Title: IMPROVED MICROALGAE STRAINS AND USE THEREOF

Abstract: The present invention relates to improved strains of microalgae, process for the same and use thereof.
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**BACKGROUND OF INVENTION**

Microalgae are unicellular organisms having the ability to adapt to various environmental conditions. Microalgae are considered as promising sources for biofuels due to their potential for attaining high yields per unit area without affecting land use patterns for food crops.

Relative to terrestrial biofuel feedstocks, microalgae can convert solar energy into lipids at higher photosynthetic efficiencies. Microalgae can be grown around the year and fix CO₂ efficiently from different sources, including industrial exhaust gases, and can thrive in wastewater streams. They also have the potential for the production of various biofuels such as biodiesel, bio-oil, bio-syngas, bio-methane, bio-ethanol and bio-hydrogen. But for commercial viability, modifications of certain traits are required.

Multiple cycles of protoplast fusion and genome shuffling are a known art for improving the traits or to acquiring the desired property.

EP1707641A2 provides methods employing iterative cycles of recombination and selection/screening for evolution of whole cells and organisms toward acquisition of desired properties. Examples of such properties include enhanced recombinogenicity, genome copy number, and capacity for expression and/or secretion of proteins and secondary metabolites.

The technique of genome shuffling combines the advantage of multiparental crossing allowed by DNA shuffling together with the recombination of entire genomes normally associated with conventional breeding, or through protoplast fusion that increases the recombination process.
US653 646 relate to method for the genetic modification and improvement of Porphyra species utilizing protoplast fusion is disclosed. The method of the invention features the use of conchoporangial branch conchoceis for at least one of the sources of protoplasts for protoplast fusion. Protoplasts fusion method involves either a chemical fusing agent like polyethylene glycol (PEG) or electrofusion.

US2010Q162620 provides systems and processes for optimizing each type of algal-based production of bioproducts (such as oil) separately and independently, thereby improving overall production of oil, lipids and other useful products. This process is advantageous because it allows the optimization of the individual steps and growth phases in the production of oil from biomass.

This also allows the use of different feedstocks and growth conditions for the different process steps.

US201 20028338 to mixed algal compositions able to proliferate on industrial waste water, and to methods of obtaining an algal biomass from such cultures for use in generating a biofuel. The invention further encompass methods of cultivating mixed populations of freshwater and marine alga comprising a plurality of genera and species to provide a biomass from Which may be extracted lipids, or converted into biodiesel by such procedures as pyrolysis.

Deng et al. (2011), African J. Agri.Res. Vol.6(16), pp. 3768-3774 is a scientific publication which relates to effects of selective medium on lipid accumulation of chlorellas and screening of high lipid mutants through ultraviolet mutagenesis

Bhatnagar et al. (2011), Applied Energy, Vol.88, Issue 10, pp. 3425-3431 relates to mixotrophic growth potential of native microalgae namely Chlamydomonas globosa, Chlorella minutissima and Scenedesmus bijugata isolated after long-term enrichments from industrial wastewater and cultured in media supplemented with different organic carbon substrates and wastewaters. The mixotrophic growth of these microalgae resulted in 3–10 times more biomass production relative to phototrophy.
Vigeolas et al. (2012), J. of Biotech. Vol.162, Issue 1, pp.3-12 is a scientific publication which relates to isolation and partial biomass characterization of high triacylglycerol (TAG) mutants of Chlorella soroMniana and Scenedesmus ohliiiis, two algal species considered as potential source of biodiesel.

Pittman J.K et al. (2010), Bioresour. Tech. Vol.102, Issue 1, pp-17-25 is another scientific publication which relates to the potential of microalgae as a source of renewable energy for microalgal biofuel production. Wastewaters derived from municipal, agricultural and industrial activities potentially provide cost-effective and sustainable means of algal growth for biofuels.

Mohan S.V et al. (2011), Bioresour. Tech. Vol.102, Issue 2, pp-1109-1117 is also a scientific publication which provides an overview on the possibility of using mixed microalgae existing in ecological water-bodies for harnessing biodiesel. Microalgal cultures from five water-bodies are cultivated in domestic wastewater in open-ponds and the harvested algal-biomass was processed through acid-catalyzed transesterification.

The prior art discloses random mutagenesis of algal strain for lipid and biomass productivity. Still, the major drawback in the microalgae for use in biofuel is the non-availability of suitable strains and cost effective method for cultivation and harvesting. Hence, there is need to develop cheaper methods for cultivation and fast growing strains with tolerance to adverse environmental conditions and ability to utilize high concentrations of CO2 and lipid productivity with composition suitable for making fuels.

**SUMMARY OF THE INVENTION**

Accordingly, the main embodiment of the present invention provides strains of microalgae belonging to green algae and blue green algae selected from the group comprising of Nannochloropsis, Chlorella, Scenedesmus and Synechococcus.

Another embodiment of the present invention provides the strains which are Nannochloropsis IOC-105, Chlorella vulgaris IOC-106, Chlorella protothecoides IOC-107, Chlorella emersonii

Another embodiment of the present invention provide for a process of preparing strains of microalgae. said method comprising the steps of:

- a) isolating the microalgae from low quality waters;
- b) culturing the microalgae in algae culture medium (AIM);
- c) adapting the microalgae by culturing in medium comprising algal isolation medium, low quality water containing heavy metals in range of 100-1000 ppm, hydrocarbons in the range of 0.001-2% and high concentration of salt of upto 3%;
- d) growing the microalgae in the culture medium for twelve cycles;
- e) treating the microalgae with mutagenzmg agents;
- f) obtaining mutagenized microalgae;
- g) isolating the protoplast from mutagenized microalgae of step (c);
- h) shuffling the protoplast obtained step (d) for fusion using 60% PEG-6000 ;
- i) culturing the microalgae of the fused protoplast in AIM media containing 0.5 M osmotic agent ;
- j) carrying out the steps (d) to (f) for six cycles or generations under stringent growth conditions; and
- k) obtaining the novel microalgae strains.

Another embodiment of the present invention provides for a process of enhancing the biomass and lipid content of microalgae, said process comprising the steps of:

- a) Culturing the microalgae in a medium comprising of algae culture medium;
- b) Adding Auxins and cytokinins in the range of 0.25-10 ppm during the lag phase of the microalgae culture;
- c) Exposing microalgae of step(b) to UV for 2 hours in log phase;
- d) Adding sodium thiosulfate in the range of 0.05-2 % in the late log phase for microalgae of step (c)
- e) Incubating the microalgae of step (d) at10°C for 6 hours;
- f) Adding hot water extract from different plants to microalgae of step (e); and
g) Obtaining microalgae with high lipid content and biomass.

DESCRIPTION OF INVENTION

While the invention is susceptible to various modifications and/or alternative processes and/or compositions, specific embodiment thereof has been shown by way of example in the drawings and tables and will be described in detail below. It should be understood, however that it is not intended to limit the invention to the particular processes and/or compositions disclosed, but on the contrary, the invention is to cover all modifications, equivalents, and alternative falling within the spirit and the scope of the invention as defined by the appended claims.

The graphs, tables, formulas, protocols have been represented where appropriate by conventional representations in the drawings, showing only those specific details that are pertinent to understanding the embodiments of the present invention so as not to obscure the disclosure with details that will be readily apparent to those of ordinary skill in the art having benefit of the description herein.

The following description is of exemplary embodiments only and is not intended to limit the scope, applicability or configuration of the invention in any way. Rather, the following description provides a convenient illustration for implementing exemplary embodiments of the invention. Various changes to the described embodiments may be made in the function and arrangement of the elements described without departing from the scope of the invention.

The terms "comprises", "comprising", or any other variations thereof, are intended to cover a non-exclusive inclusion, such that one or more processes or composition/s or systems or methods proceeded by "comprises... a" does not, without more constraints, preclude the existence of other processes, sub-processes, composition, sub-compositions, minor or major compositions or other elements or other structures or additional processes or compositions or additional elements or additional features or additional characteristics or additional attributes.
Definition:

For the purposes of this invention the following terms will have the meaning as specified therein:

As used herein, the terms "Low quality water" or "Poor quality water" or "Water containing heavy metals", when used in the context of the present invention refers water which cannot be used directly for drinking, agriculture, human or animal consumption or other purpose. Such water is a was waste from industrial effluents, water containing heavy metals, hydrocarbons, water with high salinity, sewage water, reject water of reverse osmosis (RO) plant, river water with higher COD and BOD, water with coloring agent and other industry effluent etc. Further in context of the present invention the "Low quality water" or "Poor quality water" also includes water, which is or is found to be undesirable and harmful to human, anima or aquatic life in resepect of drinking, living or for any other purpose related an organism's survival or need.

As used herein, the term "High Value Products" when used in the context of the present invention refers to vitamins, pigments, anti-oxidants, omega-3 & omega-6 polyunsaturated fatty acids, DFIA or EPA.

As used here, the term "Mutagenizing Agent/s or Mutagenic Agent/s or Mutagens ", when used in the context of the present invention refers to agent/s a chemical, ultraviolet light, or a radioactive element, that can induce or increase the frequency of mutation in an organism.

As used here the term "Strain/s or Novel Strains ", when used in the context of the present invention refers to novel/new variants/strains of the microalgae produced or developed by the process of the present invention. These variants are genetically different in their control or parent or original forms. These variants are artificially developed and survive and perform better at extreme environmental conditions.

As used here the term "Chemical oxygen Demand or COD ", when used in the context of present invention refers to the test commonly used to indirectly to measure the amount of organic compounds in water. It determines the amount of oxygen required to oxidize an organic compound to carbon dioxide, ammonia, and water.
As used here the term "Biological Oxygen Demand or BOD ", when used in the context of the present invention refers to amount of dissolved needed by aerobic biological organism in a body of water to break down organic material present in a given water sample at certain temperature over a specific period.

As used here the term "Biofuel/s ", when used in the context of the present invention refers to a fuel that uses energy from a carbon fixation produced from microalgae. These fuels are made from a microalgae biomass conversion.

As used herein the term "Mutagenesis or mutagenized", when used in the context of the present invention refers to a process by which the genetic information of an organism is changed in a stable manner, resulting in a mutation. In context of the present invention it achieved experimentally using laboratory procedure by exposing the microalgae to various mutagens.

As used herein the term "Protoplast fusion or Somatic Fusion ", when used in context of the present invention refers to genetic modification of microalgae from same species by fusing their protoplasts (for e.g. pooled samples of C. vulgaris fused with another pooled samples of C. vulgaris) to form a new hybrid plant with the characteristics of both, a somatic hybrid.

As used herein the term "Mutant strains ", when used in context of the present invention refers to modified microalgae by a mutagen and protoplast fusion, wherein the fusion has been carried out in the microalgae of same species or pool of microalgae of same species. The mutant strains of the present invention do not in any manner or meant to refer to transgenic mutants or transgenic microalgae or transgenics or transgenic material. In the present invention the strains do not comprise genes of any unrelated higher life-form's or organism/s or unrelated microorganism/s.

In the present invention even the microalgae from different genus have not be crossed or nor any genetical material from different microalgae genus have been fused.

The present invention relates to improved strains microalgae. The microalga species in the present invention were collected from various locations in India. The algal species used to develop the strains of the present invention includes fresh water green microalgae like...
Nannochloropsis spp., Chlorella species such as Chlorella vulgaris, Chlorella salina, Chlorella protothecoides, Chlorella ellipsoidea, Chlorella emersonii, Chlorella munitissima, Chlorella pyrenoidosa, Chlorella sorokiniana, Chroomonas salirata; Cyclotella sp., Dimalella sp., Botryococcus sp., Haematococcus sp., Nartnochloris sp., Neochloris, Onoraphidium sp., Scenedesmus sp., Spirulina platensis, Chlamydomonas sp., Prochlorococcus, Synechococcus, or Synechococcus sp.

Nannochloropsis sp. and Scenedesmus sp. were collected from IOCL refinery Panipat, Haryana, India. Chlorella vulgaris, Chlorella pyrenoidosa and Synechococcus sp. were collected from soil of IOCL of R&D Centre, Faridabad, Haryana, India. Chlorella protothecoides and Chlorella emersonii were collected from Yamuna River Bank, New Delhi, India.

In the present invention the preferred strains of microalgae that were developed are: Nannochloropsis sp. (referred to herein as IOC-105) which was deposited with Culture Collection of Algae and Protoza (CCAP), UK under Budapest Treaty on __________, 2013 and given accession number __________; Chlorella vulgaris (referred to herein as IOC-106) which was deposited with Culture Collection of Algae and Protoza (CCAP), UK under Budapest Treaty on __________, 2013 and given accession number __________; Chlorella protothecoides (referred to herein as IOC-107) which was deposited with Culture Collection of Algae and Protoza (CCAP), UK under Budapest Treaty on __________, 2013 and given accession number __________; Chlorella emersonii (referred to herein as IOC-108) which was deposited with Culture Collection of Algae and Protoza (CCAP), UK under Budapest Treaty on __________, 2013 and given accession number __________; Chlorella pyrenoidosa (referred to herein as IOC-109) which was deposited with Culture Collection of Algae and Protoza (CCAP), UK under Budapest Treaty on __________, 2013 and given accession number __________; Scenedesmus sp. (referred to herein as IOC-110) which was deposited with Culture Collection of Algae and Protoza (CCAP), UK under Budapest Treaty on __________, 2013 and given accession number __________ and Synechococcus sp. (referred to herein as IOC-111) which was deposited with Culture Collection of Algae and Protoza (CCAP), UK under Budapest Treaty on __________, 2013 and given accession number __________.
The improved strains so developed by the process of the present invention are useful to cultivating in various low quality waters to obtain biofuel and other value added products.

According to the present invention, there is provided a process for isolating, screening and adopting the same to obtain strains of microalgae, wherein the same have ability to grow in extreme growth conditions.

The present invention also relates to a process for improving the mutant strains of microalgae obtained by mutagenic and/or chemostat mediated adaptation. According to the invention, the microalgae strains were improved by recursive mutagenesis and protoplast fusion. The microalgae were mutagenised by chemical and radiation mutagen.

The pooled mutant population is shuffled by homologous recombination using protoplast fusion followed by selecting improved progenies and subjecting the same to next round of selection. This process was carried out for six cycles.

According to the present invention the microalgae were mutagenized by chemical (EMS, mitomycin C, N-methyl-N'-nitro-N-nitrosoguanidine, benzo(a)pyrene and 4-nitroquinoline 1-oxide) and radiation mutagen (UV, gamma-rays) or their combination. The pooled mutant population is shuffled by homologous recombination using protoplast fusion followed by selecting improved progenies and subjecting the same to next round of selection. This process was carried out for six cycles. This accelerates directed evolution through recursive recombination of improved progeny, thereby improving multiple traits. Strains with higher growth, lipid productivity, salt, pH and heavy metal tolerance and with ability to grow under autotrophic conditions were obtained.

This accelerates directed evolution through recursive recombination of improved progeny, thereby improving multiple traits like higher growth and lipid productivity, carbon di-oxide utilization ability, biomass desired lipid composition salt, pH, temperature and heavy metal tolerance. The composition of the lipid obtained from modified strains was suitable for biodiesel production.
The microalgae strains thus developed by the process of the invention have the ability to grow in presence of salt up to 3%, at a temperature range from 10-45°C, high light intensity, pH 4.5-10 and heavy metal up to 100-1000 ppm or more (TABLE-1). The strains so developed have lipid composition suitable for biofuel (TABLE-2).

Further the mutant algal strains were cultured in various bio-chemical-physical factors like growth hormones, specific bacteria, protein synthesis inhibiting chemicals- sodium thio-sulphate, ultra-violet rays; high and low temperature to improve biomass, lipid content and/or its composition (TABLE-3).

According to the present invention, various low quality water like water with hydrocarbon contamination, water from oil refinery effluent plant, reverse osmosis plant's reject, river water with higher COD and BOD, water with coloring agent and other industry effluent have been utilized for algae cultivation.

According to the invention the microalgae strains of the present invention are used to sequester carbon dioxide from various sources like flue gas, bio-gas plant exhaust and other source of concentrated CO$_2$ having CO$_2$ in the range of 0.05-50%, thereby helping in abating pollution (TABLE-4).

<table>
<thead>
<tr>
<th>Growth conditions</th>
<th>Biomass g/l</th>
<th>Lipid content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal <em>C.vulgaris</em> (IOC-106)</td>
<td>Normal <em>Nannochloropsis</em> Strain IOC-105</td>
</tr>
<tr>
<td>Media in distilled water</td>
<td>2.32</td>
<td>2.80</td>
</tr>
<tr>
<td>Media in hydrocarbon containing water</td>
<td>2.02</td>
<td>2.53</td>
</tr>
<tr>
<td>Media in reverse osmosis plant's reject water</td>
<td>2.10</td>
<td>2.17</td>
</tr>
</tbody>
</table>
Accordingly, the main embodiment of the present invention provides strains of microalgae belonging to green algae and blue green algae selected from genus comprising of *Nannochloropsis, Chioreila, Scenedesmus* and *Synechococcus*.


Yet another embodiment of the present invention provides strains having high lipid content, higher growth rate, CO₂ utilization ability and biomass desired lipid composition.

Another embodiment of the present invention provides strains wherein the strains are useful as biofueis and for value added products selected from group comprising of Vitarnms, pigments, antioxidants, omega-3 and omega-6 polyunsaturated fatty acids, Docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA).

Another embodiment of the present invention provides for strains wherein the strains tolerates high salinity, pH, heavy metal contamination, temperature and light intensity.

Yet another embodiment of the present invention provides for strains, wherein the strains have the ability to grow under salinity conditions in range of 0.5-3%.

One more embodiment of the present invention provides for strains, wherein the strains can grow in the temperature range from 10-45°C.

Another embodiment of the present invention provides for strains, wherein the strains can grow in wide range of pH from 6.5-10.
Another embodiment of the present invention provides, wherein the strains can have heavy metal tolerance in the range of 100-1000 ppm.

Another embodiment of the present invention provides microalgae strain wherein the microalgae strain have saturates in the range of 26-85%.

Another embodiment of the present invention provides microalgae strain wherein the microalgae strain have saturates in the range of 32-70%.

Yet another embodiment of the present invention provides microalgae strain wherein the microalgae strain has lipid content in the range of 20-35%.

Yet another embodiment of the present invention provides microalgae strain wherein the microalgae strain has lipid content in the range of 26-33%

One more embodiment of the present invention provides microalgae strain wherein the microalgae strain has biomass in the range of 2-7g/L.

Yet another embodiment of the present invention provides microalgae strain wherein the microalgae strain has biomass in the range of 2-6g/l.

Another embodiment of the present invention provides microalgae strain wherein the microalgae strain has biomass in the range of 2.82-5.1 g/l.

Another embodiment of the present invention provides microalgae strain wherein the microalgae strain has saturates in the range of 26-70%.

Another embodiment of the present invention provides microalgae strain wherein the microalgae strain has lipid content in the range of 21.4-30.45%.
Another embodiment of the present invention provide for a process of preparing strains of microalgae, said method comprising the steps of:

a) isolating the microalgae from low quality waters;
b) culturing the microalgae in algae culture medium (AIM);
c) adapting the microalgae by culturing in medium comprising of algal isolation medium, low quality water containing heavy metals in range of 100-1000 ppm, hydrocarbons in the range of 0.001%-2% and high concentration of salt of upto 3%;
d) growing the microalgae in the culture medium for twelve cycles;
e) treating the microalgae with mutagenizing agents;
f) obtaining mirtagenized microalgae;
g) isolating the protoplast from mirtagenized microalgae of step (e);
h) shuffling the protoplast obtained step (d) for fusion using 60% PEG-6000;
i) culturing the microalgae of the fused protoplast in AIM media containing 0.5 M osmotic agent;
j) carrying out the steps (d) to (f) for six cycles or generations under stringent growth conditions; and
k) obtaining the novel microalgae strains.

Yet another embodiment of the present invention provides for a process, wherein the microalgae is selected from the group comprising of green algae and blue-green algae.

One more embodiment of the present invention provides for a process, wherein the microalgae belong to genus selected from the group comprising of *Nannochloropsis*, *Chiorella*, *Sceriedesmus* and *Synechococcus*.

Yet another embodiment of the present invention provides for a process, wherein the mutagenizing agents in step (e) are selected from the group comprising of EMS, mitomycin C, N-methyl-N′-nitro-N-nitrosoguanidine, benzo(9)pyrene and 4-nitroquinoline 1-oxide, UV rays, gamma-rays or their combination therof, preferably EMS.

In one embodiment of the present invention provides for a process, wherein, the osmotic agent in step (i) is sucrose.

Yet another embodiment of the present invention provides a process, wherein the microalgae is useful for the production of biofuels and value added products selected from group comprising of Vitamins, pigments, antioxidants, omega-3 and omega-6 polyunsaturated fatty acids, DHA or EPA.

One more embodiment of the present invention provides for a process, wherein the microalgae has high salinity tolerate upto 3%, pH tolerance in the range of 4.5-10, heavy metal contamination tolerance in the range of 100-1000ppm, temperature tolerance in the range of 10-45°C and light intensity tolerance.

Another embodiment of the present invention provides for a process, wherein the microalgae has higher growth rate, lipid productivity, CO₂ utilization ability and biomass desired lipid composition.

Another embodiment of the present invention provides for process wherein the microalgae strain have saturates in the range of 26-85%.

Another embodiment of the present invention provides for a process wherein the microalgae strain have saturates in the range of 32-70%.

Yet another embodiment of the present invention provides for the process wherein the microalgae strain has lipid content in the range of 20-35%.
Yet another embodiment of the present invention provides for a process wherein the microalgae strain has lipid content in the range of 26-33%.

One more embodiment of the present invention provides for a process wherein the microalgae strain has biomass in the range of 2-7g/L.

Yet another embodiment of the present invention provides for a process wherein the microalgae strain has biomass in the range of 2-6g/l.

Another embodiment of the present invention provides for a process wherein the microalgae strain has biomass in the range of 2.82-5.1 g/l.

Another embodiment of the present invention provides for a process wherein the microalgae strain has saturates in the range of 26-70%.

Another embodiment of the present invention provides for a process wherein the microalgae strain has lipid content in the range of 21.4-30.45%.

Another embodiment of the present invention provides for a novel process for enhancing the biomass and lipid content of microalgae, said process comprising the steps of:

a) Culturing the microalgae in a medium comprising of algae culture medium;

b) Adding Auxins and cytokinins in the range of 0.25-10 ppm during the lag phase of the microalgae culture;

c) Exposing microalgae of step(b) to UV for 2 hours in log phase;

d) Adding sodium thiosulfate in the range of 0.05-2 % in the late log phase for microalgae of step (c);

e) Incubating the microalgae of step (d) at 10°C for 6 hours;

f) Adding hot water extract from different plants to microalgae of step (e); and

g) Obtaining microalgae with high lipid content and biomass.
Yet another embodiment of the present invention provides for a novel process for enhancing the biomass and lipid content of microalgae, wherein the microalgae are a green algae or blue-green algae.

In another embodiment of the present invention for a novel process for enhancing the biomass and lipid content of microalgae, wherein the microalgae belong to genus selected from the group comprising of *Nannochloropsis, Chlorella, Scenedesmus* and *Synechococcus*.


Another embodiment of the present invention provides for a novel process for enhancing the biomass and lipid content of microalgae, wherein the steps (b) to (f) is to be performed in a said sequential manner to obtain the strains of microalgae with high biomass and lipid content.

Another embodiment of the present invention provides for a novel process for enhancing the biomass and lipid content of microalgae, wherein the novel strains are useful for the production of biofuels and value added products selected from group comprising of Vitamins, pigments, antioxidants, omega-3 and omega-6 polyunsaturated fatty acids, DHA or EPA.

Yet another embodiment of the present invention provides for a novel process for enhancing the biomass and lipid content of microalgae, wherein the novel strains tolerate high salinity upto 3%, pH in the range of 4.5-10, heavy metal contamination in the range of 100-1000ppm, temperature in the range of 10(~)~45°C and light intensity.

One more embodiment of the present invention for a novel process for enhancing the biomass and lipid content of microalgae, wherein the stains have higher growth rate, lipid productivity, CO₂ utilization ability and biomass desired lipid composition.
Another embodiment of the present invention provides for a novel process for enhancing the biomass and lipid content of microalgae wherein the microalgae strain have saturates in the range of 26-85%.

Another embodiment of the present invention provides for a novel process for enhancing the biomass and lipid content of microalgae wherein the microalgae strain have saturates in the range of 32-70%.

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One more embodiment of the present invention provides for a novel process for enhancing the biomass and lipid content of microalgae wherein the microalgae strain has biomass in the range of 2-7g/l.

Yet another embodiment of the present invention provides for a novel process for enhancing the biomass and lipid content of microalgae wherein the microalgae strain has biomass in the range of 2-6g/l.

Another embodiment of the present invention provides for a novel process for enhancing the biomass and lipid content of microalgae wherein the microalgae strain has biomass in the range of 2.82-5.1 g/l.
Another embodiment of the present invention provides for a novel process for enhancing the biomass and lipid content of microalgae wherein the microalgae strain has saturates in the range of 26-70%.

Another embodiment of the present invention provides for a novel process for enhancing the biomass and lipid content of microalgae wherein the microalgae strain has lipid content in the range of 21.4-30.45%.

Further, cost effective media components mainly nitrogen, phosphorus and micronutrient and their concentration ranging from 5 ppm to 5% for high growth and lipid content have been used. These sources include fertilizers, biogas plant residue extract, corn steep liquor, cow dung extract, plants extract, poultry dropping extract and so forth.

Further, according to the present invention antibacterial and/or fungicidal compounds of plant origin are added to inhibit growth of bacteria/fungi in cultivation media of microalgae.

In one embodiment of the invention, during cultivation of the algae, hot water extract of plant was to inhibit growth of bacteria/fungi in cultivation media of algae. The hot water extract of plant origin, not only inhibits the growth of undesired microbes in open pond but also improves the growth of algae.

The present invention further discloses a method for cultivation in open pond/photo bioreactor/poiybags. According to the invention, the algal biomass cultivated in open pond/photo bioreactor/poiybags or in other cultivation medias is harvested followed by cell disruption by mechanical method, chemical exposure, salinity changes or pH changes and subsequently extracting the oil using combination of polar and nonpolar solvents at a suitable temperature, wherein the temperature may range between 30-80 degree C.

The following non-limiting examples illustrate specific embodiments of the present invention. They are not intended to be limiting the scope of the present invention in any way.
The invention will now be explained with the help of following examples. However, the scope of the invention should not be limited to these examples as the person skilled in the art can easily vary the proportion of the ingredients and combinations.

EXAMPLES

EXAMPLE 1
Isolation of algae:

The algae were isolated from diverse water and soil samples collected from various sources. The collected water and soil samples were inoculated in media, termed as Algal isolation media (AIM), containing (g/L) Na2 C03 (0.5-5), NaHC03 (1-5) KHzPO4 (0.5-4.8), K2HPO4 (0.5-5), MgSO4 (0.01-1.0), (NH4)2SO4 (0.25-0.50), KNO3 (0.15-4.75), Urea (0.15-4.75), Di-ammonium phosphate (0.15-4.75), ZnSO4 (0.2-2.1), NaCl (0.2-10) Trace element (2ml to 10 ml of solution). The trace element solution (gram per liter) comprises Nitrilotriacetic acid (0.1-1.0), FeSO4.7H2O (0.01-0.15), MnCl2.4H2O (0.001-0.005), CoCl2.6H2O (0.005-0.02), CaCl2.2H2O (0.01-0.5), ZnCl2 (0.01-0.15), CuCl2.H2O (0.01-0.03), H3BO3 (0.002-0.02), Na2MoO4.4H2O (0.001-0.02), Na2SeO3 (0.005-0.02), NiSO4 (0.01-0.03), SnCl2 (0.01-0.03). Each 1000 ml of flask contained 500 ml of above media was autoclaved. It was inoculated with 5-10% of soil or water sample. The flasks were incubated at 45°C for 2-10 days in presence of light and continuously CO2 was sparged.

After completion of incubation the 1 ml culture was centrifuged at 3000 rpm for 5 minutes. The cells which are floating were re-inoculated in above media where KNO3 was omitted from AIM. This was repeated for total twelve cycles. After 5th cycle the solvent used for media preparation was low quality water i.e., water containing heavy metals (100-500 ppm), hydrocarbons (0.001%-2%), & high salinity (3%). For cycle 8 & 9th cycle the incubation temperature was kept 25 degree C for next three cycle (i.e., 10, 11 & 12) was kept 45 degree C. After completion of 12 cycles culture was centrifuged at 3000 rpm for 5 minutes and after serial dilution inoculated on AIM+agarose plate. Plated were incubated at 45 degree C in presence of light. Fast growing single greenish colonies were picked and carefully transferred to a new plate. The purified colonies are selectively picked up and inoculated into flasks containing growth medium, including but not limited to components of basal medium, for further culture.
The selected algal strains were characterized according to their 18S rRNA gene sequences, using the primers and conditions known in prior art. The resulting 18S rRNA gene sequences were aligned and compared to the nucleotide sequences of some known microalgae in GenBank database of the National Center for Biotechnology Information by using Basic Local Alignment Search Tool (BLAST®).

Example 2
Preparation of Mutant Strains

EMS was added into 5 ml of the log phase culture of Chlorella vulgaris IOC-106 in a 15-ml centrifuge tube to a final concentration of 0.42 gL⁻¹ and the culture suspension was further incubated in a water bath at 45°C for 15 min. Diluting the culture 20 times with pre-chilled, fresh subsequently terminated the treatment. The mutated cells were centrifuged and transferred to the media having salt concentration 1%, heavy metal concentration (200 ppm), pH (10). After 24 h incubations at 45°C under light conditions and in presence of CO₂, the culture broth was centrifuged at 3000 rpm for 10 minutes and cells which were settled by centrifugation were plated on media agar plates having salt concentration 1%, high heavy metal concentration (200 ppm), high pH (10). The plates were incubated at 45°C for 48 hours in light and presence of CO₂. Protoplast was prepared according to method known in prior art. For shuffling, protoplasts were fused by suspension in buffer (0.5 M sucrose, 10mM Tris –HCl, 20mM MgCl₂) containing 15% dimethyl sulphoxide and 60% PEG-6000. The resulting suspension was incubated at 25°C for 50 min. The fused protoplast preparation was diluted with regeneration media (AIM media containing 0.5M sucrose) and protoplasts were harvested by centrifugation at 3500 rpm for 10 min at 25°C. The protoplast cells floating was collected and were re-suspended in regeneration media and shaken at 200 rpm for 12 h before plating on agar plates higher salt concentration 2%, heavy metal concentration (250 ppm), pH (11). The plated were scraped to generate a pooled fusion library. The formation of protoplasts, their fusion and their subsequent regeneration was repeated six times with pooled regenerated cells from one fusion being the inoculum for the subsequent protoplast culture. At each cycle cells were screened at stringent growth conditions with respect to pH, salinity, heavy metal concentration. Non-shuffled controls were prepared by
the recursive formation and regeneration of protoplasts without exposure to PEG. This process was carried out for six cycles. This accelerates directed evolution through recursive recombination of improved progeny, thereby improving multiple traits.

The microalgae strains thus developed by the process of the invention have the ability to grow in presence of salt up to 3%, at a temperature range from 10-45 degree C, high light intensity, pH 4.5-10 and heavy metal 100-1000 ppm (Table-1). The strains so developed have lipid composition suitable for biofuel (Table-2).

Table: 1. Growth and lipid content in various cycle of improvement (Strain Chlorella vulgaris IOC-106)

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Biomass yield (g/l) (Dry cell weight basis)</th>
<th>Lipid content (% DCW)</th>
<th>Temperature range where strain able to grow (degree C)</th>
<th>pH range where strain able to grow</th>
<th>Salinity range where strain able to grow (%)</th>
<th>Range of heavy metal Concentration where strain able to grow (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type and adopted</td>
<td>2.85</td>
<td>22.1</td>
<td>25-45</td>
<td>6-9</td>
<td>Up to 0.5</td>
<td>Up to 100</td>
</tr>
<tr>
<td>Cycle-1</td>
<td>3.07</td>
<td>23.4</td>
<td>20-45</td>
<td>6.5-9.0</td>
<td>Up to 1</td>
<td>Up to 250</td>
</tr>
<tr>
<td>Cycle-2</td>
<td>3.23</td>
<td>24.4</td>
<td>15-45</td>
<td>6.5-9.2</td>
<td>Up to 2</td>
<td>Up to 500</td>
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<tr>
<td>Cycle-3</td>
<td>3.29</td>
<td>25.3</td>
<td>10-45</td>
<td>5-10</td>
<td>Up to 2</td>
<td>Up to 650</td>
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<tr>
<td>Cycle-4</td>
<td>3.76</td>
<td>26.9</td>
<td>10-45</td>
<td>5-10</td>
<td>Up to 2.5</td>
<td>Up to 900</td>
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<tr>
<td>Cycle-5</td>
<td>3.94</td>
<td>27.7</td>
<td>10-45</td>
<td>5-10</td>
<td>Up to 3.0</td>
<td>Up to 1000</td>
</tr>
<tr>
<td>Cycle-6</td>
<td>4.42</td>
<td>27.7</td>
<td>10-45</td>
<td>5-10</td>
<td>Up to 3.0</td>
<td>Up to 1000</td>
</tr>
</tbody>
</table>

*no further improvement was seen after 6th cycle, so not further mutagenesis and protoplast fusion was not carried out.
Table 2 - Lipid Composition of wild and improved strain (Strain Chlorella vulgaris IOC-106)*.

Growth under light & 50% air mixed CO₂.

<table>
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<tr>
<th>SN</th>
<th>Components</th>
<th>Wild Type</th>
<th>Improved Strain</th>
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<tr>
<td>1</td>
<td>C12:0</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>C14:0</td>
<td>5.3</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>C16:0</td>
<td>10.1</td>
<td>15.8</td>
</tr>
<tr>
<td>4</td>
<td>C16:1</td>
<td>2.1</td>
<td>9.9</td>
</tr>
<tr>
<td>5</td>
<td>C18:0</td>
<td>3.6</td>
<td>13.2</td>
</tr>
<tr>
<td>6</td>
<td>C18:1</td>
<td>16.4</td>
<td>13.2</td>
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<tr>
<td>7</td>
<td>C18:2</td>
<td>9.8</td>
<td>9.9</td>
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<td>8</td>
<td>C18:3</td>
<td>15</td>
<td>12.5</td>
</tr>
<tr>
<td>9</td>
<td>&gt;C20:0</td>
<td>32.7</td>
<td>23.5</td>
</tr>
</tbody>
</table>

*More saturate is good for biodiesel production. Improved strain has higher saturates.

Example 3:

**Improvement of Lipid Contest and Biomass of Mutant Strains**

The above given factors are added in a sequential manner in suitable concentrations. The addition of the growth hormones (Auxins & Cytokinins) in 0.25 ppm to 10 ppm in media during inoculation significantly reduce the lag phase. Addition of sodium thiosulfate in late log phase of growth in 0.05-2% improved the lipid content. It was observed that exposure of the cells in UV for 2-5 min in log phase improves the growth as well as the lipid content and its fatty acid compositions. It was found that growing the algae at different temperature as well varying the temperature during growth of the microalgae improves the algal growth as well as its fatty acid content and compositions. Further it was found that addition of extracts of plants and their parts like neem, lantana etc. has shown improvement in growth of algae. The novel feature of the process relates to the sequence in which the various ingredients of the composition are added during the process. In the present invention it has been found the adding and/or mixing the various compositions of the ingredients in the sequential manner as described as herein, description of the specification and the claims allows or provides a maximum beneficial mode to enhance the biomass, lipid and saturate contents of the algal strains. It has been found that this sequential mode is not only useful for enhancing the
biomass, lipid and saturate contents of control or unmodified microalgae but also of the novel strains.

Table 3 - Modulation of growth conditions leading to improved biomass of improved strain (Strain: Chlorella vulgaris IOC-106). Sequence of addition, timing and concentration various **bio-chemical-physical** factors like growth hormones, specific bacteria, protein synthesis inhibiting chemicals- sodium thio-sulphate, ultra-violet rays; high and low temperature are used to improve biomass, lipid content and/or its composition.
Table 3: Medium for modulation of growth conditions leading to improved biomass of improved strain.

<table>
<thead>
<tr>
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<th>Normal/Control Medium</th>
<th>Medium 1</th>
<th>Medium 2</th>
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<tr>
<td></td>
<td>Biomass (g/l)</td>
<td>Lipid Content (%)</td>
<td>Saturates</td>
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<tr>
<td>C. vulgaris</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>2.85</td>
<td>22.1</td>
<td>27</td>
</tr>
<tr>
<td>IOC-106</td>
<td>4.42</td>
<td>27.7</td>
<td>32</td>
</tr>
<tr>
<td>Nannochloropsis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>2.82</td>
<td>21.4</td>
<td>26</td>
</tr>
<tr>
<td>IOC-105</td>
<td>3.17</td>
<td>26.2</td>
<td>32</td>
</tr>
</tbody>
</table>

**Medium-1**: Normal medium of Example-1 + Auxins & Cytokinins, (2.5ppm each) during lag phase, followed by 0.5% sodium thiosulfate late log phase, followed by incubation at 45°C.

**Medium-2**: Normal medium of Example-1 + Auxins & Cytokinins, (2.5ppm each) during lag phase, followed by 0.5% sodium thiosulfate late log phase, followed by incubation at 10°C.

**Medium-3**: Normal medium of Example-1 + (Novel Medium 1 or Novel Medium-2). Addition of hot.
Example 4:
Preparation of plant extract:

The composition of the invention includes extract of different parts of the plants such as lantana, tobacco, neem, manendi, vegetative and/or fruit plant material and/or mixtures thereof. Plant material includes the stem, leaves and fruit of the plant and any part of the plant. In a particularly important aspect, the plant material is dried, powdered and extracted with water, sequentially and/or simultaneously at different temperature, pressure to remove the compounds having ability to inhibit the growth of undesired bacteria and stimulate growth of algae. The temperature ranges from 40-100 °C, preferably 55-80 °C and the pressure ranges from atmospheric to 15 lbs. The extracted material was further purified using known art like column chromatography and each fraction was evaluated for their ability to inhibit growth of undesired bacteria and stimulate growth of algae. The plant extract was effective in the concentration ranging from 1-10% (v/v) in media.
We Claim:

1. Strains of microalgae belonging to green algae and blue-green algae of genus selected from the group comprising of *Nannochloropsis*, *C. Morella*, *Scenedesmus* and *Synechococcus*.


3. The strains as claimed in any one of claims 1-2, having high lipid content, high growth rate, CO₂ utilization ability and biomass.

4. The strains as claimed in any one of claims 1-2, wherein the strains tolerates high salinity, pH, heavy metal contamination, temperature and light intensity.

5. The strains as claimed in any one of claims 1-2 and 4, wherein the strains have the ability to grow under salinity conditions in range of 0.5-3%.

6. The strains as claimed in any one of claims 1-2 and 4, wherein the strains grow in the temperature range from 10-45°C.

7. The strains as claimed in any one of claims 1-2 and 4, wherein the strains grow in wide range of pH from 6.5-10.

8. The strains as claimed in any one of claims 1-2 and 4, wherein the strains have heavy metal tolerance in the range of 100-1000 ppm.

9. The process as claimed in any one of claims 1-3, wherein the microalgae has saturates in the range of 26-85%.
10. The process as claimed in any one of claims 1-3 and 9, wherein the microalgae has saturates in the range of 32-70%.

11. The process as claimed in any one of claims 1-3, wherein the microalgae has lipid content in the range of 20-35%.

12. The process as claimed in any one of claims 1-3 and 11, wherein the microalgae has lipid content in the range of 26-33%.

13. The process as claimed in any one of claims 1-3, wherein the microalgae have biomass in the range of 2-7 g/L.

14. The process as claimed in any one of claims 1-3 and 13, wherein the microalgae have biomass in the range of 2-6 g/L.

15. A process of preparing strains of microalgae, said method comprising the steps of:
   a) isolating the microalgae from low quality waters;
   b) culturing the microalgae in algae culture medium (AIM);
   c) adapting the microalgae by culturing in medium comprising of algal isolation medium, low quality water containing heavy metals in range of 100-1000 ppm, hydrocarbons in the range of 0.001%-2% and high concentration of salt of up to 3%;
   d) growing the microalgae in the culture medium for twelve cycles;
   e) treating the microalgae with mutagenizing agents;
   f) obtaining mutagenized microalgae;
   g) isolating the protoplast from mutagenized microalgae of step (c);
   h) shuffling the protoplast obtained step (d) for fusion using 60% PEG-6000;
   i) culturing the microalgae of the fused protoplast in AIM media containing 0.5 M osmotic agent;
   j) carrying out the steps (d) to (f) for six cycles or generations under stringent growth conditions; and
k) obtaining the novel microalgae strains.

16. The process as claimed in claim 15, wherein the microalgae is selected from the group comprising of green algae and blue-green algae.

17. The process as claimed in claim any one of claim 15-16, wherein the microalgae belong to genus selected from the group comprising of *Nannochloropsis, Chlorella, Scenedesmus* and *Synechococcus*.


19. The process as claimed in claim 15, wherein the mutagenizing agents in step (e) are selected from the group comprising of EMS, mitomycin C, N-methyl-N’-nitro-N-nitrosoguanidine, benzo(9)pyrene and 4-nitroquinoline 1-oxide, UV rays, gamma-rays or their combination therof, preferably EMS.

20. The process as claimed in claim 15, wherein, the osmotic agent in step (i) is sucrose.

21. The process as claimed in any one of claims 15-20, wherein the microalgae is useful for the production of biofuels and value added products selected from group comprising of Vitamins, pigments, antioxidants, omega-3 and omega-6 polyunsaturated fatty acids, DHA or EPA.

22. The process as claimed in any one of claims 15-21, wherein the microalgae has high salinity tolerate upto 3%, pH tolerance in the range of 4.5-10, heavy metal contamination tolerance in the range of 100-1000ppm, temperature tolerance in the range of 10-45°C and light intensity tolerance.
23. The process as claimed in any one of claims 15-21, wherein the microalgae has higher growth rate, lipid productivity, CO₂ utilization ability and biomass desired lipid composition.

24. The process as claimed in any one of claims 15-23, wherein the microalgae has saturates in the range of 26-85%.

25. The process as claimed in any one of claims 15-24, wherein the microalgae has saturates in the range of 32-70%.

26. The process as claimed in any one of claims 15-23, wherein the microalgae has lipid content in the range of 20-35%.

27. The process as claimed in any one of claims 15-23 and 26, wherein the microalgae has lipid content in the range of 26-33%.

28. The process as claimed in any one of claims 15-23, wherein the microalgae have biomass in the range of 2-7g/l.

29. The process as claimed in any one of claims 15-23 and 28, wherein the microalgae have biomass in the range of 2-6g/l.

30. A process of enhancing the biomass and lipid content of microalgae, said process comprising the steps of:
   a) Culturing the microalgae in a medium comprising of algae culture medium;
   b) Adding Auxins and cytokinins in the range of 0.25-10 ppm during the lag phase of the microalgae culture;
   c) Exposing microalgae of step(b) to UV for 2 hours in log phase;
   d) Adding sodium thiosulfate in the range of 0.05-2 % in the late log phase for microalgae of step (c);
   e) Incubating the microalgae of step (d) at 10°C for 6 hours;
f) Adding hot water extract from different plants to microalgae of step (e); and

g) Obtaining microalgae with high lipid content and biornass.

31. A process as claimed in claim 30, wherein the microalgae is a green algae or blue-green algae.

32. A process as claimed in any one of claims 30-31, wherein the the microalgae belong to genus selected from the group comprising of *Nannochloropsis*, *Chlorella*, *Scenedesmus* and *Synechococcus*.


34. The process as claimed in claim 30, wherein the steps (b) to (f) is to be performed in a said sequential manner to obtain the strains of microalgae with high biornass and lipid content.

35. The process as claimed in any one of claims 30-34, wherein the novel strains are useful for the production of biofuels and value added products selected from group comprising of Vitamins, pigments, antioxidants, omega-3 and omega-6 polyunsaturated fatty acids, DHA or EPA.

36. The process as claimed in any one of claims 30-34, wherein the novel strains tolerate high salinity upto 3%, pH in the range of 4.5-10, heavy metal contamination in the range of 100-1000ppm, temperature in the range of 10-45°C and light intensity.

37. The process as claimed in any one of claims 30-34, wherein the stains have higher growth rate, lipid productivity, CO₂ utilization ability and biornass desired lipid composition.
38. The process as claimed in any one of claims 30-34, wherein the microalgae has saturates in the range of 26-85%.

39. The process as claimed in any one of claims 30-34 and 38, wherein the microalgae has saturates in the range of 32-70%.

40. The process as claimed in any one of claims 30-34, wherein the microalgae has lipid content in the range of 20-35%.

41. The process as claimed in any one of claims 30-34 and 40, wherein the microalgae has lipid content in the range of 26-33%.

42. The process as claimed in any one of claims 30-34, wherein the microalgae have biomass in the range of 2-7g/l.

43. The process as claimed in any one of claims 30-34 and 42, wherein the microalgae have biomass in the range of 2-6g/l.
**INTERNATIONAL SEARCH REPORT**

**International application No**

PCT/IB2013/059407

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)

C12N C12R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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

EPO-Internal, WPI Data, BIOSIS, Sequence Search, EMBASE, CHEM ABS Data, PAJ

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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<th>Category*</th>
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[X] Further documents are listed in the continuation of Box C. [X] 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 but 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
  - "Z" document member of the same patent family

Date of the actual completion of the international search: 30 January 2014

Date of mailing of the international search report: 07/02/2014

Name and mailing address of the ISA:
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016

Authorized officer:
Seranski, Peter
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INTERNATIONAL SEARCH REPORT

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. All required additional search fees were timely paid by the applicant, this international search report covers all searchable

2. X As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.
This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-14
   Strains of microalgae belonging to green algae and blue-green algae.

2. claims: 15-29
   Process for preparing strains of microalgae.

3. claims: 30-43
   Process of enhancing the biomass and lipid content of microalgae.
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International application No: PCT/IB2013/059407