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(54) **Title**: EXTRACTION OF SCENEDESMUS CELL COMPONENTS

(57) **Abstract**: The technology disclosed herein relates to novel methods and compositions for the production processes of algae cell components from algae of the genus Scenedesmus. The extractability of algae cellular components is improved and the process is optimized and accelerated by using the enzyme compositions according to the present invention.

# Extraction of *Scenedesmus* Cell Components

## FIELD OF THE DISCLOSURE

The technology disclosed herein relates to novel methods and compositions for the production processes of algae cell components from algae belonging to the genus *Scenedesmus*. The extractability of the algae cellular components is improved and the process is optimized and accelerated by using the enzyme compositions according to the present invention.

## BACKGROUND

Generally, algae are grouped into two categories – microalgae and macroalgae – based on their morphology and size. As the name indicates, microalgae are microscopic photosynthetic organisms, many of which are unicellular. On the contrary, macroalgae which are commonly known as seaweeds, are composed of multiple cells which organize to structures resembling roots, stems, and leaves of higher plants.

Microalgae are thought to be one of the earliest life forms on earth and they are the fastest growing plants in the world. Since they can inhabit diverse ecological habitats ranging from freshwater, brackish water, or seawater, they are equipped to thrive in various extreme temperatures and pH conditions. These peculiarities make microalgae the most abundant organisms on earth.

Macroalgal in general, such as, *Laminaria*, *Saccorhiza*, *Alaria* are belonging to brown algal group and grows up to meters and their main reserved food material is laminarin and mannitol. The red algae such as *Gelidium amansii*, which is composed of cellulose, glucan and galactan, also can serve as a potential feedstock for bioconversion to ethanol.

In addition, algae yield unique biochemical substances which may be pharmacologically active, for example as viral inhibitors or cell division inhibitors, and substances which can serve as gelling and thickening agents and are used e.g. in the food industry. For example, carotenoids like astaxanthin, used as an antioxidant in human nutrition can be produced in green algae such as *Haematococcus pluvialis*. From the vast number of known marine and freshwater species of microalgae, only handfuls are currently of commercial significance. These include *Chlorella, Spirulina, Dunaliella* and *Haematococcus*.

Cultivation is the main way to generate biomass from microalgae. This has been done at industrial scale for many years. The most common production systems employed for algal cultivation are outdoor open ponds and enclosed photobioreactors (PBR). Production systems vary in terms of growth parameters control, contamination, water evaporation, productivity, downstream processing characteristics, capital and operational costs, etc.

Harvesting produces a slurry material with 2 - 7 % algal concentration. The next step is dewatering in order to get 15 to 25% concentration. This is usually achieved by pressing or centrifugation. These steps are normally integrated in the harvesting operation. Drying may be necessary for some applications. A very important issue in biomass treatment is the preservation of chemical quality.

The recovery of microalga biomass which generally requires one or more solid–liquid separation steps is a challenging phase of the algal biomass production process, and can account for 20–30% of the total costs of production. The processes involved include flocculation, filtration, flotation, and centrifugal sedimentation; some of which are highly energy consuming. Generally, microalgae harvesting is a two stage process, involving: (1) Bulk harvesting aimed at separation of biomass from the bulk suspension. The concentration factors for this operation are generally 100–800 times to reach 2–7% total solid matter. This will depend on the initial biomass concentration and technologies employed, including flocculation, flotation or gravity sedimentation. (2) Thickening—the aim is to concentrate the slurry through techniques such as centrifugation, filtration and ultrasonic aggregation, hence, are generally a more energy intensive step than bulk harvesting.

The harvested biomass slurry (typical 5–15% dry solid content) is perishable and must be processed rapidly after harvest; dehydration or drying is commonly used to extend the viability depending on the final product required. Methods that have been used include sun drying, low-pressure shelf drying, spray drying, drum drying, fluidized bed drying and freeze drying. Spray drying is commonly used for extraction of high value products, but it is relatively expensive and can cause significant deterioration of some algal pigments. Freeze drying is equally expensive, especially for large scale operations, but it eases extraction of oils. Intracellular elements such as oils are difficult to extract from wet biomass with solvents without cell disruption, but are extracted more easily from freeze dried biomass. The cost of drying is an important consideration in the processing of microalgal biomass powder for the food and feed industry and especially for the emerging biofuels industry.

Cell disruption is often required for recovering intracellular products from microalgae and performed to release intracellular products into the culture broth making them available for further separation processes. Cell disruption methods that have been used successfully include bead milling, high-pressure homogenisers, autoclaving, ultrasonication super critical CO2 extraction and addition of hydrochloric acid, sodium hydroxide, or alkaline lysis. Solvents, like e.g. hexane, are widely used to extract metabolites such astastaxanthin, beta-carotene and fatty acids from algal biomass. Properties of the cell membrane play an important part in solvent extraction process. For example, the presence of a cell wall may prevent direct contact between the solvent and the cell membrane and impede the extraction.

One of the key challenges remains in development of aqueous extraction technologies as algal cells vary greatly in compositions between the species.

A variety of aquatic microalgae, including the green alga *Scenedesmus*, have been studied for their possible efficacy as bioresources for applications as fish feed, human food, supplemental human nutrients and pharmaceutical products (Belay et al., 1993), and also for the bioremediation of polluted water (Chong et al., 2000). Scenedesmus is an ubiquitous organism, and frequently is a dominant microalga in freshwater lakes and rivers (Borowitzka and Borowitzka,1998). The green algae are among the most common and taxonomically diverse of the chloroccalean genera, within which over 200 species, and almost 1200 infraspecific taxa, have been identified (John et al., 2002). Previous studies of Scenedesmus have focused principally on continuous or semicontinuous cultures, using culture media modified in a variety of ways (Voltolina et al., 1998; Adamsson, 2000).

As mentioned above, the genus *Scenedesmus* is one of the most common genera of the green algae. The cells are usually arranged in a row to form 4- 8 cells colony. The cells in the end often have spines or the spines are present on several or all the cells. *Scenedesmus* algae are part of the Chlorophyta (= the green algae). Biochemical and physiological changes in microalgal cells, such as the cells of Scenedesmus, can be affected by the culture media, growing conditions, and nutrient compositions, in addition to gene transformation technology (McLachlan, 1973; Nichols, 1973; Kim and Giraud, 1989; Kim and Smith, 2001). *Scenedesmus* are known for its resistance to enzyme degradation.

*Scenedesmus* contains numerous bioactive compounds that can be harnessed for commercial use like chlorophyll. Chlorophyll is one of the most valuable bioactive compounds that can be extracted from microalgal biomass. It is used as a natural food coloring agent and has antioxidant as well as antimutagenic properties. The process of extracting chlorophyll from *Scenedesmus*, microalgae begins normally with dewatering and desalting the highly dilute microalgal culture (biomass concentration = 0.1–1% w/v). Chlorophyll is then extracted from the dried biomass by organic solvent extraction or supercritical fluid extraction. This process is followed by a fractionation step to separate the chlorophyll pigments and derivatives. Many studies have been carried out to optimize chlorophyll extraction and fractionation from *Scenedesmus* microalgae.

Therefore, there is a need for novel production methods for of cell components like pigments and lipids of algae belonging to the genus *Scenedesmus* and for novel methods for improving the extractability of these cell components.

## SUMMARY OF THE DISCLOSURE

The present invention provides methods for improving the extractability of cellular composition like pigments, carbohydrates, oil or lipids from algae belonging to the genus *Scenedesmus*, by subjecting the algae biomass to an enzyme composition comprising one or more specific enzymes capable to degrading the *Scenedesmus* algae cells. Furthermore, embodiments of the present invention refer to methods, uses and compositions for producing an algae cell component.

In a first aspect the present disclosure pertains to methods for improving the extractability of an algae cellular component from algae belonging to the genus *Scenedesmus* comprising:

a) subjecting the algae to an enzyme composition comprising a 1,3(4)-beta glucanase, and
b) isolating the cellular composition from the algae.

In a second aspect, the disclosure pertains to methods for producing an algae cell component by using algae belonging to the genus *Scenedesmus* comprising the steps:

a) culturing the algae in a liquid medium,
b) harvesting the algae,
c) disrupting the algae cells by enzymatic treatment with an enzyme composition comprising a comprising a 1,3(4)-beta glucanase,
d) isolating the cell component.

In a third aspect, the present disclosure pertains to methods for producing a pigment by using a photoautotrophic algae belonging to the genus *Scenedesmus* capable of producing an effective amount of a pigment comprising the steps of:

(a) culturing the algae cells under photoautotrophic conditions in a liquid medium,
(b) harvesting the algae cells within the exponential growth phase,
(c) centrifuge the harvested cells for water removal,
(d) enzymatic treatment of the algae cells or parts thereof with an enzyme composition comprising a 1,3(4)-beta glucanase,
(e) isolating the pigment from the medium by extraction and/or by centrifugation..

In another aspect, the present disclosure pertains to the uses of an enzyme composition comprising a 1,3(4)-beta glucanase, preferably an endo-1,3(4)-beta glucanase for the extraction of pigments from algae belonging to the genus *Scenedesmus*.

Furthermore, the present disclosure pertains to enzyme compositions comprising an endo-1,3(4)-beta glucanase, an endo-1,4-beta- mannanase and a protease as main enzyme activities suitable for disrupting algae cells belonging to the genus *Scenedesmus* for the extraction of algae cell components.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG.1 is a diagram showing the results of the treatment of algae biomass with different enzyme compositions

FIG.2 shows the general production process and extraction steps of microalgae

FIG.3 is a process flow chart of processing of *Scenedesmus* cells

## DETAILED DESCRIPTION OF THIS DISCLOSURE

The enzymes of the present invention may be used to improve the extractability of an algae cellular composition from algae cells belonging to the genus *Scenedesmus*.

Embodiment of the present disclosure are related to methods for producing a cell component of an algae cells belonging to the genus *Scenedesmus*, for examples natural cell components like pigments or lipids as well as products produced by the algae cells e.g. after a genetic modification of the cells. The methods according to the present disclosure may be methods for producing pigments, carotenoids, biofuels or oil products, as well as the production of pharmaceutical active components.

For example, in some embodiments, enzymes according to the present disclosure may be added to algal cells suspended in solutions to degrade the algal cell walls and release their content, whereas in some embodiments, nucleic acid molecules encoding such enzymes may be introduced into the algal cells to express the enzymes therein, so that these enzymes can degrade the algal cell walls and/or algae cyst cell walls from within.

In some embodiments, nucleic acids encoding enzymes capable of catalyzing algae cell degradation may be introduced into algal cells to express the enzymes in those cells and to degrade their cell walls, while enzymes may also added to or mixed with the cells to further promote the cell degradation.

It is an advantage of the methods and enzyme compositions according to the present disclosure that after the enzymatic degradation of the algae cells additional cell degradation methods by chemical and/or physical treatment are not needed any more or can be reduced to a minimum on time.

However, in some embodiments the methods according to the present disclosure comprises an additional non-enzymatic treatment step like heating, sonication, mechanical lysis, osmotic shock, expression of an autolysis gene, exposure to pH above 8 and exposure to pH below 6.

Disclosed herein are methods of improving the extractability of cell components of algae using enzymes and/or mixtures of enzymes according to the present disclosure. In some embodiments, methods for improving the extractability of a cellular composition from algae according to the present disclosure comprise the following steps:

a) Cultivating and growing algae cells to a desired algae biomass
b) Subjecting the algae biomass to an enzyme composition comprising one or more enzymes capable to degrading the algae cells
c) Extracting the cellular composition from the algae biomass

In advantageous embodiments, the present disclosure relates to methods for producing an algae cell component comprising the steps of:

a) culturing and growing algae in a liquid medium to a desired algae biomass,
b) harvesting the algae,

c) enzymatic treatment of the algae with an enzyme composition comprising a 1,3(4)-beta glucanase,

d) isolating the cell component from the algae biomass.

In some embodiments the cellular composition may be pigments like carotenoids, carbohydrates like starch, lipids like poly unsaturated fatty acids (PUFA), proteins, amino acids, vitamins and mineral nutrients. In an advantageous embodiment, the cellular composition is a carotenoid like astaxanthin. In yet another embodiment of the disclosure, chlorophylls and carotenoids are isolated from the neutral lipids.

In an advantageous embodiment, the cellular composition is a pigment like chlorophyll or a carotenoid. As used herein the term "carotenoid" stands for the chemical compound as such as well as for a pigment of the appropriate dye, if not otherwise stated. Carotenoids are colored lipid-soluble compounds that can be found in higher plants and algae, as well as in nonphotosynthetic organisms like animals (although they are not able to synthesize carotenoids), fungi, and bacteria. Carotenoids are responsible for the red, orange and, yellow colors of plant leaves, fruits, and flowers, as well as for the color of feathers, crustacean shells, fish flesh and skin, etc. (Gudin 2003; Johnson and Schroeder 1995; Negro and Garrido-Fernández 2000). The chemical structure of the more than 600 different carotenoids is derived from a 40-carbon polyene chain, which can be considered as the backbone of the molecule. The polyene system gives carotenoids their distinctive molecular structure, their chemical properties, and their light-absorbing characteristics. This chain may be terminated by cyclic groups (rings) and can be complemented with oxygen-containing functional groups. The hydrocarbon carotenoids are named carotenes, whereas oxygenated derivatives are known as xanthophylls. In the latter, oxygen can be present as OH groups (as in lutein), as oxi-groups (as in cantaxanthin), or in a combination of both (as in astaxanthin; Higuera- Ciapara et al. 2006).

Chlorophyll is a valuable bioactive compounds that can be extracted from microalgal biomass. It is used as a natural food coloring agent and has antioxidant as well as antimutagenic properties. The process of extracting chlorophyll from *Scenedesmus*, microalgae begins with dewatering and desalting the highly dilute microalgal culture (biomass concentration = 0.1–1% w/v). Chlorophyll is then extracted from the dried biomass by organic solvent extraction or supercritical fluid extraction. This process is followed by a fractionation step to separate the chlorophyll pigments and derivatives.

In a first step, the algae are cultured and grown in a liquid medium to a desired algae biomass. The algae are cultured the production of cellular composition {e.g., lipids, fatty acids, aldehydes, alcohols, alkanes, carotenoids etc.). The former type of culture is conducted on a small scale and initially, at least, under conditions in which the starting microorganism can grow. For example, if the starting microorganism is a photoautotroph the initial culture is conducted in the presence of light. The culture conditions can be changed if the microorganism is evolved or engineered to grow independently of light. The algae can be cultured in bioreactors or open ponds.

As mentioned above, there are two main microalgal large-scale cultivation systems: (1) open air-system and (2) photobioreactors. The selection of a cultivation system depends on several factors: the type and the biology of the algal species, the availability of sunlight, the cost of land, the water supply,

the availability of nutrients, the desired final product, the climate conditions and the supply of CO2 . The amount of nutrients and certain metals (i.e., iron and magnesium) must be optimum as they are important for the growth of microalgae and $CO_2$ fixation efficiency.

The algae according to the present disclosure are green algae with the genus *Scenedesmus,* and for instance are selected from the group comprising but not limited to *S. obliquus, S. acuminatus, S. acutiformis, S. armatus, S. costato-granulatus, S. falcatus, S. producto-capitatus, S. subcapitatus, S. wisconsiensis, S. abundans, S. acutus, S. aldevei, S. arcuatus, S. basiliensis, S. costatus, S. serratus, S. carinatus, S. maximus, S. perforatus, S. obtusus, S. dimorphus, S. ellipticus, S. quadricauda,* and *S. communis.* The list of *Scenedesmus* species is not exhaustive.

When green algae like *Scenedesmus* are subjected to stresses from the environment, such as nutrient deprivation or the presence of oxides, the green algae accumulate pigments within the cells and become resting spores. The shift to this resting state is referred to as encystment. In this specification, encystment refers to any state from the beginning of the resting state where accumulation of pigments starts, to the completely encysted state where the cells become resting spores. In order to increase the pigments content, it is preferable to use green algae in which encystment has progressed as far as possible and which has accumulated a large amount of astaxanthin. It should be noted that "cultivating encysted green algae" as used herein also includes the process of inoculating green algae containing astaxanthin that has been grown in a nutrient medium, after the algae has reached the encysted state. In the present specification, "green algae" are also intended to include encysted green algae.

In order to quantify the amount of chlorophyll in a particular species, the intracellular chlorophyll must first be extracted. The traditional method that has been employed is organic solvent extraction (S. W. Jeffrey, R. F. C. Mantoura, and S. W. Wright, Eds., Phytoplankton Pigments in Oceanography: Guidelines to Modern Methods, UNESCO, Paris, Farnce, 1997 and Simon and S. Helliwell, "Extraction and quantification of chlorophyll a from freshwater green algae" Water Research, vol. 32, no. 7, pp. 2220–2223, 1998). The extraction process involves the organic solvent penetrating through the cell membrane and dissolving the lipids as well as the lipoproteins of chloroplast membranes. It has been found that cell disruption, achieved through grinding, homogenisation, ultrasound or sonication, significantly improves the effectiveness of chlorophyll extraction using organic solvents. Simon and Helliwell found that, without cell disruption, only a quarter of the potential chlorophyll was able to be extracted by an optimal method. In addition to cell disruption, there are other parameters which affect the efficiency of organic solvent extraction, including the storage conditions of the filtered microalgae prior to the analysis, the organic solvents used, the duration of the extraction and the number of extraction steps employed in the analysis. Since chlorophyll is highly reactive, the yield of a particular extraction procedure is also affected by the formation of degradation products. Degradation products of chlorophyll are formed when their molecules are exposed to excess light, oxygen/air, high temperatures and acidic or basic conditions.

The inventors of the present invention have found that the use of the enzyme compositions and the use of the methods according to the present disclosure improve the extractability of algae cell components, in particular of pigments like chlorophyll in a production process.

There is no particular limitation on the medium used to cultivate the green algae. Generally, a medium is used that contains nitrogen, inorganic salts of trace metal (e.g., phosphorous, potassium, magnesium, and iron), vitamins (e.g., thiamine), and the like, which are essential to growth. For example, media such as the VT medium, C medium, MC medium, MBM medium, and MDM medium (see Sorui Kenkyuho, ed. by Mitsuo Chihara and Kazutoshi Nishizawa, Kyoritsu Shuppan (1979 )), the OHM medium (see Fabregas et al., J. Biotech., Vol. 89, pp. 65-71 (2001) ), the BG-11 medium, and modifications thereof may be used. In the present invention, it is preferable to use an autotrophic medium that is substantially free from organic carbon source so that contamination by bacteria can be prevented.

These media may be selected depending on their purposes, such as growth, or encystment. For example, for growth of the green algae, a medium having a large amount of components serving as a nitrogen source is used (rich medium: containing at least 0.15 g/L expressed in terms of nitrogen). For encystment, a medium having a small amount of components serving as a nitrogen source is used (encystment medium: containing less than 0.02 g/L expressed in terms of nitrogen). Alternatively, a medium containing a nitrogen source at an intermediate concentration between these media may be used (low nutrient medium: containing at least 0.02 g/L and less than 0.15 g/L expressed in terms of nitrogen).

The nitrogen source concentration, phosphorous concentration, and other properties of the medium can be determined depending on the amount of the green algae to be inoculated. For example, when a green algae count in the order of $10^5$ is inoculated in a low nutrient medium, the green algae would grow to a certain extent, but the growth may stop soon because the amount of the nitrogen source is too small. Such a low nutrient medium is suitable for performing growth and encystment continuously in a single step (in a batch manner), as described later. Furthermore, by adjusting the N/P mole ratio to value from 10 through 30, preferably 15 through 25, the green alga can be encysted.

There is no particular limitation on the apparatus for cultivating the green algae, as long as the apparatus is capable of supplying carbon dioxide and irradiating a culture suspension with light. For example, in the case of a small-scale culture, a flat culture flask may be preferably used. In the case of a large-scale culture, a culture tank that is constituted by a transparent plate made of glass, plastic, or the like and that is equipped with an irradiation apparatus and an agitator, if necessary, may be used. Examples of such a culture tank include a plate culture tank, a tube-type culture tank, an airdome-type culture tank, and a hollow cylinder-type culture tank. However, also open or closed ponds can be used for growing the algae, and seawater as a natural culture medium.

There is no particular limitation on the culture conditions, and a temperature, a pH, and the like as generally employed for cultivation of algae can be used. The green algae are cultivated at, for example, 15 to 35 °C, and preferably 20 to 25 °C. It is preferable that the pH is maintained at 6 to 8 throughout the cultivation period. Carbon dioxide is supplied by bubbling a gas containing carbon dioxide at a concentration of 1 to 3 v/v% at a rate of 0.2 to 2 wm, for example. When a plate culture tank is used, the culture suspension is stirred by supplying carbon dioxide, so that the green algae can be uniformly irradiated with light.

In the case where the green algae count for inoculation is even higher, the rich medium can be employed to perform the above-described cultivation.

In this manner, the composition of the medium can be determined in consideration of various conditions. It should be noted that the medium preferably used in the present invention, i.e., an autotrophic medium, is nearly free from an organic carbon source such as acetic acid or glucose, so that contamination by bacteria hardly occurs even in long-term cultivation.

In a second step, the algae cells are harvested and optionally centrifuged to reduce the water content. The harvested biomass can be transferred in a buffer tank (see Fig.4). In an advantageous embodiment the algae biomass is then treated with an enzyme composition.

In an advantage embodiment of the present disclosure the enzyme composition comprises a hemicellulase. "Hemicellulase" refers to a protein that catalyzes the hydrolysis of hemicellulose, such as that found in lignocellulosic materials. Hemicellulose is a complex polymer, and its composition often varies widely from organism to organism and from one tissue type to another. Hemicelluloses include a variety of compounds, such as xylans, arabinoxylans, xyloglucans, mannans, glucomannans, and galactomannans. Hemicellulose can also contain glucan, which is a general term for beta-linked glucose residues. In general, a main component of hemicellulose is beta-l,4-linked xylose, a five carbon sugar. However, this xylose is often branched as beta- 1,3 linkages or beta- 1,2 linkages, and can be substituted with linkages to arabinose, galactose, mannose, glucuronic acid, or by esterification to acetic acid. The composition, nature of substitution, and degree of branching of hemicellulose is very different in dicotyledonous plants (dicots, i.e., plant whose seeds have two cotyledons or seed leaves such as lima beans, peanuts, almonds, peas, kidney beans) as compared to monocotyledonous plants (monocots; i.e., plants having a single cotyledon or seed leaf such as corn, wheat, rice, grasses, barley). In dicots, hemicellulose is comprised mainly of xyloglucans that are 1,4- beta-linked glucose chains with 1 ,6-alpha- linked xylosyl side chains. In monocots, including most grain crops, the principal components of hemicellulose are heteroxylans. These are primarily comprised of 1 ,4-beta-linked xylose backbone polymers with 1,2- or 1,3-beta linkages to arabinose, galactose and mannose as well as xylose modified by ester- linked acetic acids. Also present are branched beta glucans comprised of 1,3- and 1,4- beta-linked glucosyl chains. In monocots, cellulose, heteroxylans and beta glucans are present in roughly equal amounts, each comprising about 15-25% of the dry matter of cell walls. Hemicellulolytic enzymes, i.e. hemicellulases, include both endo-acting and exo- acting enzymes, such as xylanases, [beta]-xylosidases. galactanases, [alpha]-galactosidases, [beta]-galactosidases, endo-arabinases, arabinofuranosidases, mannanases, [beta]-mannosidases. Hemicellulases also include the accessory enzymes, such as acetylesterases, ferulic acid esterases, and coumaric acid esterases. Among these, xylanases and acetyl xylan esterases cleave the xylan and acetyl side chains of xylan and the remaining xylo-oligomers are unsubstituted and can thus be hydrolysed with [beta]-xylosidase only. In addition, several less known side activities have been found in enzyme preparations which hydrolyze hemicellulose. Accordingly, xylanases, acetylesterases and [beta]-xylosidases are examples of hemicellulases.

In an advantage embodiment of the present disclosure the enzyme composition comprises a mannanase or a functional equivalent thereof. The term „mannanase" refers to any enzyme capable of hydrolyzing polyose chains that are composed of mannose units (mannopolymers or polymannoses). "Mannanase" therefore comprises both endomannanases and exomannanases which cleave mannopolymers internally or from the terminal ends of the polymer, respectively. In advantageous embodiments a 1,4-beta- mannanase is used, preferably a mannanase with endo-1,4-beta-mannanase main activity.

Endo-β-1,4-D-mannanase (β-mannanase; EC 3.2.1.78) catalyses the random hydrolysis of manno-glycosidic bonds in mannan-based polysaccharides. Most β-mannanases degrade oligosaccharides down to DP4 (Biely and Tenkanen (1998) *Enzymology of hemicellulose degradation*, pages 25-47. In Harman and Kubiceck (ed) *Trichoderma and Gliocladium,* vol.2, Taylor and Francis Ltd. London), however, residual activity has been demonstrated on mannotriose, indicating at least four subsites for mannose binding on the protein. The main end products of hydrolysis are often mannobiose and mannotriose, although significant amounts of mannose are also produced. Some β-mannanases are able to degrade crystalline mannan. In addition to hydrolysis, several β-mannanases including β-mannanase from *Trichoderma reesei*, have been shown to form transglycosylation products with either mannose or mannobiose as glycosidic bond acceptor.

β-mannanases have been isolated from a wide range of organisms including bacteria, fungi, plants and animals. Although mostly extracellular, some β-mannanases appear to be cell-associated. Their expression is often induced by growth on mannan or galactomannan, however, β-mannanase from *T. reesei* can also be induced by cellulose, while its expression is suppressed by glucose and other monosaccharides. Frequently multiple mannanases with different isoelectric points are found in the same organism, representing products from different genes or different products from the same gene, respectively.

In one embodiment of the present disclosure, the enzyme composition shows the mannanase enzyme activity as the main activity. In this case, even though the enzyme composition comprises other enzymes as a mannanase or a functional equivalent thereof, the mannanase activity is the main activity in the enzyme mix. For example if the enzyme composition comprises a mannanase and a cellulase, the cellulase shows only a side activity in the enzyme mixture and could be seen as a contamination of the enzyme composition. In other words, in an advantageous embodiment of the disclosure the enzyme composition shows only a small cellulase activity and a high mannanase activity.

In a further advantageous embodiment of the disclosure the enzyme composition comprises another enzyme capable to degrade the algae cells, in particular selected from the group consisting of, but not limited to other hemicellulases, alpha-galactosidases, beta-galactosidases, lactases, laminarinase, glucanases, beta-glucanases, endo-beta-1,4-glucanases, cellulases, xylosidases, xylanases, xyloglucanases, xylan acetyl-esterases, galactanases, exo-mannanases, pectinases, pectin lyases, pectinesterases, polygalacturonases, arabinases, rhamnogalacturonases, laccases, reductases, oxidases, phenoloxidases, ligninases, proteases, amylases, phosphatases, lipolytic enzymes, esterases, cutinases and/or others. In an advantageous embodiment, the glucanase is an endo and or an exo-1, 3(4)-beta glucanase, preferably an endo-1, 3(4)-beta glucanase.

The glucan endo-1,3-ß-D-glucosidases, classified as endo-1,3-beta-glucanases (EC 3.2 . 1.39) and exo-1,3-ß-glucanases (EC 3.2.1.58), are widely distributed among higher plants, fungi and bacteria. This class of enzyme catalyses the hydrolysis of 1,3-ß-D-glucosidic linkages in 1,3-ß-D-glucan, which is the main constituent of fungal cell walls and a major structural and storage polysaccharide (laminarin) of marine macro-algae. In plants, it may be involved in defence against pathogenic fungi through its ability to degrade fungal cell walls (Castresana et al., 1990Down ; Grenier et al., 1993Down ; Yi & Hwang, 1997Down ). In fungi, it may play a role in cell expansion, cell–cell fusion and spore release (de la Cruz et al., 1995Down ). In bacteria, it is related to the assimilation of fungal cell walls as a food source (Watanabe et al., 1992Down ). Recently, the first viral 1,3-ß-glucanase, from chlorella virus PBCV-1, was described (Sun et al., 2000Down ).

In another embodiment, the enzyme composition comprises a laminarinase. Laminarinase is capable of hydrolysing laminarin or callose. To date, two laminarinases have been identified, endo-β-1,3(4)-glucanase (EC 3.2.1.6) and endo-β-1,3-glucanase (EC 3.2.1.39); endo-β-1,3(4)-glucanase (EC 3.2.1.6) is capable of hydrolysing both β-1,3- and β-1,4-glycosidic bonds, while endo-β-1,3-glucanase (EC 3.2.1.39) is capable of hydrolysing mainly β -1,3-glycosidic bonds (Boeckmann et al., 2003; Terra and Ferreira, 1994). Laminarinase may also work synergistically with cellulases such as endo-β-1,4-glucanase to hydrolyse structural polysaccharides within the plant cell wall (Mansfield et al., 1999).

In a preferred embodiment, the enzyme composition comprises also lipase. A lipase is an enzyme that catalyzes the hydrolysis of ester bonds in water-insoluble, lipid substrates. Lipases catalyze the hydrolysis of lipids into glycerols and fatty acids.

In another embodiment the enzyme composition comprises at least a further enzyme in addition to the above mentioned enzymes like a protease, cellulase and/or a pectinase, or mixture thereof.

"Pectinase" according to the present disclosure refers to enzymes, such as pectinlyase, pectinesterases and polygalacturonase and combinations thereof which break down pectin.

A "Protease" according to the present disclosure is any enzyme that conducts proteolysis, that is, begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain forming the protein.

"Cellulase" according to the present disclosure is any enzymes that catalyze cellulolysis (i.e. the hydrolysis) of cellulose.

In an advantageous embodiment of the disclosure the enzyme composition comprises a 1, 3(4)-beta glucanase as main activity. In another advantageous embodiment the enzyme composition comprises a laminarinase and/or a 1,3(4) beta-glucanase as main activity.

In an advantageous embodiment of the disclosure the enzyme composition comprises a mannanase and a lipase as main activities. In a further advantageous embodiment, the enzyme composition comprises a 1,3(4) beta-glucanase and a mannanase as main activities. In another advantageous

embodiment of the disclosure the enzyme composition comprises a 1,3(4) beta-glucanase, a mannanase and a protease as main activities.

The enzyme combination comprised in the enzyme composition for extracting pigments, in particular chlorophyll from green algae, preferably from green algae of the genus *Scenedesmus* is the combination of an endo-1,3(4) beta-glucanase and/or a laminarinase, an endo-β-1,4-D-mannanase and a protease.

In another aspect, the disclosure pertains to methods for producing a pigment by using a photoautotrophic algae belonging to the genus *Scenedesmus* capable of producing an effective amount of a pigment comprising the steps of:

(a) culturing the algae cells under photoautotrophic conditions in a liquid medium,
(b) harvesting the algae cells within the exponential growth phase,
(c) centrifuge the harvested cells for water removal,
(d) enzymatic treatment of the algae cells or parts thereof with an enzyme composition comprising a 1,3(4)-beta glucanase,
(e) isolating the pigment from the medium by extraction and/or by centrifugation.

As used herein the term "isolation" as used in the present disclosure refers to a process or means that is suitable to obtain an algae cell component form the cracked algae like extraction or centrifugation. The isolation of algae cell component for further use or analysis can also carried out by known extraction procedures. In particular, the cells may be harvested by centrifugation, for example at 1900 x g for 3 minutes, and washed once or twice in water. The cell pellet that is obtained by centrifugation is broken with mortar and pestle with the aid of aluminium powder and then resuspended in a suitable organic solvent, for instance in acetone or methanol and the carotenoid extract is separated from the cell debris by centrifugation at 1900 x g, saponificated with a mixture of the same volumes of 2 percent (w/v) solution of KOH in methanol and diethyl ether, then the supernatant is evaporated under N2 and the pellet is resuspended in acetone, centrifuged and analyzed by HPLC. The process is carried out at a temperature between 0 Degrees C and 40 °C particularly 5 °C and 35 °C more particularly 10 °C and 30 °C preferably at room temperature, preferably in the dark and the carotenoid extract is kept at a temperature between -20 °C and 25 °C more particularly -20 and 4 °C preferably at -2O °C. Optionally, the samples obtained can be collected and centrifuged once more to separate undesired particles from the cells or extracts. The supernatant can be used for further spectrophotometric analysis, as mentioned above, for HPLC or other technologies concerning analysis of carotenoids or cells containing same, such as thin layer chromatography, for example using Kiesel gel plates, gas chromatography or magnetic resonance chromatography.

In general, an algae cell component, in particular a pigment or a lipid produced with a method according to the present disclosure can be isolated and/or extracted by methods known in the art. For example the carotenoids can be isolated by extraction from the microorganism or parts therefrom, such as cell debris or physically pressed cells, using an organic solvent as mentioned above.

As regards analysis by HPLC reverse phase HPLC can be used according to known procedures. In particular, a Waters Spherisorb S5 ODS 18 4.6 x 250 mm cartridge column can be used and a solvent linear gradient from 100 percent solvent A (acetonitrile: methanol: 0.1 M Tris-HCl, pH 8.0 [84: 2: 14]) to 100 percent solvent B (methanol: ethyl acetate [68: 32]) for 15 inin, followed by 3 min of solvent B, which is pumped by using a Dual Dispensity system with a flow rate of 1.2 ml min'1 from which carotenoid pigments can be eluted. The pigments can be detected by using a photodiode-array detector (Waters 2996) at 440 run. The concentration of individual carotenoids is determined using standard curves of purified pigments at known concentrations. Astaxanthin can be determined also by measuring the absorbance at 477 nm using an extinction coefficient of 2100. It is known from the literature, that the obtained carotenoid astaxanthin is achievable in the pure form of the (3S,3'S) isomer.

Furthermore, Supercritical fluid extraction (SFE) is a popular method to replace organic solvent extraction. SFE has many advantages over organic solvent extraction. One of the major advantages is the high purity of the extract. In addition to requiring less processing steps, SFE is significantly safer than organic solvent extraction and can be operated at moderate temperatures to minimize extract degradation. In the prior art, the common extraction mehods are described (e.g. F. Sahena, I. S. M. Zaidul, S. Jinap, et al., "Application of supercritical CO2 in lipid extraction—a review," Journal of Food Engineering, vol. 95, no. 2, pp. 240–253, 2009).

In some embodiments, the methods according to the present disclosure comprises an additional non-enzymatic treatment step selected from the group consisting of heating, sonication, mechanical lysis, osmotic shock, expression of an autolysis gene, exposure to pH above 8 and exposure to pH below 6. In particular, the disruption of the algae cell walls can suitably be made by one or more methods within the group consisting of ultra-sonication, liquid shear disruption, bead milling, high pressure pressing, freeze-thawing, freeze-pressing, hydrolysation, and virus degradation.

In an advantage embodiment, the non-enzymatic treatment step is a mechanical lysis, preferably milling, in particular bead-milling.

This non-enzymatic treatment step can be carried out before and/or after the enzymatic treatment. In some embodiments, the non-enzymatic treatment step is carried out before the enzymatic treatment step to disrupt the algae cell walls. The following enzymatic treatment of the non-enzymatic treated algae biomass comprising non disrupted algae cells, parts of the algae cell like compartments and/or cell walls with an enzyme composition according to the present disclosure increase the yield of the desired cell components like chlorophyll. In other embodiments, the non-enzymatic treatment step is carried out after the enzymatic treatment step. The enzymatic treatment with an enzyme composition according to the present disclosure disrupt the algae cell walls, the following energy consuming mechanical lysis e.g. via milling can be reduced in time significantly.

The use of the enzyme compositions according to the present disclosure shows a clear effect on algae cell disruption in lab scale testing as well as in pilot scale. The use results in a significant decrease of the processing time. Furthermore, the yield of the produced algae cell component is increased.

Therefore, the enzymes and enzyme compositions of the present disclosure may be used for an improved release of the contents of an algae cell. In some embodiments, contacting or mixing the algae cells with the enzymes of the present disclosure will degrade the cell walls, resulting in cell lysis and release of the cellular contents. For example, the enzymes of the present disclosure may be used to degrade the cell walls of micro- and macro algal cells in order to release the materials contained within the algal cells. In some embodiments, such materials may include, without limitation, carotenoids, alcohols and oils. The alcohols and oils so released can be further processed to produce bio-diesel, jet fuels, as well as other economically important bio-products.

The enzymes and enzyme compositions of the present disclosure may be used alone, or in combination with other enzymes, chemicals or biological materials. The enzymes of the present disclosure may be used for *in vitro* applications in which the enzymes or mixtures thereof are added to or mixed with the appropriate substrates to catalyze the desired reactions.

Additionally, the enzymes of the present disclosure may be used for *in vivo* applications in which nucleic acid molecules encoding the enzymes are introduced into algal cells and are expressed therein to produce the enzymes and catalyze the desired reactions within the cells.

For example, in some embodiments, enzymes capable of promoting cell wall degradation may be added to algal cells suspended in solutions to degrade the algal cell walls and release their content, whereas in some embodiments, nucleic acid molecules encoding such enzymes may be introduced into the algal cells to express the enzymes therein, so that these enzymes can degrade the algal cell walls from within. Some embodiments may combine the *in vitro* applications with the *in vivo* applications. For example, nucleic acids encoding enzymes capable of catalyzing cell wall degradation may be introduced into algal cells to express the enzymes in those cells and to degrade their cell walls, while enzymes may also added to or mixed with the cells to further promote the cell wall degradation. In some embodiments, the enzymes used for *in vitro* applications may be different from the enzymes used for *in vivo* applications. For example, an enzyme with the mannanase activity may be mixed with the cells, while an enzyme with the protease activity is expressed within the cells.

In one aspect, the present disclosure includes proteins isolated from, or derived from the knowledge of enzymes from a filamentous fungus such as *Aspergillum, Trichoderma* or a mutant or other derivative thereof. For example, if a filamentous fungus was cultivated on an algae species, the proteins, preferably proteins with enzymatic activity of the filamentous fungus can be isolated and used for the degradation of the same algae species in a production process.

The extraction of algae lipids for the production of algae biofuels is one example for the use of the extraction methods according to the present disclosure. Furthermore, the enzymatically degradation of the algae cell walls increases the availability of proteins and amino acids, lipids as PUFA's and/or carotenoids like astaxanthin, when they are used as animal feed. Due to the high extraction rate when using the methods according to the present disclosure, algae biomass can be used as an alternative to fish meal in aquaculture farming.

Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. Singleton, *et al.*, DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY, 20 ED., John Wiley and Sons, New York (1994), and Hale & Marham, THE HARPER COLLINS DICTIONARY OF BIOLOGY, Harper Perennial, NY (1991) provide one of skill with a general dictionary of many of the terms used in this disclosure.

The headings provided herein are not limitations of the various aspects or embodiments of this disclosure which can be had by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the specification as a whole.

The following methods and examples are offered for illustrative purposes only, and are not intended to limit the scope of the present disclosure in any way.

**Methods and Examples**

In the following examples, materials and methods of the present disclosure are provided. It should be understood that these examples are for illustrative purpose only and are not to be construed as limiting this disclosure in any manner. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

**Example 1:**

Extraction of chlorophyll from *Scenedesmus* algae cells

a) Algae

600,0 mg freeze dried Secenedesmus algae were suspended in 6 ml water. Each well was loaded with 100µl algae suspension and 5µl enzyme solution. Incubation took place at 50°C at 175 rpm on a rotary shaker for 1h. Afterwards 3,9 ml acetone 80% were added for chlorophyll extraction, and the samples were centrifuged for 30 min at 3000rpm. 200 µl of supernatant were used for chlorophyll determination by measuring absorption at 665 nm.

b) Enzymes used

| | |
|---|---|
| Viscozyme L: | endo 1,3(4)-endo glucanase from Novozymes |
| Rohalase GMP: | endo-1,4-beta-mannanase from AB Enzmyes |
| Rohapect Classic: | pectinase from AB Enzymes |
| Pectinex Ultra SPL: | pectinase from Novozymes |
| Amano 90: | hemicellulase complex form Amano Japan |

Fungal Acid Protease from BioCat US

Table 1

| | Abs 665 nm | µg Chlorophyll /ml | Improvement |
|---|---|---|---|
| no Enzyme (+ 10µl H2O) | 0,514 | 236,5737996 | |
| Viscozym L | 0,812 | 373,9964643 | 58,1 % |
| Rohalase GMP | 0,689 | 317,2998665 | 34,1 % |
| Biocat Fungal Acid Protease | 0,791 | 364,356432 | 54,0 % |
| Viscozym L,Rohalase GMP | 0,815 | 375,5161563 | 58,7 % |
| Rohalase GMP/Viscozym, Biocat Fungal Acid Protease | 0,865 | 398,5493874 | 68,5 % |

The results in table 1 show that a 1,3(4)-endo glucanase has a significant effect on the cell disruption of *Scenedesmus* algae. The combination of the 1,3(4)-endo glucanase, the mannanase and the protease and the protease shows the highest effect on the cell disruption and the extractability of chlorophyll.

Figure 1 shows additional results on the chlorophyll extractability, whereas also 1,3(4)-endo glucanase shows a significant effect on the cell disruption of *Scenedesmus* algae .

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Effective amounts of enzymes in the enzyme compositions according to the present disclosure:

Mannanase may be added in an amount effective in the range from $0.3x10^6$ - $1.6x10^6$ Units per ton algae biomass.

Protease may be added in an amount effective in the range from 0.002 $x10^6$-$314x10^6$ Units per ton algae biomass.

Xylanase may be added in an amount effective in the range from $0.16x10^6$ - $460x10^6$ Units per ton algae biomass.

1,3(4) beta glucanase may be added in an amount effective in the range from $0.2x10^6$ - $400x10^6$ Units per ton algae biomass.

Lipase may be added in an amount effective in the range from $0.1x10^6$ - $300x10^6$ Units per ton algae biomass.

Laminarinase may be added in an amount effective in the range from $0.2x10^6$ - $400x10^6$ Units per ton algae biomass.

Claims

1. A method for improving the extractability of an algae cellular component from algae belonging to the genus *Scenedesmus* comprising:
a) subjecting the algae to an enzyme composition comprising a 1,3(4)-beta glucanase, and
b) isolating the cellular composition from the algae.

2. The method according to claim 1, whereby the 1,3(4)-beta glucanase is an endo-1,3(4)-beta glucanase.

3. The method according to any one of claims 1 to 2, whereby the cellular component is selected from the group consisting of pigments, carotenoids, starch, lipids, poly unsaturated fatty acids (PUFA), proteins, vitamins and mineral nutrients.

4. The method according to any one of claims 1 to 2, whereby the cellular component is a pigment.

5. The method according to any one of claims 1 to 4, whereby the enzyme composition comprises further a mannanase, preferably a 1,4-beta- mannanase.

6. The method according to claim 5, whereby the 1,4-beta- mannanase is an endo-1,4-beta-mannanase.

7. The method according to any one of claims 1 to 6, whereby the enzyme composition comprises further a protease.

8. The method according to claim 7, whereby the enzyme composition comprises an endo-1,3(4)-beta glucanase, an endo-1,4-beta- mannanase and a protease.

9. The method according to any one of claims 1 to 8, whereby the enzyme composition comprises further cellulases and/or a pectinases and/or a laminarinase.

10. A method for producing an algae cell component by using algae belonging to the genus *Scenedesmus* comprising the steps:
a) culturing the algae in a liquid medium,
b) harvesting the algae,

c) disrupting the algae cells by enzymatic treatment with an enzyme composition comprising a comprising a 1,3(4)-beta glucanase,

d) isolating the cell component.

11. The method according to claim 10, whereby the 1,3(4)-beta glucanase is an endo-1,3(4)-beta glucanase.

12. The method according to any one of claims 10 to 11, whereby the cellular composition is selected from the group consisting of pigments, carotenoids, starch, lipids, poly unsaturated fatty acids (PUFA), proteins, vitamins and mineral nutrients.

13. The method according to any one of claims 10 to 11, whereby the cellular composition is a pigment.

14. The method according to any one of claims 10 to 13, whereby the enzyme composition comprises further a mannanase, preferably a 1,4-beta- mannanase.

15. The method according to claim 14, whereby the 1,4-beta- mannanase is an endo-1,4-beta-mannanase.

16. The method according to any one of claims 10 to 15, whereby the enzyme composition comprises further a protease.

17. The method according to claim 16, whereby the enzyme composition comprises an endo-1,3(4)-beta glucanase, an endo-1,4-beta- mannanase and a protease.

18. The method according to any one of claims 10 to 17, whereby the enzyme composition comprises further cellulases and/or a pectinases and/or a laminarinase.

19. A method for producing a pigment by using a photoautotrophic algae belonging to the genus *Scenedesmus* capable of producing an effective amount of a pigment comprising the steps of:

(a) culturing the algae cells under photoautotrophic conditions in a liquid medium,

(b) harvesting the algae cells within the exponential growth phase,

(c) centrifuge the harvested cells for water removal,

(d) enzymatic treatment of the algae cells or parts thereof with an enzyme composition comprising a 1,3(4)-beta glucanase,

(e) isolating the pigment from the medium by extraction and/or by centrifugation.

20. The method according to claim 19, whereby the 1,3(4)-beta glucanase is an endo-1,3(4)-beta glucanase.

21. The method according to any one of claims 19 to 20, whereby the enzyme composition comprises further a mannanase, preferably a 1,4-beta- mannanase.

22. The method according to claim 21, whereby the 1,4-beta- mannanase is an endo-1,4-beta-mannanase.

23. The method according to any one of claims 19 to 22, whereby the enzyme composition comprises further a protease.

24. The method according to claim 23, whereby the enzyme composition comprises an endo-1,3(4)-beta glucanase, an endo-1,4-beta- mannanase and a protease.

25. The method according to any one of claims 21 to 24, whereby the enzyme composition comprises further cellulases and/or a pectinases and/or a laminarinase.

26. The method according to any one of claims 19 to 25, whereby the method comprises an additional non-enzymatic treatment step selected from the group consisting of heating, sonication, mechanical lysis, osmotic shock, expression of an autolysis gene, exposure to pH above 8 and exposure to pH below 6.

27. The method according to claim 26, whereby the non-enzymatic treatment step is carried out before and/or after the enzymatic treatment.

28. The method according to any one of claims 26 to 27, whereby the mechanical lysis is milling, preferably bead-milling.

29. Use of an enzyme composition comprising a 1,3(4)-beta glucanase, preferably an endo-1,3(4)-beta glucanase for the extraction of pigments from algae belonging to the genus *Scenedesmus*.

30. The use according to claim 29, whereby the enzyme composition comprises further a mannanase, preferably a 1,4-beta- mannanase, more preferably an endo-1,4-beta- mannanase.

31. The use according to any one of claims 21 to 28, whereby the enzyme composition comprises further a protease.

32. The use according to claim 31, whereby the enzyme composition comprises an endo-1,3(4)-beta glucanase, an endo-1,4-beta- mannanase and a protease.

33. An enzyme composition comprising an endo-1,3(4)-beta glucanase, an endo-1,4-beta-mannanase and a protease as main enzyme activities suitable for disrupting algae cells belonging to the *genus* Scenedesmus for the extraction of algae cell components.

**Figure 1**

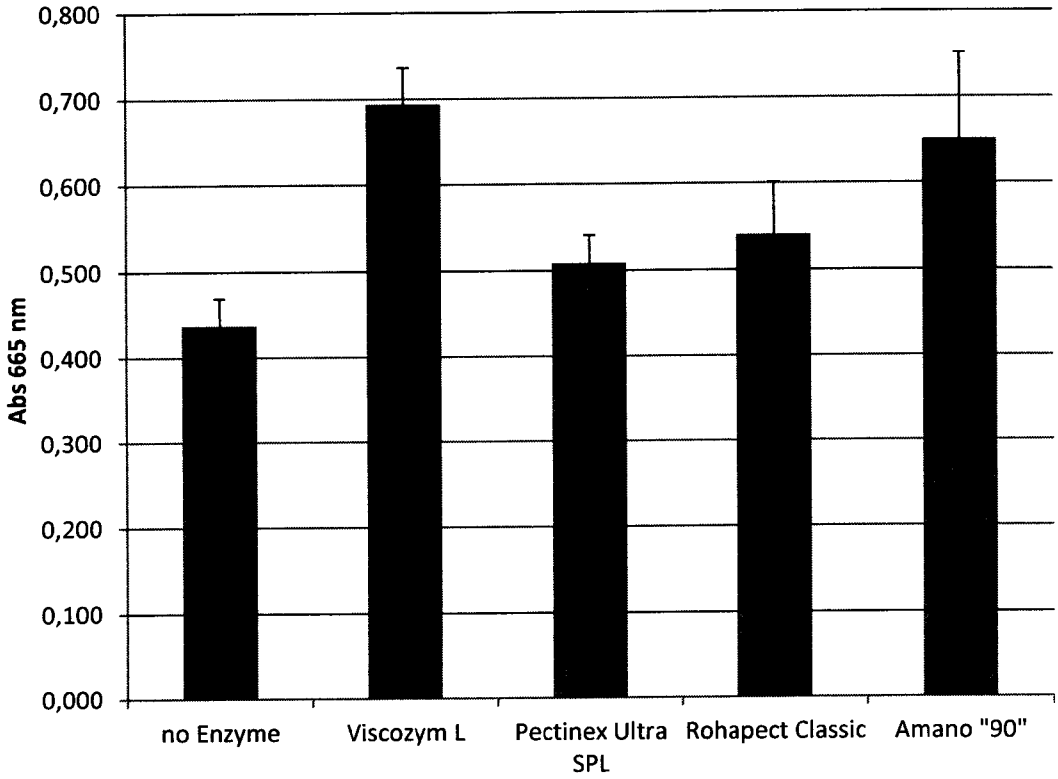

0,800
0,700
0,600
0,500
0,400
0,300
0,200
0,100
0,000
Abs 665 nm
no Enzyme
Viscozym L
Pectinex Ultra SPL
Rohapect Classic
Amano "90"

**Figure 2**

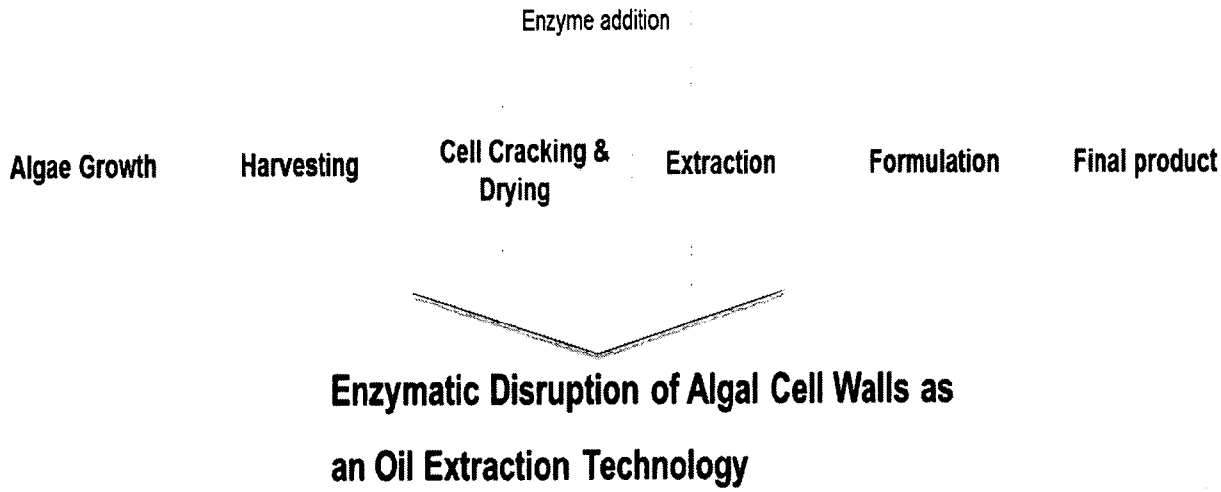
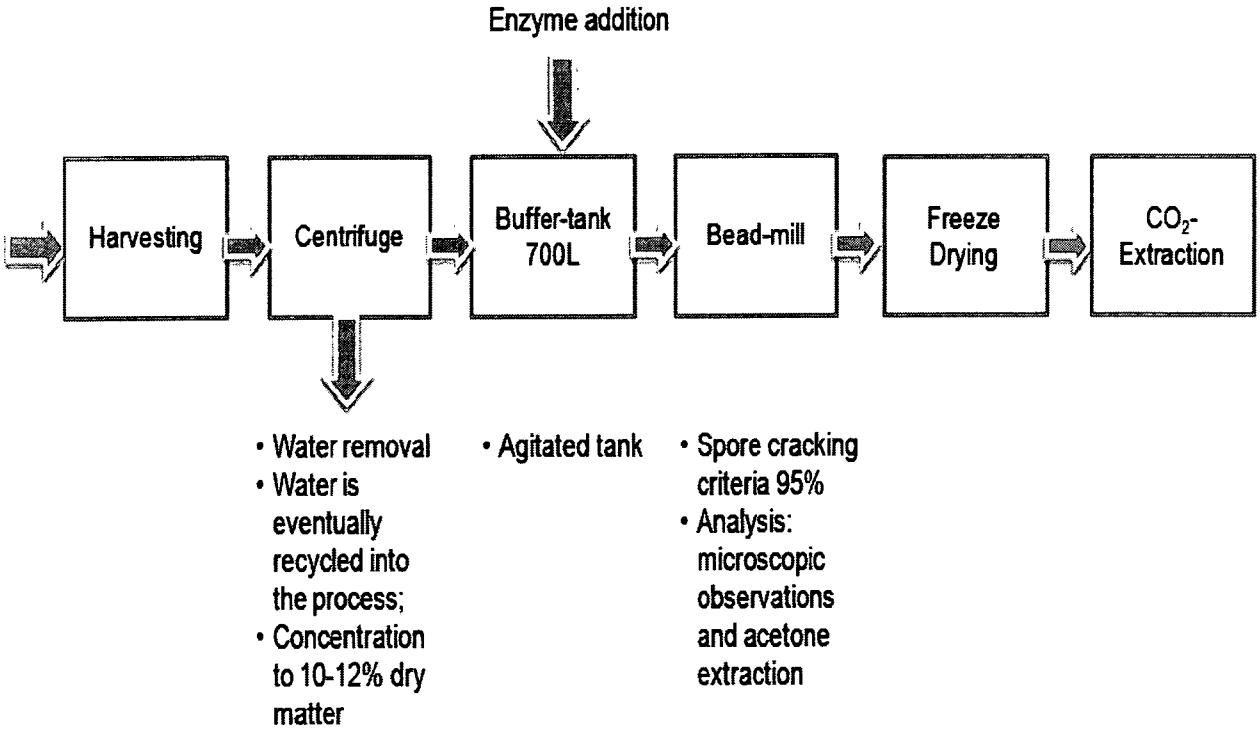

Enzyme addition
Algae Growth
Harvesting
Cell Cracking & Drying
Extraction
Formulation
Final product
Enzymatic Disruption of Algal Cell Walls as an Oil Extraction Technology

**Figure 3**


Enzyme addition
Harvesting
Centrifuge
Buffer-tank 700L
Bead-mill
Freeze Drying
$CO_2$-Extraction
• Water removal
• Water is eventually recycled into the process;
• Concentration to 10-12% dry matter
• Agitated tank
• Spore cracking criteria 95%
• Analysis: microscopic observations and acetone extraction