Effects of bacterial communities on biofuel-producing microalgae: stimulation, inhibition and harvesting

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Abstract

Despite the great interest in microalgae as a potential source of biofuel to substitute for fossil fuels, little information is available on the effects of bacterial symbionts in mass algal cultivation systems. The bacterial communities associated with microalgae are a crucial factor in the process of microalgal biomass and lipid production and may stimulate or inhibit growth of biofuel-producing microalgae. In addition, we discuss here the potential use of bacteria to harvest biofuel-producing microalgae. We propose that aggregation of microalgae by bacteria to achieve &gt;90% reductions in volume followed by centrifugation could be an economic approach for harvesting of biofuel-producing microalgae. Our aims in this review are to promote understanding of the effects of bacterial communities on microalgae and draw attention to the importance of this topic in the microalgal biofuel field.

Introduction

Considering the shrinking reserves and high price of oil and especially the environmental impact of carbon dioxide emissions, traditional fossil fuels will not be a viable transportation choice for fuels in the near future. Biofuels, derived from photosynthetic organisms, that can fix solar energy must be considered as potential alternatives to fossil fuels and have the important advantages of being renewable and environmentally benign. First and second generation biofuels are already in worldwide commercial use. These biofuels are derived from vascular plants, mainly food crops that have also been chosen as energy sources. For example, bioethanol fermented from sugarcane and other sugar-producing crops has been widely used in Brazil and, to a lesser extent, in the USA (Chiaromonti, 2007), while biodiesel made from vegetable oils, as well as animal fats, is widely used in Europe (Körbitz, 1999). Considerable disadvantages have been acknowledged for these energy sources, such as competing for arable land, increasing food prices and enhancing demand for fresh water and nitrate fertilizers. It is estimated that even if all arable lands were used to grow current oil-producing crops, less than half of the energy demand today would be met (Schenk et al., 2008). However, third generation biofuels from microalgae are considered as one of the most attractive feedstocks for high energy transportation fuels (Angermayr et al., 2009; Beer et al., 2009; Li et al., 2008a). Microalgae are prokaryotic or eukaryotic photosynthetic microorganisms that can grow rapidly and live in harsh conditions due to their unicellular or simple multicellular structure (Mata et al., 2010). Since they are rich in fatty acids, microalgae have drawn attention for their possible application for third generation biofuel production.

Here, we focus on one of the neglected critical factors to be considered if successful commercial microalgal production of biofuels is to be achieved, namely, the bacterial communities associated with biofuel-producing microalgae. We synthesize recent research advances on the effects of bacterial communities on microalgae, in particular the stimulation or inhibition of microalgal growth. Also, we emphasize a potential way to harvest biofuel-producing microalgae by the use of bacterial aggregation of the microalgae.

Biofuels from microalgae

Biofuels from microalgae have many advantages over biodiesel from oleaginous vascular plants and bioethanol, and seem to be the only realistic substitute for petroleum-based fuels because of several inherent advantages (Chisti, 2008). First, microalgae convert solar energy into biofuels as do other biofuel-producing plants, but the biomass and oil productivity of microalgae greatly exceeds that of vascular plants under suitable culture conditions (Chen et al., 2011; Chisti, 2010). Average biodiesel production yields from microalgae can be 10–20 times higher than the yield obtained...
from oleaginous seeds and vegetable oils (Gouveia & Oliveira, 2009). Second, microalgae have a very short harvesting cycle compared with conventional crops (Schenk et al., 2008). Most microalgae double their biomass in less than 24 h and can be harvested in 10 days. For vascular plants, the harvesting cycle is at least 3 months, and can be as long as 1 year. Third, cultivation of microalgae can be conducted on non-arable land and therefore avoids direct competition with agriculture for food production. Fourth, many of the microalgae that can be used for biofuel production can be grown in salt or brackish water, or wastewater, compared with the first generation of biofuel-producing plants that require fresh water.

**Challenges in microalgal biofuel production**

Even though microalgal biofuels have been widely acknowledged as an attractive substitute for petroleum, it is important to note that there are no commercial biofuels derived from microalgae available to date. Several obstacles to commercialization must be overcome by key research breakthroughs, including strain selection, lipid content improvement, cultivation system construction and microalgal culture harvesting. The concept of using microalgae as a resource for producing biofuels has at its foundation the high lipid content of some algal species. Therefore, the first critical step of exploiting microalgal biofuels is screening for high lipid producing species. The Aquatic Species Program (ASP) funded by US Department of Energy from 1978 to 1996 successfully isolated and tested considerable numbers (over 3000 strains) of potential biofuel-producing microalgae from both seawater and freshwater providing a great resource for microalgal biofuel research (Sheehan et al., 1998). The lipid content of these strains varied from 7% up to 58% of the dry weight under different growth conditions. Even higher lipid content strains that are capable of adapting to different cultivation environments remain to be isolated from the enormous diversity of microalgal present in many natural environments.

Improvement of lipid content is another key challenge for efficient microalgal biofuel production. The lipid content of microalgae can be greatly affected by nitrogen availability, light, and other environmental factors (Converti et al., 2009; Hoffmann et al., 2010). Nitrogen deficiency has been considered as one of the most efficient ways to raise the lipid content of microalgae but low nitrogen conditions reduce growth rates and subsequent microalgal biomass production (Rodolfi et al., 2009). In general, the lipid content under nitrogen deficient conditions is much higher than that achieved under nitrogen sufficient conditions (Table 1). To achieve both high biomass and high lipid accumulation, two growth stages have been widely used for cultivating biofuel-producing microalgae: a nitrogen-sufficient stage to maximize growth and a nitrogen-deficient phase for raising the lipid content of the biomass produced during the first growth phase. When oleaginous green algae with an average lipid content of 25.5% were subjected to nutrient limitation conditions, the lipid cellular content could increase 2- to 3-fold (Hu et al., 2008). _Nannochloropsis_ strain F&M-M24 could attain a 60% lipid content after nitrogen starvation, compared with 30.9% in nitrogen rich conditions (Rodolfi et al., 2009).

Genetic and metabolic engineering offers another way to promote the biofuel-producing efficiency of microalgae (Dunahay et al., 1995; Hu et al., 2008). Doebbe et al. (2007) found that by introducing HUP1 (hexose uptake protein) hexose symporter from microalga _Chlorella kessleri_ into _Chlamydomonas reinhardtii_, the mutant strain could use externally supplied glucose for hydrogen production. In a similar approach, the metabolic network could be optimized for biofuel biosynthesis by identifying key enzymes and pathways in oleaginous microalgae. Increasingly, genomic approaches will provide insights into important species with potential in biofuels production, such as _Nannochloropsis_ sp. (Wang et al., 2014) that may reveal metabolic bottlenecks that can be overcome to enhance lipid production.

Selecting appropriate cultivation systems for large-scale production of microalgal biomass is also a key challenge. Different cultivation systems have been designed based on two important criteria: biomass production efficiency and cost efficiency. The two potentially applicable systems are closed (e.g. tubular photobioreactors) and open pond systems (e.g. raceway ponds; Chisti, 2007, Sukenik et al., 2009). Open pond systems are more economical to construct (Jorquera et al., 2010; Li et al., 2008b), but are highly vulnerable to

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**Table 1. Lipid content of biofuel-producing microalgae under nitrogen sufficient and nitrogen deficient conditions.**

<table>
<thead>
<tr>
<th>Microalgal strain</th>
<th>Phylum</th>
<th>Lipid content (nitrogen sufficient)</th>
<th>Lipid content (nitrogen deficient)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ankistrodesmus sp.</td>
<td>Chlorophyta</td>
<td>23%</td>
<td>29%</td>
<td>Sheehan et al. (1998)</td>
</tr>
<tr>
<td>Boekelovia sp.</td>
<td>Phaeiasta</td>
<td>27%</td>
<td>59%</td>
<td>Sheehan et al. (1998)</td>
</tr>
<tr>
<td><em>Chlorella pyrenoidosa</em></td>
<td>Chlorophyta</td>
<td>5%</td>
<td>85%</td>
<td>Spoehr &amp; Milner (1949)</td>
</tr>
<tr>
<td><em>Chlorella vulgaris</em></td>
<td>Chlorophyta</td>
<td>6%</td>
<td>16%</td>
<td>Converti et al. (2009)</td>
</tr>
<tr>
<td><em>Chlorella vulgaris</em> ESP-31</td>
<td>Chlorophyta</td>
<td>18%</td>
<td>40%</td>
<td>Illman et al. (2000)</td>
</tr>
<tr>
<td>Isochrysis sp.</td>
<td>Haptophyta</td>
<td>7%</td>
<td>26%</td>
<td>Sheehan et al. (1998)</td>
</tr>
<tr>
<td><em>Nannochloropsis oculata</em></td>
<td>Heterokontophyta</td>
<td>8%</td>
<td>15%</td>
<td>Converti et al. (2009)</td>
</tr>
<tr>
<td><em>Nannochloris</em> sp.</td>
<td>Chlorophyta</td>
<td>20%</td>
<td>36%</td>
<td>Sheehan et al. (1998)</td>
</tr>
<tr>
<td><em>Nannochloropsis</em> sp. F&amp;M-M24</td>
<td>Heterokontophyta</td>
<td>30%</td>
<td>64%</td>
<td>Rodolfi et al. (2009)</td>
</tr>
<tr>
<td><em>Pseudochlorococcum</em> sp.</td>
<td>Chlorophyta</td>
<td>&lt;3%</td>
<td>36%</td>
<td>Li et al. (2011)</td>
</tr>
<tr>
<td><em>Scenedesmus</em> sp. LX1</td>
<td>Chlorophyta</td>
<td>20–25%</td>
<td>30%</td>
<td>Li et al. (2010)</td>
</tr>
<tr>
<td><em>Tetraselmis suecica</em> F&amp;M-M33</td>
<td>Chlorophyta</td>
<td>20%</td>
<td>38%</td>
<td>Rodolfi et al. (2009)</td>
</tr>
</tbody>
</table>
Microalgae used for biofuel production are generally small another key challenge in microalgal biofuel production. to obtain stable, long-term production on a massive scale. algae used in production, including their associated bacteria, require a profound understanding of the ecology of microalgae used in production, including their associated bacteria, to obtain stable, long-term production on a massive scale.

Harvesting of microalgae from large volumes of water is another key challenge in microalgal biofuel production. Microalgae used for biofuel production are generally small individual cells at a low concentration of approximately 0.5–10 g L⁻¹ (Chisti, 2007). This necessitates a method to concentrate microalgal cells that is low cost and energy efficient. Centrifugation is feasible for small-scale cultures, but for large scale microalgal cultures needed for biofuel production, higher capacity methods are needed in view of the prohibitive energy and capital costs associated with centrifugation (Pienkos & Darzins, 2009). Membrane filtration has been proposed for microalgal harvesting (Zhang et al., 2010), but is currently also limited to low-volume harvesting in the laboratory. When used in large-scale harvesting, especially when a vacuum is applied, filters are readily clogged by the packed cells. Gravity settling with addition of flocculation is also a primary harvesting method, but the cost is too high and water quality can be impacted by compounds used as flocculants including alum, ferric chloride and lime, which may cause some ecological problems (Schenk et al., 2008). Since harvesting can account for 20–30% of the total cost of biofuel production (Gudin & Thepenier, 1986), it is necessary to develop cost-effective methods to harvest biofuel-producing microalgae.

An often-overlooked factor that could play a critical role in the whole process of microalgal biofuel production is the bacterial communities associated with the biofuel-producing microalgae. Interactions between bacteria and microalgae are very important for both parties (Cole, 1982). Microalgae affect the abundance and growth of associated bacteria and vice versa. Mucus, polysaccharides, proteoglycans and other extracellular products produced by microalgae can serve as nitrogen and carbon sources for associated bacteria. Antibiotics excreted by microalgae can inhibit bacterial growth and influence the composition of bacterial communities. Bacteria associated with microalgal cultures grown in PBRs may result in biofilm formation that can reduce the light availability to the microalgae within the PBR. On the other hand, bacterial communities associated with algae are acknowledged to play critical roles in modulating the algal population dynamic and metabolism. The role of bacteria may be as important as factors such as inorganic nutrient supply, grazing and viral lysis in controlling algal growth, physiology and even cell differentiation (Grossart & Simon, 2007; Matsuo et al., 2005).

Abundance and diversity of bacteria associated with microalgae
Although axenic microalgal cultures can in some cases be obtained by methods that remove associated bacteria, it is completely impractical to attempt to maintain bacteria-free algal cultures in large-scale culture systems, especially in outdoor open ponds. In the microalgae–bacteria environment that has been termed as the phycosphere (Cole, 1982), microalgae always harbor an abundant variety of bacteria. In natural aquatic systems, bacteria clustered in the phycosphere around microalgae are always present at a higher concentration than found in the surrounding water (Azam & Malfatti, 2007). The bacterial count in the phycosphere can reach as high as 10⁶ bacteria cells/mL with bacteria outnumbering microalgal cells by 100- to 1000-fold (Bowen et al., 1993). In cultivation systems, the higher microalgal growth rate can increase the microalgae/bacteria ratio to 1:10. In our Nannochloropsis cultures grown on a small scale, microalgal cell densities reached 10⁷–10⁸ cells/mL in a photobioreactor system, with bacterial counts reaching up to 10⁸–10⁹ cells/mL (Wang et al., 2012).

With a range of methods now being applied to investigate the bacterial communities associated with microalgae, including culture-dependent methods, denaturing gradient gel electrophoresis (DGGE), 16S rRNA gene-based community analysis by clone libraries, fluorescence in situ hybridization (FISH), 454 pyrosequencing and other next-generation sequencing technologies, impressive progress has been made in elucidating the bacterial communities associated with microalgae. Previous studies focused mainly on the bacterial communities associated with microalgae causing harmful algal blooms, with studies on bacterial communities associated with potential biofuel-producing microalgae being limited. Sapp et al. (2007a) investigated the bacterial communities in the phycosphere of seven freshly isolated diatom and dinoflagellate species. Alphaproteobacteria, Gammaproteobacteria and Flavobacteria–Sphingobacteria affiliated with Bacteroidetes predominated in association with all seven different microalgae. Other studies indicated that different microalgal species harbor distinct bacterial communities (Goecke et al., 2013; Grossart et al., 2005; Schafer et al., 2002). In a study of the long-term association of microbes with a Chlorella culture, four bacterial species and a fungus were found to be harbored on the sheath of the Chlorella or directly attached to the Chlorella cell surface. Interestingly, the chlorophyll content of the Chlorella together with these symbionts was maintained at a high level for seven months whereas the chlorophyll content of the axenic Chlorella declined markedly (Watanabe et al., 2005). In a study of the bacteria associated with three microalgae, Chlorella saccharophila, Ulothrix variabilis and Volvox aureus, many of the bacteria were Alpha-, Beta- and Gamma-proteobacteria. The associated bacteria in this study included several strains that were only distantly related to previously described strains based on 16S rRNA gene sequence similarity (Ueda et al., 2009). Differences have been demonstrated between free-living and tightly associated bacterial assemblages, with free-living bacteria belonging mainly to Alphaproteobacteria and Gammaproteobacteria.
whereas attached bacteria were dominated by the *Cytophaga–Flavobacterium–Bacteroides* (CFB) group (Fandino et al., 2001; Grossart et al., 2005).

We investigated the diversity of bacterial communities associated with the biofuel-producing microalga *Nannochloropsis oceanica* IMET1 grown at different temperatures by a culture-dependent approach as well as the culture-independent methods, DGGE and 16S rRNA gene based clone library community analysis (Wang et al., 2012). The three methods gave the consistent result that bacterial communities varied at different temperatures and changed dramatically with the algae growing at a high temperature of 30°C. *Alphaproteobacteria*, *Gammaproteobacteria* and *Bacteroidetes* dominated in all the clone libraries. Interestingly, many novel bacteria showing less than 97% 16S rRNA gene identity with any previously cultured bacteria isolated, especially from the cultures maintained at the highest temperature of 30°C. *Alphaproteobacteria*, *Gammaproteobacteria* and *Bacteroidetes* were recognized as the dominant groups associated with microalgal cells by all aforementioned studies (Table 2). Members of these bacterial groups are therefore likely to be important players in the functional interactions between bacteria and microalgae.

The application of metagenomic approaches has great potential to reveal the diversity of bacteria associated with microalgal cultures and provide important insights into functional aspects of these bacterial communities. In the first extensive metagenomic study of the bacteria present in biofilms in a PBR in which the microalgae *Chlorella vulgaris* and *Scenedesmus obliquus* were grown, Krohn-Molt et al. (2013) found that the bacterial community comprised ca. 30 species. This community was dominated by *Alphaproteobacteria*, *Betaproteobacteria* and *Bacteroidetes*. The metagenomic analysis revealed many bacterial genes that encoded for essential B vitamins strongly suggesting that the bacteria were providing the microalgae with essential vitamins. Interestingly, a high number of genes for lipid metabolism, including esterases and lipases, were present in the bacterial genomes. This finding, coupled with lipolytic and esterolytic activities of fosmid clones containing inserts from the bacterial community DNA as well as hydrolytic activities from culture supernatants, suggests that the associated bacteria may metabolize some of the lipid produced by the microalgae.

To sum up, microalgae harbor abundant and diverse bacteria in the phycosphere. *Alphaproteobacteria*, *Gammaproteobacteria* and *Bacteroidetes* are dominant groups in this microenvironment for both natural microalgae and pilot cultures (Table 2). Bacteria associated with microalgae shows species-specific (Goecke et al., 2013; Grossart et al., 2005; Schafer et al., 2002) as well as environment-driven distribution (Wang et al., 2012). Different microalgal species harbor various bacterial communities and these bacterial communities may change as the *in situ* or culture parameters change.

**Bacterial stimulation of microalgal growth**

Bacteria can stimulate algal growth mainly by: (1) creating a favorable environment by removing excess oxygen and generating carbon dioxide; (2) supplying inorganic nutrients, vitamins and trace elements needed for microalgal growth; and (3) producing growth promoting factors, chelators and phytohormones.

**Bacterial modification of the microalgal environment**

In the phycosphere, carbon dioxide and oxygen concentrations are important for both bacterial and microalgae but particularly for microalgae. Oxygen is essential for microalgal respiration, but excess oxygen in the phycosphere can be toxic for the microalgal cells. Fast growing microalgae produce large amounts of oxygen, accumulation of which in the phycosphere can inhibit photosynthetic carbon fixation, an effect termed "photorespiration" (Cole, 1982). Aerobic bacteria associated with microalgae have been proven to stimulate algal growth by removal/consumption of oxygen in the microenvironment, thereby creating more favorable conditions for photosynthesis (Mouget, 1995). High concentrations of oxygen also negatively affect nitrogen-fixation in cyanobacteria as the nitrogenase enzyme system is highly sensitive to oxygen (Haystead et al., 1970). For biofuel-producing cyanobacteria, which have nitrogen-fixing ability, a lower oxygen concentration would be favorable for nitrogen fixation. Further, while consuming oxygen in the phycosphere, bacterial respiration produces carbon dioxide which can be immediately used for microalgal photosynthesis.

**Bacterial provision of nutrients for microalgal growth**

One of the critical issues for microalgal biofuel production is whether this is possible as that has been well-established in small-scale systems, but whether this process can produce fuel economically. The nutrients needed for microalgal growth include nitrogen and phosphorus and these inputs can have a high cost (Reijnders, 2008). Mutualistic bacteria associated with microalgae have the ability to fix or regenerate these vital elements for their host algae and potentially decrease the cost of nutrient input (Abeliovich & Weisman, 1978).

Nitrogen-fixing bacteria are capable of transforming atmospheric nitrogen into fixed nitrogen (e.g. ammonia) that is usable by other organisms without nitrogen fixing ability. Symbiotic nitrogen-fixing bacteria can play an important ecological role in providing fixed nitrogen directly to their hosts under anaerobic conditions. For example, *Rhizobium* spp. were symbionts of plants in the rhizosphere that develop root nodules in which these bacteria converted molecular nitrogen into inorganic nitrogen that was exported to the plant for assimilation (Long, 1989). Nitrogen-fixing symbiotic bacteria were also present in aquatic systems. Sponges-associated cyanobacteria had been shown to express the key nitrogen fixing gene *nifH* suggesting that these bacterial communities provided fixed nitrogen to the sponge (Mohamed et al., 2008). Cyanobacteria present in coral cells expressed *nifH*, suggesting that nitrogen fixation by symbionts might be a source of nitrogen in corals that also harbored photosynthetic zooxanthellae and grew in coral reef ecosystems that were generally nitrogen-limited (Lesser et al., 2004). Nitrogen fixing cyanobacterial symbionts also proved to be capable of providing nitrogen for the diatoms. *Richelia*
Effects of bacterial communities on microalgae

Table 2. Bacterial communities associated with microalgae.

<table>
<thead>
<tr>
<th>Chloroplast and bacterial diversity</th>
<th>Method of identification</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Method of characterization</strong></td>
<td><strong>Microalgal uses</strong></td>
<td><strong>Reference</strong></td>
</tr>
<tr>
<td>-Proteobacteria</td>
<td>-Proteobacteria</td>
<td>Hasegawa et al. (2007)</td>
</tr>
<tr>
<td>g-Proteobacteria</td>
<td>g-Proteobacteria</td>
<td>Ueda et al. (2009)</td>
</tr>
<tr>
<td>CFB Others</td>
<td>CFB Others</td>
<td>Otsuka et al. (2008)</td>
</tr>
<tr>
<td>A-Proteobacteria</td>
<td>A-Proteobacteria</td>
<td>Bruckner et al. (2008)</td>
</tr>
<tr>
<td>Chlorella vulgaris</td>
<td>Chlorella vulgaris</td>
<td>Sapp et al. (2007b)</td>
</tr>
<tr>
<td>Scenedesmus obliquus</td>
<td>Scenedesmus obliquus</td>
<td>Wang et al. (2012)</td>
</tr>
<tr>
<td>NIES-864 piliferous</td>
<td>NIES-864 piliferous</td>
<td>Ueda et al. (2009)</td>
</tr>
</tbody>
</table>
| The methods used for characterization of bacterial communities are denaturing gradient gel electrophoresis (DGGE), fluorescence in situ hybridization (FISH), direct viable count–FISH (DVC–FISH), analysis of ribosomal intergenic spacer (ARISA), pyrosequencing and Illumina-based sequencing.

Vitamin B12 (cobalamin) is one of the most important trace elements for the growth of microalgae and is necessary for biosynthesis of methionine. Microalgae cannot synthesize vitamin B12, while approximately 50% of microalgal species are dependent on vitamin B12 for growth (Croft et al., 2005). Many bacteria have the entire B12 biosynthesis pathway indicating that they are capable of producing B12 under anaerobic conditions (Rodionov et al., 2003). Microalgae can acquire vitamin B12 in the natural environment by two bacterially-mediated mechanisms (Bertrand et al., 2007). The first possibility is that microalgae obtain this vitamin by efficient uptake from the surrounding water on release of vitamin B12 through grazing and viral lysis of vitamin

intracellularis and Calothrix rhizosoleniae were always found to live with several diatom genera in close association, which was believed to be mutualistic. They could fix 171–420 times nitrogen when the cells were symbiotic compared to free-living cells. Richelia could fix 81–744% more nitrogen than needed for their own growth and up to 97.3% of the fixed nitrogen was transferred to the diatom partners (Foster et al., 2011). In addition to these large colonial cyanobacteria, unicellular diazotrophic cyanobacteria and bacterioplankton could also make a major contribution to the oceanic N budget in the open ocean (Montoya et al., 2004). In the oligotrophic Pacific, nitrogen fixed by small diazotrophs could support 10% of recent estimates of total oceanic new production and might be the largest flux of new nitrogen into the upper water column for phytoplankton growth mainly comprising microalgae (Montoya et al., 2004).

In addition to nitrogen fixation, bacteria associated with microalgae may promote microalgal growth by regenerating inorganic nutrients such as phosphate (Doucette, 1995). Phosphorus is the second major limiting nutrient for primary producers after nitrogen and is essential for synthesizing DNA, RNA, phospholipids and many other important biomolecules. Only prokaryotic microorganisms and the lower eukaryotes (e.g. yeast) have been recognized to have the ability of remineralizing phosphate (Paytan & McLaughlin, 2007). A wide range of bacteria including Escherichia coli, Pseudomonas and Bacillus can provide inorganic phosphate, especially in phosphate-depleted environments (Koromova & Nesmeyanova, 2002). Jiang et al. (2007) demonstrated that the mutualistic bacterium Pseudomonas sp. played the role of a temporary phosphorus reservoir for its host cyanobacteria Microcystis aeruginosa under phosphorus-sufficient conditions. Phosphorus transfer between M. aeruginosa and Pseudomonas sp. helped maintain appropriate concentrations of phosphorus. However, in the nutrient-deficient conditions where nitrogen was often too limited to stimulate microalgal lipid production, bacteria and microalgae competed for limiting nutrients. When the cyanobacteria were in the cultivation system, phosphorus was transferred from Pseudomonas sp. to M. aeruginosa.

**Bacterial production of trace elements, phytohormones and chelators**

Trace elements, phytohormones and chelators can be essential for microalgal growth and must receive consideration if microalgae are to be grown efficiently on a very large scale. Vitamin B12 (cobalamin) is one of the most important trace elements for the growth of microalgae and is necessary for biosynthesis of methionine. Microalgae cannot synthesize vitamin B12, while approximately 50% of microalgal species are dependent on vitamin B12 for growth (Croft et al., 2005). Many bacteria have the entire B12 biosynthesis pathway indicating that they are capable of producing B12 under anaerobic conditions (Rodionov et al., 2003). Microalgae can acquire vitamin B12 in the natural environment by two bacterially-mediated mechanisms (Bertrand et al., 2007). The first possibility is that microalgae obtain this vitamin by efficient uptake from the surrounding water on release of vitamin B12 through grazing and viral lysis of vitamin.
B12-producing bacteria and archaea. The second possible route of acquisition of vitamin B12 is from symbiotic vitamin B12-producing bacteria directly associated with the microalgae. Croft et al. (2005) isolated a vitamin B12-producing bacterium, *Halomonas* sp., which was tightly associated with its host algae. Further study indicated that vitamin B12 for microalgal growth was derived from the bacterium.

Phytohormones are vital to the growth of many plants and microalgae (Gonzalez & Bashan, 2000; Tsavkelova et al., 2006). Phytohormones include abscisic acid (ABA), cytokinins, ethylene and gibberellins, and can stimulate the growth of plants and microalgae by altering their metabolism and morphology. These chemicals were previously considered to be produced only by plants, but it is now acknowledged that bacteria associated with plants or microalgae can also produce phytohormones. Numerous cytokinin-producing marine bacteria affiliated with several different bacterial genera were isolated and considered to promote the development of algal blooms (Maruyama et al., 1986). The plant/microalgal-growth-promoting bacterium (P/MGBP) *Azospirillum brasilien* significantly increased the growth of the microalgae *Chlorella vulgaris*. The growth-promoting function was attributed to the production of indole-3-acetic acid (IAA), an important phytohormone that induces cell division and differentiation in *C. vulgaris* (De-Bashan et al., 2008).

Iron is an essential element for cellular respiration, photosynthesis and nitrate utilization of primary producers, but it is always a limiting factor for the growth of phytoplankton, due to its poor solubility and correspondingly low concentration. Bacteria can produce iron chelators, siderophores, which efficiently bind iron even at the low concentrations found in aquatic systems and solubilize precipitated iron. *Halomonas* sp., an oligotrophic bacterium associated with the red marine microalga *Dunaliella bardawil* was found to excrete siderophores. These siderophores were a factor in solubilizing a reservoir of precipitated Fe(OH)3 and consequently stimulated the growth of algae (Keshtacher-Liebso et al., 1995). Siderophores increase the solubility of iron because of their high affinity for Fe3+. Amin et al. (2009) found that some clades of *Marinobacter* produced an unusual lower-affinity dicitrate siderophore, vibrioferrin (VF), which facilitated photochemical redox cycling of Fe. By excreting vibrioferrin, these mutualistic bacteria promoted algal assimilation of iron and increased algal iron uptake by more than 20-fold.

**Application of bacterial communities in large-scale growth of microalgae for biofuel production**

The examples given above show the great potential for bacteria to stimulate microalgal growth in both cultivation systems and the natural environment. The long-term maintenance of the stability and activity of these functional bacterial communities is a key challenge for application in large-scale microalgal cultivation. Immobilization of bacterial cells is one possibility and has been well studied, especially in the field of wastewater treatment by bacteria (Feng et al., 1997; Hallas et al., 1992). In addition to the advantages of immobilized bacteria in keeping the stability and activity of bacterial assemblages, this approach also achieves minimal loss of the attached bacterial cells (Hallas et al., 1992). Further, the growth of immobilized bacteria can have a “probiotic” effect, preventing or limiting the growth of unwanted bacteria which are pathogens to microalgae or inhibit microalgal growth. There has been encouraging recent progress in immobilization of functional bacteria with the aim of stimulating microalgal growth. *Bacillus pumilus* ES4 showed the ability to fix nitrogen and when immobilized with the freshwater green alga *Chlorella vulgaris* in low nitrogen medium, algal growth was promoted two-fold in the presence of immobilized *B. pumilus* ES4 (Hernandez et al., 2009). The P/MGBP bacterium *A. brasilien* that produced the phytohormone IAA was immobilized in cultures of the microalga *C. vulgaris* in small alginate beads and greatly stimulated growth of the microalga (Gonzalez & Bashan, 2000). Wet and dry weight, total cell number and the concentrations of microalgal pigments were significantly increased (Gonzalez & Bashan, 2000).

Ideally, different functional bacteria could be selected, co-immobilized in the same matrix and applied in biofuel-producing systems to provide appropriate nutrients, trace elements, phytohormones and chelators, and help maintain the stability of the microalgal culture by inhibiting growth of unwanted microalgae and bacteria. Competition between different functional bacteria may be a problem. Careful selection of functional bacteria and optimal conditions will be important in the successful utilization of immobilized bacteria to promote growth of biofuel-producing microalgae.

**Insights into applying ecological manipulation in large-scale microalgal culture**

An alternative approach is to employ “ecological” rather than “engineering” principals and achieve a deep understanding of the natural bacterial community associated with the microalga grown on a large scale. Bacterial communities can be monitored with appropriate molecular techniques, with the option of using real-time monitoring by real-time quantitative polymerase chain reaction (qPCR). This technique has been applied in many different samples to monitor shifts in bacterial communities, including human feces (Bartosch et al., 2004), deep sea sponges (Cassier et al., 2008), high-strength organic wastewater (Lee et al., 2008), soil (Fierer et al., 2005) and other environments (Shannon et al., 2007; Wang et al., 2010a; Yergeau et al., 2009). By applying this technique, the bacterial community associated with biofuel-producing microalgae could be monitored in real-time; “desirable” and “detrimental” bacterial symbionts could then be subsequently regulated. If unwanted shifts occurred in the bacterial community, the environmental parameters could be changed or targeted addition of particular bacteria to the production facility could be performed, to restore the desired community structure. This approach requires a profound understanding of the microbial ecology of the entire production system of microalgae and associated bacteria.

**Inhibition of microalgal growth**

Microalgal-bacterial interactions are not always mutualistic. Under adverse conditions, microalgae can release substances
inhibitory (e.g. antibiotics) to inhibit bacterial growth (Borowitzka, 1995). Bacteria can in turn inhibit microalgal growth in various ways including: (1) killing or lysing microalgae by direct attack or indirectly by releasing lytic compounds; (2) changing the microenvironment in which microalgae live; and (3) competing for nutrients with microalgae.

Lysis/killing of microalgae by algicidal bacteria

Although bacterial inhibition of biofuel-producing microalgae has not been widely studied, algicidal bacteria are well known to be one of the key biological agents in the dramatic termination of microalgal blooms, especially harmful algal blooms (HABs), in natural environments (Adachi et al., 2002). Bacteria are known to actively interact with HAB species. Particular bacterial groups could increase in abundance coincident with the decline of algal blooms, suggesting that they might be responsible for the termination of algal blooms (Barlaan et al., 2007; Riemann et al., 2000; Sala et al., 2005; Wichels et al., 2004). The addition of a low concentration of marine medium to an axenic *Alexandrium tamarense* culture resulted in total lysis of algal cells within 14 h. DGGE and 16S rRNA gene sequencing suggested that two bacterial genera, *Alteromonas* sp. and *Thalassobius aestuarii* were key factors that caused the lysis (Wang et al., 2010c). Many bacterial isolates have been proved to have the capability to kill microalgae (algicidal activity) by different ways (Amaro et al., 2005; Lovejoy et al., 1998; Simon et al., 2002; Skerratt et al., 2002). Certain algicidal bacterial strains (e.g. strains in the genera *Saprospira* and *Cytophaga*) require direct contact with target microalgae to express their algicidal activity (Lewin, 1997) while algicidal bacteria mainly affiliated with *Alteromonas* and *Pseudoalteromonas* kill or lyse microalgae by producing extracellular algicidal compounds (Mayali & Azam, 2004). Doucette et al. (1999) constructed a conceptual model describing interactions between algicidal bacteria and their target algal species. At the initial stage, microalgae and algicidal bacteria co-existed in natural aquatic environments. With the development of harmful algal bloom conditions and increase in release of microalgal exudates, the concentration of algicidal bacteria also increased. These algicidal bacteria then inhibited the growth of microalgae and further development of algal blooms. The lysis of microalgae cells provided more nutrients for bacterial growth and subsequently accelerated the decline of algal blooms. At the termination of blooms and a return to low concentrations of microalgal exudates, microalgal and bacterial abundance receded back to their initial background levels.

The relationship between symbiotic bacteria and their algal hosts can change depending on the growth stage of the alga. This has been well characterized in the symbiosis between the roseobacter *Phaeobacter gallaeciensis* and the alga *Emiliania huxleyi* that could form dense blooms in many oceanic ecosystems. The *P. gallaeciensis* produced an auxin as well as a broad-spectrum antibiotic tropolothiotic acid (TDA; Geng et al., 2008; Seyedasayamdost et al., 2011) that were presumably beneficial to the host. However, as the algal culture ages, algal lignin breakdown products were released that induced the production of roseobacticides by the *P. gallaeciensis*, potent algicidal compounds that selectively lysed the *E. huxleyi* cells. This allowed the bacterium to disassociate from the dying host and the host cells provided ample nutrients for growth of the *P. gallaeciensis* (Seyedasayamdost et al., 2011).

In our previous studies, bacterial inhibition of the growth of the toxic HAB-causing alga *A. tamarense* was investigated. An axenic *A. tamarense* strain was obtained by repeated washing, lysozyme/SDS and antibiotic treatment with a mixture of gentamycin, streptomycin, cephalothin and rifampicin (Su et al., 2007a). This axenic strain was then used as a model strain to isolate bacterial strains from the East China Sea and Xiamen Bay, China, where *A. tamarense* blooms were frequently recorded, and to study the algicidal mechanism of these isolates. Twelve bacterial strains showing algicidal activity were isolated. 16S rRNA gene sequencing identification showed that 11 out of the total 12 algicidal bacteria isolated from the East China Sea were affiliated with genera *Pseudoalteromonas* (two strains), *Alteromonas* (two strains), *Idiomarina* (one strain), *Vibrio* (three strains) and *Halomonas* (two strains; Su et al., 2007b, 2011; Wang et al., 2010a), whereas the other bacterial strain isolated from Xiamen Bay were closely related to *Actinomyces* (Bai et al., 2011). All the isolates inhibited microalgal growth by secreting algicidal compounds.

Bacterial modification of the microalgal micro-environment

Microalgal growth also can be inhibited when their micro-environment changes. Low oxygen and low pH conditions were two important parameters which could influencing the stable microenvironment. In nutrient-sufficient conditions, active bacterial respiration can deplete the supply of dissolved oxygen. The growth of some microalgae could be limited, although other microalgae are capable of maintaining photosynthesis under anaerobic conditions (Cole, 1982). Concomitantly, bacterial respiration generates carbon dioxide that dissolves into the water and acidifies the microenvironment which is harmful for microalgal growth (Ferrier et al., 2002). The pH can also decreased with bacterial production of organic acid and bacterial oxidation of NH$_4^+$, H$_2$S and other compounds (Cole, 1982).

Competition for nutrients

In the nutrient-deficient phase where nitrogen is often limited to stimulate microalgal lipid production, bacteria and microalgae compete for limiting nutrients. Competitive interaction for phosphorus between *Scenedesmus acutus* and its associated heterotrophic bacteria under low phosphorus conditions has been investigated in previous studies (Currie & Kalff, 1984; Gurung et al., 1999). The bacterial competition for phosphorus was acknowledged to be higher, compared to algae (Liu et al., 2012). The phosphate uptake in the low phosphorus conditions was proved to be dominated by bacteria rather than by algae (Zubkov et al., 2007). Under high light intensity (>55 µE m$^{-2}$ s$^{-1}$) and moderate phosphorus (0.25 µM) conditions, both algal and bacterial growth was limited because of the competition for phosphorus, while under high light intensity, low phosphorus (0.1 µM) conditions, bacteria successfully competed for the phosphorus and
grew well but algal growth was limited. A similar phenomenon occurs in natural waters and in the later phases of large-scale microalgal mass culture which were starved for limiting phosphorus (Brussaard & Rieggan, 1998; Currie & Kalff, 1984).

**Harvesting of biofuel producing microalgae**

Marine snow is a term that describes the aggregation and sinking of organic materials in marine systems. In the photic zone of open oceans, a variety of organic matter including detritus from animals and plants, microalgal cells, bacteria and viral particles can aggregate and form particles. These particles slowly fall to lower layers of the water column. This process contributes greatly to the nutrient dynamics and vertical fluxes of organic materials and subsequently influences the global carbon cycle. Microalgae are an important component of marine snow, especially during algal blooms. High concentrations of microalgal cells accelerate the formation of aggregates and the sinking rate. Recent studies suggest that bacteria could be an important factor in the process of microalgal aggregation (Gardes et al., 2011; Grossart et al., 2006a). Aggregation of several potential biofuel producing microalgae in natural aquatic systems has been studied. Grossart et al. (2006b) investigated the aggregation of two high lipid content diatoms in the genera *Thalassiosira* and *Navicula*. The presence of bacteria was a pre-requisite for aggregation of *T. weissflogii* but not for *Navicula* sp. Free-living bacteria may not influence aggregation whereas bacteria attaching to these microalgal cells may increase aggregate formation (Gardes et al., 2011). Shen et al. (2011) used PCR-DGGE to reveal the role of aquatic heterotrophic bacteria in the process of development of *Microcystis aeruginosa* blooms in natural waters. Their results suggested that *Porphyrobacter, Flavobacteriaceae* and one uncultured bacterium could be specialist bacteria responsible for aggregation of *M. aeruginosa*. Aggregation of the biofuel-producing microalga *Nannochloropsis* sp. was detected in mass cultivation (Rodolfi et al., 2003). TEM images revealed that microalgal cells were stuck together with bacteria and debris. We isolated a novel bacterium from *N. oceanica* IMET1 cultures in our previous study (Wang et al., 2012). The bacterium, designated strain HW001, proved to be capable of aggregating *N. oceanica* IMET1 as well as other potential biofuel-producing microalgae including diatoms, green algae and cyanobacteria (Figure 1). After aggregation, most of the microalgal cells could be harvested from the small volume of dense culture at the bottom of the cultivation system. The lipid content of microalgal cells was not affected by aggregation. The high aggregation ratio and maintenance in lipid content meant that most of the lipid could readily be concentrated into a small volume of culture. A similar phenomenon occurs in natural waters and in the later phases of large-scale microalgal mass culture which were starved for limiting phosphorus (Brussaard & Rieggan, 1998; Currie & Kalff, 1984).

![Figure 1. Tight association between the microalga Nannochloropsis oceanic IMET1 and bacteria visualized with the fluorescent stain 4',6-diamidino-2-phenylindole (DAPI). Red (or deep color dots in print) = microalgal cells that are autofluorescent. Blue (or white dots in print) = bacteria.](image)

These studies indicate that aggregation by bacteria followed by a small volume centrifugation step might be applicable for harvesting biofuel-producing microalgae. Additional studies are needed to determine whether this approach may be commercially applicable to large-scale harvesting. Capital cost evaluation, biosafety, harvesting efficiency and other technical and economic barriers should be studied and overcome. For example, to be cost effective, the bacterial inoculation should be as small as possible to decrease the cost associated with growing the bacteria.

**Conclusion and future prospects**

Large-scale microalgal production of biofuel is receiving intense attention in academia and biotechnological industries. Some fundamental advances are still required before it is economically feasible and this should be a major driver for additional and sustained research funding, considering the high stakes in strategic and environmental terms. We speculate that within the next decade large-scale production of microalgal derived biodiesel will become a reality, and over the next 50 years, this source of biofuel will overtake petroleum as a primary energy source. One key area ripe for additional research and major practical breakthroughs is study of the interactions between biofuel-producing microalgae and bacteria. This review has summarized the important stimulating and inhibitory effects of bacteria on microalga and the potential of microalgal aggregation by bacteria for harvesting biofuel-producing microalgae (Figure 2). Bacterial communities associated with microalgae and their functions should be analyzed in more detail. This is a critical step before moving forward with large-scale culture of any particular microalgal species. By understanding and monitoring the bacterial communities associated with microalgae, chances of maintenance and long-term stability of algal cultures will be greatly improved. Functional bacteria are expected to be isolated and used in large-scale microalgal cultivation. The use of immobilized bacterial assemblages including those that enhance microalgal growth and lipid content and exclude
harmful bacteria might be useful in mass cultivation systems. We predict that use of bacteria to aggregate and harvest microalgae will be an important method for overcoming costs associated with harvesting.

**Declaration of interest**

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**References**


