Novel Chlorella sp. and Uses Therefor

Related applications

This application claims priority to U.S. Provisional Patent Application Serial No. 60/800077 filed May 12, 2006, incorporated by reference herein in its entirety.

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 among the 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, and palm seeds are a source of feedstock for biodiesel, but these crops can not adequately perform wastestream treatment.

Summary

Disclosed are isolated *Chlorella* sp. compositions, wherein the isolated *Chlorella* sp. genome comprises one or more nucleic acid sequence selected from the group consisting of SEQ ID NO:1 (ITS—1249 bp), SEQ ID NO:2 (rbcL—1393 bp), SEQ ID NO:3 (ITS1—502-739 of ITS), SEQ ID NO:4 (ITS2—899-1137 of ITS), SEQ ID NO:5 (ITS—827 bp), and SEQ ID NO:6 (rbcL—1160 bp) or complements thereof.

In a first aspect, there is provided an isolated *Chlorella* sp. composition, wherein the isolated *Chlorella* sp. genome comprises a nucleic acid sequence of SEQ ID NO:1, or complement thereof.

In a second aspect, there is provided a substantially pure culture, comprising:

(a) a growth medium; and

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

In a third aspect, there is provided an algal culture system, comprising:

(a) a photobioreactor; and

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

Also disclosed are methods for lipid isolation, wastewater remediation, waste gas remediation, and/or biomass production, comprising culturing a *Chlorella sp.*, wherein the *Chlorella* sp. genome comprises one or more nucleic acid sequence selected from the group consisting of SEQ ID NO:1 (ITS—1249 bp), SEQ ID NO:2 (rbcL—1393 bp), SEQ ID NO:3 (ITS1—502-739 of ITS), SEQ ID NO:4 (ITS2—899-1137 of ITS), SEQ ID NO:5 (ITS—827 bp), and SEQ ID NO:6 (rbcL—1160 bp) or complements thereof, under conditions suitable for lipid isolation, wastewater remediation, waste gas remediation, and/or biomass production.

In a fourth aspect, there is provided a method for lipid isolation, comprising culturing a *Chlorella* sp., wherein the *Chlorella* sp. genome comprises a nucleic acid sequence of SEQ ID NO:1, or complement thereof, wherein the culturing is done under conditions suitable for proliferation of the *Chlorella* sp., and extracting lipid from the *Chlorella* sp.

In a fifth aspect, there is provided a method for removing nutrients from wastewater, comprising culturing a *Chlorella* sp., wherein the *Chlorella* sp. genome comprises a nucleic acid sequence of SEQ ID NO:1, or complement thereof, wherein the culturing is done under conditions suitable for proliferation of the *Chlorella* 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 *Chlorella* sp.

In a sixth aspect, there is provided a method for removing nutrients from waste gas, comprising culturing a *Chlorella* sp., wherein the *Chlorella* sp. genome comprises a nucleic acid sequence of SEQ ID NO: 1, or complement thereof, wherein the culturing is done under conditions suitable for proliferation of the *Chlorella* 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 *Chlorella* sp.

In a seventh aspect, there is provided a method for producing biomass, comprising culturing a *Chlorella* sp., wherein the *Chlorella* sp. genome comprises a nucleic acid sequence of SEQ ID NO:1, or complement thereof, wherein the culturing is carried out under conditions suitable for proliferation of the *Chlorella* sp., and harvesting algal protein and/or biomass components from the cultured *Chlorella* sp.

Brief Description of the Figures

Figure 1. Effect of carbon dioxide on growth kinetics of *Chlorella* sp. cultured in 300 ml capacity glass columns (68 cm long with an inner diameter of 2.3 cm). Cultures were aerated with compressed air containing either 1% or 15% CO_2. Cultures were at 25±1°C and light intensity of 170 μmol m^{-2} s^{-1}.

Figure 2. Effect of carbon dioxide on biomass yield of *Chlorella* sp. (Culture conditions were the same as described for Figure 1).

Figure 3. Effects of carbon dioxide on the lipid content (a) and lipid yield (b) of *Chlorella* sp. (Culture conditions same as for Figure 1).

Figure 4. Effect of dairy wastewater (DWW) on growth of *Chlorella* 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 5. Effect of dairy wastewater on biomass yield of *Chlorella* sp. grown in a glass column bioreactor (Growth conditions were the same as in Figure 4).

Figure 6. Effect of dairy wastewater on lipid content of *Chlorella* sp. grown in a glass column bioreactor (Growth conditions were the same as in Figure 4).

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

Figure 8. PCR products amplified from *Chlorella* sp. A = DNA Ladder; B = ITS; and C = *rbcL.*

Figure 9. Neighbor-joining (NJ) tree based on aligned nucleotide sequences for 827 bases in the regions of ITS (internal transcribed spacer) from 22 OTUs (Operational Taxonomic Units) 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 10. 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 11. Sequence alignment of 827bp region of ITS segment for *Chlorella* sp. (CV) and its closest phylogenetically related species *Chlorella vulgaris* CBS 15-2075 (GeneBank Accession Number: AY948419). ITS1 (168-405) and ITS2 (565-803) are marked separately.

Figure 12. Sequence Alignment of 1160 bp of rbcL for *Chlorella* sp. (cv) and its closest phylogenetically related species *Chlorella pyrenoidosa* (GeneBank Accession Number: AB240145). The codon starts from 1st nucleotide.

Detailed Description of the Invention

In a first aspect, the present invention provides an isolated *Chlorella* sp. composition, wherein the isolated *Chlorella* sp. genome comprises one or more nucleic acid sequence selected from the group consisting of SEQ ID NO:1 (ITS—1249 bp), SEQ ID NO:2 (rbcL—1393 bp), SEQ ID NO:3 (ITS1—502-739 of ITS), SEQ ID NO:4 (ITS2—899-1137 of ITS), SEQ ID NO:5 (ITS—827 bp), and SEQ ID NO:6 (rbcL—1160 bp) or complements thereof. As discussed in more detail below, each of these nucleic acid sequences serves as a marker for the novel *Chlorella* sp. of the present invention, and distinguishes it from other *Chlorella* strains.

The algae of this first aspect of the invention are useful for a variety of purposes, including but not limited to lipid 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 algae of this first aspect of the invention were derived by a selection process from culture obtained from a water environment in the Phoenix metropolitan area. Thus, the *Chlorella* 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 *Chlorella* 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 algal type; in further embodiments, at least 95%, 98%, or 99% of the algae present in the composition are of the recited algal type. The isolated *Chlorella* sp. composition can be cultured or stored in solution, frozen, dried, or on solid agar plates.

The *Chlorella* 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 selected from the

group consisting of lutein, zeaxanthin, and astaxanthin, (useful, for example, as livestock or aquaculture feed additive), or combinations thereof.

As used herein, the phrase "ability to grow" means that the *Chlorella* sp. are 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. As used herein, the phrase "ability to accumulate large quantities" of biomass means 20 to 60% of dry weight.

In a second aspect, the present invention provides a substantially pure culture, comprising a growth medium and the isolated *Chlorella* sp. 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, *Chlorella* sp. isolates are usually maintained in standard artificial growth medium. For regular maintenance purposes, the *Chlorella* 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 *Chlorella* 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 *Chlorella* sp. of the present invention comprises wastewater or waste gases. This growth medium is particularly useful when the *Chlorella* 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 *Chlorella* sp. can be first cultivated in a primary growth medium, followed by addition of wastewater and/or waste gas. Alternatively, the *Chlorella* 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 *Chlorella* 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 *Chlorella* 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 *Chlorella* sp. In one embodiment, if the wastewater being treated contains sufficient nutrients to sustain the *Chlorella* sp. growth, it may be possible to use less of the growth medium. As the wastewater becomes cleaner due to *Chlorella* sp. treatment, the amount of growth medium can be increased.

The major factors affecting wastewater feeding rate include: 1) *Chlorella* 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 an 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 1000 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 *Chlorella* 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 100 millimeters and 30 centimeters, between 50 millimeters and 20 centimeters, and between 1 centimeter and 15 centimeters, and most preferably 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 *Chlorella* sp. of the present invention, wherein the *Chlorella* sp. genome

comprises one or more nucleic acid sequence selected from the group consisting of SEQ ID NO:1 (ITS—1249 bp), SEQ ID NO:2 (rbcL—1393 bp), SEQ ID NO:3 (ITS1—502-739 of ITS), SEQ ID NO:4 (ITS2—899-1137 of ITS), SEQ ID NO:5 (ITS—827 bp), and SEQ ID NO:6 (rbcL—1160 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 *Chlorella* sp. of the present invention under conditions suitable for production of total lipid content of at least 40 % of dry algal cell weight; in various embodiments, the total lipid content is at least 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 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 *Chlorella* 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 *Chlorella* 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 *Chlorella* 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 *Chlorella* 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 *Chlorella* 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 *Chlorella* 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, zeaxanthin, and astaxanthin, (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 *Chlorella* 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 oilseeds (or other) plants for limited agricultural land.

Examples

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). *Chlorella* 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 hour with magnetic stirring. The mixture was centrifuged at 3,500 rpm for ten 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^{TM} 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/minute. 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).

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 use for experiments, the dairy wastewater was 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 25% wastewater (1:3 dairy wastewater to deionized water), 50% wastewater (1:1 wastewater to deionized water), 75% wastewater (3:1 wastewater to deionized 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 a 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 vials 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.

***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 *Chlorella* species 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 *Chlorella* sp.

Primers	Sequence (5'-3')
ITS	
s15CH(F)	CTTAGTTGGTGGGTTGCC (SEQ ID NO:7)
l5pl(R)	TTCRCTCGCCGTTACT (SEQ ID NO:8)
rbcL	
RH1(F)	ATGTCACCACAAACAGAAACTAAAGC (SEQ ID NO:9)
Cel 161R(R)[2]	CATGTGCAATACGTGAATACC 9SEQ ID NO:10)

***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 Chlorella* sp.**

The starting algal culture was collected from a public water pond in city of Tempe (Arizona) and algal isolates were isolated from the water sample by standard 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 strains 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% CO2) were subjected to fatty acid content analysis. Only algal strains processing high fatty acid content (any isolates that possess a total fatty acid content of 40% or greater) were subjected to further selection for tolerance to high CO_2 concentrations, a range of temperatures and/or various wastewaters. One of algal strains derived from this selection process was identified as a *Chlorella* sp. based upon morphological features, and was subjected to further analysis.

Effect of CO_2 concentration on growth and biomass productivity

The *Chlorella* sp. derived from the selection methods of the invention has the ability to grow at a high CO_2 concentration (i.e., 15% CO_2 or more) at a growth rate similar to that at 1% CO_2 commonly applied to algal cultures (Fig. 1). This CO_2 level is equivalent to that typically occurring in flue gases emitted from fossil fuel power plants. Biomass productivity of the *Chlorella* sp. culture grown at 15% CO_2 was 420 $\pm$ 50 mg l^{-1} d^{-1}, similar to or slightly higher than 350 $\pm$ 40 mg l^{-1} d^{-1} obtained from cultures grown at 1% CO_2 (Fig. 2).

Effect of CO_2 concentration on cellular lipid content and lipid production

There was little effect of CO_2 concentrations on cellular lipid (fatty acid) content or lipid production. 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 *Chlorella* sp. When *Chlorella* sp. cultures were maintained in the glass column bioreactors supplied with 1% or 15% CO_2 under given culture conditions, the cellular lipid content was 40.5+4 % of dry weight (Fig. 3a). Likewise, the volumetric production

of lipid was about 150 ± 12 mg l^{-1} d^{-1} when *Chlorella* sp. cultures were provided with either level of CO_2 (Fig. 3b).

Effect of wastewater concentration on growth and biomass productivity

The *Chlorella* sp. 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. 4 shows growth of *Chlorella* sp. maintained in various concentrations of dairy wastewater (i.e., 25%, 50%, 75%, and 100% wastewater). When *Chlorella* sp. cells were inoculated into 100% dairy wastewater, little growth occurred for the first six days. Thereafter, *Chlorella* sp. cells began growing rapidly and reached a maximum cell density of 4.2±0.3 g l^{-1} after five more days of cultivation. When dairy wastewater was diluted from 100% to 75% or then to 50%, the lag phase of the *Chlorella* sp. culture became shorter. However, further dilution of wastewater to 25% resulted in a decline in the maximum cell density. However, compared to BG-11 growth medium, dairy wastewater did cause minor growth reduction. Among the various dilutions, 50% wastewater led to the highest volumetric biomass productivity. The wastewater concentrations either lower or higher than 50% resulted in reduction in biomass yield (Fig. 5).

Effect of wastewater concentration on lipid content and lipid production

The concentration of dairy wastewater affected not only growth but also cellular lipid content. The highest lipid content was measured in cultures grown in 25% wastewater. As the wastewater concentration increased to 50% and to 75%, the cellular lipid content decreased gradually (Fig. 6). Like the trend observed in volumetric biomass productivity, 50% wastewater sustained the highest oil productivity and a dilution rate higher (e.g., 75% DWW) or lower (e.g., 25% DWW) than that resulted in decline in lipid production (Fig. 7).

***Fatty acid composition of Chlorella* sp.**

Table 2 shows the fatty acid composition of *Chlorella* sp. grown in BG-11 growth medium. C16 and C18 are the major fatty acids consisting of more than 96% of the total fatty acids in the cell.

Table 2. Fatty acid profile of *Chlorella* sp. C16:2 and C18:4 were tentatively identified; TFA content (%) = (TFA/cell dry weight) x 100%.

Fatty acids	***Chlorella* sp.** (% of total fatty acids)
C13:0	3.3
C14:0	0.2
C16:0	24.3
C16:1	1.9
C16:2	-
C18:0	1.5
C18:1	42.7
C18:2n-6	6.7
C18:3n-3	-
C18:3n-6	-
C18:4n-6	-
C20:0	-
C20:1	-
TFA (% dry weight)	41.3

DNA markers for identification of Chlorella sp.

A 1249-bp ITS segment was amplified from *Chlorella* sp. (SEQ ID NO:1), which consists of 3' end of 18S rDNA (1-501) (SEQ ID NO:11), ITS1 (502-739) (SEQ ID NO:3), 5.8S rDNA (740-898) (SEQ ID NO:12), ITS2 (899-1137) (SEQ ID NO:4) and 5'end of 28S rDNA (1138-1249) (SEQ ID NO:13). No identical nucleotide sequence was 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 region (SEQ ID NO:5). As shown in Fig 9, *Chlorella* sp. was located in a monophyletic clade with the other 8 *Chlorella* strains. In the phylogenetic tree, *Chlorella* sp. is the sister species of *Chlorella vulgaris* CBS 15-2075 with 80% sequence identity. Based upon the sequence blasting in NCBI, the maximum identity of ITS1 share by phylogenetically most closely related species is 71% and the maximum identity of ITS2 is 85% for Chlorella sp. (See sequence alignment in Figure 11) Therefore, the closely-related species to the newly isolated *Chlorella* strain are distinguishable at the fast-evolving DNA region.

The length of the *rbcL* segment amplified from *Chlorella* sp. is 1393bp (SEQ ID NO:2), and the sequence shows, based upon a BLAST searching in NCBI, 96% identity with a strain assigned as *Chlorella pyrenoidosa*. (Figure 12) Most mutations occurred at the third position of codons among these closely-related strains. In the phylogenetic tree reconstructed on 1160 base pairs of *rbcL* from 20 Chlorophyta taxa, *Chlorella* sp. (SEQ ID NO:6) is located in a monophyletic *Chlorella* clade, which is supported by the Bootstrap analysis and is congruent with the phylogenetic relationship based on the sequences of the ITS region. Thus, the *rbcL* region can be used to distinguish the *Chlorella* sp. of the present invention from closely related organisms.

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We claim:

1. An isolated *Chlorella* sp. composition, wherein the isolated *Chlorella* sp. genome comprises a nucleic acid sequence of SEQ ID NO:l, or complement thereof.

2. A substantially pure culture, comprising:

(a) a growth medium; and

(b) the isolated *Chlorella* sp. composition of claim 1.

3. An algal culture system, comprising:

(a) a photobioreactor; and

(b) the substantially pure culture of claim 2.

4. A method for lipid isolation, comprising culturing a *Chlorella* sp., wherein the *Chlorella* sp. genome comprises a nucleic acid sequence of SEQ ID NO:l, or complement thereof, wherein the culturing is done under conditions suitable for proliferation of the *Chlorella* sp., and extracting lipid from the *Chlorella* sp.

5. A method for removing nutrients from wastewater, comprising culturing a *Chlorella* sp., wherein the *Chlorella* sp. genome comprises a nucleic acid sequence of SEQ ID NO:l, or complement thereof, wherein the culturing is done under conditions suitable for proliferation of the *Chlorella* 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 *Chlorella* sp.

6. A method for removing nutrients from waste gas, comprising culturing a *Chlorella* sp., wherein the *Chlorella* sp. genome comprises a nucleic acid sequence of SEQ ID NO: 1, or complement thereof, wherein the culturing is done under conditions suitable for proliferation of the *Chlorella* 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 *Chlorella* sp.

7. The method of any one of claims 4-6, further comprising harvesting algal protein and/or biomass components from the cultured *Chlorella* sp.

8. A method for producing biomass, comprising culturing a *Chlorella* sp., wherein the *Chlorella* sp. genome comprises a nucleic acid sequence of SEQ ID NO:1, or complement thereof, wherein the culturing is carried out under conditions suitable for proliferation of the *Chlorella* sp., and harvesting algal protein and/or biomass components from the cultured *Chlorella* sp.