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Defossiling Fuel: How Synthetic Biology Can Transform Biofuel Production

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Although crude oil production is predicted to peak soon, it is reasonable to assume that unconventional fossil fuel sources can continue to meet society's increasing energy demands for many decades to come (*1*). The real challenge is sustainability: stabilizing and reversing global climate change, minimizing political and economic energy volatility, and smoothing the transition from fossil fuels in the distant future. In response to this challenge, many are looking to biotechnology to develop biofuels, such as ethanol, butanol, biodiesel, and hydrogen (H_2), in which the energy ultimately derives from photosynthetic capture of sunlight. A fundamental issue with biofuels is efficiency. The pathway from sunlight through natural intermediates to final molecule is long, and biofuel production is perhaps the ultimate metabolic engineering problem (*2*). This challenge is made even greater by its inherent systems complexity, because any solution must be implemented in the context of an energy infrastructure with challenging engineering, economic, political, and environmental realities.

Are biofuels sustainable? Consider U.S. transportation fuels, a market poised for impact. Biofuels derive their stored chemical energy from the sun *via* photosynthesis. Biofuel use is therefore a closed carbon cycle, as carbon released during combustion is sequestered during photosynthesis. Solar radiation is clearly a sustainable energy source on human time scales, and U.S. incident solar power (~2300 TW) (*3*) greatly exceeds our transportation fuel usage (~1.0 TW) (Table 1) (*4*). The reactions of photosynthesis impose a maximal efficiency of ~12%, but final yields are significantly lower (*2*). Terrestrial plant efficiencies for solar to biomass conversion is maximally 2% (*e.g.*, for the rapidly growing *Miscanthus* (*5*)), and the subsequent conversion into biofuels is ~50% efficient (*6*). It would therefore require ~4.3% of the U.S. land area to meet our transportation energy demands, which corresponds to ~22% of current cropland. Thus, in an optimistic approximation (and ignoring social, political, and economic complexities), we can say biofuel production could be sustainable, albeit with significant challenges.

These calculations suggest that a biofuel-based energy economy is feasible but that enhancements in the efficiency of any step in energy production would be favorable from an economic and environmental standpoint. The upper bound on efficiency is set by photosynthesis—the challenge is therefore to come as close to this bound as possible. Put another way, how can one optimize metabolism to direct maximal flux from one set of metabolites to another, while still maintaining, at least partially, host fitness? Traditional industrial approaches have given us many such successes (*e.g.*, beer) and will play a major role, but the tools of systems and synthetic biology promise to deliver a degree of optimization not previously attainable (*7*).

A synthetic-biological redesign of the organisms that produce biofuels has the potential to significantly increase efficiency,

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TABLE 1. Useful biofuel numbers

U.S. incident terrestrial solar radiation, 2300 TW
U.S. transportation fuels usage (2006), ~1 TW
Miscanthus conversion efficiency of solar energy into biomass, 2%
Conversion efficiency of biomass into ethanol, ~50%
% U.S. land required to meet energetic demands, 4.3%
% of current cropland required, 22%

decrease cost, and thus enable a transition to a sustainable energy economy that is largely independent of fossil fuels. Indeed, such an approach has been underway for many years, well before the term "synthetic biology" was coined. At a certain point, strategies for biofuel production will run up against inherent limitations from the energetics of chemical reactions. From this perspective, we review major biofuels under consideration, with a focus on synthetic-biological optimizations.

Ethanol, Butanol, Biodiesel, and Hydrogen: Strategies for Biofuel Production. *Ethanol*. Ethanol is the most successful biofuel and, for example, already supplies 40% of Brazil's transportation fuel needs (*8*). Ethanol's main advantage is its established infrastructure: techniques for fermentative production of ethanol from sucrose are in place, large-scale distillation technologies have been developed, and it can be used in so-called flexible-fuel vehicles. These advantages are enabled by domesticated yeasts, which for thousands of years have been used to efficiently convert carbohydrates to ethanol. Its main disadvantages are that pure ethanol is corrosive to storage and transport equipment, ethanol is miscible with water and distillation is therefore expensive, and the energy density is somewhat lower than that of gasoline (Table 2). Furthermore, despite the successes, ethanol as derived from corn seed is not a viable long-term option—the energy return on energy invested is estimated at just over 1 (*9*).

The future of ethanol (and likely all carbohydrate-based biofuels) lies in the ability to more fully utilize plant biomass. Higher plant cells are enclosed by lignin and polysaccharide polymers (cellulose) that comprise 50–90% of plant biomass and remain inaccessible to refinement. Possible solutions to increase the amount of available carbohydrate include engineering plants to synthesize more desirable polymers, identifying and evolving novel cellulases to aid in digestion, and developing chemical processes to better depolymerize cellulose prior to fermentation (*10*).

If these techniques can be successfully implemented using energy crops, production yields would increase nearly 6-fold (from ~400 to 2300 gallons/acre) (*8*). Furthermore, from an economic standpoint, cellulosic ethanol could be competitive with fossil fuels. Fermentation converts 1 mol of glucose into 2 mol of ethanol, and many microbial systems are near this theoretical efficiency (*6*). The biomass of an energy plant such as *Miscanthus* is ~70% accessible to fermentation, so 1 ton of biomass can be converted into ~110 gallons of ethanol. If biomass could be delivered at the price of $40–50 per ton, ethanol could be produced at a price per unit energy that is comparable to that of gasoline and would have a significant impact on the energy market. It should be noted that construction of the first U.S. commercial-scale cellulosic ethanol plant began in November 2007.

Butanol. Butanol is another attractive biofuel. Its longer alkyl chain equates to an easier solvent extraction process, higher energy content per weight, and lower vapor pressure. Unlike ethanol, butanol is not corrosive (Table 2). Most biological systems are not as adept at producing butanol as ethanol, but this difference can be used to highlight how to implement a synthetic biology strategy for the creation of an optimized microbial catalyst for the conversion of biomass into a desired molecule.

Clostridia, such as *Clostridium acetobutylicum*, are known to ferment sugars into acetone, butanol, and ethanol and would serve as an excellent starting point for engineering butanol overproduction (*11*). Clostridia secrete many carbohydrate polymer degrading exoenzymes and could be engineered to secrete cellulases as well. Because Clostridia also produce the side products acetone, ethanol, and organic acids, one could use the modeling tool flux balance analysis to better understand the flow of metabolites and to design knockout mutants with flux-optimized butanol production (*12*). Finally, to overcome butanol toxicity, the heat shock response, a known remedy, could be constitutively activated, and overexpressed efflux transporters could be used to transfer butanol into the medium, where it can be extracted during an organic phase.

Alternatively, butanol could be made from a robust, highly engineerable microbe such as *Escherichia coli*. Recently, Atsumi *et al*. (*13*) introduced six genes from *C. acetobutylicum* into *E. coli* to transfer the butanol-producing metabolic pathway. In addition, they mutated a number of *E. coli* genes to enhance butanol production, some of which worked as predicted and some of which did not. In the best case, for each mole of glucose consumed, ~0.12 mol of butanol was produced, with the rest of the carbons appearing as CO_2, pyruvate, formate, ethanol, and other minor metabolites.

Biodiesel. The definition of biodiesel is somewhat ambiguous but is generally thought of as combustible fuel derived

from biological lipids. Unlike petrodiesel, which is composed of alkanes and aromatic hydrocarbons (C_{10}–C_{15}), biodiesel is composed of fatty acids with corresponding alkyl chain lengths of 16–24 carbons. This longer chain length leads to higher viscosity and high melting points and is problematic for internal combustion. Normally, biodiesel is refined by transesterification of the triglyceride fatty acids with an alcohol, often methanol (*14*). Alternatively, biodiesel can be generated by "cracking" longer alkyl chains into smaller molecules and then distilling fractions with the desired chain length, as is done to generate conventional diesel fuel and gasoline.

Biodiesel could be obtained from easily accessible waste streams, such as vegetable oil from restaurants. Unfortunately, the volume of these streams is not sufficient for large-scale production. Furthermore, compared to cellulose, it is not as easy to produce lipid at the scales required. Current oil-generating plants (*e.g.*, soybeans) have yields per acre that are 10-fold less than carbohydrate-based plants. It is possible, however, that plants such as the oil palm or the bush *Jatropha* may find success as biodiesel feedstock in tropical climates (*8*).

An alternative source of biodiesel is that derived from phototrophic algae or cyanobacteria (collectively, microalgae) grown in open "raceway" ponds (*15*). Microalgae operate closer to the theoretical maximum efficiency of photosynthesis, and large-scale experiments suggest a several-fold increase in biomass production per area over even the best terrestrial energy crops (*16*). Furthermore, this biomass can be produced with a very high lipid content, 30–80%, depending on the species, making the refinement process easier. Microalgae can also be grown on nonarable land by using wastewater streams of ocean seawater, and it is estimated that aquatic phototrophs use less resources than terrestrial plants (*15*). Perhaps most tellingly, industrial-scale experiments have found that oil can actually be produced at $84/barrel (*16*).

TABLE 2. Fuel properties

Biofuel	Relative lower heating value	Biological engineering difficulty	Infrastructure compatability
Ethanol	0.62	Low	Medium
Butanol	0.83	Medium	Medium
Biodiesel	0.85	Low	High
Hydrogen	2.76	High	Low
Gasoline	1.0		

Genetic tractability coupled with optimal biomass fixation make microalgae an intriguing synthetic biology chassis. One option would be to engineer the chain length of fatty acids for improved fuel properties. Fatty acid synthetase (synthase) produces fatty acids by adding $-COCH_3$ groups to a growing chain, reducing the C═O, then repeating this cycle. Synthesis stops when a certain chain length is reached. It may be possible to mutate fatty acid synthetase so that it releases the fatty acid at a shorter chain length (*17*). The resulting product might not be a good substrate for subsequent natural reactions (*e.g.*, esterification by glycerol) and might simply accumulate to be harvested. Depending on how the enzyme is engineered, it could produce either diesel-length or gasoline-length molecules.

Hydrogen. As a biofuel, hydrogen presents a completely different set of advantages and problems. One obvious advantage is that hydrogen is a clean-burning fuel that will not contribute to global warming upon combustion. With respect to production, hydrogen is completely nontoxic and automatically separates itself from the microbial culture. Any system that produces hydrogen from glucose will also produce CO_2, which could most likely be easily separated from H_2 based on water solubility or mass in an industrial-scale process.

Producing hydrogen has several idiosyncratic difficulties. First, a hydrogen infrastructure (storage, transport, and vehicles) is not now in place. Second, the enzymes that nature has given us to produce hydrogen, the hydrogenases, have bimetallic active sites that are extremely oxygen-sensitive. One organism, *Ralstonia eutropha*, has evolved oxygen-resistant hydrogenases, but these enzymes require a large number of maturation factors, several of which have an unclear function (*18*).

Third, the ideal overall reaction to produce hydrogen from glucose, $C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 12H_2$, is only slightly energetically favorable (–13 kJ/mol of glucose). In actual cells, the primary reducing agent is NAD(P)H, and the reaction $NADH + H^+ \rightarrow NAD^+ + H_2$ is not favorable and can only be driven forward by Le Chatelier's principle–hydrogen must continually be withdrawn. One group has actually reconstituted this reaction *in vitro*, starting with glucose 6-phosphate, enzymes of the pentose phosphate pathway (which completely converts glucose to CO_2 and uses the liberated reducing equivalents to convert NAD^+ to NADH), and a hydrogenase that can use NADH to generate H_2 (*19*). The yield was ~11 H_2/glucose, close to the theoretical maximum. However, the reaction required hydrogen to be continuously withdrawn and proceeded on a time scale of many days.

A final strategy is to harness the photosynthetic reactions themselves to directly drive the production of molecular hydrogen. The initial reactions of photosynthesis are to split water into molecular oxygen, gener-

ate a proton gradient, and generate electrons with strong reducing potential. In principle, these electrons could be directed to a hydrogenase to generate hydrogen.

Esper *et al*. (*20*) have classified the current approaches for direct photosynthesis-to-hydrogen into "engineered natural" and "bioinspired" systems. The complexity and creativity of many of these systems underscore the difficulty of this approach: strategies include electroporating a hydrogenase enzyme into a cyanobacterium, cycling the green algae *Chlamydomonas* between different growth conditions to induce photosynthesis and then eliminate the resulting oxygen by respiration, and immobilizing isolated photosystems and hydrogenases on electrode surfaces to capture the energy of transferred electrons as well as hydrogen. In one recent approach, Ihara *et al*. (*21*) expressed a fusion between an oxygen-resistant hydrogenase of *Ralstonia* and the PsaE subunit of Photosystem I, and then added this chimeric protein to a Photosystem I complex purified from a PsaE-deficient cyanobacterium. The hydrogenase in the resulting assembly appeared to produce hydrogen in a light-dependent manner at a low rate. Although technically challenging, these strategies may represent the ultimate route for operating near the upper boundary of efficiency imposed by photosynthesis.

Conclusions. On the basis of our analysis, it should be clear that a need exists for a synthetic-biological approach to biofuel production. First, every improvement of the efficiency of biofuel production will matter. Calculations on the cost of a biofuel start with the cost of biomass, and then depend on the efficiencies of subsequent conversion steps.

Second, existing approaches primarily use what nature has given us. A synthetic biology approach to biofuel production could offer substantial improvements but may require complex reengineering of natural systems. This may force us toward microbial systems for energy capture. Photosynthetic microbes growing in controlled environments may be more efficient than plants for capturing light energy, because they are fundamentally more engineerable and do not do as many wasteful things as plants. Brenner, in a U.S.-government-sponsored report that is a must-read for anyone interested in the economics of biofuels, argues this point in some detail (*2*).

Therapeutic proteins and naturally occurring molecules such as amino acids have been overproduced from engineered cells, but the goal of biofuel production goes beyond that of most biotechnology and metabolic engineering. Namely, the goal is to produce the desired products essentially at the maximum efficiency allowed by thermodynamics. The final question is whether synthetic biology is up to this challenge, which will depend as much on our creativity as on the physical constraints of nature.

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