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Current Opinion in Biotechnology

Potential of industrial biotechnology with cyanobacteria and eukaryotic microalgae

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Both cyanobacteria and eukaryotic microalgae are promising organisms for sustainable production of bulk products such as food, feed, materials, chemicals and fuels. In this review we will summarize the potential and current biotechnological developments.Cyanobacteria are promising host organisms for the production of small molecules that can be secreted such as ethanol, butanol, fatty acids and other organic acids. Eukaryotic microalgae are interesting for products for which cellular storage is important such as proteins, lipids, starch and alkanes.For the development of new and promising lines of production, strains of both cyanobacteria and eukaryotic microalgae have to be improved. Transformation systems have been much better developed in cyanobacteria. However, several products would be preferably produced with eukaryotic microalgae. In the case of cyanobacteria a synthetic-systems biology approach has a great potential to exploit cyanobacteria as cell factories. For eukaryotic microalgae transformation systems need to be further developed. A promising strategy is transformation of heterologous (prokaryotic and eukaryotic) genes in established eukaryotic hosts such as *Chlamydomonas reinhardtii*.Experimental outdoor pilots under containment for the production of genetically modified cyanobacteria and microalgae are in progress. For full scale production risks of release of genetically modified organisms need to be assessed.

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Current Opinion in Biotechnology 2013, **24**:xx–yy

This review comes from a themed issue on **Energy Biotechnology**

Edited by **Eric J Toone** and **Johannes H de Winde**

S0958-1669/$ – see front matter, Published by Elsevier Ltd.

http://dx.doi.org/10.1016/j.copbio.2013.04.004

Introduction

Algae are generally defined as photosynthetic microorganisms. They are considered as simple organisms because they are not organized into organs found in higher plants. Both prokaryotic cyanobacteria (commonly referred to as blue-green algae) and eukaryotic microalgae are commercially promising producers of chemicals and biofuels.

Commercial large-scale cultures of the eukaryotic microalga *Chlorella* were started in the early 1960s followed by the cyanobacterium *Arthrospira* in the 1970s. By 1980 large-scale algae production facilities were established in Asia, India, the USA, Israel and Australia. Food supplements from microalgae comprise an important market in which compounds such as β-carotene, astaxanthin, polyunsaturated fatty acid (PUFA) such as DHA and EPA and polysaccharides such as beta-glucan dominate [1,2].

Research on algae is not only focusing on improving production of traditional products but also on new algae products such as biodiesel, bio-ethanol and chemicals [3,4,5•]. Microalgae are considered as one of the most promising feedstock for biofuels and chemicals. Worldwide, research and demonstration programs are being carried out to develop the technology needed to expand algal energy and chemicals production from a craft to a major industrial process [6–10]. Apart from the boost in algal research generated by the oil crisis and environmental motives (climate change, CO_2 issues and land use) also technical developments such as the introduction of new generation DNA sequencers, improvements in algal genetic modification, and the rise of systems biology have contributed to the expanding of research on algae. Metabolic pathways can be introduced, deleted or changed [9,11,12].

Benefits of using both eukaryotic microalgae and cyanobacteria for sustainable production of fuels and chemicals will be summarized. We will indicate that despite being promising still a large number of bottlenecks need to be removed before eukaryotic microalgae and cyanobacteria become industrial microorganisms.

One of the items often mentioned is the use of dedicated, improved strains. In this paper we will focus on that and we will identify which features need to be improved. Furthermore we will focus on dedicated, improved strains and identify which further features need to be improved, for both cyanobacteria and eukaryotic microalgae. Transformation systems are much better developed in cyanobacteria but some products would be better to produce in eukaryotic microalgae, particularly when high intracellular product storage capacity is required. Finally aspects of scale up will be discussed.

Please cite this article in press as: Wijffels RH, et al.: Potential of industrial biotechnology with cyanobacteria and eukaryotic microalgae, Curr Opin Biotechnol (2013), http://dx.doi.org/10.1016/j.copbio.2013.04.004

Products

A number of biofuels can be produced from eukaryotic microalgae and cyanobacteria. The whole biomass can be digested into methane or processed into crude oil via pyrolysis and other thermo-chemical processes. The disadvantage of using the whole biomass for energy only is that not all the value of the biomass is used. Difficulty may reside in cost effectively producing biofuels, whereas added value compounds promise to be important drivers for microalgae development. By biorefinery it is possible to refine the biomass into different valuable products [5•,13]. Quite some efforts have been done to produce hydrogen gas, both from eukaryotic microalgae and cyanobacteria. However, this route is in a very early stage of development [14]. We believe that for biofuel purposes especially production of carbohydrates (cellulosis and starch), which can be converted into ethanol and butanol, direct production of ethanol and butanol in cyanobacteria and production of lipids which can be converted into biodiesel are the most promising routes [7,8,10,15,16].

Like for transport fuels, organic chemicals and polymers are based on fossil oil. The primary compounds are dominated by a small number of key building blocks, which are mainly converted to polymers and plastics. Eukaryotic microalgae produce hydrocarbons and polysaccharides which can be converted into building blocks such as ethylene, propylene, adipic acid and furanics [17–20] (www.eu-splash.eu). Some of these building blocks can also be directly produced by genetically engineered microalgae.

Products we would like to focus on are:

- from cyanobacteria: ethanol, butanol, fatty acids and other organic acids and accumulation of storage compounds (carbohydrates)
- from microalgae: proteins, lipids, starch, alkanes and from that the different chemicals and fuels that can be derived

Cyanobacteria

Research towards application of cyanobacteria for the production of biofuel and/or bulk chemicals has been focused on *Synechocystis* and *Synechococcus*, with much smaller additional efforts being carried out in Anabena and Cyanothece (a nitrogen-fixing strain). Representative strains from all four groups/genera have been sequenced, but for *Synechocystis* this information is available already since 1997 [21]. This has been followed up by an extensive systematic description of the organism, including application of nearly all 'omics' techniques. In both *Synechocystis* and *Synechococcus* all basic molecular-genetic techniques for metabolic engineering are available. Heterologous genes can be introduced into their genome, via natural transformation and so-called docking at a neutral site [22,23], as well as extra-chromosomally via conjugation with replicable plasmids. For each organism several resistance markers and random mutagenesis procedures are available, and markers can be deleted after mutagenesis. Basic information about weak, strong and inducible promoters is available, as well as detailed information about regulatory sequences, needed for translation. Care should be taken, after modification of the chromosome, to check proper segregation, as these cyanobacteria are polyploidic [24], although it significantly extends the time required for such modifications.

With these methods, various metabolic pathways have been introduced into *Synechocystis* and *Synechococcus*, which then allow the recombinant strains to produce any of a series of products such as: ethanol [25,16], lactic acid [26,27•], sucrose [28••], ethylene [29], isoprene [30•], iso-butyraldehyde [31••], iso-butanol [32], and 1-butanol [33•] (see Figure 1). For lactic acid and sucrose production a gene encoding a solute transporter was included in the cloned metabolic pathway [26,28••] to release the product in the extracellular medium. For other products such as lactic acid [27•] and butanediol (O Borirak, unpublished information) introduction of a such a transporter is not required. Initially product levels in the exracellualer medium were in the μg/l range, but in 2012 for several products g/l concentration were achieved (e.g. [34••,35•]). For some products this implies the need for the application of product removal (because of product toxicity, see e.g. [35•]), whereas for other, more water soluble products such as sucrose and lactic acid, these toxicity effects only occur at (sub)molar concentrations. Generally, product toxicity increases with the octanol/water partitioning, but with a complex, non-linear, relation (Table 1). Productivity of the best producing strains is still between 1 and 2 orders of magnitude below the rate of CO_2 fixation that the cells can maximally achieve.

After expansion of cyanobacterial metabolism with a heterologous metabolic pathway, and optimization of the pathway itself [33•], further optimization of product-formation and product-level can be (and has been) achieved by streamlining the cyanobacterial CO_2-fixation and intermediary metabolism. For the production of sucrose this is not even necessary, as a simple salt stress makes the cells channel nearly all (i.e. up to 90%) its fixed CO_2 into this sugar (Ducat *et al.*, 2012), which leads these authors to conclude that 'Sucrose production in engineered *S. elongatus* compares favorably with sugarcane and other agricultural crops'. However, most of the above mentioned products are derived from pyruvate. The level of pyruvate metabolite in wild type *Synechocystis* is rather low [36]. To increase product formation in that case pathways that catabolize pyruvate need to be eliminated and pyruvate synthesis pathways need to be re-enforced. This has been demonstrated amongst others for the production of ethanol, lactic acid, etc. The usefulness

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