Engineering retinal-based phototrophy via a complementary photosystem in Synechocystis sp. PCC6803

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Chapter 2

‘Direct conversion’: Artificial photosynthesis with cyanobacteria

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Abstract:

Cyanobacteria, the only bacteria that can carry out oxygenic, *i.e.* plant-type, photosynthesis, can be engineered with the methods of synthetic biology so that they acquire the ability to convert CO_2 directly into biofuel and/or commodity chemicals, *i.e.* thereby bypassing the formation of the entire complex set of (macro)molecules that jointly form biomass. This approach has become known as 'direct conversion' and has been shown to be feasible for several products already, even upon significant scale-up. Here we explain and this concept of 'direct conversion' trough natural photosynthesis and discuss its limitations and potential further improvement.

Key words:

natural photosynthesis, oxygenic photosynthesis, synthetic biology, CO_2 fixation, biofuel, commodity

I Introduction

In order not to obstruct a development towards a sustainable future, our society will have to make a fundamental transition from an economy that derives the main part of its energy from the net flow, and oxidation, of carbon from fossil sources into atmospheric $CO_{2,}$ into an economy in which the global carbon cycle will have been transformed into a closed, *i.e.* circular form, driven principally by solar energy. Many adaptations will be necessary for such a transition to become feasible, including an overall reduction in energy consumption/dissipation, increased energy efficiency of many existing activities/technologies, and the large-scale use of renewable electricity. The latter form of energy may take a primary role in future society and can and must be generated with techniques directly or indirectly tapping from solar energy, like *i.e.* with photovoltaic panels, hydroelectric- and tidal power stations, and wind turbines. In addition to electricity, the solar energy can also be directly converted into chemical (free) energy, with the use of so-called 'artificial leaves' (109) in compounds like H_2, CO_2 and CH_3OH. This approach, however, has not yet resulted in large-scale demo plants, nor has it allowed the direct synthesis of molecules containing one or more carbon-carbon bonds.

Next to these man-made processes of harvesting solar energy, there is also an additional, natural, mechanism to achieve the same tapping of solar energy for human use: photosynthesis. This process is carried out by living organisms, ranging from the smallest microorganisms to plants, and is principally aimed at producing free energy to drive the synthesis/formation of new cells. For this synthesis, next to the basic building blocks and minerals required for biomass formation, also free energy is required in two forms: redox intermediates like NAD(P)H and phosphorylated intermediates like ATP (110), with the chemiosmotic free energy of a proton gradient as an exchangeable intermediate(111). Generally, and particularly during autotrophic growth (*i.e.* with CO_2 as the carbon source), the building blocks for the synthesis of new cells will be more oxidized than the average redox level of biomass, although there are well-known exceptions to this rule when *i.e.* reduced sugars like mannitol are fermented or when photoheterotrophic bacteria grow with *i.e.* butyrate as their carbon source (see further below). But we will restrict the discussion here to photosynthesis, and more particularly to photoautotrophy.

Surprisingly, and so far little appreciated, four different types of photosynthesis have developed during evolution (Fig. 1): Generally accepted terminology

has not yet crystallized for these different types, but mostly they are referred to as: retinal-based photosynthesis (A), anoxygenic photosynthesis (B, C) and oxygenic photosynthesis (D). For the sum of (B) to (D) also the terms: chloro-photosynthesis or chloro-phototrophy are being used (*i.e.* (112). In the field of microbiology the existence and discovery of retinal-based photosynthesis has blurred the distinction between chemotrophs and phototrophs because sequence analysis only of a newly discovered strain is not enough to decide between these two categories because of the existence of retinal proteins (*i.e.* rhodopsins) with either a sensing function or a function in proton pumping (*i.e.* photosynthetic energy transduction).

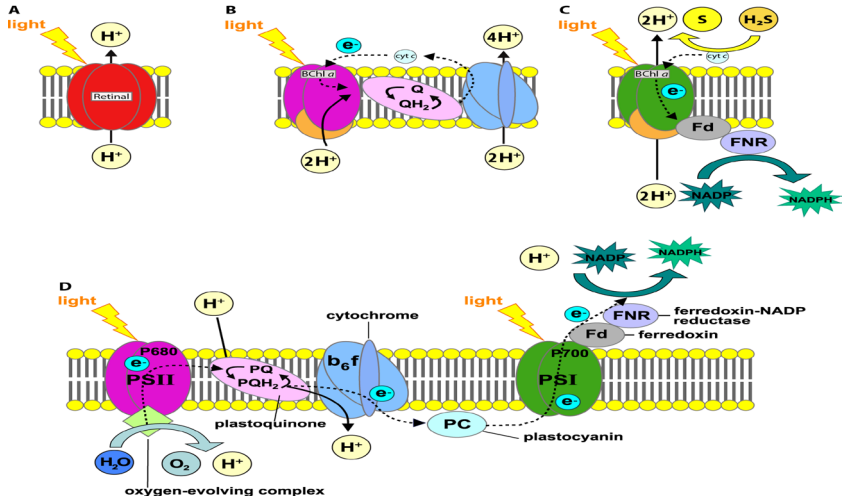

Figure 1: Four different types of photosynthesis: A: retinal photosynthesis, B, C: Anoxygenic photosynthesis and D: Oxygenic photosynthesis. The main photosynthetic complexes that operate in the four different types of photosynthesis are indicated as embedded in a bilayer membrane, connected with mobile electron/proton carriers. Also reference is made to the build-up of a proton motive force, as a result of the light-induced reactions (E/Z isomerization in A and electron transfer in B-D).

In photosynthesis of types A and B, light energy is converted into the free energy of hydrolysis of ATP only, via formation of a proton gradient as the high intermediate. The capacity to form NAD(P)H in these organisms is limited as this has to proceed via so-called 'reversed electron transfer', driven also by the energy from the proton gradient (111). In contrast, both in anoxygenic photosynthesis of type C and in oxygenic photosynthesis, light energy is directly converted not only into ATP, but also into redox carriers like NADPH. This is because the electrons activated/energized by photon energy are transported downhill through a linear pathway of electron-transfer components that even-

tually ends in the formation of a compound that is much more reduced than the respective electron donor for the process. It is also this latter aspect that distinguishes the two types of photosynthesis: For anoxygenic photosynthesis, electron donors are available like various sulfur compounds and manganese and ferrous salts, *i.e.* electron donors with a moderate redox midpoint potential and a significant environmental abundance.

Mechanistically there is a very large difference between retinal-based photosynthesis and (bacterio)chlorophyll-based photosynthesis (or: 'chloro- photosynthesis' (112)). The former process is based on transient energy storage in the re-configuration of a C=C double bond of a retinal chromophore, catalyzed by a single protein that makes very limited use of antennae pigments (113, 114). The latter makes use of complex protein-based machinery that facilitates light-driven electron transfer in one or two reaction centers and makes use of very extensive antenna systems that channel excitons to the centers (115). At the global scale chloro- photosynthesis is by far the most important, but estimates have been made indicating that retinal-based photosynthesis may contribute up to 10% of solar energy conversion (116).

II Oxygenic photosynthesis

The moderate redox midpoint potential of the electron donors that are suitable to facilitate anoxygenic photosynthesis (Fig. 1C) also assures that a single light-reaction suffices to bring an electron to a redox level so that it can be transferred spontaneously to NAD(P)H. In this latter aspect lies the principle difference with oxygenic photosynthesis. In this type of photosynthesis, water (abundantly available in many ecosystems), with its very high redox midpoint potential, can be used as the electron donor for NAD(P)H formation, which, however, because of the large redox-span, necessitates the involvement of two successively operating photosystems, *i.e.* the PSII and PSI, that together with the connecting redox components plastoquinone, the cytochrome b_6/f complex and plastocyanine, form the Z-scheme of oxygenic- or plant-type photosynthesis (Fig. 1D).

Illumination of the components of the Z-scheme embedded in the thylakoid membranes of plants and microorganisms accordingly gives rise to the formation of NADPH and ATP, such that, ideally, with the input of 8 photons, 2 molecules of NADPH and 3 molecules of ATP will be formed. Stress conditions, however, may alter this ratio (see further below under D). This 2::3 stoichiometry exactly matches the requirements of the metabolic pathway that functions

to channel the main carbon-source building block of photosynthesis, *i.e.* CO_2, into new (metabolic intermediates for) cell material/biomass. This metabolic pathway is known as the Calvin-Benson cycle (115) and involves as the key enzyme 'the most abundant protein on earth', RubisCO, that catalyzes the incorporation of CO_2 into ribulose-1, 5-bis-phosphate, to form two molecules of 3-phosphoglycerate. Accordingly, for the synthesis of one molecule of phosphoglycerate 24 photons will be necessary and its formation is accompanied by the uptake of three molecules of CO_2 and an equimolar release of oxygen. The 3-phosphoglycerate can then be converted into any of the complex set of biomolecules necessary to make a new cell, primarily via the lower part of glycolysis in combination with the tricarboxylic acid cycle, to form fatty- and amino acids, and the gluconeogenic pathway to form sugars.

Oxygenic photosynthesis has evolved to become the dominant type of photosynthesis, both in the terrestrial environment (via plants) and in the aquatic environment (via macro- and micro-algae). In cyanobacteria, this process is characterized by a relative overabundance of PSI (as compared to PSII), the occurrence of multiple forms of chlorophyll (*i.e.* a, d, f) and phycobilisome antennae that fill the gap between the two main absorption bands of chlorophyll. Their photosynthesis is very efficient, amongst others because of the presence of carboxysomes, which largely prevents photorespiration by RubisCO, but their energy metabolism is complex because of the strongly interactive nature of respiratory and photosynthetic electron flow in their interconnected thylakoid and cytoplasmic membranes (117).

Only in selected ecological niches is it that retinal-based photosynthesis and anoxygenic photosynthesis have an important role. These niches can be differentiated with respect to the specific electron donor that is available, other than water. If very little to none is available, *i.e.* in oligotrophic environments like in the open oceans or the surface of glaciers, organisms carrying out retinal photosynthesis may thrive (90). In eutrophic waters, in which a significant amount of fixed carbon is present, presumably mostly as organic acids, anoxygenic photosynthesis of type that makes use of quinone electron acceptors (displayed in Fig. 1B) will abound, whereas when reduced inorganic material is abundant, photosynthesis of the type using iron-sulfur cluster acceptors (displayed in Fig. 1C) will dominate (117).

For basic scientific research all these three forms of 'non-oxygenic' photosynthesis (*i.e.* those forms that do not use water as an electron donor and hence do not evolve oxygen) are important, particularly because they provide

well accessible model systems that allow detailed studies of the mechanisms operating in the more complex process of oxygenic photosynthesis. In this respect, the parallels between quinone-based and iron-sulfur cluster-based reaction centers (operating in purple-sulfur- and purple-non-sulfur bacteria, respectively; see Fig. 1B, C) and PSII and PSI, respectively, is very significant (two types). Regarding their biology, important unanswered basic questions still remain, particularly regarding the mutual competitiveness and coexistence of retinal-based- and (bacterio)chlorophyll-based photosynthesis (103).

III 'Direct conversion'

A closing of the global carbon cycle as referred to in part A of this **chapter** inevitably implies an intricate involvement of, and major contribution by, natural (oxygenic) photosynthesis. This process by itself is responsible for the uptake of more than 100 gigatonnes of CO_2 from the atmosphere annually (118), be it that a significant part of it is directly emitted back into the atmosphere via various mechanisms of photorespiration. Nevertheless, a large fraction of the carbon (and oxygen) of this CO_2 is converted into the multitude of molecules that jointly form biomass. Although only part of this biomass can be made available for human use, this is very a significant part. This is first and foremost because it provides us both directly and indirectly (*i.e.* as feed for husbandry animals), with the food that we humans consume.

Nevertheless, a significant second use of this fixed carbon is that it provides us with new, renewable, (*i.e.* construction) materials and with renewable energy. In the development towards a sustainable society, this second type of use has become so important that it has generated the 'food versus fuel' controversy (119) on the topic of how to best use the products of natural photosynthesis. The reason behind this is that some of the very nutritious components of plants for human consumption, like starch and tri-glycerides, are also excellent starting substrates for conversion into liquid biofuel, the form of fossil energy that is most difficult to substitute with a renewable alternative. Yet society will continue to need this form of energy for *i.e.* aviation and heavy transport. But in addition to food and fuel, there is also a need to destine a significant part of the available biomass to the production of renewable (construction) materials.

Through the use of an approach that has been labeled by some as 'bioraffinage' (120), it may be possible to bring some relief in the competition for biomass between the production processes of food, materials and fuel. This is because many forms of biomass contain parts that are less nutritional, but that are nev-

ertheless chemically reasonably well-defined and process-able, such as the cellulose-, the lingo-cellulose- and the lignin fraction. A biorefinery approach can, therefore, be applied both to the primary product of photosynthesis, as well as to the agricultural waste that remains after food- and feed production. This approach also may be an important aspect of the strategy to make new ways of production of renewable materials and liquid fuels economically competitive (121). Nevertheless, in spite of all possibilities to modify and adjust the approach of traditional crop-based photosynthesis to the future needs for production of food, materials and liquid energy carriers, the inherent maximal efficiency of plant photosynthesis – which is 4 and 6,5% for photosynthesis in C(3) and C(4) plants, respectively (14), but in practice is often below 1% (122), or even lower – will not be enough to produce sufficient supplies.

It is therefore generally assumed that further improvement in the efficiency of the application of the process of natural photosynthesis will be necessary to make a successful transition to a closed global carbon cycle. Towards this end one can follow *i.e.* the series of ideas discussed in the landmark paper of Blankenship et al (123) in which various proposals are made to modify (genetically) the basic process of oxygenic photosynthesis to allow it to use a larger part of the spectrum of electromagnetic radiation from the sun (*i.e.* use of radiation from outside the PAR window), and also more efficiently. The latter may be achieved not only through the use of far-red absorbing chlorophylls (123), but possibly also via the use of red-shifted retinal-based proton pumps (124). However, to be able to exploit these possibilities to their fullest extent will require the efforts of many, over a period of many years. Also, some of the proposed adaptations, like the adjustment of the energy gap to be bridged in PSI, may fire back on the efficiency of proton transport by the components of the central part of the Z-scheme, because these proton-pumping reactions are generally assumed to require a significant amount of excess free energy to achieve complete coupling between electron and proton transport (125). And impairment of proton pumping will directly affect the overall efficiency of photosynthesis.

For these and other reasons, we have initiated a new approach in the application of oxygenic photosynthesis for the production of materials, including liquid fuel, which has subsequently been baptized ‘direct conversion’ (11). This approach implies the use of cyanobacteria, the only prokaryotic representatives that are able to carry out oxygenic photosynthesis, in engineered form to directly synthesize the preferred product. This has a number of key advantages, as summarized in Table 1. First, regarding the organisms se-

lected: Cyanobacteria have the highest efficiency of oxygenic photosynthesis known so far (up to 10%; see ref (9); presumably in part because of their low maintenance energy requirement), and allow the use of simple and direct methods of genetic engineering, and relatively simple physiological engineering (*i.e.* because of the absence of subcellular compartments). The simple and straightforward genetic engineering is crucial because 'direct conversion' implies the application of genetically engineered cyanobacteria, such that the majority of the fixed carbon is directly channeled into a preferred product like ethanol (126), sucrose (127), butyraldehyde (128) or lactic acid (129). It can be achieved by introduction of a limited number of exogenous genes that jointly form a fermentative pathway to the desired product; usually not more than two to three genes. Its main advantage is that in this approach one bypasses the inefficiencies inherent in the anabolic reactions of biomass formation and the maintenance energy required for that (130), as well as the energy needed to process the resulting biomass to (a) desired product(s). Also, no energy needs to be invested into the synthesis of multicellular structures that do not contribute to the primary photosynthetic process (roots, trunks, *etc.*).

Table 1: Summary of the advantages of 'direct conversion' with cyanobacteria over competing applications of oxygenic photosynthesis.

• Higher absolute efficiency of photosynthesis (up to 10%)
• Smaller land use than crop-based photosynthesis because of complete growth season, completely surface-covering 'canopy', higher maximal efficiency, *etc.*
• Much lower water use than crop-based photosynthesis
• Re-use of minerals is straight-forward through processing of biomass via methane fermentation or hydro-thermal treatment of biomass
• No involvement of inefficient plant parts like roots and stems
• Straight-forward genetic and metabolic engineering because of prokaryotic nature of the organisms
• Direct conversion of CO_2 into product without the need for bioraffinage

During the past five to six years this synthetic-biology/genetic-engineering approach for direct conversion of CO_2 in cyanobacteria has been widely embraced in academia. For the synthesis of a wide range of compounds biosynthetic pathways have been added to the endogenous intermediary metabolism of particularly *Synechocystis* and *Synechococcus*, often in combination with adjustment of the endogenous metabolism to optimize the overall efficiency of the process (128). Meanwhile, for all the four products mentioned above, it has been demonstrated that the engineering was so successful that indeed

more than 50% of the fixed CO_2 molecules is directly converted into product. This makes such engineered cells qualify as a 'cell factory' for the respective product (Fig. 2), a cell factory which carries out a 'one-pot green synthesis' (131). Indeed, for some products, like ethanol and butanediol (132, 133) the partitioning of carbon over product and biomass has reached values of > 80%. Such high carbon partitioning values very significantly minimize the amount of waste biomass that is formed in parallel to the desired product and in the optimal case is processed either via anaerobic digestion or hydrothermal treatment for minerals recycling. The latter approach has the advantage that the minerals become available in their oxidized form (*i.e.* nitrate rather than ammonia), which is the most suitable form for the growth of cyanobacteria.

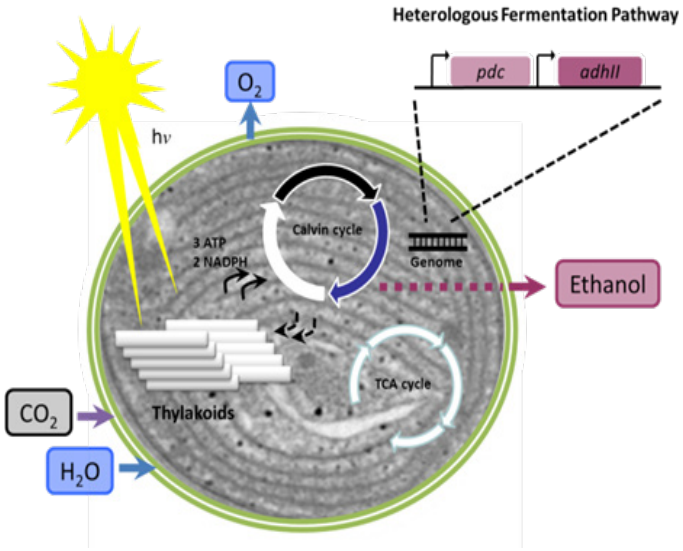

Figure 2: A schematic representation of a cyanobacterial cell factory. Exogenous genes can be added to and deleted from, the genome of a cyanobacterium in order to achieve and/or improve the production of specific chemicals. This has led to the introduction of the term "cell factory" where the bacterial metabolism can be transfigured to a factory assembly line. New assembly lines can also be added, already existing ones deleted or modified, in order to arrive at a more efficient production (system).

Concentrating on recent literature one can conclude (134-136) that the range of products that can be produced via 'direct conversion' with cyanobacteria is not limited to energy- or materials-related products. Rather, the range of products that one can make is as high as with any of the traditional organisms frequently used in biotechnology. This development has been stimulated by the recent developments in fossil fuel prices (see further below) and has amongst others led to proof that also compounds with high added value like

polyol sweeteners and flavor compounds (terpenes, alkaloids, *etc.*) are well-suited products for this approach, be it that carbon partitioning to these latter products has so far been modest at most (137-139). The versatility of cyanobacteria with respect to the provision of reducing equivalents (derived directly from the electron transfer systems in the thylakoid membrane in the form of NADPH) makes these latter organisms particularly suited for the synthesis of relatively reduced compound like the polyols and terpenes, because these phototrophic organisms are not subject to a closed redox balance like fermentative bacteria are, nor is the NAD(P)H subject to oxidation in a respiratory chain as in aerobic bacteria and yeasts.

The concept of 'direct conversion' can be extended to those applications in which a cyanobacterium is used for the light-driven conversion of CO_2 into some simple commodity chemical, like sucrose, glycerol, lactic acid, or glycolic acid, which then can be used by a second organism like *E. coli*, to synthesize a high-value-added product, for which a lot of dedicated biotechnological engineering is required that so far may not have been possible in a cyanobacterium. The two organisms can operate in separate compartments, *i.e.* to offer an anaerobic environment to delicate, *i.e.* oxygen-sensitive, biosynthetic enzymes (140). A mixed approach forms the methane directly from algal biomass (141).

IV Optimization of direct conversion' through natural photosynthesis

Aspects that can be addressed with respect to optimization of 'direct conversion' through natural photosynthesis are many-fold, varying from very specific strategies for one particular product, to very generic aspects of the efficiency of metabolism and growth of the cyanobacterial host. In this section, we will address some aspects of both. First, after a preferred product has been selected the optimal tapping point of cyanobacterial intermediary metabolism has to be selected. Very often this will be pyruvate or fructose-6-phosphate (135). The next step is optimizing the enzymology and molecular biology of the heterologous pathway that must be introduced into the cyanobacterium to allow product formation (and if necessary: product export (127)). This step includes a sufficient (but not too high, to prevent protein-burden effects) gene-expression level and enzymology of the introduced enzymes (*i.e.* substrate affinities and molecular turnover numbers. The latter may *i.e.* be a problem with the more high-value-added products like terpenes (139). Also, it is important to have sufficient excess free energy dissipation in the product-forming

pathway to drive product formation to near completion (142), for which it often helps to engineer an ATP-consuming or CO_2-liberating step. These are the first-order priorities. These steps in most cases require detailed biochemical and metabolic engineering (129, 131, 143). Beyond that, one can – if necessary – embark on further optimization via approaches like speeding-up metabolism via substrate channeling (*i.e.* via the use of fusion- or cascade proteins) or via the creation of micro-compartments (*i.e.* like those involved in ethanolamine catabolism (144) if reactions need to be catalyzed that are not directly compatible with the intracellular milieu of cyanobacteria.

Beyond the introduced, heterologous metabolic pathway, also the organisms' intrinsic metabolism can be optimized and optimally adjusted to the product forming pathway, to maximize the efficiency of the overall process. Cyanobacteria, in spite of their very strong specialization towards photoautotrophic metabolism, still have many redundant parallel metabolic pathways at their disposal, *i.e.* to catalyze multiple pathways for cyclic electron transfer (145) or sugar catabolism (*i.e.* glycolysis and the pentose-phosphate pathway). As not all these pathways have equal energetic efficiency, elimination of the least efficient ones should allow an efficiency increase. Similar arguments hold for the light-harvesting antennae of these organisms (146). Furthermore, through these adjustments, one may optimally tune the ratio of synthesis of NADPH and ATP (see the previous section), to the requirements of the specific product formed. Accordingly, it has been argued by Knoop and Steuer (147) that elimination of the pathways for cyclic electron transfer will increase the efficiency of formation of products like ethanol and ethylene, which only require input of NADPH as the high-energy intermediate in their synthesis and no ATP, because this elimination will decrease the ration of ATP over NADPH generated by the cells and therefore 'force' the cells to channel more reducing equivalents to product formation (instead of making more cells).

Also selected, *i.e.* strongly rate limiting/controlling steps, can be addressed, to remove bottlenecks in metabolism. This approach is best based on a sensitivity analysis of the main enzymes involved in product formation and can be achieved via overexpression of the relevant enzyme(s) (for published examples see *i.e.* RubisCO and pyruvate kinase (128, 132, 143). Ultimately one would like to arrive at a situation in which the control over product formation is evenly distributed over all reactions involved, but this would almost require a complete drawing-board design of such a cell factory (see further below).

V The use of large, closed, outdoor photobioreactors

These engineered cyanobacterial cell factories will then have to be grown and produce a product in large-scale photobioreactors under ambient environmental conditions of light intensity, temperature and circadian regime. Also, they will have to be incorporated into an integrated system for CO_2 supply, nutrient addition/recycling, gas (*i.e.* O_2 and CO_2) exchange, mixing and downstream processing for product recovery, to fully exploit the remainder of the advantages listed in Table 1. Nevertheless, with respect to the actual form of the photobioreactor, a large variety of choices can (and will have to) be made. First of all, because of the use of engineered, product-forming organisms, it will be unavoidable to use closed photobioreactors, rather than open ponds. The closed photobioreactors can be built in a multitude of forms, of which the basic types are: the column reactors, the flat-panel reactors and the tubular reactors (Fig. 3; for review see (148, 149). Furthermore, various modes of mixing and the introduction of CO_2, and stripping of excess oxygen, can be used (*i.e.* through gas supply in so-called "up-flow reactors" or the inclusion of a separate compartment in the reactor for gas exchange (150, 151)). Nevertheless, designs will always have to embody the restriction that the path-length of sunlight through the reactor should not be much more than several centimeters, because larger path-lengths will lead to complete darkness in a significant fraction of the photobioreactor. And in this non-illuminated volume, only energy dissipation can take place because of the maintenance energy requirement of the phototrophic organism.

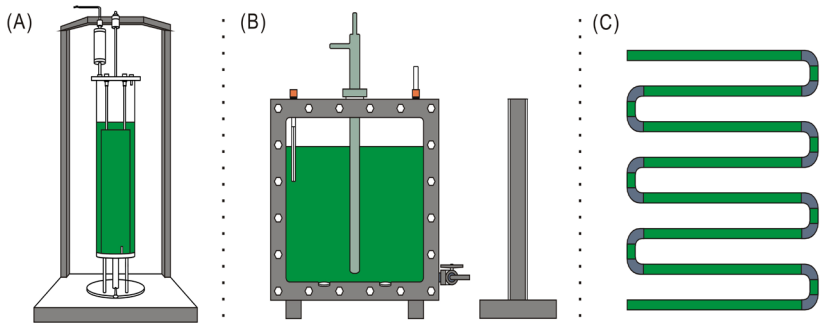

Figure 3: Schematic outline of three basic types of closed photobioreactor, suitable for upscaling. The design of three representative types of closed photo-bioreactors commonly employed for cultivation of cyanobacteria or algae. (A) Column reactor, (B) front- and side-view of a flat-panel reactor and (C) tubular reactor.

Besides the sun (light), it is also imaginable that artificial light sources, like LEDs will be used for product formation via 'direct conversion', fueled by renewable electricity (152). A speculative and simplifying calculation, which assumes that best laboratory performance now will be achievable at large-scale within a few years, leads to a very interesting conclusion: One can cover a field of non-arable land with PV panels (power efficiency 45%), and convert the electricity generated by LEDs (power efficiency 70%) to 680 nm light, which then can be used by an engineered cyanobacterium for 'direct conversion' (with 25% efficiency) in any type of photobioreactor. This then will allow photosynthesis with efficiency higher than achievable with any current crop (*i.e.* 8% versus 6.5%; (14)). The use of LEDs may also significantly simplify photobioreactor design (152), which may considerably increase the robustness of the overall production system (*i.e.* regarding the possibility to create axenic conditions; see also next paragraph).

Regarding the use of the closed, large-scale, outdoor, photobioreactors further optimization aspects are contained in the procedures to select a very robust (but accessible for molecular-genetic engineering) host strain. Also, it will be of extreme importance to develop protocols for the axenic growth of these host strains in very large photobioreactors. This robustness primarily will have to be against environmental stresses like temperature (changes), light intensity, salinity and pH, which then, in turn, may greatly facilitate procedures for their large-scale axenic growth. However, these selection criteria may also have to include increased robustness against spontaneous mutation, because the genetic instability of product-forming strains has occasionally been reported (129, 153), particularly when plasmid-based genetic engineering is used (154). For this, the natural recombination systems of the host organism may be targeted and/or its CRISPR/Cas systems (155).

In 'direct conversion' one makes use of the characteristic of cyanobacteria that many low molecular weight products, like ethanol, butanol, short-chain alkanes, monoterpenes, *etc.* (135) are rapidly secreted into the extracellular medium, and if not that this can be facilitated by engineering of a selective transporter (*i.e.* (127)). The product therefore can be recovered from the spent medium of the photobioreactor rather than from the biomass. This product can either be soluble-, non-soluble- or volatile in the photobioreactor. In the latter two cases, this will allow exploitation of the phase separation of the product, which will have a dramatic effect on the ease of its downstream processing (from either the aqueous or the gas process stream) and the associated costs thereof.

VI Outlook

The key to the further development and optimization of 'direct conversion' via natural photosynthesis is a reliable computational simulation of the process. The developments in systems biology are bringing many innovative developments in the computational simulation of intermediary metabolism and growth of life at all forms life at the cellular level. Prominent among these is flux-balance analysis, a constraint-based form of modeling of the stoichiometric metabolic network of a particular organism, any of several optimization criteria (147). Kinetic aspects of such simulations, however, so far have been very much dependent on these optimization assumptions. On the other hand, the most advanced forms of kinetic modeling of oxygenic photosynthesis in microorganisms use the assumption of functional PSII/PSI units (156), which clearly is a crude approximation of the non-integer, often very high PSI/PSII ratio observed in many cyanobacteria. We, therefore, think that the way forward in these computational simulations will be to try and derive experimental values for the light-generated fluxes of NADPH and ATP and use these as input constraints in stoichiometric metabolic network models. To derive these fluxes from modeling electron transfer in the thylakoid membranes will be extremely complicated in wild-type cyanobacteria because of the many forms of energy dissipation and cyclic electron transfer (145).

The recent surge in the generation of harvesting of renewable electricity has created yet another priority in society: The transient storage of electrical energy in more stable form, because of the limited capacity of batteries to do so. Also for this problem 'direct conversion' may offer a solution because of the ability of cyanobacteria to efficiently photosynthesize with LED-generated 690 nm light (which may be as high as 25 to 30% power conversion). This would call for a new type of volumetric (or: 3D) photobioreactor in which electricity generates LED light so that everywhere in the reactor the light intensity will be in between the compensation point (157) and the intensity at which significant photoinhibition starts. With proper design, this would allow for the most efficient use of space and convenient operation of large-scale axenic cultures of cyanobacteria, which would be able to convert peak-shaved electricity into a liquid biofuel. In such applications living organisms like engineered cyanobacteria may turn out to be much better suited than *i.e.* chemical electrolysers, because they function as almost perfect rectifiers (129), in contrast to the chemical devices which suffer significantly from corrosion under such conditions of fluctuating loads. One could even go one step further and predict that with current laboratory-type photovoltaic cells available in the field, the most

efficient form of agriculture would be to plant these photovoltaic cells on non-arable land and grow cyanobacteria or algae in such a 3D reactor. The actual efficiency of conversion of harvested sunlight into a biological product might for this type of photosynthesis turn out to be appreciably higher than for any conventional crop.

All these different aspects of 'direct conversion' make it more and more relevant to put serious effort into the design of an organism optimally equipped to the task of converting CO_2 and solar energy into a specific product. This design can be approached from multiple angles, *i.e.* using genome reduction (*i.e.* to eliminate redundancy) or a bottom-up design with synthetic biology. Also, natural selection for further optimization may have a role in this process. Currently, it cannot be decided yet which of these approaches will bring the best chances for success. Nevertheless, it is worth pointing out that this approach may have a very important secondary benefit: The more an organism is designed to specifically carry out one particular task under one specific environmental condition, the smaller the chances are that that organism will survive in the natural environment. So, it may well be that serious work to design an organism for optimally efficient 'direct conversion' also contributes to increased environmental safety of this approach.

The concept of 'direct conversion' has meanwhile been adapted by several academic and commercial researchers, leading to a situation in which the first results of commercial production of ethanol via this approach are already appearing (http://www.algenol.com/ and http://www.jouleunlimited.com/). Nevertheless, to use 'direct conversion' to compete successfully on an economic basis in the production of liquid fuel derived from fossil sources is still a major challenge. Actually many hold that this will not be possible without a significant tax on the release of fossil CO_2, because energy products are among the very cheapest products traded in our society. For that reason, a large part of the research in the field of direct conversion has shifted to the production of materials with (considerably) higher added value. This is facilitated by the developments in ongoing research in cyanobacteria, which have shown that these organisms can rival with the classical workhorses of biotechnology, *i.e. Escherichia coli* and *Saccharomyces cerevisiae*, in the versatility of their ability to make a suite of products (134, 135, 145). The main difference being that the two classical workhorses make their products from sugar, whereas cyanobacteria can do that from CO_2. The limitation of *E. coli* and *S. cerevisiae* that they need a closed redox balance under fermentative conditions does not apply to the cyanobacteria and hence the latter organisms are particularly

suited to make the more chemically reduced products, like fuels, sweeteners, and terpene-based scents.

VII Acknowledgements

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