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1 **Genetic engineering of multi-species microbial cell-factories as an**
2 **alternative for bioenergy production**

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16 **Abstract**

17 There is currently much interest in developing technology to use microalgae or
18 cyanobacteria for the production of bioenergy and biomaterials. Here we summarized
19 some remarkable achievements in strains improvement by traditional genetic
20 engineering and discussed common drawbacks for further progress. We present
21 general knowledge on natural microalgae-bacterial mutualistic interactions and discuss
22 the potential of recent developments in genetic engineering of multi-species microbial
23 cell-factories. This synthetic biology approach would rely on the assembly of complex
24 metabolic networks from optimized metabolic modules such as photosynthetic or
25 nitrogen-fixing parts.

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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 Humankind. 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. Since bioenergy is mostly captured by photosynthetic CO₂ fixation, 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 environment 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 microalgae 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, what 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 exceeds current yields may exist, it is expected that breeding and/or genetic engineering would be required to achieve industrial production [7,10-12].

This review comments 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 the host’s metabolism has prevented further progress. As a complementary alternative, it is proposed the development of multispecies microbial cell-factories comprising optimized parts as specialized metabolic modules to allow biochemical compartmentalization in complex metabolic networks.

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 quite successfully (Table 1). One of the most remarkable accomplishments has been the direct conversion of CO₂ 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 of a variety of chemicals, including isobutanol, has been produced with a projected productivity that would exceed by five-

to six-fold 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 [15]. 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 very successfully produced at up to 15 g/L in recombinant *Escherichia coli* cells from glucose [30]. Conversely, the more challenging task of producing 1-butanol from CO₂ in cyanobacteria remains more elusive since 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 PSII, optimization of the e- flux towards hydrogenase, and others [31]. However, despite many encouraging proof-of-principle demonstrations, photosynthetic hydrogen production remains low for industrial purposes.

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

In contrast to ethanolgenic microorganisms such as the yeast *Sacharomyces cerevisiae* or the bacterium *Zymomonas mobilis* that tolerate more than 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 was shown that imbalances between the recombinant enzymes activities may result in growth inhibitory concentrations of the toxic intermediate acetaldehyde [14]. 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's key metabolites [32].

Assembly of complex enzymes

Enzymes such as hydrogenases or nitrogenases, contain at their active sites very complex metal centers. Biosynthesis of the iron-molybdenum cofactor and assembly of fully functional holo-nitrogenase requires at least fifteen gene products. Although knowledge on this field has progressively increased during the last years, it is still fragmentary [33,34] and limits recombinant pathway design and introduction into selected host.

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 exo-polysaccharides production and duplication of “house keeping” genes) for its particular specialization to run strictly anaerobic pathways (mainly N₂- fixation and H₂ metabolism) together with an obligate aerobic life style [35]. Similarly, genetic, biochemical and genomic analyses uncovered unique characteristics for the remarkably high ethanol-producer *Zymomonas mobilis* including specific metabolic pathway for anaerobic fermentation of glucose and an array of genetic determinants that might account for the exceptional ethanolgenic properties [36]. 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 compromising overall productivity [37]. The kind of genetic determinants for the previous examples are currently difficult to identify since 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 microalgae-bacterial consortia as a source of biotechnological insights

In the wild, microalgae live and have evolved in the context of multi-species microbial consortia. Beneficial interactions range from mutualistic to symbiotic and their stability relies on two important organizing features: (i) trading of metabolites, mainly as cross-feeding but also exchange of dedicated molecular intra- or inter-specific signals and (ii) specialization and division of labor [38].

In most algae–bacteria consortia, microalgae provide oxygen and organic molecules. In natural systems, the algal release of dissolved organic carbon ranges from zero to 80% of photosynthates and it is around 6 to 16% in photobioreactors [39]. For bioenergy purposes, the latter can be seen as both a direct bioenergy loss and also a potential secondary loss due to the assembly of spontaneous and unattractive microorganism's consortia with low or null biotechnological value [40].

On the other hand, bacteria could provide a broader array of substances including CO₂, other nutrients, vitamins, growth-promoting substances, etc. (Fig. 2).

Carbon for nitrogen mutualisms

Although N is extremely abundant on Earth as N₂, the availability of biologically-available N constrains the productivity of both terrestrial and aquatic ecosystems. Nonetheless, N₂-fixing symbioses are common between eukaryotes and cyanobacteria or heterotrophic bacteria and represent a remarkable strategy by which some organisms can use N₂ from the air indirectly in exchange for organic C. In contrast to the photosynthetic C-fixation ability that eukaryotes have acquired through endosymbiosis with cyanobacteria during evolution, no N₂-fixing plastids or N₂-fixing eukaryotes are known [41]. In the oceans, the best-known N₂-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*) [42,43]. The cyanobacteria form short chains of vegetative cells plus a terminal heterocyst, which is a differentiated cell for N₂-fixation that provides a microaerobic environment to protect nitrogenase from inactivation by the eukaryotic host oxygenic-photosynthesis. While little is known about the molecular basis for these interactions, or how the symbionts are transmitted from generation to generation [44], 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 [44]. There appears to be some specificity between the cyanobacteria and the host genera [45]. A widely-distributed marine planktonic uncultured N₂-fixing cyanobacterium (UCYN-A) presents a dramatic reduction in genome size and lacks the genes for photosystem II (responsible for O₂ evolution), RuBisCo (ribulose- 1,5-bisphosphate carboxylase-oxygenase for C-fixation), and the tricarboxylic acid cycle (for aerobic respiration and anabolism) [46]. It was further shown that UCYN-A engages in mutualistic relationships with a prymnesiophyte exchanging fixed-N for fixed-C. Similar association appears to exist with freshwater diatoms and it was proposed that these rather simple interactions between single-celled organisms might mirror those earlier primary endosymbiotic events that gave origin to chloroplasts and mitochondria [47].

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. Algal-associated heterotrophic bacteria belonging to the

genus *Marinobacter* release the siderophore vibrioferrin (VF) in a C for Fe mutualistic relationship [48].

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 [40].

In some cases heterotrophic bacteria appeared to provide phytohormone-like substances (indole-3-acetic acid) to enhance several microalgal activities. This has been shown for the effect of co-culturing *Chlorella* spp. with the plant growth promoting bacteria *Azospirillum brasilense* for an increased pigment and lipid content, lipid variety, carbohydrates, and cell and population size of the microalgae [49-51].

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

Biotechnological use of microalgae-bacterial consortia as multi-species 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 CO₂ and pollutant removal [52,53].

The *A. brasilense* stimulation of *Chlorella* spp. growth and lipids and starch accumulation [49,54] represents a remarkable example of the biotechnological potential of microalgae-bacterial consortia pertinent to bioenergy. Also inoculation with the *Pseudomonas*-related strain GM41 increased *Synechocystis* sp PCC6803

productivity up to 8-fold by helping to degrade toxic compounds commonly found in polluted water [55].

Another application of microalgae-bacterial consortia was 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 [56].

Engineering cyanobacteria/algae-bacterial multispecies microbial cell factories

Multi-species 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 use to rely 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 of extensive genetic engineering of a single strain, sometimes very difficult to achieve, and the reusability of the parts while reducing the wiring requirement [57,58]. Thus, application of this principle might be very 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 multi-species 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 [59] and also to pursue a variety of possible applications. Some remarkable examples pertinent to biofuels production from CO₂, 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 a co-culture 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 [58,60].

Two recent examples of cyanobacteria/algae-bacteria artificial consortia comprising genetically modified microbes as CO₂-fixing or N₂-fixing synthetic parts illustrate the potential of this approach for the sustainable production of biofuels and biomaterials directly from CO₂.

Carbon-fixation cell factories

The cost of the C source in commercial fermentations can be as much as 30 to 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 of >10 mM without culture toxicity. Remarkably, the sucrose-exporting cyanobacterium exhibited increased biomass production rates relative to the wild-type strain, enhanced photosystem II activity, C-fixation, and chlorophyll content. Additional mutations to minimize C-flux towards competing glucose- or sucrose-consuming reactions further improved sucrose production up to 80% of total biomass. Such a strain/strategy may be a viable alternative to sugar synthesis by terrestrial plants, including sugarcane [61]. 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 C-source [62]. Metabolic coupling of photosynthetic modules to *E. coli*, for which advances in genetic engineering and industrial applications have few (if any) rivals, is of a remarkable importance. Co-culture of sugar-excreting cyanobacteria with any other second engineered microbe could yield a desired product without a reduced-C feedstock in situations where synthesis of the product is incompatible with cyanobacterial metabolism (Fig. 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 [63].

Nitrogen-fixing cell factories

Large-scale culture of microalgae, which have an average composition of $\text{CH}_{1.7}\text{O}_{0.4}\text{N}_{0.15}\text{P}_{0.0094}$, might represent a high-N-intensive bioprocess, if wastewater and/or other alternatives are not used as partial or complete substitutes for N-fertilizers

[64-66]. Although promotion of symbiotic N₂-fixation represents a remarkable strategy in agriculture, the sophisticated interplay of recognition signals that underlay most known N₂-fixing symbiosis [41] has complicated the development of exchangeable N₂-fixing parts for synthetic biology approaches.

Most free-living diazotrophs normally fix enough N₂ for their needs and excrete low to undetectable amounts of N₂-fixation products into the medium. Recently, it was shown that disruption of the genetic system signaling the N-status in an *A. vinelandii* mutant strain by inactivation of the *nifL* gene, expresses nitrogenase constitutively and excretes ammonium into the surrounding medium. Conversely to wild type *A. vinelandii*, the ammonium excreting strain engages in C - N mutualistic relationships with the oleaginous microalgae *Chlorella sorokiniana*, *Pseudokirchneriella* sp. and *Scenedesmus obliquus* [64] when no sources of C or N other than air were supplemented into the medium. Inoculation with the ammonium-excreting strain mimicked microalgae growth equivalent to an ammonium amendment of up to 0.5mM. In these artificial symbioses, while 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 is engaged in a more robust symbiotic relationship with microalgae. Since mostly microalgal cells proliferated during co-culture, 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 [64].

Both the metabolic pathways for biological N₂-fixation and triacylglycerol accumulation are rarely found in any single native microbial strain [67] and would likely be difficult to attain by genetic engineering of a single-strain [68]. In addition, this N₂-fixing module showed an apparently low selection of partner since it could be metabolically coupled with diverse wild-type microalgae or cyanobacteria. This case

study provides proof-of-concept for the development of N₂-fixing modules that might be coupled to photosynthetic and sugar producing synthetic cellular-parts for enhanced biomass production and a metabolically compartmentalized platform (Fig. 1B). *A. vinelandii*, already proposed as an alternative host for the expression of anaerobic pathways [35], might represent an alternative host under consideration for hydrogen production and possibly other O₂-sensitive pathways (Fig. 1C).

Living *A. vinelandii* cells have been successfully introduced into either plant or microalgae cells to construct synthetic N₂-fixing endosymbiotic systems [69] making future steps for introducing optimized N₂-fixing modules into eukaryotic cells feasible. Several filamentous heterocyst-forming cyanobacteria may also serve as a C- and N₂-fixing metabolically compartmentalized platforms. In this case the C- and N₂-fixing modules would be inseparable, what 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 [70].

Additional possibilities

Industrial production of biofuels and raw materials would require very large scale non-axenic cultivation of microorganisms, likely 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 [60]. Engineered bacteria can also be introduced to provide or amplify signals triggered by expensive chemical effectors, to aid in the utilization of recalcitrant non-expensive nutrients or stress tolerance, to facilitate harvesting or downstream processing, etc.

Beyond the biotechnological potential of artificial microbial consortia, much work is left to further 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 [71].

Concluding remarks

Proof-of-concept demonstration of C- or N-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 since bioresources that are not attractive as a single strain cell-factories might be valuable as metabolic modules. Thus, the multi-species 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.

Disclosure statement

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Legends to figures

Figure 1. Conceptual diagrams of multi-species microbial cell-factories. (a), a general overview of the principle consisting in the metabolic compartmentalization between a specialized photosynthetic module cross-feeding organic C, likely simple sugars ($C_aH_bO_c$) and O_2 to a production module for C-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 worths to further develop this principle for biotechnological purposes. (b), a model of C - N exchanging synthetic multi-species microbial cell-factory that would allow the production of biomass from C and N 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 relationships that would reduce the cost of biomass production and provide a platform with metabolic spatial compartmentalization to facilitate further optimization of specific pathways. (c), a hypothetical model derived from (b) for the nitrogenase-dependent production of H_2 . A few microbes such as *A. 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 *A. vinelandii* would make possible to challenge this hypothesis in the near future.

580 **Figure 2.** Natural oceanic N₂-fixing algal-bacterial interactions. **(a-c)** C-N mutualistic
581 interactions. **(a)** Interaction between a diatom and endosymbiotic filamentous
582 heterocyst-forming (specialized cell for N₂-fixation). **(b)** *Atelocyanobacterium*
583 *thalassa* (UCYN-A) and as yet unidentified prymnesiophyte similar to
584 *Braarudosphaera bigelowii*. **(c)** Endosymbiotic filamentous cyanobacteria and chain
585 forming diatoms (adapted from [42]). **(d)** C-Fe mutualistic interaction between the
586 dinoflagellate *Scipsiella trochoidea* and the bacterium *Marinobacter* sp. strain
587 DG879 [48]. **(e)** C-B₁₂ vitamin mutualistic relationship between the green microalgae
588 and the bacteria of the order Rhizobiales [40]. DOM, dissolved organic matter.
589

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 ten-fold that of the best oilseed crops.
- Despite being aquatic organisms, the culture systems (open ponds or photobiorreactors) demand less water than terrestrial crops minimizing pressure on freshwater resources.
- They can be cultivated in brackish water on non-arable land, not incurring in 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 (CO₂, N, P 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.

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 N and P and high nutrient-recycling capacity and/or ability to utilize abundant and/or inexpensive alternative sources of N, P and other macro-elements.
- Amenability to genetic analysis and manipulation.
- Probably less important aspects such as minimizing respiration rates to minimize C-dissimilation, decreasing the rate of photoacclimation to low light to slow down self-shading after transition to high light, lowering the C:N ratio of core nitrogenous components and the amount of C allocated to cell structure, also have potential to contribute to enhance biofuels yield.

Box 3. Multi-species 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 multi-species 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 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 multi-species microbial-cell factories to complement current efforts using more conventional genetic-engineering approaches.

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 non-conventional 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 use 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 are needed. This aspect studies should be developed back-to-back together with pilot-scale ponds/photobiorreactors design. When physical insulation could not 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.

682 • *Ecological risk*

683 As for any other genetically modified organism, the ecological risk associated
684 to its liberation to the environment, even in a semi-contained way as algae
685 culture would be, should be modeled in advance so as to be taken into account
686 while considering alternative designs [11, 72].

Table 1. Current and potential biofuels from cyanobacteria or modified microalgae			
Bioproduct	Strategy	Results/Comment	Refs
Cyanobacteria			
Ethanol	H.E. <i>pdc</i> and <i>adh</i> from <i>Zymomonas mobilis</i> into <i>Synechocystis</i> sp. PCC 6803	5.2 mmol ethanol OD ₇₃₀ unit ⁻¹ litre ⁻¹ day ⁻¹ (up to 230 mg . l ⁻¹ in 4 weeks)	13
	H.E. <i>pdc</i> from <i>Z. mobilis</i> , overexpressing <i>slr1192</i> , and disruption of the biosynthetic pathway of poly-b-hydroxybutyrate into <i>Synechocystis</i> sp. PCC 6803	212 mg l ⁻¹ day ⁻¹	14
Isobutyraldehyde	H.E. <i>kivD</i> from <i>Lactobacillus lactis</i> , <i>alsS</i> from <i>Bacillus subtilis</i> , <i>ilvCD</i> from <i>E. coli</i> and insertion of extra copies of <i>rbcLS</i> genes in <i>S. elongatus</i> PCC7942	6,230 µg isobutyraldehyde l ⁻¹ h ⁻¹ Higher than cyanobacterial productivities of hydrogen or ethanol.	15
Isobutanol	H.E. <i>kivD</i> from <i>Lactobacillus lactis</i> and <i>yqhD</i> from <i>E. coli</i> in <i>S. elongatus</i> PCC7942	450 mg isobutanol . l ⁻¹ in 6 d	15
1-butanol	H.E. <i>hbd</i> , <i>crt</i> , and <i>adhE2</i> genes from <i>Clostridium acetobutylicum</i> , <i>ter</i> from <i>Treponema denticola</i> , and <i>atoB</i> from <i>E. coli</i> in <i>S. elongates</i> PCC7942	14.5 mg 1-butanol. l ⁻¹ in 7 days	16
	H.E. <i>bldh</i> from <i>C. saccharoperbutylacetonicum</i> , <i>yqhD</i> from <i>E. coli</i> (among others) in <i>S. elongates</i> PCC7942	29.9 mg 1-butanol. l ⁻¹	17
Isoprene production	H.E. <i>ispS</i> from <i>Pueraria montana</i> expressed under regulation of the <i>psbA2</i> promoter in <i>Synechocystis</i> sp. PCC 6803	50 µg isoprene. g dw ⁻¹ . day ⁻¹ under high light conditions	18
Fatty acids	H.E. a thioesterase, among others modifications, in <i>Synechocystis</i> sp. PCC 6803	200 mg l ⁻¹ fatty acids secreted into the culture medium	19
Fatty alcohols	H.E. <i>far</i> from <i>Simmondsia chinensis</i> under the control of P _{rbc} promoter	0.2 mg l ⁻¹ fatty alcohols	20
Hydrogen	H.E. [Ni-Fe] hydrogenase	Accumulation of holo-enzyme	21

	<p><i>hynSL</i> from <i>Alteromonas macleodii</i>, and <i>Thiocapsa roseopersicina</i> and accessory genes in <i>S. elongatus</i> PCC7942</p> <p>[FeFe]-hydrogenase</p> <p><i>hydA1</i> from <i>Cr</i> in <i>Synechocystis</i> sp. PCC 6803</p> <p>Insertional disruption of the <i>hupL</i> gene in <i>Nostoc</i> sp. PCC 7422</p>	<p>that displayed hydrogenase activity <i>in vitro</i></p> <p><i>in vitro</i>-active hydrogenase</p> <p>100 $\mu\text{moles H}_2 \cdot \text{mg chlorophyll } a^{-1} \cdot \text{h}^{-1}$ (three times higher rate than that of the parental strain)</p>	<p>22</p> <p>23</p>
Microalgae			
Triacylglycerol (TAG)	<p><i>sta6</i> and <i>sta7-10</i> starchless mutants of <i>Cr</i></p> <p><i>sta7-10</i> (isoamylase mutants) complemented strains</p>	<p>2- to 10-fold increase in TAG accumulation</p> <p>$\geq 400 \text{ mg starch exceeding } l^{-1}$ in 4 days (significantly higher than those achieved by the parental strains)</p> <p>Higher values in total lipids at 96 h of nitrogen deprivation: 83-118 mg lipid. l^{-1}</p>	<p>24-26</p> <p>26</p>
Hydrogen	<p>Replacement of wild type promoter of the <i>Nac2</i> gene by a copper repressible promoter in <i>Cr</i></p> <p>Mutant strain with a double amino acid substitution (L159I-N230Y) in D1 of <i>Cr</i></p> <p>Optimization of the e- flux towards hydrogenase <i>state transitions mutant6 (stm6)</i> in <i>Cr</i></p>	<p>20 $\mu\text{moles H}_2 \cdot l^{-1}$</p> <p>1 - 3.1 $\text{mmol} \cdot \text{H}_2 \cdot \text{mol}^{-1} \cdot \text{Chl}^{-1}$</p> <p>500 ml $\text{H}_2 \cdot l^{-1}$; 5.77 ml. $l^{-1} \cdot \text{h}^{-1}$</p> <p>$\geq 10$-fold higher wild-type and 5-fold CC124 strain, mostly due to a longer production phase</p> <p>540 ml $\text{H}_2 \cdot l^{-1}$</p> <p>4 ml. h^{-1} (5-fold higher than the wild-type)</p>	<p>27</p> <p>28</p> <p>29</p>

H.E., Heterologous expression; *Cr*, *Chlamydomonas reinhardtii*

Figure 1
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Figure 2
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