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New microbial fuels: a biotech perspective

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Bioethanol and plant oil-derived biodiesel are generally considered first generation biofuels. Recognizing their apparent disadvantages, scientists and engineers are developing more sustainable and economically feasible second generation biofuels. The new microbial fuels summarized here have great potential to become viable replacements or at least supplements of petroleum-derived liquid transportation fuels. Yields and efficiencies of the four metabolic pathways leading to these microbial fuels — mostly designed and optimized in *Escherichia coli* and *Saccharomyces cerevisiae* using modern tools of metabolic engineering and synthetic biology — and the robustness of the biocatalysts that convert the metabolic intermediates to, in some cases, finished and engine-ready fuels, will determine if they can be commercially successful and contribute to alleviating our dependence on fossil fuels.

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Introduction

In an effort to combat climate change, to aid energy independence, and to counteract diminishing supplies of fossil fuels, there has been a resurgence of research on renewable and carbon-neutral energy sources. Biofuels production captures the energy of the sun as chemical energy in the bonds of biologically produced materials. All routes to biofuels hence start with photosynthesis, and it is at that point where they diverge. There are basically three routes to convert renewable resources into energy-rich, fuel-like molecules or fuel precursors: first, direct production by photosynthetic organisms, such as plants and algae; second, fermentative or nonfermentative production by heterotrophic microorganisms, such as bacteria, yeast, or fungi and third, chemical conversion of biomass to fuels. On the first view, the production of fuels directly from CO_2 using photosynthetic metabolism appears desirable as it is carbon-neutral in the strictest sense and does not rely on expensive feedstocks. However, this approach faces several major hurdles, most importantly scalability and land use issues. Several reviews have been published recently on this topic [1,2], and it will therefore not be covered. The chemical conversion of biomass to produce fuels has also been reviewed recently [3,4] and lies outside the scope of this review. The focus here will be the fermentative and nonfermentative metabolism of heterotrophic microorganisms and the different fuels they can supply. Other reviews that cover aspects of microbial fuels have been published within the last two years [5,6,7•,8]. Our emphasis will be on the pathways leading to the new generation of microbial fuels, in particular on the key biocatalysts that convert metabolic intermediates into fuel-like molecules, and on the parameters that govern their cost-effective production. Because much of this research has been carried out in biotechnology companies and is unpublished, the citation of some recent patent literature is included.

Heterotrophic microbes have been used in industrial processes for centuries, the alcohol fermentation with *Saccharomyces cerevisiae* being the oldest example. A more recent example is the production of bioplastics by *Escherichia coli* [9]. These two model organisms are being used to develop most of the new microbial fuels (see Box 1). Although heterotrophic microbes require organic feedstocks, fuels produced by these organisms approach carbon neutrality, because the feedstocks, in contrast to petroleum, are synthesized by recent plant-based carbon dioxide fixation. However, the organic feedstock is the major cost driver for using heterotrophic microbes to produce fuels. Therefore, to avoid any waste of organic carbon, the pathways and fermentation processes leading to a particular fuel must be as efficient as possible [10,11]. Another important economic factor is the recovery cost of the fuel from the fermentation broth and the cost for any additional chemical modifications necessary to convert a precursor fuel into an engine fuel. The ideal scenario is the microbial production and secretion of a water-immiscible fuel that conforms to fuel standards. Some of the new microbial fuels indeed have the potential to fulfill these criteria (see below). Currently, the new microbial fuels are mostly developed using simple carbohydrate feed stocks such as glucose. At the same time, other players in the bioenergy sector are focused on the efficient production of plant cellulosic biomass [12,13] and the breakdown of this and other lignocellulosic material into usable simple carbohydrates [14,15]. These developments go hand-in-hand, and they have great potential

Box 1 Oleaginous microbes versus *E. coli* and *S. cerevisiae*

One way to achieve high yields of microbial fuel production is to employ a microbe that naturally accumulates fuel precursors. Microbes that accumulate more than 20% of their cellular dry weight (CDW) in lipid-like oils are called oleaginous organisms. The microbes that accumulate the highest amounts of lipids in the form of triacylglycerol (TAG) and that are considered for microbial fuel production are yeast, fungi, and algae [2,55]. For example, the yeast *Rhodosporidium toruloides* accumulates 67% of CDW as lipids and can yield over 70 g/L lipids in high-density fermentation [56]. Although less common, some bacteria such as *Rhodococcus* and *Gordonia* spp. can also accumulate up to 80% CDW of TAGs [57]. The only microbes that accumulate oils other than TAGs are certain microalgae. The best-studied example is *Botryococcus braunii* [50]: depending on the strain, this microbe accumulates significant amounts of fatty-acid-derived olefins such as heptacosatriene or isoprenoid-derived olefins such as botryococcene. The challenges for microbial fuel production using oleaginous microbes are first, the lack of genetic tools and knowledge base to further improve titers and to modify the composition of the fuels and second, the production of sufficient biomass (in the case of algae). Furthermore, TAGs require two additional steps for conversion into engine fuels: isolation of the intracellular oils and chemical transesterification or hydrodeoxygenation [58].

An alternative for using naturally oleaginous microbes is to convert well-studied microbes such as *Escherichia coli* and *Saccharomyces cerevisiae* into oleaginous organisms by engineering their metabolism. The use of these industrial microbes for fuel production takes advantage of proven technologies and established processes. Furthermore, the wealth of knowledge on the metabolism and genetics of these organisms makes them ideal candidates to fully exploit new developments in metabolic engineering, systems biology [59], and synthetic biology [60]. This knowledge allows the tailoring of metabolic pathways to produce microbial fuels superior to the fuels available from natural producers (see Box 2) and at similar or even higher yields. For example, with only a few genetic changes *E. coli* could be coaxed into overproducing fatty acids at 2.5 g/L [61], which can be turned into various fuels by further genetic manipulations [39••]. Similarly, by expressing a yeast mevalonate pathway and a plant terpene synthase, *E. coli* was able to significantly overproduce an isoprene-derived olefinic fuel precursor, farnesene, at over 14 g/L [32••].

to develop processes to convert low-cost biomass into high-value microbial fuels in the future.

Metabolic pathways to new microbial fuels

As shown in Figure 1, microbial fuels can be divided into four classes depending on the pathways they are derived from: fermentative short-chain alcohols, nonfermentative short-chain alcohols, isoprenoid-derived hydrocarbons, and fatty-acid-derived hydrocarbons (see supplemental Figure 2 for more detailed pathway descriptions).

Fermentative short-chain alcohols

As ethanol is not a new microbial fuel, it will not be considered here. Butanol fermentation, though not entirely new — industrial fermentation processes using *Clostridium acetobuylicum* existed in the early 20th century — has found renewed interest and has been reviewed recently [16,17]. The butanol pathway consists of condensing two acetyl-CoA molecules (catalyzed by a thiolase) and then reducing the product to butanol (requiring four reductases and one dehydratase). It occurs in many *Clostridium* strains. One area of recent research relates to improving solvent tolerance of natural producers, because in these strains production shuts down when solvent concentrations reach 20 g/L. Other efforts are focused on minimizing the coproduction of acetone and improving productivity, or the rate of butanol production. Work has also been carried out on engineering heterologous hosts to produce butanol and successful examples include *E. coli* and *S. cerevisiae*. However, titers so far have been lower than in Clostridia [18,19•].

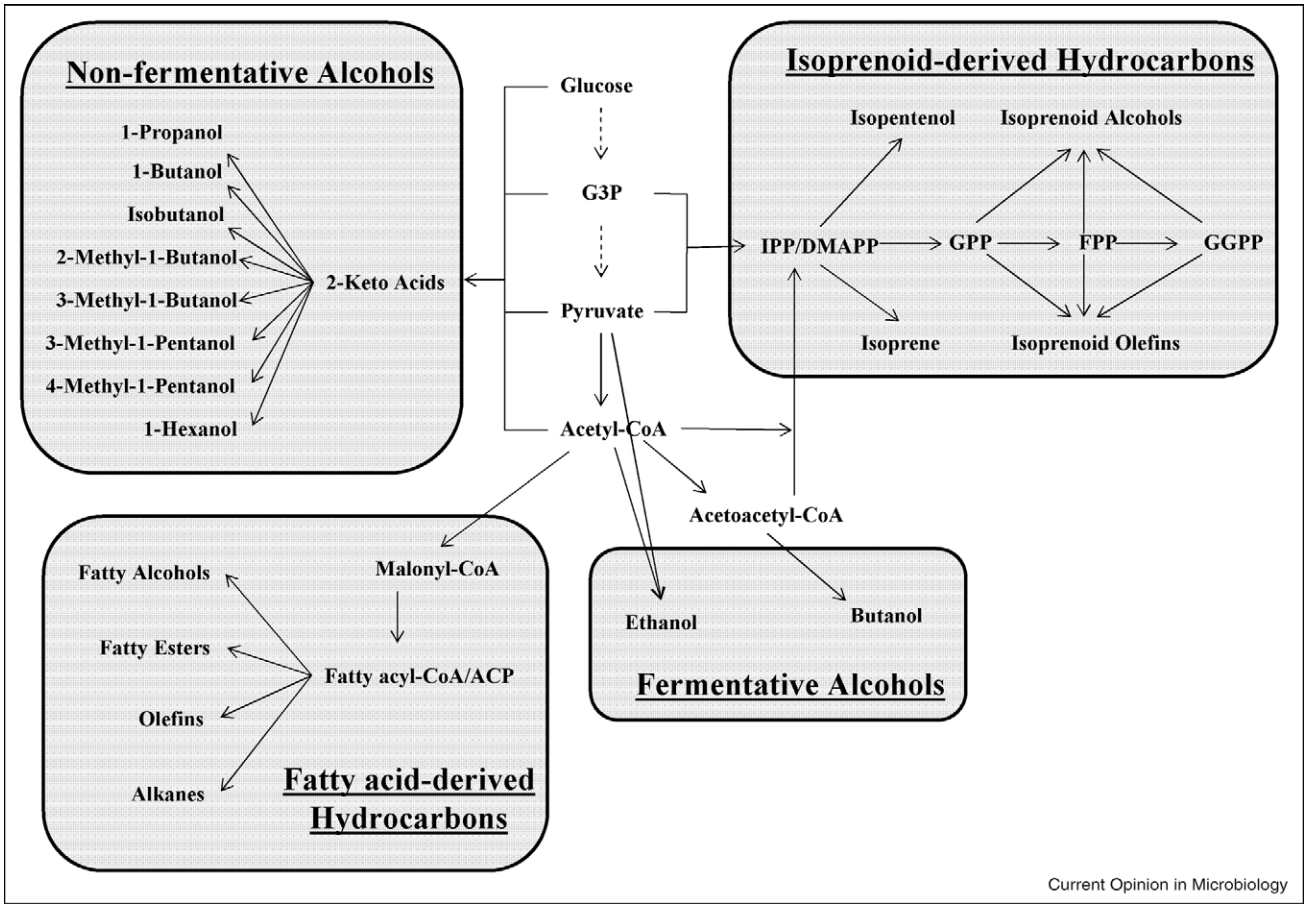
Nonfermentative short-chain alcohols

In contrast to the fermentative pathways, the nonfermentative short-chain alcohol pathways are not naturally occurring. A recent review [20] describes these pathways. The pathway consists of decarboxylating 2-keto acid intermediates from amino-acid biosynthesis and then reducing the resulting aldehyde group to an alcohol. The 2-keto acid decarboxylase from *Lactococcus lactis* Kivd and the alcohol dehydrogenase Adh2 from *S. cerevisiae* were shown to have broad substrate activity toward a variety of 2-keto acids. When these two enzymes are expressed in *E. coli*, 1-propanol, isobutanol, 1-butanol, 2-methyl-1-butanol and 3-methyl-1-butanol, and 2-phenylethanol are produced [21••]. Using classical metabolic engineering techniques, including overexpressing amino-acid biosynthesis genes, the concentration of these alcohols has dramatically increased [22,23]. In one example, isobutanol titers of 22 g/L were obtained [21••], demonstrating the promise of this pathway. Further pathway engineering yielded strains that also produced 6-carbon alcohols (4-methyl-1-pentanol, 3-methyl-1-pentanol, and 1-hexanol) [24•].

Isoprenoid-derived hydrocarbons

For many years the main interest in isoprenoid compounds has been their pharmaceutical or nutritional value. Consequently, *E. coli* and *S. cerevisiae* strains have been developed for the overproduction of isoprenoids, for example artemisinic acid [25,26]. These strains can now be adapted for producing isoprenoid-derived fuels. Two different pathways, known as the mevalonate and the deoxyxylulose pathway [27], have been exploited to overproduce the activated C5 isoprenoid units, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which are then linked with one another by a well-known head-to-tail mechanism to form the activated monoterpene (C10), sesquiterpene (C15), and diterpene precursors geranyl pyrophosphate (GPP), farnesyl phosphate (FPP), and geranylgeranyl pyrophosphate (GGPP), respectively. Whereas the biosynthesis of most pharmaceutically relevant isoprenoids requires multiple additional enzymatic steps to form the bioactive molecules, potentially fuel-like molecules, for example isoprenoid alcohols or olefins, can be synthesized from

Figure 1



Metabolic pathways leading to microbial fuels. G3P, glyceraldehyde-3-phosphate; CoA, coenzyme A; ACP, acyl carrier protein; IPP, isopentenyl pyrophosphate, DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl pyrophosphate; GPP, geranyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate.

IPP, GPP, FPP, or GGPP in just one step. The biocatalysts that convert these intermediates into fuel precursors are phosphatases, pyrophosphatases and, in particular, terpene synthases [28].

Isopentenol, a short-chain alcohol, was produced in *E. coli* from IPP using a pyrophosphatase-like enzyme from *Bacillus subtilis* [29•]. Farnesol and farnesene, which are derived from FPP, have been produced in *E. coli* and *S. cerevisiae* using a truncated yeast phosphatase [30] and certian plant terpene synthases [31], respectively. These sesquiterpenes are being developed as precursors to diesel fuels [32••]. Monoterpenes such as pinene, sabinene, or terpinene are discussed as potential next generation jet fuel components [33], and their production should be aided by the great diversity of terpene synthases [28]. Higher isoprenoid compounds (>C30) could also be explored for use as fuel feedstocks. However, these compounds are predicted to give low yields when produced in *E. coli* or *S. cerevisiae* [34,35] and would also require chemical refining (see below).

Fatty-acid-derived hydrocarbons

Fatty acid biosynthesis is the preferred pathway to accumulate energy storage compounds in many organisms. During biosynthesis, fatty acids are activated as thioesters with coenzyme A (i.e. fatty acyl-CoAs) or acyl carrier protein (i.e. fatty acyl-ACPs) [36]. Therefore, fatty acyl-CoAs or fatty acyl-ACPs are the starting molecules for the biosynthesis of fatty-acid-derived fuels. Biodiesel belongs to this class of fuels. It consists of fatty acid methyl esters (FAMEs) manufactured by chemical transesterification of plant oils. Biodiesel production faces several problems: limited supply and land yield, inconsistent performance, and challenging economics [1]. The microbial production of fatty acid esters has the potential to overcome these challenges (see also Box 2). One advantage is the enzymatic *in vivo* transesterification, which saves the costly chemical transesterification step. The enzymes capable of carrying out this reaction are certain acyltransferases [37•]. They are sometimes referred to as wax synthase/diacylglycerol acyltransferase (WS/DGAT) and have broad substrate specificity allowing

Box 2 Microbial designer fuels

Crude oil is a very complex mixture of a wide range of hydrocarbons. It is converted into a diversity of fuels and chemicals through a variety of chemical processes in refineries. The most important transportation fuels — gasoline, diesel, and jet fuel — are distinctively different mixtures of hydrocarbons tailored toward optimal engine performance. Gasoline consists of mainly straight, branched, and aromatic hydrocarbons ranging from 4 to 12 carbon atoms. In contrast, diesel consists of mainly straight hydrocarbons ranging from 9 to 23 carbon atoms. The main attractions of microbial fuels (besides their renewability) are that first, they can be produced as finished fuels that do not require additional refining or chemical modification for use in an engine and second, their composition can be tailored by genetic manipulation of the producing microbe. Although all short-chain alcohols fit these criteria as gasoline blends, the best examples for such microbial 'designer fuels' are perhaps the fatty-acid-derived monoalkylesters (fatty esters) for use as petrodiesel blends or replacement.

Fatty esters are referred to as biodiesel when they are derived from plant oils by chemical transesterification with methanol. Petrodiesel as well as biodiesel quality is evaluated by parameters such as cetane number, kinematic viscosity, oxidative stability, and cloud point [62]. Among others, these parameters are impacted by the hydrocarbon chain length as well as the degree of branching or saturation. For example, the cetane number, which is a measure of ignition quality and needs to be high, decreases with decreasing chain length, increasing branching, or increasing unsaturation. Cloud point, which is a measure of cold-flow properties, is impacted in the same way, but it needs to be low. Therefore, the hydrocarbon composition has to be well balanced to comply with the fuel specifications. One of the disadvantages of plant-derived biodiesel is that its composition, determined by the fatty acids in the plant oils, cannot be easily altered, which has been recognized as an important obstacle for optimizing 'designer biodiesel' [63•]. On the other hand, microbial fatty esters produced in *E. coli* are easily tailored. The chain length can be manipulated from 8 to 18 carbons by expressing thioesterases [64], which release free fatty acids with different chain length. The level of unsaturation can be adjusted by manipulating key regulators of the biosynthesis of unsaturated fatty acids [65]. Branch points can be introduced by expressing genes responsible for branched fatty acid biosynthesis from other microbes [66]. Taken together, metabolic engineering has enabled microbial strains to produce different mixtures of fatty esters, which can be tailored toward meeting or exceeding diesel fuel standards [39••].

for the transesterifcation of fatty acyl-CoAs of variable chain length with alcohols leading to the biosynthesis of fatty esters. This was first shown by coexpressing WS/DGAT from *Acinetobacter baylyi* and the genes encoding for ethanol biosynthesis from *Zymomonas mobilis* in *E. coli*. When such a recombinant strain was fed a fatty acid, it produced large amounts of the corresponding fatty acid ethyl ester [38]. This idea was then taken further by deregulating and increasing *de novo* fatty acid biosynthesis, which in combination with WS/DGAT expression and alcohol feeding, led to high titers of fatty esters without the need for fatty acid feeding [39••]. Fatty acids can be converted into usable fuels not only by esterification but also by reduction to the corresponding fatty alcohols. The best-studied fatty alcohol-generating enzymes are eukaryotic fatty acyl-CoA reductases (FARs). When expressed in *S. cerevisiae* FARs synthesize very-long-chain fatty alcohols (C24–C26) [40], which are unsuitable as fuel, whereas in *E. coli* they can lead to the synthesis of shorter fatty alcohols (C12–C18) but at low titers [41]. The difference in products reflects differences in the fatty acyl-CoA pools in these two microbes. The discovery of more efficient fatty alcohol-producing enzymes seems to be paramount for the commercial production of fatty alcohols.

Fatty-acid-derived alkanes could be an ideal replacement for diesel fuel, but alkane biosynthesis is poorly understood. The most plausible biochemical route to alkanes involves fatty aldehyde intermediates, which undergo loss of one carbon (decarbonylation) [42]. Consequently, most alkanes found in biological systems have an odd-numbered carbon chain (as they are derived from even-numbered fatty acids). An alternative alkane biosynthesis pathway involving the direct reduction of fatty alcohols to the corresponding alkanes is controversial and lacks credible experimental evidence [43]. The plant biosynthesis of long-chain alkanes (C24–C34), which are constituents of cuticular waxes, is the best-studied pathway [44], but neither a biochemical [45] nor a genetic approach [46] has unequivocally identified the key enzyme of alkane biosynthesis, a fatty aldehyde decarbonylase, and research over the last 10 years has not provided any new insights. Alkane biosynthesis in microbes is even less well understood than in eukaryotes, but the mechanism of fatty aldehyde decarbonylation may be preserved. The presence of alkanes ($\geq$C15) has been reported in many different microbes [47], but numerous papers report only trace amounts and should be treated with caution. Several recent papers also reported the presence of shorter chain alkanes and alkenes ($<$C15) in plant-associated microbes, for example endophytic fungi [48•] or rhizophilic bacteria [49]. It remains to be seen if any of the enzymes involved in alkane biosynthesis make robust enough biocatalysts to enable microbial fuel production.

Olefins (alkenes) can be produced by enzymatic decarbonylation of unsaturated fatty acids. However, there are at least two other distinct pathways for fatty-acid-derived olefin formation: first, terminal olefin biosynthesis and second, long-chain olefin biosynthesis by the head-to-head condensation of two fatty acids. Terminal olefins with odd-numbered carbon chain length have mostly been reported in eukaryotes, for example in algae (C23–C33), plants (C17), and insects (C15–C17) [50,51]. The genes responsible for the suggested decarboxylating biosynthesis from fatty acid-like precursors are unknown. The head-to-head condensation of two fatty acids leading to the biosynthesis of long-chain olefins (C21–C31, with internal double bonds) drew considerable attention in the 1960s and 1970s [52], but the biosynthetic pathway genes were only identified very recently [53]. Depending on the carbon chain length, these olefins can

Table 1

Theoretical yields on glucose for selected microbial fuels derived from different metabolic pathways[a]

Product	Pathway	Density[b] (g/mL)	Metabolic mass yield	Gal product/ ton glucose	Enthalpy of combustion (MJ/kg)[b]	Enthalpy of combustion yield
Gasoline replacements						
Ethanol	Fermentative	0.79	51%	155	−29.7	97%
Butanol	Fermentative	0.81	41%	121	−36.1	95%
Isobutanol	Nonfermentative	0.81	41%	122	−35.9	94%
3-Methyl-1-butanol	Nonfermentative	0.81	33%	98	−37.7	80%
Diesel replacements						
Farnesene	Mevalonate	0.84	25%	71	−47.0[c]	75%
Farnesene	Deoxyxylulose	0.84	29%	83	−47.0[c]	87%
Ethyl hexadecanoate	Fatty acid	0.86	35%	98	−39.4[d]	88%
Pentadecane	Fatty acid	0.77	29%	90	−47.0[c]	87%
Biocrudes						
Squalene	Mevalonate	0.86	25%	70	−47.0[c]	75%
Squalene	Deoxyxyulose	0.86	29%	81	−47.0[c]	87%
Hentriacontene	Fatty acid	0.78[e]	30%	92	−47.0[c]	90%

[a] Theoretical mass yields and enthalpy of combustion conversion yields were calculated using metabolic flux analysis methods (see supplementary materials for details).
[b] Values were taken from the 'Handbook of Chemistry and Physics' [67].
[c] Enthalpy of combustion data is not available for all the hydrocarbons in this table, therefore 47 MJ/kg was used which represents an approximate value for long-chain hydrocarbons.
[d] Value was taken from Ref. [63•].
[e] Density value for hentriacontane was used instead of hentriacontene.

be used as diesel fuel or intermediates for further chemical processing (see below).

Efficiencies of microbial fuel pathways and microbial fuel characteristics

As mentioned above, process feedstocks represent the largest cost component of biofuel production. Therefore, production cost is directly related to the ability of a metabolic pathway to efficiently convert sugar to fuels. Two metrics can be used to evaluate this efficiency; one is mass conversion and the other is enthalpy of combustion conversion. Table 1 shows these metrics for selected microbial fuels. For example, the theoretical mass conversion of glucose to ethanol is 51% while the theoretical conversion to butanol is only 41%. While a greater mass of ethanol can be made from the same amount of sugar, butanol has a higher enthalpy of combustion per kilogram which is not reflected in the theoretical mass yield. As a result, it is important to compare how much usable energy is captured from sugar and transferred to the new fuel. The enthalpy of combustion yield is 97% and 95% for ethanol and butanol, respectively. By this metric both pathways are equally efficient.

Microbial gasoline fuels

All short-chain alcohols described in Figure 1 can function as good gasoline replacements or blends [7•], and, with the exception of 3-methyl-1-butanol, have nearly 100% enthalpy of combustion yields attesting to the efficiency of their corresponding metabolic pathways. The C4 and C5 alcohols have distinct advantages over their first generation biofuel cousin, ethanol. First, they have higher energy densities, as defined as the enthalpy of combustion per kilogram of fuel, (Table 1) leading to reduced distribution costs. Similarly, they are less hygroscopic and corrosive than ethanol, facilitating their storage and transportation in existing distribution networks. One drawback in all the short-chain alcohols stems from post-fermentation processing. Owing to the toxicity associated with C4 and C5 alcohols, the obtainable concentrations of these alcohols in fermentations are near or slightly above their solubility in water. As a result, distillation or other energy intensive operations are required to recover the fuel [17]. The C4 and C5 alcohols also have a higher enthalpy of vaporization than ethanol, meaning their distillation will require more energy.

Microbial diesel fuels

Sesquiterpenes and most fatty-acid-derived hydrocarbons are suitable for the production of diesel fuel and include farnesene, ethyl hexadecanoate, and pentadecane (see Table 1). One advantage these molecules have over short-chain alcohols is their very low solubility in water. Therefore, centrifugation can be used to separate these compounds from fermentation broth if the molecules are excreted from the production strain resulting in considerable energy savings as compared with distillation. This feature has been demonstrated with farnesene [32••] and fatty esters [39••]. While the theoretical mass yields of these compounds are approximately 30–45% less than ethanol, their energy densities are significantly higher. As a result, the enthalpy of combustion yields for these diesel compounds approach 90%, with the exception of farnesene made from the mevalonate pathway, which is 75%.

This difference results from a 4% to 5% lower mass yield of the mevalonate pathway and equates to a ca. 20% increase in raw material costs illustrating how these efficiencies can impact overall production costs.

Farnesene itself is a fuel precursor. Its highly unsaturated nature results in a low cetane number and low oxidative stability (see Box 2). However, it can be hydrogenated chemically to produce farnesane, which has a good cetane number of 58 and excellent cold-flow properties [32••]. Although farnesane has great fuel properties, the chemical hydrogenation step can add significant cost to its manufacturing and result in an overall lower yield. As to microbial fatty esters, their composition can be tailored in order to adjust cetane number and cold-flow properties (see Box 2). Fatty-acid-derived pentadecane (conceivably produced by enzymatic decarbonylation of hexadecanoic acid, a common fatty acid in microbes) has a cetane number of 95 and would make a high quality diesel fuel blend.

Microbial biocrudes

Higher molecular weight olefins (≥C20), whether fatty acid or isoprenoid-derived, can serve as feedstocks for oil refineries. Their high molecular weight precludes them from being used as diesel or gasoline. These fuels can be catalytically cracked using existing refinery operations to make a variety of fuels [54]. These compounds are immiscible with fermentation broth and phase separate, significantly reducing their purification costs. The enthalpy of combustion yields for these compounds is approximately 90% with the exception of isoprenoids derived from the mevalonate pathway, which is energetically less efficient than the deoxyxylulose pathway. However, the need to further refine them into finished products will add additional costs and may hurt their economic viability.

Conclusions

The exploitation of the diverse metabolic pathways leading to energy-rich, fuel-like hydrocarbons opens up a path to develop renewable fuels that go far beyond the restrictions of bioethanol and plant-derived biodiesel. As novel biocatalysts leading to a greater variety of hydrocarbon products are being discovered, microbiologists will have an even more expansive tool box at their disposal to design better fuel to fit the need of different engines. The metabolic efficiency of a particular pathway has a profound impact on the economics of fuel production in a microbial host. Furthermore, microbial fuels that are easy to recover and do not require additional chemical conversion have the best chances to be developed in cost-effective and unsubsidized commercial processes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mib.2009.04.004.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Chisti Y: **Biodiesel from microalgae**. *Biotechnol Adv* 2007, **25**:294-306.

2. Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, Darzins A: **Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances**. *Plant J* 2008, **54**:621-639.

3. Demirbas A: **Progress and recent trends in biofuels**. *Progr Energy Combust Sci* 2007, **32**:1-18.

4. Huber GW, Iborra S, Corma A: **Synthesis of transportation fuels from biomass: chemistry, catalysts, and engineering**. *Chem Rev* 2006, **106**:4044-4098.

5. Antoni D, Zverlov VV, Schwarz WH: **Biofuels from microbes**. *Appl Microbiol Biotechnol* 2007, **77**:23-35.

6. Fortman JL, Chhabra S, Mukhopadhyay A, Chou H, Lee TS, Steen E, Keasling JD: **Biofuel alternatives to ethanol: pumping the microbial well**. *Trends Biotechnol* 2008, **26**:375-381.

7. • Lee SK, Chou H, Ham TS, Lee TS, Keasling JD: **Metabolic engineering of microorganisms for biofuels production: from bugs to synthetic biology to fuels**. *Curr Opin Biotechnol* 2008, **19**:556-563.
This excellent review discusses the microbial production of gasoline, diesel, and jet fuel alternatives and how metabolic engineering and synthetic biology will impact the future of these biofuels.

8. Wackett LP: **Microbial-based motor fuels: science and technology**. *Microbial Biotechnol* 2008, **1**:211-225.

9. Li R, Zhang H, Qi Q: **The production of polyhydroxyalkanoates in recombinant *Escherichia coli***. *Bioresour Technol* 2007, **98**:2313-2320.

10. Fischer CR, Klein-Marcuschamer D, Stephanopoulos G: **Selection and optimization of microbial hosts for biofuels production**. *Metab Eng* 2008, **10**:295-304.

11. Stephanopoulos G: **Challenges in engineering microbes for biofuels production**. *Science* 2007, **315**:801-804.

12. Somerville C: **Biofuels**. *Curr Biol* 2007, **17**:R115-119.

13. Zheng Y, Pan Z, Zhang R, Labavitch JM, Wang D, Teter SA, Jenkins BM: **Evaluation of different biomass materials as feedstock for fermentable sugar production**. *Appl Biochem Biotechnol* 2007, **137–140**:423-435.

14. Kumar R, Singh S, Singh OV: **Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives**. *J Ind Microbiol Biotechnol* 2008, **35**:377-391.

15. Lynd LR, Laser MS, Bransby D, Dale BE, Davison B, Hamilton R, Himmel M, Keller M, McMillan JD, Sheehan J *et al.*: **How biotech can transform biofuels**. *Nat Biotechnol* 2008, **26**:169-172.

16. Durre P: **Fermentative butanol production: bulk chemical and biofuel**. *Ann N Y Acad Sci* 2008, **1125**:353-362.

17. Lee SY, Park JH, Jang SH, Nielsen LK, Kim J, Jung KS: **Fermentative butanol production by Clostridia**. *Biotechnol Bioeng* 2008, **101**:209-228.

18. Inui M, Suda M, Kimura S, Yasuda K, Suzuki H, Toda H, Yamamoto S, Okino S, Suzuki N, Yukawa H: **Expression of *Clostridium acetobutylicum* butanol synthetic genes in *Escherichia coli***. *Appl Microbiol Biotechnol* 2008, **77**:1305-1316.

19. Steen EJ, Chan R, Prasad N, Myers S, Petzold CJ, Redding A, Ouellet M, Keasling JD: **Metabolic engineering of *Saccharomyces cerevisiae* for the production of n-butanol**. *Microb Cell Fact* 2008, **7**:36.
• The authors demonstrate high-titer production of butanol in *Saccharomyces cerevisiae* and present data that could lead the way to transform this industrial organism into a better butanol producer than Costridia.

20. Atsumi S, Liao JC: **Metabolic engineering for advanced biofuels production from *Escherichia coli***. *Curr Opin Biotechnol* 2008, **19**:414-419.

21. Atsumi S, Hanai T, Liao JC: **Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels**. *Nature* 2008, **451**:86-89.
•• This paper introduces the nonfermentative short-chain alcohol pathway and its use for producing advanced microbial fuels such as isobutanol, 2-methyl butanol, or isopentanol. The results using *Escherichia coli* as production host are very promising.

22. Cann AF, Liao JC: **Production of 2-methyl-1-butanol in engineered *Escherichia coli***. *Appl Microbiol Biotechnol* 2008, **81**:89-98.

23. Connor MR, Liao JC: **Engineering of an *Escherichia coli* strain for the production of 3-methyl-1-butanol**. *Appl Environ Microbiol* 2008, **74**:5769-5775.

24. Zhang K, Sawaya MR, Eisenberg DS, Liao JC: **Expanding metabolism for biosynthesis of nonnatural alcohols**. *Proc Natl Acad Sci U S A* 2008, **105**:20653-20658.
• The authors extend the capability of the nonfermentative short-chain alcohol pathway to producing nonnatural 6-carbon alcohols, for example 3-methyl-1-pentanol, by engineering the 2-isopropylmalate synthase and other downstream enzymes.

25. Chang MC, Keasling JD: **Production of isoprenoid pharmaceuticals by engineered microbes**. *Nat Chem Biol* 2006, **2**:674-681.

26. Ro DK, Paradise EM, Ouellet M, Fisher KJ, Newman KL, Ndungu JM, Ho KA, Eachus RA, Ham TS, Kirby J *et al.*: **Production of the antimalarial drug precursor artemisinic acid in engineered yeast**. *Nature* 2006, **440**:940-943.

27. Dewick PM: **The biosynthesis of C5–C25 terpenoid compounds**. *Nat Prod Rep* 2002, **19**:181-222.

28. Christianson DW: **Unearthing the roots of the terpenome**. *Curr Opin Chem Biol* 2008, **12**:141-150.

29. Withers ST, Gottlieb SS, Lieu B, Newman JD, Keasling JD: **Identification of isopentenol biosynthetic genes from *Bacillus subtilis* by a screening method based on isoprenoid precursor toxicity**. *Appl Environ Microbiol* 2007, **73**:6277-6283.
• The authors use an innovative approach to identify novel microbial genes which may be applicable for the production of an isoprenoid-derived short-chain alcohol.

30. Song L: **A soluble form of phosphatase in *Saccharomyces cerevisiae* capable of converting farnesyl diphosphate into E,E-farnesol**. *Appl Biochem Biotechnol* 2006, **128**:149-158.

31. Martin DM, Faldt J, Bohlmann J: **Functional characterization of nine Norway Spruce TPS genes and evolution of gymnosperm terpene synthases of the TPS-d subfamily**. *Plant Physiol* 2004, **135**:1908-1927.

32. Renninger NS, McPhee DJ: *Fuel compositions including farnesane and farnesene derivatives and methods of making and using same*. WO2008045555
•• This published patent application describes the production of the most advanced isoprenoid-derived fuel precursors, farnesene and farnesol, using *Escherichia coli* and *Saccharomyces cerevisiae* as production hosts, and how these fuel precursors can be converted into diesel fuel components.

33. Renninger NS, Ryder JA, Fisher KJ: *Jetfuel compositions and methods of making and using same*. WO2008130492.

34. Shiba Y, Paradise EM, Kirby J, Ro DK, Keasling JD: **Engineering of the pyruvate dehydrogenase bypass in *Saccharomyces cerevisiae* for high-level production of isoprenoids**. *Metab Eng* 2007, **9**:160-168.

35. Yoon SH, Kim JE, Lee SH, Park HM, Choi MS, Kim JY, Lee SH, Shin YC, Keasling JD, Kim SW: **Engineering the lycopene synthetic pathway in *E. coli* by comparison of the carotenoid genes of *Pantoea agglomerans* and *Pantoea ananatis***. *Appl Microbiol Biotechnol* 2007, **74**:131-139.

36. Zhang YM, Rock CO: **Membrane lipid homeostasis in bacteria**. *Nat Rev Microbiol* 2008, **6**:222-233.

37. Stoveken T, Steinbuchel A: **Bacterial acyltransferases as an alternative for lipase-catalyzed acylation for the production of oleochemicals and fuels**. *Angew Chem Int Ed Engl* 2008, **47**:3688-3694.
• This minireview summarizes the application of bacterial acyltransferases for *in vivo* production of fatty acid derivatives, for example fatty acid ethyl esters or 'microdiesel', in recombinant bacteria.

38. Kalscheuer R, Stolting T, Steinbuchel A: **Microdiesel: *Escherichia coli* engineered for fuel production**. *Microbiology* 2006, **152**:2529-2536.

39. Keasling JD, Hu Z, Somerville C, Church G, Berry D, Friedman L, Schirmer A, Brubaker S, Del Cardayre S: *Production of fatty acids and derivatives thereof*. WO2007136762
•• This published patent application describes in detail how the fatty acid biosynthetic pathway can be exploited to produce various microbial fuels, and how the chemical structure of these fuels can be altered by genetic manipulations of the producing microbes.

40. Rowland O, Zheng H, Hepworth SR, Lam P, Jetter R, Kunst L: **CER4 encodes an alcohol-forming fatty acyl-coenzyme A reductase involved in cuticular wax production in *Arabidopsis***. *Plant Physiol* 2006, **142**:866-877.

41. Doan TT, Carlsson AS, Hamberg M, Bulow L, Stymne S, Olsson P: **Functional expression of five *Arabidopsis* fatty acyl-CoA reductase genes in *Escherichia coli***. *J Plant Physiol* 2008 doi: 10.1016/j.jplph.2008.10.003.

42. Dennis M, Kolattukudy PE: **A cobalt-porphyrin enzyme converts a fatty aldehyde to a hydrocarbon and CO**. *Proc Natl Acad Sci U S A* 1992, **89**:5306-5310.

43. Wackett LP, Frias JA, Seffernick JL, Sukovich DJ, Cameron SM: **Genomic and biochemical studies demonstrating the absence of an alkane-producing phenotype in *Vibrio furnissii* M1**. *Appl Environ Microbiol* 2007, **73**:7192-7198.

44. Samuels L, Kunst L, Jetter R: **Sealing plant surfaces: cuticular wax formation by epidermal cells**. *Annu Rev Plant Biol* 2008, **59**:683-707.

45. Schneider-Belhaddad F, Kolattukudy P: **Solubilization, partial purification, and characterization of a fatty aldehyde decarbonylase from a higher plant, *Pisum sativum***. *Arch Biochem Biophys* 2000, **377**:341-349.

46. Aarts MG, Keijzer CJ, Stiekema WJ, Pereira A: **Molecular characterization of the CER1 gene of *Arabidopsis* involved in epicuticular wax biosynthesis and pollen fertility**. *Plant Cell* 1995, **7**:2115-2127.

47. Ladygina N, Dedykhina EG, Vainshtein MB: **A review on microbial synthesis of hydrocarbons**. *Process Biochem* 2006, **41**:1001-1014.

48. Strobel GA, Knighton B, Kluck K, Ren Y, Livinghouse T, Griffin M, Spakowicz D, Sears J: **The production of myco-diesel hydrocarbons and their derivatives by the endophytic fungus *Gliocladium roseum* (NRRL 50072)**. *Microbiology* 2008, **154**:3319-3328.
• This and other recent papers by these authors describe the occurrence of volatile, short-chain to medium-chain hydrocarbons in certain endophytic fungi. If the biosynthesis of these compounds can be elucidated, it may enable the production of microbial fuels with a wider range than currently envisioned.

49. Kai M, Effmert U, Berg G, Piechulla B: **Volatiles of bacterial antagonists inhibit mycelial growth of the plant pathogen *Rhizoctonia solani***. *Arch Microbiol* 2007, **187**:351-360.

50. Metzger P, Largeau C: ***Botryococcus braunii*: a rich source for hydrocarbons and related ether lipids**. *Appl Microbiol Biotechnol* 2005, **66**:486-496.

51. Villaverde LM, Juarez MP, Mijailovsky S: **Detection of *Tribolium castaneum* (Herbst) volatile defensive secretions by solid phase microextraction-capillary gas chromatography (SPME-CGC)**. *J Stored Prod Res* 2007, **43**:540-545.

52. Albro PW, Dittmer JC: **The biochemistry of long-chain, nonisoprenoid hydrocarbons. I. Characterization of the hydrocarbons of *Sarcina lutea* and the isolation of possible intermediates of biosynthesis**. *Biochemistry* 1969, **8**:394-404.

53. Friedman L, Da Costa B: *Hydrocarbon-producing genes and methods of their use*. WO20081487781.

54. Friedman L, Rude M: *The process of producing low-molecular weight hydrocarbons from renewable resources*. WO2008113041.

55. Li Q, Du W, Liu D: **Perspectives of microbial oils for biodiesel production**. *Appl Microbiol Biotechnol* 2008, **80**:749-756.

56. Li Y, Zhao Z, Bai F: **High-density cultivation of oleaginous yeast *Rhodosporidium toruloides* Y4 in fed-batch culture**. *Enzyme Microb Technol* 2007, **41**:312-317.

57. Gouda MK, Omar SH, Aouad LM: **Single cell oil production by *Gordonia* sp. DG using agro-industrial wastes**. *World J Microbiol Biotechnol* 2008, **24**:1703-1711.

58. Dindi H, Singupta SK, Gonzon AF, Corbin DA: *Catalytic process for converting renewable resources into parrafins for use as diesel blending stocks*. US Patent 2008.

59. Mukhopadhyay A, Redding AM, Rutherford BJ, Keasling JD: **Importance of systems biology in engineering microbes for biofuel production**. *Curr Opin Biotechnol* 2008, **19**:228-234.

60. Keasling JD: **Synthetic biology for synthetic chemistry**. *ACS Chem Biol* 2008, **3**:64-76.

61. Lu X, Vora H, Khosla C: **Overproduction of free fatty acids in *E. coli*: implications for biodiesel production**. *Metab Eng* 2008, **10**:333-339.

62. Knothe G: **Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters**. *Fuel Process Technol* 2005, **86**:1059-1070.

63. Knothe G: **''Designer'' biodiesel: optimizing fatty ester**
• **composition to improve fuel properties**. *Energy Fuels* 2008, **22**:1358-1364.
This article introduces the concept of 'designer' biodiesel, that is changing the fuel composition by genetic manipulations of the producing organism, and it recognizes the limitations of plant-derived biodiesel in this regard.

64. Mayer KM, Shanklin J: **Identification of amino acid residues involved in substrate specificity of plant acyl-ACP thioesterases using a bioinformatics-guided approach**. *BMC Plant Biol* 2007, **7**:1-11.

65. Fujita Y, Matsuoka H, Hirooka K: **Regulation of fatty acid metabolism in bacteria**. *Mol Microbiol* 2007, **66**:829-839.

66. Li Y, Florova G, Reynolds KA: **Alteration of the fatty acid profile of *Streptomyces coelicolor* by replacement of the initiation enzyme 3-ketoacyl acyl carrier protein synthase III (FabH)**. *J Bacteriol* 2005, **187**:3795-3799.

67. Lide DR (Ed): *Handbook of Chemistry and Physics*. Boca Raton, FL: CRC Press; 1995.