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A Perspective: Photosynthetic Production of Fatty Acid-Based Biofuels in Genetically Engineered Cyanobacteria

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Abstract

Biofuels are expected to play a key role in the development of a sustainable, economical and environmentally safe source of energy. Microbes offer great potential for applications in technology based biofuel production. Three fundamental questions need to be addressed in order for the development of microbial synthesis of biofuels to be successful. Firstly, what energy resource platform could be used to make biofuels. Secondly, what type of biofuel is the ideal fuel molecule that should be targeted. Finally, what microbial system could be used to transform energy resources into the targeted biofuel molecules. In this perspective, the potential of using photosynthetic microbes (cyanobacteria in particular) in the solar energy driven conversion of carbon dioxide to fatty acid-based biofuels is explored.

Key words: biofuels; cyanobacteria; fatty acid-based biofuels; synthetic biology
1. Energy resource platforms

Solar energy is captured and transformed to chemical energy by the process of photosynthesis. All plants carry out photosynthesis, yet they differ in the storage form or “biomass”. Some plants such as corn, sugarcane, and cassava specialize in starch production. Others, exemplified by switchgrass and miscanthus, are efficient cellulose producers. Starch and cellulose are both common starting materials for the biofuel ethanol. Finally, some plants are efficient in the conversion of solar energy to reduced hydrocarbons or “oils”. Soybean, palm trees, as well as microalgae, produce oils, which can be harvested and used in the production of a variety of biofuels (Fig. 1).

Biomass can be transformed into biofuel by chemical or biological conversion or a combination of both strategies. Yet, each type of biomass presents its own set challenges that must be overcome if biofuel is to be produced at low fiscal and environmental costs. For example, chemical (acid or base) or enzyme (lipase) catalysis can be used to liberate the fatty acids from triacylglycerols (TAG) produced in “oil” production plants or algae for use as the corresponding methyl or ethyl esters as biofuels. However, the inadequate supply of TAG is the bottleneck for current biodiesel production. At present, microalgae are considered to be the highest potential TAG resource because of the high oil content and faster growth compared to plants (Chisti et al., 2007).

Starch, which is a polymer of glucose, can be easily degraded into glucose monomers and used to produce ethanol through microbial fermentation. Unfortunately, cultivated starch-producing plants, such as corn, serve as an important source of food, and sugarcane and cassava planting is geographically restricted. Cellulosic biomass (cellulose and hemicellulose) is in abundant supply (Service et al., 2007), however the processes used for the separation of cellulose and hemicellulose from lignin, the depolymerization of the polysaccharide and the subsequent fermentation steps required for ethanol production are costly. The pre-treatment procedures require energy input (steam treatment) or expensive ionic solvents. The enzymes, which constitute the cocktails used for depolymerization, present the major cost barrier.

The major challenge for utilization of the biomass as an energy resource platform lies in the discovery of an efficient and environmentally sound source of TAG or polysaccharide and cost-competitive technologies for conversion to biofuel. As an
energy resource platform, the capture and conversion of solar energy should be highly efficient and adaptable to microbial systems, which can efficiently utilize solar energy and fix carbon dioxide for the synthesis of reduced hydrocarbons, without competing for land needed for food production or requiring expensive reagents.

2. Microbial systems

As a candidate for biofuel-producing microbial systems, cyanobacteria are attractive because they incorporate the favorable characteristics of prokaryotics and plants. In contrast to the generally utilized biofuel-producing microbes (E. coli, Z. mobilis, S. cerevisiae and others), cyanobacteria are photosynthetic microbes, which can absorb solar energy and fix carbon dioxide. It is more efficient to convert solar energy and carbon dioxide into biofuels in one biological system than using plants to make polysaccharides (cellulose and hemicellulose) from solar energy and carbon dioxide through photosynthesis, and microbes to make biofuels from glucose produced from the polysaccharides through fermentation (Angermayr et al., 2009).

Compared to general photosynthetic plants and eukaryotic microalgae, cyanobacteria are more amenable to genetic manipulation for installing biofuel-producing chemical pathways. In fact, the genetic engineering platform for cyanobacteria is well established and cyanobacteria have been shown to be highly tolerant to the introduction of foreign genes. The genomes of over thirty-five cyanobacteria species are known, the first being the genome of Synechocystis sp. PCC6803, which was determined in 1996 (Kaneko et al., 1996). Consequently, the genetics and metabolic regulation mechanisms of cyanobacteria are well understood. This information should facilitate the genetic engineering of cyanobacteria for biofuel production.

To date, some progress has been made in the direction of genetic engineering cyanobacteria for biofuel synthesis. In 1999, Deng et al. reported that ethanol production in genetically engineered Synechococcus elongates PCC7942 occurs in a yield of 54 nmol-OD_{730nm}/unit-liter-day. Also, the production of ethylene in Synechococcus sp. PCC 7942 has been described (Sakai et al., 1997). Recent work has focussed to the development of different types of biofuels using cyanobacteria as model microbial system. Dexter et al. have shown that bioethanol can be produced in Synechocystis sp. PCC6803 with the yield of 5.2 mmol-OD_{730nm}/unit-liter-day (2009).
Lindberg et al. has described isoprene biosynthesis in genetically engineered *Synechocystis* sp. PCC6803 (2010) and Atsumi et al. have reported on their work on isobutyaldehyde production by genetically engineered *Synechococcus elongates* PCC7942 (2009). Finally, Angermayr et al. have outlined a promising approach to biofuel production based on the redirection of cyanobacterial intermediary metabolism (2009).

Direct conversion of carbon dioxide to biofuels in photosynthetic cyanobacteria can significantly improve the efficiency of biofuel production. A theoretical calculation shows that the productivity of ethanol in a photosynthetic organism can reach *ca.* 5,280 gal/acre/year (Angermayr et al., 2009). Algenol Biofuels Inc. has developed an innovative cyanobacteria-based technology that is reported to produce ethanol at a rate of 6,000 gal/acre/year. In contrast, the annual yield of ethanol from corn is 321 gal/acre/year, from sugar cane 727 gal/acre/year (Brazil Institute Special Report, 2007), from switchgrass 330-810 gal/acre/year, and from corn stover 290-580 gal/acre/year (Sanderson, 2006). Clearly, ethanol production from cyanobacteria is significantly more efficient than is ethanol production from plant feedstocks. Moreover, the current high cost of plant biomass processing provides a strong economic incentive to switch to a cyanobacteria-based platform for ethanol production. Biofuel produced in genetically engineered cyanobacteria is not limited to ethanol. Indeed, as discussed in the following sections, cyanobacteria possess great potential for application in the production of alternative biofuels.

### 3. Biofuel molecules

Bioethanol and biodiesel (fatty acid methyl esters, or ethyl esters) are the dominate forms of biofuels currently used to address the prevailing challenges imposed by high energy demand and global warming. There is, however much room for improvement through the production of superior forms of biofuels. Ethanol, for example, is far from the ideal biofuel for several reasons. Specifically, it has a low energy density, is volatile and difficult to transport via carrier pipelines owing to its corrosive properties. Moreover, ethanol is toxic to microbes and its high solubility in the aqueous fermentation culture broth results in the exposure of the microbes to toxic levels (Somerville et al., 2007).
The exploration and development of novel biofuels, which possess high energy density, are hydrophobic and are compatible with the existing liquid fuel infrastructure (i.e., fuel engines, refinery equipment and transportation pipelines) is in great demand. In terms of fuel properties, the best replacement of petroleum fuels is “Petroleum Fuels”. In other words, ideal biofuels produced from biological systems should be chemically similar to petroleum, like fatty acid-based molecules including fatty acid esters, fatty alcohols and fatty alkanes.

Fortman et al. (2008) reviewed biofuel alternatives to ethanol, with special focus on the development of biofuels based on genetic engineering of the fatty acid and the isoprenoid pathways. A great deal of attention has been devoted to the production of butanol as a biofuel. Key advances, achieved by advanced in situ separation technology (Ezeji et al., 2007), have been made in improving acetone-butanol-ethanol (ABE) fermentation, especially with regard to reduced butanol toxicity to fermentation microbes. Higher alcohols such as C5-C8 with straight or branched chains have also been explored. Recent studies by James Liao and co-workers have shown that C4-C8 alcohols can be produced in E. coli by modifying amino acid metabolic pathways by using protein engineering and metabolic engineering and by building non-native biosynthetic pathways (Zhang et al., 2008). James Dumesic and his co-workers reported the successful conversion of cellulose-derived sugars to liquid alkanes using chemical catalysis (Huber et al., 2005).

4. Fatty acid-based biofuels in cyanobacteria

It can be concluded from above discussion that a promising strategy to develop sustainable biofuels would be to synthesize fatty acid-based compounds utilizing solar energy as the energy source, carbon dioxide as the carbon source and cyanobacteria as the biological system. The biosynthesis of fatty acid-based biofuels in cyanobacteria includes two steps, overproduction of fatty acids and transesterification of fatty acids to form fatty acid ethyl esters or reduction of fatty acids to form fatty alcohols or fatty alkanes (Fig. 2).

Fatty acid metabolism has been extensively studied. Acetyl-CoA carboxylase catalyzes the first step of the fatty acid biosynthetic pathway, involving formation of malonyl-CoA from acetyl-CoA and bicarbonate. Fatty acyl-ACPs (acyl carrier proteins) are synthesized from malonyl-CoA by a multi-subunit fatty acid synthase. The fatty acyl
moiety is eventually incorporated into phospholipids for cell membrane synthesis. The fatty acid yield ultimately depends on regulation (Magnuson et al., 1993). Feedback inhibition by fatty acyl-ACPs is considered as the main form of regulation of fatty acid biosynthesis. Feedback inhibition might be targeting the proposed rate-limiting step of fatty acid biosynthesis catalyzed by acetyl-CoA carboxylase (Davis et al., 2000), the final step of fatty acid biosynthesis catalyzed by enoyl-ACP reductase (Heath et al., 1996), or the key step catalyzed by β-ketoacyl-ACP synthase III (Heath et al., 1996). Introduction of a thioesterase can effectively release this feedback inhibition through the hydrolysis of fatty acyl-ACPs (Davis et al., 2000) (Magnuson et al., 1993).

Free fatty acids can be overproduced to reach a titer of ca. 2.5 g l\(^{-1}\) d\(^{-1}\) using fed-batch fermentation of a genetically engineered \textit{E. coli} strain. This strain over-expresses acetyl-CoA carboxylase and thioesterase, and lacks the fadD gene which encodes fatty acyl-CoA synthase (Lu et al., 2008). The synthase catalyzes the first step of fatty acid biodegradation. The results of recent work from the same group have demonstrated an improved production of fatty acids to a level of 4.5 g l\(^{-1}\) d\(^{-1}\) (Liu et al., 2010). The concentrations of free fatty acids in the culture media were significantly increased to 6.4 nmol ml\(^{-1}\) OD\(_{750}\)\(^{-1}\) for \textit{Synechocystis} sp. PCC6803 and 8.4 nmol ml\(^{-1}\) OD\(_{750}\)\(^{-1}\) for \textit{Synechococcus elongatus} PCC7942 by fatty acyl-CoA synthase gene knock-out. The amount of free fatty acids that can be detected in the culture media of wild-type cyanobacteria strains is minimal (Kaczmarzyk et al., 2010). Very recent work published in PNAS shows a successful strategy to overproduce and secret fatty acids in genetically engineered \textit{Synechocystis} sp. PCC6803 with manipulation of fatty acid metabolic pathway and deletion of S layer proteins being a protective barrier for cyanobacteria cells and the production efficiency can be up to 133 ±12 mg/L per day at a cell density of 0.23 g of dry weight per liter (Liu et al., 2010).

Control of the length of the fatty acyl chain can in principle be achieved by introducing chain-length specific acyl-ACP thioesterases. Yuan et al.’s (1995) work has demonstrated the feasibility of modifying the substrate specificity of an acyl-ACP thioesterase by protein engineering (1995). Control of the carbon chain length of the final fatty alkane product provides a mechanism to generate a biofuel having specified properties. For example, fatty alkanes with 10-14 carbons could be used as components
of renewable jet fuels and fatty alkanes with 14-18 carbons could be employed as components of renewable diesel fuels.

Recently, the biosynthesis of a fatty acid ethyl ester (FAEE) was demonstrated in genetically engineered \textit{E. coli}. Fatty acyl-CoA was transesterified with ethanol, produced by pyruvate decarboxylase (pdc) and alcohol dehydrogenase (adh), via acyltransferase catalysis. Kalscheuer and co-workers have shown that production of FAEE in \textit{E. coli} can be achieved at 1.28 g l$^{-1}$ by overexpressing pdc and adh from \textit{Zymomonas mobilis} and acyltransferase from \textit{Acinetobacter baylyi} strain ADP1 while adding exogenous oleic acid under fed-batch fermentation conditions (Kalscheuer et al., 2006). Steen et al. reported de novo biosynthesis of FAEE in \textit{E. coli} by using the same ethanol-producing pathway and acyltransferase to couple with an engineered fatty acid overproducing pathway. Fatty acid over production was achieved by overexpressing a modified \textit{E. coli tesA} gene encoding a leaderless version of the thioesterase and and \textit{E. coli} and \textit{Saccharomyces cerevisiae fadD} genes each encoding a fatty acyl-CoA ligase, and by knocking out the \textit{fadE} gene which encodes acyl-CoA dehydrogenase. The yield of de novo synthesized FAEE is 674 mg/l (Steen, 2010). Equally important, these investigators demonstrated the feasibility of in vivo production of FAEE from hemicellulose achieved via genetic engineering for production of the endoxylanase catalytic domain from \textit{Clostridium stercorarium} and the xylanase from \textit{Bacteroides ovatus} (Steen et al., 2010). The same strategy can be applied in cyanobacteria for de novo synthesis of FAEE via photosynthetic conversion of carbon dioxide. Recently, my co-workers and I have successfully employed genetic engineering to construct a FAEE biosynthetic pathway in \textit{Synechocystis} sp. PCC6803 consisting of the enzymes of the ethanol-producing pathway and the acyltransferase (unpublished data).

Reduction of the fatty acyl-CoA to the corresponding fatty alcohol is catalyzed by the fatty acyl-CoA reductase (Far). A variety of fatty acyl-CoA reductases derived from a wide range of organisms (e.g. jojoba (Metz, 2000), \textit{Arabidopsis thaliana} (Doan, 2009), mouse (Cheng, 2004)) have been characterized, and some of these have been shown to be effective in the production of fatty alcohols in genetically engineered \textit{E. coli}. Steen and coworkers combined the engineered fatty acid overproduction pathway with the Far gene derived from \textit{Acinetobacter calcoaceticus} BD413 to produce an \textit{E. coli} strain that produces fatty alcohol in a yield of 60 mg/l (Steen, 2010). We have recently found that
fatty alcohol can be produced by *Synechocystis* sp. PCC6803 engineered to contain the Far genes derived from jojoba and mouse (unpublished data).

Enzymatic conversion of fatty acid to fatty alkane can be accomplished in a two step reaction sequence, which consists of reduction of the fatty acid to the fatty aldehyde followed by conversion to the fatty alkane. Bioluminescent bacteria, such as *Vibrio harveyi* and *Photobacterium phosphoreum*, have been shown to produce fatty aldehydes using the protein products of the Lux operon (Meighen et al., 1998). Reister and coworkers (1997) reported that the *acr1* gene from *Acinetobacter calcoaceticus* encodes a reductase that catalyzes the conversion fatty acyl-CoA to fatty aldehyde. Cytochrome P450 of the housefly *Musca Domestica* is thought to catalyze the conversion of fatty aldehydes to fatty alkanes with the production of carbon dioxide (Reed et al. 1995). Furthermore, fatty aldehyde decarbonylases from *Pisum sativum* (Schneider-Belhaddad et al., 2000) and *Botryococcus braunii* (Dennis et al., 1992) have been characterized and a gene (CER1) believed to encode a fatty aldehyde decarbonylase in *Arabidopsis*, has been discovered (Aarts et al. 1995).

The demonstrated success in the overproduction of fatty acids, *de novo* biosynthesis of FAEE and fatty alcohols in genetically engineered *E. coli*, the recently published work of increasing production and secretion of fatty acids in genetically engineered cyanobacteria, and the preliminary experimental results from studies directed at producing FAEE and fatty alcohol in genetically engineered cyanobacteria are evidence that efficient photosynthetic production of fatty acid-based biofuels in cyanobacteria can be achieved through genetic engineering. The biosynthesis of fatty acid-based biofuels in cyanobacteria is expected to accelerate the application of biofuels because (1) the use of solar energy avoids the technical bottlenecks associated with biomass supply and deconstruction, (2) biofuels are directly made in a single biological system which eliminates the cost of downstream refining, and (3) fatty acid esters, long chain fatty alcohols and fatty alkanes chemically similar to fossil fuels and are thus are more compatible with current engine and transportation systems.

5. Looking forward

In order to successfully develop cyanobacteria as a platform for the production of fatty acid-based biofuels several hurdles must be overcome. Firstly, through gene
manipulation a high-efficiency expression system must be incorporated. Secondly, through enzyme and metabolic engineering metabolic flux must be redirected to feed the fatty acyl pool. Thirdly, using a synthetic biology based approach, the fatty acid converting pathway leading to fatty acid esters, long chain fatty alcohols and fatty alkanes must be constructed.

In this perspective, I have pointed out the advantages that fatty acid based biofuel production in cyanobacteria might offer and have explored the possible strategies that might be used in developing such systems. As the biotechnology moves forward, genetic engineering to increase photosynthetic efficiency of the cyanobacteria and to adapt these organisms to the unnatural environment imposed by large scale photo-bioreactors, will no doubt take central stage.

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Figure 1 Routes to biofuels, where traditional biofuels were labelled by blue lines, currently hot biofuels by green lines, and novel biofuels in cyanobacteria by red line.

Figure 2 Proposed biosynthetic pathways for production of fatty acid-based biofuels including fatty acid esters, fatty alcohols and fatty alkanes directly from solar energy and carbon dioxide in cyanobacteria.
Figure 1
Figure 2