Published in final edited form as:

Photosynth Res. 2015 March ; 123(3): 285–295. doi:10.1007/s11120-014-9980-0.

Engineering cyanobacteria as photosynthetic feedstock factories

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Abstract

Carbohydrate feedstocks are at the root of bioindustrial production and are needed in greater quantities than ever due to increased prioritization of renewable fuels and reduction of carbon emissions. Cyanobacteria possess a number of features that make them well-suited as an alternative feedstock crop in comparison to traditional, terrestrial plant species. Recent advances in genetic engineering, as well as promising preliminary investigations of cyanobacteria in a number of distinct production regimes have illustrated the potential of these aquatic phototrophs as biosynthetic chassis. Further improvements in strain productivities and design, along with enhanced understanding of photosynthetic metabolism in cyanobacteria may pave the way to translate cyanobacterial theoretical potential into realized application.

Keywords

cyanobacteria; biofuels; carbohydrates; feedstocks

Increasingly, cyanobacteria are being explored as a platform for the direct production of biofuels or other commodity products and as a strategy to address the impending crises of energy, environment, and economy (Machado and Atsumi 2012; Robertson et al. 2011; Angermayr et al. 2009). This review focuses on the use of cyanobacteria for a lesser recognized goal: the production of feedstock carbohydrates – a flexible intermediate for the downstream production of other commodities. We introduce pertinent points of basic biology that make cyanobacteria ideal for this process, touch upon some exciting advances in this field, and discuss the prospects and challenges facing this technology.

Sourcing for Bioindustrial Carbohydrate Feedstocks

Currently, nearly all scaled, commercially-viable bioindustrial efforts utilize heterotrophic organisms that convert carbohydrate feedstocks into value-added compounds. The dominant sources of carbohydrate feedstocks are traditional terrestrial crops (e.g. corn, sugarcane). While the cost of carbon feedstocks is marginal for some biologically-produced, high-value products (e.g. nutritional supplements, pharmaceuticals), it is a significant factor in

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bioindustrial production of commodities (Box 1). Without alternative sources for carbohydrates, it is inevitable that feedstock inputs will remain a significant fraction of biofuel production costs.

The use of terrestrial plants for feedstock production has raised considerable concerns about land use, sustainability, and food price instability (Wallington et al. 2012). For example, it is a widely held opinion that increased biofuel production contributed to large food price spikes in 2007–2011 although recent analysis makes it apparent that there was little connection (Baffes and Dennis 2013; Abbott et al. 2009). The complexity of other frequently-cited biofuel related land-use concerns, including the large palm oil initiatives in Malaysia and Indonesia, defy simplistic generalizations; although it is clear in certain localities that palm oil expansion has been enabled predominantly through deforestation (Wicke et al. 2011), with resulting “land use change” related increases in greenhouse emissions for the foreseeable future (Gibbs et al. 2008; Fargione et al. 2008). Furthermore, even by optimistic calculations, the amount of forest or converted agricultural lands necessary to replace a significant fraction of the current demand for petroleum-derived transportation fuels with plant-based biofuels is substantial (Chisti 2008). These points considered, it is clear that alternative carbohydrate sources will be required if biofuels are to become a substantial portion of our transport fuels in a manner that is both economically and environmentally viable (Williams et al. 2009).

The most actively pursued alternative carbohydrate source for microbial growth is sugar recovered from cellulosic materials that are normally inedible. The primary obstacles in this field relate to the recalcitrant nature of lignocellulose and the need to inexpensively treat biomass to release fermentable sugars – this topic has received excellent treatment in other recent reviews (Carroll and Somerville 2009). From an economic perspective, sugars can only realistically be derived from photosynthetic organisms; non-terrestrial options include aquatic plants (Wilkie and Evans 2010), algae and cyanobacteria. Cyanobacteria have the added benefit that they may be grown utilizing degraded land and water sources (e.g. municipal waste or brackish water) not suitable for plant agriculture (Olguín 2012; Rawat et al. 2011). Furthermore, under certain conditions cyanobacteria have higher solar conversion efficiencies than terrestrial crops and could therefore have smaller agricultural footprints. Recent life cycle analyses on the use of algae and/or cyanobacteria for biofuel production, many of which find that scaled production has the potential to significantly cut carbon emissions if certain criteria are met (Olguín 2012).

Effective aquaculture of cyanobacteria will require identifying and engineering strains that possess features necessary for high productivities and reduced costs in scaled reactors (Fig. 1). These include the capacity for rapid, robust growth under dynamic environmental conditions (e.g. temperature/light changes) with minimal and inexpensive inputs and maximized photosynthetic efficiencies. Genetic tractability, advanced molecular biology toolkits, and the capacity to rapidly recover modified strains are also important features. The capacity to flocculate into larger cell masses in an inducible fashion, for instance, would permit cell recovery by inexpensive means, such as gravity sedimentation. Finally, strains would possess the capacity to excrete or store large quantities of fixed carbon as a carbohydrate source readily convertible to a feedstock compatible with heterotrophic

microbes (e.g. glucose, fructose, glycerol). Many of the above features are found in individual species (high growth rates relative to plants/algae, nitrogen fixation for reduced fertilizer inputs, use of brackish water), but the potential for combining and improving these traits into an industrially relevant strain through breeding or engineering has hardly begun to be tapped.

Cyanobacterial carbohydrate content and natural sugars

Because of the huge variety within the phylum, cyanobacteria exhibit considerable variation with respect to chemical composition (Shih et al. 2013; Klähn and Hagemann 2011). Nonetheless, there are some useful generalized distinctions that are found with regard to the cellular content of cyanobacteria compared to algae, and plants. Under ideal growth conditions, cyanobacteria tend to be composed of relatively low carbohydrate (10–30%), intermediate lipid (5–10%) and high protein levels (40–79%), in comparison to algae (5–60% carbohydrate, 1–40% lipid, 20–50% protein) and plants (70–80% carbohydrate, 1–15% lipid, 1–10% protein) (Rajeshwari and Rajashekhar 2011; Becker 1994). With regard to carbohydrates, the dominant carbon sinks for cyanobacteria are glycogen and their exopolysaccharide layers. In contrast, plants and algae both store intracellular glucose as starch, and also invest major carbohydrate resources in a cellulose-based cell wall.

While many species of cyanobacteria have relatively low carbohydrate levels when growing rapidly in rich media, the total sugar composition can vary greatly depending on nutrient availability. Generally, carbohydrates can be ascribed as photosynthesis “carbon overflow” products in cyanobacteria. That is to say, excess fixed carbon can be stored as sugars and polysaccharides when nitrogen, sulfur, phosphate or other nutrients required for the production of proteins, nucleic acids or other biomolecules are limiting. In the case of nitrogen limitation, for instance, a global nitrogen regulator, NtcA, upregulates sucrose production while it is suggested that the same regulator may be responsible for repressing sucrose production when nitrogen is plentiful (Marcozzi 2009). In a variety of nutrient-limited media, cyanobacteria accumulate glycogen (Fig 2.) (Preiss 1984; Schwarz and Forchhammer 2005); this is in contrast to many algal species that use lipids as photosynthetic overflow products (Courchesne et al. 2009). Growth of cyanobacteria in media limited in specific nutrients can result in 50% of dry weight being stored as carbohydrates (Aoyama 1997, Carrieri 2010), but these conditions are generally accompanied by a decrease in the growth rate and photosynthetic efficiency that worsens with the duration of cultivation in nutrient poor media.

Cyanobacterial osmotic stress response pathways

Salinity is an abiotic factor that engages distinct signaling pathways and leads to significant increases in soluble sugars in cyanobacteria. Following an increase in external salt concentration, cells rapidly up-regulate activities for non-compatible ion (e.g. Na⁺) transporters within minutes, while other ions (e.g. K⁺) may be accumulated intracellularly (Nitschmann and Packer 1992). Changes in the composition of cytosolic ions generally decrease the stability or activity of many cellular proteins, but other enzymatic activities are directly activated by ions such as Na⁺ in a process that is independent of transcriptional

changes (Marin et al. 2004; Kanesaki et al. 2002; Schoor et al. 1999). Transcriptional changes are evident 30 minutes following salt shock, although it is becoming apparent that the salt stress, ionic stress, and osmotic stress, are sensed by separable factors and therefore the exact response depends on the specific ions/osmoticum used (Kanesaki et al. 2002). There is evidence that Hik sensor kinases have important roles in sensing osmotic stress and transducing the signal, though the highly integrated network of these response regulators has complicated efforts to identify the dominant response regulator(s) in the osmotic stress hierarchy (Shoumskaya et al. 2005, Paithoonrangsarid et al. 2004). Both the transcription-independent and sensor kinase strategies are involved in up-regulating the capacity of cyanobacteria to accumulate a subset of soluble, cytoplasmic sugars as compatible solutes.

Cyanobacterial sugars that are accumulated as compatible solutes include sucrose, trehalose, glucosylglycerol, glucosylglycerate, and glycine betaine (Fig. 2). These sugars are part of a universal “salt out” strategy, whereby osmotic imbalance is corrected by accumulating compatible solutes so active export systems can remove damaging ions (e.g. Na⁺) while still preventing cellular dehydration (Schoor et al. 1999; Wood 2011). Furthermore, some compatible solutes, sucrose and trehalose, act to stabilize proteins and lipids through direct interactions, substituting sugar hydroxyl groups for water in the hydration shell (Potts 1994). The dominant compatible solute accumulated depends on the species of the cyanobacteria, with some osmoprotectants able to confer tolerance to more saline environments (Fig. 2). Regardless of the final dominant compatible solute utilized at steady-state, sucrose is nearly always synthesized in a pulse ~30 min following the application of salt stress. It is thought that sucrose plays a buffering role in this context, but others have suggested that it may also act as a signaling molecule (Desplats 2005). As alluded to above, the enzyme activities responsible for compatible solute synthesis are often up-regulated directly by binding ions as well as at the level of gene expression (Marin et al. 2004; Kanesaki et al. 2002).

Engineering for enhanced sugar production and accessibility

In addition to manipulating growth conditions, directed engineering approaches can be used to improve cyanobacterial composition, accessibility, and accumulation of carbohydrates. Because of the cost of harvesting and lysing cells, the recovery and purification of cytosolic sugars (low market value) from cyanobacteria is unlikely to be an economically viable option. Instead, two dominant strategies exist for processing internal cyanobacterial carbohydrates: autofermentation of cytosolic sugars and bulk digestion of total cyanobacterial biomass. Autofermentation occurs when photosynthetically-generated carbohydrates are catabolized in the dark where prolonged periods of respiration lead to anaerobic conditions. This allows for production of useful reduced compounds that are not normally generated in significant quantities under conditions of aerobic photosynthesis (Aoyama et al. 1997). Therefore, cycling cyanobacterial cultures from periods of oxygenic carbohydrate accumulation to anaerobic carbohydrate catabolism has been proposed for the production of a variety of compounds, including hydrogen, ethanol, and organic acids (Ananyev et al. 2008). This is an especially useful approach for generating hydrogen, because it temporally restricts the activity of oxygen-sensitive hydrogenases to non-photosynthetic periods, fueling hydrogen production from reducing equivalents provided by carbohydrate catabolism. Under some conditions, the conversion of stored carbohydrates

into reduced, excreted compounds has been shown to operate at high yields (McNeely et al. 2010), though generally over relatively long periods of fermentation. To maximally utilize autofermentation, cultures could be metabolically poised for accumulation of carbohydrates during the light period of the day (with minimal culture growth), and then process all stored sugars to a desired product during the night. Generally, accelerating metabolism of stored carbohydrates through genetic means has proved difficult, though abiotic stress cycles are an alternative route to this goal (see below). Among possible cytoplasmic sugars, glycogen, and other insoluble carbohydrates are more readily mobilized for autofermentation than compatible solutes, suggesting that some sugars are less ‘available’ to cytosolic catabolism (Guerra et al. 2013).

Alternatively, bulk cyanobacterial biomass can be collected and subsequently digested by heterotrophic microorganisms. The best explored examples of this have utilized consortia of anaerobic bacteria that are similar in composition to those that are used to process municipal and industrial waste streams for the production of methane. Cyanobacterial cells must be collected, partially dewatered, and are sometimes processed to break open cells and increase carbohydrate, lipid, and protein bioavailability (Zamalloa et al. 2011). The digestion of cyanobacteria compares favorably with algal and plant biomass in that it can be demonstrated to have fairly high yields (75% of theoretical (Bohutskyi and Bouwer 2013)). However, digestion of all biomass source streams suffer from the problem that the process occurs during a long incubation (30–60 days). Though cyanobacteria are naturally less recalcitrant due to the lack of cellulosic walls, engineering strains with increased accessibility and higher carbohydrate content (Markou and Nerantzis 2013) could cut down on incubation times, eliminating a major barrier to scalability. Similarly, engineering heterotrophic species or consortia to more efficiently breakdown cyanobacterial biomass would be of interest, yet most such published research has been done to engineer strains for faster gasification of cellulosic biomass, or algae (e.g. (Wargacki et al. 2012)).

A promising third strategy for cyanobacterial sugar production is to generate strains or processes that result in the export of soluble carbon feedstocks as they are produced. Strain engineering can be accomplished either by expressing efflux transporters for specific carbohydrates, or through mutations that lead to excretion via endogenous pathways. The latter strategy has been shown in several contexts, but the mechanisms for solute export are relatively poorly characterized. One example of efflux engineering was recently reported independently by four research groups and involves inactivation of pathways leading to glycogen synthesis in cyanobacteria. In multiple model strains (*Synechococcus sp. strain PCC 7002*, *Synechococcus elongatus PCC 7942*, and *Synechocystis sp. PCC 6803*) knockout of enzymes necessary for polymerization of glucose into glycogen (glucose-1-phosphate adenylyl transferase or glycogen synthase) leads to increased export of a number of metabolites, including key intermediates of core carbon metabolism and compatible solutes (Carrieri et al. 2012; Hickman et al. 2013; Xu et al. 2012; Gründel et al. 2012). Growth of glycogen deficient strains under stress conditions (where WT cells would normally accumulate glycogen) exacerbates this non-specific “leakage” of carbohydrates and organic acids, and results in significant changes in intracellular metabolite concentrations consistent with major changes in global carbon partitioning. The mechanism for export is unknown, although expulsion of lipid vesicles has been observed in

Synechococcus sp. strain PCC 7002, and it is possible that mechanically gated transporters responsive to turgor pressure could be involved as has been observed in other prokaryotes (Levina et al. 1999). Similarly, bulk export of many soluble factors from cyanobacteria can be achieved by hypoionic shock (Reed et al. 1986; Xu et al. 2012), presumably as part of a system to rapidly shed cytosolic osmoticum and reduce water uptake. This approach is being used by the company Proterro to achieve high rates of sucrose efflux from *Synechocystis* through repeated cycling of hyper- and hypo-ionic stress (Aikens and Turner 2009). Apart from export, hypo-osmotic shock can accelerate carbon partitioning into other metabolic pathways, such as glycogen synthesis (Warr et al. 1985) or autofermentation (Carrieri et al. 2010). However, some of the aforementioned abiotic stress treatments and mutations can have ramifications on the viability and/or photosynthetic activity of the culture; as is the case in glycogen synthesis mutants (Suzuki et al. 2010; Xu et al. 2012; Miao et al. 2003; Guerra et al. 2013).

Recently, it has been demonstrated that transporters can be used to export specific carbohydrates from cyanobacteria, including glucose, fructose, lactose, and sucrose (Ducat et al. 2012; Du et al. 2013; Niederholtmeyer et al. 2010; Xuan et al. 2013). Expression of heterologous transporters allows the export process to be continual, does not require cycling of cultures between stress and recovery conditions, and leads to efflux of a defined carbohydrate pool, rather than a complex mix. Achieving significant rates of carbohydrate efflux in transporter-bearing strains requires increasing the concentration of available cytosolic sugars, through abiotic stressors or genetic mutations. For example, application of osmotic stress to sucrose transporter-bearing cyanobacteria greatly increases sugar efflux, and allows for tuning of the amount of fixed carbon that is ultimately exported as sugar (Ducat et al. 2012; Du et al. 2013; Xuan et al. 2013). Many heterologous sugar transporters have been shown to express and localize to the plasma membrane in sufficient quantities for measurable sugar export or import (McEwen et al. 2013), an indication that this approach could be expanded for the export of other sugars if strains can be engineered to reverse the chemical gradient across the plasma membrane (i.e. achieve sufficiently high cytosolic levels of target sugars to promote export). However, it is not known if heterologous transporters also can (mis)localize to thylakoids, an outcome that could dampen the effectiveness of this approach.

An interesting phenomenon that has emerged in recent articles related to engineered cyanobacteria is that enhanced rates of photosynthetic activity can be seen under conditions of continual export of target metabolites. We observed a 20–25% gain in the oxygen evolution, carbon fixation, and biomass energy content of *Synechococcus elongatus* PCC 7942 when it was induced to export high levels of sucrose (Ducat et al. 2012). Other studies have seen similar enhancements when other products (ethylene or 2, 3 butanediol) were exported from *S. elongatus* or *Synechocystis* sp. PCC 6803 (Ungerer et al. 2012; Oliver et al. 2013), suggesting that this observation may be a more general outcome of high levels of target product export under phototrophic conditions. One interpretation that is consistent with this data is that the cyanobacterial photosynthesis can become “sink limited” when grown in rich media with elevated CO₂. By this interpretation, efficient product export provides an additional metabolic sink to balance source/sink energies and relieves photosynthetic inhibition caused by insufficient end-product synthesis, analogous to

processes better-studied in plants (Weise et al. 2011). However, the precise nature of this counterintuitive effect remains to be elucidated.

While it is still difficult to obtain cross-sectional data on productivity of cyanobacteria in field trials and predicting scaled cultivation from lab experiments is often overly optimistic, the potential for cyanobacterial production is high. Realistic estimates for annual dry biomass produced from cyanobacteria are in the vicinity of 40–80 tons per hectare (Wijffels et al. 2010). This is comparable to dry biomass production rates from sugarcane, and exceeds bulk biomass production from most other terrestrial plants, while not requiring as stringent climate or arable landmass. Furthermore, many of the strains discussed above are naturally capable of partitioning >50% of their carbon towards carbohydrates under specific conditions and, post-engineering, some strains are capable of excreting carbohydrates at comparable rates (e.g. (Ducat et al. 2012; Xu et al. 2012)); this also compares favorably with the ~15% sucrose content of sugarcane (the most aerially effective carbohydrate feedstock under ideal conditions). Moreover, cyanobacterial feedstocks would have the potential to be harvested continuously rather than annually (biannually for tropical sugarcane). Yet, industrially-significant cyanobacterial cultivation faces hurdles that will need to be addressed on multiple fronts to bring practices to a level economically competitive with well-established land based agriculture practices.

Future prospects for cyanobacterial sugars

When considering the prospects for cyanobacterially-produced sugars, many of the potential limitations stem from the fact that these organisms are newcomers on the agricultural stage. Crop plants have been selected and bred in some cases for millennia, their biology has been well studied, and their scaled processing has been standardized and mechanized. Algae, and to a lesser extent, cyanobacteria, have only been under intermittent agricultural scrutiny for a couple of decades, arguably since the initiation of the Aquatic Species Program (Sheehan et al. 1998) and similar Japanese-sponsored programs in the late 1970s. Additionally, the bioengineering focus within academia and industry has been largely targeted at heterotrophic species while phototrophic metabolism has remained relatively less well explored.

The extensive use of heterotrophs in metabolic engineering has resulted in broad, systems level studies of these organisms including large numbers of sequenced genomes and a multitude of transcriptome and proteome studies enabling organism scale models, and metabolic models such as metabolic control analysis (MCA) and flux balance analysis (FBA). These systems-level efforts have proved useful in understanding and engineering heterotrophic microbes. Successful application of systems level information to cyanobacterial engineering has already been demonstrated to a degree for identification of targets to increase resistance to toxic products through transcriptome and proteome studies (Wang et al. 2012; Zhu et al. 2013; Anfelt et al. 2013) as well as fatty acid production where targets for rational engineering identified by RNA-seq increased yields up to three fold (Ruffing 2013). While ‘omics’ approaches have largely been used to interrogate cyanobacterial responses to specific stresses, further integration and application of this information for an organism level understanding is necessary. On this front, there have been

recent improvements in FBA models reflecting cyanobacteria-specific biology including photorespiration, changing metabolic state due to light accessibility or ability to adopt autotrophic, heterotrophic, or mixotrophic growth modes (Knoop et al. 2010; Montagud et al. 2010). Such models provide an avenue for understanding cyanobacterial metabolism for engineering distinct from heterotroph model organisms, though there is still much to do in this field (Steuer et al. 2012). This is well-illustrated by the fact that even the TCA cycle was only recently closed in cyanobacteria after decades of erroneous assumptions (Zhang and Bryant 2011).

Tools for engineering cyanobacteria have improved in recent years, but generally lag far behind those routinely used in heterotrophic microbes. Control over engineered genes and networks in cyanobacteria remains almost entirely limited to transcriptional induction from endogenous promoters, or a handful of inducible promoters. Furthermore, the relatively few promoters that are utilized in model cyanobacteria typically drive fairly unpredictable expression and are leaky in the ‘uninduced’ state (Kusakabe et al. 2013; Oliver et al. 2013). This is in contrast to dramatic improvements in experimenter control over the expression and activity of engineered systems in heterotrophic models, which have had many more options for years, and have seen a recent resurgence in effective control mechanisms at the level of transcription, translation, and protein stability (Khalil and Collins 2010). Some recent additions of cyanobacteria-specific inducible promoters and riboswitches have shown more reliable activity and even can exhibit tunable induction in *Synechocystis* and *S. elongatus* (Huang and Lindblad 2013; Nakahira et al. 2013). Unfortunately, cyanobacterial strains that have demonstrated high areal productivities in outdoor trials generally lack even the basic molecular tools of laboratory models, although recent prospecting efforts have identified promising new candidate species (Taton et al. 2012). Furthermore, preliminary work has been done to capitalize on the success of synthetic biology efforts in heterotrophic organisms (Berla et al. 2013; Huang et al. 2010), however, it is clear that further efforts will be necessary in order to allow for more advanced engineering efforts and to more fully open cyanobacteria as a model for the study of photosynthetic metabolism.

A large, general concern for scaled production of cyanobacteria and algae lies in engineering economically effective bioreactors. For low-value commodity products, it has been argued that even fairly minimalistic open raceway ponds may present a high enough cost to prevent viability of algal/cyanobacterial biofuels (Sheehan et al. 1998). While analysis of reactor economics is beyond the scope of this review, there are specific scalability aspects that are of value to discuss here in regard to utilizing cyanobacteria as a platform for the production of carbohydrate feedstocks. For example, while contamination is a universal problem in algal cultures, it is likely to be exacerbated when the target compound is specifically designed to be a readily metabolized carbohydrate feedstock. Because of the constraints on reactor cost, exclusion of contaminants through construction of a completely enclosed reactor will require very specialized or innovative designs. Exported sugars or cyanobacterial biomass destined for digestion would be especially vulnerable to invasion by opportunistic contaminants and predators; intrinsic properties of the cyanobacteria, bioreactor design, or culture treatment need to be considered with this in mind. For example, application of some stress conditions that increase carbohydrate production might also serve the function to select against the establishment of contaminating microbes. While the degree of salt stress applied in the

specific examples above is unlikely to provide much of a selective pressure, other growth conditions might (e.g. hypersaline media, nutrient limitation).

Reactor and organismal design also need to be compatible with the need to process cultures during growth and harvesting. In many regards, necessary reactor processing capacity is related to whether the end-product is internally retained in the cell or exported into the media. Keeping a product internalized corrects for problems that might be associated with recovery of dilute products from large volumes and minimizes the loss of product due to degradation or contaminant consumption. Yet, recovery of microscopic cells alone is a technical challenge that constitutes a large portion of production costs (Molina Grima et al. 2003) and heavy treatment or lysis of cells to access internal sugars is a further (likely prohibitive) expense. Conversely, exporting a product eliminates the need to collect/lyse the cells, can be a continuous process, and may potentially allow for higher cyanobacterial productivities (see above). But export of sugars increases contamination concerns and may make engineered strains more vulnerable to fluctuating environmental conditions. Cycling stress conditions to control the timing and rate of product export decreases contamination concerns, but at the cost of increasing bioreactor complexity and the energy necessary for liquid handling. Proterro has patented a bioreactor design that addresses a number of these problem areas (Aikens and Turner 2009). They utilize a solid-state design where dense cyanobacterial cultures are spread on an inexpensive physical support which is in turn enclosed within an air-tight, transparent chamber. These solar panel-like reactors limit liquid use by trickling liquid over the top of the cyanobacteria mat in order to both deliver nutrients and to collect exported products in a concentrated flow-through volume. It remains to be demonstrated that these chambers are inexpensive enough to recoup production costs, though their approach does illustrate how reactors can be designed to reduce many of the technical hurdles without requiring the extravagant material costs of many other advanced reactors (e.g. enclosed glass tube photobioreactors).

On the subject of product retention versus export, one area that has received little attention is engineering overproduction of extracellular polysaccharide (EPS) layers that are naturally involved in defense, attachment, or biofilm formation, although some prospecting for strains with desirable EPS sugar content has been conducted (reviewed in (Chug and Mathur)). Engineering EPS layers that are composed of sugar polymers that can be easily digested could provide an optimal compromise between cytosolic retention and export of soluble sugars. This approach might leave sugars concentrated with the cells, provide a nearly limitless sink that is not constrained by physical intracellular space, and reduce recovery costs as cell walls would not need to be broken. There has been relatively limited research in the area of engineering cyanobacterial surface sugars, although a number of recent papers have found that altering the composition of EPS layers can also provide benefits related to increasing flocculation properties or reducing susceptibility to specific predators in planktonic model cyanobacteria (Simkovsky et al. 2012; Fisher et al. 2013; Jittawuttipoka et al. 2013). Of course, engineering controlled flocculation and altered susceptibility to predation are valuable lines of research in their own right. The immediate limitations for feedstock production using this intriguing approach may ultimately be related to our lack of knowledge of fundamental biological aspects of regulation and synthesis of extracellular polysaccharides.

The efficient production of soluble sugars by cyanobacteria opens the door for the engineering cross-species microbial production schemes, whereby heterotrophic strains utilize exported carbohydrates directly, without an intervening purification step. This could take the form of direct application of cyanobacterial culture supernatants into separate bioreactors, or growth of phototroph/heterotroph consortia in a shared media. Engineered consortia have potential to ameliorate some of the challenges described above. For example, paired heterotrophic species could reduce or eliminate costs related to the harvesting and refinement of cyanobacterial carbohydrates by directly consuming them from dilute supernatants and converting them to specific target metabolites. Drawing off exported sugars by rapidly feeding them to other reactors, or by direct co-culture may also reduce contamination issues by limiting the availability of free carbohydrate in solution. A modular design would allow efficient cyanobacterial carbohydrate-producing strains to be flexibly paired with one of many possible heterotrophic modules. Such pairings would take advantage of efficient sugar producing cyanobacterial strains while allowing the use of existing, highly efficient heterotrophic strains and biological engineering technologies. Consortia of heterotrophic and autotrophic microbes are ubiquitous in nature, and, analogously to these natural populations, properly constructed consortia may prove more stable than monocultures. Artificial selected autotroph/heterotroph strains sharing the same media have been explored in only a few recent papers (Niederholtmeyer et al. 2010; Ducat et al. 2012; Ortiz-Marquez et al. 2012; Do Nascimento et al. 2013). In a notable example, *Azotobacter vinelandii* was co-cultured with algal strains where engineered strains could fix and export significant quantities of nitrogen, an example of compartmentalized metabolic reactions across different microbial species for enhanced total biomass productivities. While these preliminary studies suggest an attractive option, the difficulty of predicting the behavior of multiple species and engineering consortia should not be underestimated.

Finally, there are political and regulatory issues that cannot be ignored surrounding the scaled use of cyanobacteria, especially engineered strains. Because of the many problems above, we expect that the use of genetically engineered strains of cyanobacteria or algae will be a necessity for viable commodity production. While the conventions for using engineered organisms in the context of enclosed reactors have been in place and publically accepted for decades, photosynthetic microbes require areal scaling instead of volumetric scaling; this essentially precludes the idea of complete containment of the organism, and the genetic material contained therein. Therefore, the issue of use of genetic modification is once again under consideration, in both public and political forums. This time the discussion is occurring in the context of evidence for gene transfer from engineered to native plant species (Ellstrand 2012), during a national discussion of the promise/perils of synthetic biology (Dana et al. 2012), and the patentability of genetic material ((Oye and Wellhausen 2010)). There is need for increased engagement of academics in the dialog between public, political, and bioindustrial players, and opportunity for all parties to be thereby enriched.

Conclusions

On a variety of different fronts, cyanobacteria represent an opportunity for the efficient production of flexible carbohydrate feedstocks that are not burdened with the problems of existing land-based plant species. Yet, the economic feasibility of scaled cyanobacterial

cultivation depends on addressing the multi-faceted problems of this emerging technology. Many of the current problems of scale can be mitigated at the genetic level by careful selection or engineering of promising strains. Towards this end, recent reports have realized significant advances in the carbohydrate productivities and engineering toolkit of cyanobacteria, though significant hurdles remain at the biological, material, and political levels.

Acknowledgments

We would like to thank members of the Ducat and Silver labs for helpful discussions and critical review of the manuscript as well as Pam Silver for her support. This work was funded by the National Institutes of Health Grant GM080177, by the National Science Foundation Graduate Research Fellowship Program under Grant No. DGE1144152, and the Chemical Sciences, Geosciences and Biosciences Division, Office of Basic Energy Sciences, Office of Science, U.S. Department of Energy (award number DE-FG02-91ER20021).

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Box 1: The Cost of Carbohydrates

Simplified calculations can be used as an example to illustrate the costs of carbohydrate inputs in biofuel production. A familiar example is the fermentation of sugars to ethanol: the theoretical maximum yield of fermentation of hexose sugars into ethanol is 0.51g ethanol for every 1 g of glucose (2 mol ethanol per 1 mol glucose). Assuming interchangeability of glucose, fructose (or ½ sucrose) current world prices for refined sugar of 16.86 cents per pound (Sugar and Sweetener Outlook – September 2013, USDA: <http://www.usda.gov/>), provide the nominal cost of \$0.1686/lb glucose * 2.2 lb glucose/kg glucose * 1 kg glucose/0.51 kg ethanol = \$0.7273/kg ethanol. A total cost of \$2.75 per gallon ethanol can be derived from the density of ethanol (3.785 kg ethanol per gallon): a rather depressing number in comparison to the current average US gasoline price of about the same value (\$2.76 - without ~\$0.50 federal/state taxes). Furthermore, the energy density of gasoline is 1.6X higher than ethanol. It is therefore immediately apparent that refined/raw sugars are not a feasible biofuel feedstock – unrefined plant mass must be utilized. Here, the math becomes much more involved as, for example, the cost of raw materials (e.g. bushels of corn or sugarcane) does not include the energy/infrastructure necessary to process the biomass into a suitable bioreactor input and cannot easily account for losses due to recalcitrant biomass. The lower costs of raw plant biomass can be illustrated through the case of corn ethanol, where the upper maximal yields of ethanol produced from the extractable starch of a bushel of wet milled corn are estimated between 2.3–2.66 gallons ethanol per bushel of corn (Patzek 2006; Shapouri 2002). At the highest yield and lowest projected price per bushel of US corn (\$4.40; September 12, 2013 World Agricultural Supply and Demand, USDA) as of the time of this writing, the corn feedstock cost per gallon of ethanol is \$1.65; some of this cost may be offset by co-product creation. Feedstock prices are the largest input cost for biofuel production and a major factor hampering the economic competitiveness of biofuels with petroleum-based transportable fuels.

Figure 1.

Characteristics of the ideal microbial feedstock phototroph. The ideal carbohydrate-producing phototroph would first and foremost amass large quantities of feedstocks suited for bioindustry (i.) despite having minimal nutritional needs and utilizing non-potable water sources (ii.) limiting the amount of fertilizer needed to scale cultures. Cells would also be highly resistant to predators, phages, and contamination (iii.) while also demonstrating high efficiency of light capture and solar conversion (iv.). For industrial applications (v.) inducible regulatory promoters or networks allowing for switching from one growth mode to another would ease processing, for example by controlling product export, flocculation dynamics or self-lysis for improved product recovery. Ideally, these inducible changes would be robust and driven by inexpensive induction conditions, though accomplishing this goal (& others) will require that strains be genetically tractable (vi). Robust growth under dynamic, real-world environmental fluctuations (vii.) and overall rapid growth rates (viii.) are also essential.

– glycine, P-SER – phosphoserine, S6P – sucrose-6-phosphate, SAR- sarcosine, SER – serine, SUC – sucrose, TRE – trehalose, UDP-GLC – UDP-glucose

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