Metabolic engineering of microorganisms for biofuels production: from bugs to synthetic biology to fuels

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ABSTRACT

The ability to generate microorganisms that can produce biofuels similar to petroleum-based transportation fuels would allow the use of existing engines and infrastructure and would save an enormous amount of capital required for replacing the current infrastructure to accommodate biofuels that have properties significantly different from petroleum-based fuels. Several groups have demonstrated the feasibility of manipulating microbes to produce molecules similar to petroleum-derived products, albeit at relatively low productivity (e.g. maximum butanol production is around 20 g/L). For cost-effective production of biofuels, the fuel-producing hosts and pathways must be engineered and optimized. Advances in metabolic engineering and synthetic biology will provide new tools for metabolic engineers to better understand how to rewire the cell in order to create the desired phenotypes for the production of economically viable biofuels.

INTRODUCTION

Alternative transportation fuels are in high demand owing to concerns about climate change, the global petroleum supply, and energy security [1,2]. Currently, the most widely used biofuels are ethanol generated from starch (corn) or sugar cane and biodiesel produced from vegetable oil or animal fats [3]. However, ethanol is not an ideal fuel molecule in that it is not compatible with the existing fuel infrastructure for distribution and storage owing to its corrosivity and high hygroscopicity [1,4]. Also, it contains only about 70% of the energy content of gasoline. Biodiesel has similar problems (URL: http:// www.bdpedia.com/biodiesel/all/alt.html): it cannot be transported in pipelines because its cloud and pour points are higher than those for petroleum diesel (petrodiesel), and its energy content is approximately 11% lower than that of petrodiesel. Furthermore, both ethanol and bio-diesel are currently produced from limited agricultural resources, even though there is a large, untapped resource of plant biomass (lignocellulose) that could be utilized as a renewable source of carbon-neutral, liquid fuels [5].

Microbial production of transportation fuels from renew-able lignocellulose has several advantages. First, the production is not reliant on agricultural resources commonly used for food, such as corn, sugar cane, soybean, and palm oil. Second, lignocellulose is the most abundant biopolymer on earth. Third, new biosynthetic pathways can be engineered to produce fossil-fuel replacements, including short-chain, branched-chain, and cyclic alcohols, alkanes, alkenes, esters and aromatics. The development of cost-effective and energy-efficient processes to convert lignocellulose into fuels is hampered by significant roadblocks, including the lack of genetic engineering tools for native producer organisms (non-model organ-isms), and difficulties in optimizing metabolic pathways and balancing the redox state in the engineered microbes [6]. Furthermore, production potentials are limited by the low activity of pathway enzymes and the inhibitory effect of fuels and byproducts from the upstream biomass processing steps on microorganisms responsible for producing fuels. Recent advances in synthetic biology and metabolic engineering will make it possible to overcome these hurdles and engineer microorganisms for the cost-effective production of biofuels from cellulosic biomass. In this review, we examine the range of choices available as potential biofuel candidates and production hosts, review the recent methods used to produce biofuels, and discuss how tools from the fields of metabolic engineering and synthetic biology can be applied to produce transportation fuels using genetically engineered micro-organisms.

Liquid fuels and alternative biofuel molecules

An understanding of what makes a good fuel is important in order to retool microorganisms to produce more useful alternative biofuels. The best fuel targets for the near term will be molecules that are already found in or similar to components of fossil-based fuel in order to be compatible with existing engines (spark ignition engine for gasoline, compression ignition engine for diesel fuel, and gas turbine for jet fuel). There are several relevant factors to consider when designing biofuel candidates (Table 1). Energy contents, the combustion quality described by octane or cetane number, volatility, freezing point,

odor, and toxicity are important factors to consider. In the following section, we will consider several biofuel candidates and their properties.

Table 1

Types of liquid fuels.

Fuel type	Major components	Important property	Biosynthetic alternatives
Gasoline	C_4–C_{12} hydrocarbons	Octane number[a]	Ethanol, n-butanol and iso-butanol
	Linear, branched, cyclic, aromatics	Energy content[b]	Short chain alcohols
	Anti-knock additives	Transportability	Short chain alkanes
Diesel	C_9–C_{23} (average C_{16})	Cetane number[c]	Biodiesel (FAMEs)
	Linear, branched, cyclic, aromatic	Low freezing temperature	Fatty alcohols, alkanes
	Anti-freeze additives	Low vapor pressure	Linear or cyclic isoprenoids
Jet fuel	C_8–C_{16} hydrocarbons	Very low freezing temperature	Alkanes
	Linear, branched, cyclic, aromatic	Net heat of combustion	Biodiesel
	Anti-freeze additives	Density	Linear or cyclic isoprenoids

a A measurement of its resistance to knocking. Knocking occurs when the fuel/air mixture spontaneously ignites before it reaches the optimum pressure and temperature for spark ignition.

b The amount of energy produced during combustion. The number of C–H and C–C bonds in a molecule is a good indication of how much energy a particular fuel will produce.

c A measurement of the combustion quality of diesel fuel during compression ignition. A shorter ignition delay, the time period between the start of injection and start of combustion of the fuel is preferred, and the ignition delay is indexed by the cetane number.

Gasoline and its alternatives

Gasoline is a complex mixture of hydrocarbons including linear, branched, and cyclic alkanes (40–60%), aromatics (20–40%), and oxygenates [7]. The carbon number of hydrocarbons in gasoline varies from 4 to 12. Ethanol, the most popular additive to gasoline, has an octane number of 129, but its energy content per gallon is about 70% of that of gasoline. Ethanol also has problems as a fuel owing to high miscibility with water, which makes it difficult to distill from the fermentation broth and to transport through existing pipelines. Recently, n-butanol has received more attention as an alternative gasoline additive. Butanol has two more carbons than ethanol, which results in an energy content of about 40% higher than that of ethanol. The octane number of butanol is 96 [8], which is somewhat lower than that of ethanol but is still comparable to that of gasoline (91–99). Unlike ethanol, butanol is less soluble in water than ethanol. It can also be used not only as an additive to gasoline but also as a fuel by itself in conventional engines (URL: http://www.butanol.com).

In general, the octane number increases when the molecule has methyl branching and double bonds. Branched C_4 and C_5 alcohols are also considered potential gasoline additives. Among them, isobutanol (2-methyl-1-propa-nol) has very similar properties to n-butanol with a higher octane number, and is currently under investigation as a new biofuel target (URL: http://www.gevo.com). Other short chain alcohols, such as isopentanol (3-methyl-1-butanol or isoamyl alcohol) and isopentenol (3-methyl-3-buten-1-ol or 3-methyl-2-buten-1-ol) are also attractive gasoline fuel additives. Their octane numbers range slightly above 90, and they have higher energy contents than butanol. These alcohols can be produced from the isoprenoid biosynthetic pathway [9] or by transformation of amino acids as reported recently [4[2]]. Branched, short-chain alkanes such as isooctane are a good gasoline replacement, but the biological production of these molecules would require significant changes to existing metabolic pathways and may take significant effort to achieve.

Diesel and its alternatives

Diesel fuel is also a complex mixture of hydrocarbons including linear, branched, and cyclic alkanes (75%) and aromatics (25%). The carbon number of hydrocarbons in petrodiesel varies from 9 to 23, with an average of 16 (Table 1). Biodiesel is generally composed of fatty acid methyl esters (FAMEs), and is mostly derived from vegetable oil or animal fat. The fatty acids in FAMEs generally have a chain

length from 12 to 22, containing zero to two double bonds. Biodiesel has a comparable cetane rating and energy content to petrodiesel and additional advantages, such as higher lubricity and less emission of pollutants. Another source for biodiesel would be isoprenoids, which are naturally occurring branched or cyclic hydrocarbons mostly synthesized in plants. They usually have methyl branches, double bonds, and ring structures, which improve the fluidity at lower temperatures but lower cetane ratings. Therefore, linear or cyclic monoterpenes (C_{10}) or sesquiterpenes (C_{15}) are potential targets for biodiesel fuel, especially with complete or partial reduction of double bonds, which would improve the cetane rating.

Biojetfuels

Jet fuels (Jet A, Jet A-1, JP-8, and JP-5) are also a very complex mixture of hydrocarbons with a carbon number distribution of 8–16 and about 25% (v/v) limit of aromatics (Table 1). Jet fuel is very similar to kerosene or diesel fuel, but requires a lower freezing point since it is used under harsh conditions such as extreme cold (URL: http://www.boeing.com/commercial/environ- ment/pdf/alt_fuels.pdf). Linear or branched hydrocarbons with medium carbon chain length produced from the fatty acid or isoprenoid biosynthetic pathways are primary targets for biojetfuels. Recently, the use of isoprenoids as jet fuel has been investigated, as they have low freezing points potentially owing to their branching and cyclic structure (URL:http://business.timesonline.co.uk/tol/business/industry_sectors/natural _resources/article1844558.ece).

Production host

To convert lignocellulosic biomass into economically viable biofuels [5], the production hosts must natively have or be endowed with several characteristics (Figure 1). The user-friendly hosts (Escherichia coli and Saccharomyces cerevisiae) that have well-character-ized genetics and the genetic tools [6,10] for manipulating them are good starting points for development as production platforms. Because these host organisms are also facultative anaerobes with fast growth rates, large-scale production processes can be relatively simple and economically viable [11–13]. The successful use of E. coli or S. cerevisiae to produce alternative biofuels will require a better understanding of their physiology under a variety of conditions and subsequent strain improvements [10]. Continuous advances in 'omics' technologies, computational systems biology, and synthetic biology make it possible to better understand and engineer fuel production hosts with desired phenotypes [6,14].

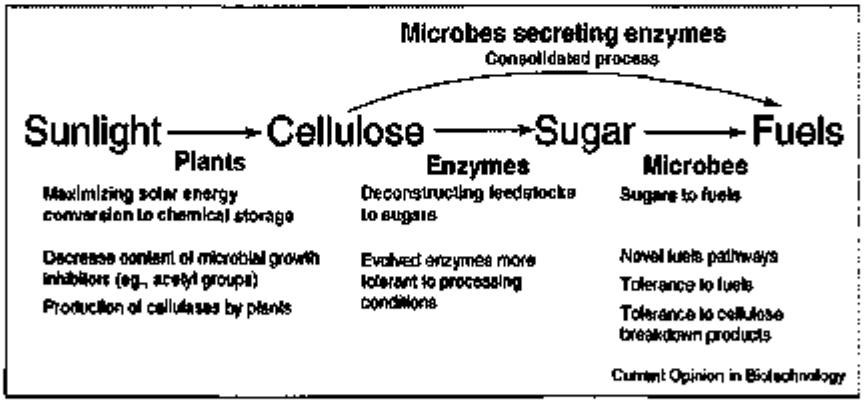

Figure 1
Route from sunlight to fuels. Conversion of biomass to fuels will involve the development of dedicated energy plants that maximize solar energy conversion to chemical storage and minimize the use of water and fertilizer, enzymes that depolymerize cellulose and hemicellulose into useful sugars, and microorganisms that produce advanced biofuels that are compatible with our existing transportation infrastructure. To achieve economically viable biofuel production, all aspects of these processes must be optimized. In particular, production hosts must natively have or be endowed with several important characteristics: extension of the substrate range, elimination of cellulose hydrolysates and biofuel product toxicity, and improvement of global regulatory functions. One method that has been proposed to reduce fuel production cost is to perform cellulose hydrolysis and fermentation in one step, called consolidated bioprocessing (CBP); this alternative approach avoids costs associated with cellulase production [14].

Metabolic engineering of fuels synthesis pathways

The synthesis pathways of some potential biofuels were recently expressed in model organisms (Table 2). For example, the genes involved in the synthesis of isopropanol [16,17] and butanol [18] from

Clostriduim were recently expressed in E. coli. Aside from producing ethanol during fermentation, S. cerevisiae is also known to produce higher alcohols and esters from amino acids [19– 21]. Recently, a similar pathway for higher alcohol production was expressed in E. coli to yield six different straight and branched-chain alcohols, and the same group has demonstrated production of 1.28 g/L of isopentanol by increasing the flux through the desired pathway [22]. Other natural pathways of interest include those for producing fatty acids in order to make biodiesel (fatty acid methyl/ethyl esters) [23] and those for producing alkanes [24]. While biodiesel production from microbial organisms has focused on the fatty acid biosynthesis pathways of E. coli and S. cerevisiae, there are efforts to expand production to explore the fatty acid biosynthesis pathway of other organisms, such as cyanobacteria and algae [25]. Looking beyond natural pathways, hybrid processes that combine both biological and chemical production steps can also lead to new chemicals that could serve as biofuels [27••].

Table 2

Examples of different metabolic engineering strategies for increasing yields of various biofuels.

Biofuel	Strategy	Yield*	Reference
Ethanol	Engineering of phosphoketolase pathway to increase the availability of NAD^+ during xylose metabolism in S. cerevisiae	0.42	[29]
	Modulation of redox metabolism by modifying ammonium assimilation in order to increase xylose utilization by deleting GDH1 and overexpressing GDH2 in S. cerevisiae	0.34	[30]
	In silico gene insertion predicted that heterologous expression of gapN would increase ethanol yield and eliminate glycerol production in S. cerevisiae during growth on glucose and xylose	0.36	[48•]
	EM analysis directed knockout strategy optimized ethanol production from pentose and hexose and removed extraneous pathways in E. coli	0.36	[42]
Butanol	Expression of different gene combinations for butanol production in E. coli modeled after the C. acetobutylicum pathway; deletion of competing pathways; increased NADH availability	.0056	[18]
	Expression of different gene combinations for isobutanol production in E. coli modeled after the amino acid catabolic pathway; deletion of competing pathways; overexpression of valine biosynthetic genes	0.35	[4••]
Pentanol	Expression of different gene combinations for isopentanol production in E. coli modeled after the amino acid catabolic pathway; deletion of competing pathways; overexpression of leucine biosynthetic genes	0.11	[22]
Propanol	Expression of different gene combinations for propanol production in E. coli modeled after the C. beijerinckii pathway	0.14	[17]

* Reported yield [g biofuel/g carbon source].

Constructing biofuel synthesis pathways is only the first hurdle to making biofuels economically viable. The expectations on yield and constraints on cost place many challenges on biofuel production [1,3•,28–30]. Some of the obstacles to achieving high yields are a result of the interdependence of metabolic networks, which are strongly influenced by the global levels of a handful of metabolites: ATP/ADP, NAD^+/NADH, $NADP^+$/ NADPH, and acyl-CoAs. These central metabolites play an important role in regulating multiple pathways in the cell, because the cell uses the relative ratios of these metabolites to regulate a pathway's activity and ultimately the physiology of the cell. For example, the redox state of a cell is essentially determined by the relative ratio of NAD^+ to NADH. The incorporation of new pathways for biofuel synthesis can destabilize the balance of these important metabolites, leading to the production of undesirable byproducts and a decrease in yield. Furthermore, the new metabolic pathway requires amino acids, redox cofactors, and energy for synthesis and function of its enzymes, which places a metabolic burden on the cell that must be minimized to maximize production of the final product.

One way to predict the impact of a new metabolic pathway on growth and product formation is through the use of metabolic models. Over the years, biochemical models of E. coli [31] and S. cerevisiae [32] metabolism have become more sophisticated. As a result, genome-scale models are playing a larger role in directing metabolic engineering efforts with more rational and systematic approaches [33]. Many of the in silico models are stoichiometric models with large solution spaces, because the equations describing the models are usually underdetermined. However, the proper use of constraints has achieved some success in producing models that can link genotype to phenotype [34]. The stoichiometic models can also be described by a set of determined equations using metabolic flux analysis (MFA), where the exchange

fluxes are measured experimentally. MFA has been useful in studying the native metabolism of E. coli under different growth conditions [35] and during recombinant protein production [36]. Many important insights into microbial metabolism have been achieved using metabolic models, such as predicting the phenotypic space of adaptive evolution in E. coli during growth on different carbon sources [37] and the topological organization of the high-flux reactions in E. coli's metabolic network [38•]. It was demonstrated that E. coli adapts to various growth conditions by reorganizing the rates through a set of high-flux reactions. Biofuel production efforts can benefit from genome-scale models, which provide a systematic framework for optimizing flux through the desired pathways, while balancing important cofactor and energy metabolites.

Recently, *in silico* models have played an important role in engineering microorganisms to utilize new substrates to produce biofuels more efficiently. The ability to use a wider array of the biomass feedstocks would help to decrease cost by reducing the number of upstream processing steps and by turning more of the biomass into biofuel. For example, S. cerevisiae has been engineered with the genes encoding xylose reductase (Xyl1p) and xylitol dehydrogenase (Xyl2p) from Pichia stipitis to enable it to utilize xylose, the second most abundant carbohydrate in nature, as a carbon source for ethanol production [39]. However, simply overexpressing the genes led to low growth and fermentation rates due to redox imbalance. Xylose reductase from P. stipitis has a higher affinity for NADPH than NADH, while xylitol dehydrogenase uses only NAD^+. Overexpression of both genes leads to an accumulation of NADH and a shortage of NADPH. Metabolic models suggested that the cofactors could be balanced by deleting $NADP^+$-dependent glutamate dehydrogenase (GDH1) and overexpressing NAD^+-dependent GDH2 to increase the specific activity of Xyl1p for NADH. This strategy led to an increase in ethanol production and a reduction in byproduct syn-thesis [40]. Interestingly, overexpression of the P. stipitis xylose reductase also increased tolerance to lignocellulosic hydrolysate [41].

Elementary mode (EM) analysis was also used to engineer a strain of E. coli that can simultaneously utilize both glucose and xylose as carbon sources to produce ethanol [42]. EM analysis was used to identify a minimum set of metabolic pathways that would support growth and ethanol production on hexoses and pentoses, by removing reactions that supported maximum ethanol and high biomass yields but whose removal led to the lowest number of remaining EMs. Using this strategy, the bur-den from extraneous pathways was reduced in the knockout mutants, so more cellular resources could be directed toward biofuel synthesis. In another example of metabolic engineering, byproduct formation was minimized in E. coli during fermentation by disrupting the tricarboxylic acid cycle in order to reduce oxygen demand for NADH oxidation and eliminating pathways for NADH oxidation other than the electron transport system [43]. Other methods for cofactor balancing include overexpressing proteins to increase NADH [44] or NADPH [45] availability, interconverting NADH and NADPH [46], and changing the coenzyme specificity of specific proteins [47]. In silico genome-scale models have also been extended beyond gene deletion or overexpression strategies to include gene insertion strategies for redox balancing. The in silico gene insertion strategy was used to improve ethanol production and decrease the production of the byproducts glycerol and xylitol. The result was a 58% reduction in glycerol, a 33% reduction in xylitol, and a 24% increase in ethanol production when glyceralde-hyde-3-phosphate dehydrogenase was introduced into S. cerevisiae [48•]. These techniques demonstrate the importance of monitoring and balancing the levels of various important metabolites in order to achieve optimal product titers. Therefore, metabolic engineering will play an important role in engineering efficient microbial pathways for the production of economically sustainable biofuels.

The role of synthetic biology in metabolic engineering for biofuels production

Synthetic biology is an emerging field that aims to bring such engineering principles as modularization and componentization to the manipulation of genetic circuitry in microorganisms, so that engineering an organism for fuel production is as easy as assembling a computer [49••]. Unlike high-value, rare products like pharmaceuticals or enzymes, fuels are commodity materials that are extremely cost sensitive. Biofuels can only be competitive when the production costs are less than or equivalent to drilling and refining petroleum, and thus the viability of biofuels depends absolutely on reducing production costs. In order to achieve this goal, any extraneous cellular processes that reduce production must be systematically controlled. To do this, technologies that enable rapid prototyping, testing, and optimization of pathways and the host organisms are crucial.

Some of the key thrusts of synthetic biology are reducing the time required to make genetic

constructs and increasing their predictability and reliability. The construction of many pathway variations and permutations [50] can be more efficiently handled by better assembly techniques rather than through synthesis of each permutation. For example, techniques such as ligation-free assembly [51,52] and BioBricks [53] are enabling rapid construction of operons and pathways from existing DNA fragments or genes. The BioBrick method, in particular, has the interesting property that the end result of assembly has the same restriction sites as at the start of the assembly—that is, each stage of assembly uses the enzymes and processes identical to the previous stage. This repetitive and cyclic nature of the method is ripe for automation, facilitating the creation of large permutation libraries quickly. Another thrust of synthetic biology is the creation of reusable parts that have useful and predictable behavior. The development of a diverse array of regulatable expression systems [54,55] that can be used to fine-tune expression is very useful in engineering metabolic pathways. Also, switches that sense and respond to environmental changes [56,57] and can subsequently initiate down-stream regulation have exciting opportunities for biofuels production. For example, it may be possible to design bacteria that switch from a cellulose digestion mode to fuel production mode by sensing the surrounding environment. Such advanced signal processes and decision-making capabilities have already been demonstrated in a remarkable way – for example, the tumor sensing bacteria that can sense and selectively invade anaerobic environments in a tumor [58] – and adopting these technologies for metabolic engineering may have fruitful results. Furthermore, the development of intra-cellular and intercellular signal processing abilities in microbes [59] as well as fine control over pathway expression are opening up the potential for coordinated gene expression and regulation for optimum production.

One of the more difficult challenges of metabolic engineering is the integration of several parts or enzymatic pathway fragments into a functioning device ('functional composition' in synthetic biology parlance). For example, Ro et al. [60] were able to take the already optimized mevalonate pathway [61] and extend it to produce artemisinic acid. As the number of biologically produced molecules increases, the need to reuse and mix enzymatic pathways or subpathways will increase as well [62]. Creation of modular subpathways that can be easily interconnected is an area of active research with much potential. To achieve this goal, a strong framework for characterization and standardization is being sought. Recent efforts in standardizing measurements [63] are encouraging, but further refinements of the methods by which these parts are tested and characterized as well as industry-wide agreement on those methods is necessary. Still, the development of synthetic gene regulation networks from scratch [64] and methods for analyzing them in silico [65] is extremely encouraging. Looking forward, the development of a 'chassis' organism for synthetic biology is a difficult and ongoing endeavor. For metabolic engineers, transferring a working pathway from one organism to another is a difficult but sometimes necessary maneuver for greater product yields. Synthetic biologists are looking to either modify existing organisms or create from scratch microorganisms with minimal genomes and therefore with a minimal set of metabolic pathways [66]. The hope is that such an organism will lead to more predictable behaviors when a foreign gene or a pathway is introduced, as any potential side pathways are known and/or controllable. In the similar vein, in vivo mutagenesis and screening techniques hold promise in whole genome engineering.

The prospect of assembling new metabolic pathways by simply putting together sub pathways into a well-defined chassis is very exciting. The rapid recent advances in synthetic biology techniques have brought new tools for assembly and control that move the entire field closer to this goal. Many of the techniques in synthetic biology are readily adaptable to metabolic engineering of microorganisms to fuels production, and already strong relationships are being forged between metabolic engineering and synthetic biology com-munities. Quick adoption of the ever-expanding sets of tools in synthetic biology will be enormously beneficial in tackling challenging metabolic engineering projects, such as biofuels production.

CONCLUSION

Recent increases in energy and fuels costs have resulted in increased attention to finding alternatives to fossil fuels. However, unlike the previous efforts to develop biofuels in the 1970s and early 1980s, we are armed with better tools to manipulate cellular metabolism in order to produce these fuels. Even more, with the advanced tools of synthetic biology, it is possible to produce biofuel candidates that are not naturally produced, enabling us to use our existing transportation infrastructure rather than replace it in order to use 'natural' biofuels like ethanol. In any case, metabolic engineering and synthetic biology will be central actors in the biofuels revolution, and the molecules and the techniques covered in this article will surely play important roles in its evolution.

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