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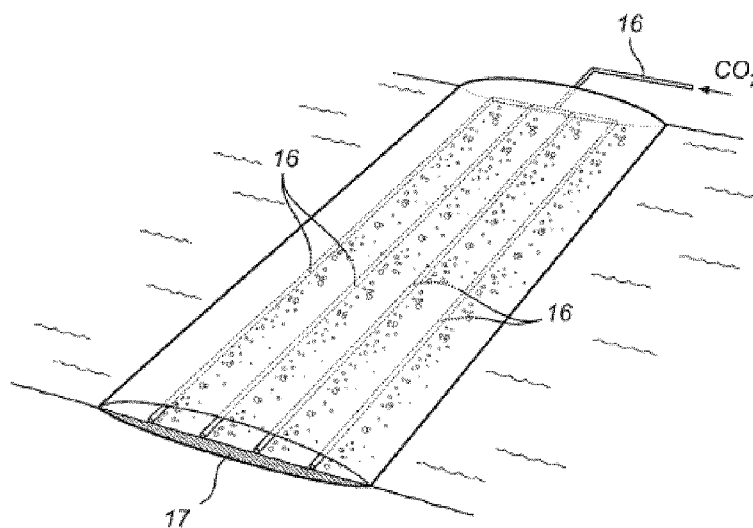
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(54) Title: HYBRID PHOTOBIOREACTOR

FIG. 2a



(57) Abstract: Herein is disclosed a method for cultivating a microorganism in a photobioreactor resulting in high biomass yields. Also provided is a system comprising a plurality of photobioreactors.

Hybrid photobioreactor

Technical field

5 The present invention relates to a method for cultivating a microorganism capable of mixotrophic growth in a photobioreactor. A system comprising a plurality of photobioreactors is also disclosed.

Background

10 Microorganisms capable of both photosynthetic and heterotrophic growth are already today found in many commercial applications, where either their photosynthetic capabilities or their fermentation capabilities are exploited. Algae are produced to manufacture β -carotene, astaxanthin, etc., or the complete algae biomass is sold as nutritional supplement. Today, the production of algae biomass faces two main
15 difficulties. First, a large part of the current production results from open systems (e.g., so-called open ponds). These open systems are sensitive to contaminations by other algae strains or by pest, therefore only algae with very specific growth requirements can be grown in these systems. Thus, for instance the alga *Dunaliella* is cultured for the production of β -carotene under very saline conditions, which are not suited for most
20 other organisms. Second, the production costs of algae biomass is rather high (> USD 2,000 per metric ton), so that a commercial production for many applications, especially in the energy sector or the transportation sector, is not profitable. In particular, production costs are often increasing even more if closed systems are used instead of open systems to avoid contaminations. Besides the open ponds, a large number of
25 various photobioreactor types are presently in use. Tube reactors, which can consist of one or more horizontal tubes, or wherein a tube is helically wound around a cylinder or cone (biocoil), are among the best known. Furthermore, flat panel reactors are often used, such reactors providing a vertical liquid layer for cultivation of algae.

30 The main challenges in the production of chemicals and energy from algae are the risk of contamination and the high cost for the manufacturing of the algae biomass. Likewise, the main challenges in the production of fine chemicals, nutritional supplements, vitamins, omega-3-fatty acids, antioxidants (e.g. carotenoids), pharmaceutically active substances or dried biomass for nutritional supplementation
35 from algae are thus the risk of contamination and the high cost for the manufacturing of

the biomass. The same challenges apply when culturing algae for biofuels, animal feed, amino acids, methane production, etc.

5 WO2009/090549 discloses a photobioreactor for cultivation of a phototrophic microorganism, where the photobioreactor is partially or completely surrounded by a body of water. The position of the photobioreactor is regulated by providing a density difference between the culture liquid comprised in the photobioreactor and the body of water.

10 Although the photobioreactor disclosed in WO2009/090549 leads to a significant reduction of the costs for producing biomass from algae, biomass production takes place via the photosynthetic route and thus only during the light hours of a day.

15 There is thus a need for methods of operating photobioreactors which further enhance productivity without increasing biomass production costs.

Summary

20 The present invention solves the problem of further reducing operating costs of operating a photobioreactor so as to obtain unprecedented high biomass yields by taking advantage of the natural daily cycles to enhance production of biomass by microorganisms capable of performing photosynthesis when exposed to light and fermentation when in the darkness. Photobioreactors can be operated for cultivation of a microorganism that is capable of mixotrophic growth and thereby grow at a
25 surprisingly high rate. Photobioreactors are operated in a way that allows the cultivated microorganism to be exposed to light and darkness in an alternating fashion and thus fully exploit the microorganism's metabolic capabilities, which leads to high biomass yield. Photobioreactors can be operated in a way that allows provision and recovery of gases such as carbon dioxide and oxygen, which can be recycled in the
30 photobioreactor operation or used for other purposes. The method is particularly advantageous when used with photobioreactors which are partially or completely surrounded by a body of water.

35 Herein is thus provided a method of operating a closed photobioreactor for cultivation of a microorganism capable of mixotrophic growth, comprising the steps of:

- i. providing at least one photobioreactor comprising a central compartment for holding a culture liquid and defined by walls of a water tight, transparent, and flexible material, said photobioreactor being partially or completely surrounded by a water body,
- 5 ii. providing a culture liquid comprising nutrients,
- iii. providing a microorganism capable of carrying out photosynthetic growth when exposed to light and heterotrophic growth when exposed to darkness,
- iv. exposing the photobioreactor to alternating periods of light and darkness.

10 Also provided is a method of producing biomass, the method comprising cultivating a microorganism capable of mixotrophic growth in a closed photobioreactor operated according to the methods disclosed herein.

15 Also provided is a system comprising at least one closed photobioreactor adapted for large-scale cultivation of a microorganism capable of mixotrophic growth as disclosed herein.

Description of Drawings

20 Figure 1a is a schematic view of a photobioreactor.

Figure 1b is a cross-sectional view of a photobioreactor.

Figure 2a shows a three-dimensional view of a photobioreactor with a CO₂ supply tube floating on the culture liquid compartment due to its low density.

25 Figure 2b displays a vertical cross-section of a photobioreactor with a CO₂ supply tube floating on the culture liquid due to its low density.

Figure 3 shows a photobioreactor with an additional compartment for controlling vertical position and or the shape of the reactor.

Detailed description

The invention is as defined in the claims.

35 In a first aspect, the present disclosure relates to a method of operating a closed photobioreactor for cultivation of a microorganism capable of mixotrophic growth, comprising the steps of:

- i. providing at least one photobioreactor comprising a central compartment for holding a culture liquid and defined by walls of a water tight, transparent, and flexible material, said photobioreactor being partially or completely surrounded by a water body,
- 5 ii. providing a culture liquid comprising nutrients,
- iii. providing a microorganism capable of carrying out photosynthetic growth when exposed to light and heterotrophic growth when exposed to darkness,
- iv. exposing the photobioreactor to alternating periods of light and darkness.

10 In a second aspect, the present disclosure relates to a method of producing biomass, the method comprising cultivating a microorganism capable of mixotrophic growth in a closed photobioreactor operated according to the methods disclosed herein.

15 In a further aspect, the present disclosure relates to a system comprising at least one closed photobioreactor adapted for large-scale cultivation of a microorganism capable of mixotrophic growth as disclosed herein.

Method of operating a closed photobioreactor for cultivation of microorganisms capable of mixotrophic growth

20 An aspect of this disclosure relates to a method of operating a closed photobioreactor for cultivation of microorganisms capable of mixotrophic growth, comprising the steps of providing at least one photobioreactor comprising a central compartment for holding a culture liquid and defined by walls of a water tight, transparent, and flexible material, the photobioreactor being partially or completely surrounded by a water body, providing
25 a culture liquid comprising nutrients, providing a microorganism capable of carrying out photosynthetic growth when exposed to light and heterotrophic growth when exposed to darkness, and exposing the photobioreactor to alternating periods of light and darkness.

30 The term "photosynthetic phase" refers to a period of time during which photosynthesis is performed by the microorganism. It does not exclude that other types of reactions may occur at the same time, for example the term "photosynthetic phase" does not exclude that chemotrophic growth, lithotrophic growth, autotrophic growth, heterotrophic growth and organotrophic growth occur simultaneously with
35 photosynthetic growth. Rather, the photosynthetic phase herein refers to a period of

time during which the culture liquid comprising the microorganism is exposed to light so that photosynthesis is possible.

5 Conversely, the term "heterotrophic phase" refers to a period of time during which the culture liquid comprising the microorganism is not exposed to light or is exposed to darkness, so that photosynthesis is not possible, but heterotrophic growth is possible. This does not exclude that chemotrophic growth, lithotrophic growth, autotrophic growth, and organotrophic growth occur simultaneously with heterotrophic growth. In one embodiment of the present disclosure, the method further comprises a step of
10 harvesting at least part of the biomass produced by the microorganism. The harvesting can take place at various time points and several means can be used, as described below.

Light and darkness

15 In order to allow the cultivated microorganism to grow photosynthetically and heterotrophically, the photobioreactor is exposed to light and darkness in alternating manner. The microorganism will grow via photosynthesis during exposure to light and via heterotrophic metabolism during exposure to darkness.

20 Preferably, the photobioreactor is exposed to light for 1 to 23 hours in a day. The term day is used to refer to a period of time consisting of 24 hours. The photobioreactor may be exposed to light for 1 hour, such as for 2 hours, such as for 3 hours, such as for 4 hours, such as for 5 hours, such as for 6 hours, such as for 7 hours, such as for 8 hours, such as for 9 hours, such as for 10 hours, such as for 11 hours, such as for 12
25 hours, such as for 13 hours, such as for 14 hours, such as for 15 hours, such as for 16 hours, such as for 17 hours, such as for 18 hours, such as for 19 hours, such as for 20 hours, such as for 21 hours, such as for 22 hours, such as 23 hours. The photobioreactor may be exposed to light for a period of time of at least 30 minutes, such as at least 1 hour, such as at least 2 hours, such as at least 3 hours, such as at
30 least 4 hour, such as at least 6 hours. The photobioreactor may therefore be exposed to light for one or more periods of time during one day, so that the total exposure time is between 6 and 18 hours per day.

Preferably, the photobioreactor is exposed to darkness for 1 hour, such as for 2 hours,
35 such as for 3 hours, such as for 4 hours, such as for 5 hours, 6 to 23 hours in a day.

The photobioreactor may be exposed to darkness for 6 hours, such as for 7 hours, such as for 8 hours, such as for 9 hours, such as for 10 hours, such as for 11 hours, such as for 12 hours, such as for 13 hours, such as for 14 hours, such as for 15 hours, such as for 16 hours, such as for 17 hours, such as for 18 hours, such as for 19 hours, such as for 20 hours, such as for 21 hours, such as for 22 hours, such as 23 hours.

The photobioreactor may be exposed to darkness for a period of time of at least 30 minutes, such as at least 1 hour, such as at least 2 hours, such as at least 3 hours, such as at least 4 hour, such as at least 6 hours. The photobioreactor may therefore be exposed to darkness for one or more periods of time during one day, so that the total exposure time is between 6 and 18 hours per day. Preferably, the photobioreactor is exposed to darkness for 6 hours. More preferably the photobioreactor is exposed to darkness for 7 hours. More preferably the photobioreactor is exposed to darkness for 8 hours. More preferably the photobioreactor is exposed to darkness for 9 hours. More preferably the photobioreactor is exposed to darkness for 10 hours. More preferably the photobioreactor is exposed to darkness for 11 hours. More preferably the photobioreactor is exposed to darkness for 12 hours.

The light duty cycle, or duty cycle, is the fraction of a total light-dark cycle in which an individual microorganism is exposed to light. In the present context, the light duty cycle is preferably the same for all microorganisms within the central compartment, although minor variations may occur in the transition periods between light and darkness.

In one embodiment, the central compartment has a duty cycle of at least 10%, such as at least 15%, for example at least 20%, such as at least 25%, for example at least 30%, such as at least 35%, for example at least 40%, such as at least 45%, for example at least 50%, such as at least 55%, for example at least 60%, such as at least 65%, for example at least 70%, such as at least 75%, for example at least 80%, such as at least 85%, for example at least 90%, such as at least 95%.

In another embodiment, the central compartment has a duty cycle of at the most 95%, such as at the most 90%, for example at the most 85%, such as at the most 80%, for example at the most 75%, for example at the most 70%, such as at the most 65%, for example at the most 60%, such as at the most 55%, for example at the most 50%, such as at the most 45%, for example at the most 40%, such as at the most 35%, for example at the most 30%, such as 29%, 28%, 27%, or 26%.

In some embodiments, the central compartment has a duty cycle between 10 and 90%, such as between 15 and 85%, for example between 20 and 80%, such as between 25 and 75%, for example between 30 and 70%, such as between 35 and 65%, for example between 40 and 60%, such as between 45 and 55%, for example 50%. Preferably the duty cycle is between 25 and 75%.

In order to expose the photobioreactor to light, different light sources can be used, for example solar radiation or various types of artificial light, provided that the light can be used by the microorganisms to carry out photosynthesis. Each period of exposure to light is followed and/or preceded by a period of exposure to darkness. The term darkness is used herein to refer to the lack of light, a condition that prevents the microorganisms to carry out photosynthesis. For example, there can be natural darkness at night or darkness can be obtained by preventing the light to reach the photobioreactor.

The photobioreactor may for example be operated in the open, where the natural daily light cycle can be taken advantage of. In such embodiments, the photobioreactor is exposed to natural light during the day, while it is in darkness during the night. The natural daily cycle may be supplemented by an artificial light source or by artificial means for keeping the photobioreactor in the dark. For example, if the photobioreactor is operated during summer, and it is desirable to have longer periods of darkness, the photobioreactor may be shielded from the light either around dawn and sunset (in order to shorten the lit period) or when the solar radiation is most intense (e.g. at midday), or at any other time. Conversely, if longer periods of light are desired, the natural day may be prolonged by using an artificial light source during the night, either around dawn and sunset, or at any other time during the night.

Preferably, the entire central compartment is exposed to either light or darkness. In a preferred embodiment, the light exposure is the same for the entire photobioreactor at a given time. In a preferred embodiment, the central compartment is not divided in lit portions and in dark portions at a given time. Changing the exposure to light may transiently result in the presence of lit and dark portions, but in its stable operating mode, the photobioreactor is either exposed to light or is kept in the dark.

35

Photobioreactor

The present method is particularly advantageous when photobioreactors partially or completely surrounded by a body of water are used. Non-limiting examples of such bioreactors are known from WO2009/090549, WO2014/209935 and WO2010/121136.

5 Such photobioreactors are advantageous for several reasons. First, operating costs are reduced because the body of water can be used to regulate temperature of the culture liquid comprised within the photobioreactor, thereby reducing energy consumption. The fact that the photobioreactor is floating on or is surrounded by a body of water automatically gives a homogenous distribution of the thickness of the culture liquid, thereby ensuring that there are no “dead volumes” in the photobioreactor – productivity is thus increased. Furthermore, as the hydrostatic inner pressure of the photobioreactor being partly compensated by the surrounding water, the strength of the walls of the photobioreactor can be reduced or a less stable material can be used, thereby also reducing production costs.

15 The term “photobioreactor”, as used herein, generally refers to a photobioreactor comprising at least one central compartment adapted for holding a culture liquid comprising a microorganism. Preferably, the photobioreactor has a flat panel shape. When the photobioreactor is operating, the central compartment holds culture liquid wherein a microorganism capable of mixotrophic growth is cultured. The central compartment may have a rectangular shape, or a circular shape, or an elliptic shape, or any other suitable shape as recognised by the skilled person. The photobioreactor may also comprise one or more peripheral compartments along the edge of the central compartment. Such photobioreactors are described in detail in the copending application filed by the same applicant and having the same filing date as the present application, entitled “Method for harvesting biomass from a photobioreactor”. The photobioreactor may also comprise additional compartments or tubes, sub-compartments or mechanical means for controlling the position and/or shape of the photobioreactor, as well as peripheral equipment such as e.g. pumps, hoses, tanks and other equipment required for operating the reactor, for example sensors, as described in the section below “Control system”.

35 The term “fresh culture liquid” or “fresh culture medium” shall be construed as any liquid which results in provision of nutrients to the central compartment. In some embodiments, the fresh culture liquid may differ from the liquid that was initially

provided in the central compartment. The fresh culture liquid may vary and will depend on the nutrient requirements of the microorganism for optimal growth under given conditions.

5 Photobioreactors are closed systems and therefore less prone to microbial contamination than open ponds and other open cultivation systems. Nevertheless, the methods disclosed herein may further comprise means to prevent contamination that will be recognized as suitable by the skilled person. In some embodiments, yeasts are provided to the culture liquid medium. The presence of yeast may help preventing
10 contamination by other microorganisms competing for the same nutrients. In fact, both yeast and microalgae together fill available ecological niches to protect against invasion.

Furthermore, yeast may utilize a different carbon source than the cultivated
15 microorganism and so contribute to the production of biomass. The introduction of yeast enables lignocellulosic sugars, which cannot be metabolized by most microalgae, to be digested and can increase algal biomass by transforming part of these carbon sources into CO₂. An example of an algal-fungal pond is described in Gomez et al., 2015. Yeast can consume some of the O₂ produced by the algae during prototrophic growth, while producing CO₂, which in turn can be consumed by the algae during
20 prototrophic growth.

In preferred embodiments, oleaginous yeast species are added to the culture liquid. Non-limiting examples of oleaginous yeast include oleaginous yeasts include
25 *Cryptococcus albidus*, *Lipomyces starkeyi*, *Rhodotorula glutinis*, *Trichosporon pullulans*, and *Yarrowia lipolytica*.

In practice, the photobioreactor is manufactured from a top sheet and a bottom sheet of a flexible, nutrient-impermeable and water-tight material, and can include a top sheet,
30 being wholly or partially a semi permeable membrane to exude or exhale or ventilate gasses, such as O₂, CO₂, N₂, H₂ which can be harmful to microalgae in different phase of their growth. The top sheet and bottom sheet are placed on each other, and the central compartment is defined by welding the sheets together close to the edge. By performing a first welding in a first position and a second welding in a second position,
35 a peripheral compartment is defined between the central compartment and the edge of

the photobioreactor. Both compartments are water-tight and nutrient-impermeable. The compartment may also be bacterial-impermeable.

5 The central compartment of a photobioreactor preferably comprises at least one first fluid connection, which can be opened and closed in the controlled manner. In the embodiments where a photobioreactor comprises a central compartment and a peripheral compartment, the compartments may be connected via one or more first fluid connections. The peripheral compartment may also comprise one or more further fluid connections, which can be opened and closed in the controlled manner, and which
10 connect the peripheral compartment to the environment, e.g. to a feeder and/or a harvester. The first and further fluid connections can be used for example in the steps of feeding and harvesting. Reference is made to the copending application entitled "Method for harvesting biomass from a photobioreactor", in particular to the sections "Connection between the central compartment and the peripheral compartment" and
15 "Connection between the peripheral compartment and the environment".

This particular structure allows operations such as the harvesting of biomass from and/or the provision of fresh culture liquid medium to the central compartment by using the peripheral compartments instead of using extensive tubing.

20

The structure of such photobioreactors allows operations such as the harvesting of biomass from and/or the provision of fresh culture liquid medium to the central compartment by using the peripheral compartments instead of using extensive tubing, as described in the sub-sections below "Harvesting" and "Feeding".

25 Density difference

In some embodiments, the disclosed method further comprises a step of providing a density difference between the culture liquid comprised in the photobioreactor and the surrounding water so that the position of the photobioreactor in the water body is controlled. Various methods to provide such a density difference are described below.

30

The vertical position of a flexible and light weight photobioreactor in a surrounding water body can be controlled by controlling the density of the photobioreactor versus the density of the surrounding water, e.g. by providing different salinity concentrations inside and outside of the reactor. Such photobioreactors assume a perfect horizontal

position regardless of their starting position and are thus very stable. Moreover, the thickness of the layer of culture liquid inside the reactor becomes very homogenous, again independent of the starting point.

- 5 The culture liquid comprised in the photobioreactor has a thickness that makes it possible for the cultivated microorganism to absorb light during the light exposure periods. In some embodiments, the thickness of the culture liquid is between 1 and 30 cm, such as between 1 and 25 cm, such as between 1 and 20 cm, such as between 1 and 15 cm, such as between 1 and 10 cm, such as between 1 and 5 cm, such as
10 between 5 and 30 cm, such as between 10 and 30 cm, such as between 15 and 30 cm, such as between 20 and 30 cm, such as between 25 and 30 cm.

Since small density differences in the water inside and outside of the photobioreactor caused by a difference in salinity and/or temperature are the only driving forces for
15 moving the reactor, it is preferable to have a thin and flexible material in the walls of the photobioreactor. Having thin and flexible walls will optimize the capability of the photobioreactor to self-stabilize. An example of a material which is suitable for use in the photobioreactor walls is polyethylene or equivalent material with a thickness of about 0.1 mm, such as 0.2 mm or thicker.

20 By provision of said density difference between the culture liquid and the surrounding water so that the position of the photobioreactor in the water body is controlled is thus created a change in buoyancy of the photobioreactor in relation to the surrounding water, this change in buoyancy being the driving force of a vertical position change of
25 the reactor. Thus, the density difference provided takes into account the weight and buoyancy of the photobioreactor itself.

The density difference may be provided by provision of a salinity difference between the culture liquid and the surrounding water. Said salinity difference may be provided
30 by increasing or decreasing the salinity of the culture liquid. Said salinity difference may also, or alternatively, be provided by increasing or decreasing the salinity of the surrounding water, in particular the surrounding water of a closed water body.

A salinity increase of the culture liquid may be provided simultaneously with a salinity decrease of the surrounding water. A salinity decrease of the culture liquid may be provided simultaneously with a salinity increase of the surrounding water.

5 The density difference may be provided by provision of a temperature difference between the culture liquid and the surrounding water. Said temperature difference may be provided by changing the temperature of the surrounding water, in particular the surrounding water of a closed water body. The meaning of "closed water body" is as
10 below.

Parameters influencing the density of the culture liquid and/or the surrounding water may be modified separately or simultaneously in order to provide a desirable density difference. The density of the culture liquid and the density of the surrounding water may be modified separately or simultaneously in order to provide a desirable density
15 difference.

The density difference may be provided so that the density of the culture liquid is increased or so that the density of the surrounding water is decreased, whereby the position of the photobioreactor in the water body is lowered. The density difference
20 may be provided so that the density of the culture liquid is decreased or so that the density of the surrounding water is increased, whereby the position of the photobioreactor in the water body is raised. The density difference may be provided so that the position of the photobioreactor in the water body is maintained. Again, the density of the culture liquid and the density of the surrounding water may be modified
25 simultaneously in order to provide a desirable density difference, either for lowering, raising or maintaining the position of the photobioreactor.

In one embodiment, the photobioreactor rests on the surface of open water as a starting position. When the photobioreactor rests, or floats, on the surface of open
30 water, the density of the photobioreactor is lower compared to the density of the water. If the position of the photobioreactor needs to be lowered, the density difference between the photobioreactor and the open water is regulated. As an example, the position of the photobioreactor may need to be lowered when the measured

temperature of the culture liquid is higher or expected to be higher than a predetermined temperature value. To regulate the density difference between the photobioreactor and the surrounding water, the salinity of the culture liquid is increased. This is achieved by replacing or complementing the culture liquid by culture liquid of higher salinity, i.e. by pumping culture liquid of higher salinity into the photobioreactor. The flow rate of the culture liquid is set so as to allow the algae to adapt to the higher salt concentrations in the culture liquid and to minimize any loss of algae in the photobioreactor. As the culture liquid is replaced or complemented by culture liquid of higher salinity, the density of the photobioreactor increases. The photobioreactor sinks in the open fresh water due to its higher density compared to the surrounding water and the position of the photobioreactor is lowered. The position of the photobioreactor may be lowered until the measured temperature of the culture liquid is within a desirable temperature range.

Other ways of operating such photobioreactors, where the position of the photobioreactor is controlled by a density difference, are described in WO2009/090549, in particular in the sections entitled "The closed photobioreactor in open water" and "The photobioreactor in a closed body of water".

Sometimes additional means, which are independent of density or salinity or temperature, for controlling the position and /or shape of the photobioreactor may be useful. This may for instance be the case when the photobioreactor needs to be submerged quickly. Such means may include additional compartments or tubes capable of being filled with high or low density medium in order to assist submersion or floatation of the photobioreactor, mechanical means for assisting submersion or floatation of the photobioreactor, and sub-compartments within the algae compartment of the photobioreactor for controlling the shape of the reactor when it is submerged. These three types of means are discussed in detail in WO2009/090549, in particular in the sections entitled "Additional means for controlling the position and/or shape of the photobioreactor", "Additional compartments or tubes capable of being filled with high or low density medium" and "Mechanical means for assisting submersion or floatation of the photobioreactor".

Water body

The photobioreactor used in the present method is partially or completely surrounded by a body of water. The body of water may be a closed body of water or an open body of water. The water may be brackish water or salt water such as sea water. Reference
5 is made in this respect to WO2009/090549, in particular to the sections entitled "The photobioreactor in a closed body of water" and "The closed photobioreactor in open water".

The term "closed water body" refers to well-defined systems of water allowing control
10 of, e.g. the amount or type, such as fresh, brackish or salt, of water therein. Examples of closed bodies of water are natural or artificial ponds or pools.

The term "open water" refers to natural bodies of water, such as lakes, rivers or the sea, wherein an effective control of the chemical or physical properties of the water is
15 difficult or impossible.

Feeding the photobioreactor with fresh culture medium

In order to cultivate the microorganism capable of mixotrophic growth, some nutrients are provided via a feeder.
20

The composition of fresh culture liquid is suitable for cultivation of the microorganism provided in the photobioreactor and is typically dependent e.g. on the type of microorganism. The skilled person will easily determine the composition of a suitable fresh culture liquid, which may comprise nutrients such as calcium, phosphorous, iron,
25 magnesium, sodium, nitrate, ammonium, sulphate, chloride, boron, manganese, copper, molybdenum among many others. The fresh culture liquid may also be a wastewater stream deriving from processing of waste, or from agricultural or industrial process, or from sewage treatment. The composition of the fresh culture medium fed to the photobioreactor during exposure to light may differ from the composition of the
30 fresh culture medium fed to the photobioreactor during exposure to darkness.

Preferably, the fresh culture liquid provided when the photobioreactor is exposed to light does not comprise carbohydrates and the cultivated microorganism will grow via photosynthetic metabolism. Likewise, the fresh culture liquid provided when the photobioreactor is exposed to darkness comprises carbohydrates and the cultivated
35 microorganism will grow via heterotrophic metabolism. The methods disclosed herein

are such that they allow the cultivated microorganism to consume at least part of the carbohydrates provided during heterotrophic growth, so that at the beginning of each photosynthetic phase at least part of the provided carbohydrates have been removed from the culture liquid. Thus in some embodiments at least 50%, such as at least 60%
5 such as at least 70% such as at least 80%, such as at least 90% such as at least 95%, such as 99% of the provided carbohydrates are consumed during heterotrophic growth.

In order to determine when the culture liquid within the central compartment should be replenished, the present method may further comprise the step of measuring process
10 parameters of the culture liquid in the photobioreactor, comparing said measured value with a reference value, and providing fresh culture liquid to the photobioreactor where the measured value is different from said reference value. The fresh culture liquid may also be harvested at predefined time intervals.

15 In preferred embodiments, oleaginous yeast species are added to the culture liquid to be provided to the photobioreactor. Non-limiting examples of oleaginous yeasts include *Cryptococcus albidus*, *Lipomyces starkeyi*, *Rhodotorula glutinis*, *Trichosporon pullulans*, and *Yarrowia lipolytica*. In embodiments where the culture liquid comprises yeast, the fresh liquid culture preferably also comprises lignocellulosic material that can
20 be used by the yeast as carbon source, e.g. cellulosic glucose, xylose or a combination thereof.

The photobioreactor can have a structure that allows the central compartment to be directly and/or indirectly connected to the feeder.

25 The step of feeding culture liquid medium may be performed in a continuous or in a semi-continuous manner. The step of feeding can be repeated to ensure optimal operation of the photobioreactor and optimal growth of the cultivated microorganism.

In embodiments where the photobioreactor comprises at least one peripheral
30 compartment, the peripheral compartment of a given photobioreactor may be used not only for harvesting biomass but also for providing nutrients to the central compartment. This can be done essentially as described for harvesting, except that the direction of fluid exchange is reversed. Preferably, the feeder comprises or is connected to a container for holding fresh culture liquid or liquid comprising nutrients. Reference is

made to the copending application entitled "Method for harvesting biomass from a photobioreactor", in particular to the section entitled "Providing fresh culture medium".

Harvesting

5 Harvesting of biomass may be performed via outlets designed for this purpose. The photobioreactor may be directly or indirectly connected to a harvester. In order to determine when said biomass should be harvested, the photobioreactor may be equipped with a sensor providing information as to the density of the culture. The sensor is preferably placed in the central compartment. The sensor may be connected
10 to a monitoring system, measuring and analysing process parameters such as temperature, salinity, pH, CO₂ concentration, O₂ concentration and biomass concentration. Suitable sensors and monitoring systems will be readily recognised by the skilled person. Preferably, the harvester comprises or is connected to a container for holding the harvested biomass.

15 In order to determine when biomass is to be harvested, the present method may further comprise the step of measuring biomass density in the photobioreactor, comparing said measured value with a reference value, and harvesting at least part of the biomass from the photobioreactor where the measured value is equal to or greater than
20 said reference value. The biomass may also be harvested at predefined time intervals.

The harvester preferably helps facilitate fluid exchange. In one embodiment, the harvester comprises a pump, such as an automatic pump or a manual pump, connected to a container for holding the harvested biomass. In other embodiments,
25 gravity is taken advantage of, and the harvester merely comprises a container for holding the harvested biomass, but do not comprise a pump. For example, a siphon may be used. Alternatively, gravity and a pump are used together. In such embodiments where gravity is taken advantage of, the compartment of the photobioreactor from which biomass is to be harvested may be divided in sub-
30 compartments, between which fluid exchange is possible in a controlled manner, e.g. by the use of a fluid connection. When the fluid connection is opened, the liquid entering the sub-compartment on one side of the photobioreactor may cause tilting of the photobioreactor, whereby the liquid, due to gravity, can flow to the container for holding the biomass more easily. In embodiments where the harvesting is facilitated by

gravity, harvesting may be initiated or further supported by a pump to facilitate initiation of the fluid exchange.

5 In systems comprising a plurality of photobioreactors, the biomass of each photobioreactor may be harvested independently of any other photobioreactor.

10 The harvester may comprise or be connected to a harvesting compartment, such as a container. The harvesting compartment may be equipped with additional devices facilitating the separation of the harvested biomass from the culture liquid, and/or facilitating the conversion of the harvested biomass into the product of interest.

15 The step of harvesting biomass may be performed in a continuous or in a semi-continuous manner. The step of harvesting can be repeated to ensure optimal operation of the photobioreactor and optimal growth of the cultivated microorganism.

20 In embodiments where the photobioreactor comprises at least one peripheral compartment, the peripheral compartment of a given photobioreactor may be used not only for providing nutrients to the central compartment but also for harvesting. This can be done essentially as described for providing the photobioreactor with fresh culture liquid, except that the direction of fluid exchange is reversed. Preferably, the harvester comprises or is connected to a container for holding the harvested biomass. Reference is made to the copending application entitled "Method for harvesting biomass from a photobioreactor", in particular to the sections entitled "Harvesting".

25 Such photobioreactors can also be used to simultaneously or sequentially providing fresh culture liquid medium to the central compartment or harvesting biomass therefrom, as described in detail in said copending application, in particular in the section entitled "Harvesting biomass and providing fresh culture medium".

30 Exchange of gases

35 Algae require for their photosynthetic growth large amounts of CO₂ since they use this as a key source of carbon. Furthermore, in the process of photosynthesis, oxygen is produced, which might be toxic to the algae. Therefore, in order to allow photosynthetic growth of the cultivated microorganism, CO₂ is supplied to and O₂ is removed from the culture medium comprised in the photobioreactor during the photosynthetic period,

which is the period of exposure to light. The mass transfer of these gases across the liquid-gas barrier is therefore crucial for high productivity. A number of possible ways of providing CO₂ to the algae culture, and for removing formed oxygen from the same will be described herein below. The methods described herein should not be construed as limiting to the present invention. Other methods apparent to a person skilled in the art in the light of the present disclosure are also considered to be within the scope of the present invention.

In an embodiment, mass transfer of CO₂ to the culture medium is achieved via passive diffusion of gaseous CO₂ over a large surface area of the culture medium. Assuming that the kinetics of diffusion processes as described by Fick's first and second law and the subsequent hydration and deprotonization processes are fast enough to provide the algae culture with enough CO₂ and to avoid toxic effects of O₂ via photo-oxidation, a passive diffusion of CO₂ via a large surface would be sufficient. Passive diffusion has the advantage that no energy is needed to move water or to force CO₂ into the water. Additionally, investment costs will be reduced since no active aeration would be required. In such a case, CO₂ transfer will take place at the interfacial layer between water and the CO₂ gas without any more energy added. In a more specific embodiment this could be realized by generating a gas bubble of CO₂ rich gas above the culture medium inside the photobioreactor.

In another embodiment, CO₂ is bubbled through the culture medium. The gaseous CO₂ may preferably be supplied by a tube or a tube-like device, extending into the culture medium. Such a system could comprise holes, through which a CO₂ rich gas may be pushed by applying pressure from an external device. The tubes or tube like devices may for example be fixed at the bottom of the reactor and the typical direction of the holes would be into the direction of the water surface.

During operation of the photobioreactor, the CO₂ rich gas and/or CO₂-enriched air may be supplied continuously to the culture medium. This embodiment also has the additional advantage that it leads to a continuous degassing of oxygen close to *statu nascendi*, i.e. the oxygen produced is removed from the culture medium shortly after it is formed.

Alternatively, the CO₂ rich gas can be added in short pulses. Various means exist to determine the length of a pulse, the amount of gas pushed in, the pressure the gas is pushed and the time between pulses. In an embodiment the gas could be pulsed by a timer, which gives a regular signal, e.g. every 5 minutes for a pulse of 1 minute. In
5 another embodiment, the pulse is controlled by a special unit which is capable of estimating the amount of CO₂ used by the algae and calculating the optimal length of the pulse, the amount of gas to be pushed in, the pressure the gas is to be pushed in with, and the time between the pulses. To estimate the amount of CO₂ required, the unit may comprise different sensors, e.g. a sensor measuring the light intensity, a
10 sensor measuring the temperature and a sensor measuring the biomass density in the photobioreactor. Using the data points received by these sensors a process controller would calculate the optimal pulse pattern for the photobioreactor system.

The amount of added CO₂ may also be related to the pH in the reactor. A pH electrode
15 is arranged in the culture medium, and this electrode continuously measures the voltage across a semi-permeable membrane allowing protons to pass the membrane against a defined redox-system, e.g. against an Ag/AgCl electrode. The voltage is registered by a process control unit. The process control unit will add a CO₂ pulse as soon as the voltage reaches a predefined point. The parameters of the pulse, such as
20 time, amount of pulses per minute, voltage to stop the pulsing can be entered into the process control unit.

In another embodiment, shown in Figs. 2a and 2b, gaseous CO₂ may be supplied to the culture medium by a tube or a tube-like device extending into the photobioreactor
25 and arranged to float on top of the surface of the culture medium due to its lower density. Bubbling of gaseous CO₂ is performed similarly as in the case described above, wherein the tube or tube-like device extends into the culture medium. However, the tube or tube-like device through which the CO₂ is supplied will be specifically designed to float on the surface of the culture medium in the photobioreactor. This is
30 achieved by the density of the whole (16) aeration system being lower than the density of the algae culture medium (17). The holes (18) in the tube or tube-like device, through which the CO₂ is pushed into the culture medium may preferably point downwards in this embodiment to achieve the best possible gas transfer. The holes will thereby be positioned at, or slightly below, the surface of the culture medium.

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During operation of the photobioreactor, the CO₂ rich gas may be supplied continuously to the culture medium. This embodiment also has the additional advantage that it leads to a continuous degassing of oxygen close to *statu nascendi*, i.e. the oxygen produced is removed from the culture medium shortly after it is formed.

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CO₂ does not necessarily have to be supplied to the culture medium in the form of gaseous CO₂ inside the photobioreactor. The CO₂ enriched medium may also be prepared outside the photobioreactor, e.g. by bubbling gaseous CO₂ through an aqueous medium. In other words, instead of supplying the CO₂ in the transparent part of the photobioreactor this could be done outside of the actual photobioreactor. In an embodiment, such a system may employ a vertical tank containing an aqueous medium wherein CO₂ rich gas is supplied at the bottom or close to the bottom of the tank. While bubbles of CO₂ rise up through the aqueous medium, CO₂ will transfer from the bubbles into the aqueous medium, and at the same time the oxygen may be removed from the culture medium. In a preferred embodiment, the aqueous medium which is enriched with CO₂ is culture medium from the photobioreactor which is enriched with CO₂ and subsequently returned into the photobioreactor. As the tank may have a height of several meters the residential time of CO₂ may be comparatively long, allowing for a good mass transfer. To bubble CO₂ into a vertical tank, energy is required to work for instance against the hydrostatic pressure. The energy, which is put in for pressurizing the gas, may also be used to move the aqueous medium from the algae compartment into the CO₂ enrichment device and back to the algae compartment.

25 In another embodiment, instead of bubbling CO₂ through the algae culture medium inside or outside of the photobioreactor, the CO₂ supply is facilitated by the use of a semi-permeable membrane. The use of such a membrane would have various advantages compared to the bubbling:

30 a) Such a membrane would work as a one-way-valve, meaning that the membrane would allow CO₂ to enter the culture medium, but prevent water from entering into the CO₂ supply system, as such a membrane would be permeable for CO₂ but not for water.

b) Lower energy consumption. Since no bubble generation is required, the membrane method allows CO₂ supply with lower energy consumption compared to the bubbling process.

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c) Low shear-stress. By avoiding the bubbling, the shear-stress on the algae cells is reduced. Less shear stress on the algae results in less dead algae cells in the algae culture medium and therefore less organic material which is prone to decomposition which may reduce the efficiency of the photobioreactor. Furthermore, this would significantly reduce the risk of contamination by heterotrophic organisms.

d) Increased mass transfer rate. The use of a membrane allows a higher CO_2 pressure than the embodiment employing passive diffusion described above, since the CO_2 pressure against the membrane is not limited to the surrounding atmospheric air pressure as it would be the case of passive diffusion. Furthermore, the membrane might have a higher surface area than a flat surface, such as the surface of the culture medium, with the same size as the membrane.

The mass transfer of CO_2 and/or oxygen may also be facilitated by moving the photobioreactor, e.g. by tilting the reactor.

The O_2 removed may be recovered and recycled, for example it may be provided to the culture medium during heterotrophic growth and/or it may be used for purposes that are outside the scope of this disclosure.

The cultivated microorganism will grow heterotrophically when exposed to darkness. At those conditions, the microorganism will use sugars and other carbohydrates as carbon source, in a fermentation process, and so it may produce CO_2 . The microorganism may also use O_2 as energy source and therefore O_2 may be provided to the photobioreactor in similar ways as described for CO_2 . Other methods apparent to a person skilled in the art in the light of the present disclosure are also considered to be within the scope of the present invention.

The O_2 produced by the microorganism during photosynthesis may be at least partly consumed by the microorganism during the heterotrophic phase. Conversely, the CO_2 produced during the fermentation phase may be at least partly consumed by the microorganism during the photosynthetic phase.

The CO_2 or O_2 produced during the fermentation phase and the photosynthetic phase, respectively, may be agitated in the culture liquid comprised in the central compartment to optimise growth and/or productivity of the microorganism.

The O₂ produced by the cultivated microorganism during the photosynthetic phase may be recovered and provided to the culture liquid during exposure to darkness, so that it can be used by the microorganism during the heterotrophic phase. Similarly, the CO₂ produced by the cultivated microorganism during heterotrophic growth may be recovered and provided to the liquid culture during exposure to light, so that the microorganism may use it for phototrophic growth. Some methods for recovering and providing a gas have been described above and other methods apparent to a person skilled in the art in the light of the present disclosure are also considered to be within the scope of the present invention.

The CO₂ or O₂ produced during the fermentation phase and the photosynthetic phase, respectively, may also be used for purposes that are apparent to the person skilled in the art. For example, the CO₂ or O₂ produced may be consumed by yeast added to the culture liquid as described above. They may also be provided to a neighbouring tank used for a different process, e.g. cultivation of another microorganism, or as a means to provide mixing/stirring to fermenters or water treatment facilities.

In some embodiments of the present disclosure, nitrogen gas that accumulates in the photobioreactor during photosynthetic growth of the cultivated microorganism may be removed and/or recovered at the time of switching conditions from exposure to light to exposure to darkness, such as when the metabolism of the cultivated microorganism switches from phototrophic to heterotrophic. The photobioreactors may thus be further equipped with a nitrogen removal device connected with the central compartment. It may be desirable to remove nitrogen in the medium after photosynthesis, and before switching to heterotrophic fermentation. In some embodiments, at least 90% of the nitrogen is removed, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as at least 99.5% or more.

Control system

In one embodiment, process parameters such as pH, pO₂, pCO₂, salinity and temperature of the culture liquid, are regulated by a multi-purpose system. As described herein, the density of the culture liquid can e.g. be used to control the position of the photobioreactor in the water body. The multipurpose system is programmed with information related to the photobioreactor, such as the overall weight

and density of the photobioreactor and the amount of biomass and culture liquid that is contained in the photobioreactor. Moreover, the system continuously measures the temperature, salinity and density of the culture liquid and the density of the surrounding water, thereby continuously determining the density difference between the
5 photobioreactor and the surrounding water. The system also controls the concentration of the different components of the culture liquid, such as the salt concentration. The system may then automatically regulate the position of the photobioreactor in the surrounding water as a response to a change in the temperature of the culture liquid, so as to keep the algae culture at a constant temperature. The system may thus be
10 equipped with known control circuits or algorithms, such as control algorithms with feedback mechanisms, to allow optimal stability when regulating the position of the photobioreactor.

In a further embodiment, when the photobioreactor is in a closed body of water, the
15 salinity and temperature of the surrounding water are regulated by the multi-purpose system described above. In an embodiment of the photobioreactor having additional compartments or tubes, the control system also regulates the filling and emptying of gas, water and other liquids of the compartments or tubes. In one embodiment, the multi-purpose system controls not only parameters related to positioning of the
20 photobioreactor but also parameters relevant to growth of the algae. Thus, the control system also measures and regulates O₂ and CO₂ contents of the algae culture.

Dimensions and materials

Photobioreactors suitable for cultivation of phototrophic microorganisms preferably let
25 light pass through, so that the microorganisms can perform photosynthesis. In one embodiment, at least part of the central compartment of the photobioreactor is manufactured from a transparent material. Preferably, the material is flexible. The water tight, transparent and flexible material may preferably further be a light weight, or low density, material. The material may preferably be a polymer based material, such
30 as a thin film of a polyolefin based polymer, e.g. polyethylene or polypropylene. Other polymers suitable for use with the present invention will be readily recognized by a person skilled in the art of polymeric materials. The thickness of the walls should be selected depending on the properties, such as flexibility, transparency and durability, of the specific material used and may for example be in the range of 10-1000 µm or in the
35 range of 25-500 µm or in the range of 50-150 µm. It is preferred, with regard taken to

the durability of the material, to make the walls of the photobioreactor as thin as possible in order to maximize the flexibility and transparency. As a non-limiting example, a polyethylene film having a thickness of about 100 μm has been found to be suitable for use in the walls of the photobioreactor.

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It will be understood that the present methods can be adapted to be used with a wide variety of photobioreactors, over a wide range of sizes. The area of the body of water covered by the photobioreactor may range from one to several hundred square meters. In some embodiments, the area covered by the photobioreactor is between 1 and 1000 m^2 , such as between 10 and 900 m^2 , such as between 25 and 800 m^2 , such as between 40 and 750 m^2 , such as between 50 and 700 m^2 , such as between 60 and 600 m^2 , such as between 70 and 500 m^2 , such as between 80 and 450 m^2 , such as between 90 and 400 m^2 , such as between 100 and 300 m^2 , such as between 150 and 275 m^2 , such as between 200 and 275 m^2 , such as about 250 m^2 . In a preferred embodiment, the area is 10 m^2 . In another preferred embodiment, the area is 40 m^2 . In another preferred embodiment, the area is 250 m^2 .

The volume of the central compartment may also vary over a wide range. In some embodiments, the volume of the central compartment of a photobioreactor is between 100 and 20,000 L, such as between 500 and 15,000 L, such as between 1,000 and 12,500 L, such as between 2,000 and 10,000 L, such as between 2,500 and 9,000 L, such as between 3,000 and 8,000 L, such as between 3,500 and 7,000 L, such as between 4,000 and 6,000 L, such as between 4,500 and 5,500 L, such as between 5,000 L. In a preferred embodiment, the volume is 500 L. In another preferred embodiment, the volume is 2,000 L. In another preferred embodiment, the volume is 12,500 L.

The average thickness or height of the central compartment may also vary. As explained herein elsewhere, the culture liquid in the central compartment in a photobioreactor completely or partially surrounded by a body of water will assume a homogenous distribution, and will be essentially even, with the exception of zones close to the edges of the central compartment, where the walls of the photobioreactor may be round to some extent. In some embodiments, the height of the central compartment is between 1 and 30 cm, such as between 1.5 and 29 cm, such as between 2 and 28 cm, such as between 2.5 and 27 cm, such as between 3 and 26 cm,

such as between 4 and 25 cm, such as between 5 and 20 cm, such as between 6 and 19 cm, such as between 7 and 18 cm, such as between 8 and 17 cm, such as between 9 and 16 cm, such as between 10 and 15 cm, such as between 11 and 14 cm, such as between 12 and 13 cm.

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In some embodiments, the thickness of the culture liquid is between 1 and 30 cm, such as between 1.5 and 29 cm, such as between 2 and 28 cm, such as between 2.5 and 27 cm, such as between 3 and 26 cm, such as between 4 and 25 cm, such as between 5 and 20 cm, such as between 6 and 19 cm, such as between 7 and 18 cm, such as between 8 and 17 cm, such as between 9 and 16 cm, such as between 10 and 15 cm, such as between 11 and 14 cm, such as between 12 and 13 cm.

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In one embodiment, the area covered by the photobioreactor is 10 m², and the volume of the central compartment is 500 L. In another embodiment, the area covered by the photobioreactor is 40 m², and the volume of the central compartment is 2000 L. In another embodiment area covered by the photobioreactor is 250 m², and the volume of the central compartment is 12,500 L.

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Microorganism capable of mixotrophic growth

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Mixotrophic microorganisms that are useful for biomass production or the production of products as described herein are known to the skilled person. Preferably, the microorganism is capable of photosynthetic growth when exposed to light, but may additionally also be capable of chemotrophic growth, lithotrophic growth, autotrophic growth, heterotrophic growth and organotrophic growth. When exposed to darkness, the microorganism is capable of heterotrophic growth, but may additionally also be capable of chemotrophic growth, lithotrophic growth, autotrophic growth and organotrophic growth.

25

The term "mixotroph" herein refers to an organism that can use a mix of different sources of energy and carbon. A mixotrophic microorganism may be autotrophic and heterotrophic, wherein autotrophy comprises photoautotrophy, and chemoautotrophy, for example lithotrophy, and wherein heterotrophy comprises photoheterotrophy, chemoheterotrophy and organoheterotrophy. Several combinations of the above trophisms are envisaged. Mixotrophs can be either eukaryotic or prokaryotic. In the present context, the mixotrophic microorganism is at least capable of performing

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photosynthesis when exposed to light. The phototrophic microorganism is preferably also capable of heterotrophic growth when not exposed to light. The mixotrophic microorganism as understood herein is thus at least capable of photosynthetic growth, i.e. the microorganisms are phototrophic, but are also one or more of chemotrophic, lithotrophic, organotrophic, and heterotrophic. Each trophic mode may be obligate, i.e. necessary for sustaining growth and/or maintenance of the microorganism, or facultative.

For example, some mixotrophic microorganisms are capable of carrying out photosynthesis when exposed to light and in presence of CO₂. They are also capable of fermenting sugars and carbohydrates.

In some embodiments of the present disclosure, microorganisms capable of mixotrophic growth are microalgae, also referred to as algae.

Non-limiting examples of suitable microorganisms are: *Dunianella salina*, *Haematococcus pluvialis*, *Neochloris oleoabundans*, *Chlorella vulgaris*, *Isochrysis galbana*, *Pavlova lutheri*, *Nanochloropsis oculata*, *Phaeodactylum tricornutum*, *Skeletonema sp.*, *Thalassiosira sp.*, *Chaetoceros sp.*, *Tetraselmis sp.* and *Spirulina platensis*.

Products

Microorganisms capable of mixotrophic growth can be used to produce compounds as described herein, which can find numerous applications in various fields, such as cosmetics and beauty products, pharmaceutical products, nutraceutical and dietary supplements, packaging and bioplastics, soil and water treatment, biofuels, pet foods and fertilisers, food and snacks. Thus in one embodiment, the method further comprises the step of extracting from the harvested biomass a biofuel, an animal feed, a protein, an amino acid, an ingredient for basic human nutrition, fine chemicals, nutritional supplements, vitamins, omega-2-fatty acids, antioxidants, such as carotenoids or beta-carotene, pharmaceutically active substances, amino acids or astaxanthin.

The described microorganisms may either be able of producing the above products, or the biomass of the cultivated microorganism may be converted to the above products.

Thus in one embodiment, the method further comprises the step of converting the

produced biomass to a biofuel, an animal feed, a protein, an amino acid, an ingredient for basic human nutrition, fine chemicals, nutritional supplements, vitamins, omega-2-fatty acids, antioxidants, such as carotenoids or beta-carotene, pharmaceutically active substances, amino acids or astaxanthin.

5

Method of producing biomass

The methods described in the present disclosure are particularly advantageous for producing biomass, preferably of a microorganism capable of mixotrophic growth, provided that the microorganism is cultivated in a closed photobioreactor operated according to the methods described herein and that the biomass is subsequently harvested, as described in detail above.

High production rates can be obtained using the disclosed methods. In some embodiments, at least 25 g/m²*day dry biomass is obtained, such as at least 30 g/m²*day dry biomass, such as at least 35 g/m²*day dry biomass, such as at least 40 g/m²*day dry biomass, such as at least 45 g/m²*day dry biomass, such as at least 50 g/m²*day dry biomass, such as at least 75 g/m²*day dry biomass, such as at least 100 g/m²*day dry biomass, such as at least 125 g/m²*day dry biomass, such as at least 150 g/m²*day dry biomass, such as at least 175 g/m²*day dry biomass, such as at least 200 g/m²*day dry biomass, such as at least 250 g/m²*day dry biomass, such as at least 275 g/m²*day dry biomass, such as at least 300 g/m²*day dry biomass, such as at least 350 g/m²*day dry biomass, such as at least 400 g/m²*day dry biomass, such as at least 450 g/m²*day dry biomass, such as at least 500 g/m²*day dry biomass.

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For example between 25 and 500 g/m²*day dry biomass can be produced, such as 50 to 500 g/m²*day dry biomass, such as 100 to 500 g/m²*day dry biomass, such as 200 to 500 g/m²*day dry biomass, such as 300 to 500 g/m²*day dry biomass, such as 400 to 500 g/m²*day dry biomass, such as 50 to 400 g/m²*day dry biomass, such as 50 to 300 g/m²*day dry biomass, such as 50 to 200 g/m²*day dry biomass, such as 50 to 100 g/m²*day dry biomass. Preferably, high production of biomass is obtained during heterotrophic growth, such as at least 5 g/m²*h dry biomass, such as at least 10 g/m²*h dry biomass, such as at least 20 g/m²*h dry biomass, such as at least 30 g/m²*h dry biomass. The surface refers to the surface of the water body covered by the photobioreactor, while the term "day" refers to a 24 hour period.

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In some embodiments, the produced biomass is further processed so that products are produced as described more in detail above. The biomass may be used for extracting various compounds. The biomass may also be converted into various products.

5 System of photobioreactors

The present methods are particularly advantageous for large-scale cultivation of microorganisms capable of mixotrophic growth, since they allow savings on the required length of tubing required for harvesting the produced biomass and/or feeding the photobioreactors with culture liquid. As will be recognised by the skilled person,
10 these savings are multiplied by the number of photobioreactors used.

Accordingly, it is an aspect of the present disclosure to provide a system comprising at least one closed photobioreactor adapted for large-scale cultivation of a microorganism capable of mixotrophic growth according to the methods described herein.

15 In some embodiments, the system comprises at least two photobioreactors, such as at least 3, at least 4, at least 5, at least 10, at least 20, at least 30, at least 40, at least 50, at least 100 photobioreactors or more.

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Detailed description of drawings

Fig. 1a is a view of a complete photobioreactor that can be adapted to be used in the methods described herein. The figure does not show the fluid connections, and is used solely to illustrate the general concept of photobioreactors. The panel shaped
25 photobioreactor 1 (also referred to herein as the “reactor”) floats on a water body, here an artificial pond 2. The size of such a photobioreactor 1 can vary. The photobioreactor 1 is in this embodiment manufactured from a flexible transparent material and within the photobioreactor is the culture liquid, in which the algae are suspended. By solar radiation on the photobioreactor 1, the algae are enabled to produce biomass via
30 photosynthesis. Carbon dioxide is used during this process and oxygen is produced. Therefore the culture medium is preferably always moving while illuminated, in order to provide new carbon dioxide and to remove oxygen which can be toxic for the algae. The culture medium is in this embodiment moved via a pump 3. The culture medium is thus moving through the photobioreactor and is brought back via a tube 4. The gas
35 exchange will take place in a tank 5, to which a tube system 6 will steadily provide a

- carbon dioxide rich gas mixture by means of a compressor 7. The carbon dioxide rich gas mixture can have its origin for instance from an electrical power plant using fossil fuels. The degassed oxygen will be lead out via a tube 8 equipped with a sterile filter. Culture liquid with algae biomass can be taken out of the system via a valve 9 and be
5 stored in a tank 10 until this harvested volume is processed further. New medium is provided to the system via a further valve 11 from a storage tank 12. This serves to level out the loss of liquid caused by the harvest and to supply culture liquid with new nutrients.
- 10 In an alternative embodiment (not shown), carbon dioxide is provided to the growing algae from a tube or hose located in the reactor, the tube or hose having one or more outlet(s) for carbon dioxide. Thus, in this embodiment the cultivation liquid must not move to pass tank 5 in order to be supplied with carbon dioxide.
- 15 Sensors 13 for determination of the salinity and the temperature of the culture liquid, and sensors 14 for determination of the salinity and the temperature of the surrounding water, are connected to a control unit 15. The control unit 15 determines the density difference between the culture liquid and the surrounding water, based on information from sensors 13, 14 as well as other parameters and stored data. The control unit
20 controls pumps (not shown) supplying seawater and fresh water, respectively, to the pond 2. In another embodiment (not shown), the control unit 15 controls means for changing the salinity of the culture liquid in the photobioreactor 1.
- Fig. 1b shows a cross section through such a system. The photobioreactor 1 is cut in a
25 lateral way, in this figure the photobioreactor floats on a water body 2. The vertical thickness of the culture liquid in the photobioreactor is typically between 1 and 30 cm. The depth of the water body 2 might vary significantly. The tube 4 which is used to circulate the culture liquid is seen in the lateral cut as well.
- 30 In an embodiment shown in Fig 3, an additional compartment is arranged on top of the photobioreactor. In this embodiment, the density of the total reactor system can be changed by adding a liquid with high density, preferably salt water, in the additional compartment (19), which is separate from the algae compartment (20). The
35 compartment, when filled, would increase the density of the whole reactor system such that the sinking process is accelerated. In this embodiment, the additional compartment

is arranged on top of the photobioreactor. The additional compartment comprises inner
gluing points (21) to provide structural stability. The additional compartments or tubes
may be connected to a supply of high density liquid by one or more hoses (22)
provided with valves (23) at one side of the reactor and a similar connection at the
5 opposite side of the reactor. When used for accelerating the submersion of the
photobioreactor according to this embodiment, the additional compartment will be filled
with water from one side and the valves at the other side will also be opened. By
starting the filling process from one side this side will become submerged first.
Remaining air in the additional compartment may thereby be collected at one side of
10 the photobioreactor and be pushed out more efficiently. The filling process will be
continued until all air is out and the complete reactor starts to sink. The valves opposite
to the filling hoses are then closed. The filling process may be stopped at this point or
the filling process may be continued for a while. Continuing the filling process increases
the pressure in the additional compartment, thus increasing the rigidity of this
15 compartment and allowing it to provide additional structural stability to the
photobioreactor during submersion and in a partially or fully submerged mode.
When the reactor system should go up, the salt water of the additional compartment
will be pumped out by a pump, having the valves opposite to the pump closed to avoid
that air bubbles enter the new compartment. To accelerate the process of going up, the
20 valves opposite to the pump will be opened and through the respective hoses
pressurized air or flue gases will be pushed in.

Using a reactor as in figure 1, we obtained 35 g biomass per square meter of covered
water per day on average all year round (day=24 h). The nocturnal growth rates, i.e.
25 the biomass production when the photobioreactor was exposed to darkness during a
period of 7 hours (night), reached up to 30 g/m²*h.

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Items

1. A method of operating a closed photobioreactor for cultivation of a microorganism capable of mixotrophic growth, comprising the steps of:
 - 5 i. providing at least one photobioreactor comprising a central compartment for holding a culture liquid and defined by walls of a water tight, transparent, and flexible material , said photobioreactor being partially or completely surrounded by a water body,
 - ii. providing a culture liquid comprising nutrients,
 - 10 iii. providing a microorganism capable of carrying out photosynthetic growth when exposed to light and heterotrophic growth when exposed to darkness,
 - iv. exposing the photobioreactor to alternating periods of light and darkness.
2. The method of item 1, wherein the alternating periods of light and darkness are
15 achieved at least partly, such as completely, by the natural day cycle.
3. The method according to any one of the preceding items, wherein the mixotrophic microorganism is an organism capable of heterotrophic growth and autotrophic growth, such as phototrophic growth.
20
4. The method according to any one of the preceding items , further comprising the step of providing a first composition comprising fresh nutrients during the periods of exposure to darkness, wherein the first composition comprises carbohydrates and providing a second composition during the periods of
25 exposure to light.
5. The method according to item 4, wherein the second composition does not comprise carbohydrates.
- 30 6. The method according to any one of the preceding items, further comprising a step of providing a density difference between said culture liquid and the surrounding water so that the position of the photobioreactor in the water body is controlled.

7. The method according to any one of the preceding items, further comprising a step of harvesting at least part of the biomass produced by the microorganism.
- 5 8. The method according to any one of the preceding items, wherein the photobioreactor is exposed to light for 4 to 20 hours in a day.
9. The method according to any one of the preceding items, wherein the photobioreactor is exposed to darkness for 4 to 20 hours in a day.
- 10 10. The method according to any one of the preceding items, further comprising the step of providing carbon dioxide to the culture liquid during the periods of exposure to light.
- 15 11. The method according to any one of the preceding items, further comprising the step of providing oxygen to the culture liquid during the periods of exposure to darkness.
- 20 12. The method according to any one of the preceding items, further comprising the step of recovering from the culture liquid the gas produced, such as carbon dioxide and/or oxygen and/or nitrogen.
- 25 13. The method according to item 10, wherein:
 - the O₂ recovered during the photosynthetic phase is recycled for use by the microorganism during the heterotrophic phase
 - and/or
 - the CO₂ recovered during the heterotrophic phase is recycled for use by the microorganism during the photosynthetic phase.
- 30 14. The method according to any one of the preceding items, wherein the microorganism is a microalga.
- 35 15. The method according to any one of the preceding items, further comprising the step of converting the produced biomass to a biofuel, an animal feed, a protein, an amino acid, an ingredient for basic human nutrition, fine chemicals, nutritional supplements, vitamins, omega-3-fatty acids, antioxidants, preferably

carotenoids or beta-carotene, pharmaceutically active substances, amino acids or astaxanthin.

- 5 16. The method according to any one of the preceding items, wherein the central compartment comprises a first fluid connection directly or indirectly connecting said central compartment to a harvester and/or a feeder.
- 10 17. The method according to any one of the preceding items, wherein the photobioreactor comprises a peripheral compartment along the edge of the central, wherein central compartment comprises at least one first fluid connection between the central compartment and the peripheral compartment and wherein the peripheral compartment comprises at least one further fluid connection directly or indirectly connecting the peripheral compartment to a harvester and/or a feeder.
- 15 18. The method according to anyone of the preceding items, wherein the opening and closing of the first fluid connection, of the further fluid connection and of any other first and further fluid connection can be controlled.
- 20 19. The method according to any one of the preceding items, wherein the step of harvesting and/or the step of feeding are repeated.
- 25 20. The method according to any one of the preceding items, wherein the step of harvesting and/or the step of feeding are continuous or semi-continuous.
- 30 21. The method according to any one of the preceding items, wherein the harvester and/or the feeder comprises or consists of a pump and/or gravity forces.
- 35 22. The method according to any one of the preceding items, wherein the harvester and/or the feeder comprises or consists of a pump directly connected to the further fluid connection.
23. The method according to any one of the preceding items, wherein the further fluid connection directly connects the peripheral compartment of one photobioreactor to the peripheral compartment of another photobioreactor.

24. The method according to any one of the preceding items, wherein the first fluid connections and the further fluid connections are independently dispersed at substantially regular intervals.
- 5 25. The method according to any one of the preceding items, wherein the photobioreactor has a flat panel shape.
26. The method according to anyone of the preceding items, wherein the thickness of the culture liquid in the photobioreactor in a vertical direction is between 1 and 30 cm.
- 10 27. The method according to any one of the preceding items, wherein the temperature of the culture liquid is regulated by the surrounding water.
- 15 28. The method according to anyone of the preceding items, wherein the salinity of the culture liquid and of the surrounding water body and parameters relevant for growth of the microorganisms can be monitored by a control unit, said control unit being operatively connected to the photobioreactor.
- 20 29. The method according to any one of the preceding items, wherein the density difference is provided by providing a salinity difference between the culture liquid and the body of water.
- 25 30. The method according to any one of the preceding items, further comprising maintaining or changing the vertical position of the photobioreactor by controlling the salinity difference.
- 30 31. The method according to any one of the preceding items, further comprising the step of measuring biomass density in the photobioreactor, comparing said measured value with a reference value, and harvesting at least part of the produced biomass from the photobioreactor where the measured value is equal to or greater than said reference value.
- 35 32. The method according to any one of the preceding items, wherein the at least one photobioreactor is a plurality of photobioreactors, wherein at least part of the biomass within the central compartment of one photobioreactor can be

harvested independently of the harvesting of the biomass within the central compartment of any other photobioreactor.

5 33. The method according to any one of the preceding items, wherein the at least one photobioreactor is a plurality of photobioreactors, wherein the central compartment of one photobioreactor can be provided with fresh culture liquid independently of the provision of fresh culture liquid to the central compartment of any other photobioreactor.

10 34. A method of producing biomass, the method comprising:
i. cultivating a microorganism capable of mixotrophic growth in a closed photobioreactor operated according to the method of any one of items 1 to 33,
ii. harvesting at least part of the biomass produced by the microorganism.

15 35. The method of item 34, wherein the mixotrophic microorganism is an organism capable of heterotrophic growth and autotrophic growth, such as phototrophic growth.

20 36. The method according to any one of items 34 and 35, wherein at least 35 g/m²*day dry biomass are produced.

25 37. A system comprising at least one closed photobioreactor adapted for large-scale cultivation of a microorganism capable of mixotrophic growth according to the method of any one of items 1 to 33 and comprising said microorganism.

30 38. The method of item 37, wherein the mixotrophic microorganism is an organism capable of heterotrophic growth and autotrophic growth, such as phototrophic growth.

Claims

1. A method of operating a closed photobioreactor for cultivation of a microorganism capable of mixotrophic growth, comprising the steps of:
 - 5 i. providing at least one photobioreactor comprising a central compartment for holding a culture liquid and defined by walls of a water tight, transparent, and flexible material, said photobioreactor being partially or completely surrounded by a water body,
 - ii. providing a culture liquid comprising nutrients,
 - 10 iii. providing a microorganism capable of carrying out photosynthetic growth when exposed to light and heterotrophic growth when exposed to darkness,
 - iv. exposing the photobioreactor to alternating periods of light and darkness, wherein the alternating periods of light and darkness are achieved at least partly by the natural day cycle.
- 15 2. The method of claim 1, further comprising the step of providing a first composition comprising fresh nutrients during the periods of exposure to darkness, wherein the first composition comprises carbohydrates and providing a second composition during the periods of exposure to light.
- 20 3. The method according to claim 2, wherein the second composition does not comprise carbohydrates.
4. The method according to any one of the preceding claims, wherein the photobioreactor is exposed to light for 4 to 20 hours in a day.
- 25 5. The method according to any one of the preceding claims, wherein the photobioreactor is exposed to darkness for 4 to 20 hours in a day.
- 30 6. The method according to any one of the preceding claims, further comprising the step of recovering from the culture liquid the gas produced, such as carbon dioxide and/or oxygen and/or nitrogen.
- 35 7. The method according to claim 6, wherein:
 - the O₂ recovered during the photosynthetic phase is recycled for use by the microorganism during the heterotrophic phase

and/or

- the CO₂ recovered during the heterotrophic phase is recycled for use by the microorganism during the photosynthetic phase.

- 5 8. The method according to any one of the preceding claims, wherein the microorganism is a microalga.
- 10 9. The method according to any one of the preceding claims, further comprising the step of converting the produced biomass to a biofuel, an animal feed, a protein, an amino acid, an ingredient for basic human nutrition, fine chemicals, nutritional supplements, vitamins, omega-3-fatty acids, antioxidants, preferably carotenoids or beta-carotene, pharmaceutically active substances, amino acids or astaxanthin.
- 15 10. The method according to any one of the preceding claims, wherein the temperature of the culture liquid is regulated by the surrounding water.
- 20 11. The method according to any one of the preceding claims, wherein the density difference is provided by providing a salinity difference between the culture liquid and the body of water.
- 25 12. The method according to any one of the preceding claims, further comprising the step of measuring biomass density in the photobioreactor, comparing said measured value with a reference value, and harvesting at least part of the produced biomass from the photobioreactor where the measured value is equal to or greater than said reference value.
- 30 13. A method of producing biomass, the method comprising:
i. cultivating a microorganism capable of mixotrophic growth in a closed photobioreactor operated according to the method of any one of claims 1 to 12,
ii. harvesting at least part of the biomass produced by the microorganism.
- 35 14. The method according to claim 13, wherein at least 35 g/m²*day dry biomass are produced.

15. A system comprising at least one closed photobioreactor adapted for large-scale cultivation of a microorganism capable of mixotrophic growth according to the method of any one of claims 1 to 12 and comprising said microorganism.

FIG. 1a

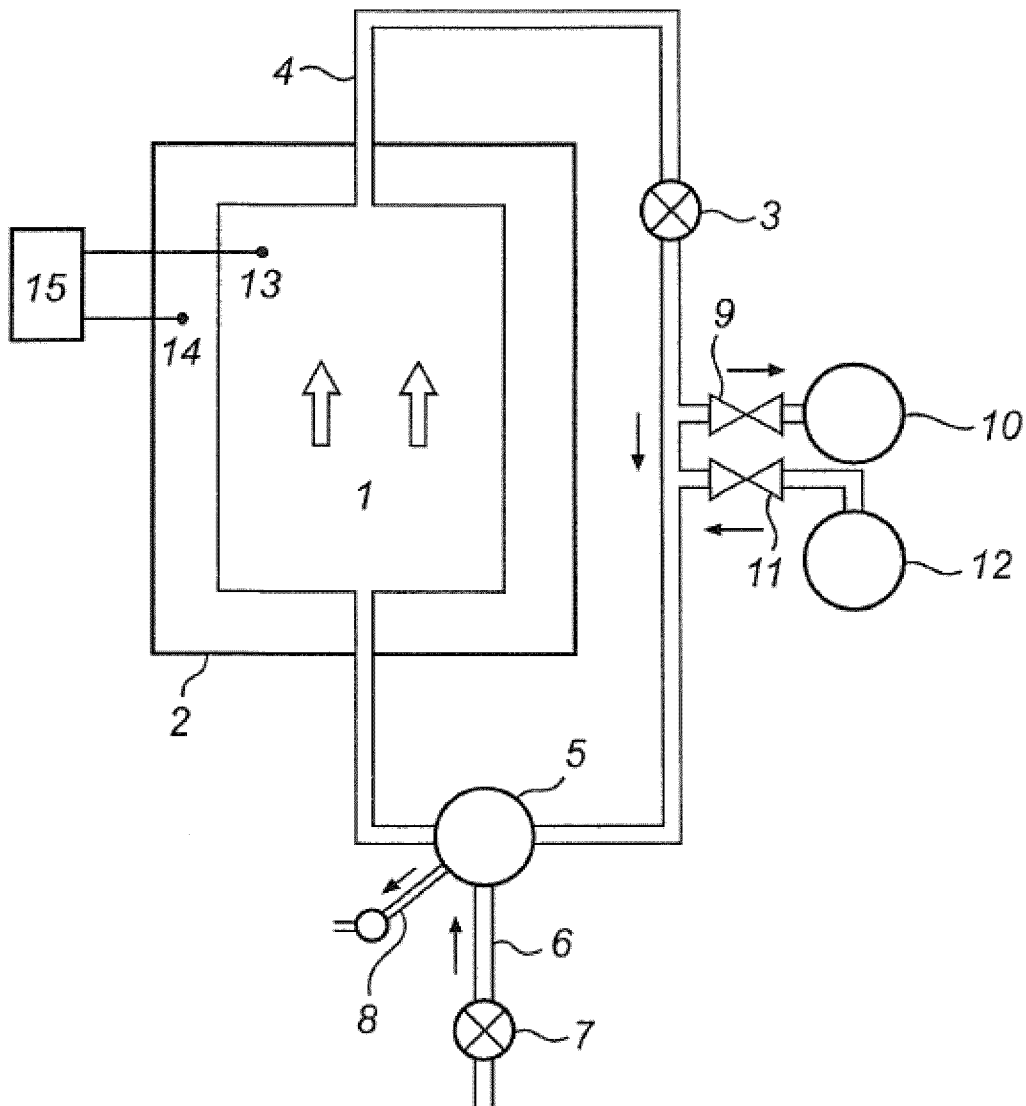


FIG. 1b

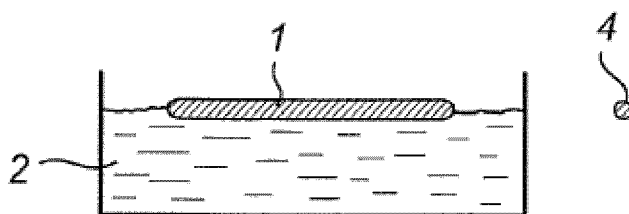


FIG. 2a

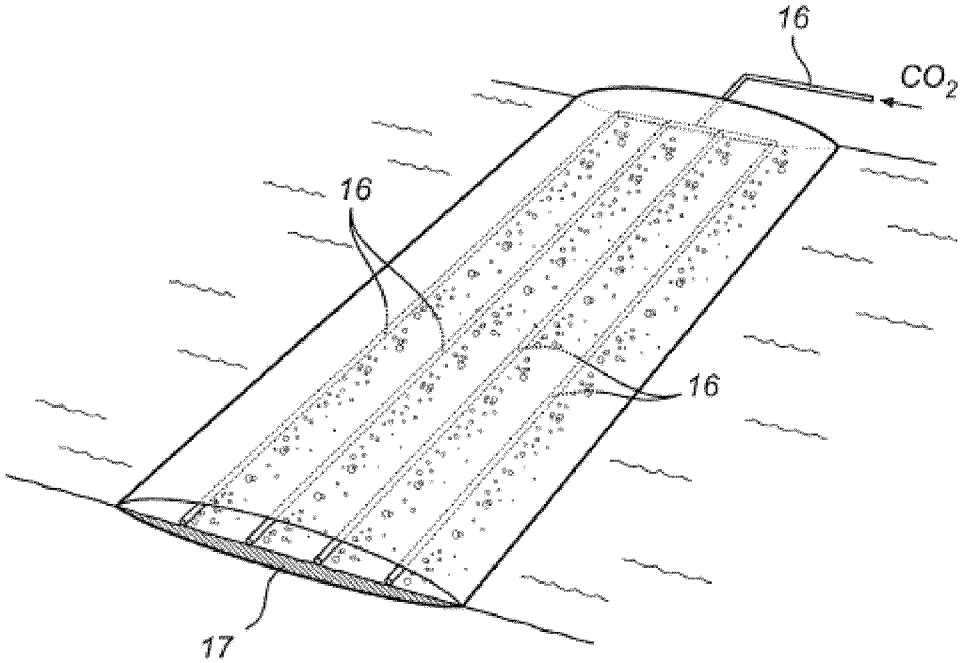
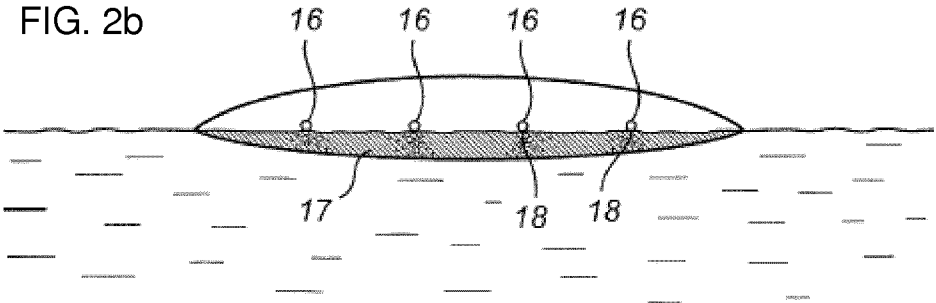
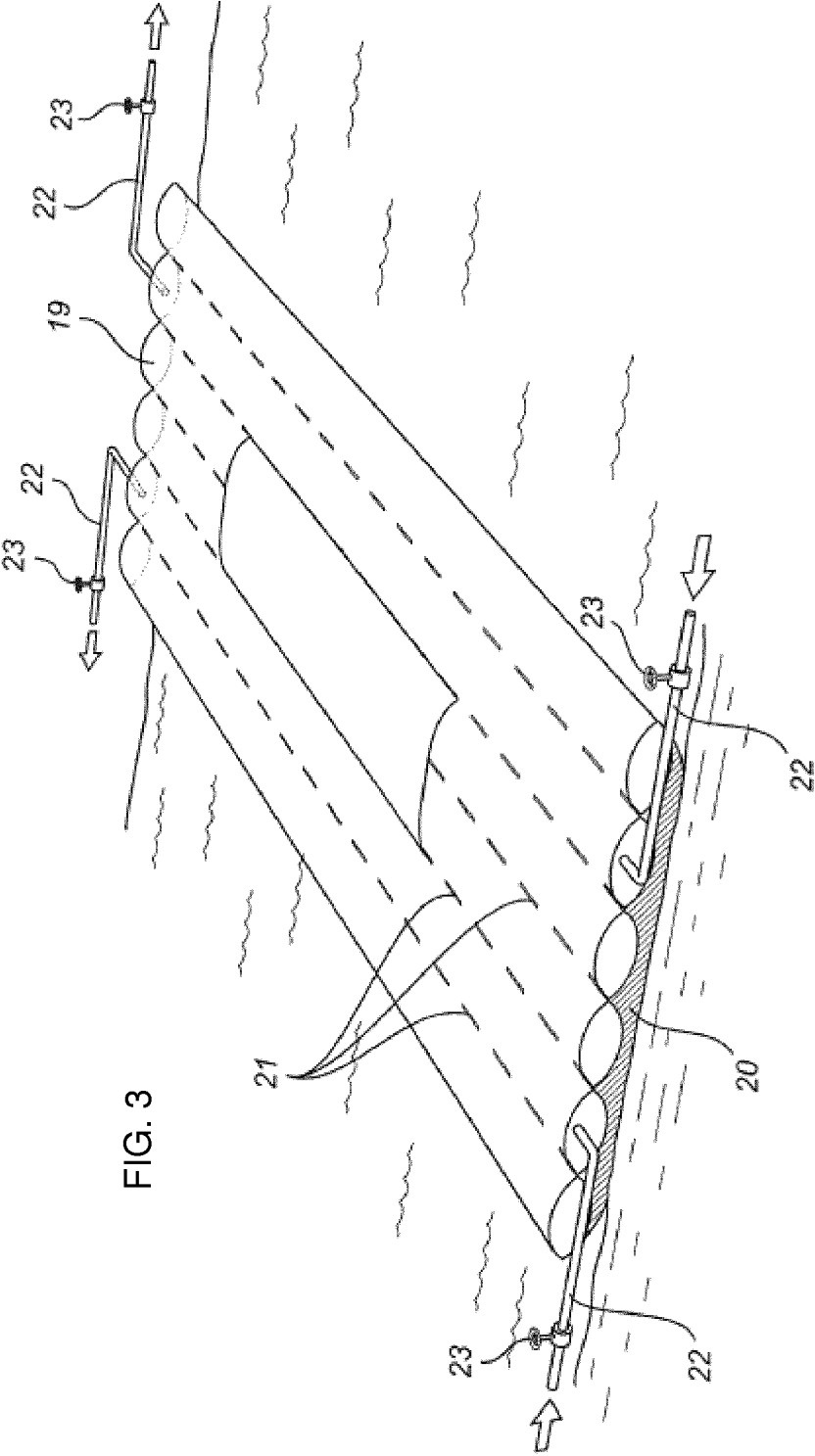


FIG. 2b





INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/054566

A. CLASSIFICATION OF SUBJECT MATTER INV. C12M1/00 C12M1/09 C12N1/12 C12M1/02 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		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2009/090549 A2 (ALGAE LTD; MEISER ANDREAS [DE]; VERHEIN MIGUEL [VE]) 23 July 2009 (2009-07-23) cited in the application figures 1-3	1-15
A	----- WO 2014/144270 A1 (HELIAE DEV LLC [US]) 18 September 2014 (2014-09-18) claim 1	1-15
A	----- US 2009/151240 A1 (KAYAMA HIROYUKI [JP] ET AL) 18 June 2009 (2009-06-18) claim 4; figure 1 -----	1-15
☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 13 April 2017		Date of mailing of the international search report 25/04/2017
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Jones, Laura

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

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