

Microbes and biofuel production

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The production of industrially important materials, via an entirely new branch of microbial biotechnology, called "white biotechnology", is currently an active field of research. The gradual exhaustion of fossil-energy resources, increasing oil price and global warming, caused by the lavish use of fossil fuels, have ignited widespread interest in utilizing biomass as an alternative and ecofriendly resource for producing several biofuel. These include bioethanol, biobutanol, alkane and especially biodiesel. Biodiesel, also called microdiesel, are non-toxic, fatty acid methyl/ethyl esters, fitting in easily into the existing infrastructure, having good lubrication properties and low flammability, and are thus non-hazardous and completely biodegradable. Oleaginous microbes, known to produce and accumulate lipids, fatty acids and triacylglycerols within their cell mass, are potent candidates for production. Such microbes include several genera of bacteria, algae, and fungi. Biodiesel production may follow both indirect and direct approaches. Metabolic-engineering, coupled with modern-day synthetic biology, and "omics" technologies, culminating in systems biology, comprises altogether a holistic approach in optimizing biofuel production, so as to complement the gradually-depleting fossil fuels. However, utilizing microorganisms is often challenging, as the production targets are sometimes toxic to the cells. Hence, engineering the strains, for not only improved biofuel overproduction, but also for tolerance, remains the ultimate goal in recent times.

Key words: Alcohol, alkyl esters, biofuel, cytotoxicity-tolerance, metabolic- engineering, oleaginous-microbes, transesterification

INTRODUCTION

The volatility of oil prices and depleting reserves, along with the major environmental concerns about climate changes, caused by the excess use of fossil fuel, have sparked renewed interest in utilizing biomass as a sustainable resource for biofuel production (Dellomonaco *et al.*, 2010). The increased use of fossil fuel has caused greenhouse gas (GHG) emissions that have already exceeded the "dangerously high" threshold of 450 ppm of CO₂ and created undesirable damages to the environment (Schenk *et al.*, 2008). Against a backdrop of current instability of oil supplies, continuously rising prices of crude oil, depletion of resources and increasing political instability in oil-producing countries, only the biomass has the potential to replace the supply of an energy-hungry civilization. The use of bio-based

alcohols as solvents or fuel is, therefore, under consideration. A new branch of biotechnology, referred to as "white biotechnology", embraces the bioproduction of fuel and other industrially significant chemicals from renewable sources (Schepens, 2003). These technologies use living cells and enzymes to synthesize products that are easily biodegradable, require less energy and create less waste during their production. Bioethanol and biodiesel are already commercialized as alternative fuel in the market worldwide. Bio-based production of butanol and alkane is also being pursued around the world. In the year 2008, fossil fuel accounted for 88% of the global primary energy consumption. The US "Energy Independence and Security Act" of 2007, has set a goal to produce 36 billion gallons of renewable fuel by 2022, with 16 billion to be obtained from cellulosic ethanol (http://energy.senate.gov/public/_files/RL342941.pdf). The US Department of Energy has established a target of supplanting 30%

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of gasoline consumption with cellulosic ethanol by 2030 (Herrera, 2006).

DIFFERENT FORMS OF BIOFUEL

Biofuel refer to the renewable fuel derived from biological sources, viz., microbes and plants. They can be utilized as transportation fuel, with little change to current technologies, reducing long-term GHG emissions (Carere *et al.*, 2008). Gasoline is a complex mixture of hydrocarbons, including linear, branched and cyclic alkanes (40–60%), aromatics (20–40%), and oxygenates (Peralta-Yahya and Keasling, 2010). The popular additives to gasoline are ethanol, n-butanol, branched C₄ and C₅ alcohols, isobutanol and other short-chain alcohols, such as isopentanol and isopentenol. Ethanol, produced from starch, is the current biofuel standard and remains the most produced biofuel in the US, with nine billion gallons produced in 2008 (<http://www.ethanolrfa.org/industry/outlook>). However, ethanol suffers from low-energy density. Its hygroscopicity and corrosivity makes it incompatible with the existing fuel storage and distribution infrastructure (Peralta-Yahya and Keasling, 2010). It cannot be piped without picking up excess water and impurities. It is costly to distil. The construction of novel infrastructure for an ethanol economy would cost hundreds of billions of dollars. Despite its high octane number, ethanol contains only 70% of the energy content of gasoline (Peralta-Yahya and Keasling, 2010). Ideally, biofuel should require minimal energy to separate from fermentation broths, be non-toxic to the host microorganism, and be efficiently produced from a variety of feedstocks. Moreover, they should have high-energy content and be compatible with the storage and transportation infrastructures, designed for petroleum-based products (Peralta-Yahya and Keasling, 2010). Compared to ethanol, n-butanol is more hydrophobic and less corrosive, has a higher energy density, has lesser predisposition for water contamination, can be suitably distributed through existing pipeline infrastructure, and can be mixed with gasoline at any ratio (<http://www.butanol.com>). Thus, n-butanol is a substantially better biofuel than ethanol (Lee *et al.*, 2008). The next-generation biofuel, also referred to as biodiesel, such as long-chain alcohols and fatty-acid-derived or isoprenoid-derived fuels, have physical properties that more closely resemble

petroleum-derived fuels. Hence, they offer promise as advanced biofuel, synthesized by microbes (Lee *et al.*, 2008). Trans-esterified fatty acids, such as fatty acid methyl ester (FAME) and fatty acid ethyl ester (FAEE), provide carbon chain-lengths compatible with compression ignition engines (Shi *et al.*, 2011). Biodiesel is better than petro-diesel in several characteristics, such as environmental friendliness, renewability, reduced emission, higher combustion efficiency, improved lubricity, higher safety, etc (Lee *et al.*, 2008). The linear or cyclic monoterpenes (C₁₀) or sesquiterpenes (C₁₅) are potential targets for biodiesel, especially with complete or partial reduction of double bonds, which would improve the cetane rating (Lee *et al.*, 2008). Long-chain alkanes are also valuable molecules for diesel and jet fuel. Jet fuels (Jet A, Jet A-1, JP-8, and JP-5) are a very complex mixture of hydrocarbons, with a carbon number distribution of 8–16, and about 25% limit of aromatics. Jet fuel is very similar to kerosene or diesel, but requires a lower freezing point. Hence, it is used under harsh conditions, such as extreme cold (http://www.boeing.com/commercial/environment/pdf/alt_fuels.pdf).

Advantages of biodiesel

(1) It helps to reduce CO₂ and other pollutant emission from engines, (2) Engine modification is not needed as it has similar properties to diesel fuel, (3) It comes from renewable sources, (4) Diesel engine performs better on biodiesel due to a high cetane number, (5) High purity of biodiesel would eliminate the use of lubricant, (6) Biodiesel production is more efficient as compared to fossil fuel, as there will be no underwater plantation, drilling and refinery, and (7) Biodiesel would make an area become independent of its need for energy, as it can be produced locally (Juan *et al.*, 2011).

BIOFUEL FROM MICROBIAL BIOMASS

Microbial factories, for the synthesis of biofuel and amenable to industrial applications, are being constructed by assembling natural and *de novo* pathways that redirect carbon to the desired products. The gene expression is modulated to fine-tune microbial metabolism for optimal production,

and the proteins engineered to acquire new catalytic activities, or to improve native properties.

Substrates used by microbes

Many biomass feedstocks can be used for the microbial production of biofuel. These include agricultural lignocellulosic residues, edible and non-edible crops, and waste streams (e.g. bagasse from sugar manufacture, industrial by-products). Starch (i.e., corn, wheat, barley, etc.) and sugar crops (i.e., cane, beet, etc.) are the primary feedstocks, currently used for bioconversion to ethanol. The triacylglycerols (TAGs), extracted from oilseed crops, are chemically esterified to biodiesel (Dellomonaco *et al.*, 2010). Microbes have evolved a plethora of enzymatic strategies for hydrolyzing xylan into its constituent sugars, for subsequent fermentation to biofuel (Dodd and Cann, 2009).

Methods used

Two main approaches for biodiesel production involve (i) indirect method, where the oil is extracted from fast-growing microbes followed by *in-vitro* catalytic trans-esterification with short-chain alcohols, also called alcoholysis, and (ii) direct method, performed from redesigned microbial cell factories. The latter method requires the synthesis of biofuel, through the development of metabolically-engineered microbes, chiefly *Escherichia coli* and *Saccharomyces cerevisiae* (Peralta-Yahya and Keasling, 2010), with emphasis on the utilization of a variety of cheap and widely-available substrates, like carbohydrates (glucose), carbohydrate mixtures, lignocellulosic mass, xylan and non-carbohydrate carbon sources. Such genetically-engineered cells can condense ethanol with fatty acids. The heterologous expression of a pyrophosphatase from *Bacillus subtilis*, or pyruvate decarboxylase and alcohol dehydrogenase genes from *Zymomonas mobilis*, in *E. coli*, enabled the production of several biofuel (Antoni *et al.*, 2007).

Microbes involved in biofuel production

Bacteria – Microdiesel is the potential future fuel produced by bacteria. The various species of *Clostridium* (e.g., *C. acetobutylicum*) produce

isopropanol and 1-butanol from acetyl-CoA. *Z. mobilis*, another ethanologenic bacterium, is able to produce high titers of ethanol from glucose and sucrose, but not pentoses (Dellomonaco *et al.*, 2010). A recent study described the efficient degradation of lignocellulosic plant material by the bacterium *Anaerocellum thermophilum* (also called *Caldicellulosiruptor bescii*). An unspecific acyltransferase, encoded by the *atfA* gene in *Acinetobacter baylyi* can esterify ethanol with the acyl moieties of CoA thioesters of fatty acids (Kalscheuer *et al.*, 2006). In addition, *Arthrobacter*, *Gordonia*, *Rhodococcus* etc. are also potential producers.

Algae - Oleaginous microalgae produce substantial amounts of TAGs or oil, which can be up to 80% of dry biomass; these oils being mostly rich in polyunsaturated fatty acids, with four or more double bonds. Algae offer many advantages in the pursuit of sustainable bioenergy source (Hannon *et al.*, 2010). They are photosynthetic organisms, known for their rapid growth (some strains capable of doubling their mass several times per day) and high content of energy-rich oil. For example, some *Botryococcus* spp. have been identified that have up to 50% of their dry mass, stored as long-chain hydrocarbons. Other desirable features include their continuous production, simple cell-division cycle, acquisition of organic compounds through photosynthesis, tolerance to varying environmental conditions (e.g., arid climate), use of waste, brackish or marine water, use of land not used for traditional agriculture (minimized land use, compared to terrestrial plants) and, when subjected to physical and chemical stress, they can be induced to produce high concentrations of specific compounds. They are efficient at removing nutrients from water, so that waste streams can be remediated. In addition, the application of microalgae for biofuel helps reduce CO₂, the main GHG (Hannon *et al.*, 2010). The groups most often considered as fuel production strains are diatoms, green algae, golden brown algae, prymnesiophytes, eustigmatophytes and cyanobacteria. Algae production strains also have the potential to be bio-engineered, allowing improvement of specific traits. The different genera involved are *Spirulina*, *Nannochloropsis*, *Neochloris*, *Dunaliella*, *Botryococcus*, *Chlorella*, *Chlamydomonas reinhardtii*, *Scenedesmus*, *Mono-*

dus, *Nitzschia*, *Phaeodactylum*, *Parietochloris*, *Schizochytrium* etc (Gouveia and Oliveira, 2009).

Fungi - The various genera involved are *Aspergillus*, *Cunninghamella*, *Humicola*, *Mortierella*, *Mucor*, *Candida*, *Cryptococcus*, *Lipomyces*, *Rhodosporidium* etc. Comparative genomics of two xylose-fermenting fungi, *Spathaspora passalidarum* and *Candida tenuis*, for enhanced biofuel production, was reported earlier (Wohlbach *et al.*, 2011). *S. cerevisiae* provides a model system for expressing heterologous biosynthetic pathways, which lead to the various forms of biofuel, as end-products (Peralta-Yahya and Keasling, 2010).

OMICS AND METABOLIC ENGINEERING

In order to establish biorefinery systems to develop economical process for biofuel production, the metabolic pathways of biofuel producers need to be optimally redesigned to achieve high performance. In present times, the "omics" technologies have been developed to analyze and model systems, as also address complex questions about the functioning of native and synthetic networks in microbial cells (Ideker *et al.*, 2001). The recent advances in synthetic biology provide new tools for metabolic-engineers to direct their strategies, and construct optimal biocatalysts for the sustainable production of biofuel (Keasling and Chou, 2008; Lee *et al.*, 2008; Mukhopadhyay *et al.*, 2008). Metabolic-engineering and synthetic biology efforts, entailing the engineering of native and *de novo* pathways, for the conversion of biomass constituents to short-chain alcohols and advanced biofuels, have gained popularity now-a-days (Dellomonaco *et al.*, 2010). This, in turn, has also paved the way to the engineering of efficient synthetic pathways for biofuel production. *E. coli*, the workhorse of modern biotechnology, has become a promising host organism in this connection. For example, the genes, involved in the synthesis of isopropanol and butanol from *Clostridium*, are overexpressed in *E. coli*. Another example is the expression of different gene combinations for butanol production in *E. coli*, modeled after the *Clostridium acetobutylicum* pathway (Hanai *et al.*, 2007; Atsumi *et al.*, 2008). In the foreseeable future, formal integration of functional genomics and systems biology tools (transcriptomics, proteomics,

metabolomics, and fluxomics) with synthetic biology and metabolic-engineering, will undoubtedly support the discovery, characterization, and engineering of new metabolic routes, more efficient microbial systems and new mutants for biofuel production (Rodriguez-Moya and Gonzalez, 2009). New sequencing technologies, enabling quick identification and analysis of genomic variations, such as single nucleotide polymorphisms (SNPs), copy number variations (CNVs), translocations, insertions and deletions (MacLean *et al.*, 2009), are being instrumental to understand complex microbial environments, unravel the diversity, and characterize the genetic makeup of various species of microorganisms, that could hold promise for generating biofuel (Ferrer *et al.*, 2009). Not only that, *in silico* models recently have helped in engineering microorganisms to utilize new substrates for more efficient biofuel formation. The ability to use a wider array of the substrates or 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 and xylitol dehydrogenase from *Pichia stipitis*, to enable it to utilize xylose (the second most abundant carbohydrate in nature), as a carbon source for ethanol production (Chu and Lee, 2007).

MITIGATION OF BIOFUEL CYTOTOXICITY

Since many biofuels are known to reduce cell viability through damage to the cell membrane and interference with essential physiological processes, the cells must trade off biofuel production and survival (Dunlop, 2011). The various forms of cytotoxicity include increase in permeability or fluidity of the membrane, allowing the release of ATP, ions, phospholipids, RNA, and proteins, diminution in energy transduction, and interference with membrane protein function or nutrient transport. Other direct effects include reduced ATP levels or ATP synthesis, and diminished proton-motive force, all of which are detrimental to energy maintenance in the cell (Dunlop, 2011). Several efforts towards engineering the microbial strains for biofuel tolerance include engineering biofuel export systems, heat shock proteins (HSPs), membrane modifications, more general stress responses, and approaches that

integrate multiple tolerance strategies. In addition, *in situ* recovery methods and media supplements can also help to ease the burden of end-product toxicity. The efflux pumps are membrane transporters that recognize and export toxic compounds from the cell, using the proton-motive force, e.g., solvent resistance pump (*srpABC*) from *Pseudomonas putida* S12 can export hexane, octanol and several other hydrocarbons (Kieboom *et al.*, 1998). Another way to improve biofuel tolerance is to overexpress HSPs, e.g., overexpression of GroESL improved tolerance in *C. acetobutylicum*, along with increased butanol yield (Tomas *et al.*, 2003). Among the membrane modifications, approaches may be targeting an increase in the ratio of *trans*- to *cis*- fatty acids (that leads to decrease in membrane fluidity) (Kiran *et al.*, 2004), alteration in the ratio of saturated to unsaturated fatty acids (for solvent exclusion) for longer-term response, or modifications to phospholipid headgroups or phospholipid chain length (Ramos *et al.*, 2002). A detailed understanding of the response to biofuel stress and the genes that are implicated in these changes may also be useful in engineering cellular control systems, e.g., reactive oxygen species were highly elevated during *n*-butanol stress, suggesting that the genes, alleviating oxidative stress, may be valuable engineering targets (Rutherford *et al.*, 2010). Classical strain improvement methods, such as chemostat-mediated adaptation, mutagenesis, and evolutionary engineering are amongst the other valuable approaches (Parekh *et al.*, 2000). It is often necessary to alter the expression of multiple genes or combine multiple strategies to provide the greatest benefit. Removing biofuel from the bioreactor (by gas stripping, liquid-liquid extraction, pervaporation, and perstraction) (Ezeji *et al.*, 2010) or supplementing the growth media with compatible-solute protectants (Nicolaou *et al.*, 2010), like trehalose, proline betaine, inositol etc. can ease the burden of cytotoxicity and improve tolerance.

CONCLUSION

Biofuel can efficiently replace the petroleum-fuels, and are associated with widespread availability, affordability, accessibility of technology, ease of transport, storage, versatility in use in engines, and socio-economic and environmental benefits. Thus, scien-

tific research, focusing in this field, has recently gained significant momentum globally. There has been a major technological advancement in the generation of biofuel from microorganisms, with some of the developed technologies on the verge of commercialization process (<http://www.gevo.com>). *E. coli*, as the host organism, have enabled the production of a wide variety of candidate biofuel compounds, while also highlighting the vast array of renewable feedstock constituents. The major goals of metabolic pathway and redesigning for biofuel producer include improved product yield, higher product concentration and productivity, and product tolerance. The production strain also should be designed so that the whole process becomes operationally inexpensive. Further biological research will be needed to contribute to an improved biofuel production, by breeding of energy plants, enzymatic hydrolysis, developing specialized fermentation strains and waste treatment.

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