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(54) Title: PROCESS FOR PRODUCTION OF ALGAL BIOMASS

(57) Abstract: Process for producing an algal biomass comprising: i)inoculating in an aqueous cultivation medium at least a first algal species and at least a first protozoan species obtaining a symbiotic co-culture, the at least first protozoan species being suitable to generate symbiosis with the at least first algal species; ii) cultivating the symbiotic co-culture obtaining an algal biomass; and iii) harvesting at least a portion of the algal biomass.

"Process for production of algal biomass"

* * *

FIELD OF THE INVENTION

The present description concerns a new process for production of an algal biomass with a high lipid content .

BACKGROUND

Algae are important resources for many beneficial bio-products .

Algae cells contain pigments and other intracellular matters useful for producing antioxidants, vitamins, aquaculture nutrients, bioplastics, dyes and colourants, feedstocks, pharmaceuticals, algae fuels and especially oils for energy and health care purposes. The biomass can give biofuel by various processes of pyrolysis (Chisti 2007, Giardi et al. 2010)

Algal cultivation, similar to culturing many other microorganisms, requires both macro and micronutrients that can be obtained from either organic or inorganic sources. Typical commercial algal growth methods, however, rely on the use of exogenously added pure chemicals and micronutrients needed to sustain the algal cultures.

Many examples of algal cultivation exist in the art. The cultivation of algae occurs in proper photobioreactors which can be closed or open systems. Examples of closed photobioreactors for algal cultures are provided in U.S. Pat. Nos. 2,732,663; 4,473,970; 4,233,958; 4,868,123; and 6,827,036. Examples of open air photobioreactor systems used for cultivation of algae are disclosed, for example, in U.S. Pat. Nos.

3,650,068; 3,468,057; 3,955,318; and 4,217,728.

These methods are nevertheless expensive.

Moreover, it is known in the literature that nitrogen, salt, heavy metal stresses in algae are used to induce algal lipid production.

The application of these kinds of stress is, nevertheless, generally limited to non-continuous batch processes in closed systems bioreactors where algae are initially grown in rich chemical medium to provide a large algal biomass followed by imposing nitrogen deprivation or other types of stresses by rapid exhaustion and/or adjustment of nutrients in the medium.

In those conditions, stress pushes the algal biosynthesis towards overproduction of lipids.

However, the disadvantage deriving from the application of different kinds of stress to the algal culture is a decrease in the yield of the overall biomass obtained from the process.

The biomass produced generally ranges from 0.1 till a maximum of 2 g/1 in autotrophic conditions; higher yields can be obtained in cases of mixotrophic conditions and only under optimal (non commercially sustainable) growth conditions (pure salts, right levels of illumination, small volumes of culture).

Moreover, algal scale up in big volumes is difficult due to several factors: i) difficulty in obtaining a correct mass movement, ii) selection of right levels of light inside the mass.

For photosynthesis to work, light must reach the algae. If a layer of algae is more than a few centimetres thick, organisms on the surface shade those underneath, blocking the sunlight. An alternative is to spread horizontally the algae. However, algae would need to cover an area of about 9 million hectares to

produce enough biodiesel to cover Europe's annual transport requirement of 370 billion litres.

Imposing light inside the biomass by various lamp sources can be also dangerous: it is known that high levels of light, instead of promoting photosynthesis, can cause an inhibition called photoinhibition at the level of the activity of photosystem II apparatus (Barber and Andersson 1992; Mattoo et al. 1999) .

In conclusion, the growth of algal biomass, particularly for biofuel production on an industrial scale, is considered still economically difficult and object of intense research (Gouveia and Oliveira 2009; Savage, 2011) .

Mutualistic symbioses are important ecological relationships that are generally defined as two or more species living together and providing benefit to each other. Several microalgal endosymbioses , for example involving flatworms and various protists contribute globally to the primary productivity of aquatic ecosystems (Fujishima 2009).

An example of a symbiotic cultivation for industrial production of an algal biomass is reported in US-A-2011/0045564 . This document discloses a co-cultivation of at least one algal species with at least one aerobic bacterial species and at least one diazotroph under continuous sustainable symbiotic conditions .

Such a cultivation process requires a minimal addition of exogenous nutrients but it is nevertheless affected by some drawbacks. This process, for example, requires a cultivation medium suitable to induce at least one nitrogen stress response in the cultured algal cells to obtain a high lipid production. Nevertheless, as already emphasized stresses applied to the algal culture can reduce the content of overall

biomass production.

There is, therefore, from both environmental and economic perspectives a pronounced need for novel methods able to i) provide high algal biomass growth with a minimal reliance on exogenous added fertilizers and chemicals to sustain the algal cultures (e.g. using surface water and/or groundwater as the primary culture medium) and ii) avoid the necessity of imposing stresses on the algal biomass for higher lipid production.

SUMMARY OF THE INVENTION

Considering these premises, the need is felt for better, more efficacious solutions able to provide an algal biomass producing process industrially sustainable .

According to the present description, the above-said object is obtained by means of the solution specifically recalled in the attached claims, which constitutes an integral part of the present description .

In one embodiment, the present description concerns a process for producing an algal biomass comprising:

i) inoculating in an aqueous cultivation medium at least a first algal species and at least a first protozoan species obtaining a symbiont co-culture, the at least first protozoan species being suitable to generate symbiosis with the at least first algal species ;

ii) cultivating the symbiont co-culture obtaining an algal biomass; and

iii) harvesting at least a portion of the algal biomass.

A preferred embodiment of the production process described herein concerns use of a symbiosis deriving from a protozoan, preferably a ciliate and/or a flagellate protozoan, containing symbiotic algal organisms inside its cytoplasm.

In one embodiment, the process herein described allows to produce algal biomass by minimal addition of exogenous nutrients (like waste organic substances and technical salts), thus maintaining production at low costs and environmentally friendly.

A further embodiment of the present description concerns further inoculating in the cultivation medium algae and/or cyanobacteria as preys which, providing nutrients deriving from their metabolic products, contribute to increase the yield of biomass production.

The production process disclosed in the present application is characterized by a continuous symbiotic co-cultivation system which provides several advantages in terms of high levels of biomass and lipid production.

Moreover, such a production process enables algal growth with an enhanced bioproduct yield (e.g., on a per-algal cell basis) with an easy scale up process. The total biomass is increased also due to the high reproduction speed of the protozoan.

In addition, both the alga and the protozoan have the ability to accumulate large amounts of lipids, which can be used as a feedstock for biofuel/bioliquid production without the need of generating any additional stress to the cultivating system.

Another advantage of the production process herein disclosed is that it is broadly applicable to different algae photosymbionts and can be practised with a broad range of suitable symbiotic protozoa.

The algal biomass obtained according to the

instant description is useful for bioproduction . The obtained algal biomass can be employed for production of (but not limited to) bioliquids, biofuel, biodiesel, bioethanol, biogasoline, biocrude, biogas, and also pharmaceuticals, therapeutics, antioxidants, nutraceuticals , cosmetics, cosmeceuticals , food, feedstock, dyes, colorants and bioplastic.

DETAILED DESCRIPTION OF SOME EMBODIMENTS

In the following description different embodiments will be described in detail, by way of non limiting example, with reference to a process for producing an algal biomass.

It is clear that while the experimental data provided below refer to use of a symbiosis between specific algal and protozoan species, the process herein described is broadly applicable to many types of algal species able to create a symbiotic system with protozoan species able to infect the algal organism in a permanent or transient manner.

In the description that follows, numerous specific details are presented to provide a thorough understanding of the embodiment. The embodiments can be practised without one or more of the specific details, or with other methods, components, materials, etc. In other instances, well-known structures, materials, or operations are not shown or described in detail to avoid obscuring aspects of the embodiments.

Reference throughout this specification to "one embodiment" or "an embodiment" means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment. Thus, the appearances of the phrases "in one embodiment" or "in a certain embodiment" in

various places throughout this specification are not necessarily all referring to the same embodiment. Furthermore, the particular features, structures or characteristics may be combined in any suitable manner in one or more embodiments.

The headings provided herein are for convenience only and do not interpret the scope or meaning of the embodiments .

An embodiment of the present description concerns a process for producing an algal biomass comprising:

i) inoculating in an aqueous cultivation medium at least a first algal species and at least a first protozoan species obtaining a symbiont co-culture, the at least first protozoan species being suitable to generate symbiosis with the at least first algal species ;

ii) cultivating the symbiont co-culture obtaining an algal biomass; and

iii) harvesting at least a portion of the algal biomass.

The process object of present description provides commercially adequate algal biomass yield with a high lipid content which, unlike stressed algae grown alone in culture without any other symbiont organism, can be sustained on a continuous symbiotic basis in both open and closed systems.

Moreover, the instant description provides a process for a sustainable continuous cultivation of algae-bearing protozoa with minimal addition of exogenous nutrients.

In the process herein described, a significant proportion of the macronutrients, necessary for the symbiotic culture, derives from the photosymbiont products continuously produced during symbiotic cultivation.

The source of a significant proportion of carbon, CO_2, magnesium, potassium, calcium, oxygen and other macronutrients for the living organisms of the symbiotic culture come, in fact, from metabolic cellular products deriving from the co-cultivation itself. More specifically, a portion of CO_2 and oxygen present in the cultivation medium is endogenously derived from the aerobic metabolism of the protozoan component. On the other hand, the algae can supply the host Paramecium with photosynthetic products, mainly maltose and sugars. Inside the host cell, the algae show a higher rate of photosynthetic oxygen production than in the isolated condition, thereby guaranteeing an oxygen supply for their host. The Paramecium can supply the algae with nitrogen components and CO_2.

Algae species suitable for the co-culture described in the present application include but are not limited to marine, brackish water and freshwater algae and include species that are derived from acidic or basic water. The algae species include micro and macro algal species like eukaryotic algae such as diatoms, green, red and brown algae and cyanobacteria.

Different algal species may require for their metabolic activity different cultivation conditions (temperature in the range from 21 to 35 °C, cultivation medium containing different salt concentrations, pH ranging from 6 to 8).

The lipid content of the algal biomass obtained by means of the process herein described is significantly higher than that obtained through stressed algae grown alone in culture without any other symbiont organism. The increased content of lipids ranges from 57-90% of the dried weight biomass, for the specific case of *Chlorella minutissima* infected by a *Paramecium sp.*, compared to the range of 30-50% obtained with the algae

alone .

A particularly preferred embodiment of the present description concerns the cultivation of symbiotic systems comprising a *Paramecium spp.* (a ciliate protozoan) with *Chlorella spp.*

In a most preferred embodiment, the process for production of an algal biomass provided very good results in terms of algal biomass yield using as the first algal species *Chlorella minutissima* and two different protozoa species able to generate symbiosis with *Chlorella minutissima,* namely *Tetrahymena pyriformis* and *Chilomonas Paramecium.*

According to an embodiment, the algal species and the protozoan species can be present in the cultivation medium in a ratio comprised between 500:0.1 and 50:5, preferably about 100:1.

According to a further embodiment, the inoculum in the cultivation medium of a second algal species and/or cyanobacteria as source of nutrients is provided. Such an addition increases the algal biomass yield in a long term cultivation. Such suitable algal species and/or cyanobacteria are not able to generate symbiosis with the protozoa species.

According to a still further embodiment, the algal biomass yield can be increased by harvesting a portion of algal biomass, optionally followed by adding new cultivation medium or water, wherein said operations may be performed repeatedly during a long term period. The harvested portion of the algal biomass may comprise algal and Paramecium biomass, as well as part of the cultivation medium.

In table 1 a list of protozoa species which can infect algal species suitable to create a symbiotic system efficiently usable in the process object of the instant application is provided (first and second

columns, respectively) . The third column provides a list of algae and cyanobacteria that can be used as exogenous nutrient of the symbiotic system.

Table 1.

Protozoa for symbiotic association	Algae for symbiotic association	Algae and Cyanobacteria as nutrient
Chilomonas Paramecium *Tetrahymena pyriformis* *Colpoda steinii* *Glaucoma scintillans* *Paramecium bursaria* *Paramecium aurelia* *Paramecium caudatum* *Climacostomum virens* *Euplotes daidaleos* *Vorticella spp.* *Stentor polymorphus* *Mayorella viridis* *Ophrydium naumanni* *Stentor araucanus* *Trebouxia impressa* *Trebouxia usneae* *Trebouxia jamesii* *Myrmecia biatorellae* *Dictyochloropsis reticulate* *Sea anemone symbiont* *Coccomyxa glaronensis* *Coccomyxa pringsheimii* *Mesodinium rubrum* *Paramecium sp.*	*Chlorella sorokiniana* *Chlorella vulgaris* *Chlorella minutissima* *Chlorella variabilis* *Parachorella kessleri* *Choricystis parasitica* *Chlorella sp.*	*c. ellipsoidea* *c. luteoviridis* *c. mirabilis* *c. saccharophila* *c. zofingiensis* *c. emersonii* *c. protothecoides* *c. pyrenoidosa* *c. salina* *Botrycoccus braunii* *Chroomonas salina* *Cyclotella cryptica* *Cyclotella spp.* *Dunaliella salina* *Dunaliella bardawil* *Dunaliella tertiolecta* *Euglena gracilis* *Gymnodinium nelsoni* *Haematococcus pluvialis* *Isochrysis galbana* *Monoraphidium minutum* *Monoraphidium spp.* *Nannochloropsis spp.* *Nannochloropsis occulata* *Nannochloropsis salina* *Tetraselmis suecica* *Tetraselmis chuii* *Nannochloris spp.* *Neochloris oleoabundans* *Nitzschia laevis* *Onoraphidium spp.* *Pavlova lutheri* *Phaeodactylum tricornutum* *Porphyridium cruentum* *Scenedesmus obliquus* *Scenedesmus quadricaula* *Scenedesmus spp.* *Skeletonema* *Stichococcus bacillaris* *Spirulina platensis* *Spirulina maxima* *Spirulina spp.* *Thalassiosira spp.*

According to the instant description, the expression "algal biomass" means the biomass obtained by the symbiont culture of the algal species and the protozoan species. Therefore, the algal biomass contains both the algal cells and the protozoan cells, as well as part of the aqueous cultivation medium.

According to the instant description, the expression "exogenous nutrients" means nutrient compounds that are added by the operator during the cultivation phase of the symbiont co-culture. Exogenous nutrients useful in the process herein described comprise, i.a. technical salts, glycerol, molasses, hormones (preferably vegetal natural hormones like for example cytochines or indoleaceutic acid), aminoacids (for example glutammic acid, asparagine, alanine, lysine), vitamins (for example A, B, C, PP, K vitamins), microelements (like boron, iron, manganese, molybdenum, zinc, copper, cobalt), humic substances like humic and fulvic acids, agro-industrial waste materials and plant detritus.

Symbioses, in general, are defined as two or more species living together in beneficial coexistence. This type of mutualistic interaction plays an important role in maintaining populations living under precarious environmental conditions.

In particular, algal-bearing protozoa are ubiquitous and abundant components in oceanic and freshwater systems of different trophic interactions.

The mixotrophic nutrition mode of algal-bearing ciliates, combining both phagotrophy and phototrophy, is considered to be an adaptation allowing exploitation of oligotrophic environments.

In symbiotic processes, phototrophic endosymbiont s are ingested by the host, but are able to escape

digestion and to utilize the waste products of the metabolism of their host. Mixotrophic organisms combine the advantages of a heterotrophic nutrition mode with autotrophic energy gain, through algal symbionts.

An example of a stable symbiosis is the mutualistic relationship between the ciliate *Paramecium bursaria* (Hymenostomatia) and unicellular green alga *Chlorella* (Trebouxiophyceae) . This symbiosis represents a permanent association with hereditary symbionts, where each algal cell is enclosed in an individual perialgal vacuole derived from the host digestive vacuole to protect from lysosomal fusion. The exclusive mutualistic relationship of *P. bursaria* with 'zoochlorellae' in natural conditions has long been considered as a fact, but aposymbiotic *P. bursaria* natural populations have been recently reported (Fujishima, 2009; Summerer, 2008).

Different *Chlorella* species (like for example, c. *vulgaris, P. kessleri*, c. *variabilis, c. sorokiniana* and c. *minutissima)* have been found to have distinct suitability for the establishment of stable symbioses in *P. bursaria* and infection rates have been shown to be affected by specificity of host and potential symbiont, such as recognition of surface antigens or by physiological conditions of the partners involved.

Actually, it is known that c. *ellipsoidea*, c. *saccharophila*, c. *luteoviridis*, c. *zofingiensis, c. mirabilis* and several other algal species are incapable to establish a symbiosis with *Paramecium bursaria.* However it is possible to adapt some *Chlorella spp*. to the host (Summerer, 2007) .

The presence of glucosamine in the rigid wall of the alga cells is a prerequisite for the realization of symbiotic association between *P. bursaria* and *Chlorella* species. On the contrary, the presence of glucose and

mannose in the rigid wall of algal cells characterizes "infection-incapable" algal species.

Many ciliates acquire photosynthesis capacity through stealing plastids or harboring intact endosymbiotic algae in not stable association. Both phenomena are a form of mixotrophy and are widespread among ciliates and flagellate.

Mixotrophic ciliates and flagellates are abundant in freshwater and marine ecosystems, sometimes making substantial contributions toward community primary productivity.

Unique adaptations may also be found in certain algal endosymbionts , facilitating establishment of symbiosis and nutritional interactions, while reducing their fitness for survival as free-living cells.

Certain strains of *M. rubrum* may have a stable association with their cryptophyte organelles, while others need to acquire a cryptophyte nucleus through feeding .

Very large numbers (3466 ml^{-1}) of ciliated protozoa were found living beneath the oxic-anoxic boundary in a stratified freshwater pond. Most ciliates (96%) contained symbiotic algae *(Chlorella* spp.). Peak abundance was in anoxic water with almost 1 mol free C02 m^{-3} and a midday irradiation of 6 μηοϊ $m^{-2}s^{-1}$. Photosynthetic rate measurements of metalimnetic water indicated a light compensation point of 1.7 μηοϊ $m^{-2}s^{-1}$ which represents 0.6% of sub-surface light. It has been calculated that photosynthetic evolution of 02 by symbionts is sufficient to meet the demand of the host ciliates for 13 to 14 hours each day. Each 'photosynthetic ciliate' may therefore become an aerobic island surrounded by anoxic water (Finlay et al., 1996) .

As previously reported, some ciliates (e.g.

Paramecium bursaria) form symbiotic relationship preferably with algae of *Chlorella* genus. Paramecium cells may harbour several hundreds of symbiotic *Chlorella* cells in their cytoplasm. Each symbiotic alga is enclosed in a special membrane called Perialgal Vacuole (PV), derived from the host Digestive Vacuole (DV) membrane of the protozoan. The Perialgal Vacuole is able to protect algal cells preventing the fusion to the host lysosoms.

By a biological point of view the symbiosis between the protozoan and the alga species takes place as follows:

– in the first stage, after mixing the two organisms, the alga appears in the cytoplasm by budding of the digestive vacuole membrane of the protozoan.

- in the second stage, after the algal appearance in the cytoplasm, the vacuole enclosing a single green alga differentiates into the perialgal vacuole from the digestive vacuole.

- in the third stage, the alga localizes beneath the host cell cortex. At about 24 h after mixing, the alga increases by cell division and establishes endosymbiosis .

- the final fourth stage is characterized by the presence of the condensed vacuolar membrane of *Paramecium* and by the brown color of digested algae which become small in diameter.

Various benefits are induced in both the host *Paramecium* and the algae by algal infection.

Alga-bearing *Paramecium* cells can divide better than the alga-free cells. Alga-bearing *Paramecium* cells show a higher survival rate than the alga-free cells under various stressful conditions, like for example administration to the culture of 0.5 mM nickel chloride

($NiCl_2$) or 150 mM hydrogen peroxide or exposing the culture to high temperatures (40°C).

Moreover, the host paramecia can receive protection against UV damage by their symbiotic algae which contain protecting substances which confers their capability to thrive in sunlit UV-exposed waters.

Furthermore, because the timing of the cell division of both algae and the host paramecia is well coordinated, the symbiotic algae can be distributed to the daughter cells.

The relationship between some species of *Paramecium* and *Chlorella* spp. is a mutualism and, therefore, the alga-free Paramecium cells and the symbiotic algae are still keeping the ability to grow without a partner (Fujishima, 2009).

Alga-free Paramecium cells can be produced easily from alga-bearing cells using one of the following methods: rapid cell division; cultivation under the constant dark condition, X-ray irradiation, treatment with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU - a blocker of electron flow in photosystem II), treatment with the herbicide paraquat or treatment with cycloheximide (an eukaryotic protein synthesis inhibitor).

On the other hand, symbiotic algae can be isolated from alga-bearing Paramecium cells by sonication, homogenization or treatment with some detergents.

Furthermore, by mixing the alga-free Paramecium and the isolated symbiotic algae, endosymbiosis can be easily established again.

Moreover, Paramecium species need simple cultivation conditions to get mass culture.

Applicants have unexpectedly discovered that symbiosis between an algal species and a protozoan species is very effective in terms of production of

algal biomass. The presence of the protozoan in the co-cultures provides for adequate and sustained algal growth and preserves the capability of high rate lipid production without needing application of any stress conditions to the culture.

Tetrahymena pyriformis is a teardrop-shaped, unicellular, ciliated freshwater protozoan about 50 µm long. *Tetrahymena* species are very common in aquatic habitats and are non-pathogenic, have a short generation time of about 2 h and can grow to high cell density in inexpensive media. *Tetrahymena pyriformis* structure is characteristic since it exhibits striking nuclear dimorphism: two types of cell nuclei, a large somatic macronucleus and a small germline micronucleus, exist in a single cell at the same time and carry out different functions with distinct cytological and biochemical properties. In addition, *Tetrahymena* possesses hundreds of cilia and microtubules in its cytoskeleton . These cylindrical polymers of tubulin can grow as long as 25 micrometers and are highly dynamic. Microtubules are important for maintaining cell structure, providing platforms for intracellular transport. Tetrahymena genus includes several species and the most common are: *T. pyriformis, T. thegewischi , T. hyperangulari , T. malaccensis , T. pigmentosa , T. thermophila and T. vorax.* Recently, the whole macronuclear genome has been sequenced for *Tetrahymena thermophila .*

Chilomonas Paramecium (19-30ym) occurs in 8 species, recently ascribed to *Cryptomonad* flagellatae. It is a colourless organism which contains a leucoplast. *Chilomonas Paramecium* shows a peculiar swaying swimming behaviour, caused by a stereospecif ic asymmetry in cell shape with clearly definable dorso-

ventral/right-left sides, and are easy to recognize due to a unique set of characters. It can be grown in relatively simple solutes even only with inorganic salts, it divides at a fairly uniform rate and it lives and thrives in a temperature range extending over twenty degrees.

Chilomonas Paramecium, when grown in a favourable environment, contains a large quantity of stored food material in the form of starch granules and neutral fats. Mast and Pace (1933) demonstrated that it produces starch, fats and proteins in a wholly inorganic medium, with one part of CO_2 added to 5 parts of air at atmospheric pressure. Starch and fat are by-products of metabolism which can be utilized as food materials under adverse conditions of nutrition.

In the following some non limiting examples of the process for producing an algal biomass according to the instant description are provided.

Production of algal biomass

Materials and methods

- **KOB medium** contains: 2g/l sodium acetate trihydrate; 2g/l yeast extract; O.lg/1 magnesium chloride anhydrous; O.Olg/1 ferrous sulphate heptahydrate ; 0.02g/l calcium chloride dihydrate.

- Chalkley's medium with grass seeds is obtained by dissolving 5ml of a stock solution (containing 20g/l NaCl; 0.8g/l KC1; 1.56g/l CaCl2 2H20) in 1L of distilled water and adding 0.7g/l of grass seeds and boiled for 15-20 min compensating water lost.

- **TAP medium** contains the following components that are added in the order: 10 ml/1 2M tris-acetate (pH 7); 10 ml/1 2M phosphate buffer (pH 7); 10 ml/1 Nutrient stock; 1 ml/1 Trace elements solution (10X dilution) . The different components are prepared as

stock solutions as follows:

→ Tris-acetate stock (100X) is obtained by dissolving 242 g Tris base in 600 mL water while titrating to pH 7 with glacial acetic acid and then brought to 1 L.

→ Phosphate buffer (100X) is obtained by dissolving in water 10.8 g K_2HPO_4, 5.6 g KH_2PO_4 and then bringing to 1 L.

→ Nutrient stock (100X) is obtained by dissolving 40 g NH4C1, 10 g MgS04 7H20 e 5 g CaCl2 2H20 in water and then bringing to 1 L.

→ Trace element solution (10X) is obtained as follows :

i. dissolving by heating 50g of acid free EDTA in 250 ml of bidistilled H2O;

ii. dissolving, in 550 mL bidistilled H2O by heating to approximately 100°C, the following components, one by one, in the following order: 11,4 g H3B03; 22,0 g ZnS04 7H20; 5,1 MnCl2 4H20; 4,9 g FeS04.7H20; 1,6 g C0CI26H2O; 1,6 g FeS04 5H20; 1,1 g $M07O2_4(NH4)_6$ 4H20;

iii. mixing the two solutions (i) and (ii) together, getting a blue-green solution;

iv. heating at 100°C,

v. cooling slightly, without letting the temperature to drop below 80°-90°C;

vi. adjusting pH to 6.5 to 6.8 with 20% KOH, while not letting the temperature dropping below 70°C until the pH is adjusted;

vii. bringing up to 1 L and let standing in a 2L Erlenmeyer flask, stopped loosely. The colour should change from green to purple over a period of days.

viii. removing the rose-coloured precipitate by

filtering, with suction, through 3 layers of Whatman #1® filter paper in a Buchner funnel. Repeat until no more precipitate is seen on the filter paper.

ix. storing in a brown bottle at 4°C.

- Glycerol used in this procedure is not pure, but derived from an industrial purification of vegetables from biodiesel plants. Before use, glycerol is purified with active carbon and after it is autoclaved at 1 Bar, 121 °C for 20 minutes.

– **Vigor ultra** is a commercial growth promoter (Hydrofert) used generally for plant growth and comprises: Aspartic acid 0.157g/100g; Glutamic acid 2.96g/100g; Alanine 0.252g/100g; Glycine 0.2g/100g; Leucine 0.112g/100g; Proline 0.139g/100g; 3-indoleaceutic acid 6.68 mg/kg.

The cultivation process occurred in a bioreactor known to the expert in the field, such as a cylindrical open plastic container containing surface water as aqueous cultivation medium. The bioreactor is provided with suitable detection systems for measuring at least pH and temperature in the cultivation medium.

Chlorella minutissima from UTEX collection with accession number Utex#2341 was used as algal species.

Chlorella minutissima was cultured using tap water sterilized by a UV lamp, and containing glycerol and vigor ultra as nutrients.

After two months culturing till a scale up of 100L, the culture resulted infected by protozoan populations, which are considered predators of algae, therefore to be avoided in growth studies.

However, it was unexpectedly found by the present inventors that when the culture was infected by

protozoa, it produced a visible higher quantity of biomass .

In order to isolate protozoan population from algae, a small part of this culture was held in the dark for 40 days at 28°C. Isolated organisms were shown to be a mixture of ciliate and flagellate protozoa of lower size than *P. bursaria*, extremely motile and easy to grow in various culture media, like TAP medium (Harris 1989), KOB medium (Kobayashi et al. 1991) or Chalkley' s medium (see composition above) .

A comparative morphological analysis involving different commercial protozoa (obtained from the Sciento and CCAP collections) showed that protozoa infecting *Chlorella minutissima* were a mixture of *Tetrahymena pyriformis* and *Chilomonas Paramecium,* present in different quantities depending on the age and aeration conditions of the culture. Under microscope analysis, both *Tetrahymena pyriformis* and *Chilomonas Paramecium* contained green *Chlorella* algae inside their body.

Chilomonas differs considerably in its metabolism from the ciliate *Tetrahymena.* The latter does not live in extremely high concentrations of CO_2 in which *Chilomonas* flourishes even when the carbon dioxide pressure reaches 400 mmHg.

However *Tetrahymena pyriformis* and *Chilomonas Paramecium* can grow together and this association is often positive.

Microstomatous forms of *Chilomonas* are observed in mixture with *Tetrahymena.* The percentage of microstomatous forms of *Chilomonas* is larger at the beginning of population growth. When the culture is young both *Tetrahymena* and *Chilomonas* mutually advantage in a sort of symbiotic association. However, disappearance of *Chilomonas* and cannibalism has been

observed in old mixed cultures.

For high algal biomass yield, the following conditions were applied.

Chlorella minutissima is stored at 26 °C in petri dishes on KOB medium. *c. minutissima* is then inoculated in TAP medium for a week at constant agitation, with temperature at 26°C and exposed to white light of 20 µmol/m²s.

Protozoa culture, containing *Tetrahymena pyriformis* and *Chilomonas Paramecium* (in the following Ps culture), is stored at 28°C in two alternative different media: TAP or Chalkley's medium. To keep the culture alive, it is advisable to refresh every week the culture medium with fresh medium.

The cultured protozoa are used to infect *Chlorella minutissima* grown in 150 ml TAP medium in the ratio (protozoa/alga) 1:10. Then, after 5 days culturing, the 150 ml mixture culture is used as inoculum to obtain a culture of 1.5L in the same TAP medium.

This new culture is kept in constant stirring at 28°C and exposed to a white light of 20-50 micromoles/m ^{2}s, for other five days.

Subsequently, this culture is used as inoculum for a new culture with a volume of 15L. This volume is obtained adding to the culture 2.5 g/L glycerol and 100 µI/L vigor ultra in tap water treated with UV and colloidal Ag (1:250).

The culture of algae plus protozoa is aerated and exposed to white environmental light at 28°C. Every day, culture is added of 2.5 g/1 glycerol; 100 µï/ï vigorultra.

After three days, ten cultures of 15L are joined together and scaled up to 200L with 2.5 g/1 glycerol, 100 µï/ï vigorultra and local water treated with UV and

overnight with colloidal Ag (ratio 1:200-500). This culture is ventilated and exposed to light of 10-15 micromoles/m ^{2}s at 28°C.

This culture can live up to 6 months or more. Every day 2.5 g/1 glycerol and 100 µï /ï vigor ultra are added to the culture.

In order to improve the vitality of microorganisms, every two or three days, the culture is refreshed harvesting the volume by 20 or 50 or 70%. The harvesting volume is taken at the bottom of the culture and can be used to fill other 200L containers to get an industrial module of hundred containers.

In case purchased *Chilomonas* and *Tetrahymena* paramecia are employed, the paramecia have to be conditioned to reach a good symbiosis with the algal species and consequently a good algal biomass yield.

Protozoa are cultured singularly in Chalkley' s medium with grass seeds or TAP medium.

After a week, *Chilomonas* and *Tetrahymena* are mixed together (ratio 1:1) or are used singularly to infect *Chlorella minutissima* in TAP medium (ratio protozoa (n) /alga 1:10) to obtain a mixed culture with all two/three microorganisms.

At this stage, mixture culture can be grown for a long time; for adaptation at least 2 months are required, refreshing it every week (half volume) by Chalkley' s medium with grass seeds or TAP medium. The quantity of algae in endosymbiosis increases with time of co-culturing and also the size of the bearing-algae protozoa and of algae increase as seen in (Nakajima T., Sano A . and Matsuoka H., 2009) . This culture can be further adapted to the nutrients glycerol and vigorultra in quantities of one fourth of the optimum concentration, increasing gradually with each refresh

with an amount of 20% until arriving at the optimum 100% (of 2.5 g/L glycerol and 100 µl/L vigor ultra) .

Different physical states for the mixed culture of microorganisms were identified:

- Motile in which the paramecia move quickly and prey algae;
- Motile in which paramecia show intact algae inside their bodies and increase greatly their size, *Tetrahymena* from 50ym increases till 150ym and *Chilomonas* from 20ym increases till lOOym or more ;
- The two paramecia can coexist in the same culture or one type, mainly *Tetrahymena ,* can be preponderant ;
- Non motile, with algae inside: in this state, called cyst, the Paramecium suspends animation and cell metabolic activities are slowed down. Unfavourable environmental conditions - such as lack of nutrients or oxygen, extreme temperatures, lack of moisture, presence of toxic chemicals, which are not conducive for the growth of the microbe - trigger the formation of a cyst;
- Algae that initially are single and have small dimension of lOym, after infection greatly increased till 3 times;
- Algae join together in big aggregations mainly over the cysts;
- The macro culture shows green, yellow and brown biomass with a state similar to glue, mostly precipitated and in part floated;
- Inside the culture it is possible to observe formation of microtubuline filaments;
- With culture refresh, encysted microrganisms reach an environment favorable to growth and

survival, the cyst wall breaks down by a process known as excystation and the forms become again motile ;

- After refreshment, culture colour pass through different colours: green, yellow and brown; for a high biomass production it is useful/advisable to maintain the co-culture in the above green or yellow stage since brown stage corresponds to partially digested alga, by means of repetitive dilutions of the culture medium. These repetitive dilutions cause an increased division of the alga-bearing Paramecium and the re-establishment of the green colour of the co-culture followed again by the various coloured phases .
- *Chlorella algae* result highly protected in this endosymbiotic process. *Chlorella* alone culture, using waste material as nutrient, does not survive over 7 and occasionally 30 days while, when infection with protozoa occurs, it can survive up to several months. Moreover, when the culture is not infected with the identified paramecia, it becomes prey of several other types of predators that do not establish endosymbiosis (e.g. rotifers) . Generally, culture of *Chlorella* alone rapidly decrease with a consequent high viscosity due to the release of microtubules in association with glycerol.

Weight of algal biomass was determined by harvesting 20% of the culture at the base of the bioreactor. 5 samples of 100 mL of algal biomass were taken, under strong aeration for homogeni zat ion, and subsequently dried at 90°C for at least 10 days till a weight constancy and production of a fine powder.

Therefore, as already stated, the algal biomass contains both the algal cells and the protozoan cells, as well as part of the aqueous cultivation medium.

Weight of algal biomass produced by the above described co-culture symbiotic system was compared to the weight of algal biomass obtained by a culture of algae without protozoa.

Results

Example 1 - Evaluation of algal biomass production by the co-culture symbiotic system (*C. minutissima* plus *Tetrahymena pyriformis* and *Chilomonas Paramecium)* vs. alga alone

Biomass yields produced by a minimal exogenous nutrition of technical degree of the above described co-culture symbiotic system (Materials and Methods section) and of the culture of the alga grown alone are reported in table 2.

Table 2.

days of culture	*Chlorella minutissima*	*Chlorella minutissima*-bearing *Paramecium sp*
Biomass produced in g/1		
1	1	1
2	0.85	1.15
3	1.51	1.82
4	1.44	2.37
5	1.77	5.82
6	2.25	5.05
7	2.43	6.88
8	2.76	7.38
9	2.93	7.96
10	3.42	7.04
11	3.64	7.32
scale up: cultivation medium volume was doubled with water		

days of culture	*Chlorella minutissima*	*Chlorella minutissima*-bearing *Paramecium sp*
12	2.08	3.49
13	2.58	8.64
14	2.89	10.52
15	3.03	12.51

The relative standard deviation, calculated on n=6 sampling of one typical pattern of growth, was less than 10%.

The results of table 2 show a higher yield of biomass (till 2-3 times) produced by the co-culture system after some days than that obtained by the culture of the alga grown alone. Particularly, the doubling of the culture medium volume leads to an increase of the yields of more than 4 times of *Chlorella-bearing* Paramecium compared to *Chlorella* grown alone.

Moreover, the experiment demonstrates that the scale up of algal symbiont is suitable more than for algae alone leading to an increase in yield of biomass.

Example 2 - Evaluation of infection specificity of different algal species

In order to define specificity of infection, Ps culture was mixed with other algae belonging to the genus *Chlorella* and comparison between different associations was performed by relative weight of the same quantity of all cultures. This test was performed using the TAP and KOB media, exposed to red light at 10 ymol/m^2 s, in continuous low agitation and harvested at the same day. The results are provided in table 3.

Table 3.

Algae	Media	Alga alone	Algae infected with Ps culture 100:1
Chlorella minutissima	TAP	+	+++

Algae	Media	Alga alone	Algae infected with Ps culture 100:1
Chlorella sorokiniana H1957		+	++
Chlorella sorokiniana H1986		+	+++
Chlorella vulgaris		++	+++
Choricystis parasitica		+	++
Chlorella minutissima	KOB	+	+
Chlorella sorokiniana H1957		+	++
Chlorella sorokiniana H1986		+	++
Auxenochlorella protothecoides		+	+
Choricystis parasitica		+	+

We observed that in presence of protozoa, algae were aggregated and under microscope they were larger than those in single culture. In this experiment, we found that in TAP medium, the aggregation is more evident in presence of *Chlorella minutissima* and *Chlorella sorokiniana* H1986, while in KOB medium formation of endosymbiosis seems to be inhibited.

Example 3 - Evaluation of further inocula of algae-bearing Paramecium(a) on overall algal biomass yield

The symbiotic culture was obtained as described in Materials and Methods section, in two months culturing. The only difference is that further inocula of algae-bearing Paramecium in TAP medium were performed to the culture medium at different time intervals.

Relative amounts of algal cells/paramecium at the beginning were maintained in a ratio of about 100:1 and addition of algae-bearing Paramecium (in the same ratio of 100:1 for the algal cell/paramecium symbiont) was repeated every 3 days. The algae-bearing Paramecium were freshly prepared and added in the second stage of infection .

The repetitive addition of symbiotic organisms provided a higher biomass yield, as shown in table 4,

wherein the biomass production after addition of fresh algae-bearing Paramecium is provided. The table represents a typical pattern of growth.

Table 4.

Day	Dried weight (g/L) Symbiont
1	0.9
2	4.9
3 - addition	6.2
4	8.9
5	10.7
6 - addition	19.2
7	22.3
8	29.4
9 - addition	20.2
10	18.0
11	19.6
12 - addition	20.5
13	20.3

The relative standard deviation, calculated on n=6 sampling of one typical pattern of growth, was less than 10%.

Example 4 - Evaluation of repetitive harvesting of algal biomass during the production process on overall algal biomass yield

The alga-bearing Paramecium was obtained as described in Materials and Methods section, in two months culturing. The culture was grown in a vessel of 15L for 6 months adding quantity of glycerol 2.5 g/1 and 100 μï /ï of vigor ultra per day; a process of harvesting 20-50-70% of the algal biomass and water re-addition to the initial volume was regularly applied on alternate days or on consecutive days.

Table 5 shows that higher biomass yields were obtained one day after harvesting the algal biomass and cultivation medium re-addition to initial volume, that was accompanied by the typical cycle of colour steps: green-faintly yellow-brown. The process could be

repeated without any production interruption.

Results reported in table 5 (representing a typical pattern of growth) show that during a 20 days production, harvesting alternatively 20-50% of the biomass, and re-addition of water in the co-culture on alternative days lead to high yield of algal biomass till about 10 g/1.

On the contrary, daily repetition of harvesting and re-addition of water leads to poorer yields of about 2g/l.

Table 5.

Day	-	Dried weight (g/L) Symbiont
1	harvesting/water re-addition	5.59
2	-	2.71
3	harvesting/water re-addition	14.57
4	-	2.01
5	harvesting/water re-addition	8.51
6	-	2.02
7	harvesting/water re-addition	4.33
8	-	4.83
9	harvesting/water re-addition	5.40
10	harvesting/water re-addition	2.32
11	harvesting/water re-addition	1.62
12	harvesting/water re-addition	2.24
13	harvesting/water re-addition	2.45
14	-	2.74
15	-	4.01
16	harvesting/water re-addition	3.23
17	-	6.06
18	harvesting/water re-addition	8.54
19	-	3.45
20	harvesting/water re-addition	10.02

The relative standard deviation, calculated on n=6 sampling of one typical pattern of growth, was less than 10%.

Interestingly, culture realized according to the process herein described can be maintained for months. As usual for biological material, seasonal differences in the yields of accumulated biomass are observed from a minimum of 1-2 g/1 and occasionally till a maximum of 20-30 g/1.

Example 5 - Evaluation of inoculum(a) of algae/ cyanobacteria in the culture medium during the production process on overall algal biomass yield

Symbiotic culture was obtained as described as described in Materials and Methods section, in two months culturing. The only differences are that glycerol was reduced to 1 g/L and *Chlamydomonas reinhardtii* cells were added to the co-culture as additional exogenous nutrient.

This example illustrates that the addition of a second alga species (in TAP or Chalkley's medium) to the co-culture is useful to reduce content of organic nutrient (in the specific case glycerol).

The results reported in table 6 (representing a typical pattern of growth) show that addition of such digested algae to the co-culture leads to higher yield of algal biomass production compared to the biomass obtained by a culture of algae grown alone (see for comparison table 2 second column).

Table 6.

Day	Biomass dried weight (g/L) Symbiont + nutrition with alga chlamydomonas
1	0.9
2	4.0
3	6.1
4	6.2

Day	Biomass dried weight (g/L) Symbiont + nutrition with alga chlamydomonas
5	8.8
6	12.1
7	11.0
8	12.3
9	11.5
10	10.1
11	15.3
12	18.7
13	19.0

The relative standard deviation, calculated on n=6 sampling of one typical pattern of growth, was less than 15%.

Example 6 - Evaluation of different paramecia/ algae combinations on overall algal biomass yield

In the present example, we have tested different paramecia/algae combinations, in order to determine if biomass production increased and if symbiosis occurs with other paramecia.

We used as microalgae *C. minutissima* (CM) and C. *sorokiniana* H1986 (CS) and as protozoa *P. bursaria*, *P. caudatum* and Ps culture.

Algae were cultivated in TAP medium at 26°C, under red light and in continuous shaking. After three days, we added at each inoculum one Paramecium culture, in a ratio alga/paramecium of 1:100. After two days, we have transferred each culture in containers of 18L and we started scaling up procedure, doubling volume every two days adding UV sterilized tap water, 2.5 g/1 glycerol and 100µ1/1 vigor ultra. After reaching the final volume to invigorate cultures, every day the harvesting of half volume was carried out, replaced by an equal volume of water. This experiment was carried out for 20 days and the results are reported in table 7.

Table 7.

Name	Biomass - dry weight on 3th day (g/1)	Biomass - dry weight on last day (g/1)
CM+Ps	1.5	2.2
CM+P. *bursaria*	4.8	1.0
CM+P. *cauda turn*	3.5	1.0
CS+Ps	1.6	2.0
CS+P. *bursaria*	1.3	2.5
CS+P. *caudatum*	1.4	2.0

The relative standard deviation, calculated on n=4 sampling of one typical pattern of growth, was less than 16%.

These data indicate that at the beginning of the experiment, associations between CM/*P. bursaria* and CM/*P. caudatum* are more productive than association with Ps, but at the end, productivity of these two cultures is reduced. CM+Ps culture instead is more productive towards the end of the experiment, indicating that the presence of these protozoa, determines an adaptation to endosymbiosis in a longer term.

In the case of the alga *C. sorokiniana,* the production of the three different cultures gives almost the same biomass.

Example 7 - Evaluation of *Chlamydomonas reinhardtii* as infected alga on overall algal biomass yield

In order to demonstrate Chlorella symbiosis specificity, we have co-cultivated the same paramecia indicated above with *Chlamydomonas reinhardtii* (CR).

In this experiment, algae were cultivated in TAP medium at 26°C, under white light and in continuous shaking. After two days, we added at each inoculum one Paramecium culture, in a ratio of 1:100. After two days, we have transferred each culture in bottles of 1L and started scaling up procedure, doubling volume every two days, till 800 mL, adding UV sterilized water,

2.5g/l glycerol and 100µ1/1 vigor ultra. After reaching the final volume of 800 mL, to invigorate the cultures, every two days the harvesting of half volume was carried out replaced by an equal volume of fresh nutrients.

This experiment was carried out for 20 days and results are reported in table 8.

Table 8.

Name	Biomass - dry weight on 7°day (g/l)	Biomass - dry weight on last day (g/l)
CR	0.9	0.8
CR+ *P. bursaria*	1.3	0.9
CR+ *P. caudatum*	0.8	0.2
CR+Ps	1.8	0.9

The relative standard deviation, calculated on n=4 sampling of one typical pattern of growth, was less than 16%.

The yield obtained in this experiment are poor compared to previous ones, especially at the end of the experiment, indicating that the presence of paramecia do not result in an increase of the productivity of the *Chamydomonas reinhardtii* culture and that this alga species is not able to establish symbiosis with the tested protozoa.

Example 8 **- Characterization of algal biomass composition**

Algal biomass has been produced according to Example 4.

The particular composition of the biomass was found to comprise cells, lipids, starch, microtubules, and soluble sugars such as glycogen, explaining that it appears as a "glue". The biomass was mostly present as precipitate in big aggregations, in part floating and in part in solution in a ratio depending on the age of

the culture. In conditions of daily nutrition of 2.5g/l glycerol and 100µ1/1 vigor ultra, with scale up to 200L, the content of lipids is preponderant and increases with the age of the culture (table 10) .

The age of the culture was calculated as days of culture starting from the first infection day, after mixing the two microorganisms, and when symbiosis was established, co-culture and algal culture were compared for the content of total lipids.

Table 10 provides the average lipid content per dried biomass in percent determined by the gravimetric method of Logan 2008.

Table 10 shows that the lipid content greatly increased in the co-cultivation system compared to the culture of the alga grown alone from 47 till 90% of lipid per dried biomass after infection of algae with Paramecium for a long time indicating that lipid content increases with the age of the culture after the infection .

Table 10.

days of culturing	% of lipids per dried biomass	
	alga alone (CM)	Symbiont (Ps+CM)
7	47	57
30	40	87
90	n.d.	90
120	n.d.	91

The relative standard deviation, calculated on n=6 sampling of one typical pattern of growth, was less than 10%.
n.d. not determined since the culture of alga alone did not resist 90-120 days in culture. Most of the cultures with algae alone and waste material die after only 7 days and occasionally lasted till 30 days.

For the characterization of the samples, analysis by gas chromatography of the single lipid components was performed by lyophilizing the culture samples.

Then, the samples were extracted by organic solvents (methanol :chloroform 1:2, see extraction method Al in Table 12) . The organic phase contains neutral lipids, colesteryl esters and phospholipids (indicated with PC, PsE, PnE, PI, PS) . The analyses were repeated twice in double. The extracts were transesterif ied with NaOH/MeOH for 25 min. The lipids containing fatty acids were transformed into the corresponding fatty acid methyl esters (FAME) with a quantitative yield (>97%) . Quantification was performed by calibration using standards. *Method GC:* Injection split (50:1) of FAME on GC Agilent 6850 column 60m × 0.25mm χ 0.25ym of (50%-cyanopropyl) -methylpolysiloxane DB23, Agilent) with flame ionization as revealing system. Temperature of the injector: 230 °C. Conditions: initial temperature 195 °C for 26 min, increased by 10 °C/min till 205 °C, maintained for 13 min, followed by a second increment of 30 °C/min till 240 °C, maintained for 10 min. Constant pressure (29 psi) with hydrogen gas carrier.

In table 11 the quantities of fatty acids contained in the biomass treated samples are expressed in percentage to obtain a whole vision of the categories of produced fatty acids. It is clear that there is a slight different distribution of the various lipid classes.

In the tested conditions of temperature, nutrition and selected extraction method, the first sample (Ps+CM) is richer on PUFA (about 35%) than the CM sample (about 26%) . In details, the sample PS+CM has a slightly major production of arachidonic acid omega-6 (about 29% against 20% for CM) and similar production of DHA omega-3 (about 6%) . *CM+caudatum* lipid distribution is more similar to CM+Ps production than *CM+bursaria.*

Table 11.

Fatty acids	Ps+CM	Bursaria+CM	Caudatum+CM	CM
14:0	10.16	1.20	7.81	12.27
16:0	24.88	30.73	25.52	23.69
9c-16:1	1.45	1.58	2.01	2.19
17:0	1.13	3.41	1.49	2.02
18:0	6.26	7.89	5.32	6.42
9c-18:1	8.93	14.21	12.35	13.29
11c-18:1	4.99	6.42	4.99	6.99
18:2 omega-6	17.87	11.85	19.36	13.91
18:3 omega-6	2.84	1.09	1.66	1.60
18:3 omega-3	3.28	4.56	4.38	2.62
18:4 omega-3	0.95	0.48	0.72	0.57
20:3 omega-6	1.51	2.91	1.33	2.05
20:4 omega-6	6.61	0.75	5.32	2.77
20:5 omega-3	0.79	2.65	0.75	0.23
22:0	2.43	6.07	2.50	4.20
22:6 omega-3	0.89	2,05	1.14	2.21
24:0	5.04	2.16	3.35	2.96
SFA	49.89	51.46	46.00	51.57
MUFA	15.36	22.21	19.35	22.48
OMEGA-6	28.83	16.60	27.67	20.33
OMEGA-3	5.92	9.74	6.98	5.63
PUFA	34.75	26.34	34.65	25.96

In Table 11 the yield of FAME obtained by three methods of extraction are reported for CM+Ps. We performed a comparison of lipid production using different extractions and esterification methods:

- Method A1: extraction with methanol-chloroform 1:2 and esterification by NaOH in methanol;
- Method A2: extraction with methanol-chloroform 1:1 and esterification by NaOH in methanol;
- Method A3: extraction with methanol-chloroform 1:1 and esterification by NaOH in methanol/benzene .

Between the two extractive methods A2 and A3 we obtained major components with more alcoholic functions (mono-glycerids and diglycerids) compared to the method A1 that is for apolar and phospholipidic components. The total lipid amount extracted with method A1 and method A2 is similar while, fatty acid composition

changes after the esterification procedure due to the different composition of lipids. The third column on the right shows how the quantity of fatty acids varies using the second method of esterification that allows to transform efficiently the cholesterol components. The method A1 has allowed to derivatize phospholipid components and triglycerides, while the method A3 has allowed to derivatize well the components of cholesterol .

Table 12.

Fatty acids	% Fatty acids		
	Extraction method		
	Method A1	Method A2	Method A3
14:0	10.16	6.03	5.92
16:0	24.88	18.16	18.46
9c-16:1	1.45	2.16	1.63
17:0	1.13	0.59	0.70
18:0	6.26	4.72	4.52
9c-18:1	8.93	17.70	13.84
11c-18:1	4.99	7.55	4.55
18:2 omega-6	17.87	18.36	19.02
18:3 omega-6	2.84	5.60	8.09
18:3 omega-3	3.28	2.29	2.41
18:4 omega-3	0.95	0.65	0.70
20:3 omega-6	1.51	7.45	9.98
20:4 omega-6	6.61	3.96	3.66
20:5 omega-3	0.79	0.56	0.44
22:0	2.43	0.74	1.53
22:6 omega-3	0.89	0.51	0.48
24:0	5.04	2.93	4.05
SFA	49.89	33.17	35.18
MUFA	15.36	27.41	20.02
OMEGA-6	28.83	35.37	40.75
OMEGA-3	5.92	4.01	4.03
PUFA	34.75	39.38	44.78

In addition, to screening microalgae for elevated biomass productivity and intrinsic cellular lipid content, the fatty acid profile of microalgae is an

important characteristic as it ultimately affects the quality of biodiesel production. The length of carbon chain of saturated and unsaturated fatty acids affects biodiesel properties such as cetane number, oxidative stability and cold-flow properties. Generally, high proportion of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) are preferred for increased energy yield, superior oxidative stability, and higher cetane numbers. However, oils dominated by these fatty acids are prone to solidify at low temperature. While oils rich in polyunsaturated fatty acids (PUFAs) have very good cold-flow properties, they are, on the other hand, more susceptible to oxidation.

Results in tables 11 and 12 show that the majority of fatty acids presents in isolated cultures were C16:0 (about 23-25%), 16:1 (about 10-12%) and omega-3 and 6 which comprised 35-40% of the total FAME. SFA and MUFA were predominant at >60% of the total lipid content which is favourable for high cetane number. However, also PUFAs in range of 35-45% exceeded the requirements in the International biodiesel Standard for Vehicles (EN14214) . PUFA are high-value fatty acids for nutrition, food additives and aquaculture nutrients. In order to comply with biodiesel standard on the PUFA ratio, these components can be hydrogenated or extracted before the rest of oil is converted into biodiesel. That makes the oils derived biodiesel less susceptible to oxidation in storage and takes full advantage of algae-paramecium symbiotic culture.

Naturally, details of implementation and the embodiments may vary widely with respect to what is described and illustrated without thereby departing from the scope of protection of the present invention, as defined in the annexed claims.

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CLAIMS

1. Process for producing an algal biomass comprising :

i) inoculating in an aqueous cultivation medium at least a first alga species and at least a first protozoan species obtaining a symbiotic co-culture, the at least first protozoan species being suitable to generate symbiosis with the at least first algal species;

ii) cultivating the symbiotic co-culture obtaining an algal biomass;

iii) harvesting at least a portion of the algal biomass .

2. Process according to claim 1, wherein step i) provides for inoculating at least a second protozoan species, the at least second protozoan species being different from the first protozoan species and being suitable to generate symbiosis with the at least first algal species.

3. Process according to claim 1 or claim 2, wherein the at least first algal species and the at least first protozoan species are present in the cultivation medium in a ratio alga/protozoan comprised between 500:0.1 and 50:5, preferably about 100:1.

4. Process according to claim 2, wherein the at least first algal species and the at least second protozoan species are present in the cultivation medium in a ratio alga/protozoan comprised between 500:0.1 and 50:5, preferably about 100:1.

5. Process according to any one of the preceding

claims, wherein in phase i) or in phase ii) a further inoculum of a) at least a second alga species and/or b) at least one cyanobacterium species is provided, wherein the at least second alga species is not suitable to generate symbiosis with the at least first protozoan species.

6. Process according to any one of the preceding claims, wherein the aqueous cultivation medium is selected among at least one of ground water, surface water, brackish water, salt water, sea water, marine water, lake water, river water, waste water, and tap water .

7. Process according to any one of the preceding claims, wherein at least one exogenous nutrient is added to the cultivation medium in phase i) and/or phase ii) .

8. Process according to claim 7, wherein the at least one exogenous nutrient is selected among salts, glycerol, vegetal natural hormones, aminoacids, vitamins, microelements, humic substances, agro-industrial waste materials.

9. Process according to any one of the preceding claims, wherein during phase ii) a gentle stirring of the cultivation medium is carried out.

10. Process according to any one of the preceding claims, wherein phase ii) is realized alternating exposure to light and dark.

11. Process according to any one of the preceding claims, wherein phase ii) is carried out at a

temperature between 21 and 35°C, preferably between 25 and 30°C.

12. Process according to any one of the preceding claims, wherein the aqueous cultivation medium has a pH ranging from 6 to 8, preferably from 6.8 to 8.

13. Process according to any one of the preceding claims, wherein during phase ii) one or more further inocula of the at least first alga species and the at least first protozoan species is/are performed.

14. Process according to any one of claims 2 to 13, wherein during phase ii) one or more further inocula of the at least first alga species and the at least second protozoan species is/are performed.

15. Process according to any one of the preceding claims, wherein during phase ii) at least one dilution of the aqueous cultivation medium is performed by adding further aqueous cultivation medium.

16. Process according to any one of the preceding claims, wherein phase iii) is carried out by repetitive harvesting a portion of the algal biomass.

17. Process according to claim 16, wherein subsequently to each harvesting operation an addition of further aqueous cultivation medium is performed.

18. Process according to any one of the preceding claims, wherein the at least first protozoan species is selected from the list in table 1.

19. Process according to any one of claims 2 to

18, wherein the at least second protozoan species is selected from the list in table 1.

20. Process according to any one of the preceding claims, wherein the at least first protozoan species is a Paramecium species.

21. Process according to any one of the preceding claims, wherein the at least first algal species is a Chlorella genus species.

22. Process according to any one of claims 2 to 21, wherein the at least first protozoan species is *Chilomonas Paramecium* and the at least second protozoan species is *Tetrahymena pyriformis*.

23. Process according to any claim 21, wherein the at least first algal species is selected among *Chlorella sorokiniana*, *Chlorella vulgaris*, *Chlorella minutissima*, *Chlorella variabilis*, *Parachorella kessleri*, *Choricystis parasitica*.

24. Process according to any one of claims 3 to 23, wherein the at least second alga/cyanobacteria species is selected from the list in table 1.

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2012/057336

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N1/10 C12N1/12 C12P7/64
ADD.

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)
C12M C12N C12P

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 , BIOSIS, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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A	page 65 - page 74	1-17

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 April 2013	16/04/2013

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

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INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2012/057336

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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