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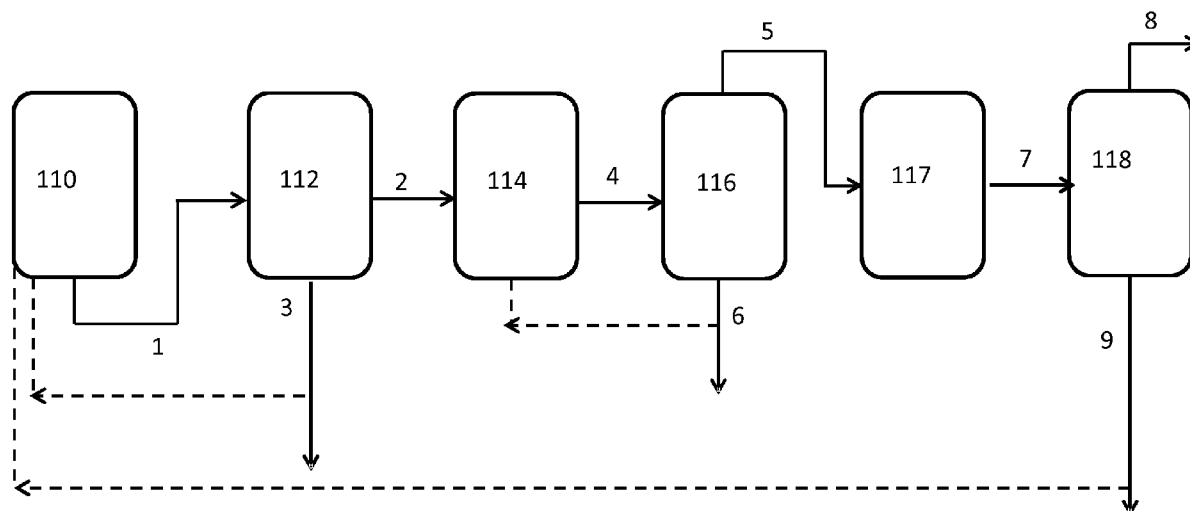


FIGURE 1

(57) Abstract: The invention concerns a process for recovering lipids produced by fermentation of microbial cells, comprising: (a) providing a fermentation medium containing microbial cells capable of producing lipids comprising mainly one or several fatty acids chosen from C18 and C19 fatty acids, (b) optionally mechanically lysing the microbial cells resulting in a lysed fermentation medium, optionally separated into a solid/liquid light phase containing lipids and a solid/liquid heavy phase containing the lysed cells, (c) demulsifying the fermentation medium, the lysed fermentation medium or the solid/liquid light phase by heating at a temperature from 30 to 80°C under agitation for at least 2 hours thereby generating a stream having a first phase containing mainly the lipids and a second phase containing water, and (d) separating the first phase containing the lipids. The process is performed without addition of chemical compound(s) at any stage of the process from step (b) to step (d).

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WET PROCESS FOR RECOVERING OIL PRODUCED BY MICROORGANISM

[Field of the invention]

The present invention relates to the recovery of oil produced by microorganisms. In particular, the invention provides methods for recovering oil produced by a microorganism through a wet process. The invention accordingly relates to the fields of biology, microbiology, fermentation technology and oil and fuel production technology.

[Background of the invention]

Lipids for use in biofuels can be produced in microorganisms, such as algae, fungi and bacteria.

Many oleaginous microorganisms, including the well characterized yeast *Yarrowia lipolytica*, produce lipids.

Microorganisms synthesize lipids with distinct carbon chain lengths and degrees of unsaturation. The lipid may be excreted by the microorganism or may remain inside the cell, stored in organelles, termed lipid bodies or lipid droplets, as storage lipids. The lipids can be produced as fatty acids, triacylglycerides (TAG) or ester of fatty acids. The lipid profile of a cell, i.e., the relative amounts of fatty acid species that make up the total lipids in the cell, is determined by the activities and substrate specificities of various enzymes that synthesize fatty acids (fatty acid synthase, elongase, desaturase), various enzymes that stabilize fatty acids by incorporating them into storage lipids (acyltransferases), and various enzymes that degrade fatty acids and storage lipids (e.g. lipases). The lipid yield of oleaginous organisms can be increased by the up-regulation, down-regulation, or deletion of genes implicated in a lipid pathway.

Typically, producing a lipid using a microorganism involves growing microorganisms which are capable of producing a desired lipid in a fermentor or bioreactor, lysing the microorganisms if the lipids are intracellular products, and recovering the lipids produced by breaking the emulsion obtained.

US2011295028A1 discloses processes for obtaining a lipid from a cell by lysing the cell, contacting the cell with a base and/or salt, and separating the lipid. The disclosed processes include raising the pH of the cell composition to 8 or above and separating lipid from the cell composition. The microbial cells suitable are organisms

such as algae, bacteria, fungi and protist. Yeast such as Ascomycetes or Basidiomycetes are mentioned. The salt used is selected from alkali metal salts, alkali earth metal salts, sulfate salts and their combination. In the examples, NaCl and $\text{Na}_2(\text{SO}_4)_2$ salts have been tested on microalgae.

- 5 However, the Applicant observed no lipids could be recovered at pH above 9.5 from an emulsion containing lipids obtained from microorganisms producing mainly C18 and/or C19 fatty acids, in particular oleic acid (C18:1).

WO2015/095696 discloses processes for obtaining microbial oil from microbial cells by lysing the cells to form a lysed cell composition, treating the lysed cell composition
10 to form an oil-containing emulsion and then recovering the oil from the oil-containing emulsion. The heating to at least 50°C is provided for demulsifying the oil-containing emulsion. However, the processes disclosed still provide addition of salts and of acid or basis for either lysing or demulsifying. Such addition of chemicals increases considerably the cost of the process and also requires treatment of the waste waters
15 produced, which can be complicated and costly.

WO2018013670A1 discloses a method for extracting a microbial oil comprising polyunsaturated fatty acids from a ferment broth containing oleaginous microorganism. The fatty acids produced include C18 and C20 fatty acids. No mention of production of C19 fatty acids is made. The method disclosed concerns
20 only oils recovered by a lysing step. To improve demulsification, the method provides a dewatering step before extraction. A typical solventless extraction method is disclosed which involves the following steps : pasteurizing or heating the cell-containing broth; lysing the cells to release microbial oil from the cells to form a lysed cell composition, which is in the form of a solution; treating the lysed cell solution by
25 heat, salt and pH adjustment in order to coalesce the oil droplets and remove emulsion from the solution; centrifuging the demulsified solution.

WO2015095690A2 discloses processes for obtaining microbial oil from microbial cells without using solvents. The fatty acids produced include C18, C20, C22 fatty acids. No mention of production of C19 fatty acids is made. The methods disclosed
30 concern only oils recovered by a lysing step. An emulsifier is added prior to, during or after the lysing of the cells to improve demulsification.

A process has here been found able to recover lipids containing mainly one or several fatty acids chosen from C18 and/or C19 fatty acids, without addition of any

chemical compound(s), contrarily to the above prior art disclosure. In particular, the process disclosed is a solventless extraction process.

[Brief summary of the invention]

An object of the present invention is a process for recovering lipids produced by fermentation of microbial cells from a fermentation medium, said lipids comprising

mainly one or several fatty acids chosen from C18 and C19 fatty acids, comprising:

(a) providing a fermentation medium containing microbial cells capable of producing said lipids by fermentation,

(b) optionally lysing the microbial cells contained in the cell fermentation medium to release the intracellular lipids into the fermentation medium resulting in a lysed fermentation medium containing lysed cells, wherein lysing is performed by mechanically treating the fermentation medium, and optionally submitting the lysed fermentation medium to :

(i) a solid/liquid separation into a solid/liquid light phase containing lipids and a solid/liquid heavy phase containing the lysed cells,

(c) demulsifying the fermentation medium of step (a), the lysed fermentation medium of step (b) or the solid/liquid light phase containing lipids of step (b)(i), wherein said fermentation medium of step (a) or solid/liquid light phase containing lipids of step (b)(i) is heated at a temperature from 30 to 80°C under agitation for at least 2 hours, thereby generating a stream having a first phase containing the lipids and a second phase containing water, and

(d) separating the first phase containing the lipids from the stream obtained in step (c),

and wherein said process is performed without addition of chemical compound(s) at any stage of the process from step (b) to step (d).

In particular, chemical compound include organic and inorganic compound.

The present invention is useful for recovering at least 25wt%, 30 wt%, 35wt%, 40 wt%, 45wt%, 50wt%, 55wt%, 60wt%, 65 wt%, 70 wt%, 75 wt%, 80 wt%, 85 wt%, 90 wt%, 95 wt%, or higher, of lipids as measured by % dry cell weight. In preferred embodiments, 45wt%, 55wt%, 65 wt%, 70 wt%, 75 wt%, 80 wt%, 85 wt%, 90 wt%, 95 wt% or higher of lipids are recovered.

A recovery as high as 100wt%, 99wt%, 98wt%, 97 wt%, 96 wt%, 95 wt% of lipids as measured by % dry cell weight can be obtained by the process of the invention.

In preferred embodiments, from 45 to 100wt% or from 60 to 99wt% or from 65 to 99wt% of lipids as measured by % dry cell weight are recovered.

- 5 The present invention allows recovering lipids comprising mainly C18 and/or C19 fatty acids. In the present invention “comprising mainly C18 and/or C19 fatty acids” refers to a C18 or C19 or C18+C19 fatty acids proportion of at least 75% or higher as a weight percentage of total fatty acids.

10 In some embodiments, the separation step (d) includes a liquid/liquid separation, a solid/liquid separation, or the both.

In one embodiment, the process further comprises, prior to the demulsification step (c), a separation step without addition of chemical compound(s) in which microbial cells contained in the fermentation medium are removed. This separation step can be performed by a solid/liquid separation. This embodiment is particularly
15 advantageous for a process recovering an extracellular production of lipids, in which the lysing step (b) is not performed.

In such a case, or for a process in which the lysing step is not optional and the lysed fermentation medium has been submitted to the solid/liquid separation (b)(i), in the separation step (d), the first phase containing the lipids may be separated without
20 addition of chemical compound(s) into a liquid/liquid light phase containing the lipids and a liquid/liquid heavy phase by a liquid/liquid separation.

In another embodiment, no solid/liquid separation is performed on the fermentation medium or on the lysed fermentation medium. In such a case, in the separation step (d), the first phase containing the lipids may be separated without addition of
25 chemical compound(s) into a solid/liquid light phase containing the lipids and a solid/liquid heavy phase by a solid/liquid separation, and the solid/liquid light phase is separated without addition of chemical compound(s) into a liquid/liquid light phase containing the lipids and a liquid/liquid heavy phase by a liquid/liquid separation.

In some embodiments, the fermentation medium provided at step (a) is submitted to
30 a dewatering step without addition of chemical compound(s) prior the lysing step. The dewatering step may be a solid/liquid separation step selected from centrifugation, filtration, and decantation.

In particular, the dewatering step may be a solid/liquid separation step selected from centrifugation or cross flow filtration. When cross flow filtration is performed, at least one tubular inorganic membrane having a pore size of 0.5µm at most is used to separate the microbial cells from the fermentation medium or the lysed fermentation medium.

In some embodiments, the stream obtained in step (c) is heated at a temperature from 30 to 90°C prior to the separation step (d).

[Brief description of the figures]

The teaching of the application is illustrated by the following Figure which is to be considered as illustrative only and do not in any way limit the scope of the claims.

Figure 1: Schematic representation of a process for the recovery of lipids from a fermentation mixture according to an embodiment of the method disclosed herein.

[Detailed description of the invention]

Unless otherwise defined, all terms used in disclosing the invention, including technical and scientific terms, have the meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. By means of further guidance, term definitions are included to better appreciate the teaching of the present invention.

As used herein, the singular forms "a", "an", and "the" include both singular and plural referents unless the context clearly dictates otherwise.

The terms "comprising", "comprises" and "comprised of" as used herein are synonymous with "including", "includes" or "containing", "contains", and are inclusive or open-ended and do not exclude additional, non-recited members, elements or method steps. Where reference is made to embodiments as comprising certain elements or steps, this encompasses also embodiments which consist essentially of the recited elements or steps.

The recitation of numerical ranges by endpoints includes all numbers and fractions subsumed within the respective ranges, as well as the recited endpoints.

All documents cited in the present specification are hereby incorporated by reference in their entirety. In particular, the teachings of all documents herein specifically referred to are incorporated by reference.

With “bio-organic compound” is meant herein an organic compound that is made by microbial cells, including recombinant microbial cells as well as naturally occurring microbial cells.

5 The term “free bio-organic compound” refers to bio-organic compound which is not in an emulsion and forms a continuous phase, usually supernatant.

The term “cell” refers to a microorganism, capable of being grown in a liquid growth medium.

10 The term “dry weight” or “dry matter” means weight determined in the relative absence of water. For example, reference to oleaginous cells as comprising a specified percentage of a particular component by dry weight means that the percentage is calculated based on the weight of the cell after substantially all water has been removed (until constant weight).

The “dry cell weight” or “dry cell matter” means weight determined in the relative absence of water after sample washing for insoluble solids removal.

15 The term “microbial cell” refers to organisms such as algae, bacteria, fungi, protest and combinations thereof, e.g. unicellular organisms.

The term “cell-associated” as used herein in connection to fermentation products refers to fermentation products that are associated to the host cell or host cell debris.

20 The term “emulsion” generally refers to a mixture of two immiscible liquids, such as water and an oil. As used herein, it particularly refers to a mixture of a bio-organic compound envisaged herein and water.

25 The term “host cell” as used herein refers to a microbial cell which is used for the production of a bio-organic compound. The host cell may be a recombinant cell, which implies that it has been genetically modified to induce or increase the production of the bio-organic compound. In particular embodiments, the host cell contains a foreign DNA and/or has one or more genetic modifications compared to the wild type organism which affects the production of the bio-organic compound. However, also considered host cells are microbial cells naturally producing a bio-organic compound of interest.

30 A. Microbial fermentation

The bio-organic compounds envisaged herein (lipids containing mainly C18 and/or C19 fatty acids) are produced by microbial fermentation. Microbial production of

organic compounds is well known in the art, and the invention is applicable to any technique deemed suitable by a skilled person involving microbial fermentation. Typically, micro-organisms are cultured under conditions suitable for the production of the organic compounds by the microbial host cells. Suitable conditions include many parameters, such as temperature ranges, levels of aeration, pH and media composition. Each of these conditions, individually and in combination, is typically optimized to allow the host cell to grow and/or to ensure optimal production of the organic compound of interest. Exemplary culture media include broths or gels. The host cells may be grown in a culture medium comprising a carbon source to be used for growth of the host cell. Exemplary carbon sources include carbohydrates, such as glucose, fructose, cellulose, or the like, that can be directly metabolized by the host cell. In addition, enzymes can be added to the culture medium to facilitate the mobilization (e.g., the depolymerization of starch or cellulose to fermentable sugars) and subsequent metabolism of the carbon source. A culture medium may optionally contain further nutrients as required by the particular microbial strain, including inorganic nitrogen sources such as ammonia or ammonium salts like ammonium sulfate, and minerals like phosphates, potassium salts, magnesium salts, manganese salts, iron salts, copper salts, zinc salts, calcium salts or sodium salts. Other growth conditions, such as temperature, cell density, and the like are generally selected to provide an economical process. Temperatures during each of the growth phase and the production phase may range from above the freezing temperature of the medium to about 50°C. The fermentation may be conducted aerobically, anaerobically, or substantially anaerobically. Briefly, anaerobic conditions refer to an environment devoid of oxygen. Substantially anaerobic conditions include, for example, a culture, batch fermentation or continuous fermentation such that the dissolved oxygen concentration in the medium remains between 0 and 10% of saturation. Substantially anaerobic conditions also includes growing or resting cells in liquid medium or on solid agar inside a sealed chamber maintained with an atmosphere of less than 1% oxygen. The percent of oxygen can be maintained by, for example, sparging the culture with an N₂/CO₂ mixture or other suitable non-oxygen gas or gasses. The fermentation can be conducted continuously, batch-wise, or some combination thereof. Conventional fermentation bioreactors, shake flasks, test tubes, microtiter dishes, and petri plates can be used.

Examples of culture conditions are provided in US2012130099A1, incorporated therein by reference.

This step does not require addition of chemical compound(s) other than those necessary for the fermentation of the medium. The chemical compounds necessary for the fermentation of the medium are listed above.

- 5 In other words, this step does not require addition of any chemical compound(s) such as a surfactant, an emulsifier, a solvent, a basic compound, an acid compound.

B. Microbial Cell

- 10 Suitable micro-organisms for fermentation are known in the art. Non-limiting examples of suitable micro-organisms include bacteria such as *Escherichia* (e.g. *E. coli*), *Bacillus* or *Lactobacillus* species, fungi, in particular yeasts such as *Saccharomyces* (e.g. *S. cerevisiae*) species, or algae such as *Chlorella* species. In particular embodiments, the microbial host cell is a fungus, preferably a yeast. The micro-organisms may naturally produce the bio-organic compound of interest, or they may have been genetically modified (i.e. recombinant micro-organisms) to ensure production of the bio-organic compound of interest.

- 15 Suitable micro-organisms for use in the present invention are capable to produce lipids comprising mainly C18 and/or C19 fatty acids; in particular, the micro-organism is capable to excrete (extracellular production) or secrete (intracellular production) the lipids.

- 20 In some embodiments, the microbial cell is selected from the group consisting of algae, bacteria, molds, fungi, plants, yeasts and combination thereof.

In some embodiments, a microbial cell is an eukaryotic cell, such as a yeast cell, a fungi cell, an algae cell. Advantageously, the microbial cell is a yeast or an algae.

- 25 Examples of suitable cells include, but are not limited to, fungal or yeast species, such as *Arxula*, *Aspergillus*, *Aurantiochytrium*, *Candida*, *Claviceps*, *Cryptococcus*, *Cunninghamella*, *Geotrichum*, *Hansenula*, *Kluyveromyces*, *Kodamaea*, *Leucosporidiella*, *Lipomyces*, *Mortierella*, *Ogataea*, *Pichia*, *Prototheca*, *Rhizopus*, *Rhodospiridium*, *Rhodotorula*, *Saccharomyces*, *Schizosaccharomyces*, *Tremella*, *Trichosporon*, *Wickerhamomyces*, and *Yarrowia*.

- 30 In some embodiments, the microbial cell is *Saccharomyces cerevisiae*, *Yarrowia lipolytica*, or *Arxula adeninivorans*.

In some embodiments, the host cell is a microbial cell selected from the genus *Escherichia*, *Bacillus*, *Lactobacillus*, *Pantoea*, *Zymomonas*, *Rhodococcus*,

Pseudomonas, Chroococcales, Chroococcidiopsidales, Chroococcidiopsidales, Chroococcidiopsidales, Gloeobacterales, Nostocales, Oscillatoriales, Pleurocapsales, Spirulinales, Synechococcales, Incertae sedis.

In certain embodiments in which the lipid production is intracellular, the microbial cell
 5 comprises at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, or more lipid as measured by % dry cell weight.

Example of suitable genetically modified cells include, but are not limited to, the cells
 10 obtained by the processes disclosed in WO2016/094520 A1 or WO2016/014900 A2 (both documents incorporated therein by reference).

C. Bio-organic compound

The bio-organic compound envisaged therein is a lipid containing mainly C18 and/or C19 fatty acids. The lipid may also contain other fatty acids such as C16 fatty acids
 15 In some embodiments, the proportion of C18 and C19 fatty acids is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, or higher as a weight percentage of total fatty acids produced by the microbial cell.

In one embodiment, the lipids produced contain mainly C18 fatty acids. The proportion of C18 fatty acids is at least 75wt% or higher as a weight percentage of
 20 total fatty acids produced by the microbial cells.

In some embodiments, the proportion of C18 fatty acids is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, or higher as a weight percentage of total fatty acids produced by the microbial cell.

In preferred embodiments, the proportion of C18 fatty acids is at least 85% or higher
 25 as a weight percentage of total fatty acids produced by the microbial cell.

In some embodiments, C18 fatty acids include oleic acid.

In some embodiments, the microbial cell produces oleic acid at a concentration of at least 50%, 51 %, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%,
 30 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, or higher as a weight percentage of total fatty acids in the cell.

In some embodiments, the proportion of C19 fatty acids is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, or higher as a weight percentage of total fatty acids produced by the microbial cell.

In some embodiments, C19 fatty acids include 10-methyloctadecanoic acid.

5 D. Overall recovery process

Provided herein are methods for recovering bio-organic compounds, in particular lipids containing mainly C18 and/or C19 fatty acids, from a fermentation medium. A fermentation medium (also referred to herein as fermentation mixture, fermentor
10 broth or whole cell broth (WCB)) typically comprises micro-organisms, a culture medium and, once cultivation has started, the fermentation products or bio-organic compounds produced by the micro-organisms. These bio-organic compounds can be intracellular products or can be released by the micro-organisms in the culture medium from which they may be recovered.

Emulsion formation can be promoted in the fermentation medium by the mechanical
15 energy from fermentation (e.g. from agitators or fermentation gases produced by the microbial host cells), or by the microbial host cells or various bio-molecules therein. In the case of excreted bio-organic compounds, formation of an emulsion is observed along with production of bio-organic compounds. In case of secreted bio-organic compounds, emulsion can occur in the recovery process once the cells are
20 lysed and the bio-compounds freed into the fermentation medium.

Emulsion formation, which is inherent to microbial production systems, is a source of loss of bio-organic compounds to recover.

Provided herein are recovery processes for bio-organic compounds from a fermentation medium characterized in that they comprise the recovery of said bio-
25 organic compounds comprised in emulsions without addition of any chemical compound(s) in particular for lysing if required, for demulsification and for separation of the lipids from the demulsified medium..

Accordingly, in an aspect, the invention comprises methods for the (improved) recovery of lipids containing mainly C18 and/or C19 fatty acids from a fermentation
30 mixture or a lysed fermentation mixture, which methods comprise recovering the lipids which are present in the fermentation medium in emulsions without addition of any chemical compound(s) in particular for lysing if required, for demulsification and for separation of the lipids from the demulsified medium.

Optional dewatering step

The objective of this step is to reduce the moisture content of the fermentation medium provided by step (a), prior a lysing step, thereby generating a dewatered fermentation medium. In other words, this step performs a concentration of the fermentation medium. This step applies for intracellular production of bio-organic compounds. This step does not require addition of any chemical compound(s).

This step also generates a water stream which can be used for the fermentation.

Typically, this dewatering step is a solid/liquid separation step.

The solid/liquid separation of the fermentation mixture may be achieved by well-known techniques, including, without limitation, centrifugation, filtration, and decantation, preferably by centrifugation and filtration, in particular cross flow filtration.

A centrifuge can separate the fermentation mixture in batch or on a continuous flow basis. Preferably continuous flow centrifugation is used in the methods described herein. A non-limiting example of a centrifuge suitable for solid/liquid separation of a fermentation mixture as taught herein is a disk stack centrifuge, such as a disk stack centrifuge with nozzles. Centrifugation conditions can be suitably determined by the skilled person to achieve the desired solid/liquid separation.

Tangential flow filtration (TFF), also known as cross-flow filtration, is a separation technique that uses membrane system(s) and flow force to purify solids from liquids. TFF generates a retentate and a permeate. Typically, the dewatered fermentation medium is recovered as the retentate.

The retentate should not pass through the membrane system(s) at a significant level. The retentate also should not adhere significantly to the membrane system(s) material.

In some embodiments, the dewatering step is performed by cross flow filtration using one or more tubular inorganic membranes, in other words, non-organic membrane(s). In a preferred embodiment, the inorganic membrane is a ceramic membrane.

Non-limiting examples of tangential flow filtration include those involving the use of a membrane with a pore size of at most 0.5 micrometer, at most 0.4 micrometer, at most 0.3 micrometer, at most 0.2 micrometer, at most 0.18 micrometer. In some

embodiments, the pore size of the membrane is of 0.1 micrometer or more. The pore size may be within a range defined by any combination of the above limits. Preferred pore sizes of TFF allow solutes and debris in the fermentation broth to flow through, but not microbial cells.

- 5 In some embodiments, a membrane has at least one channel of tubular shape.

In some embodiments, the surface of the membrane in contact with the fluid has an active layer which determines the porosity of the membrane. The active layer is full of holes, the diameters of which do not allow the pass through of the microbial cells.

- 10 In some embodiments, a membrane with a multi-layer configuration for filtration of a medium is provided with at least one first layer which is a carrier layer and a second layer which is a separation layer that filters the medium and generates a retentate and a permeate. In such a case, the separation layer is a ceramic or carbon material, in other word a non organic material.

- 15 In some embodiments, the inorganic material of the membrane is a ceramic. The inorganic material may be selected from titanium oxide (TiO_2), zirconium oxide (ZrO_2), alumina (Al_2O_3), silicon carbide (SiC), boron carbide (B_4C), silicon nitride (Si_3N_4), aluminium nitride (AlN), boron nitride (BN), agglomerated carbone or mixtures thereof. In some embodiments, the material of the membrane is selected from titanium oxide (TiO_2), zirconium oxide (ZrO_2), alumina (Al_2O_3).

- 20 In some embodiments, conditions of filtrations are chosen to reduce the moisture content of the fermentation medium so as to obtain a dewatered fermentation medium having a moisture content of 80%wt or less, of 78%wt or less, of 75%wt or less, or of 70%wt or less or even of 65%wt or less. Those conditions will be determined by the man skilled in the art by controlling the moisture content while
25 monitoring one or several of the following parameters: the transmembrane pressure (TMP), the differential pressure, the feed flowrate, the cross flow velocity. The moisture content / dry matter can be measured by monitoring the weight in function of time at a temperature suitable for water evaporation (e.g. 100-110°C) until the weight is constant.

- 30 The use of the above inorganic membrane(s) allows steady flow rate without clogging despite fouling.

The at least one tubular inorganic membrane can be provided in a filtration module. In particular, a filtration module may comprise one or more tubular inorganic

membranes, each membrane may itself comprise several tubular channels arranged in a bundle. By way of example, membranes having from 4 to 10 tubular channels may be used, but the invention is not limited by a number of tubular channels in a tubular membrane or by the number of the tubular membranes used. The man skilled
5 in the art knows how to choose the number of membranes and tubular channels in the membrane used depending on the filtration surface required to perform the filtration. Several filtration modules may be provided, some of which being cleaned so as to restore their permeability while the others are used for filtration. A continuous treatment of the dewatered broth can then be performed. The cleaning
10 treatment to recover some or all of the initial permeability of the membrane(s) may include one or several of the following actions: flushing the membranes with water, rinsing the membrane(s) with water, washing the membrane(s) using alkaline and/or acid solution. In other words, in one embodiment, the temperature is raised during the cleaning treatment, for example up to 50°C.

15 Optional lysing step

Such lysing step is performed when the lipids are intracellular products. Lysing may be performed on the fermentation medium obtained from the fermentor. In a preferred embodiment, the lysing step is performed on a dewatered fermentation medium.

20 The lysing step, which is a widely established step in the extraction of bio-organic compounds from fermentation medium, ruptures the cell wall and/or cell membrane of a cell to release their cytoplasmic content into the fermentation medium.

A lysed fermentation medium is obtained by lysing the microbial cells contained in the fermentation medium or the dewatered fermentation medium. Such lysed
25 fermentation medium comprises lysed cells, including cell debris, lipids (initially comprised in the cells), other natural contents of the cells and optionally, aqueous components from the fermentation broth.

According to the invention, lysing is performed by mechanically treating without addition of chemical compound(s).

30 As used herein, mechanically treating includes, but is not limited to, homogenizing a cell, applying ultrasound to a cell, cold-pressing a cell, milling a cell or the like, and combinations thereof.

Mechanical devices for treating a cell can include, but is not limited to, processes utilizing a French pressure cell press, a sonicator, a homogenizer, a ball mill, a rod mill, a pebble mill, a bead mill, a high pressure grinding roll, a vertical shaft impactor, an industrial blender, a high shear mixer, a paddle mixer, a polytron homogenizer or
5 the like, and combinations thereof.

In a preferred embodiment, lysing is performed by milling, in particular using a bead or ball mill, for example an agitator bead or ball mill or an accelerator bead or ball mill, or using a homogenizer.

An agitator bead or ball mill has separating system formed from a separating
10 component which has two circular discs co-axial to the chamber axis, and between which are installed several transporting or vane elements distributed symmetrically around the disc center point and leading inwards from the disc edge. During the operation of the separating device the transporting or vane elements create a back pressure on the material and milling body mix so by centrifugal force and different
15 specific densities the milling bodies are separated from the product and transported back to the inner chamber of the mill. A suitable agitator bead or ball mill is for example described in EP1468739.

An accelerator bead or ball mill has a milling chamber and an agitator having a rotatably mounted and driven agitator shaft and accelerators arranged thereupon. A
20 suitable agitator bead or ball mill is disclosed in WO2010112274A1, its accelerator that is arranged furthest downstream, that is, the accelerator closest to the milling material outlet, lengthens axially and extends along the axial length of the sieve.

In a preferred embodiment, lysing is performed by milling using an accelerator bead or ball mill, in particular an accelerator bead mill.

25 The lysed fermentation medium resulting from the lysing step (b) may then be submitted to a solid/liquid separation step prior to the demulsification step.

Optional solid/liquid separation step prior to the demulsification step

This optional separation allows obtaining a fraction of the fermentation medium or of the lysed fermentation medium with reduced amounts of microbial cells. In other
30 words, one of the streams generated by the optional separation has reduced dry matter content. This step does not require addition of any chemical compound(s).

In some embodiments, the solid/liquid light phase has a dry matter content from 25 to 50wt% or from 30 to 45wt%. The dry matter content may be within a range defined by any combination of these limits.

In a preferred embodiment, this separation is performed by a solid/liquid separation.

5 This optional separation step separates the fermentation medium or the lysed fermentation medium into a solid/liquid light phase containing the lipids and a solid/liquid heavy phase containing the microbial cells. When the fermentation medium is separated by this step, in particular in the case of extracellular lipid production, the dewatering step is not necessary.

10 In a preferred embodiment, the solid/liquid light phase obtained is submitted directly to the demulsification step (c).

The solid/liquid separation step, which is a widely established step in the extraction of bio-organic compounds from fermentation medium, separates the micro-organisms from the fermentation mixture. The stream comprising the micro-organisms is also referred to herein as "microbial pellet", "cell slurry", "solid/liquid underflow", "solid/liquid centrifuge waste stream" or "solid/liquid heavy phase". This stream comprises the micro-organisms and cell-associated bio-organic compounds, and may further comprise host cell debris, culture medium and some bio-organic compounds. The solid/liquid heavy phase is preferably a liquid stream. The generally supernatant or light phase obtained by solid/liquid separation of the fermentation mixture, also referred to herein as "solid/liquid light phase", comprises the culture medium, free bio-organic compounds and bio-organic compounds comprised in an emulsion, and may further comprise host cell debris.

25 The solid/liquid separation of the fermentation mixture may be achieved by well-known techniques, including, without limitation, centrifugation, filtration, and decantation, preferably by centrifugation.

Aging step

Prior to the demulsification step, the fermentation medium of step (a), the lysed fermentation medium of step (b) or the solid/liquid light phase containing lipids of step (b)(i) can be submitted to an aging step of at least 48 hours, without agitation. This step does not require either addition of chemical compound(s).

The aging step may last from 48 hours to 5 days.

The aging step is preferably performed at a positive ambient temperature, for example from 0°C to 40°C depending on the season.

In some embodiments, the aging step may be performed at a temperature from 0 to 40°C or from 0 to 30°C.

- 5 Such aging step is therefore performed without any heating. This aging step is not a pasteurization step in which heat is applied to inactivate undesirable enzymes such as the ones that might degrade the oil.

10 In other words, during the aging step, the fermentation medium of step (a), the lysed fermentation medium of step (b) or the solid/liquid light phase containing lipids of step (b)(i) is left at rest, without any heating, agitation and addition of chemical compounds.

Demulsification step

15 The demulsification step generates a stream having a first phase containing the lipids and a second phase containing water by breaking the emulsions containing the lipids previously formed.

According to the invention, the breaking of the emulsions is performed without addition of any chemical compound(s), thus without addition of any surfactant compounds, salt, alkaline compound, acid compound. In particular, there is no need to control the pH of the emulsion.

20 According to the invention, the fermentation medium of step (a), or solid/liquid light phase containing lipids of step (b)(i) is heated for demulsifying.

In one embodiment, the heating is from 30 to 80°C or from 35°C to 70 °C or from 40°C to 60°C, or within a range defined by any combination of these limits.

25 Such heating at any of the above mentioned temperature ranges is performed under agitation for at least 2 hours, preferably for at least 3 hours.

For example, heating may be performed from 2 hours to 24 hours or from 3 hours to 23 hours or within a time range defined by any combination of these limits.

30 The terms “agitating” and “agitation” refer to a process to impart motion within a medium through application of a force. The agitation may be performed by stirring, mixing, blending, shaking, vibrating or combination thereof.

In some embodiments, the agitation may be performed by a rotational stirring, an impeller or a spiral stirrer.

Examples of suitable impellers include propellers with blades, propellers with blades and counter blades, propellers with blades inside a guide tube. Counter blades are
5 usually fixed at a predetermined distance of the walls of a tank, whereas the blades rotate around a central axis of the tank.

The rotation speed, and eventually the features of impellers to use, can be determined by the man skilled in the art by monitoring the quantity of oil recovered.

Demulsification step may be performed in a container, for example in a container
10 equipped with heating means. Such heating means include, without limitation, a double wall for hot fluid circulation, double wall containing electric heating, impellers with heating blades.

At the end of the demulsification step, a stream having a first phase containing the lipids and a second phase containing the water is generated. In general, in the
15 absence of solvent lighter than the lipids, the first phase is supernatant and the second phase is heavier than the supernatant phase. The second phase may also contain cell debris and/or some left over lipids.

More particularly, the demulsified stream may contain: an oil light phase containing the lipids (generally supernatant phase) and, in the second phase (generally heavier
20 than the first phase), eventually an emulsion phase, a heavy phase containing mainly water, and eventually, at the bottom, deposits (debris if any and dead cells).

In one embodiment, the second phase may be recycled until it contains no more left over lipids, such recycle being send to any step prior to the demulsification step or at the beginning of the demulsification step.

25 Optional heating step

In some embodiments, the separation step (d) is preceded by a heating step in which the stream generated by the demulsifying step is heated at a temperature from 30 to 90°C, or from 30 to 85 °C or from 40 to 70°C to improve the oil recovery. The temperature may be within a range defined by any combination of the above limits.

30 This step does not require addition of any chemical compound(s).

In a preferred embodiment, the heating is performed on the stream generated by the demulsifying step.

This heating can be performed by passing the stream in a heat exchanger.

Separation step (d)

This step allows separation of the first phase containing the lipids from the stream generated at the demulsifying step, eventually heated by the optional heating step, and thus the recovery of the lipids.

This step does not require addition of any chemical compound(s). This separation step (d) may include a solid/liquid separation, a liquid/liquid separation, or a solid/liquid separation followed by a liquid/liquid separation.

In some embodiments, the first phase containing the lipids is separated by a liquid-liquid separation. The liquid/liquid separation step, which is a widely established step in the extraction of bio-organic compounds, separates the bio-organic compound from the second phase. The light phase obtained comprising the bio-organic compound is also referred to herein as "crude", "supernatant phase" or "liquid/liquid light phase". This stream comprises the bio-organic compounds produced by the fermentation and may further comprise some dead cells. The heavy phase recovered, also referred to herein as "phase heavier than the supernatant phase" or "liquid/liquid heavy phase", comprises the culture medium, dead cells and may further comprise host-cell debris, eventually some free bio-organic compounds and bio-organic compounds comprised in an emulsion. A further solid phase may be obtained, also referred to herein as "discharged phase", "discharge composition" which comprises the culture medium, host-cell debris, dead cells and may further comprise free bio-organic compounds and bio-organic compounds comprised in an emulsion.

The liquid/liquid separation may be achieved by well-known techniques, including, without limitation, centrifugation, filtration, and decantation, preferably centrifugation. A centrifuge can separate liquid/liquid light phase in batch or on a continuous flow basis. Preferably continuous flow centrifugation is used in the methods described herein. A non-limiting example of a centrifuge suitable for liquid/liquid separation of a fermentation mixture as taught herein is a disk stack centrifuge. Centrifugation conditions can be suitably determined by the skilled person to achieve the desired liquid/liquid separation.

In a preferred embodiment, the separation step (d) is a liquid/liquid separation, and prior to the demulsification step (c), the fermentation medium or the lysed

fermentation has undergone a solid/liquid separation into a solid/liquid light phase containing the lipids and a solid/liquid heavy phase containing the microbial cells, and the solid/liquid light phase is further submitted to the demulsification step (c).

5 In other embodiments, the first phase containing the lipids is separated by a solid/liquid separation followed by a liquid/liquid separation. This solid/liquid separation may be performed as described above for the optional solid/liquid separation. This is particularly advantageous when the fermentation medium or the lysed fermentation medium has not been submitted to a solid/liquid separation prior to the demulsification step (c).

10 In such a case, the first phase containing the lipids is separated into a solid/liquid light phase containing the lipids and a solid/liquid heavy phase by a solid/liquid separation, and the solid/liquid light phase is separated into a liquid/liquid light phase containing the lipids and a liquid/liquid heavy phase.

15 The heavy phases recovered by the solid/liquid and/or liquid/liquid separations can be recycled in the process partly or completely.

In some embodiments, the heavy phase of the liquid/liquid separation which is aqueous can be submitted, partly or completely, to a chemical or coalescence treatment in a further step (e) to recover residual lipids droplets and then recycled into the fermentor of the fermentation step.

20 In some embodiments, alternatively or in combination with the previous embodiment, the heavy phase of the solid/liquid separation can be recycled partly or completely to the optional lysing step and/or submitted to a solvent extraction for more lipids recovery, in a further step (f).

25 The invention will be further understood with reference to the following non-limiting examples.

[Examples]

Example 1 : description of the process according to an embodiment

Figure 1 shows a schematic representation of a process for the recovery of lipids from a fermentation mixture according to an embodiment of the present invention. A
30 fermentation mixture (whole cell broth, stream #1) prepared in a bioreactor 110 is fed into in a dewatering device 112, such as a centrifuge or a cross flow filtration device for reduction of its moisture. The permeate (stream #3) obtained is discarded

or preferably recycled partly or in totality in the process, for example in the bioreactor 110. The retentate (dewatered fermentation medium-stream #2) is conducted to a device 114 which is a lysing equipment or a tank in which the cells are lysed. The lysed fermentation medium (stream #4) is then separated in a separation solid-liquid
5 separation device 116 into a solid/liquid light phase (stream #5) and a solid/liquid heavy phase (stream #6).

The solid/liquid light phase (stream #5) containing the lipids is then demulsified by heating at a temperature from 30 to 80°C under agitation for at least 2 hours. In the figure, this demulsification is performed in a device 117, such as a tank, reactor or
10 similar, equipped with an agitating element (not represented) and a heating device (not represented). The stream #7 generated is then separated in a liquid-liquid separation device 118 into a liquid/liquid light phase (stream #8 crude containing the lipids) and a liquid/liquid heavy phase (stream #9). The process thus allows recovering the lipids (stream #7).

15 The light phase (crude containing most of the lipids, stream #8) can be further treated. It can for example be submitted to a polishing centrifuge to remove some dead cells and dissolved lipids. The lipid stream obtained may then be purified appropriate treatments such as refining, bleaching and deodorization. These treatments are usual and not described herein. The lipid stream may further be
20 submitted to chemical reactions.

The liquid/liquid heavy phase #9 which is an aqueous phase can be recycled partly or in totality in the process, for example in the bioreactor 110.

Prior to the demulsification step, the lysed fermentation medium #4 or the solid/liquid light phase containing lipids #5 can be optionally submitted to an aging step without
25 addition of chemical compound(s). Such aging step can be performed in a dedicated tank for example placed between devices 114 and 116 or between devices 116 and 117. This aging step can also be performed in the tank 117 used for demulsification. For more lipids recovery, the solid/liquid heavy phase #6 may be recycled to the lysed tank 114 or may be submitted to a further treatment such as a
30 solvent extraction using a suitable solvent, such as alkanes, alcohols, in particular anhydrous alcohols, aromatic compounds, chlorinated compounds, ethers, ketones, esters, aldehyde, sulfides.

The process described in figure 1 is particularly adapted for intracellular production of lipids. In case of extracellular production, the dewatering and lysing steps (devices 112 and 114) should be omitted.

Example 2 :Tangential flow filtration test.

- 5 A 20 L broth of genetically modified *Yarrowia lipolytica* has been fermented in a bioreactor for 5 days.

In this example, unless otherwise indicated :

- the lipid content has been determined by gravimetric analysis of extracted lipids. Lipids have been extracted by lyophilisation until weight stabilization followed by disruption and then solvent extraction in hexane.
- The dry matter (DM) content has been measured by gravimetric analysis until weight stabilization,
- The moisture content has been determined from the dry matter content,
- The turbidity has been measured by a turbidimeter which sends a beam of light through a water sample and measures the amount of light passing through the water in relation to the amount of light that is reflected by the particles in the water,
- The density at 20°C using an oscillating tube density meter,
- The fatty acids by reacting the fatty acids with methanol and heptadecanoic acid or tridecanoic acid as internal standard, to form methyl esters of the fatty acid to measure and the ester of heptadecanoic acid or of tridecanoic acid, followed by gas chromatography analysis of the esters using the ester of heptadecanoic acid or of tridecanoic acid as internal standard.

The features of the broth are collected in the below table 1. The fatty acids content of the broth is given below in table 2.

25 Table 1:

Parameter	Unit	Fermentation broth
lipid content	%wt	66,2
Total suspended solids (TSS) (NF T 90-105-2)	g/kg	88.7
Turbidity	NTU	>4000
Conductivity at 25°C	mS/cm	11.43
pH at 18.2 °C	-	4.82
Density at 20°C	g/L	995
Granulometry		
d ₁₀	µm	1.32
d ₅₀		4.43

d ₉₀		7.72
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Filtration Tests: Tubular ceramic membranes have been tested in a cross-flow filtration bench.

Membranes INSIDE CéRAM™ from Tami Industries have been tested, both of which have an external diameter of 10mm and a length of 250mm. Their properties are presented in the below table 3.

Table 2

Fatty acid		Fermentation broth	
		g/L	%wt TSS
lauric acid	C12:0	nd	nd
myristic acid	C14:0	nd	nd
palmitic acid	C16:0	5.56	5.607
palmitoleic acid	C16:1	7.25	7.309
stearic acid	C18:0	2.38	2.403
oleic acid	C18:1	74.27	74.888
linoleic acid	C18:2	Nd	Nd
Total fatty acids		89.46	90.206

Nd: non detected

10

Table 3

Membrane	Material	Properties
M1	TiO ₂ and ZrO ₂	1 single channel Hydraulic diameter : 6mm Area : 47 cm ² Pore size : 0.14µm
M2	TiO ₂ and ZrO ₂	1 single channel Hydraulic diameter : 6mm Area : 47 cm ² Pore size : 0.8µm

The retentate flowrate is determined by volume sampling (retentate weight sampled during a determined period of time); this measurement has been performed with water at a determined set of parameters (of the pump and the back pressure valve), before the tests and it has been performed for different set of parameters during the tests.

15

The permeate flowrate is measured, either by a balance (and recorded with time) for the tests with permeate production, or by volume sampling (permeate weight sampled during a determined period of time) for the tests with no permeate production (recycling of permeate; variations vs time or operating conditions). Then, the instantaneous and average permeate flowrate per filtration area is calculated as well as, for tests with permeate production, the mass concentration factor ($MCF = \text{initial weight of sample} / \text{retentate weight} = \text{initial weight of sample} / (\text{initial weight of sample} - \text{permeate weight})$).

The transmembrane pressure is calculated with the pressure sensors: $TMP = (\text{retentate pressure before membrane} + \text{retentate pressure after membrane}) / 2 - \text{permeate pressure}$ (which can be taken equal to 0). The concentration factor (CF) for TSS can be also calculated as $CF (TSS) = \text{retentate TSS} / \text{broth TSS}$.

The two membranes M1 and M2 have been tested.

Table 4 : Operating conditions

	Test 1	Test 2
Membrane	M1	M2
Initial weight (g)	2847.5	2847.5
Average TMP (bar)	2.1	1.3
Feed Flowrate (kg/h)	450	450
Cross flow velocity (m/s)	4.4	4.4
Temperature (°C)	26	25

The results are shown in the below table 5.

For the membrane 0,8 μm (M2), the permeate flowrate decreases a lot (the test is stopped before reaching a MCF of 2,5): internal fouling have probably occurred. Moreover, the permeate is not clear at the beginning (as shown by TSS values). The water content for the retentate is 80.9wt%.

For the membrane 0,14 μm (M1), the permeate flowrate is steady during the increase of MCF (until 2,5) and the permeate has a good quality (TSS equal to 0). The final DM of retentate reaches 23,3wt% which corresponds to a water content of 76.7 %wt.

Test with membrane M2 was expected to have better filtration behavior. In particular, higher flowrates were expected due to the greatest pore size. Surprisingly, the membrane with the smallest pore size (M1) gives the higher steady permeate flowrate, along with the lower water content. It would not be surprising to obtain much

lower water content, in particular less than 75%wt or 70%wt or 65%wt, by optimizing the filtration conditions.

Table 5

		Test 1	Test 2
Production results			
Final MCF		2.5	2
Final average permeate flowrate	kg/h/m ²	139	87
Final instantaneous permeate flowrate	kg/h/m ²	158	25
Permeate			
TSS	mg/kg	0	7-17
DM	wt %	2	2.1-2.2
Retentate (final)			
TSS	g/kg	195	169
DM	wt %	23.3	19.1
CF (TSS)		2.2	1.9
Washing (after flushing and rinsing) Ultrasil P11 (0.5%wt) at 66°C during 30min with feed circulation ~ 300-350 kg/h of and TMP = 0.7 bar			
initial permeability	L/h/m ² /bar at 20°C	1996	3746
permeability after test (loss)	L/h/m ² /bar at 20°C (%)	95 (95%)	54 (99%)
Permeability after washing (loss)	L/h/m ² /bar at 20°C (%)	1336 (33%)	3268 (13%)

The results show fouling of the membranes leading to a loss of permeability. A washing step allows recovering the permeability. An alkaline washing enables to recover 67% of the initial water permeability.

The following washing has been performed on the membranes:

- Water flushing (non-recycling) and water rinsing (retentate recycling/5 min/1.2 bar/ feed circulation 450kg/h)

- Washing: Ultrasil P11 (0.5wt%) at 66°C during 30min with feed circulation 300-350kg/h and TMP : 0.7 bar

Ultrasil P11 is a chlorinated, powdery alkaline detergent composed of emulsifiers, degreasers and surfactants for the cleaning of membrane systems.

Analysis of the flushing, rinsing and washing waters have shown that about 8%wt fatty acids from the initial sample are found in these streams, which should therefore

be recycled to recover the maximum of lipids, at least for the flushing and rinsing streams.

Microbial cell composition tested in examples 3-14:

A broth containing *Yarrowia Lipolytica* cells has been prepared in a fermentor.

- 5 The cell used is a genetically modified cell, which produces oil having a typical composition shown in the below table 1.

Table 6 : Typical composition of oil produced by *Yarrowia Lipolytica* genetically modified

		% (wt) with respect to total lipid weight
Palmitic acid	C16:0	5.9-6.3
7-cis-Hexadecenoic acid	C16:1 Δ 7	0.6-6.1
Palmitoleic acid	C16:1 Δ 9	0.3-6.1
Stearic acid	C18:0	1.2-2.8
Oleic acid	C18:1 Δ 9	84.2-86.5

- 10 The broth has been dewatered to obtain a dewatered broth B1. The dewatered broth B1 corresponds to stream #2 in figure 1.

In examples 4-7, this dewatered broth B1 has been submitted to a cell disruption by bead milling in a DYNO-MILL MULTI LAB®, in the following conditions :

- 4 Agitator discs
- Agitator tip speed at 14m/sec
- 15 - 0.4-0.6 mm Zirconium Beads
- Grinding chamber 85% v/v beads.

A lysed cell composition LC1 is obtained. LC1 corresponds to stream #4 in figure 1.

A batch of lysed cell composition (LC1) has been submitted to a liquid-solid separation to remove cell debris.

- 20 This separation has been performed using centrifuge Alfa-Laval DX203.

A lysed cell composition without cell debris LC2 is obtained. LC2 corresponds to stream #5 in figure 1.

In examples 8-14, the dewatered broth B1 has been submitted to a cell disruption by bead milling in a DYNO-MILL MULTI LAB®, in the following conditions :

- 25 - 3 Dyno®-accelerators
- Agitator tip speed at 10m/sec
- 0.8 mm Zirconium Beads

- Grinding chamber 65% v/v beads.

A lysed cell composition LC'1 is obtained.

The oil recovery yield (wt%) is calculated as the ratio between free oil (supernatant oil) weight (g) and total oil weight in the emulsion (g). The total oil weight in the emulsion is obtained by assuming that the oil accounts for all the dry matter of the emulsion, even when the emulsion contains debris. Real yields are therefore higher than calculated yields. The measured error on the yields values is about 5%.

In Examples 4-7, the oil recovery yield has been calculated from measured volume, considering an oil density of about 912g/L at 20°C.

In Examples 8-14, each of the samples has been centrifuged at 4500g (g being the earth gravity constant) at 25°C for 10 min using a Rotanta 460 RF Hettich centrifuge, before measuring the oil recovery yield.

From Examples 10 to 14, all the samples were stored at 4°C less than 2 days before each measurement.

15 **Example 3 : comparison of lysing using bead mill**

The dewatered broth B1 has been submitted to a cell disruption by bead milling in a DYNO-MILL MULTI LAB®, with two different milling tools, as shown in table 7. Milling tool of process#1 is an agitator bead mill whereas the milling tool of process #2 is an accelerator bead mill.

20 Table 7 : Operating conditions for cells milling

	Process #1	Process #2
Milling tool	4 Agitator discs	3 Dyno®-accelerators
Agitator tip speed	14 m/sec	10 mec/sec
Beads	Zirconium	Zirconium
Bead diameters	0.4-0.6 mm	0.8 mm
Grinding chamber	85%v/v beads	65% v/v beads

The lysed cells have been submitted to a liquid-solid separation using centrifuge Rotanta 460 RF Hettich at 4600 G for 10 min. Three different phases are observed from up to down: a cream, an aqueous phase and a debris phase. The oil titer of the different phases is measured, which makes it possible to quantify the oil distribution from the volume of each phase.

As shown in table 8 and 9, the lysis with process#2 allows recovering about 6 wt% more oil than the process #1. This extra-recovered oil comes from the debris phase. Whatever the process, the amount of oil in the aqueous phase remains the same.

Table 8 : Oil repartition after lysis with process #1

	Volume (mL)	Oil Titer (mg/mL)	Total Oil (mg)	Oil Fraction (wt%)
Sample	40	150	6000	100
Debris	27.5	37	1025	17
Aqueous phase	7.5	70	525	9
Cream	5	890	4450	74

5 Table 9 : Oil repartition after lysis with process #2

	Volume (mL)	Oil Titer (mg/mL)	Total Oil (mg)	Oil Fraction (wt%)
Sample	40	150	6000	100
Debris	20	37	660	11
Aqueous phase	10	52.5	525	9
Cream	10	480	4800	80

Example 4 –effect of pH on oil recovery

A batch of LC2 (without cell debris) has been separated in several samples for which the pH has been adjusted at different pH ranging from 4.5 to 11 by addition of appropriate amounts of NaOH. Each of the samples has been centrifuged at 4500g (g being the earth gravity constant) for 10 min or heated at 50 °C for at least 60 min and then centrifuged at 4500g, for 10min, using a Rotanta 460 RF Hettich centrifuge.

The characteristics of the tested LC2 emulsion are the following :

- Oil in water dispersion,
- Mean diameter of oil droplets : 3 microns (size range : 0.5 to 20 microns)
- 15 - Dry matter : 40wt% (wt% of solid, corresponds to the wt% of oil)
- Initial pH : 4.5± 0.3

The results are shown in the table 10. Very low oil recovery was observed for pH above 9. The higher oil yields are recovered at pH 8 and 9 after centrifugation. The temperature makes it possible to slightly increase the quantity of oil recovered and also to accelerate demulsification.

Table 10 : effects of pH on oil recovery yield (wt%)

pH	Centrifugation (wt%)	Heating 50°C + Centrifugation (wt%)
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4.5	0	0
5	0	0
5.5	2.3	2.3
6	4.6	6.8
7	4.6	6.8
8	22.8	27.4
9	11.4	13.7
10	2.3	2.3
11	2.3	2.3

Example 5—effect of pH and surfactants on oil recovery

A batch of LC2 (without cell debris) has been separated into several samples for which the pH has been set to 8 and 9 by addition of an appropriate amount of NaOH. LC2 has the same characteristics as in example 4.

- 5 Different surfactants have been added to LC2, each to obtain a 1wt% concentration of surfactant. The 3 following surfactants have been tested:

TA1: Sorbitan monoleate, a non-ionic surfactant commercialized under the name Span® 80. TA1 is an oil-soluble surfactant. It dissolves in organic solvents including ethanol, toluene and xylene. TA1 is dispersible in water.

- 10 TA2 is a polyether polyol (non-ionic surfactant) with a molecular weight of 2750g/mol, commercialized under the name TERGITOL™ L-62. TA2 has a large hydrophobic section surrounded by two hydrophilic sections. TA2 is soluble in water and soluble in organic solvents, including ethanol, toluene and xylene.

- 15 TA3: Alkyldiphenyloxide disulfonate, anionic surfactant, commercialized under the name of DOWFAX™-2A1. This compound comprises two sulfonated aromatic rings linked by an ether function. Two C6 to C16 hydrocarbon chains are branched on these aromatic rings. At least 20% of the chains are C16 chains. TA3 is a water-soluble surfactant. TA3 is highly soluble in strong acid and alkali solutions.

- 20 Each of the samples has been mixed for two hours using a magnetic bar and then centrifuged at 4500g for 10min or heated at 50°C for 60 to 120 min and centrifuged at 4500g, for 10min, using a Rotanta 460 RF Hettich centrifuge.

The addition of each of these surfactants at alkaline pH allows improving the yield of oil recovered (table 11). The best results are obtained with TA3 at pH8.

Table 11: effects of pH and surfactants on oil recovery yield (wt%)

TA1	TA2	TA3	TA1	TA2	TA3
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	Centrifugation (wt%)			Heating 50°C+ Centrifugation (wt%)		
pH 8	27.4	27.4	27.4	25.1	25.1	29.6
pH 9	13.7	13.7	13.7	16	16	16

Example 6 –effect of pH and salts on oil recovery

A batch of LC2 (without cell debris) has been separated into several samples for which the pH has been set to 8 and 9 by addition of an appropriate amount of NaOH. LC2 has the same characteristics as in example 4.

- 5 Different salts were added : NaCl, MgCl₂, CaCl₂, Na₂SO₄, (NH₄)SO₄.

Table 12 collects the results obtained for different salts with a pH set at 8 by addition of NaOH. The salt concentration is 0.01mol/L. Each of the samples has been mixed for two hours using a magnetic bar and then centrifuged at 4500g for 10 min, using a Rotanta 460 RF Hettich centrifuge.

- 10 Salts with divalent cations, CaCl₂ and MgCl₂, are the most efficient salts for oil recovery.

- 15 Table 12: effects of pH and salts on oil recovery

Salt	Salt concentration (M)	Oil yield (wt%)
NaCl	0.01	16.4
Na ₂ SO ₄	0.01	10.9
(NH ₄)SO ₄	0.01	10.9
MgCl ₂	0.01	29.2
CaCl ₂	0.01	65.7

Example 7–effect of pH, salts and surfactants on oil recovery

A batch of LC2 (without cell debris) has been separated into several samples for which the pH has been set to 8 or 9 by addition of an appropriate amount of NaOH. LC2 has the same characteristics as in example 4.

- 20 Different salts have been added to obtain a salt concentration of 0.01M. Additionally, surfactant TA3 has been added to obtain a concentration of 1wt%.

The oil has been recovered after mixing with a magnetic bar for 2 hours followed by centrifugation at 4500g, for 10min, optionally after heating at 50°C for at least 60 min. The oil yields recovered are shown in table 13.

Table 13: effects of pH, salts and surfactant TA3 on oil recovery yield (wt%)

	NaCl	CaCl ₂	(NH ₄) ₂ SO ₄	NaCl	CaCl ₂	(NH ₄) ₂ SO ₄
	Centrifugation (wt%)			Heating 50°C+ Centrifugation (wt%)		
Initial pH	/	4.6	/	/	6.8	/
pH 8	18.2	38.8	16	25.1	45.6	27.4
pH 9	6.8	27.4	9.1	16	43.3	16

Other tests have been done on a different batch of LC2 (without cell debris). Each of the samples has been centrifuged at 4500g (g being the earth gravity constant) for 10 min or heated at 50 °C for at least 60 min and then centrifuged at 4500g, for 10min. The oil yields recovered are shown in table 14.

The characteristics of the tested LC2 emulsion are the following:

- Oil in water dispersion,
- Mean diameter of oil droplets : 4 microns (size range : 0.5 to 20 microns)
- Dry matter : 31wt%(wt% of solid, corresponds to the wt% of oil)
- Initial pH : 4.2± 0.3

Table 14: effects of pH, CaCl₂ and surfactant TA3 on oil recovery yield (wt%)

	Centrifugation (wt%)	Heating 50°C + Centrifugation (wt%)
Initial pH	3.3	6.5
pH 8	52.1	65.1
pH 9	39.1	65.1

Example 8- effect of aging on oil recovery

After each lysis, LC'1 samples are kept cold at 4°C in order to be used later for other measurements.

A batch of LC'1 has been separated in several samples, 30mL of LC'1 in 50mL Falcon centrifugation tubes. These tubes have been submitted to a rotational stirring using a Stuart Rotator SB3 at three different rotation speeds 10; 20 and 40 rpm at room temperature (between 20 and 25°C).

An effect of sample storage time has been noticed on oil recovery yield after stirring. Table 15 and table 16 show the difference in oil recovery yields between samples stored less than 2 days and more than 4 days.

Table 15: effects of rotation speed on a freshly lysed sample, stored less than 2 days at 4 ° C.

Time (h)	10 rpm	20 rpm	40 rpm
6	9 wt%	35 wt%	45 wt%
12	45 wt%	67 wt%	66 wt%

Table 16: effects of rotation speed on a lysed sample, stored more than 4 days at 4 °C).

Time (h)	10 rpm	20 rpm	40 rpm
6	54 wt%	83 wt%	54 wt%
12	69 wt%	78 wt%	55 wt%

5 **Example 9 –effect of storage temperature on oil recovery**

The effect of storage temperature on oil recovery yield has been studied after spiral stirring.

A batch of LC'1 has been filled in a tank equipped with a spiral stirrer. The spiral stirrer (R 3003.1, IKA) has been used at 1 m/s. The ratio D/T of the diameter of the stirrer (D) to the width of the tank (T) is of 0.9. The ratio H/T of the height (H) of liquid to the width of the tank (T) is equal to 1.

The results are shown in table 17. Samples with aging times from 1 to 7 days (after lysing) at 4°C and 20°C have been demulsified by stirring at 20°C for 6 hours.

Table 17 : Effects of aging for lysates stored without agitation at 2 different temperatures on oil recovery yield (wt%) after a stirring at 20°C for 6 hours

Aging time (days)	+1	+2	+3	+4	+7
Stored at 4°C	0	0	60 ± 8	77 ± 10	80 ± 18
Stored at 20°C	0	53	81	82	95

Example 10 –effect of time, temperature and agitation on oil recovery

Samples of a batch of LC'1 freshly lysed have been filled in a tank. Some of the samples have been stored without agitation at different temperatures, other samples have been stored under agitation at the same temperatures. The results are collected in table 18.

Table 18 : Oil recovery yields (in wt%) as a function of time, agitation and temperature on freshly lysed samples.

	Static				Magnetic bar stirring			
Time (h)	20°C	50°C	60°C	80°C	20°C	50°C	60°C	80°C
1	0	0	10	17	0	10	33	38
2	0	0	17	16	0	19	32	52

3	0	0	22	17	0	23	56	84
4	0	8	32	18	0	82	90	95
5	0	10	27	17	0	95	95	95
12	0	65	67	/	0	95	95	/

Example 11 –effect of time and temperature on oil recovery - stirring by rotation

A batch of LC'1 has been separated in several samples, 30mL of LC'1 in 50mL Falcon centrifugation tubes. These tubes have been submitted to a rotational stirring using a Stuart Rotator SB3 at a rotation speed of 20 rpm. Different agitation durations at different temperatures have been monitored. The oil recovery yields are collected in table 19.

Table 19 : Oil recovery in wt%

Time (h)	20°C	50°C	60°C	80°C
1	0	48	72	66
2	0	68	81	70
3	0	78	81	77
4	13	81	93	81
5	35	85	95	80
12	75	/	/	/

Example 12 –effect of time and temperature on oil recovery - stirring with propeller

A batch of LC'1 has been filled in a tank comprising a 4-blades propeller (R 1342, IKA) with the following characteristics:

- T, width of the tank
- H, height of liquid
- D, diameter of the propeller
- C, height of the impeller from the bottom of the tank

In all experiments H is equal to T and the rotation speed of the propeller is 1.5 m/s.

The oil recovery yields are collected in table 20.

Table 20 : oil recovery yields (in wt%) at 1.5m/s – D/T=0.66 & C/T=0.5

Time (h)	20°C	30°C	50°C	60°C	80°C
1	0	0	6	72	75
2	0	0	51	78	81
3	0	0	74	84	83
4	0	0	95	93	/
5	0	50	95	91	/

Example 13 –effect of time and temperature on oil recovery - stirring with spiral stirrer

A batch of LC'1 has been filled in a tank equipped with a spiral stirrer which generates an axial flow. A spiral stirrer (R 3003.1, IKA) has been used.

The oil recovery yields are collected in table 21.

Table 21 : Oil recovery yield (wt%) at 1m/s (D/T=0.9 & H/T=1)

Time (h)	20°C	50°C	60°C	80°C
1	0	0	0	45
2	0	0	67	62
3	0	70	85	83
4	0	84	89	70
5	0	95	95	90

Example 14 –comparison with formulation

A batch of lysed cell composition (LC'1) freshly lysed (less than two days of storage) has been separated in several samples of 30mL in 50mL tubes submitted to the following conditions:

- 5 hours at 20 rpm using a Stuart Rotator SB3 at ambient temperature (20°C)
- 5 hours at 20 rpm using a Stuart Rotator SB3 at 50°C

The best results are obtained without pH control and addition of salt by heating the emulsion at a temperature of 50°C under agitation (see table 22).

Table 22 : Oil repartition (wt%)

	Debris	Aqueous phase	Cream	Free Oil
LC'1	16 ± 5 wt%	5 ± 2 wt%	80 ± 10 wt%	0 wt%
LC'1 + 5 hours at 20 rpm at 20°C	19 ± 4 wt%	8 ± 2 wt%	38 ± 4 wt%	35 ± 5 wt%
LC'1 + 5 hours at 20 rpm at 50°C	5 ± 3 wt%	4 ± 2 wt%	0 ± 0.5 wt%	89 ± 6 wt%

Example 15 –pilot test

A fermentation broth from a culture of oleaginous yeast *Yarrowia lipolytica* has been harvested and sent to a centrifuge (Alfa Laval model DX-203) to increase the cell concentration from an initial Dry Cell Weight of 84,2 (g/kg) out of the fermentor to 212,4 (g/kg) after concentration by centrifugation.

It should be noted that a tangential flow filtration unit could be used instead of a centrifuge for this first step of concentration.

The dry cell weight has been measured with a moisture analyzer (Metler Toledo model HB43): a sample is placed on a Glass Fiber 1µm Filters (Millipore APFD9050) and weighted. The filter is then placed on an Erlenmeyer vacuum flask equipped with a fritted glass funnel and rinsed 3 times with deionized water to eliminate any residual soluble salts or sugar. The filter is then placed in the moisture analyzer where an infrared lamp is drying the sample up to 130°C until weight of the filter is reaching an asymptote. The Dry Cell Weight concentration is then calculated from the difference with the initial sample weight.

A sample of the concentrated broth was analyzed to measure a cell lipid content of 65.5% w/w.

The analytical procedure used can be summarized as follows: The sample is first dried by lyophilization and then subjected to acid-catalyzed transesterification with a solution of HCl/Methanol. After the transesterification is completed, the lipid-soluble components of the reaction mixture are separated from the water-soluble components using a two-phase liquid extraction and subsequently analyzed with a capillary gas chromatograph (GC) equipped with a robotic injector and a flame ionization detector (FID). Quantification of the methyl-ester products is achieved with use of both an internal standard and various concentrations of an external standard mixture of fatty acid methyl esters (FAMES).

A quantity of 170,2 kg of concentrated broth, representing a total lipid quantity of 23.68 kg of lipid ($127.2 \times 0.2124 \times 0.655$), is then lysed using a bead mill (DYNO®-MILL Model KDL-Pilot) filled with zirconium beads 0.6 – 0.8mm in diameter and fed with a flowrate of 0.8 L/min of concentrated broth.

The lysed material has then been placed in a mixing tank equipped with a mixer (Feldmeier Farmamixer with 16" diameter impeller) and a double jacketed for temperature control. The sample was let overnight under moderate mixing (90 RPM) at 60°C for a 14 Hr.

After this "maturation" period, the lysed material was diluted with 50L of deionized water and sent to a centrifuge Alfa Laval model DX-203 equipped with 3 nozzles of 0.5 mm fed with a gear pump (max feed rate of 4.5 L/min). The feed rate of the pump is first manually adjusted so that only oil is getting out of the light phase outlet (during

this phase all the outlets are recirculated to the feed tank). The oil phase is then collected and the heavy phase (containing water, cell debris and some left over lipids) is recirculated to the feed tank until no oil is coming out of the light phase outlet.

- 5 A total of 19.93 kg of oil has been collected, representing an overall recovery yield of 84.2 % ($= 19.93 / 23.68$).

CLAIMS

1 Process for recovering lipids produced by fermentation of microbial cells from a fermentation medium, said lipids comprising mainly one or several fatty acids chosen from C18 and C19 fatty acids, said process comprising:

5 (a) providing a fermentation medium containing microbial cells capable of producing said lipids by fermentation,

(b) optionally lysing the microbial cells contained in the fermentation medium to release intracellular lipids into the fermentation medium, resulting in a lysed fermentation medium containing lysed cells, wherein lysing is performed by
10 mechanically treating the fermentation medium, and optionally submitting the lysed fermentation medium to :

(i) a solid/liquid separation into a solid/liquid light phase containing lipids and a solid/liquid heavy phase containing the lysed cells,

(c) demulsifying the fermentation medium of step (a), the lysed fermentation medium
15 of step (b) or the solid/liquid light phase containing lipids of step (b)(i), wherein said fermentation medium of step (a) or solid/liquid light phase containing lipids of step (b)(i) is heated at a temperature from 30 to 80°C under agitation for at least 2 hours, thereby generating a stream having a first phase containing mainly the lipids and a second phase containing water, and

20 (d) separating the first phase containing the lipids from the stream obtained in step (c),

and wherein said process is performed without addition of chemical compound(s) at any stage of the process from step (b) to step (d) and step (a) does not require addition of chemical compound(s) other than those necessary for the fermentation
25 of the medium.

2 Process according to claim 1, wherein during the demulsifying step, said fermentation medium of step (a), lysed fermentation medium of step (b) or solid/liquid light phase containing lipids of step (b)(i) is heated at a temperature from 40 to 60°C.

30 3. Process according to any one of claims 1 or 2, wherein during the demulsifying step, said fermentation medium of step (a), lysed fermentation medium

of step (b) or solid/liquid light phase containing lipids of step (b)(i) is heated under agitation for at least 3 hours.

4. Process according to any one of claims 1 to 3, wherein prior to the demulsification step, said lysed fermentation medium of step (b) or solid/liquid light phase containing lipids of step (b)(i) is submitted to an aging step without addition of chemical compound(s).

5. Process according to any one of claims 1 to 4, wherein, prior to the demulsification step (c), the fermentation medium undergoes a solid/liquid separation without addition of chemical compound(s) into a solid/liquid light phase containing the lipids and a solid/liquid heavy phase containing the microbial cells and the solid/liquid light phase is further submitted to the demulsification step (c) without lysing step (b).

6. Process according to any one of claims 1 to 5 wherein the lysed fermentation medium has been submitted to the solid/liquid separation step (b)(i) or, wherein the fermentation medium has been submitted to the solid/liquid separation step prior to the demulsification step (c) according to claim 5, and wherein in the separation step (d), the first phase containing the lipids is separated without addition of chemical compound(s) into a liquid/liquid light phase containing the lipids and a liquid/liquid heavy phase by a liquid/liquid separation.

7. Process according to any one of claims 1 to 4, wherein, no solid/liquid separation is performed on the fermentation medium or on the lysed fermentation medium prior to the demulsification step (c) and wherein in the separation step (d), the first phase containing the lipids is separated without addition of chemical compound(s) into a solid/liquid light phase containing the lipids and a solid/liquid heavy phase by a solid/liquid separation, and the solid/liquid light phase is separated without addition of chemical compound(s) into a liquid/liquid light phase containing the lipids and a liquid/liquid heavy phase by a liquid/liquid separation.

8. Process according to any one of claims 1 to 7, wherein the fermentation medium provided at step (a) is submitted to a dewatering step without addition of chemical compound(s), prior the lysing step.

9. Process according to claim 8, wherein the dewatering step is a solid/liquid separation step selected from centrifugation, filtration, and decantation.

10. Process according to claim 9, wherein the filtration is a cross-flow filtration performed using at least one tubular inorganic membrane having a pore size of 0.5µm at most.

11. Process according to any one of claims 1 to 10, wherein the lysing step is performed by milling, in particular using a bead or ball mill or an accelerator bead or ball mill, or using a homogenizer.

12. Process according to any one of claims 1 to 11, wherein the stream obtained in step (c) is heated at a temperature from 30 to 90°C prior to the separation step (d).

13. Process according to any one of claims 1 to 12, wherein the proportion of C18 and/or C19 fatty acids is at least 75wt% as a weight percentage of total fatty acids produced by the microbial cell.

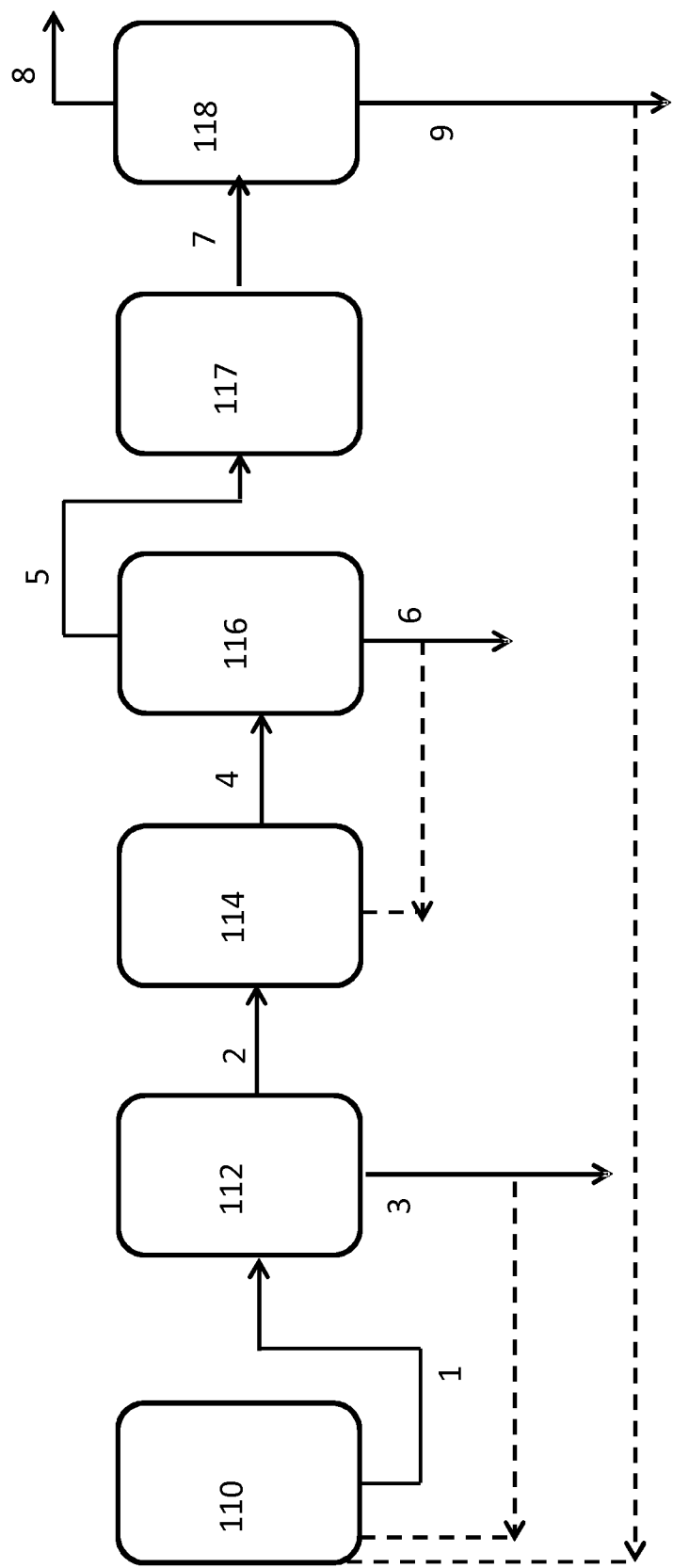


FIGURE 1

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2019/069383

A. CLASSIFICATION OF SUBJECT MATTER
 INV. C12P7/64 C11B3/00
 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)

C12P C11C C11B

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

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

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Further documents are listed in the continuation of Box C.



See patent family annex.

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

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