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The Effects of Plant Growth Substances and Mixed Cultures on Growth and Metabolite Production of Green Algae *Chlorella* sp.: A Review

John J. Tate · M. Teresa Gutierrez-Wing · Kelly A. Rusch · Michael G. Benton

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Abstract Recent interest in the use of microalgae for the production of biofuels and bioproducts has stimulated an interest in methods to enhance the growth rate of microalgae. This review examines past work involving the stimulation of *Chlorella* sp. growth and metabolite production by plant growth substances as well as by mixed cultures of *Chlorella* sp. with bacteria. Plant growth substances known to regulate *Chlorella* sp. growth and metabolite production include auxins, cytokinins, abscisic acid, polyamines, brassinosteroids, jasmonic acid, salicylic acid, and combinations of two or three of the aforementioned substances. Mixed cultures of bacteria are examined, including both natural bacteria–algae consortia and artificially induced symbioses. For natural consortia, commonly occurring bacterial species, including the genera *Brevundimonas* and *Sphingomonas*, are discussed. For artificially induced symbioses, the use of the nitrogen-fixing bacterium *Azospirillum* is examined in detail. In particular, a variety of studies have involved the coimmobilization of *Chlorella* sp. with *Azospirillum* sp. in alginate beads, with the goal of using the mixed culture to treat wastewater. In summary, the use of plant growth substances and mixed cultures provides two methods to increase the growth of *Chlorella* sp., whether for the production of lipids for biofuels, the production of bioproducts, the treatment of wastewater, or a variety of other reasons.

J. J. Tate · M. G. Benton (✉)
Department of Chemical Engineering, Louisiana State University, Baton Rouge, LA 70803, USA
e-mail: benton@lsu.edu

M. T. Gutierrez-Wing · K. A. Rusch
Department of Civil and Environmental Engineering, Louisiana State University, Baton Rouge, LA 70803, USA

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Introduction

In the past few years, considerable interest has been devoted to biofuels. Although a variety of terrestrial plant crops have been used for the production of biofuels, microalgae have increasingly been considered for biofuels production for a variety of reasons. First, theoretical yields of lipids from microalgae for biofuels exceed lipid yields of terrestrial crops by 10–100 times (Greenwell and others 2009). Second, outdoor cultivation of microalgae does not require arable land (Huang and others 2010). Furthermore, saline or brackish water can be used for microalgae cultivation (DOE 2010). Finally, microalgae are even able to provide added benefit through the removal of nutrients such as nitrogen and phosphorus from wastewater, in addition to capturing CO_2 from the atmosphere (Tam and Wong 1990; Li and others 2008).

Microalgae also provide a number of valuable products other than lipids for biofuels. These include food supplements, animal feed additives, and aquaculture feed (DOE 2010). Some important bioproducts produced by microalgae include the high-value carotenoid astaxanthin (Kang and Sim 2007); bioplastics (Zabochnicka-Swiatek 2010); omega-3 fatty acids, including eicosapentaneoic acid (EPA) and docosahexaenoic acid (DHA) (Harwood and Guschina 2009); and medicines and cosmetics (Zabochnicka-Swiatek 2010). Coproduction of these products along with lipids will be important to the economic viability of biofuels, as production costs for algal biofuels currently exceed those of conventional fuels (Thurmond 2009; Singh and Gu 2010; Gallagher 2011; Sun and others 2011).

The production of these products begins with the selection of an appropriate strain and its cultivation at optimal conditions. Desired strain characteristics include rapid growth and adaptation to growth conditions, a high content of lipids or the desired bioproduct, and strain robustness (DOE 2010). A variety of studies have focused on enhancing growth and metabolite production by varying culture conditions of the algae, including pH, temperature, irradiance level, carbon source, aeration, and concentrations of specific nutrients (Illman and others 2000; Liu and others 2008; Converti and others 2009; Hsieh and Wu 2009; Widjaja and others 2009; Bhola and others 2011; Multu and others 2011; Bai and others unpublished). Other studies have focused on genes involved in the biosynthesis of fatty acids in microalgae (Khozin-Goldberg and Cohen 2011; Merchant and others 2011; Rismani-Yazdi and others 2011). Thus, growth conditions and the genetics of lipid production have been explored with the goal of enhancing the economic production of microalgae for bioproducts or lipids for biofuels.

Key components that have been explored are plant growth substances and their effects on the growth and metabolite production of *Chlorella* sp., a common genus of green algae that has been used as a model for plant studies (Machida and others 2008) and studied for biofuels (DOE 2010). Microalgae of the genus *Chlorella* possess a variety of desired traits that make them advantageous for the study of plant growth substances. First, the algae are easily grown in the laboratory and outdoors and can be grown rapidly (Andersen 2005). Second, the genome of a *Chlorella* species has been completely sequenced (Blanc and others 2010). Finally, some species of *Chlorella* are prolific lipid producers (DOE 2010).

This review explores the following two areas: the effects of the addition of one or more plant growth substances to the growth media of *Chlorella* sp., and the stimulation of *Chlorella* sp. growth using mixed cultures of the algae and bacteria and/or cyanobacteria.

Background

The possibility that plant growth substances affect algal growth has been considered for several decades. Studies on the effects of auxins on *Chlorella* sp. date as far back as the 1930s (Yin 1937; Pratt 1938). The field has flourished more recently due to the development of analytical techniques such as gas chromatography (GC), mass spectroscopy (MS), high-performance liquid chromatography (HPLC), and nuclear magnetic resonance spectroscopy (NMR) for the study of phytohormones (Bradley 1991; Evans and Trewavas 1991). Most recently, interest in biofuels has prompted diverse study of methods for optimizing algal growth, including using plant growth substances. Phytohormones, including auxins, cytokinins, gibberellins, ethylene, abscisic acid (ABA), polyamines, brassinosteroids, jasmonides, salicylates, and signal peptides, have been found in a variety of algae (Tarakhovskaya and others 2007). Furthermore, heterotrophic bacteria, cyanobacteria, algae, and fungi are known to synthesize phytohormones (Tsavkelova and others 2006). Finally, genes orthologous to phytohormone receptors in *Arabidopsis* for auxins, cytokinins, and abscisic acid were found in the genome of *Chlorella variabilis* NC64A (Blanc and others 2010). In addition, genes orthologous to those in *Arabidopsis* were identified for receptors or biosynthetic pathways for polyamines (specifically spermidine), brassinosteroids, and jasmonic acids (Blanc and others 2010). The presence of phytohormones in algae as well as genes orthologous to those for phytohormone receptors in *Arabidopsis* suggests that these substances have an effect on algae.

Effects of Plant Growth Substances on *Chlorella* sp.

Auxins

Auxins are one of the most commonly studied phytohormones and are involved in promoting plant growth by controlling cell division and cell elongation (George and others 2008a). In higher plants, auxins are responsible for the differentiation of cells and tissues (Tsavkelova and others 2006). Early studies on the addition of auxins to the growth media of *Chlorella vulgaris* noted that the auxin was consumed by the cells and resulted in enlarged cells (Yin 1937). However, higher concentrations of auxin were found to have an inhibitory effect on growth (Pratt 1938).

The application of auxins (Czerpak and others 1999), auxin precursors and analogs (Czerpak and others 1994), and indomethacin (IM) (Piotrowska and others 2008) at concentrations of 10^{-8}–10^{-4} M has been found to stimulate *Chlorella* sp. growth and increase cell counts, with the most stimulatory effects observed for concentrations of 10^{-5}–10^{-4} M. The auxins indole-3-acetic acid (IAA), indole-3-*n*-propionic acid (IPA), and indole-3-*n*-butyric acid (IBA), when added at a 50-ppm concentration (2.85×10^{-4} M IAA, 2.64×10^{-4} M IBA, and 2.46×10^{-4} M IBA) to the growth media of *C. vulgaris*, increased cell counts per unit volume by 11–19 times after 26 days of growth (Pratt 1938). Addition of the auxin precursors or analogs anthranilic acid (AA), tryptamine (Trp-NH_2), 2, 4-dichlorophenoxyacetic acid (2,4-D), phenylacetic acid (PAA), naphthyl-3-acetic acid (NAA), naphthyl-3-sulfonic acid (NSA), and IAA to the growth media of *C. pyrenoidosa* stimulated growth, resulting in an increase of fresh weight of 184–297 % compared to a control (Czerpak and

others 1994). Addition of NAA to a culture of *C. sorokiniana* increased biomass productivity compared to a control without NAA (Hunt and others 2011). Finally, exogenously applied IM increased cell counts and dry mass of *C.vulgaris* compared to a control (Piotrowska and others 2008).

Increased metabolite production has also been widely observed in *Chlorella* sp. due to exogenously applied auxins (Czerpak and others 1999), auxin precursors and analogs (Czerpak and others 1994), and IM (Piotrowska and others 2008). The addition of seven auxin precursors and analogs to the growth media of *C. pyrenoidosa* resulted in mean increases over a control with no addition of the substances of 213–273 % for chlorophyll *a* and *b* content and 164–258 % for carotenoid content (Czerpak and others 1994). Stimulatory effects were also observed for aldohexoses and water-soluble protein content. In another study (Czerpak and others 1999), application of the auxins IAA, indole-3-lactic acid (ILA), and IBA to the growth media of *C. pyrenoidosa* increased the content of chlorophyll *a* and *b*, aldohexoses, and water-soluble proteins, with the strongest effect observed for IAA. Finally, addition of IM (Piotrowska and others 2008) resulted in stimulatory effects compared to a control for per-cell contents of DNA, RNA, carotenoids, water-soluble proteins, extracellular excretion of proteins, inorganic phosphate, organic phosphate. The most stimulating effect was observed on the fifth day of culture growth, corresponding to the highest photosynthetic oxygen evolution rate during the seven-day growth period.

In summary, several different auxins and auxin-related substances were found to have stimulatory effects at concentrations of 10^{-5}–10^{-4} M on *Chlorella* sp. growth and metabolite production. One auxin-related substance, IM, had an inhibitory effect on growth and metabolite production at a concentration of 10^{-3} M. The observed increases in protein, chlorophyll, and carotenoid content due to the effects of exogenously applied auxins added to the growth media would be of value in cultivation of *Chlorella* for commercial production of animal feed or bioproducts.

Cytokinins

Cytokinins are involved in control of the cell cycle in plants (George and others 2008b), and they stimulate protein synthesis and chloroplast production (Tsavkelova and others 2006). The effects of cytokinins on *Chlorella* sp. have been observed by several research groups. An early study (Burkiewicz 1987) observed that addition of two cytokinins to the growth media of *C. vulgaris* stimulated cell division and increased the dry weight. Additionally, supplementation of the growth media with the cytokinin kinetin at a concentration of 4.6×10^{-5} M increased cell division rates of *C. pyrenoidosa* by 12–16 % compared to a control in which no kinetin was added (Vance 1987).

More recent research observed the effects on *Chlorella* sp. of a variety of cytokinins at multiple concentrations. A total of six different cytokinins and their effects have been studied, three for *C. pyrenoidosa* (Czerpak and others 1999) and four for *C. vulgaris* (Piotrowska and Czerpak 2009). For *C. pyrenoidosa*, the cytokinins *N*-6-benzylaminopurine (BAP), *N*-6-furfurylaminopurine (kinetin, Kin), and allantoin/5-ureidohydantoin (AT) were added to the growth media at concentrations of 10^{-5} and 10^{-4} M (Czerpak and others 1999). For *C. vulgaris*, N^6-benzyladenine (BA), *N*,*N*′-diphenylurea (DPU), kinetin (Kin), and *trans*-zeatin (Z) were applied exogenously at eight different concentrations from 10^{-10}–10^{-3} M (Piotrowska and Czerpak 2009), and the most pronounced stimulatory effects were observed for the concentrations of 10^{-8}–10^{-6} M.

Application of BAP, Kin, and AT to *C. pyrenoidosa* each increased the content of chlorophyll *a* and *b*, aldohexoses, and water-soluble proteins (Czerpak and others 1999). Effects of the different cytokinins differed only slightly. Compared to a control, chlorophyll content was increased 183–256 %, aldohexose content was increased 182–249 %, and water-soluble protein content was increased 208–233 %. For *C. vulgaris*, addition of BA, DPU, Kin, and Z to the growth media displayed stimulatory effects that followed the trend of DPU > Z > Kin > BA (Piotrowska and Czerpak 2009). Measured variables included cell number, protein content, and amount of secreted proteins, and the activity of the enzyme NADH-dependent glutamate dehydrogenase (NADH-DGH). Stimulatory effects were also observed for per-cell contents of RNA, chlorophyll *a* and *b*, carotenoids, and monosaccharides in both the light and the dark. The increase in levels of monosaccharides, chlorophyll, and carotenoids suggested that cytokinins promote photosynthesis. Additionally, an increase in the levels of NADH-DGH was observed for the cytokinin-treated cultures compared to the control and was speculated to cause the increase in protein content in the cells as well as the content of excreted proteins. Finally, *C. vulgaris* was suggested for use as a model for studying the effects of cytokinins on higher plants.

Clearly, a variety of cytokinins have a stimulatory effect on *Chlorella* sp. when added to the growth media, as measured by a variety of variables, including cell number and chlorophyll content. An increase in the content of metabolites due to the presence of cytokinins suggests that they stimulate photosynthesis. The variety of stimulatory effects observed for cytokinin application to *Chlorella* suggests that further study of cytokinin effects would be

invaluable in optimizing growth of *Chlorella* for production of bioproducts and biofuels.

Other Plant Growth Substances

Although auxins and cytokinins are the most studied of the plant growth substances in studies performed on *Chlorella* sp., several other plant growth substances are known to have an effect on *Chlorella* growth and metabolite production. These include abscisic acid (ABA), polyamines, brassinosteroids, jasmonides, and salicylic acid. Notable exceptions among the plant growth substances studied for *Chlorella* sp. are ethylene and the gibberellins. Although at least one study suggests that gibberellins increased dry weight in *Chlorella* cultures (Burkiewicz 1987), the genomic sequence of *C. variabilis* NC64A did not contain any known genes orthologous to those in *Arabidopsis* for ethylene or gibberellin biosynthesis (Blanc and others 2010).

Although ABA is known to be synthesized in *Chlorella* (Bajguz 2009a), its function is not well understood (Tarakhovskaya and others 2007). In higher plants, ABA controls the uptake of water and ions (George and others 2008c) and is involved in resistance to temperature and salinity stresses (Mauseth 1998). Nonetheless, despite the unknown function of ABA in *Chlorella*, five ABA-related genes orthologous to genes found in *Arabidopsis* have been identified in the *C. variabilis* NC64A genome (Blanc and others 2010). A study on *C. fusca* revealed that nitrate uptake was stimulated by 210 % at an ABA concentration of 500 μM compared to a control with no ABA (Ulrich and Kunz 1984). At a physiological concentration of 20 μM, nitrate uptake was also stimulated, although at a level only 20 % greater than that for the control. Rates of respiratory oxygen consumption in the dark were increased with an ABA physiological concentration of 20 μM. Additionally, for ABA at a 500 μM concentration, respiratory oxygen consumption rates exceeded that of the control by 13 times. The role of ABA in nitrate uptake in the dark should be further investigated due to the importance of nitrate as a key nutrient in the growth media for commercial cultivation of algae.

Stimulatory effects of diamines and polyamines on *C. vulgaris* growth and metabolism have also been observed (Czerpak and others 2003). Two diamines (agmatine and putrescine) and two polyamines (spermine and spermidine) were added to the algal growth media in concentrations of 10^{-6}–10^{-3} M. All four substances were found to be inhibitory at the 10^{-3} M concentration, and the most pronounced stimulatory effects on metabolism were found for spermidine and putrescine applied at the 10^{-4} M concentration. Diamines and polyamines increased cell count per unit volume and the per-cell contents of monosaccharides, protein, and chlorophyll. The cell count for an initial spermine concentration of 10^{-6} M was more than triple the cell count of the control after 9 days of growth. The effects of diamines and polyamines on *Chlorella* are confirmed by the presence of at least five genes in the *C. variabilis* NC64A genome that are orthologous to polyamine-related genes in *Arabidopsis* (Blanc and others 2010).

One of the lesser-known types of plant growth substances that have been studied in *Chlorella* are brassinosteroids. Currently, at least seven brassinosteroids are known to be synthesized by *C. vulgaris* (Bajguz 2009b). The addition of two brassinosteroids, brassinolide and 24-epibrassinolide, to the growth media of *C. vulgaris* in concentrations ranging from 10^{-15}–10^{-8} M increased the dry weight of the culture by more than twofold over a period of 48 h (Bajguz and Czerpak 1996). A later study involving three additional brassinosteroids found that the previous two substances had the greatest stimulatory effect among the five studied (Bajguz 2000). The effect was most pronounced for concentrations of 10^{-12}–10^{-8} M. Addition of brassinolide to the culture medium at a 10^{-8} M concentration resulted in cell counts per unit volume of 330–337 % over those of a control without brassinolide after 36 h of growth and increased the DNA, RNA, and protein contents per cell (Bajguz 2000). This concentration of exogenously added brassinolide also enhanced the ABA content of the cells in response to short-term heat stress (1–3 h) (Bajguz 2009a). This effect could be valuable for outdoor cultivation of the algae, in which temperatures as high as 45 °C might be encountered (Bajguz 2009a). Finally, support for the biosynthesis and effects of brassinosteroids in *Chlorella* is found in the NC64A genome, in which at least two related genes have been identified (Blanc and others 2010).

Another plant growth substance found in *Chlorella* is jasmonic acid. Considering its connection to stress response and the accumulation of free fatty acids in higher plants (Czerpak and others 2006), an understanding of its presence and effects will likely be valuable for the growth of algae for biofuels. Both jasmonic acid and methyl jasmonate have been found in *Chlorella* (Ueda and others 1991). Addition of jasmonic acid to the growth media of *C. vulgaris* at concentrations ranging from 10^{-8}–10^{-6} M resulted in increases in cell number, carotenoids, and chlorophyll content and in the amount of excreted protein (Czerpak and others 2006). The greatest increases were observed after 7 days of growth. Jasmonic acid is believed to be involved in the stress response to UV light, reactive oxygen species, and heavy metals in *Chlorella* (Czerpak and others 2006). It is known to be involved in response to heat stress as well, as is brassinolide, and thus a better understanding of its role could be invaluable in the outdoor

commercial cultivation of microalgae. Finally, support for the biosynthesis and effects of jasmonic acid in *Chlorella* is found in the NC64A genome, in which at least two related genes have been identified (Blanc and others 2010).

Finally, the effects of salicylic acid on the growth and metabolism of *C. vulgaris* have been researched (Czerpak and others 2002). Salicylic acid added to the growth media of *C. vulgaris* at concentrations ranging from 10^{-6}–10^{-4} M resulted in increased cell number per unit volume and increased per-cell amounts of monosaccharides, chlorophyll, carotenoids, and excreted protein content. Although the most pronounced stimulatory effects were observed for the 10^{-4} M concentration, higher concentrations were not tested as they are known to inhibit the key photosynthetic enzyme RuBP carboxylase.

The effects of multiple plant growth substances applied at once have also been investigated. A study examined the effects of 12 different substances on *C. sorokiniana* (Hunt and others 2010). These authors conducted a series of 20 experiments with a growth period of 10 days to examine the effects of the 12 substances individually as well as the combination of two or three substances on biomass productivity, chlorophyll *a* productivity, biomass density, protein content, and lipid content of the *Chlorella*. They found that the most effective single substance was the auxin NAA at an initial concentration of 5 ppm, which increased biomass productivity over a control by 133 %. Two combined treatments of NAA with the gibberellin GA_3 (10 ppm) and/or the cytokinin zeatin (1 ppm) increased biomass productivity over the control by 138 and 136 %, respectively. Additionally, treatments of NAA, NAA and the auxin 2-phenylacetic acid, and NAA combined with zeatin and the gibberellin GA_3 had higher chlorophyll *a* production than the control samples. Nonetheless, the protein content remained around 50 % for all of the different treatments. In terms of biomass productivity, the greatest amount observed was an increase of 170 % over the control resulting from the combined treatment of NAA (5 ppm), GA_3 (10 ppm), and zeatin (1 ppm). Finally, after 10 days, the highest net lipid content found was for the treatment of NAA (5 ppm) with zeatin (1 ppm). Considering the advantage of treatment with combined plant growth substances in promoting biomass productivity, more studies involving multiple substances are merited. In particular, the economics of enhanced growth and metabolite production must be considered, as the addition of plant growth substances will result in additional cost to culture the algae that must be offset by the stimulatory effects observed due to the addition of these substances to the growth medium.

Synopsis

An examination of the experimental studies involving the effects of plant growth substances on *Chlorella* sp. reveals that a variety of these substances stimulate the growth and metabolism of the algae (Table 1). Furthermore, several of the substances, including brassinosteroids and jasmonic acid, may confer heat tolerance to the algae, which would be important in outdoor cultivation for the production of biofuels and bioproducts. Considering the importance of rapid growth and high metabolite content in commercial microalgal cultivation, more study to gain a better understanding of these plant growth substances is warranted.

Mixed Cultures of *Chlorella* sp.

It has been commonly observed for purified algal cultures that cultures containing contaminating bacteria grow better (Andersen 2005), and algae and bacteria are widely known to form consortia in nature (Subashchandrabose and others 2011). A number of reasons for the enhanced growth of algae in mixed cultures, that is, cultures containing a single species of microalgae and one or more species of bacteria, have been proposed. One key theory is that many algae require vitamin B_{12} but do not synthesize it on their own, instead acquiring it from symbiotic bacteria (Croft and others 2005). A second explanation is that aerobic heterotrophic bacteria use organic compounds that are produced by the algae and whose accumulation in the growth media can inhibit photosynthesis in the algae (Subashchandrabose and others 2011). A third possible reason is that bacteria growing in association with algae can consume dissolved oxygen that has evolved from photosynthesis, thus raising the photosynthetic efficiency by allowing Rubisco to preferentially fix CO_2 rather than O_2 (Subashchandrabose and others 2011).

Although the reasons for enhanced growth of algae when present in mixed cultures with bacteria are not completely known, the phenomenon is nonetheless useful for a variety of applications. First, enhanced growth contributes to better productivity of lipid for biofuels and metabolites for bioproducts. Second, consortia of bacteria and algae have been widely studied for removal of pollutants from wastewater, including organic, metal, and nutrient pollutants (Subashchandrabose and others 2011). A number of studies have focused on mixed cultures of *Chlorella* sp. and bacteria, in addition to a study focusing on *Chlorella* sp., cyanobacteria, and bacteria. In many cases, *Chlorella* sp. is known to naturally occur with certain strains of bacteria, although the introduction of foreign bacteria to a *Chlorella* culture has also been performed in many studies. Thus, the two main groups of *Chlorella* sp.–bacteria studies are those focusing on naturally occurring associations and those focusing on artificially induced associations involving nitrogen-fixing bacteria and *Chlorella* sp.

Table 1 Studies documenting the effects of plant growth substances on *Chlorella* sp.

Plant growth substance	Ideal concentration	Effects/comments	Reference(s)
Auxin precursors and analogs (7 total)	5×10^{-6}–5×10^{-4} M	Increased content of chlorophylls *a* and *b*, carotenoid; increased fresh weight	Czerpak and others (1994)
Auxins (3)	10^{-4} M, 10^{-5} M	Increased content of chlorophylls *a* and *b*, aldohexoses, and water-soluble proteins	Czerpak and others (1999)
Indomethacin	10^{-7} M	Increased cell count, dry mass, cell contents of DNA, RNA, proteins, several other metabolites	Piotrowska and others (2008)
Cytokinins (3)	10^{-4} M, 10^{-5} M	Increased content of chlorophylls *a* and *b*, aldohexoses, and water-soluble proteins	Czerpak and others (1999)
Cytokinins (4)	10^{-8}–10^{-6} M	Increased cell number, protein content and secretion, NADH-DGH activity, and the contents of RNA, chlorophylls *a* and *b*, carotenoids, and monosaccharides	Piotrowska and Czerpak (2009)
Abscisic acid	5×10^{-4} M	Increased nitrate uptake 210 %, respiratory oxygen consumption in the dark 13 times compared to a control	Ulrich and Kunz (1984)
Diamines and polyamines	10^{-4} M	Increased cell count; increased content of monosaccharides, protein, and chlorophyll	Czerpak and others 2003
Brassinosteroids (2)	10^{-15}–10^{-8} M	Increased the dry weight of the culture by over 2 times over a period of 48 h	Bajguz and Czerpak (1996)
Brassinosteroids (7)	10^{-8} M	Increased cell count; increased content of DNA, RNA, and proteins	Bajguz (2009a)
Jasmonic acid	10^{-8}–10^{-6} M	Increases in cell number, carotenoids, and chlorophyll content, and the amount of excreted protein	Czerpak and others (2006)
Salicylic acid	10^{-6}–10^{-4} M	Increased cell count and content of monosaccharides, chlorophyll, carotenoids, and excreted proteins	Czerpak and others (2002)
1-Naphthalene acetic acid (NAA)	5 ppm	Increased biomass productivity by 133 %	Hunt and others (2010)
NAA, gibberellins GA_3	5 ppm, 10 ppm	Increased biomass productivity by 138 %	Hunt and others (2010)
NAA, zeatin	5 ppm, 1 ppm	Increased biomass productivity by 136 %; produced highest net lipid productivity of treatments tested	Hunt and others (2010)
NAA, gibberellins GA_3, zeatin	5 ppm, 10 ppm, 1 ppm	Increased biomass productivity by 170 %	Hunt and others (2010)

Natural Associations of *Chlorella* sp. and Bacteria

Laboratory cultures of *Chlorella* sp. have long been known to contain a variety of bacteria. In any early study involving *C. sorokiniana*, 29 strains of bacteria present in nonaxenic continuous cultures of the algae were isolated (Litchfield and others 1969). Following taxonomic classification, the isolated bacterial strains were found to belong to the genera *Pseudomonas*, *Acinetobacter*, *Flavobacterium*, and *Bacillus*.

More recently, various studies have investigated the variety of bacteria found in xenic, unialgal laboratory cultures of *Chlorella* sp. (Watanabe and others 2005, 2008; Park and others 2008; Vu and others 2010). A natural consortium of *C. sorokiniana* IAM C-212 was found to contain four bacterial strains and a fungal strain (Watanabe and others 2005). The four bacterial strains were isolated and matched by 16S rDNA sequences to *Ralstonia pickettii*, *Sphingomonas* sp. DD38, *Microbacterium trichothecenolyticum*, and *Micrococcus luteus*. The isolated fungal strain matched *Acremonium*-like hyphomycete KR21-2, as determined by 18S rDNA sequence. Although the *Micrococcus* strain could not be cultivated in a pure culture, the other four strains were separated and cultivated alone with the *Chlorella*. The *Ralstonia* strain and the fungal strain promoted *Chlorella* growth, whereas the *Sphingomonas* and *Microbacterium* strains had no effect. Scanning electron microscopy (SEM) was employed to determine the nature of the association; the first bacterial strain and the fungal strain directly adhered to the *Chlorella* cells, whereas the second and third bacterial strains were found on the sheath of the *Chlorella*. The Watanabe study (Watanabe and others 2005) was the first in which a *Chlorella*–bacteria–fungi consortium was documented as

well as the first in which a fungal strain was found to adhere to *Chlorella*.

The extracellularly released organic carbon (EOC) synthesized by *C. sorokiniana* in the Watanabe consortium was also investigated (Watanabe and others 2008). Analysis of compounds secreted into the media revealed 12 different dissolved carbohydrates, with sucrose being the most prevalent. Additionally, 27 dissolved organic nitrate substances were identified. Nonetheless, these 39 compounds comprised only 11.1 % of the total EOC content in the media, suggesting that many other compounds are present. The cell-free broth resulting from 2 weeks' growth of *Chlorella* was used as an artificial EOC medium, suitable for the cultivation of heterotrophic bacteria isolated from environmental samples.

A long-term xenic laboratory culture of *C. ellipsoidea* (UTEX 247) was found to contain eight different bacterial strains (Park and others 2008). Each strain was coinoculated separately with the *Chlorella*, and one strain was found to promote the growth of the *Chlorella* by three times compared to growth of the *Chlorella* strain alone. This strain was identified as *Brevundimonas* sp. and was found by SEM to be directly adhered to the *Chlorella* cells. Additionally, the *Chlorella* cells in the coculture of *Brevundimonas* sp. had a crinkled appearance compared to the cells in the axenic *Chlorella* culture. Notably, both the bacteria and the algae displayed higher cell counts per unit volume when cultivated together.

Bacteria of the *Brevundimonas* genus were also isolated from a culture of *C. vulgaris* NIES-227 (Vu and others 2010). Although bacterial strains from six other genera were isolated from the culture, only *Brevundimonas* appeared to promote the growth of the algae. The *Brevundimonas* strain increased the culture lifetime to 8 weeks, whereas several of the other strains decreased the culture lifetime to 5–7 weeks.

Bacterial communities in a *C. vulgaris* culture grown in municipal tap water were also identified (Lakaniemi and others 2011). Bacterial genera, as identified by genetic analysis of 16S rDNA, included *Brevundimonas*, *Sphingomonas*, *Blastomonas*, *Porphyrobacter*, and *Mesorhizobium*. The first two genera have been found in other bacterial communities associated with mixed cultures of *Chlorella* and bacteria (Park and others 2008; Watanabe and others 2005, 2008; Vu and others 2010), although *Sphingomonas* did not have a significant effect on *Chlorella* growth (Watanabe and others 2005; Lakaniemi and others 2011). Several of the species identified were unknown and may be species not previously found in *Chlorella* cultures.

A final study involves the cocultivation of *C. vulgaris* and a cyanobacterium *Leptolyngbya* sp. (Rusch and Gutierrez-Wing unpublished data; Tate and others unpublished). The alga–cyanobacterium coculture was isolated from an environmental sample and is known to contain bacteria, although the strains have not yet been identified. The coculture possessed higher biomass productivity than a unialgal culture of the *Chlorella* and has proven resistant to contamination by fungi and other airborne contaminants (Rusch and Gutierrez-Wing unpublished data).

In summary, many long-term laboratory cultures that are unialgal for *Chlorella* sp. have been found to contain a variety of bacteria (Table 2). In some cases, the bacteria are known to enhance the growth of the *Chlorella*, as determined by cultivating the desired bacterial strain and the *Chlorella* without the other species in the consortium. Furthermore, bacteria from several genera, including *Brevundimonas* and *Sphingomonas*, have been found by multiple studies that identified bacterial strains present in xenic *Chlorella* cultures. A better understanding of the role of bacteria in mixed cultures may prove invaluable in promoting *Chlorella* sp. growth, especially for commercial cultivation.

Artificially Induced Associations of *Chlorella* sp. and Bacteria/Yeast

The second group of mixed cultures of *Chlorella* sp. and bacteria are artificially induced associations. These mixed cultures are generally synthesized with the goal of treating wastewater, although the goal of promoting *Chlorella* sp. growth by artificially induced symbioses would have more general commercial application. Common characteristics among the artificially induced associations are the use of nitrogen-fixing bacteria and the coimmobilization of the *Chlorella* and the bacteria.

One of the early studies involving nitrogen-fixing bacteria and *Chlorella* used a culture isolated from an agro-industrial wastewater pond (Gonzalez-Bashan and others 2000; Lebsky and others 2001). *C. vulgaris* Beijerinck and N_2-fixing bacterium *Phyllobacterium myrsinacearum* were isolated from the pond and cultivated separately. The two species were coimmobilized in alginate beads, a common strategy used for microorganisms used to treat wastewater. Production of the pigments chlorophyll *a* and *b*, violoxanthin, lutein, and β-carotene increased in the mixed culture compared to in a *Chlorella* culture without the bacteria, although the increase was not statistically significant for chlorophyll *a* and β-carotene. Nonetheless, the ability of the *Chlorella* to remove ammonium and phosphorus from the wastewater was reduced in the mixed culture, possibly suggesting that in the wastewater pond a similar phenomenon of reduced ability to assimilate ammonium and phosphorus may occur. Similarly, coimmobilization of *C. vulgaris* Beijerinck with the N_2-fixing soil bacteria *Bacillus pumilus* was tested, but the mixed culture did not remove pollutants from the wastewater

Table 2 Bacteria/fungi in mixed cultures with *Chlorella* sp.

Chlorella species	Bacteria/fungus genera	Natural/ artificial consortium	Comments	Reference
sorokiniana	*Pseudomonas Acinetobacter Flavobacterium Bacillus*	Natural	Taxonomic classifications performed without genetic techniques	Litchfield and others (1969)
sorokiniana	*Ralstonia Spingomonas Microbacterium Micrococcus Acremonium* (fungus)	Natural	*Ralstonia* and *Acremonium* promoted *Chlorella* growth; *Sphingomonas* and *Microbacterium* had no effect; *Micrococcus* not able to be cultivated by itself	Watanabe and others (2005)
ellipsoidea	*Brevundimonas*	Natural	7 other strains, but *Brevundimonas* promoted most increase in growth of *Chlorella*	Park and others (2008)
vulgaris	*Brevundimonas*	Natural	6 other strains, but only *Brevundimonas* promoted increased growth of *Chlorella* compared to a control	Vu and others (2010)
vulgaris	*Brevundimonas Sphingomonas Blastomonas Porphyrobacter Mesorhizobium*	Natural	Several unknown strains as well; bacterial isolates were from municipal tap water	Lakaniemi and others (2011)
vulgaris	*Leptolyngbya*	Natural	Unique in that consortium is alga with cyanobacteria	Rusch and Gutierrez-Wing, in review
vulgaris	*Phyllobacterium*	Artificial	Ability of *Chlorella* to remove ammonium and phosphorus from wastewater hindered in the mixed culture	Gonzalez-Bashan and others (2000)
vulgaris	*Bacillus*	Artificial	No promotion of nutrient removal from the wastewater over *Chlorella* without the bacteria	Hernandez and others (2009)
vulgaris	*Azospirillum*	Artificial	Increased dry weight, fresh weight, cell number, size, and number of algae clusters in the alginate beads, and levels of 5 pigments	Gonzalez and Bashan (2000)
vulgaris and *sorokiniana*	*Azospirillum*	Artificial	In mixed culture two *Chlorella* species increased population size, one increased cell size; increased lipid productivity	de-Bashan and others (2002)
vulgaris	*Azospirillum*	Artificial	*Azospirillum* mutants deficient in IAA production stimulated growth of *Chlorella* little to none	de-Bashan and others (2008)
vulgaris	*Azospirillum*	Artificial	Examined effects of heterotrophic, mixotrophic, and autotrophic growth	Perez-Garcia and others (2010)
vulgaris	*Rhodotorula*	Artificial	Oleaginous yeast and *Chlorella* in mixed culture; both produce lipids	Cheirsilp and others (2011)
sorokiniana	*Microbacterium*	Artificial	Artificial symbiosis induced by addition of $CaCl_2$ to *Chlorella* growth medium, causing augmentation of sheath	Imase and others (2008)
vulgaris	*Azospirillum*	Artificial	Biofilm observed even when alginate beads dissolved; could be produced by either species or a combination of both	de-Bashan and others (2011)

better than did the unialgal *Chlorella* culture (Hernandez and others 2009).

A comparison of mixed cultures of *C. vulgaris* with *P. myrsinacearum* and *C. vulgaris* with *Azospirillum brasilense*, which has been studied extensively in light of plant–bacteria relationships (Bashan and others 2004), revealed that coinoculation with *Azospirillum* allowed the *Chlorella* to remain growing for a longer period of time (Lebsky and others 2001). As a result, *Azospirillum* was used in subsequent studies on mixed *Chlorella* cultures. The N_2-fixing bacteria *A. brasilense* was coimmobilized in alginate beads with *C. vulgaris* Beijerinck (Gonzalez and

Bashan 2000). Coculturing the *Chlorella* in this mixed culture increased the dry weight, fresh weight, cell number per unit volume, size of the algae clusters in the beads, number of algae clusters per bead, and levels of the pigments chlorophyll *a* and *b*, violoxanthin, lutein, and β-carotene. Notably, a similar effect to that of coculturing with *A. brasilense* was observed by the addition of the auxin IAA to a liquid culture of *Chlorella* without *Azospirillum* before immobilization in the alginate beads.

Further research was performed to study the coimmobilization of *Chlorella* sp. and *Azospirillum* sp. in alginate beads (de-Bashan and others 2002, 2008, 2011; Perez-Garcia and others 2010). First, three different strains of *Chlorella*, including the previously used *C. vulgaris* Beijerinck UTEX 2714 in addition to *C. vulgaris* UTEX 395 and *C. sorokiniana* Shih. and Kraus UTEX 1602, were tested (de-Bashan and others 2002). Each was separately immobilized with *A. brasilense* in the alginate beads. The first and third *Chlorella* strains displayed an increase in population size, whereas the second strain displayed 62 % larger cells but not a significantly larger number of cells. After 3 days of growth, all three strains displayed an increase in pigment content for chlorophylls *a* and *b*, violoxanthin, lutein, and β-carotene. Furthermore, lipid content was increased for each, and in the mixed cultures eight different fatty acids were synthesized as compared to only five for each *Chlorella* culture without *Azospirillum*.

Different strains of *Azospirillum* have also been tested in the mixed cultures, using *C. vulgaris* Beijerinck UTEX 2714 for each (de-Bashan and others 2008). Three wild-type and four mutant *Azospirillum* strains were used; the mutants had a deficiency that reduced their production of the auxin IAA to less than 5 % of that of the wild-type strains. The mixed cultures of *Chlorella* and the mutant strains displayed little to no increased growth compared to a unialgal *Chlorella* culture, suggesting that auxin production explains growth promotion of the *Azospirillum*.

Finally, the effects of heterotrophic, mixotrophic, and autotrophic growth on the mixed cultures containing *C. vulgaris* and *A. brasilense* have been examined (Perez-Garcia and others 2010). Important findings were that heterotrophic growth is best when the *Chlorella* is cultivated alone. Additionally, growth promotion by *Azospirillum* occurs only in the mixed culture for mixotrophic and autotrophic conditions. The use of a mixed culture versus a *Chlorella* culture without *Azospirillum* and the best mode of growth were determined for whatever target parameter for which the system would be designed, such as ammonium removal, phosphorus removal, or *Chlorella* growth.

One final mixed *Chlorella* culture involved *C. vulgaris* and the oleaginous yeast *Rhodotorula glutinis* (Cheirsilp and others 2011). The mixed culture was grown with the goal of maximizing lipid productivity, using industrial wastes as nutrient sources. It was postulated that the enhanced biomass yield and lipid production of the mixed cultures occurred because the alga consumed CO_2 produced by the yeast and generated O_2 for the yeast to consume. Optimal conditions for the mixed culture, including yeast:alga ratio (1:1), initial pH (5.0), molasses concentration in the growth media (1 %), agitation speed of the bioreactor impeller culture (200 rpm), and light intensity (0.5 klux), were reported. The ability of both species in the mixed culture to produce lipids for biofuel use is an advancement, as opposed to a mixed culture in which only the alga produces most of the lipids.

Because a variety of mixed cultures promote *Chlorella* growth, a better understanding of the cell–cell interactions causing growth promotion of these cultures is desired. When *Chlorella* is cultivated under photoautotrophic conditions in a mixed culture, a sheath around the cells has been observed (Watanabe and others 2006; Imase and others 2008; de-Bashan and others 2011). The sheath consists of carbohydrates, protein, and metal ions for *C. sorokiniana*, and it is known that the sheath can be artificially augmented by the addition of $CaCl_2$ to the growth media (Imase and others 2008). The enhanced sheaths were able to bind bacteria in the culture and create an artificial consortium that displayed higher growth and allowed *Chlorella* to be grown in a wastewater medium containing propionate, which would normally inhibit *Chlorella* growth.

Alginate beads were used to coimmobilize *C. vulgaris* and *A. brasilense* (de-Bashan and others 2011). A thorough study of the biofilm found in the alginate beads was conducted, and it was suggested that the system could be used as a model to investigate eukaryote–prokaryote cell–cell interactions (de-Bashan and others 2011). As previously mentioned, larger *Chlorella* clusters were observed when *Azospirillum* was present. When the alginate bead was dissolved, a biofilm remained, which could have been composed of polysaccharides known to be produced by *Azospirillum* and that aid in the attachment of the bacterium to plant roots, that is, the sheath possibly synthesized by the *Chlorella* cells (Imase and others 2008), or some mixture of the two. The biofilm and the resulting attachment were compared to those of *Azospirillum* to plant roots in which the attachment prevents the bacterium from being washed off, prevents other bacteria from consuming substances that the plant excretes to promote the symbiosis with *Azospirillum*, and prevents the colonization of the root sites by other, potentially harmful, bacteria. The *Azospirillum–Chlorella* association was suggested as a good model for the general study of bacterium–plant interactions.

In summary, various studies have been performed using artificial symbioses involving coimmobilization of *Chlorella* sp. with nitrogen-fixing bacteria, particularly *A. brasilense*. Nonetheless, it has also been shown that formation

can be promoted by inducing augmentation of the sheath of the *Chlorella* cells by the addition of calcium chloride to the growth medium. Such a sheath could promote the attachment of possibly growth-promoting bacteria to the *Chlorella* cells. Although the benefit of the bacteria to the *Chlorella* is not well understood, reasons including the removal of dissolved oxygen from the medium (Subashchandrabose and others 2011), production of vitamin B_{12} for the alga (Croft and others 2005), and synthesis of the auxin IAA (de-Bashan and others 2008) have been suggested. Considering the practical value of the mixed cultures in treating wastewater and promoting growth of the *Chlorella* in general, further study of mixed *Chlorella* cultures is expected.

Conclusions and Future Prospects

Recently, interest in microalgae has greatly increased by an interest in biofuels. Additionally, microalgae, including the commonly cultivated *Chlorella* sp., have been sought as "cell factories" of bioproducts, including food supplements, supplements of animal feed, pigments, and cosmetics. Important to the commercial cultivation of microalgae is the optimization of algal growth. Traditional approaches to optimization of growth and metabolite production have included varying growth parameters, including pH, temperature, irradiance level, carbon source, aeration, and concentrations of specific nutrients. Nonetheless, two other attractive approaches are the use of plant growth substances and the use of mixed cultures. Considering the ability of plant growth substances and mixed cultures to have a large effect on the stimulation of growth and metabolite production, more work in these two areas is warranted. An additional benefit of studying these topics using microalgae is that a better understanding of higher plants and of plant–bacteria interactions will be gained.

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References

Andersen RA (ed) (2005) Algal culturing techniques. Elsevier Academic Press, Burlington

Bai R, Silaban AG, Gutierrez-Wing MT, Benton MG, Rusch KA (in review) Effects of nitrogen and irradiance on lipid content and composition of a Louisiana native *Chlorella vulgaris*/*Leptolyngbya* sp. co-culture

Bajguz A (2000) Effect of brassinosteroids on nucleic acids and protein content in cultured cells of *Chlorella vulgaris*. Plant Physiol Biochem 38:209–215

Bajguz A (2009a) Brassinosteroid enhanced the level of abscisic acid in *Chlorella vulgaris* subjected to short-term heat stress. J Plant Physiol 166:882–886

Bajguz A (2009b) Isolation and characterization of brassinosteroids from algal cultures of *Chlorella vulgaris* Beijerinck (Trebouxiophyceae). J Plant Physiol 166:1946–1949

Bajguz A, Czerpak R (1996) Effect of brassinosteroids on growth and proton extrusion in the alga *Chlorella vulgaris* Beijerinck (Chlorophyceae). J Plant Growth Regul 15:153–156

Bashan Y, Holguin G, de-Bashan LE (2004) *Azospirillum*–plant relationships: physiological, molecular, agricultural, and environmental advances (1997–2003). Can J Microbiol 50:521–577

Bhola V, Desikan R, Santosh SK, Subburamu K, Sanniyasi E, Bux F (2011) Effects of parameters affecting biomass yield and thermal behaviour of *Chlorella vulgaris*. J Biosci Bioeng 111:377–382

Blanc G, Duncan G, Agarkova I, Borodovsky M, Gurnon J, Kuo A, Lindquist E, Lucas S, Pangilinan J, Polle J, Salamov A, Terry A, Yamada T, Dunigan DD, Grigoriev IV, Claverie J-M, Van Etten JL (2010) The *Chlorella variabilis* NC64A genome reveals adaptation to photosymbiosis, coevolution with viruses, and cryptic sex. Plant Cell 22:2943–2955

Bradley PM (1991) Plant hormones do have a role in controlling growth and development of algae. J Phycol 27:317–321

Burkiewicz K (1987) The influence of gibberellins and cytokinins on the growth of some unicellular Baltic algae. Bot Mar 30:63–69

Cheirsilp B, Suwannarat W, Niyomdecha R (2011) Mixed culture of oleaginous yeast *Rhodotorula glutinis* and microalga *Chlorella vulgaris* for lipid production from industrial wastes and its use as biodiesel feedstock. New Biotechnol 28:362–368

Converti A, Casazza AA, Ortiz EY, Perego P, Del Borghi M (2009) Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production. Chem Eng Prog 48:1146–1151

Croft MT, Lawrence AD, Raux-Deery E, Warren MJ, Smith AG (2005) Algae acquire vitamin B12 through a symbiotic relationship with bacteria. Nature 438:90–93

Czerpak R, Bajguz A, Bialecka B, Wierzcholowska LE, Wolanska MM (1994) Effect of auxin precursors and chemical analogues on the growth and chemical composition in *Chlorella pyrenoidosa* Chick. Acta Soc Bot Pol 63:279–286

Czerpak R, Krotke A, Mikal A (1999) Comparison of stimulatory effect of auxins and cytokinins on protein, saccharides, and chlorophylls content in *Chlorella pyrenoidosa* Chick. Pol Arch Hydrobiol 46:71–82

Czerpak R, Bajguz A, Gromek M, Koztowska G, Nowak I (2002) Activity of salicylic acid on the growth and biochemism of *Chlorella vulgaris* Beijerinck. Acta Physiol Plant 24:45–52

Czerpak R, Bajguz A, Pietrowska A, Dobrogowska R, Matejczyk M, Weislawski W (2003) Biochemical activity of di- and polyamines in the green alga *Chlorella vulgaris* Beijerinck (Chlorophyceae). Acta Soc Bot Pol 72:19–24

Czerpak R, Pietrowska A, Szulecka K (2006) Jasmonic acid affects changes in the growth and some components content in alga *Chlorella vulgaris*. Acta Physiol Plant 28:195–203

de-Bashan LE, Bashan Y, Moreno M, Lebsky VK, Bustillos JJ (2002) Increased pigment and lipid content, lipid variety, and cell and population size of the microalgae *Chlorella* spp. when co-immobilized in alginate beads with the microalgae-growth-promoting bacterium *Azospirillum brasilense*. Can J Microbiol 48:514–521

de-Bashan LE, Antoun H, Bashan Y (2008) Involvement of indole-3-acetic acid produced by the growth-promoting bacterium *Azospirillum* spp. in promoting growth of *Chlorella vulgaris*. J Phycol 44:938–947

de-Bashan LE, Schmid M, Rothballer M, Hartmann A, Bashan Y (2011) Cell–cell interaction in the eukaryote–prokaryote model of the microalgae *Chlorella vulgaris* and the bacterium *Azospirillum brasilense* immobilized in polymer beads. J Phycol 47:1350–1359

Evans LV, Trewavas AJ (1991) Is algal development controlled by plant growth substances? J Phycol 27:322–326
Gallagher BJ (2011) The economics of producing biodiesel from algae. Renew Energy 36:158–162
George EF, Hall MA, de Klerk GJ (eds) (2008a) Plant growth regulators I: introduction; auxins, their analogues and inhibitors. Plant propagation by tissue culture. Springer, Berlin
George EF, Hall MA, de Klerk GJ (eds) (2008b) Plant growth regulators II: cytokinins, their analogues and antagonists. Plant cell propagation by tissue culture. Springer, Berlin
George EF, Hall MA, de Klerk GJ (eds) (2008c) Plant growth regulators III: gibberellins, ethylene, abscisic acid, their analogues and inhibitors; miscellaneous compounds. Plant cell propagation by tissue culture. Springer, Berlin
Gonzalez LE, Bashan Y (2000) Increased growth of the microalga *Chlorella vulgaris* when coimmobilized and cocultured in alginate beads with the plant-growth-promoting bacterium *Azospirillum brasilense*. Appl Environ Microbiol 66:1527–1531
Gonzalez-Bashan LE, Lebsky VK, Hernandez JP, Bustillos JJ, Bashan Y (2000) Changes in the metabolism of the microalgae *Chlorella vulgaris* when coimmobilized in alginate with the nitrogen-fixing *Phyllobacterium myrsinacearum*. Can J Microbiol 46:653–659
Greenwell HC, Laurens LML, Shields RJ, Lovitt RW, Flynn KJ (2009) Placing microalgae on the biofuels priority list: a review of the technological challenges. J R Soc Interface 7:703–726
Harwood JL, Guschina IA (2009) The versatility of algae and their lipid metabolism. Biochimie 91:679–684
Hernandez J, de-Bashan L, Rodriguez D, Rodriguez Y, Bashan Y (2009) Growth promotion of the freshwater microalga *Chlorella vulgaris* by the nitrogen-fixing, plant growth-promoting bacterium *Bacillus pumilus* from arid zone soils. Eur J Soil Biol 45: 88–93
Hsieh CH, Wu WT (2009) Cultivation of microalgae for oil production with a cultivation strategy of urea limitation. Bioresour Technol 100:3921–3926
Huang G, Chen F, Wei D, Zhang X, Chen G (2010) Biodiesel production by microalgal biotechnology. Appl Energy 87:38–46
Hunt RW, Chinnasamy S, Bhatnagar A, Das KC (2010) Effect of biochemical stimulants on biomass productivity and metabolite content of the microalga, *Chlorella sorokiniana*. Appl Biochem Biotechnol 162:2400–2414
Hunt RW, Chinnasamy S, Das KC (2011) The effect of naphthaleneacetic acid on biomass productivity and chlorophyll content of green algae, coccolithophore, diatom, and cyanobacterium cultures. Appl Biochem Biotechnol 164:1350–1365
Illman AM, Scragg AH, Shales SW (2000) Increase in *Chlorella* strains calorific values when grown in low nitrogen medium. Enzyme Microb Technol 27:631–635
Imase M, Watanabe K, Ioyagi H, Tanaka H (2008) Construction of an artificial symbiotic community using a *Chlorella*–symbiont association as a model. FEMS Microbiol Ecol 63:273–282
Kang CD, Sim SJ (2007) Direct extraction of astaxanthin from *Haematococcus* culture using vegetable oils. Biotechnol Lett 30: 441–444
Khozin-Goldberg I, Cohen Z (2011) Unraveling algal lipid metabolism: recent advances in gene identification. Biochimie 93: 91–100
Lakaniemi AM, Intihar VM, Tuovinen OH, Puhakka JA (2011) Growth of *Chlorella vulgaris* and associated bacteria in photobioreactors. Microb Biotechnol 5:69–78
Lebsky VK, Gonzalez-Bashan LE, Bashan Y (2001) Ultrastructure of interaction in alginate beads between the microalga *Chlorella vulgaris* with its natural associative bacterium *Phyllobacterium myrsinacearum* and with the plant growth-promoting bacterium *Azospirillum brasilense*. Can J Microbiol 47:1–8
Li Y, Horsman M, Wu N, Lan CQ, Dubois-Calero N (2008) Biofuels from microalgae. Biotechnol Prog 24:815–820
Litchfield CD, Colwell RR, Prescott JM (1969) Numerical taxonomy of heterotrophic bacteria growing in association with continuous-culture *Chlorella sorokiniana*. Appl Microbiol 18:1044–1049
Liu ZY, Wang GC, Zhou BC (2008) Effect of iron on growth and lipid accumulation in *Chlorella vulgaris*. Bioresour Technol 99: 4717–4722
Machida T, Murase H, Kato E, Honjoh K-i, Matsumoto K, Miyamoto T, Iio M (2008) Isolation of cDNAs for hardening-induced genes from *Chlorella vulgaris* by suppression subtractive hybridization. Plant Sci 195:238–246
Mauseth JD (1998) Botany: an introduction to plant biology. Jones and Bartlett, Sudbury
Merchant SS, Kropat J, Liu B, Shaw J, Warakanont J (2011) TAG, You're it! *Chlamydomonas* as a reference organism for understanding algal triacylglycerol accumulation. Curr Opin Biotechnol 23:352–363
Multu YB, Isik O, Uslu L, Koc K, Durmaz Y (2011) The effects of nitrogen and phosphorus deficiencies and nitrite addition on the lipid content of *Chlorella vulgaris* (Chlorophyceae). Afr J Biotechnol 10:453–456
Park Y, Je KW, Lee K, Jung SE, Choi TJ (2008) Growth promotion of *Chlorella ellipsoidea* by co-inoculation with *Brevundimonas* sp. isolated from the microalga. Hydrobiologia 598:219–228
Perez-Garcia O, De-Bashan LE, Hernandez JP, Bashan Y (2010) Efficiency of growth and nutrient uptake from wastewater by heterotrophic, autotrophic, and mixotrophic cultivation of *Chlorella vulgaris* immobilized with *Azospirillum brasilense*. J Phycol 46: 800–812
Piotrowska A, Czerpak R (2009) Cellular response of light/dark-grown green alga *Chlorella vulgaris* Beijerinck (Chlorophyceae) to exogenous adenine- and phenylurea-type cytokinins. Acta Physiol Plant 31:573–585
Piotrowska A, Czerpak R, Pietryczuk A, Olesiewicz A, Wedołowska M (2008) The effect of indomethacin on the growth and metabolism of green alga *Chlorella vulgaris* Beijerinck. Plant Growth Regul 55:125–136
Pratt R (1938) Influence of auxins on the growth of *Chlorella vulgaris*. Am J Bot 25:498–501
Rismani-Yazdi H, Haznedaroglu BZ, Peccia J (2011) Transcriptome sequencing and annotation of the microalgae *Dunaliella tertiolecta*: pathway description and gene discovery for production of next-generation biofuels. BMC Genomics 12:148
Rusch KA, Gutierrez-Wing MT (unpublished data) A protocol for native mixed algae-cyanobacteria selection for biofuels and bioproducts feedstock
Singh J, Gu S (2010) Commercialization potential of microalgae for biofuels production. Renew Sustain Energ Rev 14:2596–2610
Subashchandrabose SR, Ramakrishnan B, Megharaj M, Venkateswarlu K, Naidu R (2011) Consortia of cyanobacteria/microalgae and bacteria: biotechnological potential. Biotechnol Adv 29:896–907
Sun A, Davis R, Starbuck M, Ben-Amotz A, Pate R, Pienkos PT (2011) Comparative cost analysis of algal oil production for biofuels. Energy 36:5169–5179
Tam NFY, Wong YS (1990) The comparison of growth and nutrient removal efficiency of *Chlorella pyrenoidosa* in settled and activated sewages. Environ Pollut 65:93–108
Tarakhovskaya ER, Maslov YI, Shishova MF (2007) Phytohormones in algae. Russ J Plant Physiol 52:163–170
Tate JJ, Gutierrez-Wing MT, Rusch KA, Benton MG (2012) Gene expression analysis of a Louisiana native *Chlorella vulgaris* (Chlorophyta)/*Leptolyngbya* sp. (cyanobacteria) co-culture using suppression subtractive hybridization. Eng Life Sci. doi:10.1002/elsc.201200063

Thurmond W (2009) Algae 2020: advanced biofuel markets and commercialization outlook, 1st edn. Emerging Markets online, Houston

Tsavkelova EA, Klimova SY, Cherdyntseva TA, Netrusov AI (2006) Microbial producers of plant growth stimulators and their practical use: a review. Appl Biochem Microbiol 42:117–126

Ueda J, Miyamoto K, Aoki M, Hirata T, Sato T, Momotani Y (1991) Identification of jasmonic acid in *Chlorella* and *Spirulina*. Bull Univ Osaka Prefect Ser B 43:103–108

Ulrich WR, Kunz G (1984) Effect of abscisic acid on nitrogen uptake, respiration and photosynthesis in green algae. Plant Sci Lett 37:9–14

US DOE (Department of Energy) (2010) National Algal Biofuels Technology Roadmap. Available at http://www1.eere.energy.gov/biomass/pdfs/algal_biofuels_roadmap.pdf. Accessed 1 Sept 2011

Vance BD (1987) Phytohormone effects on cell division in *Chlorella pyrenoidosa* Chick (TX-7-11-05) (Chlorellaceae). J Plant Growth Regul 5:169–173

Vu HT, Otsuka S, Ueda H, Senoo K (2010) Cocultivated bacteria can increase or decrease the culture lifetime of *Chlorella vulgaris*. J Gen Appl Microbiol 56:413–418

Watanabe K, Takihana N, Aoyagi H, Hanada S, Watanabe Y, Ohmura N, Saiki H, Tanaka H (2005) Symbiotic association in *Chlorella* culture. FEMS Microbiol Ecol 51:187–196

Watanabe K, Imase M, Sasaki K, Ohmura N, Saiki H, Tanaka H (2006) Composition of the sheath produced by the green alga *Chlorella sorokiniana*. Lett Appl Microbiol 42:538–543

Watanabe K, Imase M, Aoyagi H, Ohmura N, Saiki H, Tanaka H (2008) Development of a novel artificial medium based on utilization of algal photosynthetic metabolites by symbiotic heterotrophs. J Appl Microbiol 105:741–751

Widjaja A, Chien CC, Ju YH (2009) Study of increasing lipid production from fresh water microalgae *Chlorella vulgaris*. J Taiwan Inst Chem Eng 40:13–20

Yin HC (1937) Effect of auxin on *Chlorella vulgaris*. Proc Natl Acad Sci USA 23:174–176

Zabochnicka-Swiatek M (2010) Algae–feedstock of the future. Arch Combust 30:225–236