocesses, depend largely on biochemical pathway yields and feedstock costs. Bioprocesses that utilize as feedstock cellulosic biomass—agricultural or wood industry by-products, grasses growing on marginal land, etc.—offer maximal CO₂ offsets, and don't compete with food production [70]. In the future it may be possible to engineer strains that grow directly on cellulosic biomass, or other abundant and inexpensive substrates, such as methane or CO₂. Or it may be possible to produce molecules with better performance, or as yet unimagined uses. As synthetic biology matures, this young technology holds vast potential to supplant the fossil economy with a sustainable and versatile biomanufacturing platform.

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(Literature highlights are included in the separate “highlights” file)

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FIGURE 1

A

Isooctane
(Gasoline)

Hexadecane
(Diesel)

Fatty acyl alkyl ester
(FAAE, or Biodiesel)¹

B

Triacylglyceride (TAG)

Acyl thioester
(X=ACP or CoA)²

Free fatty acid

Phospholipid

C

Ladderane fatty acid
(Annamox bacteria)

Isoprene-chain phospholipid
(Archaea)

FIGURE 2

Dear Editor and Reviewer,

Below please find a version of the manuscript showing changes over the original.

Best,
Leo

Synthetic biology for microbial production of lipid-based biofuels

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Abstract

The risks of maintaining current CO₂ emission trends have led to interest in producing biofuels using engineered microbes. Microbial biofuels reduce emissions because CO₂ produced by fuel combustion is offset by CO₂ captured by growing biomass, which is later used as feedstock for biofuel fermentation. Hydrocarbons found in petroleum fuels share striking similarity with biological lipids. Here we review synthetic metabolic pathways based on fatty acid and isoprenoid metabolism to produce alkanes and other molecules suitable as biofuels. We further discuss engineering strategies to optimize engineered synthetic biosynthetic routes, as well as the potential of synthetic biology for sustainable manufacturing.

Highlights

- Biological lipids are naturally energy-dense and several make good biofuels.
- ~~Metabolic~~ We discuss microbial-based biofuels as replacements to petroleum fuels
- ~~We review synthetic~~ pathways to produce these for production of fatty acid and isoprenoid ~~biofuels have been engineered in microbial hosts.~~
- A wide range of tools and methodologies are available ~~We highlight several strategies~~ for improving biofuel production.
- Synthetic biology offers great ~~much untapped~~ potential for sustainable manufacturing.

Introduction

Over the last century, human use of fossil fuels has raised atmospheric CO₂ to levels 40% higher than at any other time in the 800,000-year record[1,2]. The rising CO₂ can be linked to global

climate change, including more frequent and intense extreme weather events, and rising mean global temperatures. The already-observed temperature increase of 0.7°C is projected to reach 2–8 °C by the end of the century, with potentially catastrophic consequences for our biosphere [1,3]. Over the last century, human activity has released 1 teraton of CO₂ into the atmosphere, resulting in levels 33% higher than any time in the preceding 800,000 years [1]. The rising CO₂ can be linked to global climate change, including rising temperatures and sea levels. Climate models indicate that global mean temperature today is 0.7 °C higher than it would be absent human activity [1]. This temperature anomaly is expected to increase to at least 2 °C—and possibly 8 °C—by the end of this century. The consequences of such unabated climate change are daunting [2].

Of all anthropogenic CO₂ emissions, one quarter originate from the liquid fuel combustion of liquid in the transportation fuels sector [4]. Curbing [3]. Strategies to curb these emissions will require a multi-faceted approach, including improved include instituting tighter gas mileage and emission standards for vehicle fuel economy and emissions, new vehicles, increasing usage of electric and other alternative-powered vehicles, and usage of biofuels. Biofuels reduce emissions because CO₂ produced by fuel combustion is offset by CO₂ captured by growing biomass, which is in turn used to produce more fuel (Figure 1). “Drop-in” biofuels that can be used with existing vehicles—especially trucks and planes that are impractical to power using current fuel cell technology—are especially desirable [5]. Stemming from these concerns, several. Many governments including those of the United States, China, and the European Union have instituted mandates for biofuels to constitute an increasing percentage of total transportation fuel usage in the coming years [6][4].

Liquid transportation fuels

Today, only 2% of all transportation fuel is bio-based. By far the most prevalent biofuel is ethanol produced by microbial fermentation of sugars blended into gasoline as a volume booster and oxygenate [7]. [5]. However, ethanol can only be used as 10% of the blend due to its low energy density (and other factors). In the United States, most gasoline is already blended at this 10% ethanol limit.

Besides ethanol, the other predominant biofuel today is fatty acid alkyl ester (FAAE, marketed as biodiesel). FAAE is produced by thermochemical esterification of plant oils with alcohol—typically methanol or ethanol—and used as a diesel substitute. FAAE is chemically

different from petroleum diesel, and from gasoline. Gasoline is primarily composed of linear and ringed C4-C9 hydrocarbons, diesel and jet fuel C8-21. FFAE, on the other hand, is composed of methyl- or ethyl-esters of linear C16-C22 ~~alkyl~~ chains (Figure 2). The increased oxygen content of FFAE leads to more complete fuel combustion, decreasing particulate and CO emissions [8]. However, oil crops used as feedstocks for FFAE production have low yields and ~~divert~~ agricultural resources from food crops.

The limitations of first-generation biofuels have generated interest in genetically engineering microorganisms to perform the bioconversion of an abundant and inexpensive feedstock into a biofuel. Here we discuss how various enzymes can be combined to biologically produce molecules suitable as transportation fuels, focusing on lipid-based replacements for diesel and jet fuel produced in the two most well-known microbial hosts, *Escherichia coli* and *Saccharomyces cerevisiae*. We also provide an overview of the tools and methodologies of synthetic biology for creating and optimizing biological designs, and outlooks on its potential for future biomanufacturing.

~~Bio-based replacements for diesel and jet fuel are especially desirable since the vehicles they power operate for long lifetimes, and demand high power outputs not easily achievable with current fuel-cell technologies. The limitations of oil crop-based biodiesel production have generated interest in employing microorganisms to perform the bioconversion of a renewable feedstock into a desired biofuel (Figure 1). This presents several potential advantages. First, many organisms are known to grow on a number of abundant and inexpensive feedstocks. Second, biochemical pathways often exhibit exquisite specificity and complexity, at times producing compounds that are difficult—or impossible—to produce through synthetic chemistry. Combining activities into a microbial cell factory has the potential to produce bespoke fuel molecules with improved performance, perhaps cheaply. Lastly, this synthetic biology can, in perturbing and analyzing living systems, offer insights into their inner workings. The ramifications for sustainability, health, and other areas are immense. In this review, we highlight how the tools and methodologies of synthetic biology have been applied to the microbial production of biofuels, with a focus on lipid-based replacements for diesel and jet fuel.~~

Figure 1. (high-def color image in separate file)

Carbon cycle for a microbial biofuel. Biofuels reduce emissions because CO₂ produced by fuel combustion is offset by CO₂ captured by growing biomass, which is in turn used to produce more fuel. With synthetic biology, it may be possible to produce fuel from various sources of carbon and energy. It may also be possible to produce fuels, or other molecules, with improved properties, or other compounds, using the diverse bioconversions observed in living organisms.

Hydrocarbons and lipids

The hydrocarbons we use as fuels today share have striking similarity with some of the lipids most organisms use to store energy (Figure 2). Lipids ~~These compounds~~ are naturally energy-dense, and many exhibit other properties desirable in a biofuel, making them attractive as biofuels. In fact, early demonstrations of the internal combustion (Diesel) engine used peanut oil as fuel [91, 77]. The triacylglycerides (TAGs) predominant in oils, and other molecules structurally similar to fuels, are produced through the fatty acid biosynthetic pathway. This nearly universally conserved pathway produces some of the major cellular components—e.g., phospholipids and TAGs—and also a great variety of other molecules.

Figure 2 (high-def image in separate file)

Hydrocarbons and lipids. Compounds present in liquid transportation fuels are chemically similar ~~to with~~ compounds produced through fatty acid biosynthesis. (A) Iso-octane is a major component of gasoline, hexadecane of diesel fuel, and FFAE of biodiesel. ¹FAAE is typically produced using methanol and producing fatty ~~acids~~ methyl ester (FAME). (B) TAGs are produced in many organisms as energy storage molecules, and phospholipids as the main structural components of cellular membranes. Fatty acid biosynthesis produces acyl thioester intermediates that give rise to phospholipids, TAGs, and other molecules. ²The acyl chains in these thioesters can be bound to acyl carrier protein (ACP) or CoA, depending on the organism. Generally bacteria employ acyl-ACPs and eukaryotes acyl-CoAs. (C) ~~Lesser~~ ~~Less well~~-known microorganisms contain intriguing lipids. Annamox bacteria produce fatty acids containing linearly concatenated cyclobutane rings termed “ladderanes”. Archaeal membranes are composed of phospholipids containing isoprene-chains linked through ether (rather than ester) linkages.

Fatty acid metabolism

~~The biosynthesis of the diverse fatty acid metabolism-derived molecules shares a (nearly) universally conserved pathway. It~~ begins with the carboxylation of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase (ACC). Fatty acid synthase (FAS) then condenses one acetyl-CoA starter unit and several malonyl-CoA extender units iteratively to produce a linear acyl chain typically 12-22 carbons long, ~~depending on the organism.~~ Fatty acids are released as acyl thioesters, bound to either coenzyme A (CoA, in type I FAS) or to acyl carrier protein (ACP, in type II FAS). Most fungi and mammals employ type I FAS, most bacteria type II. Type II FAS is organized as discrete multifunctional polypeptides. By contrast type I FAS is organized as multimeric complexes of one or more polypeptides, each containing multiple enzyme activities. ~~The length of the product acyl thioesters (acyl-CoA or acyl-ACP) varies by organism, but is typically C12-22.~~

~~After synthesis, acyl thioesters are mainly routed biologically toward membrane phospholipids (containing two acyl chains bound to a glycerol backbone) or energy storage TAGs (containing three). Most organisms can degrade TAGs or other fatty acids through β -oxidation. Some organisms perform additional bioconversions, such as consuming alkanes [10], or producing wax esters, polyhydroxyalkanoates, fatty alcohols, or other compounds through variations of this versatile pathway. After synthesis, acyl thioesters are mainly routed biologically toward membrane phospholipids (containing two acyl chains bound to a glycerol backbone) or energy storage TAGs (containing three). Most organisms can degrade TAGs or other fatty acids through β -oxidation. Some organisms perform additional bioconversions, including consuming alkanes [8], or producing wax esters, polyhydroxyalkanoates, fatty alcohols, and other compounds through this versatile pathway.~~

Engineered microbial production of fatty-acid derived biofuels

~~Genes encoding enzymes~~ ~~The fact that perform desired chemical conversions can be introduced into an easy-microorganisms employ fatty acid biosynthesis to-culture-produce high quantities of membrane phospholipids and other molecules has led to interest in diverting the pathway to produce biofuels. A well-known and genetically tractable microbial host, allowing the such as *Escherichia coli* or *Saccharomyces cerevisiae*, can be engineered strain to~~ convert a simple feedstock—e.g., glucose—~~through fatty acid metabolism into an acyl thioester, then into a target molecule, biofuel through a heterologous terminal enzyme(s).~~ A number of enzymes have been identified that catalyze the conversion of fatty acids or their intermediates into different products ~~with good fuel properties.~~

FAAE—molecularly identical to oil-crop biodiesel—has been produced by heterologously expressing a wax ester synthase (WS) catalyzing the esterification of an acyl thioester with ethanol [11,12]. Several WS enzymes have been shown to catalyze this reaction [13]. FAEE, for example, has been produced by heterologously expressing a wax ester synthase (WS) catalyzing the esterification of an acyl thioester with ethanol [9,10]. Several WS enzymes have been shown to catalyze this reaction [11].

Alkanes and alkenes—the major constituents of petroleum diesel—have been produced through various bioengineered routes, such as the reduction of acyl-thioesters (or free fatty acids) into fatty aldehydes followed by decarbonylation [14–16]. Other routes include the decarboxylation of free fatty acids directly into α -alkenes by a bacterial cytochrome P450 [16], or polyketide synthase(PKS)-mediated extension-decarboxylation[17].

Similarly, different pathways can be assembled to produce molecules not currently used as fuels, but with likely suitable properties, including fatty alcohols [12,18], methyl ketones [19,20], ω -hydroxy and dicarboxylic acids [21], and other fatty acid-derived products (Figure 3). It should be noted that different tailoring enzymes have different preferences for substrate chain length and terminal moiety, *e.g.*, acyl-ACP, acyl-CoA, or free fatty acids. These can mirror the nature of their host's central FAS pathway—type I produces acyl-CoA, type II acyl-ACP. However, it is possible to introduce a type I FAS pathway into a host that natively employs type II FAS [22], or vice-versa [23]. Additionally, the FAS pathway can be modified to incorporate branched amino acids and produce branched-chain fatty acids[24]. Chain branching lowers freezing point, which is important for fuel performance in high altitudes or cold weather. Alkanes and alkenes—the major constituents of petroleum diesel—have been produced through various engineered routes. One route is the reduction of acyl thioesters (or free fatty acids) into fatty aldehydes followed by decarbonylation[12–14]. A second route to alkanes was demonstrated by Liu *et al.* in which the bacterial cytochrome P450 OleT₁₆ decarboxylates free fatty acids directly into α alkenes [14]. For yet a third route, polyketide synthases (PKSs) have been observed to perform an extension-decarboxylation reaction on acyl ACPs—involving malonyl CoA as the extender unit—to produce α alkenes [15]. Liu *et al.* expressed an engineered iterative type I PKS from *Streptomyces globisporus*, SgeE, and its cognate thioesterase SgeE10, in *E. coli* to produce polyenes [16].

Similarly, different pathways have been shown to produce molecules not currently used as fuels, but with likely suitable properties, including fatty alcohols [10,17], methyl ketones [18,19], ω -hydroxy and dicarboxylic acids [20], and other fatty acid-derived products (Figure 3).

It should be noted that different tailoring enzymes have different preferences for substrate chain length and terminal moiety, e.g., acyl-ACP, acyl-CoA, or free fatty acids. These can mirror the nature of the central FAS pathway—type I produces acyl-CoA, type II acyl-ACP. However, it is possible to introduce a type I FAS pathway into a host that natively employs type II FAS [21], or vice versa [22]. This can serve as a way to control product chain length, or perhaps to decouple production from endogenous regulation. Additionally, chain branching—which lowers the biofuel freezing point to improve cold-flow properties—can be introduced by incorporating branched amino acids as FAS starter units (rather than acetyl-CoA) [23].

Isoprenoid metabolism

Branching is a defining feature of isoprenoids, another main class of lipids that produces molecules similar to those found in fuels. Also called terpenoids, these molecules are defined by being formed from 5-carbon isoprene building blocks into thousands of molecules, encompassing 60% of known natural products [25]. Isoprenoids are better known as plant secondary metabolites. However they perform diverse functions in all kingdoms of life. In archaea, for example, isoprenoids are part of primary metabolism and comprise the hydrophobic chains of cell membranes (Figure 2). Branching is a defining feature of another main class of lipids that produces molecules similar to those found in fuels, the isoprenoids. These molecules are defined by formed from 5-carbon isoprene building blocks, and make up 60% of known natural products including sterols, cannabinoids, and several vitamins [24]. Isoprenoids, rather than fatty acids, comprise the hydrophobic chains of archaea cell membranes.

Isoprenoids are synthesized through one of two pathways: the 1-deoxy-D-xylulose 5-phosphate (DXP) pathway (native to most bacteria) or the mevalonate (MVA) pathway (native to most eukaryotes and archaea). The MVA pathway begins with three acetyl-CoA molecules, which combine to form mevalonate through and consists of six enzyme-catalyzed steps, enzymes. The DXP pathway begins with pyruvate and glyceraldehyde-3-phosphate, which combine to form DXP through and consists of seven steps, enzymes. Both pathways proceed further to form and with the production of the five-carbon building blocks isopentenyl pyrophosphate (IPP) and

dimethylallyl pyrophosphate (DMAPP). IPP and DMAPP can be condensed and modified in various ways to produce geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP) by various kinds of terpene synthases (TPSSs) to form thousands. Further modification (e.g., hydrolysis, cyclization, hydroxylation) creates a wide array of products. C10 and C15 isoprenoids, called respectively: monoterpenes (C10, derived from GPP), sesquiterpenes (C15, from FPP), and diterpenes (C20, from GGPP), as well as those from greater multiples of five carbons. Monoterpenes and sesquiterpenes have appropriate carbon numbers for a liquid biofuel as diesel grade biofuels.

Engineered microbial production of isoprenoid-derived biofuels

Monoterpenes (C10) are produced by the condensation of IPP and DMAPP into geranyl pyrophosphate (GPP) catalyzed by GPP synthase, and further modified by any number of monoterpene synthases. Synthetic pathways to monoterpenes such as α -pinene [26], sabinene [27], limonene [28], and geraniol [29], have been successfully constructed in microbial hosts. Monoterpene production has been engineered by overexpressing GPP synthase (GPPS) and different monoterpene synthase genes. Yang *et al.* produced α -pinene in *E. coli* by expressing a heterologous *S. cerevisiae* MVA pathway combined with pine GPPS and α -pinene synthase genes [25]. Combining GPPS and different monoterpene synthases has led to production of other monoterpenes including sabinene [26], limonene [27], and geraniol [28].

Sesquiterpenes (C15) are produced by the condensation of two IPP molecules and one DMAPP into farnesyl pyrophosphate (FPP) by FPP synthase, and subsequent modification by sesquiterpene synthases. Combining pathways to FPP with various sesquiterpene synthases in microbial hosts has produced bisabolene [30] and α -farnesene [31].

Sesquiterpenes (C15) can be produced by overexpression of FPP synthase (FPPS) and sesquiterpene synthases. In an early example, Peralta-Yahya *et al.* produced bisabolene by combining the MVA pathway, FPPS, and a bisabolene synthase in *E. coli* [29]. The same pathway was successfully ported to *S. cerevisiae* [29]. Following a similar strategy (of using a heterologous MVA and FPPS in *E. coli*) Wang *et al.* employed an α -farnesene synthase to produce α -farnesene [30].

Figure 3 (high-def color image in separate file)

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Biosynthetic routes for the production of natural and synthetic fuels from glucose. Fatty acid biosynthesis (pink) naturally produces phospholipids for membrane composition, and TAGs for energy storage. Isoprenoid biosynthesis (green) naturally produces sterols and other compounds. These pathways can be coopted using heterologous genes to produce a number of biofuel molecules. From acyl thioesters: 1, Esterification with ethanol by wax synthase [11] to produce FAEE; 2, Reduction followed by decarbonylation [14–16], or PKS-mediated extension-decarboxylation [32] to produce alkanes/enes; 3, Reduction either directly [22] or through fatty aldehydes intermediates [18] to produce fatty alcohols; 4, Other routes to other products [12,19]; 5, Heterologous FAS pathways [22,23]; 6, Monoterpene synthases can modify C10 geranyl-PP to produce pinene, limonene, or other monoterpenes [28,33]; 7, Sesquiterpene synthases can modify C15 Farnesyl-PP to form farnesene, bisabolene, or other sesquiterpenes [30,31]. Unsaturated lipids can be chemically hydrogenated for biofuel production (e.g., farnesene to farnesane). ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase. The isoprenoid pathway shown is the mevalonate (MEV) pathway. Bacteria employ an alternative route, the DXP pathway, not shown for simplicity. Biosynthetic routes for the production of natural and synthetic fuels from glucose. Fatty acid biosynthesis (pink) naturally produces phospholipids for membrane

composition, and TAGs for energy storage. Isoprenoid biosynthesis (green) naturally produces sterols and other compounds. These pathways can be coopted using heterologous genes to produce a number of biofuel molecules. From acyl thioesters: 1, Esterification with ethanol by wax synthase [9] to produce FAE; 2, Reduction followed by decarbonylation [12–14], or PKS-mediated extension-decarboxylation [15] to produce alkanes/enes; 3, Reduction either directly [21] or through fatty aldehydes intermediates [17] to produce fatty alcohols; 4, Other routes to other products [10,18]; 5, Heterologous FAS pathways [21,22]; 6, Monoterpene synthases can modify C10 geranyl PP to produce pinene, limonene, or other monoterpenes [27,31]; 7, Sesquiterpene synthases can modify C15 Farnesyl PP to form farnesene, bisabolene, or other sesquiterpenes [29,30]. Unsaturated lipids can be chemically hydrogenated for biofuel production (e.g., farnesene to farnesane). ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase. The isoprenoid pathway shown is the mevalonate (MEV) pathway. Bacteria employ an alternative route, the DXP pathway, not shown for simplicity.

Alkanes and alkenes—the major constituents of petroleum diesel—have been produced through various engineered routes. One route is the reduction of acyl thioesters (or free fatty acids) into fatty aldehydes followed by decarbonylation. The reduction can be catalyzed by a carboxylic acid reductase (CAR, on free fatty acids) [32], an acyl-ACP reductase (AAR) [12], or an acyl-CoA reductase (ACR) [13]. In *E. coli*, acyl-ACP (the product of FAS) can be converted to acyl-CoA by the combined activity of ‘TesA (a cytoplasmic version of the native acyl-ACP thioesterase) and FadD (the native acyl-CoA ligase) [10]. A second route to alkanes was demonstrated by Liu *et al.* in which the bacterial cytochrome P450 OleT_{HS} decarboxylates free fatty acids directly into α -alkenes [14]. For yet a third route, polyketide synthases (PKSs) have been observed to perform an extension-decarboxylation reaction on acyl-ACPs—involving malonyl-CoA as the extender unit—to produce α -alkenes [15]. Liu *et al.* expressed an engineered iterative type I PKS from *Streptomyces globisporus*, SgcE, and its cognate thioesterase SgcE10, in *E. coli* to produce polyenes [16].

Metabolic engineering Engineering strategies for lipid-based biofuels microbial bioproduction

Constructing and optimizing an engineered biosynthetic pathway is not a trivial feat. The distribution of molecular fluxes through metabolism depends on many factors, including the thermodynamics of the constituent reactions, kinetics of pathway enzymes—including their

affinity for substrates, or inhibitors—and complex multi-level regulation. How these factors interact to coordinate metabolism is an active area of research [33]. Yet, several tools and methodologies are available to guide efforts to rewire metabolism.

First, having decided on a pathway to employ, a common tool for improving heterologous gene expression is codon optimization—ostensibly to change gene sequences to match the new host's codon usage, although the exact relationship between codon usage and active expression is poorly understood. Improving an enzyme's activity—or changing its substrate preference—can also be achieved through directed evolution [34].

Random mutagenesis can also be performed on the whole genome, and combined with high-throughput screening to identify beneficial mutations. There are now an increasing number of computational tools to simulate and optimize metabolic flux distributions, with mixed success [35]. Traditional approaches include a “pull-push-block” strategy to maximize metabolic flux toward the product. Blocking consumption of products or intermediates can be achieved by deleting genes catalyzing undesirable reactions [10,36,37]. “Pulling” on a pathway can be achieved by overexpressing terminal enzymes, or providing a sink, which create a thermodynamic driving force for forward flux.

“Pushing” flux involves overcoming bottlenecks that could form along the pathway. For example, carboxylation of acetyl-CoA into malonyl-CoA by ACC is known to be the rate-limiting step of fatty acid biosynthesis in many organisms [38]. The regulation of entry into this energy draining pathway can occur at various steps. ~~In *E. coli*, ACC activity is inhibited by the product acyl-ACP. This inhibition can be minimized by converting acyl-ACP to other products—e.g., by expressing a thioesterase [10].~~ In *S.* Having chosen a set of enzyme activities as a biosynthetic route to a biofuel, achieving good production levels is often challenging and time-consuming. Traditional metabolic engineering often employs a “pull-push-block” approach. “Pulling” on a pathway by overexpressing terminal enzymes or providing an irreversible sink—such as partitioning into a separate phase [12]—can create a thermodynamic driving force for product formation. “Blocking” consumption of products or intermediates can be achieved by deleting genes catalyzing undesirable reactions [12,34,35]. “Pushing” flux involves overcoming bottlenecks that may form along the pathway. The problems, and solutions, are often specific to the pathway and host organism.

In fatty acid biosynthesis, for example, a common bottleneck is the carboxylation of acetyl-CoA into malonyl-CoA by ACC [36]. In *S. cerevisiae*, ACC transcription is inhibited by elevated fatty acid levels. Replacing the native ACC promoter with a strong constitutive promoter has led to improved fatty product titers [36]. Another layer of ACC regulation occurs at the post-translational level, where phosphorylation in response to signals—such as glucose depletion— inhibits enzyme activity. Mutating the phosphorylation sites in ACC has resulted in increased titers of fatty acid products [39].

In *E. coli*, ACC activity is inhibited by the product acyl-ACP. This inhibition can be minimized by converting acyl-ACP to other products that do not inhibit ACC—e.g., by expressing a thioesterase [12]. In *S. cerevisiae*, ACC activity is inhibited post-translationally by phosphorylation in response to signals—such as glucose depletion. Mutating phosphorylation sites in ACC has resulted in increased titers of fatty acid products [37]. Another layer of regulation in yeast is transcriptional inhibition of ACC in response to elevated fatty acid levels. Replacing the native ACC promoter with one that is constitutively active has led to improved fatty product titers [34].

The availability of the central metabolite acetyl-CoA is important not only for fatty acid-derived products, but also isoprenoids produced through the mevalonate pathway, and many other targets. An additional bottleneck for biosynthesis of fatty acid and many other bio-products—e.g., isoprenoids produced through the MVA pathway—is the availability of cytoplasmic acetyl-CoA. In *S. cerevisiae* this metabolite is provided to different subcellular pools through various biosynthetic routes [40]. The route to cytoplasmic acetyl-CoA is energetically draining and suppressed in many conditions. This yeast is known to operate a metabolic switch that favors the formation of ethanol over that of acetate—and later acetyl-CoA—in the high-glucose conditions commonly encountered in laboratory and industrial conditions [41]. Strategies to overcome this bottleneck have included expressing heterologous genes or pathways for the provision of acetyl-CoA [42–45].

In *S. cerevisiae*, acetyl-CoA is provided to different subcellular pools through various biosynthetic routes [38]. The route to cytoplasmic acetyl-CoA is energetically draining and suppressed in the high-glucose conditions typical of laboratory and industrial cultivation (for reasons that are still debated [39,40]). Strategies to overcome this bottleneck have included expressing heterologous pathways for the provision of this central building block [41–44].

A number of other strategies (reviewed elsewhere [45–48]) have been used to improve production levels of many biofuel molecules (Table 1). While many improvements in production levels have been realized, there is much room for further optimization to approach maximum theoretical yields.

TABLE 1. Yields of lipid fuels produced in engineered *E. coli* and *S. cerevisiae*

<u>Biofuel</u>	<u>Max Theoretical Yield^A (g/g glucose)</u>	<u>Host</u>	<u>Titer (g/L)</u>	<u>Yield (g/g glucose)</u>	<u>Percent of Max Theoretical Yield^F</u>	<u>Reference</u>
<u>FFA (C16)</u>	0.37	<u><i>E. coli</i></u>	<u>7.0</u>	<u>0.28</u>	<u>76%</u>	<u>[49]</u>
		<u><i>S. cerevisiae</i></u>	<u>2.2</u>	<u>0.11</u>	<u>30%</u>	<u>[35]</u>
<u>FAEE (C18)</u>	0.36	<u><i>E. coli</i></u>	<u>1.5</u>	<u>0.075</u>	<u>21%</u>	<u>[50]</u>
		<u><i>S. cerevisiae</i></u>	<u>0.034</u>	<u>0.0017</u>	<u><1%</u>	<u>[13]</u>
<u>Fatty alcohol (C16)</u>	0.34	<u><i>E. coli</i></u>	<u>3.8^B</u>	<u>0.13</u>	<u>38%</u>	<u>[22]</u>
		<u><i>S. cerevisiae</i></u>	<u>1.1</u>	<u>0.055</u>	<u>16%</u>	<u>[18]</u>
<u>Alkanes (C15)</u>	0.30	<u><i>E. coli</i></u>	<u>0.58</u>	<u>0.029</u>	<u>8%^C</u>	<u>[15]</u>
		<u><i>S. cerevisiae</i></u>	<u>0.0037</u>	<u>0.00019</u>	<u><1%</u>	<u>[51]</u>
<u>Bisabolene (C15)</u>	0.27	<u><i>E. coli</i></u>	<u>1.1</u>	<u>0.055</u>	<u>20%</u>	<u>[52]</u>
		<u><i>S. cerevisiae</i></u>	<u>1.0</u>	<u>0.050</u>	<u>19%</u>	<u>[30]</u>

Titers and yields of current laboratory-scale demonstrations of selected microbial biofuels produced in *E. coli* and *S. cerevisiae*. In general, experiments were performed using 2% glucose in shake flask fermentations.

^A Maximum theoretical yields are calculated using an *in silico* optimization algorithm employing a whole genome-scale reconstruction [53,54].

^B This example used 3% glucose rather than 2%.

^C This example produced C8 alkanes, with a different maximum theoretical yield, which is nevertheless accounted for in the percent yield calculation.

Synthetic biology tools and methodologies

Synthetic biology today encompasses an increasing number of tools and methodologies to facilitate strain construction and optimization. Synthesizing, sequencing, and introducing DNA sequences into living cells [55] is cheaper and easier than ever. Codon-optimization, directed evolution [56], screening enzyme libraries, and incorporating non-natural amino acids [57] all provide ways of improving or generating novel enzymatic activities.

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Moving beyond which genes to express (or delete), how to express them—when, where, and at what level—can have significant effects on the growth rate and product titer of engineered strains. It is a defining feature of biological systems that their constituent parts are interconnected—as compared to electrical circuits or chemical engineering unit operations. More fully characterizing and modeling biological systems will reveal principles and design rules for synthetic biology [52,58]. These can be implemented using an increasing number of tools to program gene expression: ribosome binding sites (RBSs) [59], promoters [60], trans-acting activators [61], sensors and switches [50,62], enzyme fusions [63], scaffolds [64], localization tags [65], and other genetic “parts”. Employing these in ways approaching the complex natural orchestration of metabolism [66] will be necessary for more sophisticated and better performing biological designs. Metabolic bottlenecks can arise due to thermodynamic constraints, and from multi-level cellular control. Powerful gene regulatory networks can present obstacles, and perhaps opportunities. In yeast, Feng *et al.* found several transcription factors which when deleted improved fatty chemical yields [17]. In *E. coli*, deletion of the gene encoding the transcriptional regulator FadR—which regulates fatty acid biosynthesis and degradation depending on the availability of fatty acids—has been employed by several groups to improve fatty chemical titers. Dellomonaco *et al.* instead overexpressed FadR—as well as other regulatory genes—to achieve a functional reversal of β oxidation producing a number of fatty chemicals from glucose [46]. Zhang *et al.* created a sensor-regulator system comprising FadR and cognate synthetic promoters which work together to regulate the expression of *atfA* and *fadD* (a pathway to produce FFAE) based on levels of fatty acid intermediates [47]. This dynamically responsive pathway produced the highest FFAE titer reported to date (Table 1).

TABLE 1. Yields of lipid fuels produced in engineered *E. coli* and *S. cerevisiae*

<i>Biofuel</i>	<i>Max Theoretical Yield^a (g/g glucose)</i>	<i>Host</i>	<i>Titer (g/L)</i>	<i>Yield (g/g glucose)</i>	<i>Percent of Max theoretical yield^b</i>	<i>Reference</i>
<i>FFA (C16)</i>	0.37	<i>E. coli</i>	7.0	0.28	76%	[46]
		<i>S. cerevisiae</i>	2.2	0.11	30%	[37]
<i>FAEE (C18)</i>	0.36	<i>E. coli</i>	1.5	0.075	21%	[47]
		<i>S. cerevisiae</i>	0.034	0.0017	<1%	[11]
<i>Fatty alcohol (C16)</i>	0.34	<i>E. coli</i>	3.8 ⁴⁶	0.13	38%	[21]
		<i>S. cerevisiae</i>	1.1	0.055	16%	[17]

<i>Alkanes</i> (C15)	0.30	<i>E. coli</i>	0.58	0.029	8% ^c	[13]
		<i>S. cerevisiae</i>	0.0037	0.00019	<1%	[48]
<i>Bisabolene</i> (C15)	0.27	<i>E. coli</i>	1.1	0.055	20%	[49]
		<i>S. cerevisiae</i>	1.0	0.050	19%	[29]

~~Titers and yields of current laboratory scale demonstrations of selected microbial biofuels produced in *E. coli* and *S. cerevisiae*. In general, experiments were performed using 2% glucose in shake flask fermentations.~~

~~^a Maximum theoretical yields are calculated using an *in silico* optimization algorithm employing a whole genome scale reconstruction~~

~~While we have constrained our discussion to *S. cerevisiae* and *E. coli*, several other microorganisms are already used as hosts for engineered bioproduction [67]. A different host organism may have a significantly different base metabolism, availability of substrates and cofactors, or compatibility with heterologous genes, which can lead to better pathways. Exploring and developing genetic tools for non-traditional hosts will open possibilities for novel metabolic pathways.~~

~~[50,51].~~

~~^b This example used 3% glucose rather than 2%.~~

~~^c This example produced C8 alkanes, with a different maximum theoretical yield, which is nevertheless accounted for in this value.~~

Conclusions

~~With more powerful synthetic biology tools, the concept of “host” gives way to a fundamentally different synthetic organism. It’s worth considering how the design objectives differ between a natural organism that evolved to maximize fitness in a given ecological niche, and one synthetically constructed to convert a feedstock into a product. How to realize these radically different objectives is a current aim in synthetic biology. The propensity of organisms to grow—rather than to necessarily produce a target molecule—can lead to lower product yields. The propensity to mutate—perhaps necessary to adapt to changing environmental conditions—may not be desirable in constant fermentation conditions, and in fact yield to strain instability. Many of these concerns have significant impacts on the viability of microbial bioproduction.~~

~~For biofuels, strain performance is paramount. A gallon of petroleum gasoline sells for less than a gallon of water [68]. Techno-economic analysis shows that microbial biofuels provide for significant reductions in CO₂ emissions over using petroleum fuels [69]. However, these savings,~~

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and the economic viability of such bioprocesses, depend largely on biochemical pathway yields and feedstock costs. Bioprocesses that utilize as feedstock cellulosic biomass—agricultural or wood industry by-products, grasses growing on marginal land, etc.—offer maximal CO₂ offsets, and don't compete with food production [70]. In the future it may be possible to engineer strains that grow directly on cellulosic biomass, or other abundant and inexpensive substrates, such as methane or CO₂. Or it may be possible to produce molecules with better performance, or as yet unimagined uses. As synthetic biology matures, this young technology holds vast potential to supplant the fossil economy with a sustainable and versatile biomanufacturing platform.

The extent to which biofuels produced from engineered microorganisms replace fossil fuels involves complex cost vs. benefits questions in areas beyond science and engineering. Techno-economic analysis—including the effects of feedstock production—shows that biofuels produced from sugarcane or cellulosic biomass by microbial fermentation provide for significant reductions in CO₂ emissions over using petroleum fuels [52]. Cellulosic biomass—agricultural or wood industry by-products, grasses growing on marginal land, etc.—can be broken down into sugars by thermochemical means and then used as fermentation feedstocks. A US Department of Energy report concluded that biomass is abundant and can replace 30% of transportation fuel by 2030 [53]. Cellulosic bioethanol production is already in commercial development. Other biofuels in industrial production include isobutanol, alkanes, and farnesane.

Limitations yet remain in developing biomanufacturing processes that compete with fossil fuels on cost. A gallon of petroleum gasoline sells for less than a gallon of water [54]. Biofuel strains can take years to develop, and yields are often low. Yet this is more a limitation of our current sophistication than a fundamental one. Many of the engineered pathways discussed in the text employ a handful of promoters and terminators, compared to the thousands natively employed by these host organisms. Utilizing a larger repertoire of natural [55] and synthetic [57] parts, along with better gene editing tools [60], will expand the capabilities of synthetic biological designs.

What will tomorrow's microbial fuel producers do? Will they be *E. coli*, yeast, or something else? Will they grow on unprocessed waste [61]? On methane? CO₂? And what will they produce? Life in our world has branched into myriad manifestations we've only just begun to catalogue. These designs and their capabilities are encoded using four simple letters. We're just now learning how to write.

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(Literature highlights are included in the separate “highlights” file)

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