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(54) Title: NOVEL *PSEUDOCHLOROCOCCUM* SPECIES AND USES THEREFOR

(57) Abstract: The present invention relates to algal species and compositions, methods for identifying algae that produce high lipid content and possess CO_2 tolerance, and methods for using such algae for lipid isolation, wastewater remediation, waste gas remediation, and/or biomass production.

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Novel *Pseudochlorococcum* species and uses therefor

Field of the Invention

The invention relates to algae, algae selection methods, and methods for using algae to make various products.

Background of the Invention

Global warming due to increases in CO_2 and other greenhouse gases (methane, chloroflurocarbons, etc.) in the atmosphere, and widespread water pollution with nutrients (such as nitrogen and phosphate) and other contaminants, are major environmental concerns. Although many conventional techniques and approaches are available for pollution prevention and control, these methods are usually very expensive with high energy consumption. Large quantities of sludge and/or liquid wastes generated from these systems are difficult to deal with and may also pose the risk of creating secondary contamination. Oil, natural gas, coal, and nuclear energy are the predominant sources of energy used today and they are not sustainable. As energy consumption increases, the natural reserves of these nonrenewable fossil fuels shrink drastically. For instance, at the current rate of consumption, currently identified oil reserves will last approximately 50 years or less. Production and consumption of fossil fuels are also the major causes of regional and global air and water pollution.

Engineered bacterial system may be designed that can breakdown and remove nutrients and other contaminants from waste streams, but can not effectively convert and recycle waste nutrients into renewable biomass. Many oil crops such as soy, rapeseeds, sunflower seeds, palm seeds are a source of feedstock for biodiesel, but these crops can not adequately perform wastestream treatment.

Summary of the Invention

In a first aspect, the present invention provides isolated *Pseudochlorococcum* sp. compositions, wherein the isolated *Pseudochlorococcum* sp. genome comprises one or more nucleic acid sequence selected from the group consisting of SEQ ID NO:1 (ITS—1622 bp), SEQ ID NO:2 (rbcL—1160 bp), SEQ ID NO:3 (ITS1—928-1082 of ITS), SEQ ID NO:4 (ITS2—1247-1487 of ITS), and SEQ ID NO:5 (ITS—827 bp), or complements thereof.

In a second aspect, the present invention provides a substantially pure culture, comprising:

(a) a growth medium; and

(b) the isolated *Pseudochlorococcum* sp. composition of the first aspect of the invention.

In a third aspect, the present invention provides an algal culture system, comprising:

(a) a photobioreactor; and

(b) the substantially pure culture of the second aspect of the invention.

In a fourth aspect, the present invention provides methods for lipid isolation, wastewater remediation, waste gas remediation, and/or biomass production, comprising culturing a *Pseudochlorococcum* sp., wherein the *Pseudochlorococcum* sp. genome comprises one or more nucleic acid sequence selected from the group consisting of SEQ ID NO:1 (ITS—1622 bp), SEQ ID NO:2 (rbcL—1160 bp), SEQ ID NO:3 (ITS1—928-1082 of ITS), SEQ ID NO:4 (ITS2—1247-1487 of ITS), and SEQ ID NO:5 (ITS—827 bp), or complements thereof, wherein the culturing is carried out under conditions suitable for fatty acid isolation, wastewater remediation, waste gas remediation, and/or biomass production.

Brief Description of the Figures

Figure 1 Typical GC chart of fatty acid profile of *Pseudochlorococcum* sp.

Figure 2 Effect of carbon dioxide on growth of *Pseudochlorococcum* sp. aerated with air containing either 1% or 15% CO_2. Cultures were maintained at 25±1°C and light intensity of 175μmol m^{-2} s^{-1}. Cultures were grown in 300 ml capacity glass columns 68 cm long with an inner diameter of 2.3 cm.

Figure 3 Effect of carbon dioxide on biomass yield of *Pseudochlorococcum* sp. (Culture conditions were the same as described for figure 1).

Figure 4 Effects of carbon dioxide on the lipid content (a) and lipid yield (b) of *Pseudochlorococcum* sp (Culture conditions same as for figure 1).

Figure 5 Effect of dairy wastewater (DWW) on growth of *Pseudochlorococcum* sp. grown in 300 ml capacity glass columns (68 cm long with an inner diameter of 2.3 cm) at 25±1°C, 1% CO_2, and continuous illumination of 170 μmol m^{-2} s^{-1}.

Figure 6 Effect of dairy wastewater on biomass yield of *Pseudochlorococcum* sp. grown in a glass column bioreactor (Growth conditions were the same as for figure 4).

Figure 7 Effect of dairy wastewater on lipid content of *Pseudochlorococcum* sp. grown in a glass column bioreactor (Growth conditions were the same as for figure 4).

Figure 8 Effect of dairy wastewater on lipid production by *Pseudochlorococcum* sp. grown in a glass column bioreactor (Growth conditions were the same as for figure 4).

Figure 9 Growth kinetics of *Pseudochlorococcum* strain grown outdoors in flat panel bioreactors varying in light path. Culture conditions: maximum daily culture temperature was maintained at 29±2°C by evaporative cooling. pH was 7.0 ~ 8.0. Mixing and additional CO_2 supply were provided by compressed air stream enriched with 1% CO_2 through a perforated tube running through the bottom of the reactor.

Figure 10 Lipid content of *Pseudochlorococcum* cells grown outdoors in the flat panel bioreactors of various light paths. (Culture conditions described in Fig. 8)

Figure 11 Areal (a) and volumetric (b) production of *Pseudochlorococcum* biomass outdoors in the flat panel bioreactors of various light paths. (Culture conditions described in Fig. 8)

Figure 12 Fig. 12. Areal lipid yield and volumetric lipid yield of *Pseudochlorococcum* sp. grown in the different light-paths of the flat-panel photobioreactors outdoors. (Culture conditions described in Fig. 8)

Figure 13 PCR products amplified from *Pseudochlorococcum* sp. A: DNA Ladder; B: ITS; and C: *rbcL*.

Figure 14 Neighbor-joining (NJ) tree based on aligned nucleotide sequences for 827 bases in the regions of ITS from 22 OTUs belonging to Chlorophyta. The numbers above branches indicate the bootstrap values resolved in the majority-rule consensus tree of a bootstrap analysis based on 1000 replications. The non-significant values below 50 were not shown.

Figure 15 Neighbor-joining (NJ) tree based on aligned nucleotide sequences for 1129 base pairs in the regions of *rbcL* from 20 OTUs belonging to Chlorophyta. The numbers above branches indicate the bootstrap values resolved in the majority-rule consensus tree of a bootstrap analysis based on 1000 replications. The non-significant values below 50 were not shown.

Figure 16 Sequence alignment of 827 bp region of ITS-rDNA segment for *Pseudochlorococcum* sp. and its phylogenetically closest-related species *Desmodesmus multivariabilis* var. *turskensis* Mary 8/18 T-1W (GeneBank Accession Number: DQ417). ITS1 (171-325) and ITS2 (488-729) are marked separately.

Figure 17 Sequence Alignment of 1160 bp of *rbcL* for *Pseudochlorococcum* sp. (PSP) and its phylogenetically closest-related species *Neochloris sp.* LCR (GeneBank Accession Number: EF012704).

Detailed Description of the Invention

In a first aspect, the present invention provides an isolated *Pseudochlorococcum* sp. composition, wherein the isolated *Pseudochlorococcum* sp. genome comprises one or more nucleic acid sequence selected from the group consisting of SEQ ID NO:1 (ITS—1622 bp), SEQ ID NO:2 (rbcL—1160 bp), SEQ ID NO:3 (ITS1—928-1082 of ITS), SEQ ID NO:4 (ITS2—1247-1487 of ITS), and SEQ ID NO:5 (ITS—827 bp), or complements thereof. As discussed in more detail below, each of these nucleic acid sequences serves as a marker for the novel *Pseudochlorococcum* sp. of the present invention, and distinguishes it from other *Pseudochlorococcum* strains.

The isolated *Pseudochlorococcum* sp. is useful for a variety of purposes, including but not limited to oil production, wastewater remediation, waste gas remediation, and production of other value-added biomass which can be used, for example, as animal feed and organic fertilizer. These uses are described in more detail below.

The alga of this first aspect of the invention was derived by a selection process from culture obtained from a water environment in the Phoenix metropolitan area. Thus, the *Pseudochlorococcum* sp. derived may be naturally occurring, but previously not isolated, or may be derived by mutation caused by selective pressure during the selection process. As used herein, the *Pseudochlorococcum* sp. includes any strain with the identifying characteristics recited.

As used herein the term "isolated" means that at least 90% of the algae present in the composition are of the recited *Pseudochlorococcum* genotype; in further embodiments, at least 95%, 98%, or 99% of the algae present are of the recited *Pseudochlorococcum* genotype. The isolated *Pseudochlorococcum* sp. can be cultured or stored in solution, frozen, dried, or on solid agar plates.

The *Pseudochlorococcum* sp. of this first aspect of the invention is characterized by (i) significant ammonia uptake, (ii) an ability to assimilate large quantities of nutrients selected from the group consisting of nitrogen, phosphorous, and inorganic carbon, and (iii) an ability to accumulate large quantities of biomass (including, but not limited to crude proteins, total lipids, total polysaccharides, and/or carotenoids (useful, for example, as livestock or aquaculture feed additive), or combinations thereof.

As used herein, the phrase "ability to grow" means that the algae capable of reproduction adequate for use in the methods of the invention under the recited conditions. As used herein, the phrase "an ability to assimilate large quantities of nutrients" means the following: for nitrogen (nitrate or ammonia/ammonium) removal from contaminated water and wastewater, at least 2 mg per liter of nitrogen as nitrate or ammonia per hour of treatment is regarded as a high removal rate (ie: assimilating large quantities of nutrients). In the case of CO_2 removal from power plant flue gas emissions of at least 2 grams of CO_2 per liter of algal culture per hour of cultivation time is regarded as a high removal rate.

In a second aspect, the present invention provides a substantially pure culture, comprising a growth medium; and isolated algae of the first aspect of the invention. As used herein, the term "growth medium" refers to any suitable medium for cultivating algae of the present invention. The algae of the invention can grow photosynthetically on CO_2 and sunlight, plus a minimum amount of trace nutrients. The volume of growth medium can be any volume suitable for cultivation of the algae for any purpose, whether for standard laboratory cultivation, to large scale cultivation for use in, for example, bioremediation, lipid production, and/or algal biomass production. Suitable algal growth medium can be any such medium, including but not limited to BG-11 growth medium (see, for example, Rippka, 1979); culturing temperatures of between 10° and 38° C are used; in other embodiments, temperature ranges between 15° and 30° are used. Similarly, light intensity between 20 μmol $m^{-2}s^{-1}$ to 1000 μmol $m^{-2}s^{-1}$ is used; in various embodiments, the range may be 100 μmol $m^{-2}s^{-1}$ to 500 μmol $m^{-2}s^{-1}$ or 150 μmol $m^{-2}s^{-1}$ to 250 μmol $m^{-2}s^{-1}$. Further, aeration is carried out with between 0% and 20 % CO_2; in various embodiments, aeration is carried out with between 0.5% and 10 % CO_2, 0.5% to 5 % CO_2, or 0.5% and 2 % CO_2.

For maintenance and storage purposes, *Pseudochlorococcum* sp. isolates are usually maintained in standard artificial growth medium. For regular maintenance purposes, the *Pseudochlorococcum* sp. isolates can be kept in liquid cultures or solid agar plates under either continuous illumination or a light/dark cycle of moderate ranges of light intensities (10 to 40 μmol m^{-2} s^{-1}) and temperatures (18°C to 25°C). The culture pH may vary from pH 6.5 to pH 9.5. No CO_2 enrichment is required for maintenance of *Pseudochlorococcum* sp. isolates. In various non-limiting examples, the temperature of culture medium in growth tanks is preferably maintained at from about 10°C to about 38°C, in further embodiments, between about 20°C to about 30°C.

In various embodiments, the growth medium useful for culturing *Pseudochlorococcum* sp. of the present invention comprises wastewater or waste gases. This growth medium is particularly useful when the *Pseudochlorococcum* sp. is used in a waste remediation process, although use of this growth medium is not limited to waste remediation processes. In one embodiment when wastewater is used to prepare the medium, it is from nutrient-contaminated water or wastewater (e.g., industrial wastewater, agricultural wastewater domestic wastewater, contaminated groundwater and surface water), or waste gases emitted from power generators burning natural gas or biogas, or flue gas emissions from fossil fuel fired power plants. In this embodiment, the *Pseudochlorococcum* sp. can be first cultivated in a primary growth medium, followed by addition of wastewater and/or waste gas. Alternatively, the *Pseudochlorococcum* sp. can be cultivated solely in the wastestream source. When a particular nutrient or element is added into the culture medium, it will be taken up and assimilated by the *Pseudochlorococcum* sp., just like other nutrients. In the end, both wastewater-containing and spiked nutrients are removed and converted into macromolecules (such as lipids, proteins, or carbohydrates) stored in *Pseudochlorococcum* sp. biomass. Typically, the wastewater is added to the culture medium at a desired rate. This water, being supplied from the waste water source, contains additional nutrients, such as phosphates, and/or trace elements (such as iron, zinc), which supplement growth of the *Pseudochlorococcum* sp. In one embodiment, if the wastewater being treated contains sufficient nutrients to sustain the *Pseudochlorococcum* sp. growth, it may be possible to use less of the growth

medium. As the wastewater becomes cleaner due to *Pseudochlorococcum* sp. treatment, the amount of growth medium can be increased.

The major factors affecting wastewater feeding rate include: 1) *Pseudochlorococcum* sp. growth rate, 2) light intensity, 4) culture temperature, 5) initial nutrient concentrations in wastewater; 5) the specific uptake rate of certain nutrient/s; 6) design and performance of a specific bioreactor and 7) specific maintenance protocols.

In a third aspect, the present invention provides an algal culture system, comprising:

(a) a photobioreactor; and

(b) the substantially pure culture of the second aspect of the invention.

As used herein, a "photobioreactor" is a lab-scale or industrial-scale culture vessel in which algae grow and proliferate. For use in this aspect of the invention, any type of photobioreactor can be used, including but not limited to open raceways (i.e. shallow ponds (water level ca. 15 to 30 cm high) each covering an area of 10 to 5000 m^2 or larger, constructed as a loop in which the culture is circulated by a paddle-wheel (Richmond, 1986), closed systems, i.e. photobioreactors made of transparent tubes or containers in which the culture is mixed by either a pump or air bubbling (Lee 1986; Chaumont 1993; Richmond 1990; Tredici 2004), tubular photobioreactors (for example, see Tamiya et al. (1953), Pirt et al. (1983), Gudin and Chaumont 1983, Chaumont et al. 1988; Richmond et al. 1993) and flat plate-type photobioreactors, such as those described in Samson and Leduy (1985), Ramos de Ortega and Roux (1986), Tredici et al. (1991, 1997) and Hu et al. (1996, 1998a,b). In this third aspect, the present invention provides systems of various designs, which can be used, for example, in methods for nutrient removal (described below) using the *Pseudochlorococcum* sp. of the invention.

The distance between the sides of a closed photobioreactor is the "light path," which affects sustainable algal concentration, photosynthetic efficiency, and biomass productivity. In various embodiments, the light path of a closed photobioreactor can be between approximately 5 millimeters and 40 centimeters; between 50 millimeters and 30 centimeters, between 100 millimeters and 30 centimeters, between 1 centimeter and 30 centimeters, between 2 centimeters and 30 centimeters; between 2 centimeters and 20 centimeters, or between 2 centimeters and 10 centimeters. The most optimal light path for a given application will depend, at least in part, on factors

including the specific algal strains to be grown and/or specific desired product/s to be produced.

In a fourth aspect, the present invention provides methods for lipid isolation, wastewater remediation, waste gas remediation, and/or biomass production, comprising culturing the *Pseudochlorococcum* sp. of the present invention, wherein the *Pseudochlorococcum* sp. genome comprises one or more nucleic acid sequence selected from the group consisting of SEQ ID NO:1 (ITS—1622 bp), SEQ ID NO:2 (rbcL—1160 bp), SEQ ID NO:3 (ITS1—928-1082 of ITS), SEQ ID NO:4 (ITS2—1247-1487 of ITS), and SEQ ID NO:5 (ITS—827 bp) or complements thereof, under conditions suitable to promote algal proliferation, and isolating lipids, removing nutrients from wastewater or waste gas, and/or extracting algal biomass. The methods can be carried out alone, or carried out in any combination. In one embodiment, methods for lipid isolation are carried out, where the lipid isolated can be a single lipid type, including, but not limited to, isolation of fatty acids, pigments (chlorophyll, carotenoids, etc.), sterols, vitamins A and D, or hydrocarbons, or combination thereof (such as total lipid). In a further embodiment, the methods comprise culturing the *Pseudochlorococcum* sp. of the present invention under conditions suitable for production of total lipid content of at least 20 % of dry algal cell weight; in various embodiments, the total lipid content is at least 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, or more of the dry algal cell weight.. As used herein, the "dry cell weight" is the total weight of the algal culture after concentrating and drying the algae from the culture. As discussed above, the methods of the first aspect of the invention can be used to select for algal isolates that produce a total lipid content of at least 40 % of dry algal cell weight. Thus, those of skill in the art will be able to use such novel algae for lipid isolation, using any lipid extraction technique known in the art, including but not limited to the methods described below. Lipids, isolated via this method can be used for any purpose, including but not limited to biofuel production (including but not limited to biodiesel), detergent, biopolymers, and bioplastic.

In another embodiment, the methods comprise removing nutrients from a wastestream, comprising culturing the algal strain in a culture medium comprising at least 5% wastestream water, under conditions whereby nutrients in the wastestream are removed by the *Pseudochlorococcum* sp. of the present invention. In further

embodiments, the culture medium comprises 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% wastewater. Through this process up to 95% or more of the nutrients can be removed from the wastewater, resulting in nutrient levels below maximum contaminant levels set for individual contaminants by the U.S. Environmental Protection Agency (EPA). One non-limiting example of such wastewater is groundwater that may contain tens or hundreds of milligrams per liter of nitrogen as nitrate. The amounts of nitrate can be removed to below 10 mg nitrate-N L^{-1} within one or several days, depending on initial nitrate concentration in the groundwater. The amounts of groundwater that can be purified by the methods of the invention depend on the initial concentrations of nutrients to be removed and the size of photobioreactor system used. In some cases, the groundwater may be spiked with trace amounts of phosphate (in a range of micro- or milligrams per liter) or microelements (such as Zn, Fe, Mn, Mg) in order to enable the algae to completely remove nitrate from the groundwater.

In another non-limiting embodiment, wastewater comes from Concentrated Animal Feeding Operations (CAFOs), such as dairy farms, which may contain high concentrations of ammonia (hundreds to thousands of milligrams per liter of nitrogen as ammonia) and phosphate (tens to hundreds of milligrams per liter of phosphorous as phosphate). Full-strength CAFO wastewater can be used as a "balanced growth medium" for sustaining rapid growth of selected algal strains in photobioreactors as described above. In some cases the CAFO wastewater can be diluted to a certain extent to accelerate growth and proliferation of the *Pseudochlorococcum* sp. of the present invention. As a result, ammonia and phosphate concentrations can be removed within one or several days, depending on initial concentrations of these nutrients. In contrast to the groundwater situation, no chemicals are required to be introduced into CAFO wastewater in order to reduce or eliminate ammonia and phosphate levels to meet the U.S. EPA standards. In another embodiment, wastewater is agricultural runoff water that may contain high concentrations (in a range of several to tens of milligrams per liter) of nitrogen in forms of nitrate and ammonia and phosphates. The *Pseudochlorococcum* sp. of the present invention can remove these nutrients to below the U.S. EPA's standards within one day or two, depending on initial concentrations of these nutrients and/or weather conditions. In case the nitrogen to phosphorous ratio is distant from the ratio of 15:1, addition of one chemical (either nitrates or

phosphates) to balance the ratio is necessary to remove these nutrients from the wastewater.

In another embodiment of this fourth aspect, the methods comprise removing nutrients from a waste gas, comprising culturing the *Pseudochlorococcum* sp. of the present invention in a culture medium comprising waste gas, under conditions whereby nutrients in the waste gas are removed. In one embodiment, flue gas emissions provide a carbon source (in a form of carbon dioxide, or CO_2) for algal photosynthesis and waste nutrient removal. Flue gases may be those from any source, including but not limited to fossil fuel-burning power plants. Through the photosynthetic machinery, the *Pseudochlorococcum* sp. of the present invention cells fix CO_2 and convert it into organic macromolecules (such as carbohydrates, lipids, and proteins) stored in the cell. As a result, molecular CO_2 entering into the culture system disclosed above is removed and converted into algal biomass, and thus the gas released from the photobioreactor is significantly reduced in CO_2 (at least a 50% reduction).

In one embodiment, flue gases are delivered into a photobioreactor as disclosed above. One method involves injection of the flue gas directly into the photobioreactor at a flow rate that will sustain (0.1 to 0.5 liter of flue gas per liter of culture volume per minute) vigorous photosynthetic CO_2 fixation while exerting minimum negative effects due to lowering culture pH by dissolved NO_x and SO_x and/or certain toxic molecules such as the heavy metal mercury. Alternatively, the flue gas may be blended with compressed air at a certain ratio (flue gas to compressed air ratio may range from 0.1~0.6 volume to 1 volume) and delivered into the photobioreactor through an aeration system. In a further embodiment, a liquid- or gas-scrubber system may be introduced to reduce or eliminate contaminant transfer from the gas-phase and accumulation of toxic compounds in the algal growth medium. In a further preferred embodiment, flue gases coming out from the power generator may be pre-treated with proton-absorbing chemicals such as NaOH to maintain an essentially neutral pH and turn potentially harmful NO_x and SO_x compounds into useful sulfur and nitrogen sources for algal growth. For example, a commercially available gas-scrubber can be incorporated into the photobioreactor system to provide algae with pretreated flue gas. In case of liquid wastes, pre-treatment includes but is not limited to 1) treat wastewater first through an anaerobic digestion process or natural or constructed wetland to remove most of the organic matter; 2) dilute

wastewater 10% to 90% with regular ground or surface water, depending on concentrations of potential toxic compounds; 3) add certain nutrients (such as phosphorous and/or trace elements) to balance the nutrient composition for maximum sustainable nutrient removal and/or biomass production.

In a further embodiment of this fourth aspect of the invention, methods for producing biomass are provided, comprising culturing the *Pseudochlorococcum* sp. of the present invention and harvesting algal biomass components from the cultured algae. Such biomass can include, but is not limited to, crude proteins, total lipids (such as fatty acids), total polysaccharides, and/or carotenoids selected from the group consisting of lutein and beta-carotene (useful, for example, as livestock or aquaculture feed additive), or combinations thereof. In one embodiment, a multi-stage maintenance protocol is described to remove waste nutrients at the early stages, while inducing and accumulating high-value compounds (such as fatty acids, carotenoids) at later stages. In a further embodiment, algal biomass produced from the photobioreactor is used as feedstock for biodiesel production. In a further preferred embodiment, residues of algal mass after extraction of algal fatty acids will be used as animal feed or organic fertilizer additive. In another embodiment, carotenoid-rich algal biomass as a by-product of waste-stream treatment by algal strains grown in the photobioreactors described above is used as an animal feed additive or a natural source of high-value carotenoids. Methods for algal biomass production and/or protein expression are well known in the art. See, for example: Hu, Q. (2004) Chapter 5: pp. 83-93. *In* Richmond A. (ed.) *Handbook of Microalgal Culture*, Blackwell Science Ltd, Oxford OX2 0EL, UK; Hu, Q. (2004) Chapter 12: *Arthrospira (Spirulina) platensis*, pp. 264-272. *In* Richmond A. (ed.) *Handbook of Microalgal Culture*, Blackwell Science Ltd, Oxford OX2 0EL, UK; Hu, Q., et al. (2000) *Appl. Env. Microbiol.* 66: 133-139; Hu, Q., et al. (1999) *Acaryochloris marina. Biochim. Biophys. Acta,* 1412: 250-261; Hu, Q., et al. (1998) *Proc. Natl. Acad. Sci. USA,* 95: 13319-13323; Hu, Q., et al. (1998) *Acaryochloris marina. In*: Garab G. (ed.) *Photosynthesis: Mechanisms and Effects*, Vol. I. 437-440, Kluwer Academic Publishers, Dordrecht, The Netherlands; Hu, Q., et al. (1998) *J. Ferment. Biotechnol.* 85: 230-236; Hu, Q., et al. (1998) *Eur. J. Phycol.* 33: 165-171; Hu, Q., et al. (1998) *Appl. Microbiol. Biotechnol.* 49: 655-662; Iwasaki, I., et al. (1988) *J. Photochem. Photobiol.* B: Biology 44: 184-190; Hu, Q., et al. (1997) *Eur. J. Phycol.* 32: 81-86; Richmond, A. and Hu, Q. (1997) *Appl. Biochem. Biotechnol.* 63-65: 649-658; Hu, Q.,

et al. (1996) *Biotechnol. Bioeng.* 51: 51-60; Hu, Q., et al. (1996) *J. Phycol.* 32: 1066-1073; Hu, Q. and Richmond, A. (1996) *J. Appl. Phycol.* 8: 139-145; Gitelson, A., et al. (1996) *Appl. Env. Microbiol.* 62: 1570-1573; Hu, Q. and Richmond, A. (1995) In: Mathis P. (ed.) *Photosynthesis: from Light to Biosphere*, Vol. IV, 1037-1040, Kluwer Academic Publishers, The Netherlands; and Hu, Q. and Richmond, A. (1994) *J. Appl. Phycol.* 6: 391-396.

The present invention addresses environmental pollution control while producing renewable energy through novel algal reagents and methods. The *Pseudochlorococcum* sp. of the present invention can be used to produce biofuel (such as biodiesel) and/or rapidly remove nutrients from wastewater and/or waste gases (including but not limited to wastewater and power plant flue gases) and convert them into value-added compounds stored into algal biomass. The biomass can then be used, for example, as feedstock for production of liquid biofuel and/or fine chemicals, and used as animal feed, or organic fertilizer. The major advantages of the reagents and methods of the present invention over conventional bacteria-based systems are that it they only remove nutrients from wastewater or waste gas, but also recycle them in form of renewable biomass and fine chemicals, whereas bacterial systems strip off potentially valuable nitrate and/or ammonia into the atmosphere through nitrification and de-nitrification processes. Bacterial systems also usually generate large amounts of sludge which require proper disposal. Compared to natural and constructed wetland systems, the algae-based reagents and methods of the present invention are more efficient in terms of nutrient removal and biomass production. From the energy production side, the reagents and methods of the present invention are more efficient than conventional lipid crop production, producing up to 20 to 40 times more feedstock per unit area of land per year. The reagents and methods of the present invention can be applied in non-agricultural environments, such as arid and semi-arid environments (including deserts). Thus, the present technology will not compete with food/energy crop (or other) plants for limited agricultural land.

Materials and Methods

The Organism and growth conditions:

Starting algal cultures were obtained from a water environment in the Phoenix metropolitan area and maintained at 25°C in BG-11 growth medium (Rippka, 1979).

Optical density and dry weight Measurements:

Algal cell population density was measured daily using a micro-plate spectrophotometer (SPECTRA max 340 PC) and reported as optical density at 660 nm wave length. The dry weight of algal mass was determined by filtration from 10-20 ml culture through a pre-weighed Whatman GF/C filter. The filter with algae was dried at 105°C overnight and cooled to the room temperature in a desiccator and weighed.

Chlorophyll measurement:

A hot methanol extraction method was used (Azov (1982). The concentration was calculated using the Talling coefficient:

$$\text{Chlorophyll a (mg/L)} = 13.9\ (DO_{665} - DO_{750})\ V/U$$

where DO665 = optical density measured at 665 nm wavelength, DO750 = optical density measured at 750 nm wavelength, V= total volume of methanol (ml), and U = volume of algal suspension (ml).

Lipid extraction:

The lipid extraction procedure was modified according to Bigogno, et al. (2002). *Pseudochlorococcum* cell biomass (100 mg freeze-dried) was added to a small glass vial sealed with Teflon screw cap and was extracted with methanol containing 10% DMSO, by warming to 40°C for 1 h with magnetic stirring. The mixture was centrifuged at 3,500 rpm for five minutes. The resulting supernatant was removed to another clean vial and the pellet was re-extracted with a mixture of hexane and ether (1:1, v/v) for 30 minutes. The extraction procedure was repeated several times until negligible amounts of chlorophylls remained in the pellet. Diethyl ether, hexane and water were added to the combined supernatants, so as to form a ratio of 1:1:1:1 (v/v/v/v). The mixture was hand-shaken and then centrifuged at 3,500 rpm for 5 minutes. The upper phase was collected. The lower water phase was re-extracted twice with a mixture of diethyl ether:hexane (1:1, v/v). The organic phases were combined, and the solvents in the oil extract were completely removed by bubbling with nitrogen gas until the weight of the remaining oil extract was constant.

Fatty acid analysis:

Fatty acids were analyzed by gas chromatography (GC) after direct transmethylation with sulphuric acid in methanol (Christie, 2003). The fatty acid methanol esters (FAMEs) were extracted with hexane containing 0.8% BHT and analyzed by a HP-6890 gas chromatography (Hewlett-Packard) equipped with HP7673 injector, a flame-ionization detector, and a HP-INNOWAX™ capillary column (HP 19091N-133, 30 m × 0.25 mm × 0.25 μm). Two (2) μL of the sample was injected in a split-less injection mode. The inlet and detector temperatures were kept at 250°C and 270°C, respectively, and the oven temperature was programmed from 170°C to 220°C increasing at 1°C/min. High purity nitrogen gas was used as the carrier gas. FAMEs were identified by comparison of their retention times with those of the authentic standards (Sigma), and were quantified by comparing their peak areas with that of the internal standard (C17:0).

A typical GC chart of fatty acid profile of *Pseudochlorococcum* sp. is shown in Figure 1. Each peak was marked as retention time and name of individual fatty acid. Some minor peaks between C16:1 and C18:0 (i.e., on both sides of the C17:0 peak) and between C18:3 (n-3) and C20:1 were not identifiable with the available standards and therefore were not labeled.

Collection of dairy wastewater:

Dairy wastewater was collected at a dairy in Mesa, Arizona (latitude N 33.35030, longitude W 111.65837) from a shallow wastewater pond consisting of piped dairy stall waste and overland runoff. A composite wastewater sample was collected from no fewer than three access points along the bank of a shallow wastewater pond. Wastewater was stored in a plastic container (5 gallons or larger) at 4°C.

Wastewater, in raw form, was brownish-red colored and contained undigested grains, grasses, soil and other unidentified solids. Before used for experiments, the dairy wastewater was filtered through a filtration system or centrifuged to remove particles and native species of algae at 5,000 rpm. The clear brown dairy wastewater was collected for assigned experiments. The wastewater was diluted to 5% wastewater (1: 20 dairy wastewater to water), 25% wastewater (1:3 dairy wastewater to water), 50% wastewater (1:1 wastewater to water), 75% wastewater (3:1

wastewater to water), and 100% wastewater (undiluted wastewater) to meet various experimental needs.

Experimental Design:

A 300-ml capacity glass column (68 cm long with an inner diameter of 2.3 cm) with a glass capillary rod placed down the center of the column to provide aeration was used to grow the alga. The top of the column was covered with a rubber stopper surrounded by loosely-fitting aluminum foil to prevent contamination among columns. Unless otherwise stated, a culture temperature of 25°C, a light intensity of 170 μmol m^{-2} s^{-1}, and compressed air of 1% CO_2 were applied to glass columns throughout the experiment.

For experiments, log-phase cultures were harvested and centrifuged to remove the culture medium and re-suspended into small volume of sterilized distilled water for inoculation. Each treatment was run in triplicate. Deionized water was added daily to the column to compensate for water loss due to evaporation.

For nutrient removal experiments, 10 ml of culture suspension was collected from the column daily and centrifuged at 3,500 rpm for 10 minutes. The supernatant was pooled into small vial and frozen in a -20°C freezer for nutrient analysis. The pellets were re-suspended into distilled water for dry weight measurement.

High carbon dioxide treatment:

For CO_2 treatment experiments, algal cells were grown in BG-11 growth medium either bubbled with air enriched with 1% CO_2, or air enriched with 15% CO_2.

Outdoor mass culture experiments:

To prepare a seed culture, 150 ml of stock culture of *Pseudochlorococcum* sp. was transferred from a flask to a 750 ml capacity glass column (68 cm long with an inner diameter of 5.7 cm), agitated with compressed air enriched with 1% CO_2. The seed culture was illuminated with a bank of daylight fluorescent lamps from one side of the column at a photon flux density of 100 μmol m^{-2} s^{-1} and at 25°C. When cell density of the culture reached 5 x 10^7/ml, the culture was transferred to a flat-plate reactor measuring 210 cm × 40 cm × 13 cm, and containing 100 liters of BG-11 growth medium. The culture conditions for the flat-plate reactor were same as for the

glass column reactors. When cell density of the flat-plate reactor reached 5 x 10^6/ml, the 100 liters of culture was transferred to an outdoor thin panel photobioreactor.

The outdoor thin panel photobioreactor consisted of individual culture units varying in light path (i.e., culture depth), as desired. In this particular case, five different light-paths were used (2.5 cm, 5.0 cm, 10.0 cm, 20.0 cm and 30.0 cm). Given that all the individual reactors measured 210 cm long and 40 cm height, the total culture volume for the five different light-path reactors (e.g., 2.5 cm, 5.0 cm, 10.0 cm, 20.0 cm and 30.0 cm) was 21, 42, 84, 168 and 252 liters of culture, respectively.

Maximum daily culture temperature was maintained at 29 ± 2°C by evaporative cooling. Culture pH was maintained at 7.0~8.0. Culture mixing and CO_2 supply were provided by compressed air enriched with 1 % CO_2 through a perforated tube running through the bottom of the reactor.

***DNA extraction, amplification, and sequencing*:**

Fifty (50) ml of cell culture was collected and centrifuged (3000 rpm × 5 minutes) and then homogenized into powder in liquid nitrogen. Genomic DNA was extracted and purified with NucleoSpin Plant kit (MACHEREY-NAGEL Inc.). The ribosomal DNA internal transcribed spacer (ITS) (SEQ ID NO:1) and the large subunit of the Rubisco (*rbcL*) gene (SEQ ID NO:2) were used as the molecular markers for *Pseudochlorococcum* sp identification. PCR reactions contained 12.5µl GoTaq Green Master Mix (Promega), 200ng template DNA and 0.5 µM primers (see Table1) and H_2O in a final volume of 25 µl. PCR cycles for amplification of the region ITS were as follows: 1 cycle of 94°C, 5 min, 35 cycles of 94 °C 30s, 50 °C 30s, 72 °C 1 min 30s and 1 cycle of 72 °C 10 min. PCR cycles for the amplification of *rbcL* were as follows: 1 cycle of 94°C, 5 min, 35 cycles of 94 °C 30s, 55 °C 30s, 72 °C 1 min 30s and 1 cycle of 72 °C 10 min. PCR products are examined on 1.5% agarose. Two (2) µl of PCR products were cloned into the pCR®4-TOPO vector (Invitrogen). Plasmids for sequencing were extracted from the positive clones with the PureLink Quick Plasmid Miniprep kit (Invitrogen). The primers M13R and M13F were used for sequencing.

Table 1. Primers used for amplification of ITS and *rbcL* from *Pseudochlorococcum* sp.

Primers	Sequence (5'-3')
ITS	
s15CH(F)	CTTAGTTGGTGGGTTGCC (SEQ ID NO:9)
15pl(R)	TTCRCTCGCCGTTACT (SEQ ID NO:10)
rbcL	
RH1(F)	ATGTCACCACAAACAGAAACTAAAGC (SEQ ID NO:11)
Cel 161R(R)[2]	CATGTGCAATACGTGAATACC 9SEQ ID NO:12)

***Phylogenetic analysis methods*:**

DNA sequences were aligned with Clustal W 1.83 and verified manually with Seaview. Phylogenetic trees were reconstructed with neighbor-joining (NJ) algorithm as implemented in Mega 3. The Kimura 2-parameter model was applied to calculate the substitution rate for reconstructing the phylogenetic trees.

Results and Discussion:

***Isolation and morphological description of Pseudochlorococcum* sp.**

The starting algal culture was collected from a public water pond in the Phoenix metropolitan area (Arizona) and isolated from the water sample by agar plating. Individual green colonies were then transferred into test tubes with screw-cap containing 10 ml BG-11 growth medium. Cultures were maintained at 20-25°C with a light intensity of 20-40 μmol photons m^{-2} s^{-1}. Cultures were examined weekly for growth by microscopy and spectrophotometry. Those mono-algal isolates that exhibited rapid growth and reproduction (any isolates that exhibited 1 to 3 doubling times per day under our culture conditions (e.g., BG-11 growth medium, 25°C, at light intensity of 170 μmol m^{-2} s^{-1}, and aeration with 1~2% CO_2)) were subjected to lipid content analysis. Only algal strains processing high lipid content (any isolates that possess a total lipid content of 40% or greater) were subjected to further screening for tolerance to high CO_2 concentrations and various wastewaters. One

isolate that passed this screening process was identified as *Pseudochlorococcum* sp. based upon morphological features.

Pseudochlorococcum cells in a 2-week-old culture were ellipsoidal, with a single, thin parietal chloroplast (having the appearance of a thin, green rim) with at least 1 pyrenoid (additional pyrenoids may or may not occur with age). Cells were spherical in stationary phase cultures, and the chloroplast increased in size and filled the lumen, thereby causing old cells to resemble *Chlorococcum*. In a stationary phase, chloroplast was fissured but in young cells the chloroplast was always continuous. Large vacuoles were present, usually 1-2 in young cells, and additional vacuoles developed in older, spherical cells. Reproduction occurred only by 2 to 8 autospores, formed by successive bipartition (s). The genus *Pseudochlorococcum* was established by Archibald in 1970. At the beginning, only two species, *Pseudochlorococcum typicum* and *Pseudochlorococcum polymorphum*, were assigned to this genus. The major morphological features of this new isolate are similar to both *Pseudochlorococcum typicum* and *Pseudochlorococcum polymorphum*. However, the new isolate culture was dark green, whereas that of *Pseudochlorococcum typicum* and *Pseudochlorococcum polymorphum* were grass green during log- and stationary-phases. Under various stress conditions (such as high light, nutrients depletion or drought), cells of the new isolate tended to accumulate extraplastidic secondary carotenoids and high lipid content , making the culture orange to red color, whereas *Pseudochlorococcum typicum* and *Pseudochlorococcum* cells turned only a yellow color. Accordingly, this new isolate was assigned to be a new species of *Pseudochlorococcum*.

Effect of CO_2 concentration on growth and biomass productivity

The *Pseudochlorococcum* strain can grow at a high CO_2 concentration (i.e., 15% CO_2) at a growth rate similar to that at 1% CO_2 commonly applied to algal cultures (**Fig. 2**). This CO_2 level is equivalent to that typically occurring in flue gases emitted from fossil fuel power plants. In a batch culture mode, the biomass productivity of the *Pseudochlorococcum* strain grown in a glass column reactor at 15% CO_2 was 570 ± 50 mg l^{-1} d^{-1}, similar to 610 ± 70 mg l^{-1} d^{-1} obtained from cultures grown at 1% CO_2 (**Fig. 3**).

Effect of CO_2 concentration on cellular lipid content and lipid productivity

There was little effect of CO_2 concentrations on cellular lipid (fatty acid) content or lipid production of *Pseudochlorococcum* sp. As used herein, "content" refers to cellular lipid content at a point in time; lipid "production rate" or lipid "productivity" or "yield" refers to amount of lipid produced per unit culture volume or reactor illuminated surface area per time (day) of *Pseudochlorococcum* sp. When *Pseudochlorococcum* cultures were maintained in the glass column bioreactors supplied with 1% or 15% CO_2 under given culture conditions, the cellular oil content was 51~56% of dry weight (**Fig. 4a**). Likewise, the volumetric productivity of oil was about 320 ± 40 mg l^{-1} d^{-1} when *Pseudochlorococcum* cultures were provided with either level of CO_2 (**Fig. 4b**).

Effect of wastewater concentration on growth and biomass productivity

The *Pseudochlorococcum* strain has the ability to thrive in wastewater from various sources, such as nutrient-contaminated groundwater, agriculture runoff, and animal feeding operation wastewater. No additional nutrient chemicals were added to the culture, suggesting that the dairy wastewater contained nutrients necessary for sustaining algal growth and reproduction. **Fig. 5** shows growth of *Pseudochlorococcum* sp. maintained in various concentrations of dairy wastewater (i.e., 25%, 50%, 75%, and 100% wastewater). While little growth occurred in cultures supplied with 100% dairy wastewater, *Pseudochlorococcum* cells did grow in 75% wastewater, albeit at much reduced growth rate. As the concentration of the wastewater decreased from 75% to 50% and further to 25% by dilution with tap water, growth was much improved (**Fig. 5**). As a result, a reverse relationship between wastewater concentration and biomass productivity of *Pseudochlorococcum* cells was observed: biomass productivity increased from 290 ± 40 mg l^{-1} d^{-1} to about 800 ± 60 mg l^{-1} d^{-1} as the wastewater concentration decreased from 100% to 25% (**Fig. 6**).

Effect of wastewater concentration on lipid content and lipid productivity

The concentration of dairy wastewater did affect the lipid content of algal biomass. The highest percentage of lipid was obtained in cultures maintained in 25% dairy wastewater. As the concentration of wastewater increased from 25% to 50% and to 75%, the cellular lipid content decreased (**Fig. 7**). As the cellular lipid content of the cells from 25% wastewater was significantly higher than that from BG-11 growth

medium, it suggests that the wastewater may contain certain elements/compounds that stimulate biosynthesis and accumulation of lipid while at the same time somewhat inhibited growth. As a tradeoff, the lipid productivity was similar in cultures maintained in both BG-11 growth medium and 25% wastewater (**Fig. 8**).

Fatty acid composition of Pseudochlorococcum sp.

Table 2 shows the fatty acid composition of *Pseudochlorococcum sp.* grown in BG-11 growth medium. The major fatty acids (more than 95% of the total fatty acids in the cell) were C16 and C18.

Table 2. Fatty acid profile of *Pseudochlorococcum* sp.

Fatty acids	***Pseudochlorococcum* sp.** (% of total fatty acids)
C13:0	2.5
C14:0	0.2
C16:0	21.3
C16:1	3.5
C16:2	1.3
Unidentified peak-1	3.3
Unidentified peak-2	2.0
C18:0	8.1
C18:1	48.6
C18:2n-6	5.7
C18:3n-3	9.5
C18:3n-6	0.1
C18:4n-6	0.5
C20:0	0.4
C20:1	0.5
TFA (% dry weight)	52.4

***Outdoor mass culture of Pseudochlorococcum* sp.**

The *Pseudochlorococcum* strain was able to grow vigorously in photobioreactors of various designs (such as open raceway pond, vertical columns, and large flat panel reactors) to produce lipid-rich cell biomass under outdoor environmental conditions. The cultivation of the *Pseudochlorococcum* strain has been evaluated in a flat panel photobioreactor outdoors throughout the year. The results obtained indicate that *Pseudochlorococcum* sp. can thrive at a minimum culture temperature as low as 0 °C or even below 0 °C during the winter season, or in solar radiation as high as ca. 2,500 μmol m^{-2} s^{-1} at noon of a typical summer day in the Phoenix metropolitan area. **Fig. 9** shows algal growth as a function of reactor light paths ranging from 2.5 cm to 30.0 cm. As indicated by the increase in algal dry biomass per culture volume over a 13-day period, the shorter the reactor light path,

the greater the specific growth rate and thus the greater the maximum volumetric cell density reached at the end of cultivation.

Reactor light path not only affected growth and final cell density of *Pseudochlorococcum* sp., but also affected cellular biochemical composition of the alga. **Fig. 10** provides an example of total lipid content being largely affected by reactor light path. As reactor light path decreased from 30.0 cm to 2.5 cm, the total cellular lipid content increased from 18 $\pm$ 2% to 51 $\pm$ 6% of dry weight during a 13 day period of cultivation.

As a result, biomass productivity of *Pseudochlorococcum* or total lipid content was largely affected by reactor light path. On a per culture surface area basis, the difference in terms of productivity of *Pseudochlorococcum* biomass was minimum as the reactor light path decreased from 30.0 cm to 2.5 cm. However, on a volumetric basis, the shorter the reactor light path the greater the volumetric biomass yield (**Fig. 11 a** and **b**).

Photobioreactor light path exerted a more profound effect on productivity of total cellular lipids/oil of *Pseudochlorococcum* cultures. Both areal and volumetric productivities of total lipids were higher as a flat panel reactor decreased from a broader light path (e.g., 30.0 cm) to a narrower light path (e.g., 2.5 cm). Accordingly, over 100% increase in areal productivity of total lipids was obtained in cultures maintained in a 2.5 cm flat panel reactor compared to a 30.0 cm reactor, or more than 10 times higher total lipids productivity when calculated on a volumetric basis (**Fig.12 a** and **b**).

In conclusion, the *Pseudochlorococcum* strain can grow and produce lipid as high as ca. 50% of dry weight with a production rate of more than 7 g m^{-2} d^{-1} in the flat panel photobioreactor tested for this disclosure under outdoor environmental conditions.

DNA markers for identification of Pseudochlorococcum sp.

A 1623-bp ITS segment (SEQ ID NO:1) was amplified from *Pseudochlorococcum* sp., indicated by agarose gel electrophoresis (**Fig. 13**). The ITS segment consists of 3' end of 18S rDNA (1-927) (SEQ ID NO:6) with an intron of 409bp (491-899), ITS1 (928-1082) (SEQ ID NO:3), 5.8S rDNA (1083-1246) (SEQ ID NO:7), ITS2 (1247-1487) (SEQ ID NO:4) and 5'end of 28S rDNA (1489-1622) (SEQ ID NO:8). No identical nucleotide sequence can be found by a BLAST

searching in the National Center for Biotechnology Information (NCBI) databases. The phylogenetic relationships of 22 Chlorophyta taxa were inferred based on 827 base pairs of the ITS regions (SEQ ID NO:5). As shown in **Fig 14**, *Pseudochlorococcum* sp. was clustered with *Desmodesmus* forming a clade supported by the Bootstrap analysis with high confidence. Based upon the morphological characteristics, the *Pseudchlorococcum* genus belongs to the class Chlorococcales, characterized with a single parietal chloroplast and being azoosporic. However, the ITS sequence analysis suggests that the *Pseudchlorococcum* strain may be phylogenetically related to Sphaeropleales species and supposedly originated from an ancestor shared with *Desmodesmus*. Whether all *Pseudochlorococcum* species fall into the same clade remains to be determined. It is also possible that the *Pseudochlorococcum* genus may be composed of a number of species that are genetically heterogenous.

A 1160-bp *rbcL* segment (SEQ ID NO: 2) was also amplified from *Pseudochlorococcum* sp. and the sequence showed high identity with the strains belonging to the Sphaeropleales as indicated by a BLAST search in NCBI. Most mutations occurred at the third position of codons among closely-related strains. The phylogenetic tree reconstructed on 1160 base pairs of 20 different Chlorophyta taxa (**Fig. 15**) supports *Pseudochlorococcum* sp. being related to some Sphaeropleales species, which is congruent with the phylogenetic relationship based on the sequences of the ITS region.

The internal transcribed spacer 1 (ITS1) is the non-coding segment located between 18S rDNA and 5.8S rDNA; the internal transcribed spacer 2 (ITS2) is located between 5.8S rDNA and 28S rDNA. The ITS1 of the newly-isolated *Pseudochlorococcum* sp. being 155 bp in length shares 95% identity with the sequence of its closely-related *Desmodesmus multivariabilis* var. *turskensis* Mary 8/18 T-1W. As shown in **Figure 16** six indels (insertion and deletion) occurred in the ITS1 region. The identity of ITS2 sequence shared by these two species is about 99%, slightly more conserved than ITS1. Therefore, *Pseudochlorococcum* sp. is distinguishable to its closely-related species at the fast-evolving DNA region ITS1.

The *rbcL* sequence of *Pseudochlorococcum* sp. shows 97% identity with *Neochloris sp.* LCR (**Figure 17**). Only two of the mutations (843A/G, 1153T/G) are non-synonymous and others that occurred at the third position of the codons are

synonymous. Thus, the *rbcL* region can be used to distinguish *Pseudochlorococcum* sp. from its closely-related species.

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We claim:

1. An isolated *Pseudochlorococcum* sp. composition, wherein the isolated *Pseudochlorococcum* sp. genome comprises one or more nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5, or complements thereof.

2. The isolated *Pseudochlorococcum* sp. of claim 1, wherein the isolated *Pseudochlorococcum* sp. genome comprises the nucleic acid sequence of SEQ ID NO:3.

3. The isolated *Pseudochlorococcum* sp. of claim 1, wherein the isolated *Pseudochlorococcum* sp. genome comprises the nucleic acid sequence of SEQ ID NO:4.

4. The isolated *Pseudochlorococcum* sp. of claim 1, wherein the isolated *Pseudochlorococcum* sp. genome comprises the nucleic acid sequence of SEQ ID NO:5.

5. The isolated *Pseudochlorococcum* sp. of claim 1, wherein the isolated *Pseudochlorococcum* sp. genome comprises the nucleic acid sequence of SEQ ID NO:1.

6. A substantially pure culture, comprising:

(a) a growth medium; and

(b) the isolated *Pseudochlorococcum* sp. of any one of claims claim 1-5.

7. An algal culture system, comprising:

(a) a photobioreactor; and

(b) the substantially pure culture of claim 6.

8. A method for lipid isolation, comprising culturing a *Pseudochlorococcum* sp., wherein the *Pseudochlorococcum* sp. genome comprises one or more nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5, or complements thereof, wherein the culturing is done under conditions suitable for proliferation of the *Pseudochlorococcum* sp., and extracting lipid from the *Pseudochlorococcum* sp.

9. A method for removing nutrients from wastewater, comprising culturing a *Pseudochlorococcum* sp., wherein the *Pseudochlorococcum* sp. genome comprises one or more nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5, or complements thereof, wherein the culturing is done under conditions suitable for

proliferation of the *Pseudochlorococcum* sp., and wherein the culturing is carried out in a culture medium comprising at least 5% wastewater, under conditions whereby nutrients in the wastewater are removed by the *Pseudochlorococcum* sp.

10. A method for removing nutrients from waste gas, comprising culturing a *Pseudochlorococcum* sp., wherein the *Pseudochlorococcum* sp. genome comprises one or more nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5, or complements thereof, wherein the culturing is done under conditions suitable for proliferation of the *Pseudochlorococcum* sp., and wherein the culturing is carried out in a culture medium comprising waste gas, under conditions whereby nutrients in the waste gas are removed by the *Pseudochlorococcum* sp.

11. The method of any one of claims 8-10, further comprising harvesting algal protein and/or biomass components from the cultured *Pseudochlorococcum* sp.

12. A method for producing biomass, comprising culturing a *Pseudochlorococcum* sp., wherein the *Pseudochlorococcum* sp. genome comprises one or more nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5, or complements thereof, wherein the culturing is carried out under conditions suitable for proliferation of the *Pseudochlorococcum* sp., and harvesting algal protein and/or biomass components from the cultured *Pseudochlorococcum* sp.

13. The method of any one of claims 8-12, wherein the *Pseudochlorococcum* sp. genome comprises the nucleic acid sequence of SEQ ID NO:3.

14. The method of any one of claims 8-12, wherein the *Pseudochlorococcum* sp. genome comprises the nucleic acid sequence of SEQ ID NO:4.

15. The method of any one of claims 8-12, wherein the *Pseudochlorococcum* sp. genome comprises the nucleic acid sequence of SEQ ID NO:5.

16. The method of any one of claims 8-12, wherein the *v* sp. genome comprises the nucleic acid sequence of SEQ ID NO:1.

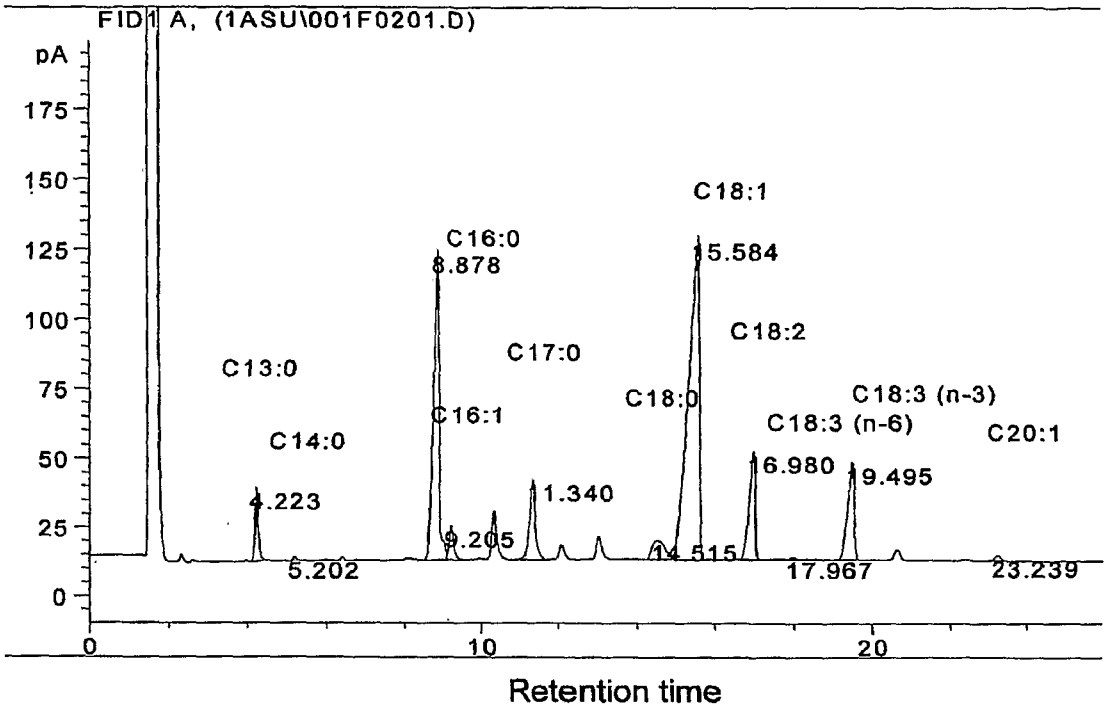

FID1 A, (1ASU\001F0201.D)
pA
175
150
125
100
75
50
25
0
C13:0
C14:0
4.223
5.202
C16:0
8.878
C16:1
9.205
C17:0
11.340
C18:0
14.515
C18:1
15.584
C18:2
16.980
C18:3 (n-3)
C18:3 (n-6)
19.495
C20:1
23.239
17.967
0
10
20
Retention time

Figure 1

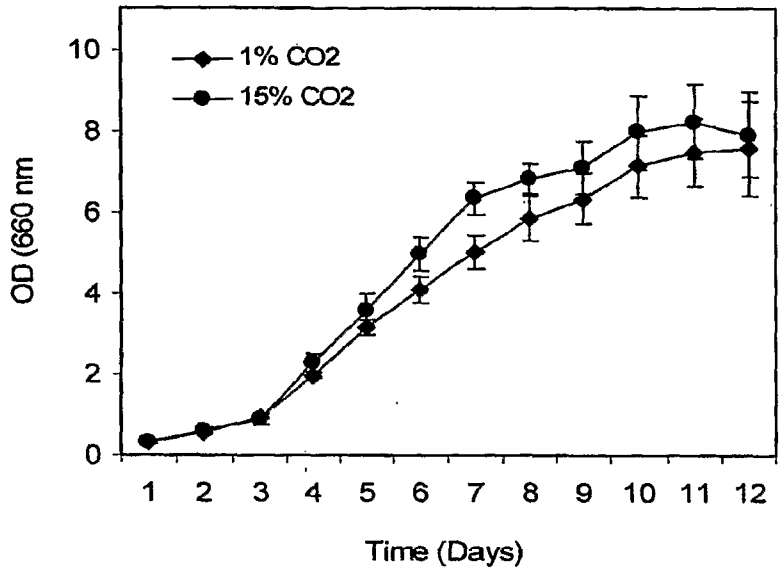


Figure 2

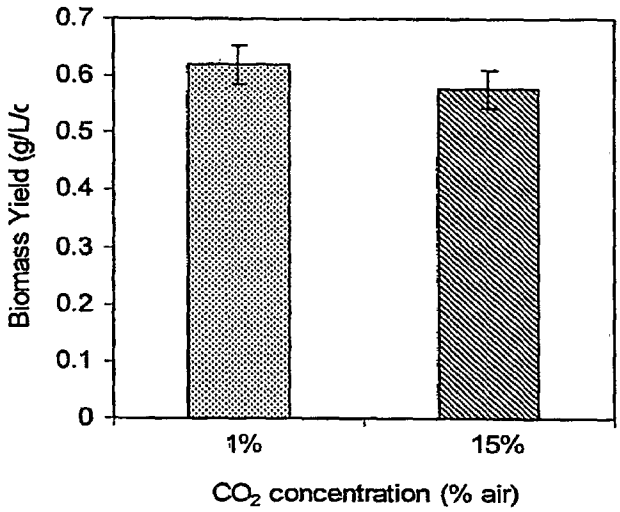

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Biomass Yield (g/L/c
1%
15%
CO2 concentration (% air)

Figure 3

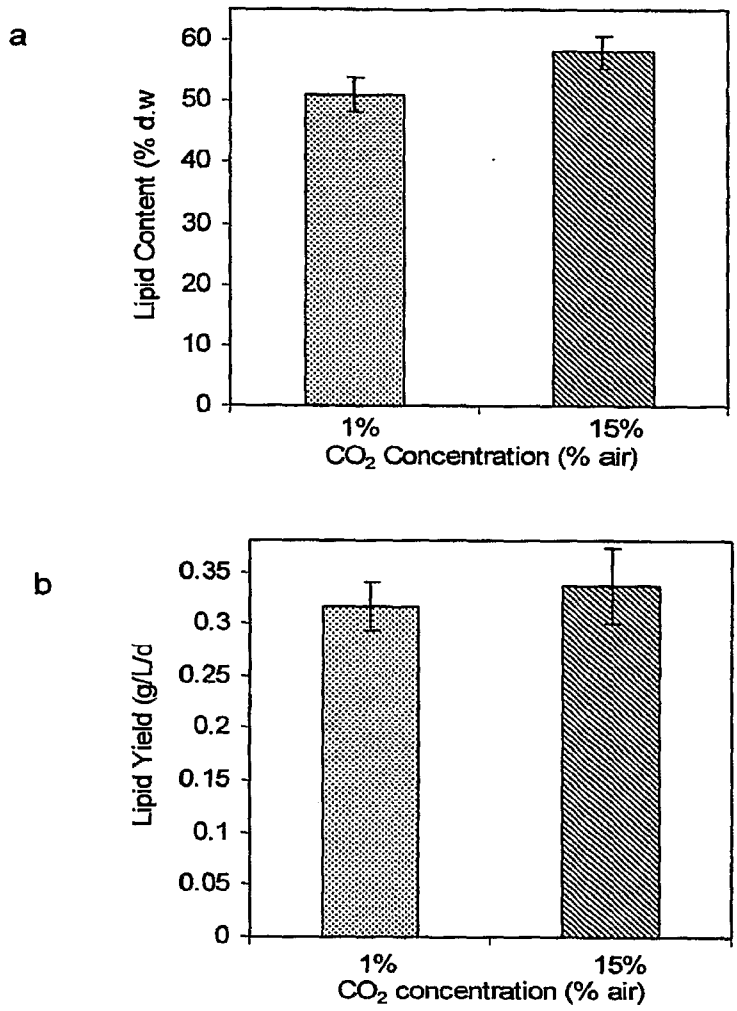

a
Lipid Content (% d.w
60
50
40
30
20
10
0
1%
15%
CO_2 Concentration (% air)
b
Lipid Yield (g/L/d
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
1%
15%
CO_2 concentration (% air)

Figure 4

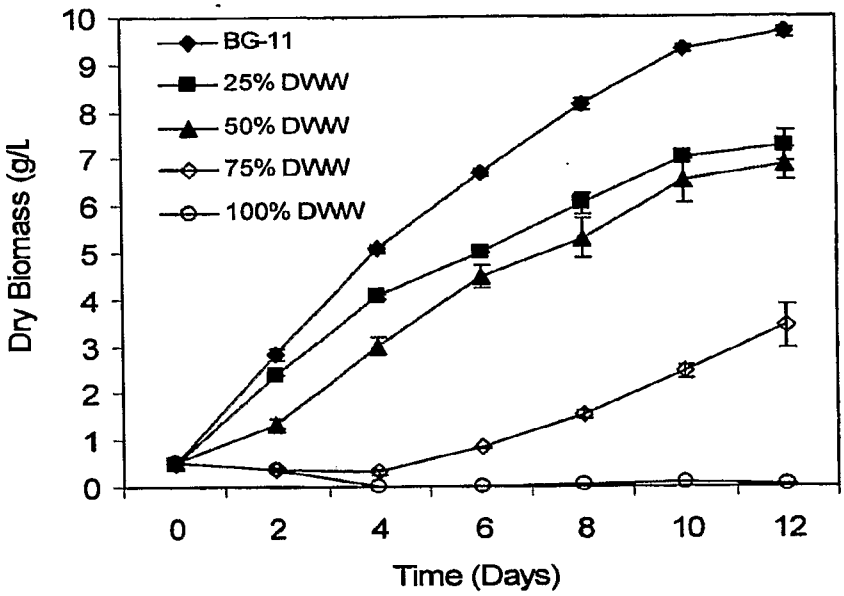

BG-11
25% DWW
50% DWW
75% DWW
100% DWW
Dry Biomass (g/L
Time (Days)
0
1
2
3
4
5
6
7
8
9
10
0
2
4
6
8
10
12

Figure 5

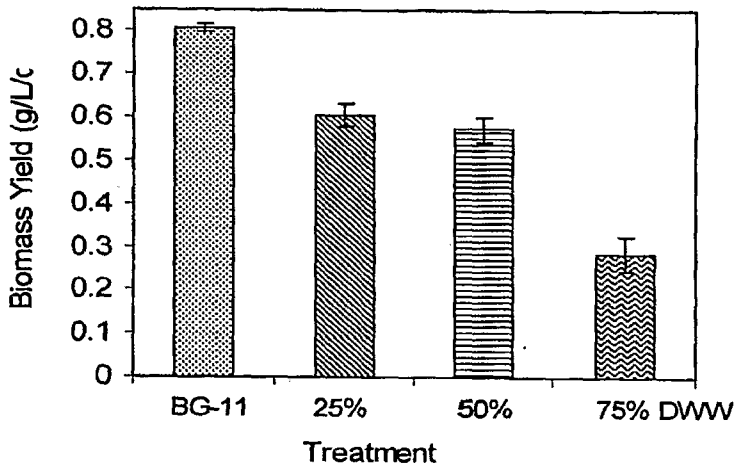

Biomass Yield (g/L/c
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
BG-11
25%
50%
75% DWW
Treatment

Figure 6

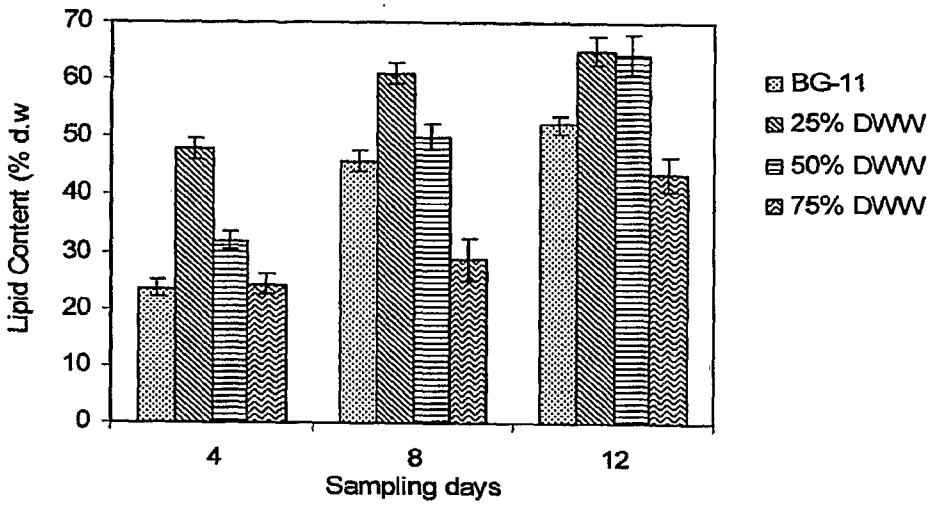

70
60
50
40
30
20
10
0
Lipid Content (% d.w
4
8
12
Sampling days
BG-11
25% DWW
50% DWW
75% DWW

Figure 7

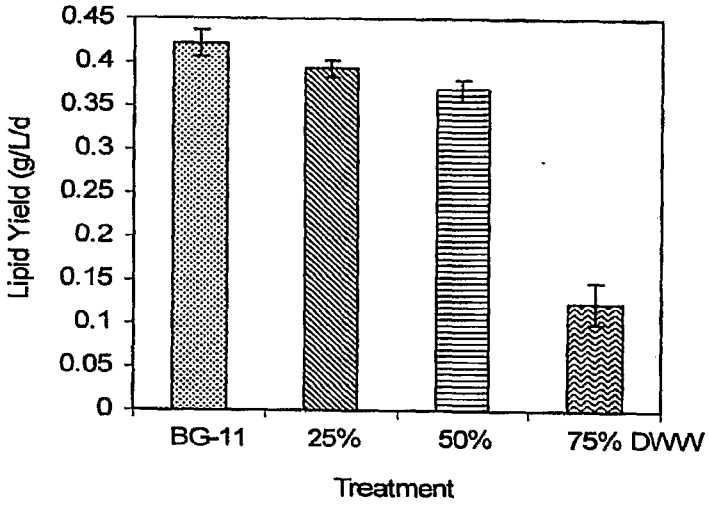

0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Lipid Yield (g/L/d
BG-11
25%
50%
75% DWW
Treatment

Figure 8

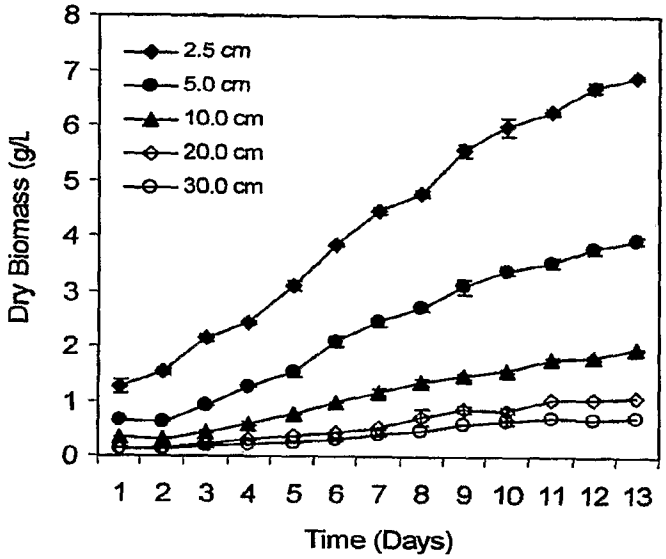

2.5 cm
5.0 cm
10.0 cm
20.0 cm
30.0 cm
Dry Biomass (g/L
Time (Days)
0
1
2
3
4
5
6
7
8
1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 9

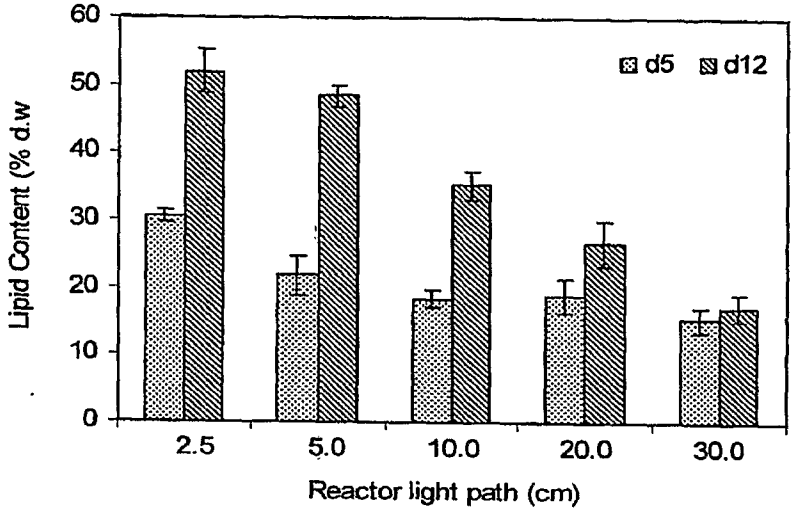

60
50
40
30
20
10
0
Lipid Content (% d.w
d5
d12
2.5
5.0
10.0
20.0
30.0
Reactor light path (cm)

Figure 10

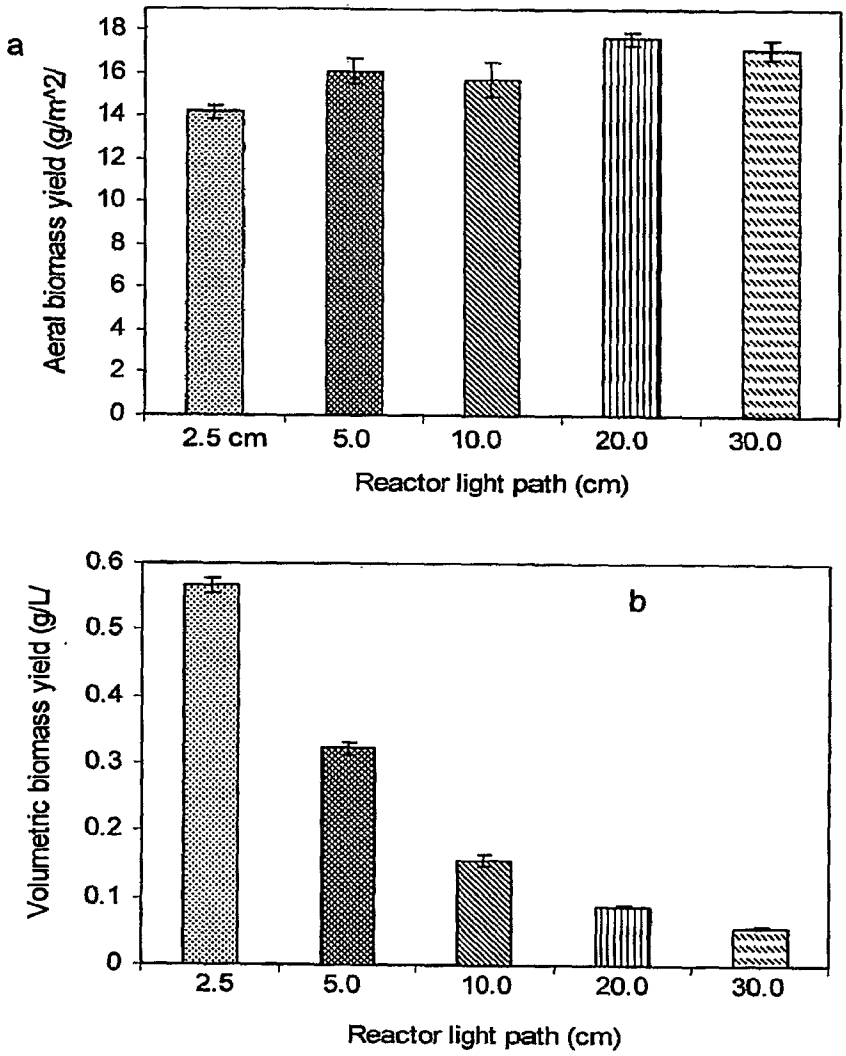
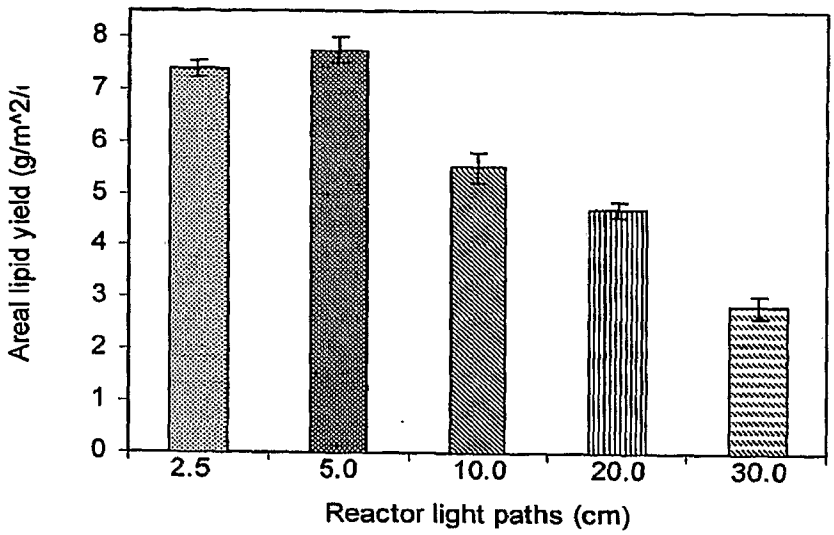

a
Aeral biomass yield (g/m^2/
18
16
14
12
10
8
6
4
2
0
2.5 cm
5.0
10.0
20.0
30.0
Reactor light path (cm)
b
Volumetric biomass yield (g/L/
0.6
0.5
0.4
0.3
0.2
0.1
0
2.5
5.0
10.0
20.0
30.0
Reactor light path (cm)

Figure 11


Areal lipid yield (g/m^2/
8
7
6
5
4
3
2
1
0
2.5
5.0
10.0
20.0
30.0
Reactor light paths (cm)

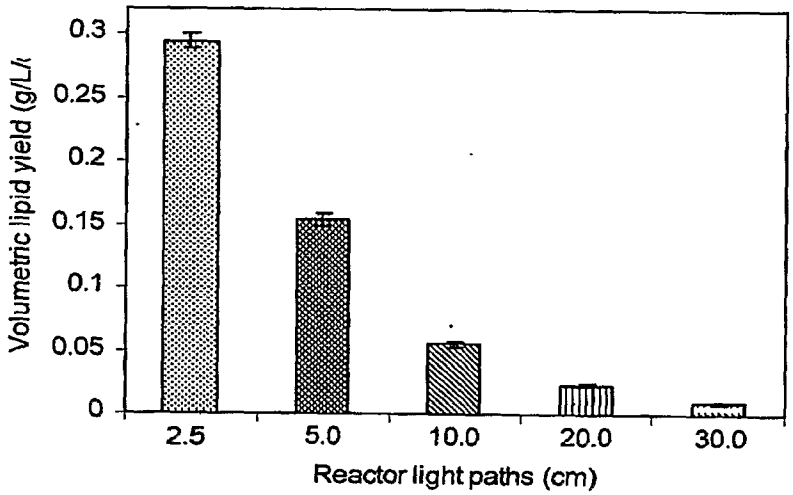

Volumetric lipid yield (g/L/
0.3
0.25
0.2
0.15
0.1
0.05
0
2.5
5.0
10.0
20.0
30.0
Reactor light paths (cm)

Figure 12

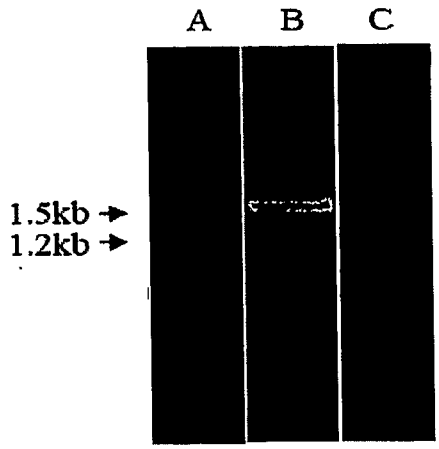


Figure 13

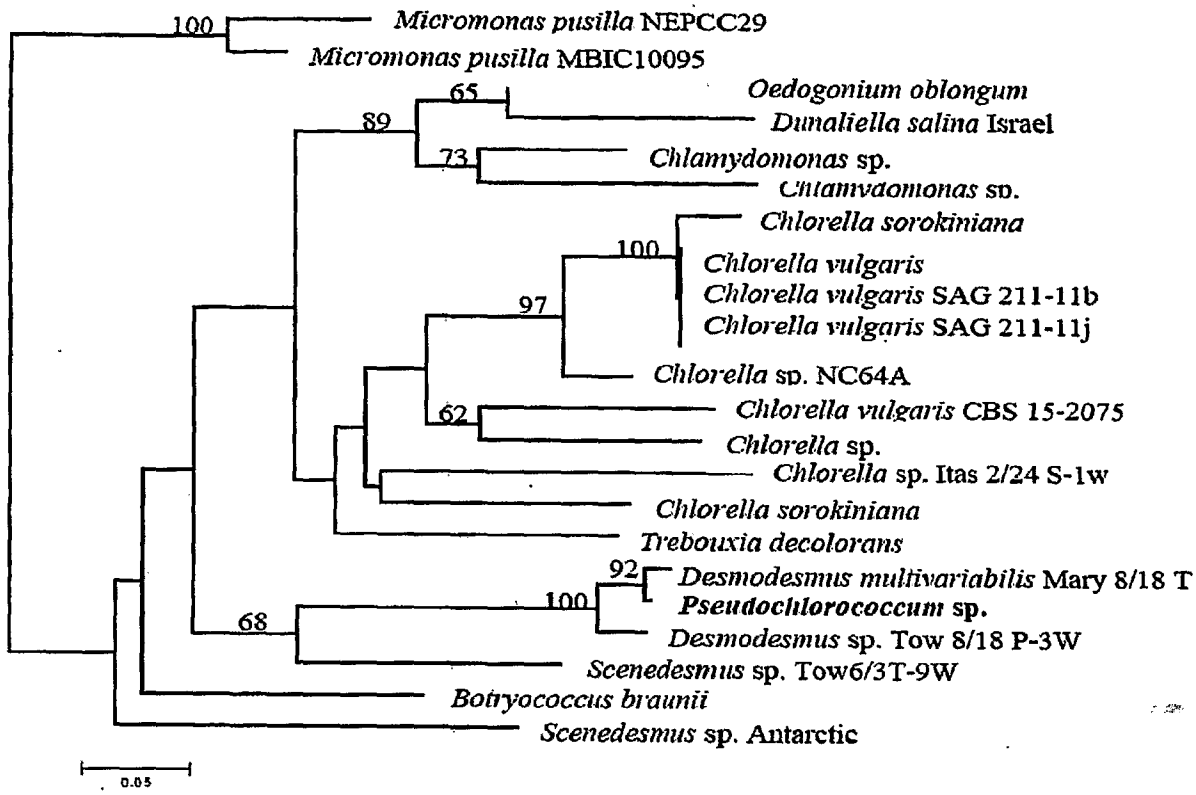

100
Micromonas pusilla NEPCC29
Micromonas pusilla MBIC10095
65
89
Oedogonium oblongum
Dunaliella salina Israel
73
Chlamydomonas sp.
Chlamydomonas sp.
Chlorella sorokiniana
100
Chlorella vulgaris
Chlorella vulgaris SAG 211-11b
97
Chlorella vulgaris SAG 211-11j
Chlorella sp. NC64A
62
Chlorella vulgaris CBS 15-2075
Chlorella sp.
Chlorella sp. Itas 2/24 S-1w
Chlorella sorokiniana
Trebouxia decolorans
92
Desmodesmus multivariabilis Mary 8/18 T
100
Pseudochlorococcum sp.
68
Desmodesmus sp. Tow 8/18 P-3W
Scenedesmus sp. Tow6/3T-9W
Botryococcus braunii
Scenedesmus sp. Antarctic
0.05

Figure 14

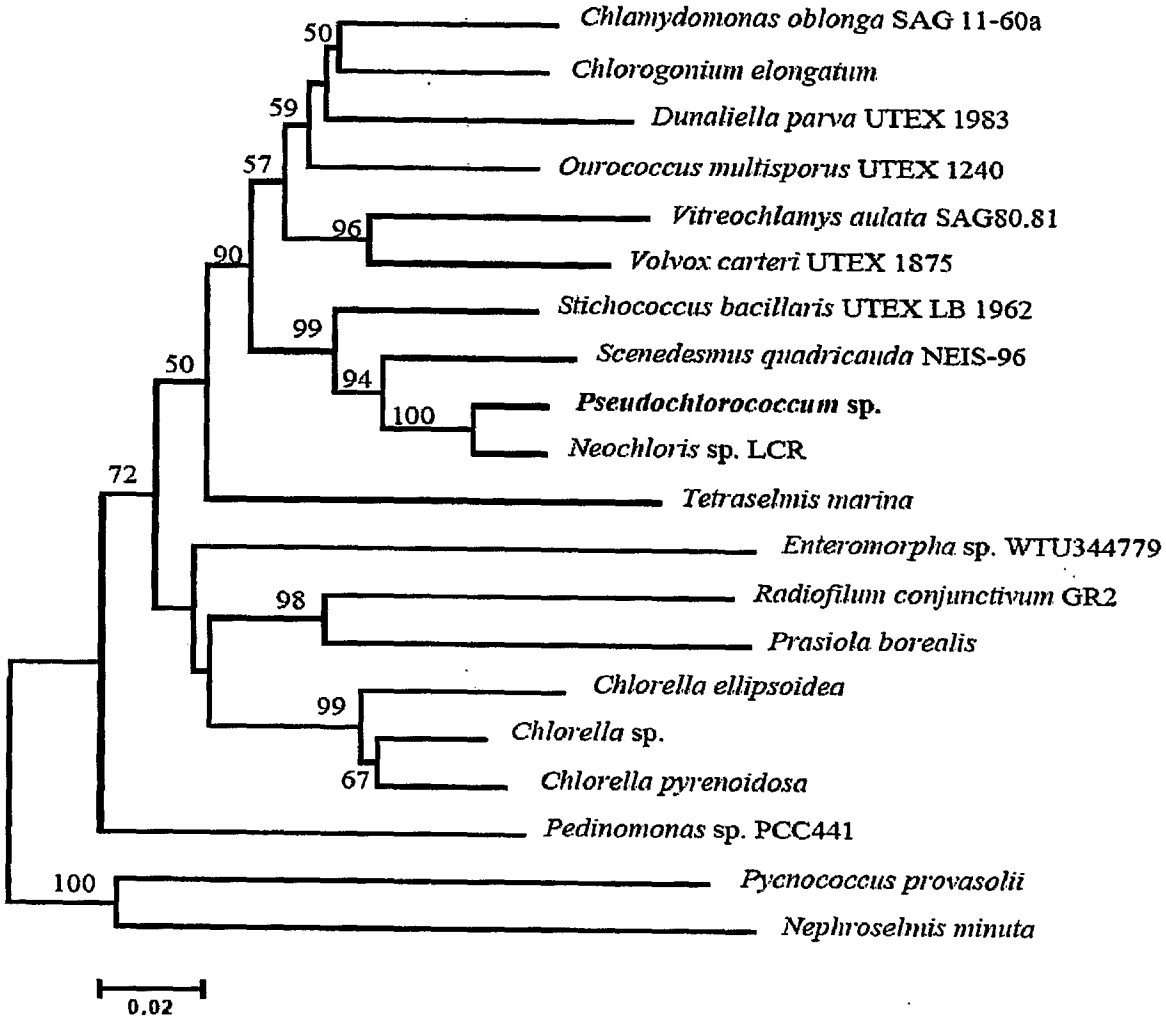
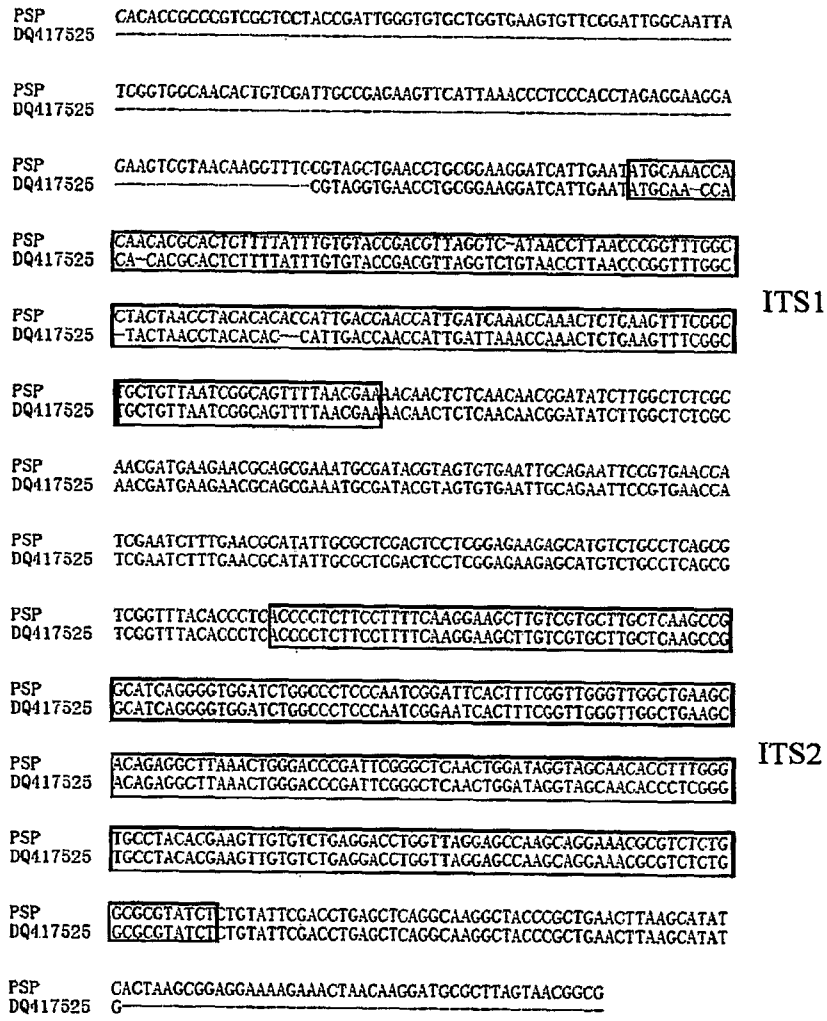

Chlamydomonas oblonga SAG 11-60a
Chlorogonium elongatum
Dunaliella parva UTEX 1983
Ourococcus multisporus UTEX 1240
Vitreochlamys aulata SAG80.81
Volvox carteri UTEX 1875
Stichococcus bacillaris UTEX LB 1962
Scenedesmus quadricauda NEIS-96
Pseudochlorococcum sp.
Neochloris sp. LCR
Tetraselmis marina
Enteromorpha sp. WTU344779
Radiofilum conjunctivum GR2
Prasiola borealis
Chlorella ellipsoidea
Chlorella sp.
Chlorella pyrenoidosa
Pedinomonas sp. PCC441
Pycnococcus provasolii
Nephroselmis minuta
50
59
57
96
90
99
50
94
100
72
98
99
67
100
0.02

Figure 15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DQ417525 ——
PSP TCGGTGGCAACACTGTCGATTGCCGAGAAGTTCATTAAACCCTCCCACCTAGAGGAAGGA
DQ417525 ——
PSP GAAGTCGTAACAAGGTTTCCGTAGCTGAACCTGCGGAAGGATCATTGAATATGCAAACCA
DQ417525 ——————————————CGTAGGTGAACCTGCGGAAGGATCATTGAATATGCAA-CCA
PSP CAACACGCACTCTTTTATTTGTGTACCGACGTTAGGTC-ATAACCTTAACCCGGTTTGGC
DQ417525 CA-CACGCACTCTTTTATTTGTGTACCGACGTTAGGTCTGTAACCTTAACCCGGTTTGGC
ITS1
PSP CTACTAACCTACACACACCATTGACCAACCATTGATCAAACCAAACTCTGAAGTTTCGGC
DQ417525 -TACTAACCTACACAC--CATTGACCAACCATTGATTAAACCAAACTCTGAAGTTTCGGC
PSP TGCTGTTAATCGGCAGTTTTAACGAAAACAACTCTCAACAACGGATATCTTGGCTCTCGC
DQ417525 TGCTGTTAATCGGCAGTTTTAACGAAAACAACTCTCAACAACGGATATCTTGGCTCTCGC
PSP AACGATGAAGAACGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCCGTGAACCA
DQ417525 AACGATGAAGAACGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCCGTGAACCA
PSP TCGAATCTTTGAACGCATATTGCGCTCGACTCCTCGGAGAAGAGCATGTCTGCCTCAGCG
DQ417525 TCGAATCTTTGAACGCATATTGCGCTCGACTCCTCGGAGAAGAGCATGTCTGCCTCAGCG
PSP TCGGTTTACACCGTCACCCCTCTTCCTTTTCAAGGAAGCTTGTCGTGCTTGCTCAAGCCG
DQ417525 TCGGTTTACACCGTCACCCCTCTTCCTTTTCAAGGAAGCTTGTCGTGCTTGCTCAAGCCG
PSP GCATCAGGGGTGGATCTGGCCCTCCCAATCGGATTCACTTTCGGTTGGGTTGGCTGAAGC
DQ417525 GCATCAGGGGTGGATCTGGCCCTCCCAATCGGAATCACTTTCGGTTGGGTTGGCTGAAGC
ITS2
PSP ACAGAGGCTTAAACTGGGACCCGATTCGGGCTCAACTGGATAGGTAGCAACACCTTTGGG
DQ417525 ACAGAGGCTTAAACTGGGACCCGATTCGGGCTCAACTGGATAGGTAGCAACACCCTCGGG
PSP TGCCTACACGAAGTTGTGTCTGAGGACCTGGTTAGGAGCCAAGCAGGAAACGCGTCTCTG
DQ417525 TGCCTACACGAAGTTGTGTCTGAGGACCTGGTTAGGAGCCAAGCAGGAAACGCGTCTCTG
PSP GCGCGTATCTCTGTATTCGACCTGAGCTCAGGCAAGGCTACCCGCTGAACTTAAGCATAT
DQ417525 GCGCGTATCTCTGTATTCGACCTGAGCTCAGGCAAGGCTACCCGCTGAACTTAAGCATAT
PSP CACTAAGCGGAGGAAAAGAAACTAACAAGGATGCGCTTAGTAACGGCG
DQ417525 G————————————————————————————————————

Figure 16

```
PSP         TGTCACCACAAACAGAAACTCGTGCAGGTGCTGGATTCAAAGCCGGTGTTAAAGACTATC
EF012704    TGTCACCACAAACAGAAACTCGTGCAGGTGCTGGATTCAAAGCCGGTGTTAAAGACTATC

PSP         GTTTAACATACTACACTCCAGACTACGTTGTAAAAGATACTGATATCTTAGCAGCATTCC
EF012704    GTTTAACATACTACACTCCAGACTACGTTGTAAAAGATACTGATGTATTAGCAGCATTCC

PSP         GTATGACTCCTCAACCAGGTGTTCCTGCTGAAGAATGTGGTGCAGCGGTTGCTGCTGAAT
EF012704    GTATGACTCCTCAACCAGGTGTTCCTGCTGAAGAATGTGGTGCAGCGGTTGCAGCTGAAT

PSP         CATCAACAGGTACTTGGACAACAGTATGGACTGACGGTCTTACTACTTTAGACCGATACA
EF012704    CATCAACAGGTACTTGGACAACAGTATGGACTGACGGTCTTACTACTTTAGACCGATACA

PSP         AAGGACGTTGTTACGATATTGAGCCAGTACCCGGTGAAGACAACCAGTACATTGCATATG
EF012704    AAGGACGTTGTTACGATATCGAGCCGGTACCTGGTGAAGACAACCAGTACATTGCATACG

PSP         TTGCGTACCCTATTGACTTATTTGAAGAAGGATCTGTTACTAACTTATTCACTTCAATCG
EF012704    TTGCGTACCCTATCGACTTATTTGAAGAAGGATCAGTTACTAACTTATTCACTTCAATCG

PSP         TAGGTAACGTATTCGGTTTCAAAGCTCTTCGTGCTTTACGTTTAGAAGACTTACGTATTC
EF012704    TAGGTAACGTATTCGGTTTCAAAGCTCTTCGTGCTTTACGTTTAGAAGACTTACGTATTC

PSP         CTCCAGCTTACGTTAAAACTTTCCAAGGTCCTCCTCACGGTATTCAAGTTGAACGTGACA
EF012704    CTCCAGCTTACGTTAAAACTTTCCAAGGACCTCCTCACGGTATCCAAGTTGAACGTGACA

PSP         AATTAAACAAATACGGGCGTGGTTTATTAGGTTGTACAATTAAACCAAAATTAGGTTTAT
EF012704    AATTAAACAAATATGGTCGTGGTTTATTAGGTTGTACAATTAAACCAAAATTAGGTTTAT

PSP         CAGCAAAAAACTACGGGCGTGCTGTTTACGAGTGTTTACGTGGTGGGTTAGACTTTACGA
EF012704    CAGCTAAAAACTACGGACGTGCTGTTTACGAGTGTTTACGTGGTGGTTTAGACTTCACGA

PSP         AAGATGATGAAAACGTAAACTCTCAACCATTCATGCGTTGGAGAGACCGTTTCTTATTTG
EF012704    AGGATGACGAAAACGTTAACTCTCAACCATTCATGCGTTGGAGAGACCGTTTCTTATTCG

PSP         TAGCTGAAGCTTGTTACAAAGCTCAAGCAGAAACTGGTGAAATCAAAGGGCACTACTTAA
EF012704    TAGCTGAAGCTTGTTACAAAGCTCAAGCAGAAACTGGTGAAATCAAAGGGCACTACTTAA

PSP         ACGCTACTGCAGGTACTTGTGAAGAAATGCTTAAACGTGCTCAGTGTGCTAAAGATTTAG
EF012704    ACGCTACTGCAGGTACTTGCGAAGAAATGCTTAAACGTGCTCAGTGTGCTAAAGACTTAG

PSP         GTGTACCTATTGTTATGCACGACTACTTAACTGGTGGTTTCACTGCAAACACTTCTTTAG
EF012704    GTGTACCTATTGTTATGCATGACTACTTAACTGGTGGTTTCACTGCAAACACTTCTTTAG

PSP         CTACTTACTGTCGTGACCACGGTTTATTATTACACATCCACCGTGCTATGCACGCTGTAA
EF012704    CAGCTTACTGTCGTGACCATGGTTTATTATTACACATTCACCGTGCTATGCACGCTGTAA

PSP         TTGACCGTCAAAGAAACCACGGTATCCACTTCCGTGTATTAGCGAAAGCTTTACGTATGT
EF012704    TTGACCGTCAAAGAAACCACGGTATCCACTTCCGTGTATTAGCGAAAGCTTTACGTATGT

PSP         CAGGTGGTGACCACTTACACAGTGGTACAGTAGTAGGTAAATTAGAAGGTGAACGTGAAG
EF012704    CAGGTGGTGACCACTTACACAGTGGTACAGTAGTAGGTAAATTAGAAGGTGAACGTGAAG

PSP         TTACTTTAGGTTTCGTAGACTTAATGCGTGACGACTACATCGAAAAAGACCGTAGCCGTG
EF012704    TTACTTTAGGTTTCGTAGACTTAATGCGTGACGACTACATCGAAAAAGACCGTAGCCGTG

PSP         GTATTTACTTCACTCAAGACTGGTGTTCTATGTCAGGAACAATGCCAGTAGCTTCAGGTG
EF012704    GTATTTACTTCACTCAAGACTGGTGTTCTATGTCAGGAACAATGCCAGTAGCTTCAGGAG

PSP         GTATTCACGTATTGCACATG
EF012704    GTATTCACGTATGGCACATG
```

Figure 17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US07/15199

A. CLASSIFICATION OF SUBJECT MATTER
IPC: **C12N 1/00**(2006.01),**1/12**(2006.01),**1/04**(2006.01),**1/02**(2006.01)

USPC: 435/243,257.1,260,261
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/243,257.1,260,261

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Archibald P.A. Pseudohlorococcum, A New Chlorococcalean Genus Archibald Journal of Phycology, Vol 6, No 2, Pages 127-132, 1970. see entire document	*1-16*

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
28 August 2008 (28.08.2008)

Date of mailing of the international search report
25 SEP 2008

Name and mailing address of the ISA/US
Mail Stop PCT, Attn: ISA/US
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
Facsimile No. (571) 273-3201

Authorized officer
Sumesh Kaushal

Telephone No. (571) 272-0547

Form PCT/ISA/210 (second sheet) (April 2007)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US07/15199

Continuation of B. FIELDS SEARCHED Item 3:
STN: Medline, Caplus, Scisearch, Biosis, Mediconf. EAST: Uspat, Pgpub, Epo, Jpo, Derwent. STIC:Sequence search in SEQ databases.
SEARCH TERMS: Nucleotide Sequences of SEQ ID NO:1,2,3,4 and 5.