Review

Cell PRESS

Genetic engineering of multispecies microbial cell factories as an alternative for bioenergy production

Juan Cesar Federico Ortiz-Marquez[1], Mauro Do Nascimento[1], Jonathan Philip Zehr[2], and Leonardo Curatti[1]

[1] Instituto de Investigaciones en Biodiversidad y Biotecnología – Consejo Nacional de Investigaciones Científicas y Técnicas. Mar del Plata, Buenos Aires, Argentina
[2] Ocean Sciences, University of California, Santa Cruz, CA 95064, USA

There is currently much interest in developing technology to use microlgae or cyanobacteria for the production of bioenergy and biomaterials. Here, we summarize some remarkable achievements in strains improvement by traditional genetic engineering and discuss common drawbacks for further progress. We present general knowledge on natural microalgal–bacterial mutualistic interactions and discuss the potential of recent developments in genetic engineering of multispecies microbial cell factories. This synthetic biology approach would rely on the assembly of complex metabolic networks from optimized metabolic modules such as photosynthetic or nitrogen-fixing parts.

Photosynthetic microbes for the production of bioenergy and biomaterials

Increasing food and energy demand, global climate change, and general environmental decay are some of the main current challenges for humans. Biofuels, among other alternative energy sources, have great potential to harmonize the food–energy–environment trilemma [1,2]. Photosynthetic organisms including plants, algae, and cyanobacteria capture solar energy and store it as chemical energy of their biomass (bioenergy). Thus, agriculture might serve as a source of food and bioenergy. Bioenergy is mostly captured by photosynthetic CO_2 fixation, therefore, carbon-containing biofuels have a varied tendency to be carbon neutral after combustion. The extent to which this is accomplished largely depends on the nature of the feedstock, the agricultural practice, and the industrial process, and thus it might proportionally contribute to climate change mitigation [3]. First-generation biofuels were based on edible feedstocks. Consequently, several alternative feedstocks have been proposed, mainly to alleviate food-bioenergy competition and land use change, leading to second generation or more advanced biofuels [4].

The use of microbial cell factories for bioenergy or related purposes, although not new, has regained attention as a result of increasing pressure for higher productivities, novel bioproducts, and environmental protection. It is widely appreciated that the microbial world contains by far the greatest fraction of biodiversity in the biosphere, and thus a corresponding innovation potential [5]. According to the advantages listed in Box 1, there is currently much interest in developing the technology for the use of photosynthetic microorganisms, such as eukaryotic microlgae or cyanobacteria [6,7]. Although some startup companies are already attempting to commercialize algal fuels [8], their actual potential is still a matter of debate [9].

Identifying suitable microalgae strains is usually a starting point in the roadmap towards microalgae-based technology development. The most appreciated traits for the 'ideal microalga' are listed in Box 2 [6,7]. Currently, there are no available strains excelling in all these traits, which is not surprising considering that, conversely to the development of modern plant crops, there have been no breeding programs for microalgae [10]. Although natural microalgae or cyanobacterial isolates that exceed current yields may exist, it is expected that breeding and/or genetic engineering would be required to achieve industrial production [7,10–12] Box 3.

This review comments on some of the most significant achievements in genetic engineering of microalgae and cyanobacteria and discusses some cases in which biochemical incompatibility between the recombinant pathways and host metabolism has prevented further progress. As a complementary alternative, we propose development of multispecies microbial cell factories comprising optimized parts as specialized metabolic modules to allow biochemical compartmentalization in complex metabolic networks (Figure 1).

Progress and constraints of genetic engineering of photosynthetic microbial cell factories

Microalgae and cyanobacteria may be engineered to produce a target product and there are numerous examples where this has been achieved (Table 1). One of the most remarkable accomplishments has been the direct conversion of CO_2 into alcohols, partially bypassing the complexity of the formation of biomass. Recombinant ethanol production up to 5.5 g/l has been obtained in cyanobacteria [13]. Also, isobutyraldehyde that can be used as a precursor

Corresponding author: Curatti, L. (lcuratti@fiba.org.ar).

0167-7799/$ – see front matter
 http://dx.doi.org/10.1016/j.tibtech.2013.05.006

Box 1. Main advantages of microalgae or cyanobacteria as biofuels feedstock

- At their exponential growth rate they can double their biomass in periods as short as 3.5 h.
- They can be produced almost year round under favorable weather.
- For oleaginous microalgae, the projected oil productivity per hectare would exceed by tenfold that of the best oilseed crops.
- Despite being aquatic organisms, the culture systems (open ponds or photobioreactors) demand less water than terrestrial crops, minimizing pressure on freshwater resources.
- They can be cultivated in brackish water on nonarable land, not incurring land-use change and associated environmental damage, and competence with the production of food, fodder, and other benefits from crops.
- They can take the most vital nutrients (CO_2, nitrogen, phosphorus, and others) from industrial or municipal waste, helping out to manage waste disposal.
- Microalgae cultivation does not require herbicides or pesticides application.
- They can also produce valuable co-products such as proteins and residual biomass after oil extraction, which may be used as feed or fertilizer, or fermented to produce ethanol or methane.
- Some strains produce biohydrogen either as a result of their metabolism, by means of hydrogenase and/or nitrogenase enzymes or indirectly by biomass fermentation with appropriate microorganisms.

of a variety of chemicals, including isobutanol, has been produced with a projected productivity that would exceed by five- to sixfold estimates for corn and cellulosic ethanol production on a land area basis. Isobutyraldehyde-overproducing strains were further modified to produce isobutanol as a better substitute for gasoline than ethanol [14].

However, the success of importing a heterologous pathway is influenced by a variety of drawbacks that may include combinations of different aspects.

Incompatibility of oxygenic photosynthesis with anaerobic metabolism

1-Butanol, a likely even-better substitute of gasoline than isobutanol, was successfully produced at up to 15 g/l in recombinant *Escherichia coli* cells from glucose [15]. Conversely, the more challenging task of producing 1-butanol from CO_2 in cyanobacteria remains more elusive because only up to 0.0145 g/l over 7 days could be produced and only under dark, anaerobic conditions at the expense of internal carbohydrate storage [16].

Hydrogen production is catalyzed by combinations of oxygen-sensitive hydrogenases and/or nitrogenases. Hydrogen production is normally low in cyanobacteria and microalgae and thus genetic engineering approaches have been pursued to boost that capacity by means of heterologous expression and/or hydrogenase engineering for oxygen tolerance or increased activity, inactivation of the uptake [NiFe]-hydrogenase in cyanobacteria, fine-tuning of the oxygen-evolving activity of photosystem II (PSII), optimization of the e-flux towards hydrogenase, and others [17]. However, despite many encouraging proof-of-principle demonstrations, photosynthetic hydrogen production remains low for industrial purposes.

Box 2. Most desired characteristics of the ideal photosynthetic microbial cell factory

- High efficiency of light capture and biomass yield (growth rate and final culture density).
- High production of lipid or any other useful energy carrier. Production of biomass and energy carrier at the same time.
- Large cells and/or flocculation properties to facilitate harvesting.
- Thin cell walls or with structural characteristics for easy intracellular products extraction.
- Tolerance to high light intensity and oxygen concentration.
- Resistance to contamination with other microorganisms or predators.
- High efficiency of the use of cellular nitrogen and phosphorus and high nutrient-recycling capacity and/or ability to utilize abundant and/or inexpensive alternative sources of nitrogen, phosphorus, and other macroelements.
- Amenability to genetic analysis and manipulation.
- Probably less important aspects such as minimizing respiration rates to minimize carbon dissimilation, decreasing the rate of photoacclimation to low light to slow down self-shading after transition to high light, lowering the carbon:nitrogen ratio of core nitrogenous components and the amount of carbon allocated to cell structure, and the potential to contribute to enhance biofuels yield.

Box 3. Multispecies microbial cell factories

Microbial consortia usually perform more complex tasks than monocultures and can perform functions that are difficult or even impossible for individual strains or species.

This approach may alleviate pressure towards increasing yields of heterologous pathways in selected expression platforms, for which overall metabolic coupling is weak and/or mostly poorly understood. In turn, the concept of genetic engineering of multispecies microbial cell factories might rely, as a starting point, on microbes with one or a few outstanding properties as metabolic/production modules, which can be further optimized for the same or other 'easier-to-accomplish' tasks. This approach takes advantage of the possibility of metabolic compartmentalization between or among cells with different characteristics and/or onto which the burden of overexpressing multiple genes can be split among the partners of the consortium. Communication for trading extracellular metabolites is a key aspect of the principle which on one hand increases the level of complexity and on the other hand might provide opportunities for additional possibilities of regulation of the productive platforms and/or removing toxic byproducts (if any).

It is expected that better understanding of the natural assemblages of microbial communities and detailed knowledge of naturally occurring symbiosis will represent a lead for biologically inspired design of multispecies microbial cell factories to complement current efforts using more conventional genetic engineering approaches.

Host tolerance to high concentration of recombinant-pathway product and/or precursors

In contrast to ethanologenic microorganisms such as the yeast *Saccharomyces cerevisiae* or the bacterium *Zymomonas mobilis* that tolerate >15% ethanol in the external medium, the cyanobacterium *Synechocystis* sp PCC 6803 tolerates only up to 1%. Although such inhibitory concentrations have not been met from ethanol producing cyanobacteria [13], it has been shown that imbalances between the recombinant enzymes activities may result in growth inhibitory concentrations of the toxic intermediate acetaldehyde [18]. Interestingly, a recent study showed improved recombinant-biodiesel yields in *E. coli* by a dynamic sensor–regulator system that adjusts recombinant enzymes activities according to the levels of the host key metabolites [19].

Table 1. Current and potential biofuels from cyanobacteria or modified microalgae[a]

Bioproduct	Strategy	Results/comment	Refs
Cyanobacteria			
Ethanol	H.E. *pdc* and *adh* from *Zymomonas mobilis* into *Synechocystis* sp. PCC 6803	5.2 mmol ethanol OD_{730}/u.l.d (up to 230 mg/l in 4 weeks)	[13]
	H.E. *pdc* from *Z. mobilis*, overexpressing *slr1192*, and disruption of the biosynthetic pathway of poly-b-hydroxybutyrate into *Synechocystis* sp. PCC 6803	212 mg/l.d	[18]
Isobutyraldehyde	H.E. *kivD* from *Lactobacillus lactis*, *alsS* from *Bacillus subtilis*, *ilvCD* from *Escherichia coli* and insertion of extra copies of *rbcLS* genes in *Synechococcus elongatus* PCC7942	6230 μg isobutyraldehyde/l.h Higher than cyanobacterial productivities of hydrogen or ethanol.	[14]
Isobutanol	H.E. *kivD* from *L. lactis* and *yqhD* from *E. coli* in *S. elongatus* PCC7942	450 mg isobutanol/l in 6 d	[14]
1-Butanol	H.E. *hbd*, *crt*, and *adhE2* genes from *Clostridium acetobutylicum*, *ter* from *Treponema denticola*, and *atoB* from *E. coli* in *S. elongates* PCC7942	14.5 mg 1-butanol /l in 7 d	[16]
	H.E. *bldh* from *Clostridium saccharoperbutylacetonicum*, *yqhD* from *E. coli* (among others) in *S. elongates* PCC7942	29.9 mg 1-butanol/l	[59]
Isoprene production	H.E. *ispS* from *Pueraria montana* expressed under regulation of the *psbA2* promoter in *Synechocystis* sp. PCC 6803	50 μg isoprene/g dw.d under high light conditions	[60]
Fatty acids	H.E. a thioesterase, among others modifications, in *Synechocystis* sp. PCC 6803	200 mg/l fatty acids secreted into the culture medium	[61]
Fatty alcohols	H.E. *far* from *Simmondsia chinensis* under the control of P_{rbc} promoter	0.2 mg/l fatty alcohols	[62]
Hydrogen	H.E. [Ni-Fe] hydrogenase *hynSL* from *Alteromonas macleodii*, and *Thiocapsa roseopersicina* and accessory genes in *S. elongatus* PCC7942	Accumulation of holo-enzyme that displayed hydrogenase activity *in vitro*	[63]
	[FeFe]-hydrogenase *hydA1* from *Cr* in *Synechocystis* sp. PCC 6803	*in vitro*-active hydrogenase	[64]
	Insertional disruption of the *hupL* gene in *Nostoc* sp. PCC 7422	100 μmoles H_2/mg chlorophyll a.h (three times higher rate than that of the parental strain)	[65]
Microalgae			
Triacylglycerol (TAG)	*sta6* and *sta7-10* starchless mutants of *Cr*	2- to 10-fold increase in TAG accumulation	[66–68]
	sta7-10 (isoamylase mutants) complemented strains	≥400 mg/l starch exceeding in 4 d (significantly higher than those achieved by the parental strains) Higher values in total lipids at 96 h of nitrogen deprivation: 83–118 mg lipid/l	[68]
Hydrogen	Replacement of wild type promoter of the Nac2 gene by a copper repressible promoter in *Cr*	20 μmoles H_2/l 1–3.1 mmol/H_2 mol. Chl	[69]
	Mutant strain with a double amino acid substitution (L159I-N230Y) in D1 of *Cr*	500 ml H_2/l; 5.77 ml/l.d ≥10-fold higher wild type and fivefold CC124 strain, mostly due to a longer production phase	[70]
	Optimization of the e- flux towards hydrogenase *state transitions mutant6* (*stm6*) in *Cr*	540 ml H_2/l 4 ml/h (fivefold higher than the wild type)	[71]

[a] *Cr*, *Chlamydomonas reinhardtii*; H.E., heterologous expression; u.l.d, unit.litre.day.

Assembly of complex enzymes

Enzymes such as hydrogenases or nitrogenases contain at their active sites complex metal centers. Biosynthesis of the iron–molybdenum cofactor and assembly of fully functional holo-nitrogenase requires at least 15 gene products. Although knowledge on this field has progressively increased during recent years, it is still fragmentary [20,21] and limits recombinant pathway design and introduction into selected hosts.

General physiological and metabolic adaptations

During the course of evolution, some microbes become exquisitely specialized to carry out some metabolic pathways for which they orchestrate sophisticated arrays of physiological and metabolic adaptations. *Azotobacter vinelandii* displays a concerted set of mechanisms (increased aerobic respiration and exopolysaccharide production and duplication of housekeeping genes) for its particular specialization to run strictly anaerobic pathways (mainly N_2- fixation and H_2 metabolism) together with an obligate aerobic life style [22]. Similarly, genetic, biochemical and genomic analyses uncovered unique characteristics for the remarkably high ethanol producer *Z. mobilis*, including a specific metabolic pathway for anaerobic fermentation of glucose and an array of genetic determinants that might account for the exceptional ethanologenic properties [23]. In some other cases, knowledge of the genetic basis of some metabolic switches is not available to design strategies genetic improvement. This might be the case of those targeted bioenergy carriers that

constitute the natural carbon reserves of cyanobacteria and microalgae (carbohydrates or lipids) that normally accumulate under unbalanced growth after nutritional or environmental adverse conditions compromise overall productivity [24]. The kind of genetic determinants for the previous examples are currently difficult to identify because they used to be encoded by a multiplicity of genes that contribute to the trait in an additive fashion; sometimes synergistic or even as emergent properties of the system. This fact highlights the challenge of optimizing recombinant pathways in hosts selected for other beneficial traits.

Limitation of molecular biology toolkit for cyanobacteria and microalgae

This aspect also precludes a faster improvement of strains by genetic engineering in the short term. Thus, recombinant pathway–host overall incompatibilities may include combinations of the previously discusses aspects in addition to unforeseen ones.

Natural microalgal–bacterial consortia as a source of biotechnological insights

In the wild, microalgae live and have evolved in the context of multispecies microbial consortia. Beneficial interactions range from mutualistic to symbiotic, and their stability relies on two important organizing features: (i) trading of metabolites, mainly as crossfeeding but also exchange of dedicated molecular intra- or interspecific signals; and (ii) specialization and division of labor [25].

In most algae–bacteria consortia, microalgae provide oxygen and organic molecules. In natural systems, the algal release of dissolved organic carbon ranges from 0 to 80% of photosynthates and it is 6–16% in photobioreactors [26]. For bioenergy purposes, the latter can be seen as both a direct bioenergy loss and a potential secondary loss due to the assembly of spontaneous and unattractive microbial consortia with low or null biotechnological value [27].

By contrast, bacteria could provide a broader array of substances including CO_2, other nutrients, vitamins, and growth-promoting substances (Figure 2).

Carbon for nitrogen mutualisms

Although nitrogen is extremely abundant on Earth as N_2, the availability of biologically available nitrogen constrains the productivity of both terrestrial and aquatic ecosystems. Nonetheless, N_2-fixing symbioses are common between eukaryotes and cyanobacteria or heterotrophic bacteria and represent a remarkable strategy by which some organisms can use N_2 from the air indirectly in exchange for organic carbon. In contrast to the photosynthetic carbon-fixation ability that eukaryotes have acquired through endosymbiosis with cyanobacteria during evolution, no N_2-fixing plastids or eukaryotes are known [28]. In the oceans, the best-known N_2-fixing symbioses are between several species of pennate diatoms and heterocyst-forming cyanobacteria. These associations span the range of epibionts (*Calothrix–Chaetoceros*) to endosymbionts (*Richelia–Rhizosolenia* and *Richelia–Hemiaulus*) [29,30]. The cyanobacteria form short chains of vegetative cells plus a terminal heterocyst, which is a differentiated cell for N_2 fixation that provides a microaerobic environment to protect nitrogenase from inactivation by the eukaryotic host oxygenic photosynthesis. Although little is known about the molecular basis for these interactions, or how the symbionts are transmitted from generation to generation [31], there is genome streamlining associated with the endosymbiotic species. The closely associated species within the frustule lack ammonium transporters, and one species is the only known cyanobacterium that lacks glutamate synthase [31]. There appears to be some specificity between the cyanobacteria and the host genera [32]. A widely distributed marine planktonic uncultured N_2-fixing cyanobacterium (UCYN-A) presents a dramatic reduction in genome size and lacks the genes for photosystem II (responsible for O_2 evolution), RuBisCo (ribulose-1,5-bisphosphate carboxylase-oxygenase for carbon fixation), and the tricarboxylic acid cycle (for aerobic respiration and anabolism) [33]. It was further shown that UCYN-A engages in mutualistic relations with a prymnesiophyte exchanging fixed nitrogen for fixed carbon. A similar association appears to exist with freshwater diatoms and it is proposed that these rather simple interactions between single-celled organisms might mirror those earlier primary endosymbiotic events that gave origin to chloroplasts and mitochondria [34].

Carbon for other nutrients or growth-promoting substances

Iron is an essential element for life and its low bioavailability also limits productivity in large areas of the oceans. Alga-associated heterotrophic bacteria belonging to the genus *Marinobacter* release the siderophore vibrioferrin (VF) in a carbon for iron mutualistic relationship [35].

A large proportion of microalgae are auxotrophs or facultative auxotrophs for vitamins, especially B12, and at least some of them can acquire B12 from more or less specific interactions with heterotrophic bacteria [27].

In some cases heterotrophic bacteria appear to provide phytohormone-like substances (indole-3-acetic acid) to enhance several microalgal activities. This has been shown for the effect of coculturing *Chlorella* spp. with the plant growth-promoting bacterium *Azospirillum brasilense* for an increased pigment and lipid content, lipid variety, carbohydrates, and cell and population size of the microalgae [36–38].

Such associations between unicellular microorganisms may provide models for means to interact with cyanobacterial/microalgal metabolism for biotechnological applications.

Biotechnological use of microalgal–bacterial consortia as multispecies microbial cell factories

The combined use of algae and bacteria in microbial consortia is gaining renewed interest as a biotechnological alternative for the enhancement of yields and reducing production costs of algal biomass for bioenergy purposes in integrated bioprocesses together with CO_2 and pollutant removal [39,40].

The *A. brasilense* stimulation of *Chlorella* spp. growth and lipids and starch accumulation [36,41] represents a

remarkable example of the biotechnological potential of microalgal–bacterial consortia pertinent to bioenergy. Also, inoculation with the *Pseudomonas*-related strain GM41 increased *Synechocystis* sp PCC6803 productivity up to eightfold by helping to degrade toxic compounds commonly found in polluted water [42].

Another application of microalgal–bacterial consortia is the development of microbial solar cells that comprise photoautotrophic microorganisms to harvest solar energy and release organic compounds that are used by electrochemically active microorganisms to generate electricity [43].

Engineering cyanobacterial/algal–bacterial multispecies microbial cell factories

Multispecies microbial consortia can be subjected to traditional genetic engineering or modern synthetic biology approaches to improve current applications of microbial consortia or envision new ones. Synthetic biology relies in analogies between biological networks and electronic circuits comprising computing reusable parts and connectors (wires). However, both are current major concerns for synthetic circuits design and intended function in single cells. As part of a solution, it has been shown how the distribution of simple computations among the members of non-uniform populations would increase exponentially the complexity of circuits that could be executed, bypassing the need for extensive genetic engineering of a single strain, sometimes very difficult to achieve, and the reusability of the parts while reducing the wiring requirement [44,45]. Thus, application of this principle might be appealing for genetic engineering of cyanobacteria/algae, for which the genetic toolkit is limited. This approach may alleviate pressure towards increasing yields of heterologous pathways in selected expression platforms, for which overall metabolic coupling (wiring) is weak and/or mostly poorly understood. In turn, the concept of genetic engineering of multispecies microbial cell factories might rely on microbes with outstanding properties as metabolic/production modules, which can be further optimized for the same or other 'easier-to-accomplish' tasks.

Microbial consortia comprising heterotrophic cells have been engineered to disclose some organizational features of multicellularity, ecology, and evolution [46], and to pursue a variety of possible applications. Some remarkable examples pertinent to biofuels production from CO_2, although indirectly, are: (i) the assembly of functional minicellulosomes by intercellular complementation using a synthetic yeast consortium that divides the metabolic burden of expressing high levels of recombinant proteins among the members of the consortium; (ii) simultaneous and efficient fermentation of hexoses and pentoses from lignocellulose by coculture of engineered strains specialized in the fermentation of each sugar; and (iii) engineering of two strains of *E. coli* that cooperate in the transformation of xylan into ethanol; while one strain secretes two hemicellulases the other uses the released sugars to produce ethanol [45,47].

Two recent examples of cyanobacteria/algae–bacteria artificial consortia comprising genetically modified microbes as CO_2- or N_2-fixing synthetic parts illustrate the potential of this approach for the sustainable production of biofuels and biomaterials directly from CO_2.

Carbon-fixation cell factories

The cost of the carbon source in commercial fermentations can be as much as 30–50% of the overall operating cost, and hence the interest in developing photosynthetic microbial-cell factories primarily based on cyanobacteria or microalgae. Recent progress in the heterologous expression of the *E. coli* sucrose permease *cscB* in the cyanobacterium *Synechococcus elongatus* has allowed the irreversible export of sucrose into the medium at concentrations >10 mM without culture toxicity. Remarkably, the sucrose-exporting cyanobacterium exhibits increased biomass production rates relative to the wild type strain, and enhances photosystem II activity, carbon fixation, and chlorophyll content. Additional mutations to minimize carbon flux towards competing glucose- or sucrose-consuming reactions further improves sucrose production up to 80% of total biomass. Such a strain/strategy may be a viable alternative to sugar synthesis by terrestrial plants, including sugar cane [48]. Similarly, *S. elongatus* transformed with *Z. mobilis invA* and *glf* genes, encoding invertase and a glucose/fructose facilitator, respectively, excreted sugars into the medium in such a way that it supported *E. coli* growth in the absence of supplementation with a carbon source [49]. Metabolic coupling of photosynthetic modules to *E. coli*, for which advances in genetic engineering and industrial applications have few (if any) rivals, is of remarkable importance. Coculture of sugar-excreting cyanobacteria with any other second engineered microbe could yield a desired product without a reduced-carbon feedstock in situations where synthesis of the product is incompatible with cyanobacterial metabolism (Figure 1A). Moreover, attempts to introduce wild type cyanobacteria as 'synthetic chloroplasts' into animal cells were successful as a proof-of-principle. However, calculations indicated that even if the sugar excreting strains were introduced, a significantly higher ratio of bacterial-to-animal cells would be required to provide an adequate supply of sugar to the heterotrophic host [50].

Nitrogen-fixing cell factories

Large-scale culture of microalgae, which have an average composition of $CH_{1.7}O_{0.4}N_{0.15}P_{0.0094}$, might represent a high-nitrogen-intensive bioprocess, if wastewater and/or other alternatives are not used as partial or complete substitutes for nitrogen fertilizers [51–53]. Although promotion of symbiotic N_2 fixation represents a remarkable strategy in agriculture, the sophisticated interplay of recognition signals that underlay most known N_2-fixing symbiosis [28] has complicated the development of exchangeable N_2-fixing parts for synthetic biology approaches.

Most free-living diazotrophs normally fix enough N_2 for their needs and excrete low to undetectable amounts of N_2-fixation products into the medium. Recently, it was shown that disruption of the genetic system signaling the nitrogen status in an *Az. vinelandii* mutant strain by inactivation of the *nifL* gene, expresses nitrogenase constitutively and excretes ammonium into the surrounding medium. Con-

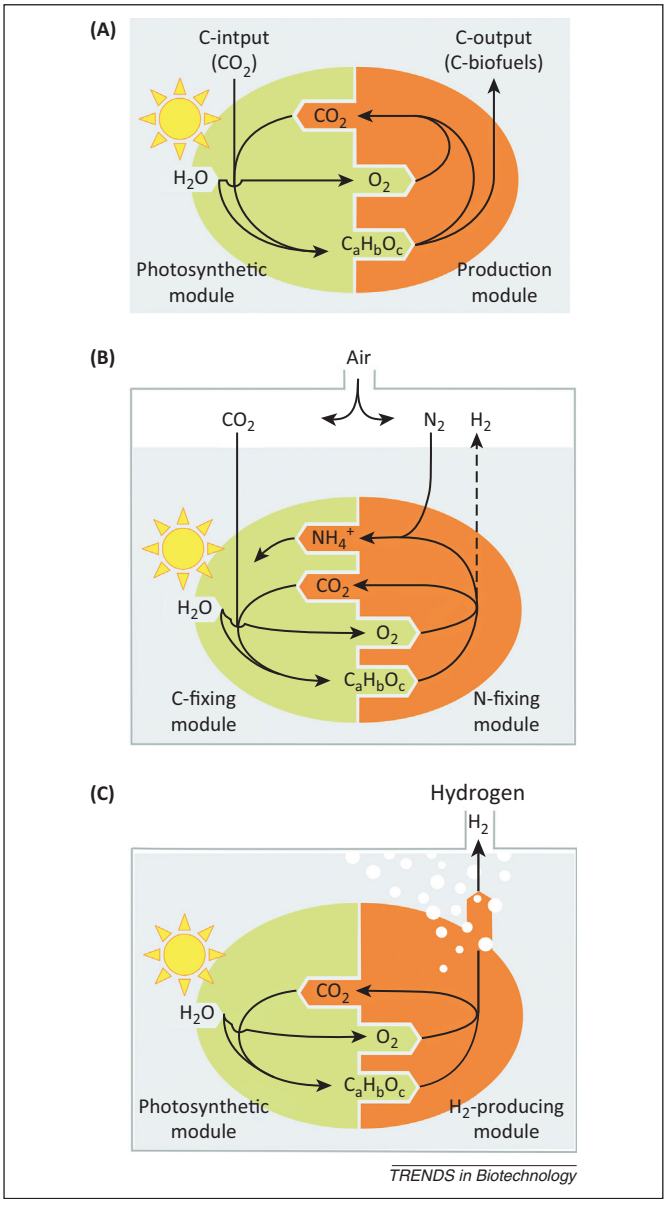

Figure 1. Conceptual diagrams of multispecies microbial cell factories. **(A)** General overview of the principle consisting in the metabolic compartmentalization between a specialized photosynthetic module cross-feeding organic carbon, likely simple sugars ($C_aH_bO_c$), and O_2 to a production module for carbon-containing biofuels with some CO_2 recycling. Although this is a widespread metabolic loop in natural communities, recent improvement by genetic engineering has represented valuable proof-of-concept suggesting that it is worth further development of this principle for biotechnological purposes. **(B)** Model of carbon–nitrogen exchanging synthetic multispecies microbial cell factory that would allow the production of biomass from carbon and nitrogen from the air as an alternative platform to diazotrophic cyanobacteria. A recent demonstration showed how rather simple genetic engineering manipulations can make microbes more prone to engage in mutualistic relations that would reduce the cost of biomass production and provide a platform with metabolic spatial compartmentalization to facilitate further optimization of specific pathways. **(C)** Hypothetical model derived from (B) for the nitrogenase-dependent production of H_2. A few microbes such as *Azotobacter vinelandii* produce H_2 in the air by means of nitrogenases. In the absence of N_2, more electrons are diverted to the reduction of H^+ by nitrogenases. Although we are not aware of such a demonstration, we anticipate that both the available knowledge on N_2 and H_2 metabolisms and molecular tools for genetic manipulation of *Az. vinelandii* would make it possible to challenge this hypothesis in the near future.

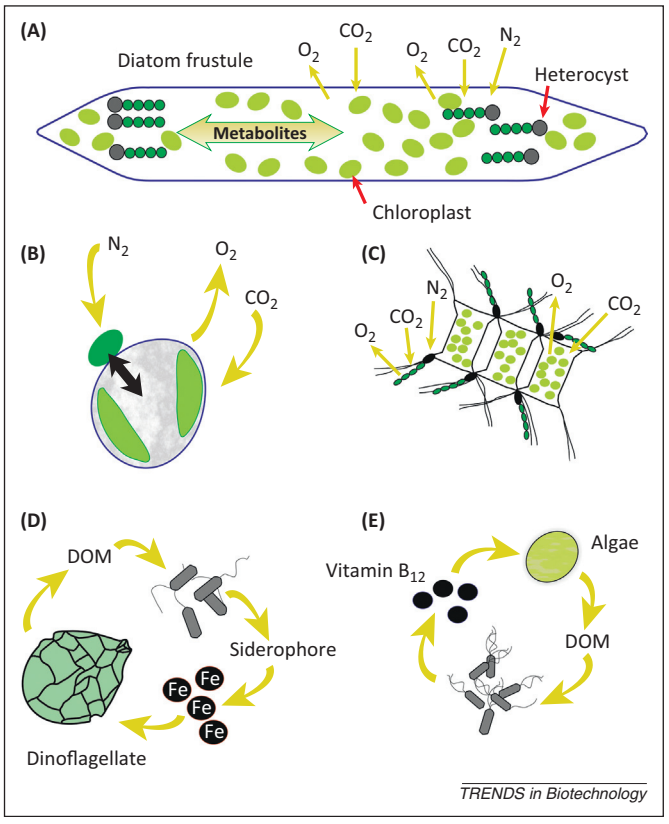

Figure 2. Natural oceanic N_2-fixing algal–bacterial interactions. (A–C) Carbon–nitrogen mutualistic interactions. **(A)** Interaction between a diatom and endosymbiotic filamentous heterocyst-forming (specialized cell for N_2 fixation). **(B)** *Atelocyanobacterium thalassa* (UCYN-A) and as yet unidentified prymnesiophyte similar to *Braarudosphaera bigelowii*. **(C)** Endosymbiotic filamentous cyanobacteria and chain forming diatoms (adapted from [29]). **(D)** Carbon–iron mutualistic interaction between the dinoflagellate *Scripsiella trochoidea* and the bacterium *Marinobacter* sp. strain DG879 [35]. **(E)** Carbon–B_{12} vitamin mutualistic relation between the green microalgae and the bacteria of the order Rhizobiales [27]. Abbreviation: DOM, dissolved organic matter.

versely to wild type *Az. vinelandii*, the ammonium-excreting strain engaged in carbon–nitrogen mutualistic relations with the oleaginous microalgae *Chlorella sorokiniana*, *Pseudokirchneriella* sp., and *Scenedesmus obliquus* [51], when no sources of carbon or nitrogen other than air were supplemented into the medium. Inoculation with the ammonium-excreting strain mimicked microalgal growth equivalent to an ammonium amendment of up to 0.5 mM. In these artificial symbioses, although the number of viable bacterial cells decreased dramatically for the wild type strain, it remained more stable for the ammonium-excreting cells, suggesting that it was engaged in a more robust symbiotic relation with microalgae. Mostly microalgal cells proliferated during coculture, therefore, the resulting biomass mirrored the composition of that of the oleaginous microalgae, which attained lipid contents of up to 30% on a dry biomass basis [51]. Both the metabolic pathways for biological N_2 fixation and triacylglycerol accumulation are rarely found in any single native microbial strain [54], and would likely be difficult to attain by genetic engineering of a single strain [55]. In addition, this N_2-fixing module showed an apparently low selection of partner because it could be metabolically coupled with diverse wild type microalgae or cyanobacteria. This case study provides proof-of-concept for the development of N_2-fixing modules that might be coupled to photosynthetic and sugar-producing synthetic cellular parts for enhanced biomass production and a metabolically compartmentalized

platform (Figure 1B). *Az. vinelandii*, already proposed as an alternative host for the expression of anaerobic pathways [22], might represent an alternative host under consideration for hydrogen production and possibly other O_2-sensitive pathways (Figure 1C).

Living *Az. vinelandii* cells have been successfully introduced into either plant or microalgal cells to construct synthetic N_2-fixing endosymbiotic systems [56], making future steps for introducing optimized N_2-fixing modules into eukaryotic cells feasible.

Several filamentous heterocyst-forming cyanobacteria may also serve as carbon- and N_2-fixing metabolically compartmentalized platforms. In this case the carbon- and N_2-fixing modules would be inseparable, which would be only useful for some potential applications. Nevertheless, it would be desirable to encourage the development of the required genetic tools to bring into play these naturally compartmentalized metabolic platforms [57].

Additional possibilities

Industrial production of biofuels and raw materials would require large scale nonaxenic cultivation of microorganisms, probably in open ponds. This fact would open additional possibilities for synthetic consortia, for example, to control microbial competitors or grazers for crop protection [6,10] by engineered microbes producing specific antimicrobials similarly as has been shown for biomedical applications [47]. Engineered bacteria can also be introduced to provide or amplify signals triggered by expensive chemical effectors, to aid in the utilization of recalcitrant nonexpensive nutrients or stress tolerance, to facilitate harvesting or downstream processing.

Box 4. Outstanding questions

The most significant aspects that need to be addressed to advance the field should be approached multidisciplinary:

- *Synthetic biology aspects*
  The genetic toolkit for nonconventional microbes (other than *E. coli* or yeast) naturally efficient to perform specific tasks needs to be improved and shared among public and private sectors. More specific cases that would benefit from the used of synthetic microbial consortia should be identified to stimulate creative thinking on synthetic circuits design. The real challenge in design would be to account for all the aspects below.
- *Cultivation strategies*
  Physical separation of the consortium partners might not be practical for large-scale cultivation. Thus, detailed characterization of prototype synthetic consortia including nutritional and cultivation requirements is needed. This aspect should be developed back-to-back together with pilot-scale ponds/photobiorreactors design. When physical insulation cannot be bypassed, synthetic biology designs for self structured microbial communities and engineering alternatives for inexpensive reactors construction might be considered.
- *Life cycle analysis*
  The previous activities should complete the set of data to assess the economic and environmental sustainability and benefits of the alternative use of different synthetic consortia for biofuels or bulk chemicals production for a variety of hypothetical scenarios.
- *Ecological risk*
  As for any other genetically modified organism, the ecological risk associated to its liberation to the environment, even in a semicontained way as algal culture would be, should be modeled in advance so as to be taken into account while considering alternative designs [11,72].

Beyond the biotechnological potential of artificial microbial consortia, much work is left to optimize custom-made parts to fit a variety of purposes. Detailed knowledge of naturally occurring symbiosis might represent a lead for biologically inspired design without resigning the innovation capacity [58].

Concluding remarks

Proof-of-concept demonstration of carbon- or nitrogen-releasing modules may catch the attention of more researchers to develop and apply the concept of multispecies microbial cell factories. Much work is ahead to continue exploring its potential as well as to identify specific drawbacks (Box 4). Consideration of this alternative might also have some influence on strain selection and bioprospecting because bioresources that are not attractive as single strain cell factories might be valuable as metabolic modules. Thus, the multispecies microbial cell factories concept is a promising alternative to long-standing problems in genetic engineering of complex metabolic pathways to improve sustainable production of bioenergy and other commodities.

Acknowledgements

Many pioneering and/or relevant articles could not be cited because of space limitations. We are very grateful to the editor and reviewers for constructive and insightful comments. This work will be included in the Ph.D. thesis of J.C.F.O-M (UNMdP). J.C.F.O-M and M.D.N. are fellows and L.C. is a career researcher at the CONICET, Argentina. Supported by ANPCyT Grants PICT-2007 01717 and PICT-2011 2705, CONICET Grant PIP 2012-2014 N° 1032 and FIBA. This work is dedicated to the memory of Julio Curatti, who passed away on February, 27th, 2013.

Disclaimer statement

The authors declare no conflict of interest.

References

1 Tilman, D. *et al.* (2009) Beneficial biofuels: the food, energy, and environment trilemma. *Science* 325, 270–271
2 Harvey, M. and Pilgrim, S. (2010) The new competition for land: food, energy, and climate change. *Food Policy* 36 (Suppl. 1), S40–S51
3 Johnson, E. (2009) Goodbye to carbon neutral: getting biomass footprints right. *Environ. Impact Assess. Rev.* 29, 165–168
4 Naik, S.N. *et al.* (2010) Production of first and second generation biofuels: a comprehensive review. *Renew. Sustain. Energy Rev.* 14, 578–597
5 Beloqui, A. *et al.* (2008) Recent trends in industrial microbiology. *Curr. Opin. Microbiol.* 11, 240–248
6 Wijffels, R.H. and Barbosa, M.J. (2010) An outlook on microalgal biofuels. *Science* 329, 796–799
7 Rasala, B.A. *et al.* (2013) Genetic engineering to improve algal biofuels production. In *Algae for Biofuels and Energy* (Borowitzka, M.A. and Moheimani, N.R., eds), pp. 99–113, Springer
8 Chisti, Y. and Yan, J. (2011) Energy from algae: current status and future trends: algal biofuels: a status report. *Appl. Energy* 88, 3277–3279
9 Petkov, G. *et al.* (2012) A critical look at the microalgae biodiesel. *Eur. J. Lipid Sci. Technol.* 114, 103–111
10 Georgianna, D.R. and Mayfield, S.P. (2012) Exploiting diversity and synthetic biology for the production of algal biofuels. *Nature* 488, 329–335
11 Flynn, K.J. *et al.* (2013) Monster potential meets potential monster: pros and cons of deploying genetically modified microalgae for biofuels production. *Interface Focus* 3, http://dx.doi.org/10.1098/rsfs.2012.0037
12 Larkum, A.W.D. *et al.* (2012) Selection, breeding and engineering of microalgae for bioenergy and biofuel production. *Trends Biotechnol.* 30, 198–205
13 Dexter, J. and Fu, P. (2009) Metabolic engineering of cyanobacteria for ethanol production. *Energy Environ. Sci.* 2, 857–864
14 Atsumi, S. *et al.* (2009) Direct photosynthetic recycling of carbon dioxide to isobutyraldehyde. *Nat. Biotechnol.* 27, 1177–1180

15 Shen, C.R. *et al.* (2011) Driving forces enable high-titer anaerobic 1-butanol synthesis in *Escherichia coli*. *Appl. Environ. Microbiol.* 77, 2905–2915
16 Lan, E.I. and Liao, J.C. (2011) Metabolic engineering of cyanobacteria for 1-butanol production from carbon dioxide. *Metab. Eng.* 13, 353–363
17 Srirangan, K. *et al.* (2011) Biochemical and genetic engineering strategies to enhance hydrogen production in photosynthetic algae and cyanobacteria. *Bioresour. Technol.* 102, 8589–8604
18 Gao, Z. *et al.* (2012) Photosynthetic production of ethanol from carbon dioxide in genetically engineered cyanobacteria. *Energy Environ. Sci.* 5, 9857–9865
19 Zhang, F. *et al.* (2012) Design of a dynamic sensor-regulator system for production of chemicals and fuels derived from fatty acids. *Nat. Biotechnol.* 30, 354–359
20 Rubio, L.M. and Ludden, P.W. (2008) Biosynthesis of the iron-molybdenum cofactor of nitrogenase. *Annu. Rev. Microbiol.* 62, 93–111
21 Peters, J.W. *et al.* (2013) Hydrogenases, nitrogenases, anoxia, and H_2 production in water-oxidizing phototrophs. In *Algae for Biofuels and Energy* (Borowitzka, M.A. and Moheimani, N.R., eds), pp. 37–75, Springer
22 Setubal, J.o.C. *et al.* (2009) Genome sequence of *Azotobacter vinelandii*, an obligate aerobe specialized to support diverse anaerobic metabolic processes. *J. Bacteriol.* 191, 4534–4545
23 Seo, J-S. *et al.* (2005) The genome sequence of the ethanologenic bacterium *Zymomonas mobilis* ZM4. *Nat. Biotechnol.* 23, 63–68
24 Do Nascimento, M. *et al.* (2012) Bioprospecting for fast growing and biomass characterization of oleaginous microalgae from South-Eastern Buenos Aires, Argentina. *Bioresour. Technol.* 125, 283–290
25 Brenner, K. *et al.* (2008) Engineering microbial consortia: a new frontier in synthetic biology. *Trends Biotechnol.* 26, 483–489
26 Lakaniemi, A-M. *et al.* (2012) Eukaryotic and prokaryotic microbial communities during microalgal biomass production. *Bioresour. Technol.* 124, 387–393
27 Kazamia, E. *et al.* (2012) Synthetic ecology-a way forward for sustainable algal biofuel production? *J. Biotechnol.* 162–169
28 Charpentier, M. and Oldroyd, G. (2010) How close are we to nitrogen-fixing cereals? *Curr.Opin. Plant Biol.* 13, 556–564
29 Zehr, J.P. (2013) Interactions with partners are key for oceanic nitrogen-fixing cyanobacteria: ocean-dwelling cyanobacteria associate with a variety of other microorganisms, including those that are photosynthetic. *Microbe* 8, 117–122
30 Foster, R.A. *et al.* (2010) Isolation of *Calothrix rhizosoleniae* (cyanobacteria) strain SC01 from *Chaetoceros* (Bacillariophyta) spp. diatoms of the subtropical northern pacific ocean. *J. Phycol.* 46, 1028–1037
31 Hilton, J.A. *et al.* (2013) Genomic deletions disrupt nitrogen metabolism pathways of a cyanobacterial diatom symbiont. *Nat. Commun.* http://dx.doi.org/10.1038/ncomms2748
32 Zehr, J.P. (2011) Nitrogen fixation by marine cyanobacteria. *Trends Microbiol.* 19, 162–173
33 Tripp, H.J. *et al.* (2010) Metabolic streamlining in an open-ocean nitrogen-fixing cyanobacterium. *Nature* 464, 90–94
34 Thompson, A.W. *et al.* (2012) Unicellular cyanobacterium symbiotic with a single-celled eukaryotic alga. *Science* 337, 1546–1550
35 Amin, S.A. *et al.* (2012) Siderophore-mediated iron uptake in two clades of *Marinobacter* spp. associated with phytoplankton: the role of light. *Biometals* 25, 181–192
36 Choix, F.J. *et al.* (2012) Enhanced accumulation of starch and total carbohydrates in alginate-immobilized *Chlorella* spp. induced by *Azospirillum brasilense*: II. Heterotrophic conditions. *Enzyme Microb. Technol.* 51, 300–309
37 de-Bashan, L.E. *et al.* (2008) Involvement of indole-3-acetic acid produced by the growth promoting bacterium *Azospirillum* spp. in promoting growth of *Chlorella vulgaris*. *J. Phycol.* 44, 938–947
38 Tate, J.J. *et al.* (2012) The effects of plant growth substances and mixed cultures on growth and metabolite production of green algae *Chlorella* sp.: a review. *J. Plant Growth Regul.* 1–12
39 Olguin, E.J. (2012) Dual purpose microalgae-bacteria-based systems that treat wastewater and produce biodiesel and chemical products within a biorefinery. *Biotechnol. Adv.* 30, 1031–1046
40 Subashchandrabose, S.R. *et al.* (2011) Consortia of cyanobacteria/microalgae and bacteria: biotechnological potential. *Biotechnol. Adv.* 29, 896–907
41 de-Bashan, L.E. *et al.* (2002) Removal of ammonium and phosphorus ions from synthetic wastewater by the microalgae *Chlorella vulgaris* coimmobilized in alginate beads with the microalgae growth-promoting bacterium *Azospirillum brasilense*. *Water Res.* 36, 2941–2948
42 Abed, R.M.M. (2010) Interaction between cyanobacteria and aerobic heterotrophic bacteria in the degradation of hydrocarbons. *Int. Biodeterior. Biodegrad.* 64, 58–64
43 Strik, D.P.B.T.B. *et al.* (2011) Microbial solar cells: applying photosynthetic and electrochemically active organisms. *Trends Biotechnol.* 29, 41–49
44 Macía, J. *et al.* (2012) Distributed computation: the new wave of synthetic biology devices. *Trends Biotechnol.* 30, 342–349
45 Shong, J. *et al.* (2012) Towards synthetic microbial consortia for bioprocessing. *Curr. Opin. Biotechnol.* 23, 798–802
46 Tanouchi, Y. *et al.* (2012) Engineering microbial systems to explore ecological and evolutionary dynamics. *Curr. Opin. Biotechnol.* 23, 791–797
47 Chuang, J.S. (2012) Engineering multicellular traits in synthetic microbial populations. *Curr. Opin. Chem. Biol.* 16, 370–378
48 Ducat, D.C. *et al.* (2012) Rerouting carbon flux to enhance photosynthetic productivity. *Appl. Environ. Microbiol.* 78, 2660–2668
49 Niederholtmeyer, H. *et al.* (2010) Engineering cyanobacteria to synthesize and export hydrophilic products. *Appl. Environ. Microbiol.* 76, 3462–3466
50 Agapakis, C.M. *et al.* (2011) Towards a synthetic chloroplast. *PLoS ONE* 6, e18877
51 Ortiz-Marquez, J.C.F. *et al.* (2012) Association with an ammonium-excreting bacterium allows diazotrophic culture of oil-rich eukaryotic microalgae. *Appl. Environ. Microbiol.* 78, 2345–2352
52 Hulatt, C.J. *et al.* (2012) Energy demands of nitrogen supply in mass cultivation of two commercially important microalgal species, *Chlorella vulgaris* and *Dunaliella tertiolecta*. *BioEnergy Res.* 5, 669–684
53 Peccia, J. *et al.* (2013) Nitrogen supply is an important driver of sustainable microalgae biofuel production. *Trends Biotechnol.* 31, 134–138
54 Hu, Q. *et al.* (2008) Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *Plant J.* 54, 621–639
55 Raven, J.A. (2010) Why are mycorrhizal fungi and symbiotic nitrogen-fixing bacteria not genetically integrated into plants? *Ann. Appl. Biol.* 157, 381–391
56 Preininger, É. and Gyurján, I. (2001) Trials to create artificial nitrogen-fixing symbioses and associations using in vitro methods: an outlook. *In Vitro Cell. Dev. Biol. Plant* 37, 139–148
57 Ducat, D.C. *et al.* (2011) Engineering cyanobacteria to generate high-value products. *Trends Biotechnol.* 29, 95–103
58 Agapakis, C.M. *et al.* (2012) Natural strategies for the spatial optimization of metabolism in synthetic biology. *Nat. Chem. Biol.* 8, 527–535
59 Lan, E.I. and Liao, J.C. (2012) ATP drives direct photosynthetic production of 1-butanol in cyanobacteria. *Proc. Natl. Acad. Sci. U.S.A.* 109, 6018–6023
60 Lindberg, P. *et al.* (2010) Engineering a platform for photosynthetic isoprene production in cyanobacteria, using *Synechocystis* as the model organism. *Metab. Eng.* 12, 70–79
61 Liu, X. *et al.* (2011) Fatty acid production in genetically modified cyanobacteria. *Proc. Natl. Acad. Sci. U.S.A.* 108, 6899–6904
62 Tan, X. *et al.* (2011) Photosynthesis driven conversion of carbon dioxide to fatty alcohols and hydrocarbons in cyanobacteria. *Metab. Eng.* 13, 169–176
63 Weyman, P.D. *et al.* (2011) Heterologous expression of *Alteromonas macleodii* and *Thiocapsa roseopersicina* [NiFe] hydrogenases in *Synechococcus elongatus*. *PLoS ONE* 6, e20126
64 Berto, P. *et al.* (2011) The cyanobacterium *Synechocystis* sp. PCC 6803 is able to express an active [FeFe]-hydrogenase without additional maturation proteins. *Biochem. Biophys. Res. Commun.* 405, 678–683
65 Yoshino, F. *et al.* (2007) High photobiological hydrogen production activity of a *Nostoc* sp. PCC 7422 uptake hydrogenase-deficient mutant with high nitrogenase activity. *Mar. Biotechnol.* 9, 101–112

66 Li, Y. *et al.* (2010) *Chlamydomonas* starchless mutant defective in ADP-glucose pyrophosphorylase hyper-accumulates triacylglycerol. *Metab. Eng.* 12, 387–391

67 Wang, Z.T. *et al.* (2009) Algal lipid bodies: stress induction, purification, and biochemical characterization in wild-type and starchless *Chlamydomonas reinhardtii*. *Eukaryot. Cell* 8, 1856–1868

68 Work, V.H. *et al.* (2010) Increased lipid accumulation in the *Chlamydomonas reinhardtii sta7-10* starchless isoamylase mutant and increased carbohydrate synthesis in complemented strains. *Eukaryot. Cell* 9, 1251–1261

69 Surzycki, R. *et al.* (2007) Potential for hydrogen production with inducible chloroplast gene expression in *Chlamydomonas*. *Proc. Natl. Acad. Sci. U.S.A.* 104, 17548–17553

70 Torzillo, G. *et al.* (2009) Increased hydrogen photoproduction by means of a sulfur-deprived *Chlamydomonas reinhardtii* D1 protein mutant. *Int. J. Hydrogen Energy* 34, 4529–4536

71 Kruse, O. *et al.* (2005) Improved photobiological H_2 production in engineered green algal cells. *J. Biol. Chem.* 280, 34170–34177

72 Snow, A.A. and Smith, V.H. (2012) Genetically engineered algae for biofuels: a key role for ecologists. *Bioscience* 62, 765–768