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(54) **METHOD FOR PRODUCING BIODIESEL
USING HIGH-CELL-DENSITY CULTIVATION
OF MICROALGA CHLORELLA
PROTOTHECOIDES IN BIOREACTOR**

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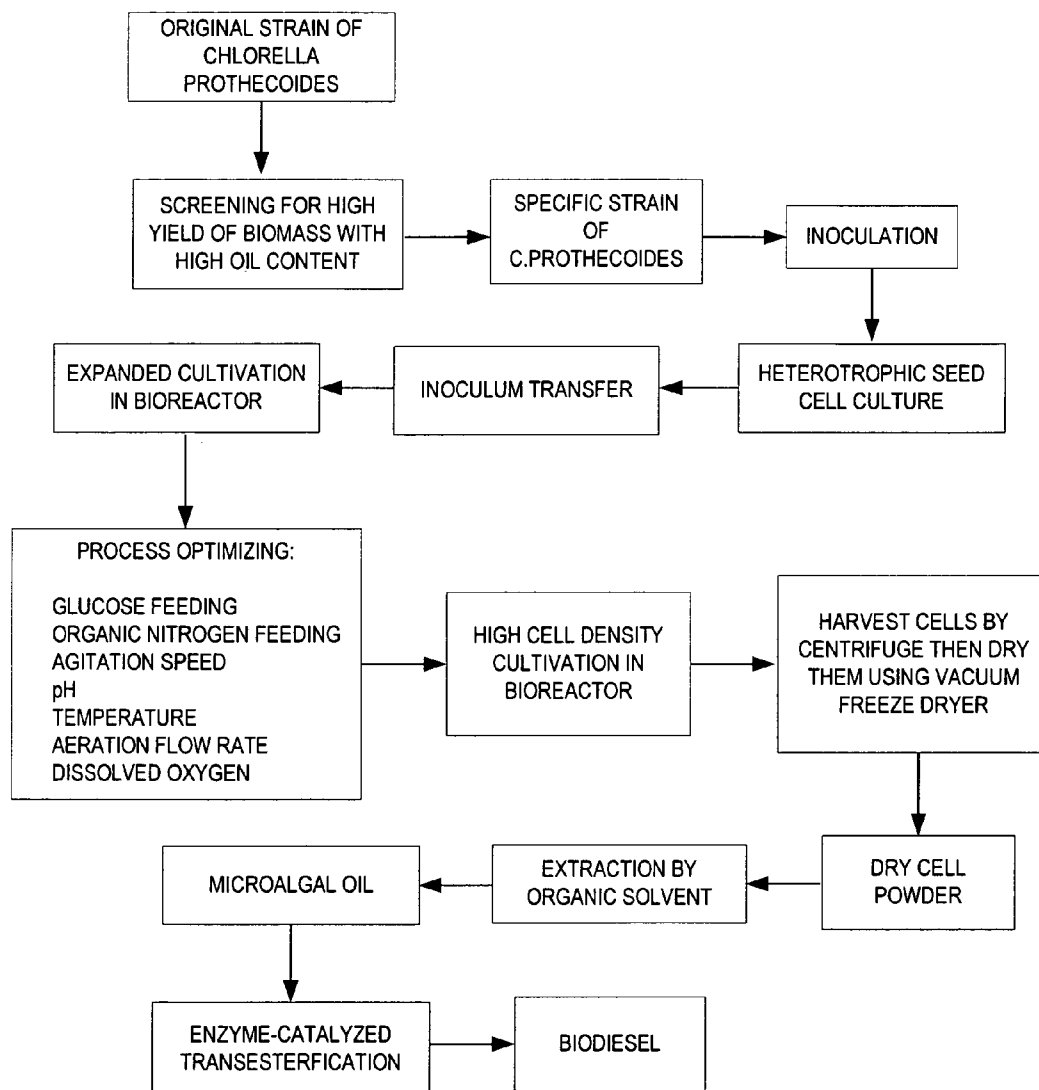
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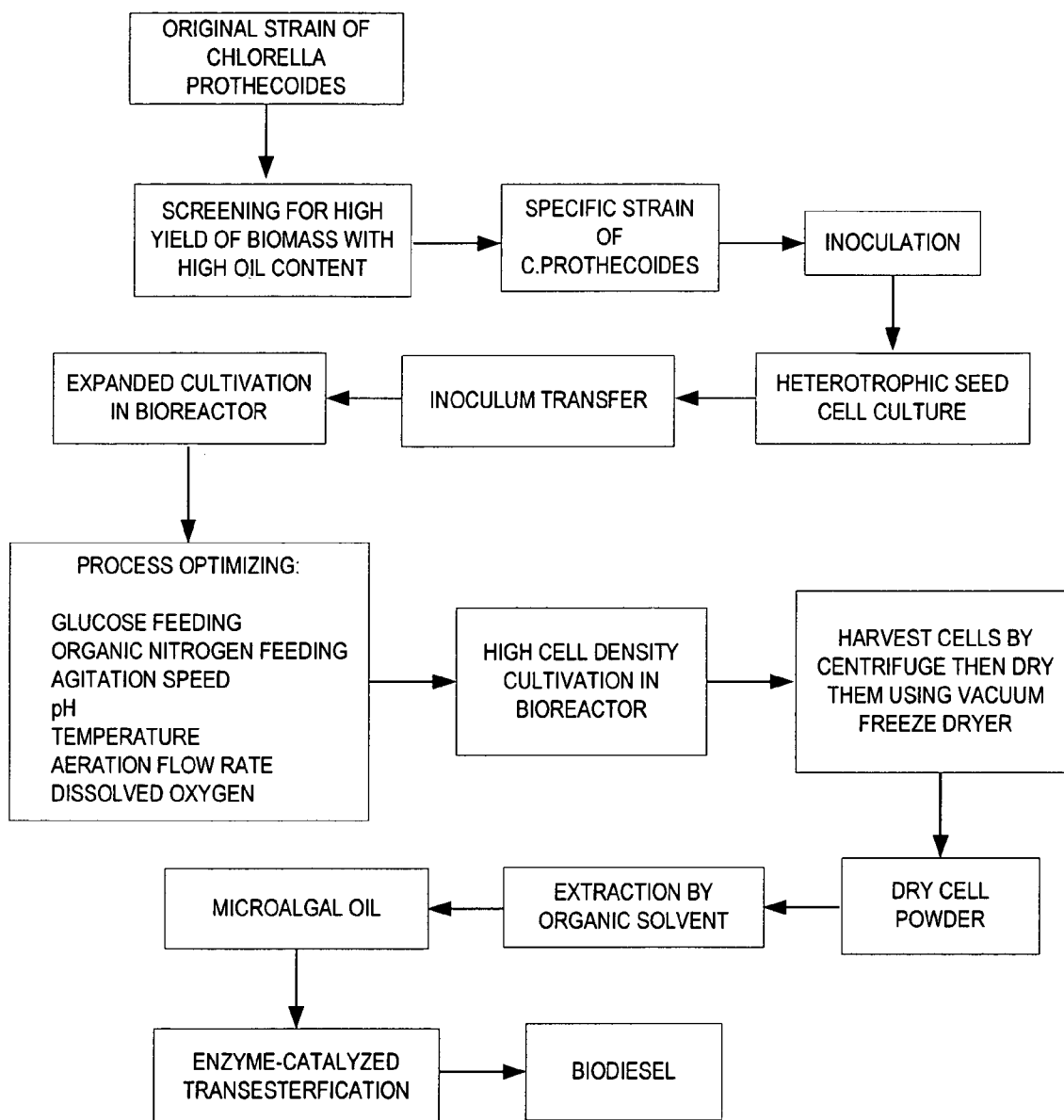
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C10L 1/18 (2006.01)(52) **U.S. Cl.** **44/308**(57) **ABSTRACT**

A method is provided to produce biodiesel from algae using a strain of microalga *chlorella protothecoids*, by screening a specific strain with characteristics of high yield of biomass and high oil content, cultivating the screened strain for high-cell-density growth for up to 108 grams of dry cell weight per liter of the suspension in a bioreactor using solutions containing carbohydrates as feed, harvesting and drying the high density cultivated algal cells to extract oil from the dried algal cells, and producing the biodiesel by reaction of catalyzed transesterification using the extracted oil as feedstock.

(76) Inventors: **Qingyu Wu**, Beijing (CN);
Wenguang Zhou, Beijing (CN);
Wei Xiong, Beijing (CN)

Correspondence Address:
Carlineo, Spicer & Kee, LLC
2003 S. Easton Road, Suite 208
Doylestown, PA 18901 (US)

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**Fig. 1**

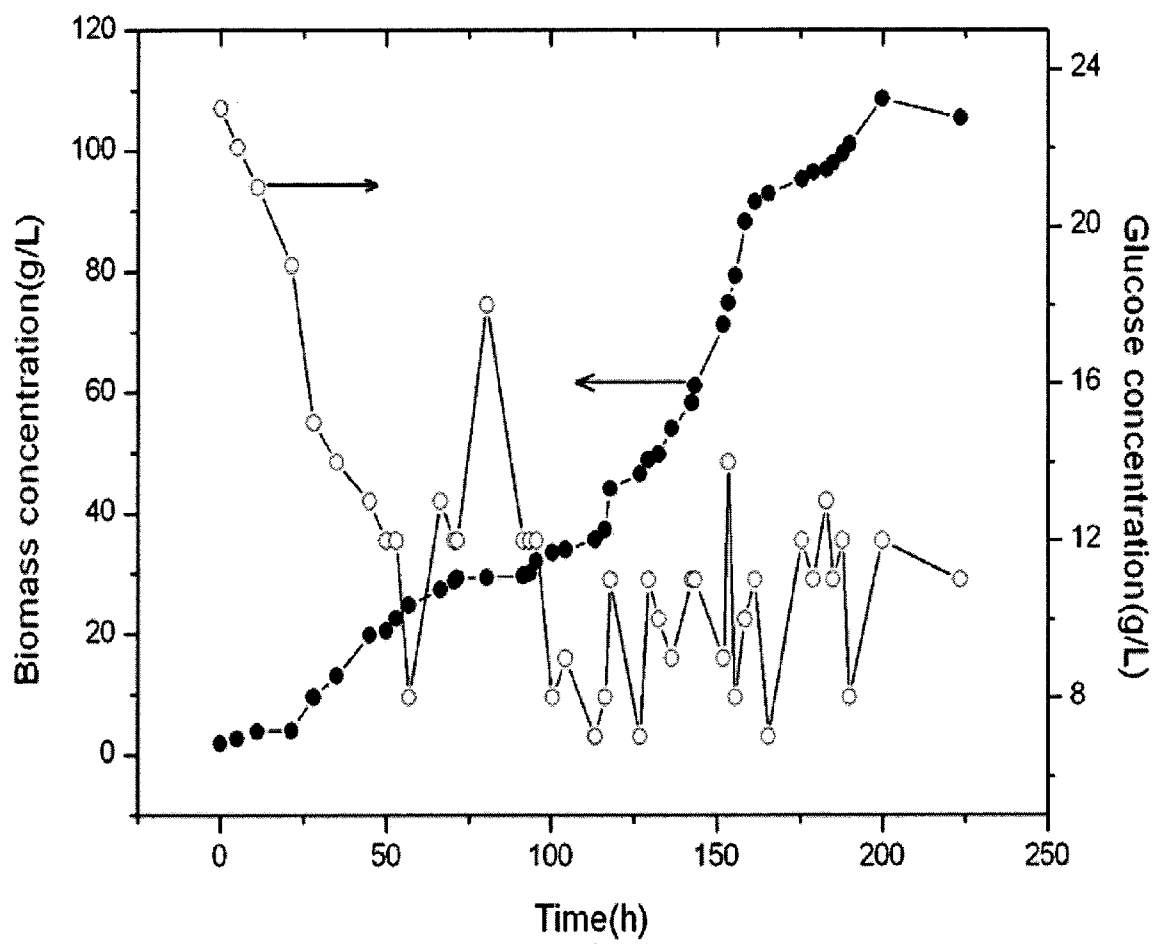
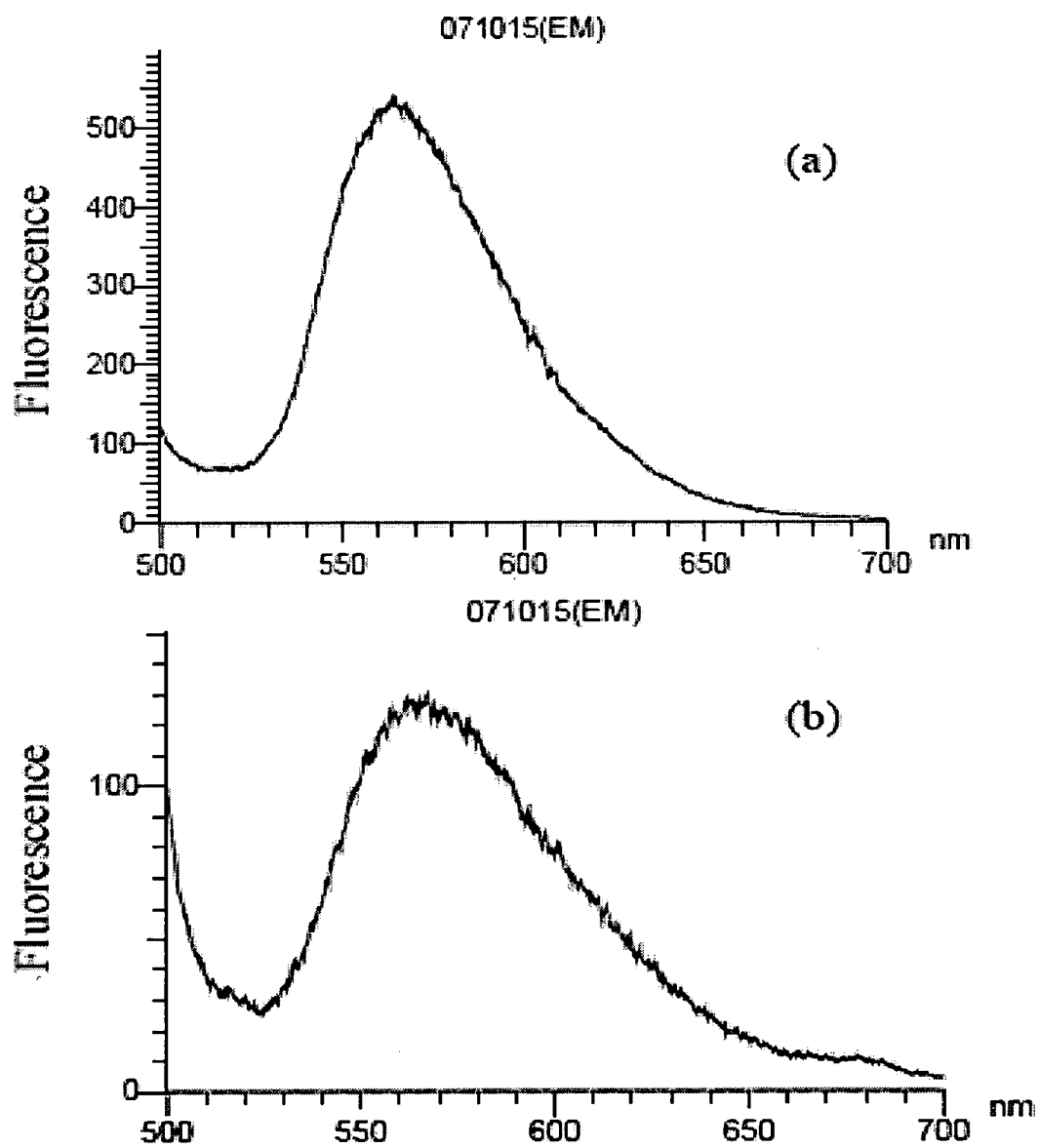
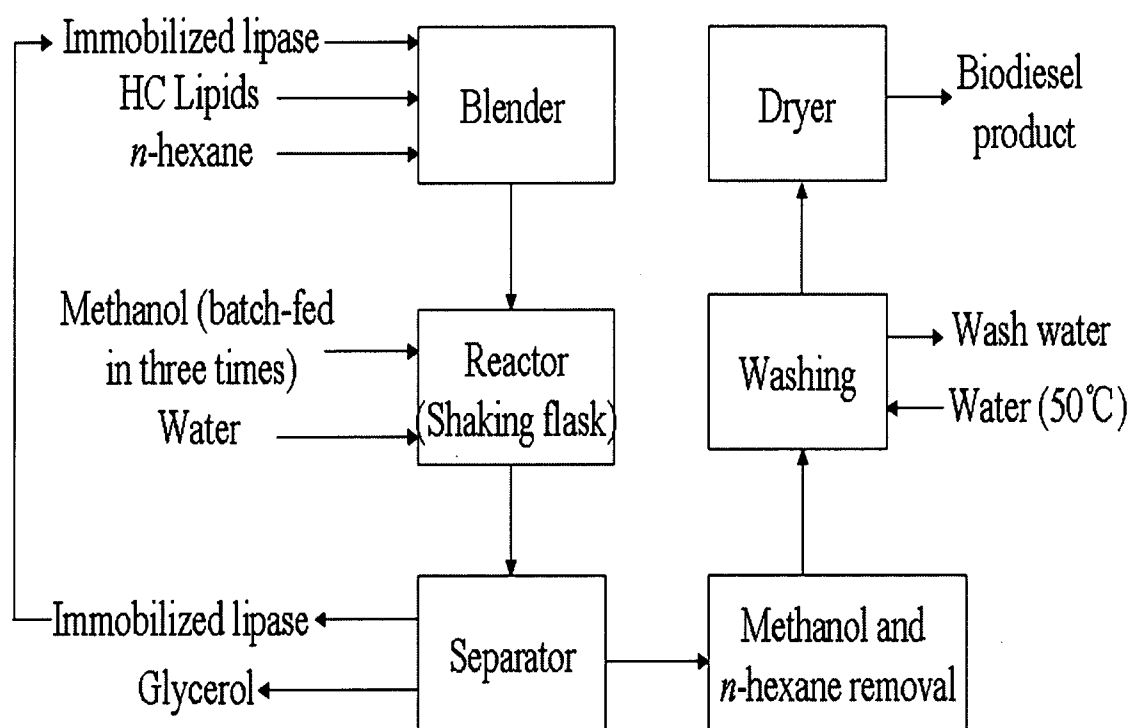
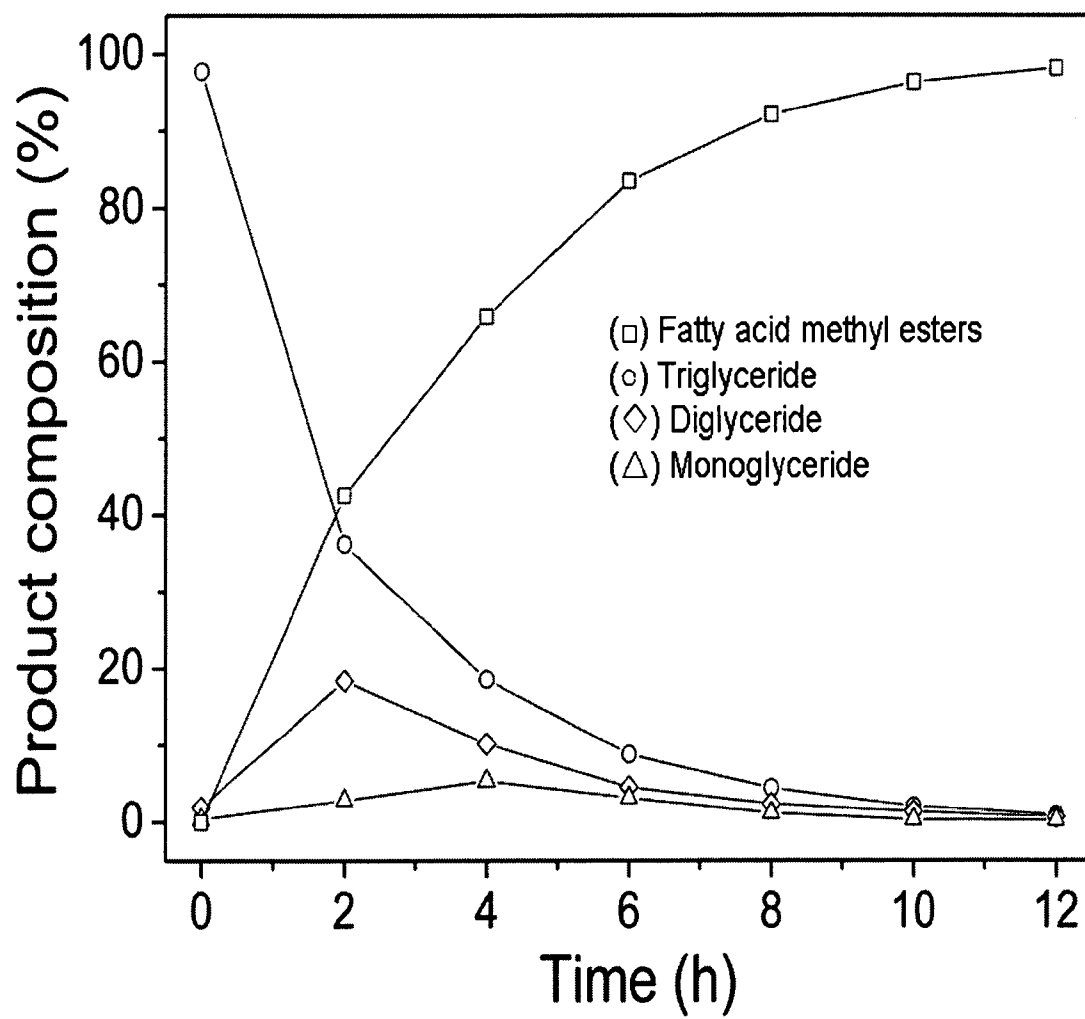


Fig. 2

**Fig. 3**

**Fig. 4**

**Fig. 5**

**METHOD FOR PRODUCING BIODIESEL
USING HIGH-CELL-DENSITY CULTIVATION
OF MICROALGA CHLORELLA
PROTOTHECOIDES IN BIOREACTOR**

**CROSS REFERENCE TO RELATED
APPLICATION**

[0001] This application claims the benefit of China Patent Application No. 200810100871.5, filed on Feb. 25, 2008, and entitled "Method for Producing Biodiesel using High-Cell-Density Cultivation Of Microalga *Chlorella Protothecoides*", the entire disclosure of which is incorporated herein by reference.

FIELD OF THE INVENTION

[0002] The present invention relates to area of regenerable biofuel, in particular to a method for producing biodiesel from microalga oil by high-cell-density heterotrophic cultivation of a specific strain of green alga *Chlorella protothecoides* rich in lipids coupled with biodiesel production by transesterification reaction.

BACKGROUND OF THE INVENTION

[0003] Renewable carbon-neutral fuels, such as biodiesel, are considered necessary replacement of traditional petroleum fuel for environmental and economic sustainability. Biodiesel is one such fuel composed of monoalkyl esters of long chain fatty acids transesterified from vegetable oil or animal fat. Biodiesel has been used in many areas, especially in the United States and Europe. The high cost of biodiesel, which is mainly due to the high cost of oil feedstock, is the main obstacle to its broader commercialization. Therefore, there have been many research efforts to minimize oil feedstock costs.

[0004] Currently, soybean, rapeseed and sunflower seeds along with several other oil/food crops are the major sources of feedstock for biodiesel production. Due to their low oil yield potential and high demands on land, water, and fertilizer, which create competition with food/feed industries, the conventional oil/food crop-based biodiesel production system cannot meet the growing demand on sustainable feedstock for biodiesel production.

[0005] Microalgae are a group of photosynthetic microorganisms. The concept of 'algae for fuels' has been evaluated over the past years. However, no technology of biodiesel production by microalgae is economically feasible because rapidly growing cells contain low amounts of lipids and algal cells accumulating high lipid content exhibit little growth.

[0006] Wu and his coworkers first reported bio-oil and biodiesel production from the green microalga by means of heterotrophic cultivation of *C. protothecoides*. However, both microalgal cell yield and oil content in harvested dry cells are not high enough for its industrial application.

[0007] Therefore, there is a need to further increase the biomass, such as a process for high-cell-density cultivation of microalga with high oil content, to make industrial application feasible.

SUMMARY OF THE INVENTION

[0008] The present invention provides a novel method to produce oil feedstock for biodiesel production from the heterotrophic microalga *C. Protothecoides* by high-cell-density cultivation in a bioreactor. The present invention comprises

the steps of screening a specific strain with characteristics of high yield of biomass and high oil content; inoculating the strain in a first bioreactor for algal-seed-cells cultivating; feeding a first solution containing carbohydrates into the first bioreactor; transferring the cultivated algal-seed-cells into a second bioreactor for high-cell-density cultivation; feeding a second solution containing carbohydrates into the second bioreactor; harvesting the high density cultivated algal cells from the second bioreactor; drying the high density cultivated algal cells; extracting oil from dried algal cells; and producing the biodiesel by reaction of transesterification using the extracted oil as feedstock.

[0009] The high-cell-density cultivation of the present invention further comprises heterotrophic cultivation of *C. Protothecoides* with high oil content, preferably between 20% to 61% dry cell weight.

[0010] The present invention further comprises a screening step using centrifuge to select cells with high oil content. The cells in the supernatant (upper layer) of centrifuge tubes, which have high oil content and low relative gravity, are inoculated and spread in agar plates to form a single algal colony. The larger colonies with higher cell dividing rate are selectively picked up and inoculated into a flask containing growth medium, including but not limiting to components of basal medium, for further culture.

[0011] The cultivated cell density in said first bioreactor or said second bioreactor of the present invention is controlled between 15 to 108 (dry weight) g/L.

[0012] The screening step of the present invention further comprises to select a large single algal colony with a high growth rate from agar plates.

[0013] The present invention comprises a specific strain of microalga *C. protothecoides*, *Chlorella. Protothecoides* sp 0710, with characteristics of high growth rate and high yielding oil.

[0014] The present invention further comprises a batch feeding operation for feeding the first solution containing carbohydrates into the first bioreactor.

[0015] The present invention further comprises a continuous feeding operation for feeding the second solution containing carbohydrates into the second bioreactor.

[0016] The carbohydrates solutions include but not limit to glucose or other monosaccharides, and/or disaccharides, or polysaccharides, preferably with the concentration of glucose or other monosaccharides, disaccharides, polysaccharides, or hydrolysates of corn starch, wheat flour, cassaya starch and tapioca starch, or juices of sorghum or broomcorn, in the first or the second bioreactor, preferably with concentration of the carbohydrates solutions being controlled between 0.01 and 100 gL⁻¹.

[0017] In a preferred embodiment of the present invention, the amount of inoculum in the first bioreactor or the second bioreactor is controlled between 0.01% and 50% by volume.

[0018] Another preferred embodiment of the present invention further comprises agitating the medium in the first or the second reactor, preferably with a speed of agitation maintained between 5 and 1000 rpm.

[0019] Yet another preferred embodiment of the present invention further comprises aerating in the first or the second bioreactor, wherein the dissolved oxygen in cultivation medium in the first or the second bioreactor is maintained at least 5% of saturation, preferably at least 20% of saturation.

[0020] Another preferred embodiment of the present invention further comprises feeding organic nitrogen into the first

and the second bioreactor, wherein the organic nitrogen includes but not limit to glycine, yeast powder, yeast extract, or corn syrup.

[0021] In a preferred embodiment of the present invention, pH value in the first or the second bioreactor is preferably maintained between 6.0 and 8.0.

[0022] Yet another preferred embodiment of the present invention further comprises adding a base to reduce acidity and regulate the pH of the culture medium in the first or the second bioreactor, preferably KOH or other bases.

[0023] In a preferred embodiment of the present invention, the temperature in the first or the second bioreactor is preferably controlled between 20° C. to 45° C.

[0024] Yet in another preferred embodiment of the present invention, the cell density of *C. protothecoides* in the second bioreactor is preferably between 20 to 108 g (dry weight)/L with the oil content preferably between 40% to 61% of dry cell weight.

[0025] The present invention further comprises a process of enzyme-catalyzed transesterification, preferably using catalyzed by immobilized lipase from *candida* sp. 99-125. The enzyme-catalyzed transesterification further comprises adding said lipase and methanol into said feedstock.

[0026] Other objects, advantages, and features of the present invention will become apparent from the following detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0027] The following detailed description will be better understood when read in conjunction with the appended drawings, in which there is shown one or more of the multiple embodiments of the present invention. It should be understood, however, that the various embodiments of the present invention are not limited to the precise arrangements and instrumentalities shown in the drawings.

[0028] In the Drawings:

[0029] FIG. 1 is a process flow schematic for biodiesel production from high-cell-density heterotrophic cultivation of microalga *C. protothecoides*.

[0030] FIG. 2 is a graph of cell growth and glucose consumption in high-cell-density heterotrophic cultivation of *C. protothecoides* in a 5 L bioreactor.

[0031] FIG. 3 shows the relative fluorescence peak in Nile Red dyed cells under fluorescence microscope. Relative fluorescence in Nile Red dyed cells under fluorescence: (a) Test sample from high-cell-density heterotrophic cultivation of the specific strain of *C. protothecoides*; (b) Control sample from former heterotrophic cultivation of the original strain.

[0032] FIG. 4 is a process flow schematic for biodiesel production by immobilized lipase.

[0033] FIG. 5 shows product composition for transesterification during the reaction time.

DEFINITIONS

[0034] Unless otherwise stated, the following definitions provide meanings and examples to terms used herein. Such definitions are also intended to encompass any meaning that may be contemplated or could be appreciated by a person of ordinary skill with the art.

[0035] Biodiesel: refers to a diesel-equivalent processed fuel derived from biological sources (such as vegetable oils, animal fats or microbial lipids) which can be used in unmodified diesel-engine vehicles.

[0036] The terms “mono-alkyl ester” or “monoester” and derivations thereof including, for example, mono-alkyl esters, monoesters or esters generally refer to a type of fatty acid ester biodiesel or biodiesel fuel. For example, a fatty acid mono-alkyl ester biodiesel comprises fatty acids such as oleic, stearic, linoleic or palmitic acids as well as any sort of combinations of these fatty acids.

[0037] Alga, algae, algal: Any of various chiefly aquatic, eukaryotic, photosynthetic organisms, ranging in size from single-celled forms to the giant kelp. Algae are once considered to be plants but are now classified separately because they lack true roots, stems, leaves, and embryos.

[0038] Microalgae: Unicellular aquatic algae.

[0039] Heterotrophic: requiring organic compounds of carbon and nitrogen for nourishment.

[0040] Transesterification: a process of exchanging the alkoxy group of an ester compound by another alcohol.

[0041] Monoalkyl esters of long chain fatty acids, fatty acid methyl esters: a class of organic compounds formed from methanol and fatty acid by removal of water.

[0042] Bioreactor: a vessel or instrument including fermenter for cell growth or a chemical process involving organisms or biochemically active substances derived from such organisms.

[0043] Heterotrophic cultivation or fermentation: both of terms refer to highly oxygenated and aerobic growth of microbial cells through consuming glucose or other organic carbon sources.

[0044] Yeast fermentation: refers to the conversion process of sugar to alcohol using yeast under anaerobic conditions.

[0045] Fuel ethanol: an alcohol fuel that's derived mainly from grain through the process of microbial fermentation.

[0046] Oil, lipid: a class of hydrophobic, naturally-occurring molecules, which is characterized as their long hydrocarbon chain structure. Here, both oil and lipid are used to refer to fatty-acids and their derivatives (including tri-, di-, and monoglycerides and phospholipids) as well as other fat-soluble sterol-containing metabolites such as cholesterol.

[0047] Nile Red: a lipophilic dye which is intensely fluorescent with a strong yellow-gold emission when in a lipid-rich environment.

[0048] Pyrolysis: is the chemical decomposition of organic materials by heating in the absence of oxygen or any other reagents except possibly steam.

[0049] Single algal colony: a single cluster of alga in agar plate culture that is produced from a single starting algal cell.

[0050] Incubator: An apparatus in which environmental conditions, such as temperature and humidity, can be controlled, used for growing microbial cultures or providing suitable conditions for a chemical or biological reaction.

[0051] Organic carbon: a class of carbon compounds that had its origin in living material such as sugar or lipid. It is distinguished from carbon dioxide which belongs to inorganic carbon.

[0052] Organic nitrogen: We use the term “organic nitrogen” to describe a nitrogen compound that had its origin in living material such as the nitrogen in protein and urea.

[0053] Broomcorn: A variety of sorghum (*Sorghum bicolor*) having a stiff, erect, much-branched flower cluster, the stalks of which are often used to make brooms.

[0054] Sweet broomcorn: refers to the broomcorn which has sweet juicy stems. In this patent, sweet broomcorn is processed by pressing the juice out of the stems and used as feedstock for algae cultivation.

[0055] Cassaya: Cassaya is a shrubby, tropical, perennial plant, whose roots are high in starch.

[0056] Soxhlet extractor: A fast semi-automated solvent extraction unit for separating lipid from algal cells.

[0057] Heating value: Amount of heat generated by a given mass of fuel when it is completely burned.

[0058] Monosaccharide: a simple sugar such as glucose, fructose, xylose et al., having the general formula $C_nH_{2n}O_n$, that cannot be decomposed by hydrolysis.

[0059] Disaccharide: molecule formed by two simple sugar units linked together.

[0060] Polysaccharide: molecule formed by more than two simple sugar units linked together.

[0061] Hydrolysate: any compound produced by hydrolysis, the process of decompose a chemical compound by reaction with water. Herein, refers to the catalytic conversion of starch, cellulose or other polysaccharides to glucose or other mono-saccharides.

DETAILED DESCRIPTION

[0062] Certain terminology is used herein for convenience only and is not to be taken as a limitation on the embodiments of the present invention. It should be appreciated that the particular embodiments shown and described herein are examples of the invention and are not intended to otherwise limit the scope of the present invention in any way.

[0063] One objective of the present invention is to provide a novel method of biodiesel production from heterotrophic microalga *C. protothecoides* by high-cell-density cultivation in bioreactor.

[0064] Referring generally to FIG. 1, a preferred embodiment of the present invention is illustrated:

(1) Screening a Specific Strain with Characteristics of High-Cell-Density Growth Rate and High Oil Content

[0065] Microalgal strain of *C. protothecoides*, originally obtained from the Culture Collection of Alga at the University of Texas (Austin, Tex.), is screened for heterotrophic growth. The resulted heterotrophic cultured cells are centrifuged twice in different centrifuge rate, preferably at 8000-12000 g (earth gravity) for 30 min at 4° C. The cells in the supernatant (upper layer) of centrifuge tubes, which have high oil content, are inoculated and spread in agar plates to form a single algal colony, preferably at a temperature of $28 \pm 0.5^\circ$ C. for 240 to 360 hours. Then larger colonies are selectively picked up and inoculated into flasks containing growth medium, including but not limiting to components of basal medium, for further culture. Glucose with a concentration of 30 g/L and yeast extract with a concentration of 4 g/L are added into the basal medium. The cell growth rates and cellular oil contents in different culture are then compared with each other to determine a specific strain with characteristics of the highest oil content and a high cell growth rate.

(2) Inoculating the Specific Strain into a First Bioreactor for Algal-Seed-Cells Cultivation

[0066] The above specific strain of *C. protothecoides* is inoculated into a shaking flask, containing culture medium for algal-seed-cells cultivation, which serves as the first bioreactor, in an incubator. The components of basal culture medium are:

[0067] KH_2PO_4 : 0.7 g/L,

[0068] K_2HPO_4 : 0.3 g/L,

[0069] $MgSO_4 \cdot 7H_2O$: 0.3 g/L,

[0070] $FeSO_4 \cdot 7H_2O$: 3 mg/L,

[0071] Glycine: 0.1 g/L,

[0072] vitamin B_1 : 0.01 mg/L,

[0073] A5 trace mineral solution 1.0 ml/L, wherein the A5 trace mineral solution comprises H_3BO_3 , $Na_2MoO_4 \cdot 2H_2O$, $ZnSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, and $CuSO_4 \cdot 5H_2O$.

[0074] A preferred A5 trace mineral solution comprises:

[0075] H_3BO_3 : 2.86 g/L,

[0076] $Na_2MoO_4 \cdot 2H_2O$: 0.039 g/L,

[0077] $ZnSO_4 \cdot 7H_2O$: 0.222 g/L,

[0078] $MnCl_2 \cdot 4H_2O$: 1.81 g/L,

[0079] $CuSO_4 \cdot 5H_2O$: 0.074 g/L.

[0080] Algal cell yield can be determined using various methods, including but not limiting to light intensity measurement of the cell suspension, such as OD540 nm of cell suspension. Preferable conditions such as glucose concentration, different nitrogen sources in the basal medium, temperatures, and flask shaking rate during algal-seed-cells cultivation in shaking flasks are determined by real-time light intensity measurement of the cell suspension.

[0081] A preferable glucose concentration in a range of 5 to 30 g/L glucose is added in the basal medium.

[0082] A preferable yeast extract in a range of 1 to 10 g/L yeast extract is added in the basal medium.

[0083] A temperature in incubator is set between 20-45° C., preferably at 28° C.

[0084] The flask shaking rate is controlled between 50 to 300 rpm, preferably at 200 rpm.

[0085] A weak light at $5 \text{ mol m}^{-2} \text{ s}^{-1}$ is also adopted.

[0086] The cells are harvested till the culture of algal seed cell enters into late-exponential-phase. The cell harvesting time before reaching the late-exponential-phase is approximately at 168 hours.

(3) Transferring the Algal-Seed-Cells from the First Bioreactor to a Second Bioreactor for High-Cell-Density Cultivation by Process Control and Optimization

[0087] The late-exponential-phase algal-seed-cells in the flask are transferred to a second bioreactor, which is larger than the shaking flask, preferably between 5 L to 11,000 L, for high-cell-density cultivation by process control and optimization.

[0088] Glucose and yeast extract solutions are added into the basal culture medium initiate in the second bioreactor, preferably with 5 to 50 g/L glucose and 1 to 10 g/L yeast extract, further preferably with 30 g/L glucose and 4 g/L yeast extract.

[0089] Parameters, such as the amount of inoculum, substrate (organic carbon and nitrogen) feeding, oxygen supply, stirring rate, temperature, pH, and time of cell harvest, and adjusted to optimize cell growths in the second bioreactor.

[0090] Among the parameters, dissolved oxygen (DO) in the fermentation suspension for high-cell-density cultivation of heterotrophic algal cells in the bioreactor can be used to monitor the growth conditions, such as organic carbon sources in the reactor, biomass and accumulation of lipids. On-line monitoring of the DO parameter is preferably used to monitor the growth conditions, and to adjust agitation speed and aerating rate.

[0091] The glucose added into the basal culture medium can be substituted by fructose, sucrose, corn powder hydrolysate, cassaya powder hydrolysate, wheat starch hydrolysate, juice of broomcorn, juice of sugar cane and waste water containing sugar from the food and beverage industry.

[0092] The conditions for high-cell-density heterotrophic cultivation of the specific strain of *C. protothecoides* are automatically monitored and set as follows:

- [0093] inoculum amount of seed algal cells (V/V): 20-30%, preferably of 25%;
- [0094] temperature at 20-45° C., preferably at 28±0.5° C.;
- [0095] aerobic rate at 100-200 L/h (1:1vvm), preferably at 180 L/h;
- [0096] pH 6.0 to 8.0, preferably at 6.3±0.1;
- [0097] concentration of glucose in medium: 5 to 18 g/L;
- [0098] continuous feeding organic nitrogen (yeast extract) at 0.5 to 1.0 g/L/h, preferably at 0.75 g/L/h;
- [0099] DO over 20% controlled by increasing agitation and airflow, gradually increasing agitation speed from 100 to 600 rpm after a period of cultivation for about 88 hours, to maintain the dissolved oxygen at above 20% of saturation;
- [0100] Continuous feeding both organic nitrogen and glucose at the same time after about 128 hours of cultivation.
- [0101] When the cell density of *C. protothecoides* and/or the oil content reach desired values, preferably with the dry cell density reaching 108 g/L and the oil content reaching 61% of dry cell weight, the growth of the cells in the second reactor is terminated. The growth duration in said second bioreactor lasts about 213 hours.
- (4) Harvesting the High Density Microalgal Cells from Bioreactor
- [0102] After determining a sample of the high-cell-density heterotrophic cultivation to reach a desired dry biomass concentration, preferable between 20 to 108 g/L, dry biomass of the algal cell suspension from bioreactor is separated using a separation process, including but not limiting to filtration or centrifuge. The separated dry biomass may be in a form of powder or other solid forms.
- (5) Extracting the Oil from Dried Algal Cells
- [0103] Lipids (oil) in heterotrophic *C. protothecoides* cell powder are subsequently extracted. Techniques for extraction comprises the Soxhlet method, wherein N-hexane is used as the standard Soxhlet solvent for extracting oil from heterotrophic cells of *C. protothecoides*. Extraction is achieved by washing the cells repeatedly with pure solvent until no lipid is left in cells. Then the solvent in the extract is removed under reduced pressure.
- (6) Producing the Biodiesel by Transesterification Reaction Using the Microalgal Oil as Feedstock
- [0104] The heterotrophic algal fermentation based biodiesel is produced from the feedstock of microalgal oil by transesterification reaction. Transesterification can be catalyzed using strong acids, such as sulfate acid as a catalyst, or a lipase as a catalyst. A lipase extracted from *Candidia* sp. 99-125, triacylglycerol acylhydrolase, EC 3.1.1.3, is a preferred catalyst. Catalyst activity of the triacylglycerol acylhydrolase is about 12,000 U/g. Preferable conditions for transesterification are: pH value of 7.0, 60% immobilized lipase (w/w, 12,000 U/g) relative to the microalgal oil mass, 10% water content (w/w) relative to the microalgal oil mass, reaction temperature 38° C., 160 rpm, adding methanol to the microalgal oil three times to reach 3:1 methanol to the microalgal oil mass ratio.
- [0105] The process flow schematic for enzymatic biodiesel production is shown in FIG. 4.

EXAMPLE

Materials and Methods

- [0106] The original strain of *C. protothecoides* came from the Culture Collection of Alga at the University of Texas

(Austin, Tex.), which has been kept in Department of Biological Science and Biotechnology in Tsinghua University since 1990. The compositions of the culture medium for this strain are:

- [0107] KH_2PO_4 : 0.7 g/L,
 [0108] K_2HPO_4 : 0.3 g/L,
 [0109] $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$: 0.3 g/L,
 [0110] $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$: 3 mg/L,
 [0111] Glycine: 0.1 g/L,
 [0112] vitamin B_1 : 0.01 mg/L,
 [0113] A5 trace mineral solution 1.0 ml/L.

[0114] By oriented selection and screening as described in step (1) above, the specific strain with characteristics of high cell growth rate and high oil content is obtained as *Chlorella. Protothecoides* sp 0710. Comparing with the original cells, the *Chlorella. Protothecoides* sp 0710 is in golden color, cell dry weight approximately 108 g/l with oil/lip content at about 61% of cell dry weight. Table 1 illustrates compositions of biodiesel produced by the *Chlorella. Protothecoides* sp 0710 and the original *Chlorella. Protothecoides* cells.

[0115] The selected strain is inoculated in a first bioreactor, comprising 500 ml aeration flasks, for algal-seed-cells cultivating at 28° C. and 200 rpm in batch cultures. 30 g/L glucose and 4 g/L yeast extract are added in the basal medium. After 168 hours of cultivation, cell density reaches about 15 g/L and cell growth reaches an end of exponential-phase grow. Weak light (5 $\mu\text{mol}/\text{m}^2\text{s}$) is adopted during cultivation.

[0116] High-cell-density growth is performed in a 5 L stirred bioreactor (MINIFORS, Switzerland) with turbine impellers. The *C. Protothecoides* cell in exponential phase is inoculated in the basal medium with 30 g/L glucose and 4 g/L yeast extract and an inoculation ratio of 25% (V/V). The conditions for high-cell-density heterotrophic cultivation of our specific strain *C. protothecoides* are set as follows: temperature at 28±0.5° C., aerobic rate at 180 L/h (1:1vvm), pH 6.30±0.1, 25% inoculum amount of seed algal cells (V/V), concentration of glucose controlled between 5 and 18 g/L, and dissolved oxygen (DO) over 20% controlled by increasing agitation and airflow, with feeding organic nitrogen (yeast extract) at 0.75 g/L/h and glucose in the same time after 128 h cultivation. The strategy of high-cell-density cultivation is set up through substrate feeding and process control in the 5 L bioreactor. The consumptions of glucose, glycine, phosphate, and the changes of pH and DO during the growth of *C. Protothecoides* are also monitored in bioreactor. After fermentation about 213 hours, dry cell density reaches 108 g/l, as illustrated in FIG. 2. The cells are harvested by centrifugation and dried by vacuum freeze dryer.

[0117] Cell growth is measured by the absorbance of the suspension at 540 nm. Biomass concentration is calculated from OD540 by a regression equation calculated from our experiment: $y=0.4155x$, ($R^2=0.9933$, $P<0.05$), where y (g/L) is the cell concentration, x is the absorbance of the suspension at 540 nm.

[0118] After finishing high-cell-density heterotrophic cultivation of specific strain of *C. protothecoides*, the algal cell suspension from bioreactor is centrifuged at 10,000 rpm, 4° C. for 2 min and the cell pellets are collected for vacuum-freeze drying. The dry biomass of 108 g/L is harvested in high-cell-density heterotrophic cultivation of specific strain of *C. protothecoides* (FIG. 2).

[0119] The Soxhlet method is adopted to extract total lipids in the dried *C. Protothecoides* cells. N-hexane is used as the standard Soxhlet solvent. Extraction is achieved by washing the sample repeatedly with pure solvent.

[0120] Molecular weights of oil of the *C. protothecoides* are calculated from saponification and the acid values determined using the formula: $M=168,300 (SV-AV)-1$, where M is molecular weight of lipids, SV is the saponification value, and AV is the acid value.

[0121] The oil extracts by the Soxhlet method are dried by distillation under a reduced pressure. The oil content is about 61% of cells dry weight.

[0122] The transesterification is catalyzed by immobilized lipase from *Candidia* sp. 99-125 in shaker flasks on a constant temperature shaker, with the rotation rate of 180 rpm. The reaction mixture is left to separate into two layers in a separating funnel after reaction. The upper layer (biodiesel) is separated and washed with hot water (50° C.). Biodiesel product is obtained after the solvent is evaporated.

[0123] Variables are studied in order to determine the operation conditions that could maximize the yield of biodiesel and fulfill the specifications of the US Standard for Biodiesel (ASTM 6751) related to mono, di, and triglycerides, methanol, free glycerol, and fatty acids contents. The variables are the amount of immobilized lipase related to the quantity of lipids, the quantity of methanol (methanol to lipids), the feeding frequency of methanol, the quantity of organic solvents, the water content in reaction mixtures, reaction temperature, pH value, and reaction time. The conversion rate of lipids to biodiesel was estimated by gas chromatography and mass spectrometry (GC-MS), through which the relative contents of fatty acid esters, free fatty acids, mono, di, and triglycerides could be determined. A DSC GC system (Thermo, USA, VARIAN VF-5 ms, capillary cylinder 30 m×0.25 mm) is used for the chromatography work and data analysis. The GC was operated in constant flow mode with a flow rate of 10 mL/min. The oven is initially held at 70° C. for 2 min and then elevated to 300° C. at a rate of 10° C./min, and held at this temperature for 20 min. A split injector is used with a split ratio of 30:1 and a temperature of 250° C.

[0124] The high-cell-density cultivation of heterotrophic cultivation for 213 hours under above conditions, the cell density of *C. protothecoides* reached up to 108 g/L, and the oil content reached 61% of dry cell weight. During transesterification, 98.15% of the oil is converted to monoalkyl esters of fatty acids (biodiesel) in 12 hours. The biodiesel production rate is 64.66 g/L in bioreactors. Nine kinds of biodiesels are detected. Oleic acid methyl ester, linoleic acid methyl ester and cetane acid methyl ester are the major components, over 80% of the total content. Main results are illustrated in Table 1.

TABLE 1

Components	Relative fatty acid methyl ester content (%)		
	Sample from seed cell in flask	Sample from former cultivation in bioreactor	Sample from high-cell-density cultivation in bioreactor using specific strain screened
Methyl tetradecanoate (C ₁₅ H ₃₀ O ₂)	0.27	1.31	2.45
Hexadecanoic acid methyl ester (C ₁₇ H ₃₄ O ₂)	13.42	12.94	16.46
Heptadecanoic acid methyl ester (C ₁₈ H ₃₆ O ₂)	2.08	0.89	1.75

TABLE 1-continued

Components	Relative fatty acid methyl ester content (%)		
	Sample from seed cell in flask	Sample from former cultivation in bioreactor	Sample from high-cell-density cultivation in bioreactor using specific strain screened
9,12-Octadecadienoic acid methyl ester (C ₁₉ H ₃₄ O ₂)	19.86	17.28	16.24
9-Octadecenoic acid methyl ester (C ₁₉ H ₃₆ O ₂)	58.94	60.84	53.34
Octadecanoic acid methyl ester (C ₁₉ H ₃₈ O ₂)	3.4	2.76	4.98
10-Nonadecenoic acid methyl ester (C ₂₀ H ₃₈ O ₂)	1.41	0.36	2.48
11-Eicosenoic acid methyl ester (C ₂₁ H ₄₀ O ₂)	—	0.42	1.26
Eicosanoic acid methyl ester (C ₂₁ H ₄₂ O ₂)	0.62	0.35	1.04

Components and Relative Content of Biodiesel from Heterotrophic *C. protothecoides* Lipids

[0125] While specific embodiments have been described in detail in the foregoing detailed description and illustrated in the accompanying drawings, it will be appreciated by those skilled in the art that various modifications and alternatives to those details could be developed in light of the overall teachings of the disclosure and the broad inventive concepts thereof. It is understood, therefore, that the scope of the present invention is not limited to the particular examples and implementations disclosed herein, but is intended to cover modifications within the spirit and scope thereof as defined by the appended claims and any and all equivalents thereof.

What is claimed is:

1. A method for producing biodiesel from algae using microalga *Chlorella Protothecoides* in bioreactors, comprising the steps of:

screening a specific strain of said *Chlorella Protothecoides* with characteristics of high yield of biomass and high oil content;
inoculating the strain in a first bioreactor for algal-seed-cells cultivating;
feeding a first solution containing carbohydrates into the first bioreactor;
transferring the cultivated algal-seed-cells into a second bioreactor for high-cell-density cultivation;
feeding a second solution containing carbohydrates into the second bioreactor;
harvesting the high density cultivated algal cells from the second bioreactor;
drying the high density cultivated algal cells;
extracting oil from dried algal cells; and
producing the biodiesel by reaction of transesterification using the extracted oil as feedstock.

2. The method of claim 1, wherein said high-cell-density is between 15 to 108 g/L.

3. The method of claim 1, wherein the high-cell-density cultivation further comprises heterotrophic cultivation of *C. Protothecoides* with a high oil content 40-61% dry cell weight.

4. The method of claim 1, wherein the screening step further comprises selecting cells with lower gravity and/or higher oil content using a centrifuge.

5. The method of claim 1, wherein said feeding the first solution containing carbohydrates into the first bioreactor and/or said feeding the second solution containing carbohydrates into the second bioreactor further comprises a batch feeding operation.

6. The method of claim 1, wherein said feeding the first solution containing carbohydrates into the first bioreactor and/or said feeding the second solution containing carbohydrates into the second bioreactor further comprises a continuous feeding operation.

7. The method of claim 1, wherein the first or the second sugar solution further comprises at least one of glucose or other monosaccharides, disaccharides, and polysaccharides.

8. The method of claim 7, wherein the concentration of said at least one of glucose or other monosaccharides, disaccharides, and polysaccharides, in the first or the second bioreactor is controlled between 0.01 and 100 g/L.

9. The method of claim 1, wherein the amount of inoculum in the first bioreactor or the second is between 0.01% and 50% by volume.

10. The method of claim 1, further comprising agitating the medium in the first or the second reactor with a preferable speed of agitation between 5 and 1,000 rpm.

11. The method of claim 1, further comprising a step of aerating in the first or the second bioreactor, wherein the

dissolved oxygen (DO) in cultivation medium in the first or the second bioreactor is maintained at least 5% of DO saturation.

12. The method of claim 1, wherein the source of carbohydrates fed into the first or the second bioreactor comprises at least one of plants containing glucose, fructose, corn starch hydrolysate, cassaya starch hydrolysate, wheat starch hydrolysate, and juice of broomcorn.

13. The method of claim 1, further comprising feeding organic nitrogen into the first and the second bioreactor.

14. The method of claim 13, wherein the organic nitrogen comprises glycine, yeast powder, yeast extract, or corn syrup.

15. The method of claim 1, wherein pH value in the first or the second bioreactor is maintained between 6.0 and 8.0.

16. The method claim 1, further comprising adding a base to reduce acidity and regulate the pH of the culture medium in the first or the second bioreactor.

17. The method of claim 16, wherein the base comprises KOH.

18. The method of claim 1, wherein the temperature in the first or the second bioreactor is between 25 to 33° C.

19. The method of claim 1, wherein said transesterification further comprises catalyzation by immobilized lipase from *candida* sp. 99-125.

20. The method of claim 19, further comprising adding said lipase and methanol into said feedstock.

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