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Engineering microbes to produce biofuels

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The current biofuels landscape is chaotic. It is controlled by the rules imposed by economic forces and driven by the necessity of finding new sources of energy, particularly motor fuels. The need is bringing forth great creativity in uncovering new candidate fuel molecules that can be made via metabolic engineering. These next generation fuels include long-chain alcohols, terpenoid hydrocarbons, and diesel-length alkanes. Renewable fuels contain carbon derived from carbon dioxide. The carbon dioxide is derived directly by a photosynthetic fuel-producing organism(s) or via intermediary biomass polymers that were previously derived from carbon dioxide. To use the latter economically, biomass depolymerization processes must improve and this is a very active area of research. There are competitive approaches with some groups using enzyme based methods and others using chemical catalysts. With the former, feedstock and end-product toxicity loom as major problems. Advances chiefly rest on the ability to manipulate biological systems. Computational and modular construction approaches are key. For example, novel metabolic networks have been constructed to make long-chain alcohols and hydrocarbons that have superior fuel properties over ethanol. A particularly exciting approach is to implement a direct utilization of solar energy to make a usable fuel. A number of approaches use the components of current biological systems, but re-engineer them for more direct, efficient production of fuels.

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''Advances in the petroleum-hydrocarbon industry, more than anything else, may be credited to the high standard of living we enjoy.''
-George Olah, in *Hydrocarbon Chemistry*

Introduction

George Olah, and a legion of chemists and engineers, taught us how to wring fuels and chemicals out of 'rock;' the word petroleum derives from Latin roots meaning 'rock oil' [1]. In the current world, amid concerns that our sources of petroleum are dwindling [2], we are turning over all stones for suitable alternatives to petroleum. We will have to search in creative ways. Olah and his associates were very adept in developing the tools to efficiently crack and reformulate petroleum into gasoline, jet fuel, diesel, and heating oil.

The exchange that we envision is to replace an essentially non-renewable resource, petroleum, with renewable fuels and thus make our future sustainable. The major renewable materials on Earth are derived from biological systems that reproduce and replenish themselves. So it is natural to turn to biological systems for producing renewable fuels. The present review focuses on recent advances in engineering organisms and processes to make renewable fuels. In his book *Hydrocarbon Chemistry*, George Olah stated, ''... in the not too distant future, we will need to make synthetic hydrocarbons on a large scale.'' Using renewable biological resources, the conversions may be synthetic or biosynthetic.

Biomass deconstruction

The primary source of most biofuels is the biomass of photosynthetic organisms. Thus, efficient biomass deconstruction is one of the fundamental goals of the biofuels industry. Plants evolved to resist attack from insects and microbes. The barriers erected are formidable. Lignin is a heterogeneous polymer and difficult to depolymerize. Plant cellulose occurs in both crystalline and amorphous form, the former is degraded relatively slowly by biological systems. There has been significant progress in recent years in better understanding the microbial mechanism for degrading recalcitrant cellulose; a mechanism that appears to require direct cell contact [3] and structures known as cellulosomes [4]. Cellulosomes are extracellular microbial machines. It is now appreciated that the complex structural arrangement of cellulosome component proteins leads to an enhancement of cellulose degradation compared to catalysis carried out by individual enzymes free in solution. Thus, the field needs to go beyond individual high-resolution X-ray structures of component proteins and begin solving structures of protein aggregates that comprise the cellulosome structure. Specifically, the recent study by Adams *et al.* [5••] has demonstrated the high-resolution structure of the type-II cohesin module of the cell surface protein SdbA connected to a fragment of the scaffoldin protein CipA.

The studies suggested that individual scaffoldins associate to generate cellulosome arrays, and this may be important in efficient cell-surface cellulose hydrolysis.

In the practical application of cellulose depolymerization, the addition of exogenous enzymes would probably prove both inefficient and costly. In this context, there are continuous efforts to find microbes that can directly degrade plant biomass without acid or enzyme pre-treatment. One recent study describes the efficient degradation of lignocellulosic plant material by the bacterium *Anaerocellum thermophilum* [6••] that has now been reclassified as *Caldicellulosiruptor bescii* [7]. *C. bescii* strain DSM 6725T is an extreme thermophile, with the ability to grow at temperatures as high as 90 °C. Of even greater interest, the microorganism degrades the crystalline cellulose and xylan of untreated plant biomass. The thermophile has been shown to be active with plants that are being targeted as 'bioenergy' plants; for example, poplar and switchgrass. The complete genome sequence of this bacterium was recently determined [8].

Biotechnological and chemical engineering hybrid approaches

Another approach for dealing with biomass complexity is to transform biomaterial with chemical catalysts directly. The fundamental problem to be solved is converting the highly oxygenated biomass feedstock into a significantly less oxygenated compound(s), characteristic of liquid fuels [9]. One issue of biofuels that is often glossed over is the combustion properties of different molecules that can be generated from bio-feedstocks. This was recently addressed by Kohse-Höinghaus *et al.* [10]. With respect to the actual transformation reactions, there is a great deal of diversity in terms of feedstock composition and the fuel produced. An earlier effort described a mixed enzymatic and catalyst transformation to produce fructose and, ultimately, dimethylfuran as the fuel [11]. Another study describes the conversion of γ-valerolactone into liquid alkenes that can be hydrogenated [12]. Lennen *et al.* have taken the approach of engineering *Escherichia coli* cells to produce fatty acids, molecules that are more highly reduced than the average biological material [13]. The fatty acids can then be catalytically converted to alkanes. Many of the chemical catalytic approaches suffer from the requirement to start with a single, or specific type of biological molecule, for example γ-valerolactone, or a class of molecules like medium chain-length fatty acids. This requires significant upfront engineering, either biological or chemical, to produce a simple feedstock stream from a complex carbon source (biomass). Several approaches are being developed to be able to use much more heterogeneous, and even undefined, biomass feedstock streams. In one example, Binder and Raines are producing furans from lignocellulosic biomass [14•]. Colby *et al.* have described a process of partial catalytic oxidation in which biomass carbon is taken to carbon monoxide in 100% yield [15•]. This is significant for producing a synthesis gas stream without loss of biomass carbon as carbon dioxide or char.

In silico guided metabolic engineering

When metabolic engineering is required, efficiency in design and execution is important to make a relatively low-priced commodity chemical or a fuel. Recently, there have been dramatic improvements in modeling metabolism computationally and this can translate into improving the biofuels effort in a major way. Some recent examples of this have been trained on an *E. coli* metabolic model and used for making ethanol [16] and butanol [17], respectively.

Mitigation against fuel cytotoxicity

Alcohols have long been known to be toxic to microbial cells [18] and this still remains a key issue in expressing higher titers of alcohol fuels via microbial fermentation processes. In an effort to overcome this problem, new methods are being brought to bear. Genomic methods have been used to explore the resistance of *E. coli* to n-butanol [19]. A traditional fermentation for fuels and chemicals is the butanol fermentation of *Clostridium acetobutylicum*. Gene-expression tools have been used recently in an effort to enhance the production of butanol [20].

Fuels from phototrophs

Conceptually, it is attractive to produce fuels biologically from sunlight and carbon dioxide. One of the key issues that many people are considering is the overall efficiency of the photosynthetic apparatus of different microorganisms for capturing the total photon energy available in a given area of irradiation. The review by Larkum [21] provides a useful overview of this concept.

Several groups are forging ahead to genetically engineer cyanobacteria to produce fuels and chemicals directly from carbon dioxide, thus obviating the cycle of growing plants and deconstructing their lignin and cellulose. A paper by Atsumi *et al.* exemplified the best of this approach [22••]. They engineered a non-native metabolic pathway in *Synechococcus elongatus* to produce the four carbon aldehyde isobutyraldehyde. While the preferred fuel is isobutanol, stopping at the aldehyde stage was a clever strategy as it mitigated against the product toxicity of alcohols and the aldehyde is sufficiently volatile to allow it to be stripped from the growth medium through the gas phase. This latter point is very important, as product recovery of alcohols from fermentation broths is very energy intensive. Recovery of the product from the gas phase thus makes the process much more attractive economically. This study leads the way in using innovative bioprocess technology for producing fuels and chemical feedstocks via engineered photosynthetic bacteria.

Another process describes the production of isoprene from carbon dioxide by the cyanobacterium *Synechocystis* sp. PCC 6803 [23]. Like isobutyraldehyde, isoprene is volatile and gases out of the culture broth, making product recovery easier. The yield was modest, 50 μg isoprene per gram dry weight of biomass per day. A much greater yield and productivity was obtained in a glucose fermentation with an engineered *E. coli* strain [24]. Isoprene is highly combustible but is not being considered to be used as a motor fuel. Rather, its utility lies in its use as a monomer for making rubber products, principally tires.

A different utility of light harvesting is suggested by the observation that many non-photosynthetic marine bacteria contain proteorhodopsin and light stimulates the growth of these strains [25]. This has prompted groups to examine the utility of using proteorhodopsin to increase the energy efficiency of heterotrophic fermentative strains [26]. While this is not a biotechnological replacement for photosynthetic energy harvesting coupled to carbon dioxide reduction, increasing energy efficiency ultimately improves industrial processes and saves energy for society.

Extreme bioengineering for direct solar conversion

Given questions regarding the photon efficiency of naturally evolved photosynthetic systems, it is being considered whether biological systems may be fundamentally altered for improving light harvesting capabilities for generating fuels at higher photon efficiencies. One extreme case of bioengineering for improved solar conversion is described by Conlan *et al.* [27•]. Cytochrome *b*1 from *E. coli* was redesigned for light-driven electron transport by replacing the heme with the photoactive zinc–chlorin cofactor and the di-iron site was repopulated with two manganese ions. This is the first artificial reaction center carrying out the photo-catalytic oxidation of a di-metal center in a re-engineered protein designed by nature for other function. It paves the way for other innovative approaches for improving nature's protein designs for more efficient solar conversion of carbon dioxide into fuel chemicals.

In general, direct routes are more efficient and this is the concept underlying the rewiring of photosystem I for the direct solar production of hydrogen gas [28••]. The premise is that the practical production of biofuels from light energy requires a photochemical unit that generates, ideally, a low-redox potential reductant, a catalytic unit that uses the electrons to do reductive chemistry, and a means for electron transfer between the units. The present study demonstrated feasibility in linking a low potential iron–sulfur redox center in photosystem I via a tether linked through sulfhydryl groups. The tether could transfer electrons to a non-biological platinum nanoparticle or to a biological hydrogenase. The latter represents a direct rerouting of electrons from the photosystem to an enzyme capable of making hydrogen gas from protons.

Fatty acid-derived fuels—general

The biological production of fuel molecules that most resemble petroleum gasoline and diesel will probably come from the fatty acid biosynthetic pathways of bacteria. The fatty acids that comprise the major carbon chain of biodiesel today are derived largely from plants. The fundamental mechanism of fatty acid synthesis of plants and microbes is the same, but microbes offer greater metabolic engineering opportunities. The fatty acid pathway of *E. coli* was extensively engineered in the work described by Steen *et al.* [29]. The best strains were made defective in fatty acid degradation and contained a plant thioesterase that would liberate fatty acids from fatty acyl thioesters. The latter strategy serves to deregulate fatty acid biosynthesis and makes for continued synthesis significantly beyond the needs of the organism and for excretion of product into the growth medium.

In other studies with *E. coli* [30], 2.5 g of fatty acids per liter of culture was achieved with similar genetic manipulations as carried out by Steen *et al.* [29]. To increase fatty acid production further, additional knowledge was needed regarding the optimum levels of enzymes and the key regulatory steps controlling them. To attain this knowledge, a complete set of enzymes for cell-free fatty acid synthesis was generated by overexpressing and purifying the relevant enzymes [31••]. This allowed a determination of the kinetic constants of the relevant enzymes that could be used with data on intracellular pools of metabolites to gain insight into bottlenecks in biosynthesis. As a result, an *E. coli* strain was engineered to produce 4.5 g of total fatty acids per liter of culture per day in a fed batch fermentation. There are also key patents in this area of fatty acid overproduction [32].

Fatty acid-derived fuels—esters

The section on nomenclature made the point that biodiesel can be derived totally from biomass carbon, or, more likely, from commodity methanol esterified to biological fatty acids. Several years ago, Steinbüchel and coworkers [33] discovered acyltransferases that can transfer biologically derived ethanol to fatty acids to make fatty acid ethyl esters. More recently, the *E. coli* p(Microdiesel) strain has been used in moderately large scale fermentations to yield a dry cell mass of 61 gram per liter with a fatty acid ethyl ester content as high as 25.4% of the culture dry mass [34]. These fed batch cultivations were supported initially with glycerol and then with glucose and oleic acid as the carbon sources.

Fatty acid-derived fuels—hydrocarbons

There have been many reports of microbial hydrocarbon biosynthesis through the years [35,36], but some have proven to be irreproducible [37]. However, it is clear that

microbes biosynthesize alkanes and alkenes. The biological functions and the biochemical mechanisms have largely remained obscure but, with renewed effort, they are now being elucidated.

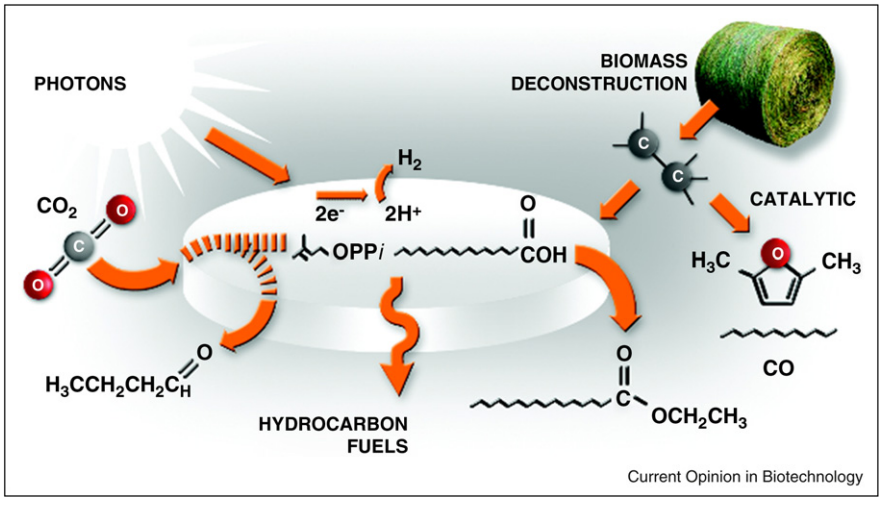
A recent breakthrough was made in understanding how cyanobacteria biosynthesize diesel-length alkanes [38••]. These compounds are derived from fatty acids via a two-step metabolic pathway in which an acyl group is reduced to an aldehyde followed by an apparent decarbonylation to generate the C-1 alkane, or alkene if the fatty acyl starter group were unsaturated. The decarbonylase gene was discovered by a substractive genomic approach, looking for key genes that were absent in *Synechococcus* PC 7002, that does not make alkanes, but are present in other cyanobacteria that make alkanes. The decarbonylase gene encodes a protein representing a large class whose members contain a binuclear metal center, exemplified by ribonucleotide reductase. It was further of interest that a high-resolution X-ray structure of the protein was already available from a structural genomics project. In that structural genomics research, the function of the protein had not been determined. There are still some outstanding questions remaining with respect to the requirement for NADPH in what appears to be an overall redox-neutral reaction, and the precise nature of the binuclear metal center. Nonetheless, this represents an exciting development in the overall process of engineering bacteria to produce biofuel hydrocarbons.

The decarbonylase mechanism is intriguing but microbial hydrocarbons arising via a fatty acid head-to-head condensation mechanism appear to be more widespread phylogenetically. The latter mechanism generates olefins with a double bond at or near the center of the chain. These head-to-head olefins are derived from fatty acids with the fatty acids being condensed at the carboxyl carbon of one chain and the α-carbon of another [39]. This suggests that a Claisen-type condensation reaction is occurring. In biology, both decarboxylative and non-decarboxylative Claisen condensations are well known [40]. The former is exemplified by the fatty acid elongation reaction in which a malonyl group provides an activated methylene carbon for carbanion generation and attack at the electrophilic acyl carbon of a fatty acyl-ACP undergoing elongation. The non-decarboxylative Claisen condensation is represented by the biosynthetic thiolase reaction catalyzed in the initial step of polyhydroxybutyrate biosynthesis. The enzymes catalyzing each type of Claisen condensation are in the same protein family, the thiolase superfamily [40], and contain many of the same active site groups for bringing about catalysis. In this context, it is difficult to discern between an decarboxylative and non-decarboxylative enzyme by sequence analysis alone.

Recently, Beller *et al.* identified the *ole* genes involved in head-to-head olefin biosynthesis in *Micrococcus luteus* and proposed a decarboxylative fatty acid condensation mechanism [41]. Sukovich *et al.* described the *ole* genes in *Shewanella oneidensis* and 68 other bacterial strains and proposed a non-decarboxylative Claisen condensation mechanism for fatty acid joining [42,43]. Further research is necessary to resolve the details of the olefin biosynthetic mechanisms. Most recently, the crystallization and preliminary X-ray structure of the OleC protein was described [44].

Another major focus in biohydrocarbon production is trained on the isoprenoid biosynthetic pathways [45]. One example of a diesel-type fuel that is being developed

Figure 1



Multiple approaches for making next generation fuels from renewable resources. The input can be directly from the sun (photons) or from biomass. The output can be alcohols, aldehydes, esters, or hydrocarbons.

commercially by Amyris is farnesane, which is derived from the chemical hydrogenation of the bioproduct farnesene [46]. Other terpenoid bioproducts being considered for use as precursors to jet fuels include pinene, sabinene, and terpinene.

Conclusions

The present era of biofuels research is marked by great creativity. In this context, multiple technologies are being pursued (Figure 1). Approaches range from biomass fermentations for producing alcohols to modified photosynthetic systems for producing second generation fuels. It is likely that one or more of these technologies will take root. Ultimately, the chances for success appear to be good as society increasingly looks to replace non-renewable fuels, for both economic and environmental reasons.

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