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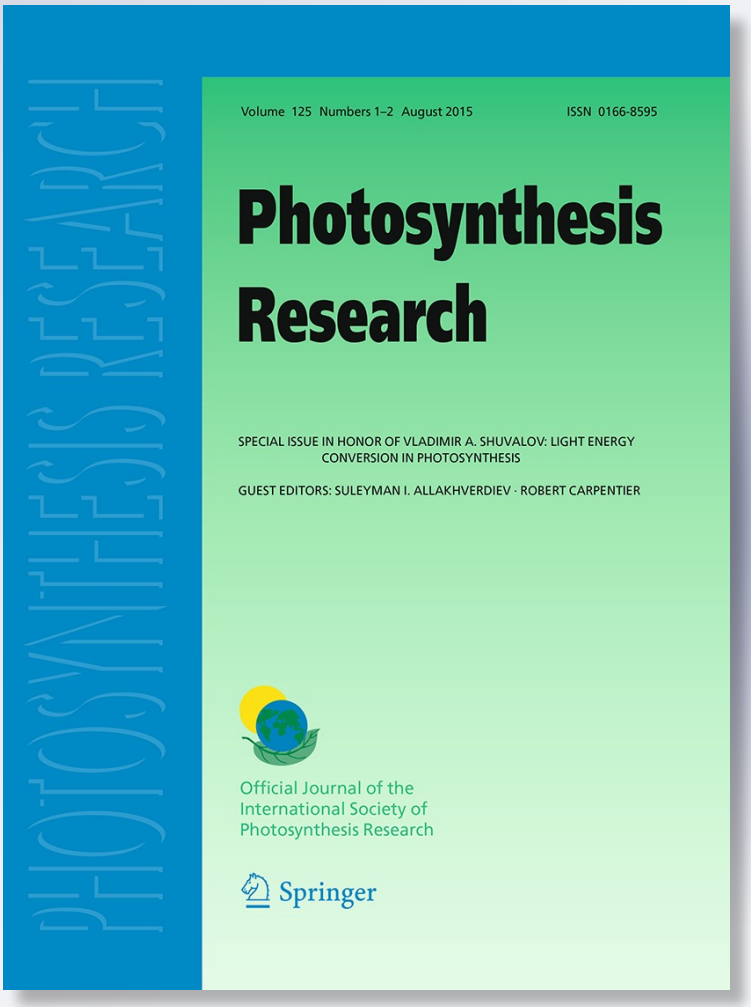

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REVIEW

Cyanofuels: biofuels from cyanobacteria. Reality and perspectives

Fariza Sarsekeyeva · Bolatkhan K. Zayadan · Aizhan Usserbaeva · Vladimir S. Bedbenov · Maria A. Sinetova · Dmitry A. Los

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Abstract Cyanobacteria are represented by a diverse group of microorganisms that, by virtue of being a part of marine and freshwater phytoplankton, significantly contribute to the fixation of atmospheric carbon via photosynthesis. It is assumed that ancient cyanobacteria participated in the formation of earth's oil deposits. Biomass of modern cyanobacteria may be converted into bio-oil by pyrolysis. Modern cyanobacteria grow fast; they do not compete for agricultural lands and resources; they efficiently convert excessive amounts of CO_2 into biomass, thus participating in both carbon fixation and organic chemical production. Many cyanobacterial species are easier to genetically manipulate than eukaryotic algae and other photosynthetic organisms. Thus, the cyanobacterial photosynthesis may be directed to produce carbohydrates, fatty acids, or alcohols as renewable sources of biofuels. Here we review the recent achievements in the developments and production of cyanofuels—biofuels produced from cyanobacterial biomass.

Keywords Biofuels · Biomass · Cyanobacteria · Cyanofuels · Fatty acids · Photosynthesis

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Abbreviations

ACP	Acyl carrier protein
DAG	Diacylglycerol
FA	Fatty acid
FFA	Free fatty acid
PM	Photosynthetic machinery
TAG	Triacylglycerol

Introduction

Biofuels are derived from a biomass—the product of carbon fixation by modern living photosynthetic organisms—plants, microalgae, and cyanobacteria. Traditional natural organic fossil fuels (coal, peat, oil, and gas) are the products of long-term geological deposition of the plant-type biomass consisting of carbon and hydrogen. Fossil fuels were formed from a biomass by its anaerobic degradation under heat and pressure in the earth's crust over millions of years. Obviously, in terms of human life (or even of tens or hundreds of generations) scale, such fuels are considered as nonrenewable natural resources.

According to data, provided by the International Energy Agency (http://www.iea.org), the recently consumed primary energy sources are mainly represented by oil (36.0 %), coal (27.4 %), and natural gas (23.0 %) that add up a total of 86.4 % of the energy (fossil and nonfossil) consumed in the world. The nonfossil energy sources include hydropower plants (6.3 %), nuclear (8.5 %), and other sources of energy (geothermal, solar, tidal, wind, wood, and waste burning) with a sum of 0.9 % (Fig. 1a). However, over the last 5 years, a trend has been observed toward reduction of the consumption of traditional energy

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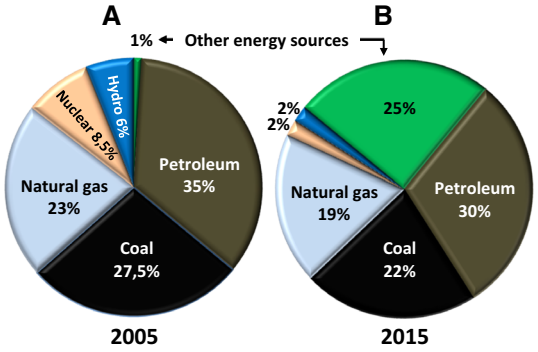

Fig. 1 World's primary energy demands in 2005 (**a**) and 2015 (**b**). The data of International Energy Agency (IEA): http://www.iea.org/publications/freepublications/publication/kwes.pdf

sources, with a simultaneous increase in the use of non-traditional sources (up to 25 % in 2015), including solar energy, wind, biomass use, etc. (Fig. 1b).

Growing energy demands raise a concern about the imminent depletion of nonrenewable energy sources and stimulate the search for new, alternative ways of producing energy. A biochemical approach to produce alternative fuels implies the use of biomass, which can be converted into energy by thermal, chemical, or biochemical conversion. The purpose of this review is to estimate the achievements in the field of production of alternative biofuels and to assess the prospects of research in the field of applied biology of cyanobacteria as potential producers of renewable substances—bio-oils and biofuels (cyanofuels).

Biomass as a source of energy

The "*biomass*" is a renewable energy source derived from living or recently living organisms. Cyanobacterial, algal, and plant biomasses are produced due to atmospheric or water-dissolved CO_2 fixation during the process of photosynthesis. As a renewable energy source, biomass can be used directly to produce heat via combustion, or after its conversion to biofuels. Biomass resources can be divided into four general categories (McKendry 2002):

1. Forest products: wood, logging residues, trees, shrubs and wood residues, sawdust, bark etc. from forest clearings.
2. Wastes: agricultural production and processing wastes, urban wood, and organic wastes.
3. Energy crops: short rotation woods, herbaceous woody crops, grasses, starch crops (corn, wheat, and barley), sugar crops (cane and beet), forage crops (grasses, alfalfa, and clover), oilseed crops (soybean, sunflower, and safflower).
4. Aquatic photosynthetic organisms: cyanobacteria, algae, water weeds, water hyacinth, reed, and rushes.

Types of biofuels

As a product of biomass conversion, biofuels are represented by biogas or liquid biofuels. Sometimes, solid biofuels are also considered: these biofuels are produced by combustion of agricultural waste biomass (rice husk, wheat straw, coconut shell, corn waste, etc.) or imply the cultivation of the so-called energy plantations of fast-growing woods for production of firewood, briquettes, and pellets from waste wood (McKendry 2002).

Biogas is a mixture of gases (primarily methane and CO_2) produced by the breakdown of biomass in the absence of oxygen. On the contrary, hydrogen derived from water by algae and cyanobacteria in the light should not be considered as biogas, and it is usually termed as biohydrogen (Allakhverdiev et al. 2010). The thermal treatment of biomass, known as gasification, produces synthesis gas (syngas) (Rauch et al. 2014).

Liquid biofuels are represented by bio-alcohols (ethanol, butanol, and methanol) and biodiesel (Singh Nigam and Singh 2011). In 2011, global production of bioethanol, mainly from corn and sugar bean, reached about 10 billion liters, and the expected volume of production in 2020 is 281.5 billion liters.

Biodiesel is a product of esterification of oils derived from vegetables, microalgae, or other microorganisms. Triacylglycerides (TAGs) and diacylglycerides (DAGs) may be also converted to gasoline (petrol) or jet fuel through distillation and cracking (Georgianna and Mayfield 2012).

Types of biofuel also vary by source of raw materials and processing technologies.

First-generation biofuel was obtained from edible crops with a high content of oil, starch, or sugars suitable for processing into biodiesel or bioethanol (Naik et al. 2010). These biofuels are rather expensive because of land processing with water, fertilizers, pesticides, etc. In addition, withdrawal of these lands from agricultural use greatly increases the price of food.

Second-generation biofuels is derived from nonfood materials (grass, straw, timber) containing cellulose and lignin (Naik et al. 2010). These products can be burned directly, or converted to flammable gases, or pyrolyzed. The main disadvantage of second-generation biofuels is that these raw materials occupy a substantial land area with relatively low returns per unit area.

Third-generation biofuels produced from microalgae (unicellular algae), which can be cultivated in open ponds or closed photobioreactors (Přibyl et al. 2014). Microalgae do not compete for crop land, they have high growth rates, and can reach high density of biomass, from which biofuels or their precursors may be produced. Third-generation biofuels usually refer to microalgal triacylglycerols (TAGs),

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which are extracted from cells and used for biodiesel production (Chisti 2007).

Fourth-generation biofuels combine the properties of third-generation biofuels with an advantage of genetic optimization of their producers (Al-Thani and Potts 2012; Nozzi et al. 2013). Cyanobacteria differ from microalgae and plants by the relative ease of genetic manipulations. Genetic modifications of cyanobacterial cells allow for conversion of the fixed atmospheric carbon not just into a crude biomass, but also into desired end-products, which are most suitable for the production of biofuels (Quintana et al. 2011). Furthermore, genetically engineered cyanobacteria can secrete metabolic end-products, alkanes, or free fatty acids (FFAs), into the culture medium. Thus, costly stages of cell collection and disruption to extract the products may be skipped (Peralta-Yahya et al. 2012). At present, such biofuels are considered as most effective in terms of cost and processing technologies.

Cyanobacteria as a feedstock for the production of cyanodiesel and other cyanofuels

The popularity of biofuels is growing due to the depletion of oil and gas reserves, rising prices for these resources, and the need to ensure energy security. Microalgae and cyanobacteria are considered as a suitable sources of renewable liquid biofuels (Angermayr et al. 2009) consisting of hydrocarbon chains that can replace petroleum hydrocarbons as fuels, lubricants, plastics, etc.

At present, three types of diesel fuels are distinguished according to their origin and processing technology (Knothe 2010; Bezergianni and Dimitriadis 2013).

1. *Petrodiesel* is the diesel fuel that is derived from petroleum (standards ASTM D975 in the USA and EN 590 in EU).
2. *Biodiesel* is a renewable fuel composed of mono-alkyl esters of long chain FAs derived from vegetable oils or animal fats and meeting the strict industry specifications (standards ASTM D6751 and EN 14214).
3. *Renewable diesel* is also made from biomass by catalytic reaction of FAs and FA esters with hydrogen, hydrodeoxygenation (Stumborg et al. 1996), or by cracking and pyrolysis of biomass (Zeng et al. 2013) (standards are the same as for petrodiesel—ASTM D975 and EN 590).

The term "*biodiesel*" was first pronounced in an article published in Chinese (Wang 1988), although the concept of biodiesel (the use vegetable oil as a fuel for engines) was first proposed by the inventor of the conventional diesel engine Rudolf Diesel in 1895. In 1900, at the World Exhibition in Paris, the first car engine that operated on peanut oil was already presented (Knothe 2001). Thus, the *first-generation biofuel* was created at the edges of nineteenth and twentieth centuries.

Today we stand on the threshold of creating and using a fourth-generation biofuels, which is based on fast growing, rapidly renewable, and genetically engineered resource—prokaryotic photosynthesizing cells of cyanobacteria.

Cyanobacteria have a high potential for the production of cyanodiesel because of their significant advantageous properties:

1. Cyanobacteria do not claim arable lands and do not compete with farmers for crop areas and other resources.
2. Cyanobacteria grow accumulate biomass rapidly.
3. Cyanobacteria directly fix atmospheric or water-dissolved CO_2, and they require only sunlight, water, and a minimum set of inorganic trace elements for growth. Cyanobacteria are capable of handling the excess CO_2 (the emissions we are trying to avoid) directly into hydrocarbons for biofuels. They can be also used to recover the water wastes from organic and inorganic contaminants (Fig. 2).
4. Plasticity of cyanobacterial metabolism allows for directed biosynthesis of lipids in controlled photobioreactors.
5. Many strains of cyanobacteria may be easily and stably transformed. Therefore, they can be used as convenient platforms for the genetic modification of metabolic pathways. Some researchers also favor cyanobacteria because fuel from engineered cyanobacteria is excreted outside the cell, in contrast to eukaryotic algae, in which fuel production occurs inside the cell.

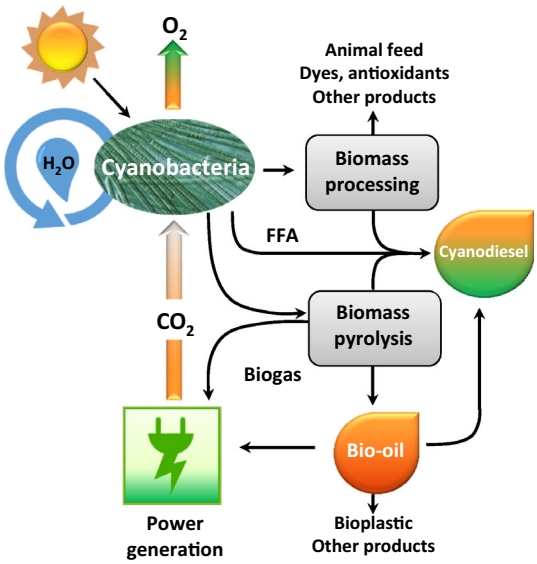

Fig. 2 The use of cyanobacteria to produce cyanodiesel and other valuable products. *FFA* free fatty acids

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Advantages of genetic and metabolic engineering, Part 1: Alcohols and isoprene

Cyanobacteria are widely used as model organisms to study photosynthesis, lipid metabolism, stress responses, etc. Methods of transformation and of targeted gene knockouts became available long time ago (Shestakov and Khyen 1970; Grigorieva and Shestakov 1982). The complete genome of the model strain *Synechocystis* sp. PCC 6803 was published in 1996 (Kaneko et al. 1996). Nowadays, the genomes of about 150 cyanobacterial strains are available in GenBank database (Shih et al. 2013). Commercial or custom made DNA microarrays are available for several cyanobacterial strains, i.e., *Anabaena*, *Synechocystis*, and *Synechococcus*. Differential RNA-seq-type transcriptomic analyses are also applicable to cyanobacteria (Ruffing 2013; Kopf et al. 2014).

Cyanobacteria increasingly attract the attention as promising cell factories for the production of renewable biofuels and chemicals from just CO_2 and water by the expense of sunlight. The theoretical possibilities of genetic alterations in the cyanobacterial metabolic pathways look somewhat amazing (Erdrich et al. 2014). Although not all hopes and calculations ultimately lead to viable and productive phenotypes, some progress has been achieved in cyanobacterial biofuel-related biotechnology. The biochemical pathways that have been modified to produce cyanofuels are depicted in Fig. 3.

Strategies for metabolic optimization in cyanobacteria can be grouped into four areas:

1. Improvement of CO_2 fixation;
2. Optimization of pathway flux;
3. Improvement of tolerance toxic products;
4. Elimination of competing pathways.

In addition to three general biotechnological strategies (2–4), the first strategy is specific to photosynthetic organisms, particularly to cyanobacteria. Theoretical maximum efficiency of solar energy conversion by photosynthetic machinery (PM) is 12–14 %. The enzyme responsible for CO_2 fixation, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), lacks high specificity, and it can bind O_2 instead CO_2. Genetically improved specificity of this enzyme often leads to a decrease in its catalytic rate (Tcherkez et al. 2006). Nevertheless, RuBisCO may be optimized to the best possible compromise between specificity and catalytic efficiency (Rosgaard et al. 2012). Among photosynthetic organisms, cyanobacteria have already evolved very efficient carbon-concentrating mechanisms (CCMs), which allow RuBisCO to operate near maximum rates by lowering the need for high specificity (Burnap et al. 2015).

PM of cyanobacteria, which generates oxygen, limits the list of potential heterologous enzymes available for metabolic engineering. Many enzymes expressed in cyanobacteria had low activity and therefore limited production (Lan and Liao 2011; Ungerer et al. 2012). Changing enzymes, as well as their co-factors (e.g., NADPH versus NADH) may improve the yield (Lan and Liao 2011).

Improved tolerance of photosynthetic cells to end-products of gene expression should allow for higher productivity. Such studies have already begun on cyanobacteria (Ruffing 2013; Dienst et al. 2014). Alternatively, removal of toxic products can also increase production (Ungerer et al. 2012).

The elimination of competing pathways in cyanobacteria is almost uncharted (Nozzi et al. 2013). Rapidly growing data on cyanobacterial "omics," however, may soon provide the necessary background for metabolic modeling and construction.

Production of ethanol

One example is the transgenic *Synechococcus elongatus* PCC 7942, which was engineered to produce ethanol by transformation with two genes for pyruvate decarboxylase and alcohol dehydrogenase II from *Zymomonas mobilis*, which degrades sugars to pyruvate and then ferments it to produce ethanol and carbon dioxide as the only products (Deng and Coleman 1999). The yield of ethanol produced by the transformed strain was 5 mM (0.23 g L^{-1}). The same genes expressed in *Synechocystis* sp. PCC 6803 yielded 10 mM (0.46 g L^{-1}) (Dexter and Fu 2009). A mutant of *Synechocystis* with the disrupted biosynthetic pathway for poly-β-hydroxybutyrate synthesis, which expressed a pyruvate decarboxylase from *Z. mobilis* and overexpressed the endogenous alcohol dehydrogenase (*slr1192*), reached significantly higher ethanol-producing efficiency (5.50 g L^{-1}) (Gao et al. 2012b). It is important that the molecular responses of engineered *Synechocystis* during long-term ethanol production and cultivation are rather specific and appear well manageable (Dienst et al. 2014; Song et al. 2014).

Production of isobutanol

Another example is the transgenic *S. elongatus* PCC 7942, which was constructed to produce isobutanol via a ketoacid pathway (Atsumi et al. 2008). Isobutanol is considered as a promising gasoline substitute and competes with ethanol and 1-butanol for gasoline replacement. However, no organism can naturally synthesize isobutanol at high yield and productivity. *Synechococcus*, however, was able to adopt an artificial metabolic pathway to isobutanol produce at 0.450 g L^{-1} (Atsumi et al. 2009).

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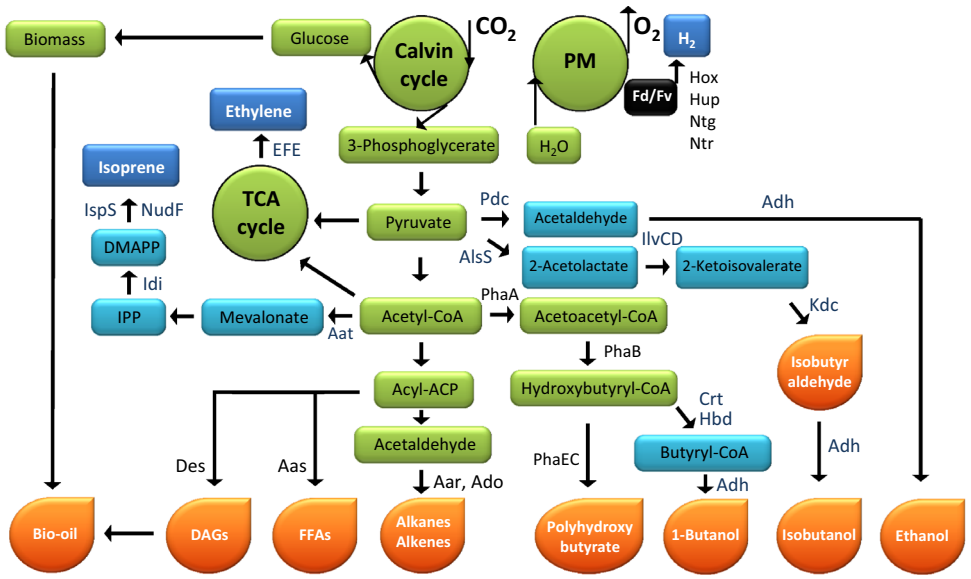

Fig. 3 Engineered and natural biochemical pathways of cyanobacteria that are employed for production of valuable compounds. *Aas* acyl-ACP synthase, *Aat* acetoacetyl-CoA transferase, *Adh* aldehyde/alcohol dehydrogenase, *AlsS* acetolactate synthase, *AtoB* acetyl-CoA thiolase, *Crt* crotonase, *Des* fatty acid desaturases, *DMAPP* dimethylallyl-diphosphate, *EFE* ethylene-forming enzyme, *Fd* ferredoxin, *Fv* flavodoxin, *Hbd* 3-hydroxybutyryl-CoA, *Hox* bidirectional hydrogenase, *Hup* uptake hydrogenase, *IlvC* acetohydroxy acid isomeroreductase, *IlvD* dihydroxy-acid dehydratase, *Idi* IPP isomerase, *IPP* isopentenyl diphosphate, *IspS* isoprene synthase, *Kdc* 2-keto acid decarboxylase, *NudF* ADP-ribose pyrophosphatase, *Ntr* nitrogenase reductase, *Ntg* nitrogenase, *PhaA* β-ketothiolase, *Pdc* pyruvate decarboxylase, *PhaB* acetoacetyl-CoA reductase, *PhaEC* PHB synthase, *PM* photosynthetic machinery, *TCA cycle* tricarbonic acid cycle

Production of 1-butanol

Synechococcus elongatus PCC 7942 was also able to produce 1-butanol (which is usually produced by anaerobic bacteria such as *Clostridium*) via a modified CoA-dependent pathway (Lan and Liao 2011). To synthesize 1-butanol (13.16 mg L^{-1}), five genes were integrated into the genome of *S. elongatus*: the genes for aldehyde/alcoholdehydrogenase (*adhE2*), crotonase (*crt*), hydroxybutyryl-CoA dehydrogenase (*hbd*) from *Clostridium acetobutylicum*, modified *trans*-enoyl-CoA-reductase (*ter*) from *Treponema denticola*, and acetyl-CoA acetyltransferase or thiolase (*atoB*) from *Escherichia coli*. The engineered *S. elongatus* strain did not produce 1-butanol in the aerobic conditions in the light. However, inhibition of photosynthetic electron transport by DCMU allowed 1-butanol production during light exposure (Lan and Liao 2011). It was suggested that oxygen, rather than light, is an important factor for 1-butanol production in cyanobacteria. However, 1-butanol synthesis in the dark was not tested.

Non-photosynthesizing heterotrophic bacteria, like *E. coli*, may be also engineered to produce alcohols, butanol (Pásztor et al. 2015), 1-octanol (Akhtar et al. 2015), or even gaseous propane (Kallio et al. 2014). However, heterotrophs just convert one form of energy (carbohydrates) into another (hydrocarbons), whereas photoautotrophic biosynthesis implies conversion of inorganic CO_2 into organic matter.

Production of isobutyraldehyde

Synechococcus elongatus was engineered to produce isobutyraldehyde (Atsumi et al. 2009). This pathway employed three additional genes from the pyruvate–valine biosynthesis pathway to direct the flux of carbon toward the synthesis of the ketoacid precursor, 2-ketoisovalerate: *alsS* from *Bacillus subtilis,* and *ilvCD* from *Escherichia coli*. The resulting strain was capable of producing isobutyraldehyde at 1.1 g L^{-1}. In this strain, the CO_2-fixing activity of RuBisCo was also enhanced by the introduction of extra copies of *rbcLS* operon derived from a close relative of *S. elongatus* PCC 7942, *S. elongatus* PCC 6301 (Machado and Atsumi 2012).

Production of isoprene

Synechocystis sp. PCC 6803 was engineered to produce isoprene (50 μg g^{-1} dry cell weight per day) by the expression of the *ispS* gene for the isoprene synthase from the isoprene-emitting kudzu vine, *Pueraria montana* (Lindberg et al. 2010; Melis 2012; Chaves et al. 2014). An increase in photosynthetic carbon partitioning toward production of isoprene (120 μg g^{-1}) was achieved by the heterologous expression of the mevalonic acid pathway in *Synechocystis*, which enriched the pool of precursors to isoprene, isopentenyl-diphosphate and dimethylallyl-diphosphate (Bentley et al. 2014).

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Production of ethylene

Similar to isoprene, ethylene is a simple C_2 volatile hydrocarbon that can be used as raw material to produce various petrochemical products. Cyanobacteria and higher plants have similar two-component ethylene-sensing systems based on sensory histidine kinases (Kehoe and Grossman 1996). Unlike in higher plants, where ethylene regulates a number of physiological processes (Schaller and Kieber 2002), the role of ethylene signaling in cyanobacteria is not yet defined. The latter, however, is not an obstacle for engineering ethylene-producing cyanobacterial strains. Originally, the strategy was based on the introduction of the enzyme known as the ethylene-forming enzyme (EFE), which transforms 2-oxoglutarate from TCA cycle into ethylene and CO2 in an O2-dependent manner (Fig. 3). A gene for EFE from *Pseudomonas syringae* (or other bacterial and plant source) was introduced into *S. elongatus* PCC 7942 (Sakai et al. 1997; Takahama et al. 2003), and *Synechocystis* sp. PCC 6803 (Guerrero et al. 2012; Ungerer et al. 2012).

Another approach was based on the synthetic enzyme complex that places the enzymes in close proximity to each other and thus facilitates substrate channeling (Jindou et al. 2014). *S. elongatus* PCC 7942 was transformed with tomato genes for 1-aminocyclopropane-1-carboxylate ACC synthase and aminocyclopropane carboxylate oxidase. In this way, ethylene was produced from endogenous *S*-adenosylmethionine derived from the methionine cycle. Significant enhancement of ethylene production was achieved by complexing these two plant ethylene biosynthesis enzymes by cohesin–dockerin cellulosome from cellulolytic anaerobic bacteria (Jindou et al. 2014). However, the overall production of ethylene was lower that with the use of EFE, probably, due to a limited amount of substrate.

Production of hydrogen

Hydrogen is one of the most promising clean fuels, since its combustion yields only water. Among oxygenic phototrophs, only green microalgae and cyanobacteria have been shown to sustain hydrogen production. The most effective process involves a direct transfer of electrons from water to hydrogen-evolving enzymes accompanied by oxygen and hydrogen evolution—the process called direct biophotolysis (Allakhverdiev et al. 2010). Normally hydrogen production in phototrophic organisms is coupled to the photosynthetic electron transport chain via specific enzymes, which catalyze generation of molecular hydrogen under certain conditions using reduced ferredoxin or NAD(P)H as the electron donors (for a review, see Antal et al. 2011). In cyanobacteria, three types of enzymes participating in hydrogen metabolism have been reported: a nitrogenase, a reversible bidirectional hydrogenase (Hox), and an uptake hydrogenase (Hup) (Hallenbeck 2012). The most efficient H_2-producing species contain nitrogenase enzymes that produce H_2 as a by-product of N_2 fixation (Allahverdiyeva et al. 2010). The bidirectional hydrogenases can either produce or consume H_2 according to the cellular redox environment (Carrieri et al. 2011). The uptakes by hydrogenases catalyze the consumption of H_2 and, thus, should be removed from cells for efficient H_2 production to occur (Masukawa et al. 2002).

A crucial problem associated with the use of cyanobacteria for hydrogen production is simultaneous production of oxygen and hydrogen by the two photosystems. The evolved O_2 inhibits hydrogenase activity and suppresses its synthesis. However, several natural and technological strategies are available to protect hydrogen-evolving enzymes from O_2.

Natural strategies employ spatial separation of O_2 and H_2 production: nitrogen-fixing cyanobacteria perform H_2 generation in specialized compartments—heterocysts. Temporal separation implies O_2 production in the light, and H_2 production in the dark.

Technological approaches are mainly directed toward

1. Enhancement of the O_2-tolerance and efficiency of hydrogenases by genetic engineering (Hallenbeck 2012);
2. The use of immobilized cyanobacterial cells: this provides an improved light access and directs the absorbed light energy into H_2 production, rather than to biomass accumulation (Kosourov et al. 2014);
3. Optimization of culturing conditions, including the development of automated photobioreactors to increase the efficiency of hydrogen photoproduction (Sakurai et al. 2013).
4. Development of artificial water-splitting systems to enhance the production of H_2 (Najafpour and Allakhverdiev 2012).

Advantages of genetic and metabolic engineering, Part 2: alkanes and free fatty acids

Production of alkanes and alkenes

Recently, a two-step alkane biosynthetic pathway was identified in cyanobacteria (Schirmer et al. 2010). In *Synechocystis* sp. PCC 6803, heptadecane and heptadecene are the major constituents of alka(e)nes, and their total content does not exceed 0.15 % of the cell dry weight. Alka(e)nes are produced by reduction of acyl–acyl carrier protein (ACP) to aldehyde by an acyl-ACP reductase (AAR, Sll0209 in *Synechocystis* sp. PCC 6803) with subsequent oxidation of aldehyde to alkane or alkene by an aldehyde-deformylating oxygenase (ADO, Sll0208). This

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pathway employs the fatty acid substrates that are synthesized by fatty acid synthase (FAS) as acyl chains of membrane lipids (Wang et al. 2013). Alternatively, FFAs generated by lipolytic enzymes during degradation of membrane lipids may be used as substrates (Kaczmarzyk and Fulda 2010).

Alkane biosynthetic genes isolated from *Synechocystis* were expressed in *E. coli* and marine *Synechococcus* sp. PCC 7002, and alka(e)ne production ranged from 5 to 40 mg L^{-1} in *E. coli* and reached 5 % of DW in *Synechococcus* sp. PCC 7002 (Schirmer et al. 2010). While the production of alka(e)nes by wild-type cells of *Synechocystis* sp. PCC 6803 was near 0.13 %, the engineered *Synechocystis* with overexpressed alkane biosynthetic genes (*sll0209* and *sll0208*) produced 1.1 % of alka(e)nes of cell dry weight (Wang et al. 2013).

Production of free fatty acids

FAs derived from the membrane lipids during their degradation by lipases are converted to fatty acyl-thioesters by fatty acyl-CoA- or acyl-ACP synthetase (the process of FA *activation*) prior to recycling to DAGs or synthesis of alkanes. In cyanobacteria, the activating enzyme is represented by the acyl-ACP synthetase (Slr1609 in *Synechocystis* sp. PCC 6803). The *slr1609*-deficient mutant of *Synechocystis* was unable to activate endogenous FAs, secreting them into the culture medium (Kaczmarzyk and Fulda 2010). It was also unable to utilize the exogenous FAs (von Berlepsch et al. 2012). Thus, the acyl-ACP synthetase appeared metabolically crucial for production of FFAs and their derivatives in cyanobacteria. The amount of FFAs can be doubled in the *slr1609* knockout of *Synechocystis* (Gao et al. 2012a, b). Secretion of hydrophobic FFAs into culture medium simplifies the process of their separation and eliminates the stages of biomass recovery and cell disruption. The FFA-secreting culture may be used continuously for a long time (Liu et al. 2011a).

Further genetic modifications of the FAAs synthesis pathway in *Synechocystis* included several steps: (1) the gene for the acyl-ACP synthetase (*slr1609*) was deleted; (2) the genes for acetyl-CoA carboxylase (*fabH* and *fadI*) were introduced and expressed; (3) the modified genes for acyl-ACP thioesterases (*tesA*, *fatB1,* or *fatB2*) that control the chain length of FAs were expressed (Liu et al. 2011b). Two poly-3-hydroxybutyrate synthesis genes (*slr1993* and *slr1994*) were deleted to prevent the production of by-products that would compete with production of FFAs. Peptidoglycan layer was weakened by deletion of the *sll1951* gene for a surface protein, and this deletion enhanced FFAs secretion.

Such massive genetic intervention into a helpless single-cell organism (called *metabolic engineering* or *designing*) leads to the enhanced production of FFAs that precipitated as a granular ring on the glass flask wall above the aqueous phase (Liu et al. 2011b)—a dream of a separation technologist. Finally, due to thioesterase activities, the amount of secreted medium chain FFAs (C_{12} and C_{14}) increased from 16.5 % (*tesA* of *E. coli*) to 47.3 % (*fatB1* of a higher plant, *Umbellularia californica*). The highest amount of secreted FFAs was observed in codon-optimized *tesA* expressing strain with an additional knock out of *slr2132*, a *pta* gene coding for phosphotransacetylase ($\sim$200 mg L^{-1} compared to 2 mg L^{-1} in wild-type and 84 mg L^{-1} in the *slr1609* knockout-expressing *tesA*).

Another model cyanobacterium, *S. elongatus* PCC 7942, was engineered to produce secreted FFAs, via similar strategy: knockout of a gene for an acyl-ACP synthetase and expression of a thioesterase. Similar to *Synechocystis*, the engineered *Synechococcus* produced and excreted FFAs, although the yields were rather small (Ruffing and Jones 2012). The engineered strains demonstrated reduced photosynthetic yields, relocation of the light-harvesting pigments, chlorophyll degradation—the effects similar to that observed in wild-type *Synechococcus* supplemented with α-linolenic acid ($C_{18:3}$). It was suggested that the excess of FFAs may affect cell and thylakoid membranes' integrity and structure and interfere with the functioning of the photosynthetic apparatus.

Further detailed investigation of the molecular basis of such physiological phenomena by RNA-Seq analysis (Ruffing 2013) revealed that FFA's production affects transcription of several categories of genes in *Synechococcus*, namely, genes for general stress response (*groELS*, *sigD*, *hspA*, *sodB*, *trx*) nitrogen metabolism, protein-folding genes, and some others (Los et al. 2010). The induction of genes for general stress response during FFA's production implies the possible involvement of reactive oxygen species (ROS) in regulation of cellular processes and a stressor nature of the excessive FFAs. Accordingly, the overexpression of ROS-degrading proteins reduced the toxic effects of FFA's production, allowing for improved growth, physiology, and FFA yields (Ruffing 2013). A total of 15 gene target genes were identified to reduce the toxic effects of FFA's production. However, single-gene-targeted mutagenesis led to minor changes in FFA's production. Further efforts are expected to clarify whether the combination of mutations may enhance the production of FFAs by stabilization of physiological conditions of the FFA-producing strains.

Quality of fatty acids

In addition to the engineering of cyanobacteria to increase production or yield of FFAs, it is also desirable to take care of the quality of FAs and lipids, which will serve as a

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source for cyanodiesel. Lipid properties are determined by its acyl composition: both carbon chain length and the number of double bonds (i.e., degree of unsaturation). Naturally occurring cyanobacterial species synthesize a vast range fatty acid structures. The carbon chain length and degree of unsaturation of FAs in each cyanobacterial species may affect the flow of cold and oxidative stability properties of cyanodiesel obtained from the raw material. Cyanobacterial FAs are mainly represented by C_{16} and C_{18} species with 0–3 double bonds: 16:0, 16:1, 18:1, 18:2, 18:3 (Murata et al. 1992; Los and Mironov 2015). Some species, however, may have predominant C_{14} and C_{16} saturated and monounsaturated FAs (Sarsekeyeva et al. 2014). Ideally, for the production of cyanodiesel, a cyanobacterial strain should be reaching C_{12}–C_{18} medium-chain saturated and monounsaturated FAs. Thus, the FA profiles of engineered *Synechococcus* (Ruffing and Jones 2012) or naturally occurring *Cyanobacterium* sp. (Sarsekeyeva et al. 2014) look quite appropriate for conversion to cyanodiesel. Such cyanofuel, composed of C_{14} and C_{16} saturated and monounsaturated FFAs, should yield high cetane number (an indicator of the combustion speed of diesel fuel) and low iodine values (that reflect the degree of unsaturation). The latter parameters are the indicators of proper ignition and combustion quality, low deposit formation, and reduced lubricant degradation of a biofuel (Hoekman et al. 2012).

It is known that viscosity of liquid biofuels increases with FA chain length, and that their oxidative stability decreases with an increase in FA unsaturation. Thus, biodiesel with saturated or monounsaturated FAs of shorter chain length are preferable for production of high-quality cyanodiesel. To meet these criteria, at least, two complementary approaches may be used: (1) a continuous search and isolation of new natural cyanobacterial strains with various length and unsaturation of FA chains in their lipids; and (2) genetic modification that employs genes for short chain-specific acyl-ACP thioesterases. The latter approach may be useful for reducing the chain length of FAs (Voelker and Davies 1994; Cantu et al. 2010; McMahon and Prathera 2014). If necessary, the level of FA unsaturation may be reliably altered in some cyanobacterial strains by expression or knock-out of genes for FA desaturases (Wada et al. 1990; Tasaka et al. 1996; Mironov et al. 2012).

Advantages of conventional (nonengineering) approaches

Along with great and unquestionable opportunities of genetic engineering, there are several traditional ways to improve lipid and FA production by cultures of cyanobacteria. This includes (1) a search and selection of natural strains; (2) random mutagenesis, cell sorting, and selection; and (3) control of culture conditions.

Cyanobacteria inhabit a wide range of ecosystems, from fresh to marine or soda water environments, from depleted soils, rocks or arid deserts, and from the cold polar waters to geothermal hot springs. Many cyanobacterial species are adapted to survive under various extreme conditions, including high and low O_2 or CO_2 levels, different light intensities, temperatures, salinity, pH, and desiccation. Some strains are obligate photoautotrophs, some are facultative heterotrophs, and others can fix nitrogen via heterocysts. In general, the metabolic and genetic diversity of cyanobacteria reflects the diversity of their habitats. Therefore, extensive search, isolation, and identification of new cyanobacterial species are necessary to explore their abilities to produce valuable compounds.

Random mutagenesis (chemical, UV, or antibiotic cartridge) followed by cell sorting may be applied to cyanobacteria in order to select most productive strains in terms of lipid or FA synthesis, similar to microalgal strains (Doan and Obbard 2012; Xie et al. 2014). However, the development of the next generation sequencing (NGS) technology simplifies the whole-genome scale analysis of cyanobacterial strains, making them an easier platform to targeted metabolic engineering.

Maintenance of the proper culture conditions is the simplest way to enhance the yield of the desired product; however, it is often ignored. The control over culture conditions includes light quality and intensity, CO_2 concentration, nutrients, and temperature (for a recent review, please, see Sharma et al. 2012).

Light

Photobioreactors equipped with recently developed light-emitting diode (LED) illuminating systems produce significantly more biomass (244,668 kg ha^{-1}) than solar-illuminated photobioreactors (8262 kg ha^{-1}) and open ponds (4957 kg ha^{-1}) (Přibyl et al. 2014). However, the production cost of biomass in LED-lit photobioreactors is 8–10 times higher (25–30$) than in open ponds ($3 per kilogram). Although production in open ponds is much cheaper, the use of transgenic cyanobacteria for mass cultivation raises additional environmental issues, because, in this case, the escape of genetically modified organisms into the environment is unavoidable (Henley et al. 2013).

In eukaryotic algae, low light intensities (20–50 μmol m^{-2} s^{-1}) drive lipid biosynthesis toward the accumulation of polar DAGs, whereas moderate light (100–150 μmol m^{-2} s^{-1}) promotes the accumulation of neutral TAGs. In cyanobacteria, no TAG accumulation has been detected (Hu et al. 2008). Instead, the majority cyanobacterial strains are packed with photosynthetic thylakoid membranes, which contain about 40 % of DAGs

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under regular growth conditions (Quinn and Williams 1982).

Nutrients

In cyanobacterial cells, lipid content may be further increased by nitrogen deprivation—a well-known (Spoehr and Milner 1949; Piorreck and Pohl 1984), but recently rediscovered (Martin et al. 2014; Weng et al. 2014), phenomenon. An increase in iron and NaCl concentration in the growth medium or sulfur deficiency may also induce lipid accumulation (Přibyl et al. 2014). Mixotrophic growth may enhance biomass and lipid production by cyanobacteria (Hamed and Klöck 2014). Mixotrophically grown *Arthrospira* had higher growth speed, faster accumulation of biomass, and enhanced resistance to high light intensities (Vonshak et al. 2000).

Co_2

Carbon dioxide (gaseous CO_2 or water-dissolved bicarbonate) is accumulated in cyanobacteria via CO_2 concentrating mechanism (CCM) and fixed into biomass by RuBisCO. It is difficult to expect any lipid accumulation under conditions of CO_2 limitation, e.g., continuous growth of cyanobacteria for 30 days in Erlenmeyer flasks in BG11 medium without CO_2 supplementation (Karatay and Dönmez 2011), since cell's energy will be consumed mainly for the synthesis of the components of CCM and carboxysomes. It is also hard to imagine the production of very long chain FAs (C_{20}–C_{22}) by cyanobacterial cells, since they do not possess C_{18}- or C_{20}-elongases characteristic for some eukaryotic algae, fungi, and higher plants. Engineered C_{20}–C_{22} biosynthesis in cyanobacteria faces difficulties because those FA elongases are intrinsic proteins of the endoplasmic reticulum, which is obviously absent in prokaryotes.

Temperature

Low temperatures induce unsaturation of fatty acids (Wada and Murata 1990). Thus, temperature of cultivation is the important factor if cyanobacterial strains are used with several fatty acid desaturases, which produce polyunsaturated FAs. To avoid this problem, the strains with only one FA desaturase may be employed, in which the desaturase expression is temperature-independent, e.g., *S. elongatus* or *Cyanobacterium* sp. (Sarsekeyeva et al. 2014).

In some cases, however, cultivation of a specific strain is not necessary, since biomass may be obtained from, for example, harmful biomass waste generated by water blooms. This biomass may be catalytically pyrolyzed and converted to a high quality bio-oil (Zeng et al. 2013).

Conclusion

Cyanobacteria are a promising source of biomass for production of biofuel (cyanofuel) because of their fast growth, high productivity, and tolerance to genetic manipulations. Extensive search and characterization of new natural cyanobacterial species with certain biotechnological properties are highly desirable. Genetic modification of strains is a powerful tool to redirect the biosynthetic pathways of cyanobacteria to desirable end-products, including those that have never been produced by these organisms (alcohols, isoprene). The technology of cyanobacterial biomass conversion to bio-oil and/or cyanodiesel should be further developed and standardized to meet the criteria of the today's demands.

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