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(54) **METHOD FOR WET EXTRACTION OF LIPID COMPONENTS FROM MICROORGANISMS**

(57) The invention relates to a method for wet extraction of lipid components from microorganisms in a bi-phasic extraction mixture comprising an aqueous and an organic phase.

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Description**INTRODUCTION**

[0001] The invention relates to a method for wet extraction of lipid components from microorganisms in a bi-phasic extraction mixture comprising an aqueous and an organic phase.

[0002] Some microorganisms have the capacity to accumulate huge amounts of lipids i.e. more than 40% of their dry weight and sometimes even up to 80%. Such microorganisms can be microalgae, yeast, fungi, bacteria or cyanobacteria. The type of lipids produced, and in particular the fraction of neutral or polar lipids, the length of the fatty acid chains and the degree of saturation / unsaturation, are specific to each organism and to the mode of cultivation. Such lipids have a commercial and industrial value. Neutral lipids with low levels of unsaturation are for example the preferred precursors for biodiesel or bio-lubricants. Conversely, lipids with high levels of unsaturation can be profitably used by the food and feed industry or by the chemical industry for the production of biopolymers.

[0003] The present invention relates to a new method of extracting the above-mentioned lipids from microorganisms in microbial biomass with an improved liquid extraction method.

BACKGROUND OF THE INVENTION

[0004] Lipids are important components of human and animal nutrition and are widely used in the pharmaceutical and cosmetic field as well as in the chemical industry as precursors in the development of biobased plastics, foams and polymers, of biobased lubricants or in the production of biodiesel.

[0005] In the human and animal nutrition, fatty acids and triacylglycerides are of particular importance, such as in particular polyunsaturated fatty acids (PUFAs), omega-3 fatty acids (n3-fatty acids), which are essential components of the human or animal nutrition. They can be isolated from natural sources or ingested with the nutrition. However, in most industrially developed countries, the supply with n3-fatty acids is inadequate, while the total fat content in the nutrition and the intake of saturated fatty acids and n6-fatty acids is too high. This results from a change in the composition of nutrition over the last 150 years and is correlated to the remarkable increase of chronic diseases, such as cardiovascular diseases - the main cause of death in industrialised nations. The targeted intake of n3-fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), for example, can reduce the cardiovascular risk and the consumption of n3-fatty acids is therefore recommended by health organisations such as the WHO.

[0006] However, such lipids also play an important role in the pharmaceutical and cosmetic industry, for example as carriers, excipients and lubricants in formulating active ingredient application forms but also for acting themselves as ingredients with beneficial physiological properties.

[0007] The use of such lipids in the chemical industry offers further important application fields, including - without being limited thereto - the development of biobased plastics, foams, polymers, lubricants, and biodiesel.

[0008] Due to the broad variety of application fields and their significance for products of the consumer market, it is desirable to provide large amounts of such lipids by economically feasible manufacturing processes, i.e. by processes which are easy and reliable, cheap and environmentally safe.

[0009] As a cheap and easy to provide source of such lipids, microorganisms with the capacity to accumulate lipids are known and of high interest, which can easily be provided by common methods for cultivation of microorganisms as biomass. The lipids accumulated in the cultivated (harvested) microorganisms need to be extracted and isolated from the biomass containing the microorganisms and, in this respect, different extraction methods exist.

PRIOR ART

[0010] Extraction of lipids from microbial biomass is usually performed using organic solvents on dry biomass. Indeed, organic solvents work very well in the absence of water [Dong, Tao, Eric P. Knoshaug, Philip T. Pienkos, and Lieve M. L. Laurens. 2016. "Lipid recovery from wet oleaginous microbial biomass for biofuel production: A critical review". Applied Energy 177 (September): 879-95. <https://doi.org/10.1016/j.apenergy.2016.06.002>].

[0011] In particular, this approach is almost systematically used in the laboratory in all analytical methods of lipid extraction aiming at quantification and characterization. However, such an approach is not realistic for an industrial application and large-scale production due to the extremely high costs of drying. This especially applies for microalgae [Sander, Kyle, and Ganti S. Murthy. 2010. "Life Cycle Analysis of Algae Biodiesel". The International Journal of Life Cycle Assessment 15 (7): 704-14. <https://doi.org/10.1007/s11367-010-0194-1>; Razon, Luis F., and Raymond R. Tan. 2011. "Net Energy Analysis of the Production of Biodiesel and Biogas from the Microalgae: Haematococcus Pluvialis and Nannochloropsis". Applied Energy, Special Issue of Energy from algae: Current status and future trends, 88 (10): 3507-14. <https://doi.org/10.1016/j.apenergy.2010.12.052>; Lam, Man Kee, and Keat Teong Lee. 2012. "Microalgae Bio-fuels: A Critical Review of Issues, Problems and the Way Forward". Biotechnology Advances 30 (3): 673-90. <https://doi.org/10.1016/j.biotechadv.2012.02.001>].

[tps://doi.org/10.1016/j.biotechadv.2011.11.008](https://doi.org/10.1016/j.biotechadv.2011.11.008).] but it also applies to other microbial biomass [Dong *et al.*, 2016].

[0012] To avoid the high costs for drying biomass, permanent efforts are made to develop approaches that operate directly on wet biomass. The known methods can be separated into two categories, "mono-phasic" approaches and "bi-phasic" approaches.

[0013] In the so-called "mono-phasic" approaches solvents are used, which are perfectly miscible with water under the applied extraction conditions, resulting in an extraction mixture in the form of a single phase. As wet biomass is hydrophilic due to the high amount of water or aqueous biomass medium, these approaches systematically use a polar solvent for extraction. Consequently, the polar solvents are then difficult to separate from the remaining water or aqueous phase and are generally problematic with respect to recycling [Dong *et al.*, 2016]. In the worst case, the extraction solvent forms an azeotrope with the water of the aqueous biomass medium, for example when using ethanol, which considerably increases the separation costs.

[0014] In the so-called "bi-phasic" approaches the wet biomass is contacted with a water-immiscible component e.g. a non-polar solvent or a solvent of very low polarity, such as in particular hexane or heptane. Even if such approaches are aiming at performing lipid extraction on wet biomass with non-polar solvents only, the prior art describes to keep the amount of water in the wet biomass low to allow sufficient contact of the organic phase with the biomass or such biphasic extraction so far requires a preliminary step of disrupting the cells or cell walls so that the solvents get access to the lipids inside the cells [Halim, Ronald, Michael K. Danquah, and Paul A. Webley. 2012. "Extraction of oil from microalgae for biodiesel production: A review". *Biotechnology Advances* 30 (3): 709-32. <https://doi.org/10.1016/j.biotechadv.2012.01.001>; Yap, Benjamin H. J., Geoff J. Dumsday, Peter J. Scales, and Gregory J. O. Martin. 2015. "Energy evaluation of algal cell disruption by high pressure homogenisation". *Bioresource Technology, Advances in biofuels and chemicals from algae*, 184 (Mai): 280-85. <https://doi.org/10.1016/j.biortech.2014.11.049>; Halim, Ronald, Ioannis Papachristou, George Q. Chen, Huining Deng, Wolfgang Frey, Clemens Posten, and Aude Silve. 2022. "The Effect of Cell Disruption on the Extraction of Oil and Protein from Concentrated Microalgae Slurries". *Bioresource Technology* 346 (Februar): 126597. <https://doi.org/10.1016/j.biortech.2021.126597>.]

[0015] Cell-disruption comprises any measure which destroys the mechanical integrity of the cell and in particular the integrity of the cell wall. Commonly used technologies are high pressure homogenization (HPH) and bead-milling. Several authors demonstrate the correlation between lipid extraction yield and disruption rate [Angles, Emilie, Pascal Jaouen, Jérémy Pruvost, and Luc Marchal. 2017. "Wet Lipid Extraction from the Microalga *Nannochloropsis* Sp.: Disruption, Physiological Effects and Solvent Screening". *Algal Research* 21 (Januar): 27-34. <https://doi.org/10.1016/j.algal.2016.11.005>; Halim, Ronald, David R. A. Hill, Eric Hanssen, Paul A. Webley, Susan Blackburn, Arthur R. Grossman, Clemens Posten, and Gregory J. O. Martin. 2019. "Towards Sustainable Microalgal Biomass Processing: Anaerobic Induction of Autolytic Cell-Wall Self-Ingestion in Lipid-Rich *Nannochloropsis* Slurries". *Green Chemistry* 21 (11): 2967-82. <https://doi.org/10.1039/C8GC03186J>.]

[0016] Therefore, such approaches are typically limited by the high energetic costs associated with the disruption technique. Additionally, disruption of cells of the biomass leads to the generation of extremely small debris which are difficult to separate from the extraction mixture and is prone to form extremely stable emulsions which is detrimental to phase separation and therewith the lipid isolation and organic solvent recycling. The ultimate consequence is an increase in the complexity of the further downstream processes and therefore in the costs, which prevents industrial implementation and large-scale production.

[0017] WO2020/248020 describes such a method of recovering lipid components from wet biomass by disrupting the cell structure and extracting the lipids with water-immiscible solvents, such as heptane and hexane. Therein, the use of bi-phasic extraction mixtures using for example hexane as non-polar extraction solvent is discussed as having several disadvantages. In the method described therein, a water-in-oil emulsion of the disrupted aqueous biomass with the water-immiscible solvent is formed. It is described therein, that the cell disruption is needed to enable the release of the intracellular organic components of the biomass to form a biomass suspension. The extraction method described therein particularly relates to the implementation of process steps allowing to deal with the emulsion formed in the extraction process.

[0018] The work of König-Mattern and colleagues [König-Mattern, Laura, Steffen Linke, Liisa Rihko-Struckmann, and Kai Sundmacher. 2021. "Computer-Aided Solvent Screening for the Fractionation of Wet Microalgae Biomass". *Green Chemistry* 23 (24): 10014-29. <https://doi.org/10.1039/D1GC03471E>.] describes an extraction method using solvents which are at least partially miscible with water and which form a mono-phasic system containing the organic solvent and very low amounts of water that can be dissolved in the organic solvent. Therein, non-polar solvents such as hexane or heptane are excluded. It is mentioned by the authors that their approach is generally not well suited for neutral lipids.

[0019] WO2014/096024 describes a method for extracting lipids from wet biomass, wherein at least one polar organic solvent is used in the extraction mixture. The extraction mixture is mono-phasic in cases of using a polar solvent which is completely miscible with water or it is bi-phasic in cases of using a polar solvent which is partially miscible and saturated with water. The preferred polar solvent is ethyl-acetate. It is not described therein, to use only non-polar solvents in a bi-phasic system or solvents, which are immiscible with the aqueous phase.

[0020] The document discusses prior art extracting methods, wherein hexane is used as a non-polar extraction solvent, which all use dry biomass and require a step of disrupting the cell walls, such as for example WO2003/039832.

[0021] Angles and colleagues [*Angles et al.*, 2017] have published a detailed solvent screening study according to which the presence of water in a wet extraction method improves lipid extraction efficiency for immiscible solvents like heptane. However, this observation was done on biomass after HPH treatment, i.e. again following cell disruption. The authors of this publication concluded that for non-polar solvents which are immiscible with water or solvents with only low miscibility with water, extraction rates are at best similar to disruption rates and usually lower.

OBJECT OF THE INVENTION

[0022] It was the object of the invention to provide a new and improved method for extracting lipid components from wet microbial biomass, which lacks the disadvantages of the so far available wet extraction methods. It was a further object of the invention to provide new wet extraction methods wherein the disruption of cells and therewith the formation of emulsions can be avoided. It was a further object of the invention to provide new wet extraction methods which provides high yields. It was a further object of the invention to provide new wet extraction methods wherein the amount of organic solvent can be reduced to a minimum. It was a further object of the invention to provide new wet extraction methods which allow easy separating of the organic solvents used for the lipid extraction and therewith allow easy recycling of the solvents. It was a further object of the invention to provide new wet extraction methods which are cost effective, easy to implement and apply, reliable and safe for humans and nature. It was a further object of the invention to provide new wet extraction methods which are suitable for upscaling and industrial large-scale processes.

[0023] The inventors of the present invention developed a new extraction method which enables to extract lipids from microbial biomass, at reasonable costs. In particular, in the new method there is no need to dry the biomass and therefore the extremely energetically and financially costly step of drying can be avoided. Further, the solvents are selected to avoid formation of a mono-phasic extraction mixture with their difficulties in the separation of the solvent/water mixture or azeotrope which allows lowering the costs of solvent/water separation and makes solvent recycling easier. Further, the amount of solvent required for efficient lipid extraction can be reduced, which also reduces production and recycling costs [Martin, Gregory J. O. 2016. "Energy Requirements for Wet Solvent Extraction of Lipids from Microalgal Biomass". *Bioresource Technology* 205 (April): 40-47. <https://doi.org/10.1016/j.biortech.2016.01.017>].

[0024] The new method further needs no disruption techniques, such as bead-milling or high-pressure homogenization, which are expensive in terms of energy and which generate cells debris which complicates the further processing and separation steps [Dong et al., 2016; Law, Sam Q. K., Binbo Chen, Peter J. Scales, and Gregory J. O. Martin. 2017. "Centrifugal Recovery of Solvent after Biphasic Wet Extraction of Lipids from a Concentrated Slurry of *Nannochloropsis* Sp. Biomass". *Algal Research* 24 (Juni): 299-308. <https://doi.org/10.1016/j.algal.2017.04.016>].

[0025] Surprisingly, the new method is suitable to extract the lipid components at high yields although the measures, described in the prior art as necessary to achieve suitable extraction yields, can be avoided.

SUMMARY OF THE INVENTION

[0026] The present invention includes, without being limited thereto, the following aspects:

[1] A method for extracting lipid components from biomass, the method comprising the steps of:

- (i) preparing or providing an aqueous microbial biomass containing microorganisms, wherein the aqueous biomass is adjusted to a content of dry weight of the microorganisms in the aqueous biomass of 30 wt.% or less to obtain a liquid microorganism suspension;
- (ii) adding one or more organic solvents to the aqueous microbial biomass to prepare a bi-phasic extraction mixture comprising the microbial biomass in an extraction mixture with an aqueous phase and an organic phase;
- (iii) shaking or mixing the bi-phasic extraction mixture to extract the lipid components from the cells of the microorganisms in the aqueous biomass;
- (iv) separating the organic phase with the extracted lipid components dissolved therein from the bi-phasic extraction mixture; and
- (v) removing the organic solvent to isolate the lipid components.

[2] The method according to Embodiment 1, wherein the one or more organic solvent is selected

- (a) from the group of non-polar solvents,
- (b) from the group of solvents with low polarity, or
- (c) mixtures thereof.

[3] The method according to Embodiment 1 or 2, wherein the pH of the aqueous phase of the bi-phasic extraction mixture is adjusted to a pH of 6.0 or higher.

[4] The method according to Embodiments 1 to 3, wherein the one or more organic solvent is selected from the group of solvents with low-polarity and wherein the pH of the aqueous phase of the bi-phasic extraction mixture is adjusted to a pH of 6.0 or higher.

[5] The method according to any one of the preceding Embodiments, wherein the content of dry weight of the microorganisms in the aqueous biomass is adjusted to a range of 5 to 30 wt.%.

[6] The method according to Embodiments 1 to 5, wherein the adjustment of the dry weight content of the microorganisms is carried out by:

- (a) concentrating the aqueous microbial biomass containing microorganisms of step (i) or
- (b) suspending or diluting the microbial biomass of step (i) further by adding an aqueous solution, including water, culture medium and buffer solutions for pH adjustment

to obtain a liquid microorganism suspension.

[7] The method according to any one of the preceding Embodiments, wherein a dewatered microorganism pellet derived from centrifugation of the harvesting yield is provided and an aqueous solution, including water, is applied onto the dewatered pellet until a liquid microorganism suspension is obtained to provide the aqueous biomass of step (i).

[8] The method according to any one of the preceding Embodiments, which is controlled to preserve the overall structure of the microorganisms and to avoid disruption of cell walls of the microorganisms.

[9] The method according to Embodiments 1 to 8, further comprising one or more mild pre-treatment steps to the aqueous biomass, wherein mild pre-treatment steps comprise washing, pulsed electric field treatment, dark anoxia treatment, osmotic stress induction, incubation, and which are controlled to preserve the overall structure of the microorganisms and to avoid cell disruption.

[10] The method according to Embodiments 3 to 9, wherein the pH adjustment is carried out by adding a buffer, which may be selected from Mc Illaine buffer, MES buffer, Tris buffer.

[11] The method according to any one of the preceding Embodiments, wherein the aqueous biomass contains at least 70 wt.% of aqueous solution based on the mixture of biomass and aqueous solution.

[12] The method according to any one of the preceding Embodiments, wherein the ratio of aqueous solution : organic solvent is in the range of 0.005 : 5.0, preferably 0.5 : 3.0.

[13] The method according to any one of the preceding Embodiments, wherein the microorganisms in the aqueous biomass are selected from the groups of microalgae, yeasts, fungus, bacteria and cyanobacteria.

[14] The method according to any one of the preceding Embodiments, wherein the microorganisms in the aqueous biomass are selected from the groups of microalgae and yeasts, preferably from microalgae.

[15] The method according to any one of the preceding Embodiments, wherein the aqueous biomass is the aqueous harvesting mixture directly derived from microorganism harvesting.

[16] The method according to any one of the preceding Embodiments, wherein

- microorganisms from the group of microalgae are selected from *Auxenochlorella Protothecoides* (*A. Protothecoides*), *Nannochloropsis Gaditana* (*N. Gaditana*),

or from the group of *Stramenopiles*, *Chromista* or *Heterokonta*, including *Dinophyta*, such as *Cryptocodinium* with *C. cohnii*, or *Stramenopiles*, such as *Pinguiphyceae* with *Glossomastix*, *Phaeomonas*, *Pinguicoccus*, *Pinguicoccus* and *Polydochrysis*, including *Japonochytrium*, *Schizochytrium*, *Thraustochytrium*,

Althornia, *Labyrinthuloides*, *Aplanochytrium* and *Ulkenia*,
or from the group of *Nannochloropsis*, *Chlorella*, *Haematococcus*, *Dunaliella*, *Scenedesmus*, *Isochrysis*,
Phaeodactylum, *Chlamydomonas*, *Navicula*, *Porphyridium*, *Botryococcus* and *Thrausfochytrium*, including
Porphyra, *Macrocystis*, *Spirogyra*, *Ulva*, *Sargassum*, *Augophyllum*, and *Oedogonium*;

- microorganisms from the group of yeasts are selected from oleaginous yeasts, including *Cutaneotrichosporon oleaginosum*, *Apiotrichum porosum*, *Scheffersomyces segobiensis*, *Rhodotorula glutinis*, *Yarrowia lipolytica*, *Rhodospiridium toruloides*, *Cryptococcus curvatus*, *Cryptococcus victoriae*, *Lipomyces starkeyi*, *Saitozyma podzolica* (*S. Podzolica*);

- microorganisms from the group of and cyanobacteria are selected from *Spirulina*, *Microcystis*, *Anabaena*, *Prochlorococcus*, *Nosfoc* and *Synechocystis*.

[17] The method according to any one of the preceding Embodiments, wherein the non-polar solvents are selected from the group comprising pentane, hexane, heptane, cyclohexane, chloroform, toluene, p-xylene and mixtures thereof.

[18] The method according to any one of the preceding Embodiments, wherein the non-polar solvent is hexane and/or heptane, preferably hexane.

[19] The method according to any one of the preceding Embodiments, wherein the low-polarity solvents are selected from the group comprising methyl-tert-butylether (MTBE), methyltetrahydrofuran (MeTHF), 2-butanol, ethylacetate and mixtures thereof.

[20] The method according to any one of the preceding Embodiments, wherein the shaking or mixing of the bi-phasic extraction mixture in step (iii) is carried out either in batch mode or in continuous flow.

[21] The method according to any one of the preceding Embodiments, wherein the shaking or mixing of the bi-phasic extraction mixture in step (iii) is carried out using a magnetic agitator, an orbital shaker, a rotation wheel, or similar suitable mixing devices, and wherein the shaking or mixing is controlled to preserve the overall structure of the microorganisms and to avoid cell disruption.

[22] The method according to any one of the preceding Embodiments, wherein a treatment for disrupting the cells of the microorganisms in the biomass, including one or more of high-pressure homogenisation, bead milling, sonication, pulsed electric fields, osmotic stressing, enzymatic treatment, microwave irradiation, mechanical pressing or pureeing, is excluded.

[23] The method according to any one of the preceding Embodiments, wherein the lipid components extracted from the microorganisms comprises neutral and polar lipids, including triglycerides, phospholipids and glycolipids, pigments, carotenoids, di-glycerides, monoglycerides, free fatty-acids and mixtures thereof.

[24] Lipid components obtainable by a method according to any one of the preceding Embodiments.

[25] The use of the lipid components obtainable by a method according to any one of the preceding Embodiments

- in the food and feed industry, e.g. as food supplements or for feed products (e.g. for fish farming),
- in the chemical industry, e.g. for the manufacturing of biobased plastics, foams and polymers,
- in the cosmetic and pharmaceutical industry,
- in the aviation and construction industry, e.g. as precursors for biofuel and biodiesel or bio-lubricants.

[0027] The present invention is described in more detail as follows.

DETAILED DESCRIPTION OF THE INVENTION

[0028] The inventors of the present invention surprisingly found a new method for wet extraction of lipids from aqueous

biomass at high yields from bi-phasic extraction mixtures using non-polar solvents or solvents with only low polarity and without disrupting the cell structure of the biomass, therewith avoiding the undesired emulsion formation. By increasing the water content of the aqueous microbial biomass suspension to work with an excess of water (or aqueous solution) the inventors found that the two phases present during the extraction (aqueous and organic phases) can efficiently come into contact during gentle mixing and no cell disruption is required to extract the lipids from the cells. With this surprising approach, which is in contrast to the so far described wet extraction methods and their obstacles, solvents which so far were considered as totally inefficient to extract lipids from aqueous biomass were surprisingly found to become highly efficient, even at high biomass : solvent ratios i.e. by using a low amount of organic solvent. Therewith the new method of the present invention deviates from the so far followed approaches of reducing water content as much as possible [Dong et al., 2016] and provides several advantages over the methods applied in the prior art.

[0029] For example, the inventors observed that the new method as described herein is well suited for all types of lipids accumulating microorganisms especially (but not limited to) microalgae, yeast, cyanobacteria. The biomass can be used directly in the form of aqueous (wet) biomass, e.g. directly after harvesting, no drying step is required any more. No cell disruption is required, which avoids the emulsion formation and the resulting difficulties in lipid isolation and solvent recycling. The new method surprisingly allows to use non-polar solvents, such as hexane or heptane or solvents with very low polarity, which form a bi-phasic extraction mixture, and which can therefore be separated and recycled easily. The new method described herein further enables to reduce the amount of organic solvent remarkably, which offers further benefits with respect to costs, safety and handling effort.

Definitions:

[0030] As far as used herein, the terms "comprise / comprising", "contain / containing", and "include / including" are intended to maintain the possible presence of other components, or additional process steps.

[0031] As used herein the term "biomass" or "microbial biomass" refers to a mass of living or dead biological material and includes materials in their natural or native states and materials that have been subjected to processing to produce a semi-processed biomass. A biomass as used in the method of the present invention contains microorganisms or mixtures of different microorganisms. In principle, a biomass / microbial biomass can be present in dry or wet form, however, wet forms of biomass are preferred.

[0032] When the invention refers to "aqueous biomass" the microbial biomass material contains water or an aqueous solution or the biomass material is present in an aqueous environment. That means, the biomass material may either *per se* contain water, e.g. from the harvesting or cultivation process, or is mixed, diluted or suspended with water or an aqueous solution.

[0033] When used herein, the expressions "aqueous solution", "aqueous phase" or "aqueous medium" are intended to include water as well as aqueous solutions, e.g. from the microorganism harvesting or cultivation process (culture medium), physiological solutions and aqueous buffer solutions.

[0034] Using the expression "excess of water" in context with the method of the invention is intended to cover using a wet microbial biomass with high amounts of water or aqueous solution (e.g. culture medium, physiological solutions or aqueous buffer solution), wherein the dry weight content of the biomass is adjusted to the ranges as defined herein and wherein the water / aqueous solution exceeds the amount of the biomass material.

[0035] As used herein the term "culturing" or "harvesting" refers to common methods of growing, cultivating and multiplication of cells or organisms by providing suitable growth conditions for the cell or organism to carry out some or all of its natural biological processes such as reproduction or replication, such that the total amount of biomass increases. Usually, the harvested microorganisms are then available in the form of dewatered pellets obtained by centrifugation and removal of the cultivation medium. The water content of such dewatered pellets varies among the harvested species and may depend on the dewatering (centrifugation) conditions.

[0036] References to "emulsion(s)" made herein refer to emulsions in the sense of the general understanding of the skilled person, including a mixture of two or more liquids that are normally immiscible, and wherein one of the liquids forms a dispersed phase and the other liquid forms a dispersion medium. In accordance with the general understanding, two liquids can form different types of emulsions, including oil-in-water emulsions wherein the oil is dispersed in water as the dispersion medium as well as water-in-oil emulsions wherein water is dispersed in oil as the dispersion medium.

[0037] The term "miscibility" or "miscible" as used herein refers to the property or ability of substances to mix in all proportions, or to fully dissolve in each other at any concentration.

[0038] Accordingly, the term "immiscible" means that the substances do not mix or dissolve in each other to form one unseparated liquid phase but form a bi-phasic system with a phase separation wherein the immiscible phases are present as distinct phases (phase separation).

[0039] A "mono-phasic system" or "mono-phase" means a single liquid phase wherein all liquid components are completely and homogeneously miscible with each other and wherein no phase separation occurs. In the sense of the present invention, a homogenous mixture in the form of a stable-emulsion is considered as a mono-phasic extraction

mixture.

[0040] Accordingly, a "bi-phasic system" or "bi-phase" means a system comprising (at least) two separated liquid phases wherein the liquid components of the separated phases are not or not completely and homogeneously miscible with each other and wherein phase separation occurs.

[0041] As used herein, the term "non-polar organic solvents" refers to solvents which are known to be immiscible with polar solvents, water or aqueous solutions (polar liquids) and accordingly form bi-phasic systems when added to polar liquids.

[0042] As used herein, the term "low-polarity", in particular when used for "solvents with low polarity", refers to solvents which are only poorly miscible with polar solvents, water or aqueous solutions (polar liquids) and therewith also form bi-phasic systems when added to polar liquids.

[0043] That means, both, the non-polar and the low-polarity organic solvents used in the method of the present invention form bi-phasic systems or extraction mixtures with phase separation between organic solvent(s) and the aqueous phase of the aqueous biomass.

[0044] The term "lipid(s)" or "lipid components", in particular when used with respect to the lipid components to be recovered or extracted from the microbial biomass, generally refers to any nonpolar chemical substance that is hydrophobic and/or lipophilic.

[0045] In the sense of the present invention "extraction" or "extracting" means dissolving and/or recovering and/or removing and isolating lipid components from inside the cells of the microorganisms of the biomass.

[0046] The method for extracting lipid components from biomass according to the present invention comprises the following steps of:

(i) preparing or providing an aqueous microbial biomass containing microorganisms, wherein the aqueous biomass is adjusted to a content of dry weight of the microorganisms in the aqueous biomass of 30.00 wt.% or less to obtain a liquid microorganism suspension;

(ii) adding one or more organic solvents to the aqueous microbial biomass to prepare a bi-phasic extraction mixture comprising the microbial biomass in an extraction mixture with an aqueous phase and an organic phase;

(iii) shaking or mixing the bi-phasic extraction mixture to extract the lipid components from the cells of the microorganisms in the aqueous biomass;

(iv) separating the organic phase with the extracted lipid components dissolved therein from the extraction mixture; and

(v) removing the organic solvent to isolate the lipid components.

[0047] A particular aspect of the invention relates to providing a wet extraction method using a wet biomass with an excess of water.

[0048] in step (i) of the method of the invention, the aqueous microbial biomass can be prepared by providing a dry biomass and adding water or an aqueous solution. It is also possible to use directly an aqueous microbial biomass, e.g. by generally using biomass containing the microorganisms and water or by using the wet biomass derivable from harvesting or cultivation systems. Therein, the aqueous biomass used in step (i) shall be present in the form of a liquid microorganism suspension with an excess of water, which means that no dry or dewatered microorganism pellet or highly viscous microorganism mass is used.

[0049] In one aspect of the invention, the aqueous biomass is the aqueous harvesting or cultivation mixture directly derived from microorganism harvesting or cultivation.

[0050] To carry out the method in the above-mentioned excess of water, the aqueous biomass is controlled or adjusted to contain the microorganisms in an amount of ≤ 30.00 wt.% of dry weight (30.00 wt.% or less) relative to the aqueous biomass. Preferably the aqueous biomass is controlled or adjusted to contain the microorganisms in an amount of ≤ 20.00 wt. % of dry weight (20.00 wt. % or less), or in an amount of ≤ 10.00 wt. % of dry weight (10.00 wt. % or less).

[0051] The content of dry weight of the microorganisms in the aqueous biomass can be adjusted or controlled to be within the range of 5.00 to 30.00 wt.%, preferably 5.00 to 20.00 wt. %, more preferably 5.00 to 10.00 wt. %, relative to the aqueous biomass.

[0052] When using a dewatered microorganism pellet derived from centrifugation of the harvesting yield the suitable dry weight value is adjusted in accordance with the microorganism species by applying as much aqueous solution, including water, onto the dewatered pellet of microorganism until the resulting aqueous biomass is present in liquid form and no solid pellet and no highly viscous microorganism mass remains but a liquid microorganism suspension is prepared.

[0053] Adjusting the dry weight of the biomass within the ranges as described herein allows to obtain an extraction system with an excess of water (aqueous solution), which has surprisingly been found to enable increased miscibility of the biomass suspension with the non-polar or low-polarity solvents in the step of gentle mixing. The control of an excess of water adjusted in the wet undisturbed microbial biomass surprisingly turned out to improve the lipid yield by solvent extraction with non-polar or low-polarity organic solvents in a bi-phasic extraction system and allows to recover

the lipids even without cell disruption. The content of water or aqueous solution should be high enough to ensure a fluid water phase. Typical values of dry weight of biomass in the total aqueous phase after addition of water should be lower than 33.00 wt.%, preferably equal to or lower than 30.00 wt.%, more preferably equal to or lower than 20.00 wt.%, equal to or lower than 10.00 wt.%, or even equal to or lower than 5.00 wt.%. An aqueous biomass with about 10.00 wt.% or less of dry weight is most preferred. In case the biomass to be used in step (i) is dry or not sufficiently liquid, the water content is increased until the dry weight content of biomass is adjusted to the ranges defined herein and until the liquid state is achieved for providing the liquid microorganism suspension.

[0054] An excess of water in accordance with the present invention covers a wet (aqueous) biomass with a content of water (aqueous solution) in the microbial biomass of 70 wt% or more, relative to the aqueous biomass. By using a wet biomass with such high amount of water / aqueous solution, the method of the invention distinguishes from extraction methods wherein a dry biomass or a concentrated biomass with reduced water content is used.

[0055] The dry weight content or amount of aqueous solution in the aqueous biomass can be controlled or adjusted by adding fresh water, culture medium or buffer solutions. The adjustment of the dry weight content of the microorganisms and/or excess of water can be achieved by:

- (a) concentrating the aqueous microbial biomass containing microorganisms of step (i), by suitable measures well known to a skilled person, or
- (b) suspending or diluting the microbial biomass of step (i) by adding an aqueous solution, including water, culture medium and buffer solutions for pH adjustment

to obtain the liquid microorganism suspension.

[0056] The organic solvents used in the method of the invention are immiscible with the polar aqueous biomass and therefore form bi-phasic extraction mixtures when added to the aqueous biomass. The one or more organic solvent is preferably selected from the group of non-polar solvents. Non-polar solvents are preferably selected from the group comprising pentane, hexane, heptane, cyclohexane, chloroform, toluene, p-xylene and mixtures thereof. More preferably, the non-polar solvent is hexane and/or heptane, most preferred is hexane.

[0057] It is also possible to carry out the method of the invention by using organic solvents with very low polarity, which likewise form a bi-phasic extraction mixture but which dissolve in the aqueous phase only to a low degree. Low polarity can be defined by a relative static permittivity at 25°C of lower than 20, preferably lower than 10, more preferably lower than 8. Examples of low-polarity solvents are selected from the group comprising methyl-tert-butylether (MTBE), methyltetrahydrofuran (MeTHF), 2-butanol, ethylacetate and mixtures thereof. From the group of low-polarity solvents ethylacetate is less preferred and in a particular aspect of the invention, ethylacetate is excluded.

[0058] It is also possible to use mixtures of the aforementioned organic solvents, including mixtures from the groups of non-polar solvents and solvents with low-polarity.

[0059] In any case, a bi-phasic system must be formed when adding low-polarity solvents to the aqueous biomass.

[0060] The inventors further found that the pH of the aqueous phase can influence the lipid extraction. In a further aspect of the invention the extraction method as described herein therefore comprises a further step of controlling and/or adjusting the pH of the aqueous phase of the bi-phasic extraction mixture to pH of 6.0 or higher, preferably of 7.0 or higher.

[0061] In particular, when using low-polarity solvents, the pH of the aqueous phase should be controlled and/or adjusted in the above-mentioned range to support lipid extraction in sufficiently high and even increased yields. Therefore, in a further aspect of the invention the extraction method as described herein comprises the use of low-polarity solvents in combination with a control and/or adjustment of the pH of the aqueous phase to a pH of 6.0 or higher, preferably of 7.0 or higher.

[0062] In principle, the pH adjustment can be carried out by adding acids and alkali as well as any suitable aqueous buffer solution, including a buffer selected from Mc Illaine buffer, MES buffer, Tris buffer and any other buffer suitable in cell culture media. The pH control and choice of pH adjusting agent should be carried out under sufficiently mild conditions avoiding cell disruption or lysis. The use of buffer solutions is preferred.

[0063] The method of the invention can be carried out with an extraction mixture, wherein the ratio of aqueous solution : organic solvent is in the range of 0.005 : 5.0, preferably 0.5 : 3.0.

[0064] As explained above, a significant advantage of the method of the invention can be seen in that no cell disruption is required to extract the lipids from the biomass. And in view of the above discussed disadvantages deriving from cell disruption, the method of the invention shall be carried out under mild conditions, allowing to control and preserve the integrity and overall structure of the microorganisms and avoid disruption of cell walls of the microorganisms.

[0065] In a particular aspect, in the method of the invention any kind of treatment leading to or effecting disrupting the cells of the microorganisms in the biomass, including one or more of high-pressure homogenisation, bead milling, sonication, pulsed electric fields, osmotic stressing, enzymatic treatment, microwave irradiation, mechanical pressing or pureeing, is excluded.

[0066] The method of the invention comprises a step (iii) of shaking and/or mixing the bi-phasic extraction mixture to

bring the organic solvent in contact with the cells of the biomass. The shaking and/or mixing should ensure a good contact between the aqueous phase and the organic solvent phase. A poor mixing will not result in high extraction yields, however, the shaking and mixing must be controlled to avoid disruption of the cells. Suitable mixing devices comprise magnetic agitator, orbital shaker, rotation wheel, or similarly suitable mixing devices, provided that the shaking or mixing is controlled to preserve the integrity and overall structure of the microorganisms and to avoid cell disruption. Preferably, the mixing enables to apply an enhanced shear stress to the cells, which supports lipid extraction. Preferably, the mixing provides enough shear-stress to promote intense contact between the organic phase and the aqueous phase. Shear stress should not be disruptive for the microorganisms to be extracted.

[0067] The shaking or mixing of the bi-phasic extraction mixture in step (iii) can be carried out either in batch mode or in continuous flow.

[0068] The method of the invention may further comprise one or more mild (pre-)treatment steps carried out on the aqueous biomass, preferably prior to adding the organic solvent. Such mild pre-treatment steps comprise washing, pulsed electric field treatment, dark anoxia treatment, osmotic stress induction, incubation, and others, provided that such pre-treatment steps are controlled to preserve the integrity and overall structure of the microorganisms and to avoid cell disruption. Such pre-treatment steps may be beneficial to achieve extraction of high yields of lipids.

[0069] The separation of the organic phase in step (iv) can be carried out by well-known techniques, including centrifugation or gravitational settling.

[0070] The removal of the organic solvent and the isolation of the lipid components in step (v) can be achieved by well-known techniques, including distillation.

[0071] In principle, all microorganisms which accumulate lipids or polar components, such as for example neutral and polar lipids, including triglycerides, phospholipids and glycolipids, pigments, carotenoids, di-glycerides, monoglycerides, free fatty-acids and mixtures thereof, can be used as biomass material.

[0072] Examples of microorganisms for use in the method of the present invention can be selected from the groups of microalgae, yeasts, fungus, bacteria and cyanobacteria. Preferably, the microorganisms in the aqueous biomass are selected from the groups of microalgae and yeasts, more preferred from microalgae.

[0073] Examples of microorganisms comprise:

- Microorganisms from the group of microalgae, which are selected from *Auxenochlorella Protothecoides* (*A. Protothecoides*), *Nannochloropsis Gaditana* (*N. Gaditana*),

or from the group of *Stramenopiles*, *Chromista* or *Heterokonta*, including *Dinophyta*, such as *Cryptothecodinium* with *C. cohnii*, or *Stramenopiles*, such as *Pinguiphyceae* with *Glossomastix*, *Phaeomonas*, *Pinguiochrysis*, *Pinguicoccus* and *Polydochrysis*, including *Japonochytrium*, *Schizochytrium*, *Thraustochytrium*, *Althornia*, *Labyrinthuloides*, *Aplanochytrium* and *Ulkenia*,

or from the group of *Nannochloropsis*, *Chlorella*, *Haematococcus*, *Dunaliella*, *Scenedesmus*, *Isochrysis*, *Phaeodactylum*, *Chlamydomonas*, *Navicula*, *Porphyridium*, *Botryococcus* and *Thrausfochytrium*, including *Porphyra*, *Macrocystis*, *Spirogyra*, *Ulva*, *Sargassum*, *Agrophillum*, and *Oedogonium*;

- Microorganisms from the group of yeasts, which are selected from oleaginous yeasts, including *Cutaneotrichosporon oleaginosum*, *Apiotrichum porosum*, *Scheffersomyces segobiensis*, *Rhodotorula glutinis*, *Yarrowia lipolytica*, *Rhodospiridium toruloides*, *Cryptococcus curvatus*, *Cryptococcus victoriae*, *Lipomyces starkeyi*, *Saitozyma podzolica* (*S. Podzolica*);

- Microorganisms from the group of cyanobacteria, which are selected from *Spirulina*, *Microcystis*, *Anabaena*, *Prochlorococcus*, *Nostoc* and *Synechocystis*.

[0074] A further aspect of the invention covers the lipid components obtainable by the method as described herein.

[0075] The lipid components obtainable by the method as described herein can be used in one or more of the following technical, industrial and commercial fields and applications:

- in the food and feed industry, e.g. as food supplements or for feed products (e.g. for fish farming),
- in the chemical industry, e.g. for the manufacturing of biobased plastics, foams and polymers useful e.g. in the aviation and construction industry. Also for production of bio-lubricants.
- in the cosmetic and pharmaceutical industry,

- in the energy sector e.g. as precursors for biofuel and biodiesel.

[0076] The method of the invention is, in particular, suitable for large-scale applications.

DESCRIPTION OF THE FIGURES

[0077]

Fig. 1 Comparison of the efficiency of solvents with minimum amount of water in the biomass remaining after dewatering with centrifugation (left) and with excess water according to the invention (right). The crude yields are expressed as percentage of cell dry weight (CDW). The experiments were performed on fresh *A. Protothecoides* with 15 g respectively 30 g of dry biomass per litre of solvent for the experiment without additional water respectively with excess water. Solvent extraction was either applied directly after harvesting (Control) or after pulsed electric field (PEF) (pre-) treatment. Results are the average $\pm$ std of three independent experiments. The y-axis is labelled crude yield but analyses have demonstrated a lipid purity in the extract above 98%.

Fig. 2 Influence of mixing on the lipid yield when extracting with hexane and an excess of water. Experiments were performed on fresh *A. Protothecoides*, previously submitted to PEF-treatment. Extraction was performed using 30 g of dry biomass per litre of solvent during 2h or 24h. Results are the average $\pm$ std of 2 independent experiment.

Fig. 3 Impact of the biomass: solvent ratio on the extraction yield using either n-hexane (left) or 2-butanol (right) as an organic solvent and excess of water. Experiments were performed on fresh *A. Protothecoides*, previously submitted to PEF-treatment. Results are the average $\pm$ std of 2 independent experiments.

Fig. 4 Impact of the amount of water during an extraction performed with n-hexane and water. Experiments were performed on fresh *A. Protothecoides*, previously submitted to PEF-treatment. Extraction was performed using 15 g of dry biomass per litre of solvent. Results are the average $\pm$ std of 4 independent experiments.

Fig. 5 Comparison of the efficiency of solvents with minimum amount of water in the biomass and with excess water. The experiments were performed on fresh *N. Gaditana* with 15 g respectively 30 g of dry biomass per litre of solvent for the experiment without additional water respectively with excess water. Results are the average $\pm$ std of 2 independent experiments.

Fig. 6 Comparison of the efficiency of solvents with minimum amount of water in the biomass and with excess water. The experiments were performed on fresh *S. Podzolica* with 30 g of dry biomass per litre of solvent. Results are the average of duplicates in one single experiment.

Fig. 7 Influence of the pH of the aqueous phase during extraction using n-hexane and water. Experiments were performed on fresh *A. Protothecoides* at 30 g of dry biomass per litre of hexane. The extraction was performed after PEF treatment. Results are the average $\pm$ std of duplicates from one experiment.

EXAMPLES

[0078] The invention is described further by the following examples, without being limited thereto.

Wet Extraction of Lipid Components from Microorganisms

[0079] The method as described herein was performed at small scale, using typical overall extraction volumes between 20mL and 50 mL.

Example 1A Lipid Extraction with Excess of Water from Microalgae *Auxenochlorella Protothecoides* (*A. Protothecoides*)

[0080] In a first aspect, the method of the invention was carried out using the microalgae *Auxenochlorella Protothecoides* (*A. Protothecoides*), which is a model microalga well known for its high lipid content [Sun, Zheng, Zhi-gang Zhou, Henri Gerken, Feng Chen, and Jin Liu. 2015. "Screening and characterization of oleaginous Chlorella strains and exploration of photoautotrophic Chlorella protothecoides for oil production". Bioresource Technology, Advances in biofuels

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[0081] The microalgae are cultivated in the laboratory and processed immediately after harvesting. The harvest consists in dewatering to approximately 10% dry weight (DW) using a centrifuge or a disc separator. Further dewatering to approximately 33% DW was performed before proceeding with the extraction with organic solvents.

[0082] The two dewatering steps can easily be combined in one single step without any influence on the results. The lipid extraction was performed with 4 different solvents using wet biomass, either directly after harvest or after a mild pre-treatment using pulsed electric field (PEF).

Solvents Used:

[0083]

Hexane
Heptane
MeTHF
2-Butanol

[0084] Results of the extraction using no additional water (i.e. only the water remaining in the biomass pellet) and results with addition of excess water are shown in the tables below and displayed on Figure 1:

Without Excess of Water (extraction of wet pellet) - Left				
	Control		PEF	
	average	std	average	std
Hexane	0,1	0,2	0,4	0,0
Heptane	0,0	0,0	0,3	0,1
MeTHF	4,8	1,3	9,7	2,2
2-Butanol	8,8	3,0	3,5	0,4

With excess of Water - Right				
	Control		PEF	
	average	std	average	std
Hexane	0,3	0,1	27,2	3,6
Heptane	0,2	0,2	25,0	4,2
MeTHF	1,1	2,3	17,5	2,3
2-Butanol	6,1	0,9	33,3	0,8

[0085] In both cases, extraction performed on microalgae which did not receive a mild pre-treatment (control) gave lower lipid yield. If microalgae were submitted to the PEF treatment, most solvents remained not efficient in cases of not adding water (i.e. if not carried out with an excess of water) (Figure 1 left).

[0086] In case water is added in excess, the efficiency of all tested solvents is greatly increased. In particular, the non-polar solvents hexane and heptane, which are non-miscible solvents and therefore very easy to recycle, perform well with extraction yields above 25%. From the group of low-polarity solvents MeTHF and 2-butanol showed increase efficiency in the presence of an excess of water.

Example 1B Influence of the Mixing on Lipid Extraction with non-polar solvents

[0087] The influence of the mixing step of the bi-phasic extraction mixture on the extraction yields was evaluated using the non-polar solvent hexane.

[0088] Results are shown in the table below and displayed on Figure 2:

Extraction time		2h extraction		24h extraction	
Mixing device	Speed in rpm	average	std	average	std
Orbital Shaker	200 rpm	1,48	0,72	8,37	0,08
	350 rpm	12,01	0,69	32,59	4,88
	500 rpm	9,97	3,32	29,35	3,11
Rotation Wheel	40 rpm	10,51	1,54	29,10	6,49
Magnetic Agitator	350 rpm	9,18	0,59	30,51	5,93

[0089] The results show that all three tested extraction techniques appeared efficient: orbital shaker, rotation wheel and magnetic agitator. However, orbital shaker at low speed, i.e. 200 rpm, was not sufficient for a good lipid extraction to take place and crude yield was below 10 % after 24h of mixing. These results underline the importance of mixing during the extraction to bring the organic phase in contact with the microalgae.

Example 1C Influence of the biomass : solvent ratio

[0090] Additional experiments were performed to test if the process remains efficient at high biomass to solvent ratio, a prerequisite for the process to be viable at industrial scale. The results for hexane and 2-butanol are shown in the tables below and displayed on Figure 3:

Hexane	g _{DW} /L _{hexane}	average	std
Control	30	0,20	0,28
PEF	30	31,33	2,61
	60	30,53	3,41
	150	26,33	3,69
	300	22,67	3,25
2-Butanol	g _{DW} /L _{2-Butanol}	average	std
Control	30	5,50	n.d
PEF	30	35,50	1,41
	60	35,05	1,63
	150	31,15	0,78
	300	28,75	1,63

[0091] The water was maintained in excess and the biomass : solvent ratio was increased from 30 g/L to 300 g/L. Increasing the biomass : solvent ratio resulted in a slight decrease of the yields: from 32% to 24% in the case of hexane and from 35% to 29% in the case of 2-butanol. However, the high yield still obtained at 300g/L solvent allow to consider an upscaling and an industrial implementation.

Example 1D Evaluation of DW content

[0092] Finally, the minimum amount of water necessary to add to enable an efficient lipid extraction (excess of water amount) was evaluated using hexane as the organic solvent.

[0093] Results are shown in the table below and displayed on Figure 4:

	%DW in biomass	average	std
Fresh water	33	0,29	0,28
	27	4,28	7,44
	23	9,32	9,39
	20	10,33	7,20
	18	12,60	7,09
	16	13,26	7,01
	10	17,62	4,01
Supernatant	10	21,51	2,02

[0094] The results show the crude yield as a function of the biomass concentration in the biomass pellet. The first data point to the left i.e. 33 % of dry weight corresponds to the pellet as it can be obtained after dewatering by centrifugation i.e. maximum dewatering but without applying any expensive drying technique. If such a wet biomass is mixed with pure hexane, the extraction is inefficient and the crude yield is close to zero. Adding water to the pellet i.e. reducing progressively the DW in the biomass from 33 % to 10 % induces a progressive increase of the lipid yield which reaches in that case 18 %.

[0095] Instead of dewatering to 33 % DW and adding fresh water, it is also possible to simply keep more of the initial medium from the cultivation so that the dewatering only brings the biomass to 10% DW. This is shown on Figure 4 on the right (data point "Supernatant") and appears even more efficient since the yield obtained was 22 %. This strategy, which demands less fresh water is interesting for industrial implementation to save water resource.

Example 2 Lipid Extraction with Excess of Water from Microalga *N. Gaditana*

[0096] The influence of an "excess of water" in the extraction method of the present invention was further tested on *Nannochloropsis Gaditana* (*N. Gaditana*) a model microalga intensively studied for lipid production, and very well known for its robust cell wall. *N. Gaditana* is moreover a saltwater microalga and from that point of view an interesting comparison organism. The microalgae were again cultivated in the laboratory and processed fresh directly after harvest.

[0097] Results of lipid yield after extraction from wet biomass using hexane and heptane as non-polar organic solvents are displayed on the left graph in Figure 5.

Solvents Used:

[0098]

Hexane
Heptane

[0099] The results obtained when an excess of water is used are shown in the table below and displayed on Figure 5:

		average	std
Hexane	Wet Pellet	6,66	8,71
	Excess Water	34,55	1,64
Heptane	Wet Pellet	2,63	3,61
	Excess Water	34,19	1,25

on the graph to the right. As can be seen, when no extra water is added to the wet pellet, the non-polar solvents hexane and heptane are poorly efficient in extracting lipids. The addition of excess water results in a much higher extraction efficiency for those solvents, with yields reaching 35% and 34% for hexane and heptane respectively. The experiments show that also for *N. Gaditana*, the excess water strategy is particularly efficient for the non-polar organic solvents.

Example 3 Lipid Extraction with Excess of Water from Yeast *S. Podzolica*

[0100] The influence of an "excess of water" in the extraction method of the present invention was further tested on a completely different microorganism, the oleaginous yeast *Saitozyma podzolica* DSM 2719 (*S. Podzolica*). The yeast was cultivated and processed fresh directly after harvest.

Solvents Used:**[0101]**

Hexane
Heptane
MTBE
MeTHF
2-Butanol

[0102] Results of lipid yield after extraction on wet biomass using only organic solvent are shown in the table below and displayed on the left graph in Figure 6. The results obtained when an excess of water is used together with the solvent are displayed on the graph to the right:

		average
Hexane	Wet Pellet	0,24
	Excess Water	0,36
Heptane	Wet Pellet	0,00
	Excess Water	0,71
MTBE	Wet Pellet	1,55
	Excess Water	31,08
MeTHF	Wet Pellet	13,22
	Excess Water	30,73
2-Butanol	Wet Pellet	9,41
	Excess Water	11,43

[0103] Figure 6 illustrates that when solvent only is used on the wet pellet, none of the solvent tested is able to extract high level of lipids. The best performing solvent was MeTHF with an achieved yield of 13%. Adding an excess of water again achieved a significant improvement in extraction yields, in particular with low-polarity solvents like MTBE and MeTHF, which enabled to reach lipid yields of 31% in both cases.

Example 4 Influence of the pH control on Lipid Extraction

[0104] The influence of a pH control in the aqueous phase in the bi-phasic extraction mixture has been evaluated with the non-polar solvent n-hexane. The results are shown in the table below:

		average	std
Mc Illaine Buffer	pH 3	1,72	0,99
	pH 4	2,39	2,03
	pH 5	2,67	0,72
	pH 6	8,25	0,14
	pH 7	20,63	0,18
	pH 8	26,91	0,23

(continued)

		average	std
MES Buffer	pH 6	10,09	1,13
Tris Buffer	pH 8	27,26	0,36

[0105] Figure 7 displays the lipid yield obtained when performing extraction on *A. Protothecoides* using water and n-Hexane and with a pH buffering of the water. In this example, all the buffers were prepared with identical ionic strength. Results clearly show that lipid yield increases with pH. The examples further show, that the type of buffer has no impact as can be seen by comparing Mc Illaine pH6 versus MES pH6 or Mc Illaine pH8 versus TRIS pH 8.

Conclusion

[0106] The Examples provided herein show, that the new method of wet lipid extraction from microorganisms with an excess of water works well in bi-phasic extraction systems using non-polar solvents or solvents with low-polarity, even without cell disruption for releasing the lipid components from within the cells. Further, the influence of the pH of the aqueous phase has been shown.

Claims

1. A method for extracting lipid components from biomass, the method comprising the steps of:

- (i) preparing or providing an aqueous microbial biomass containing microorganisms, wherein the aqueous biomass is adjusted to a content of dry weight of the microorganisms in the aqueous biomass of 30 wt.% or less to obtain a liquid microorganism suspension;
- (ii) adding one or more organic solvents to the aqueous microbial biomass to prepare a bi-phasic extraction mixture comprising the microbial biomass in an extraction mixture with an aqueous phase and an organic phase;
- (iii) shaking or mixing the bi-phasic extraction mixture to extract the lipid components from the cells of the microorganisms in the aqueous biomass;
- (iv) separating the organic phase with the extracted lipid components dissolved therein from the bi-phasic extraction mixture; and
- (v) removing the organic solvent to isolate the lipid components.

2. The method according to claim 1, wherein the one or more organic solvent is selected

- (a) from the group of non-polar solvents,
- (b) from the group of solvents with low polarity, or
- (c) mixtures thereof.

3. The method according to claim 1 or 2, wherein the pH of the aqueous phase of the biphasic extraction mixture is adjusted to a pH of 6.0 or higher.

4. The method according to claims 1 to 3, wherein the one or more organic solvent is selected from the group of solvents with low polarity and wherein the pH of the aqueous phase of the bi-phasic extraction mixture is adjusted to a pH of 6.0 or higher.

5. The method according to claims 1 to 4, wherein the adjustment of the dry weight content of the microorganisms is carried out by:

- (a) concentrating the aqueous microbial biomass containing microorganisms of step (i) or
- (b) suspending or diluting the microbial biomass of step (i) further by adding an aqueous solution, including water, culture medium and buffer solutions for pH adjustment,

to obtain a liquid microorganism suspension.

6. The method according to any one of the preceding claims, which is controlled to preserve the overall structure of the microorganisms and to avoid disruption of cell walls of the microorganisms.
7. The method according to claims 1 to 6, further comprising one or more mild pre-treatment steps to the aqueous biomass, wherein mild pre-treatment steps comprise washing, pulsed electric field treatment, dark anoxia treatment, osmotic stress induction, incubation, and which are controlled to preserve the overall structure of the microorganisms and to avoid cell disruption.
8. The method according to any one of the preceding claims, wherein the ratio of aqueous solution : organic solvent is in the range of 0.005 : 5.0, preferably 0.5 : 3.0.
9. The method according to any one of the preceding claims, wherein the microorganisms in the aqueous biomass are selected from the groups of microalgae, yeasts, fungus, bacteria and cyanobacteria; preferably from the groups of microalgae and yeasts; more preferably from microalgae.
10. The method according to any one of the preceding claims, wherein the aqueous biomass is the aqueous harvesting mixture directly derived from microorganism harvesting.
11. The method according to any one of the preceding claims, wherein
 - Microorganisms from the group of microalgae are selected from *Auxenochlorella Protothecoides* (*A. Protothecoides*), *Nannochloropsis Gaditana* (*N. Gaditana*),
 - or from the group of *Stramenopiles*, *Chromista* or *Heterokonta*, including *Dinophyta*, such as *Cryptocodinium* with *C. cohnii*, or *Stramenopiles*, such as *Pinguiphyceae* with *Glossomastix*, *Phaeomonas*, *Pinguiochrysis*, *Pinguicoccus* and *Polydochrysis*, including *Japonoichytrium*, *Schizochytrium*, *Thraustochytrium*, *Althornia*, *Labyrinthuloides*, *Aplanochytrium* and *Ulkenia*,
 - or from the group of *Nannochloropsis*, *Chlorella*, *Haematococcus*, *Dunaliella*, *Scenedesmus*, *Isochrysis*, *Phaeodactylum*, *Chlamydomonas*, *Navicula*, *Porphyridium*, *Botryococcus* and *Thraustochytrium*, including *Porphyra*, *Macrocystis*, *Spirogyra*, *Ulva*, *Sargassum*, *Agophyllum*, and *Oedogonium*;
 - Microorganisms from the group of yeasts are selected from oleaginous yeasts, including *Cutaneotrichosporon oleaginosum*, *Apiotrichum porosum*, *Scheffersomyces segobiensis*, *Rhodotorula glutinis*, *Yarrowia lipolytica*, *Rhodospiridium toruloides*, *Cryptococcus curvatus*, *Cryptococcus victoriae*, *Lipomyces starkeyi*, *Saitozyma podzolica* (*S. Podzolica*);
 - Microorganisms from the group of and cyanobacteria are selected from *Spirulina*, *Microcystis*, *Anabaena*, *Prochlorococcus*, *Nostoc* and *Synechocystis*.
12. The method according to any one of the preceding claims, wherein the non-polar solvents are selected from the group comprising pentane, hexane, heptane, cyclohexane, chloroform, toluene, p-xylene and mixtures thereof; preferably from hexane and heptane, more preferably hexane; and wherein the low-polarity solvents are selected from the group comprising methyl-tert-butylether (MTBE), methyltetrahydrofuran (MeTHF), 2-butanol, ethylacetate and mixtures thereof.
13. The method according to any one of the preceding claims, wherein a treatment for disrupting the cells of the microorganisms in the biomass, including one or more of high-pressure homogenisation, bead milling, sonication, pulsed electric fields, osmotic stressing, enzymatic treatment, microwave irradiation, mechanical pressing or pureeing, is excluded.
14. The method according to any one of the preceding claims, wherein the lipid components extracted from the microorganisms comprises neutral and polar lipids, including triglycerides, phospholipids and glycolipids, pigments, carotenoids, di-glycerides, monoglycerides, free fatty-acids and mixtures thereof.
15. The use of the lipid components obtainable by a method according to any one of the preceding claims
 - in the food and feed industry, e.g. as food supplements or for feed products (e.g. for fish farming),
 - in the chemical industry, e.g. for the manufacturing of biobased plastics, foams and polymers e.g. for aviation or construction industry, or bio-lubricants,

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- in the cosmetic and pharmaceutical industry,
- in the energy sector e.g. as precursors for biofuel and biodiesel.

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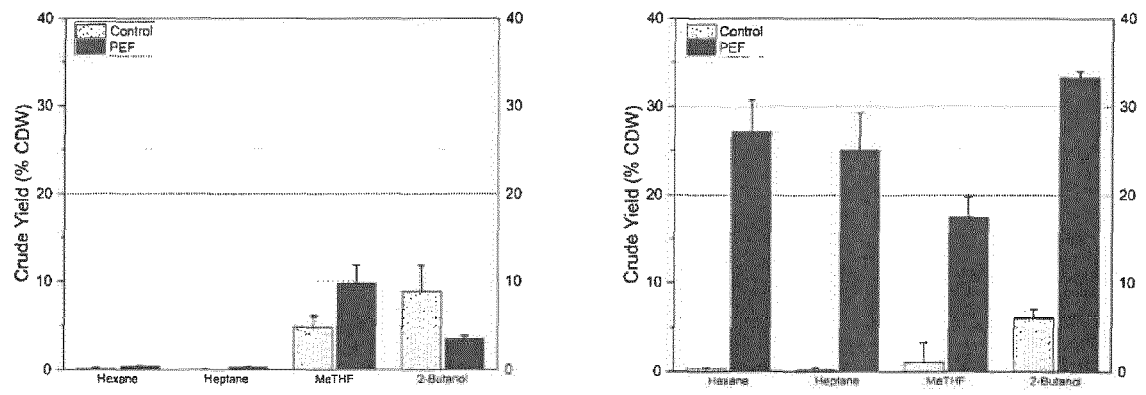


FIG. 1

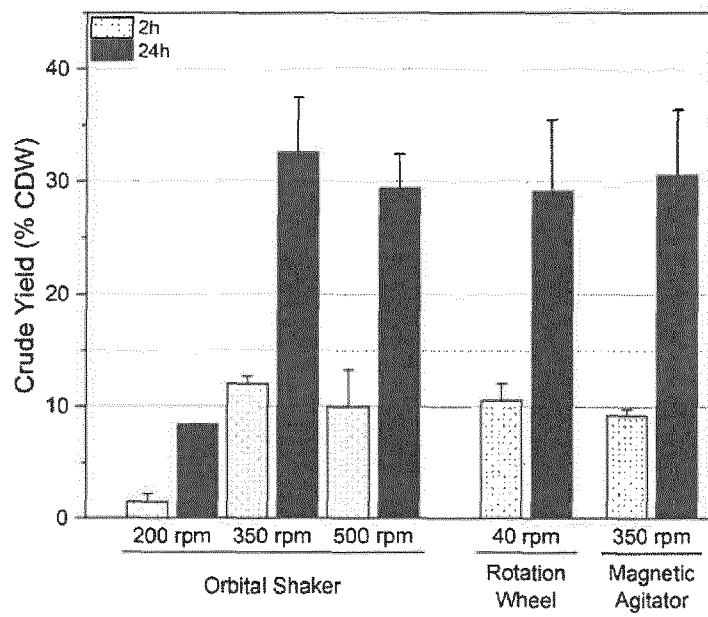


FIG. 2

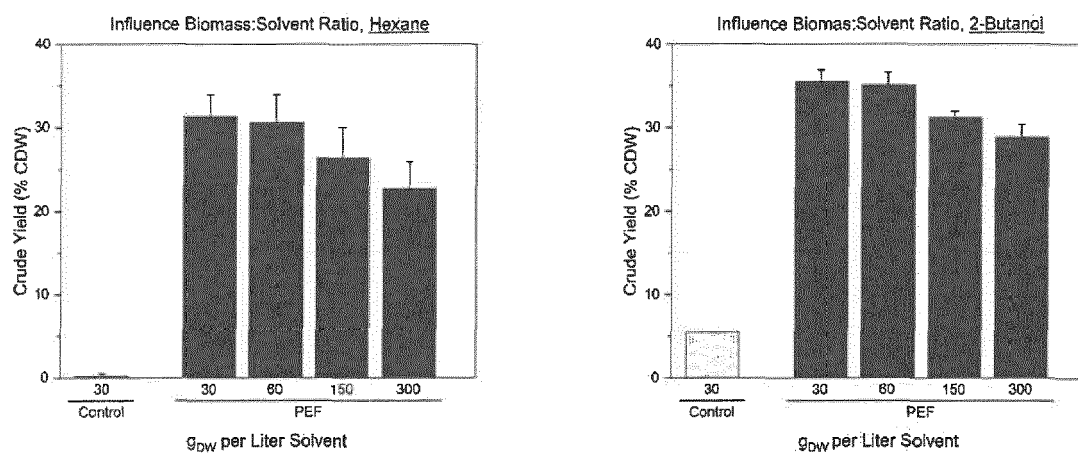


FIG. 3

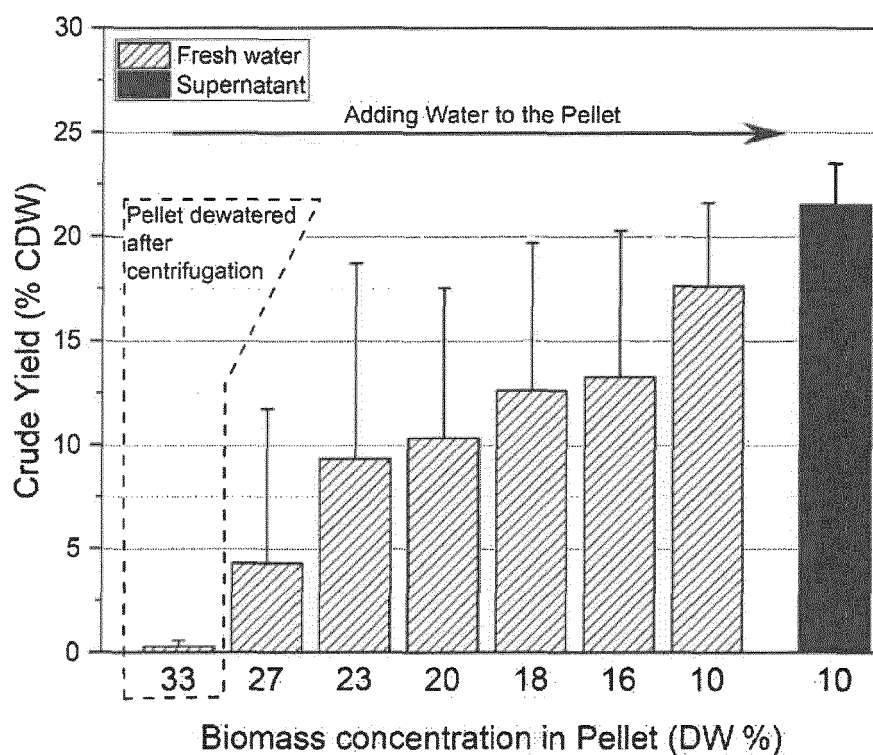


FIG. 4

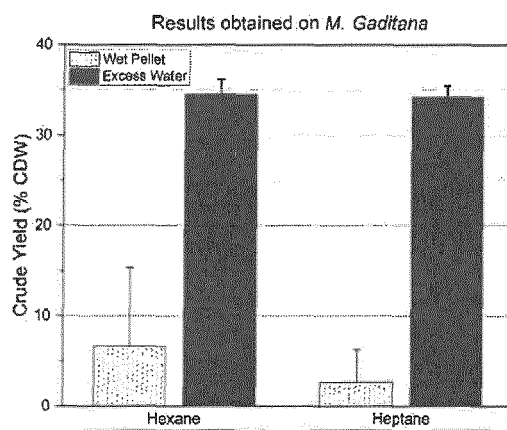


FIG. 5

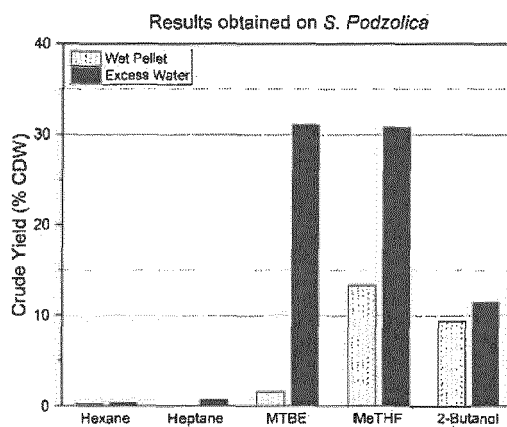


FIG. 6

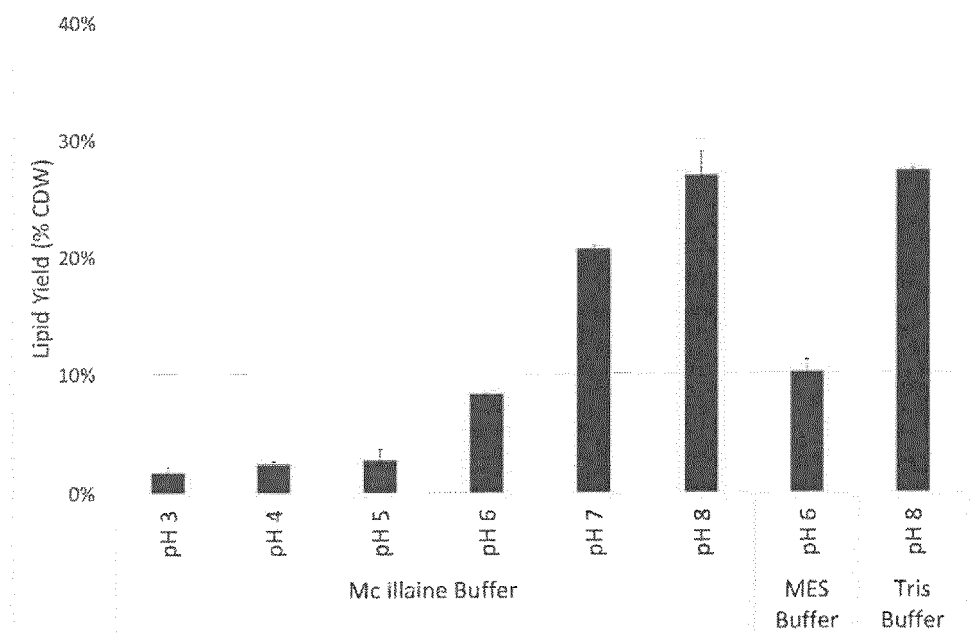


FIG. 7



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The present search report has been drawn up for all claims			
Place of search Munich		Date of completion of the search 14 June 2023	Examiner Smeets, Dieter
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			TECHNICAL FIELDS SEARCHED (IPC)
The present search report has been drawn up for all claims			
Place of search Munich		Date of completion of the search 14 June 2023	Examiner Smeets, Dieter
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	

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