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(71) Applicant *(for all designated States except US)*: **DSM N.V.** [NL/NL]; Het Overloon 1, NL-6411 TE Heerlen (NL).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **HENDRIK, Louis** [NL/NL]; Insulindestraat 72, NL-3131 ZD Vlaardingen (NL). **SCHAAP, Albert** [NL/NL]; Smitshoek 13, NL-2993 BG Barendrecht (NL).

(74) Agent: **WRIGHT, Simon, Mark**; J.A. Kemp & Co., 14 South Square, Gray's Inn, London, WC1R 5JJ (GB).

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(54) Title: ISOLATION OF MICROBIAL OILS

(57) Abstract: The extraction of a microbial or single cell oil, for example comprising one or more polyunsaturated fatty acids (PUFAs), directly from microbial cells is disclosed which avoids the need for solvents. After fermentation, the microbial cells are pasteurised, washed and the cell walls lysed or disrupted by a mechanical (e.g. homogenisation), physical (boiling or drying), chemical (solvents) or enzymatic (cell wall degrading enzymes) technique. The oil (containing the PUFA) is then separated from the resulting cell wall debris. This is achieved by centrifugation, which results is an oily phase (top layer) that contains the oil which that can be separated from an aqueous phase (containing the cell wall debris). The oil can then be extracted and if necessary the PUFA can be purified or isolated from the oil.

ISOLATION OF MICROBIAL OILS

The present invention relates to the extraction (and then isolation) of a microbial (or single cell) oil, preferably comprising one or more polyunsaturated fatty acids (PUFAs), from single cell (or micro-) organisms. The process of the invention involves the disruption or lysis of microbial cell walls, followed by separating the oil from the resulting cell debris. The invention additionally relates to a microbial oil recovered by this process, preferably having a PUFA.

Polyunsaturated fatty acids, or PUFAs, are found naturally and a wide variety of different PUFAs are produced by different single cell organisms (algae, fungi, etc). They have many uses, for example inclusion into foodstuffs (such as infant formula), nutritional supplements and pharmaceuticals.

In most microbial PUFA production processes a microorganism is first cultured in a fermenter in a suitable medium. The microbial biomass is then harvested and treated to enable subsequent extraction of a lipid from the biomass with a suitable solvent. The lipid is usually subjected to several refining steps. Care must be taken during the process because degradation can occur if the lipids are subjected to lipolysis or oxidising conditions, for example heating (in the presence of oxygen) and/or due to lipases or lipoxygenases. The art teaches that to avoid oxidation (such as resulting from breaking open the cells and so exposing the contents to oxygen) PUFAs can be extracted from whole intact cells using a solvent (see WO-A-97/36996 and WO-A-97/37032). The use of solvents is a common way of removing lipids from microbial biomass (WO-A-98/50574).

Although these extraction processes have been used for several years, the solvent needs to be removed and this results in extra cost. In addition, if the lipid is to be used in a foodstuff, it is important that certain solvents, such as hexane, are removed completely, or only remain in very small quantities. If the hexane is removed by evaporation then this may involve heating, and that not only adds to costs but can cause lipid degradation. Furthermore, with increasing environmental

considerations, the use of solvents for the extraction of lipids is becoming increasingly expensive and unpopular.

The present invention therefore seeks to solve or at least mitigate these problems. The applicant has found that lipids, such as those comprising a PUFA, can be efficiently extracted from microbial cells without the need for solvent(s).

Therefore, according to a first aspect of the present invention there is provided a process for obtaining an oil from microbial cells, the oil comprising one or more polyunsaturated fatty acids (PUFAs), the process comprising (a) disrupting the cell walls of the microbial cells to release the oil; and (b) separating, by centrifugation, the oil from at least part of the cell wall debris formed in (a). One can then recover, purify and/or isolate the (microbial) oil (or one or more PUFAs). A good yield of the oil can be achieved using this process without the need for a solvent Preferably this process (including stages (a) and (b)) is solvent-free.

According to a second aspect of the present invention there is provided a microbial or single cell oil comprising a PUFA, or a PUFA, obtained by a process as defined above. Preferably the oil will comprise a PUFA, namely one or more PUFAs.

Recent PUFA preparation processes advocate keeping the microbial cells intact (WO-A-97/36996). This publication describes a PUFA production process where a microbial biomass is generated by fermenting a microorganism, and following fermentation the cells are heated. Water is removed from the biomass, and the resulting material extruded to form porous granules. The PUFA is then extracted from the intact cells inside the granules by contact with a solvent, usually hexane.

The hexane is then evaporated to produce a crude oil. Throughout this process the cells are kept intact to prevent oxygen in the atmosphere contacting the PUFAs as it was thought that this would cause undesirable oxidation. However, it has now been found that a good quality PUFA oil can be achieved if the cells are in fact lysed: any potential oxidation by the atmosphere can be more than compensated by the advantage of avoiding the need for solvents.

PUFAs and microorganisms

The PUFA can either be a single PUFA or two or more different PUFAs.

The or each PUFA can be of the n-3 or n-6 family. Preferably it is a C18, C20 or C22 PUFA or a PUFA with at least 18 carbon atoms and 3 double bonds.

The PUFA(s) can be provided in the form of a free fatty acid, a salt, as a fatty acid ester (e.g. methyl or ethyl ester), as a phospholipid and/or in the form of a mono-, di- or triglyceride.

Suitable (n-3 and n-6) PUFAs include:

docosahexaenoic acid (DHA, 22:6 Ω3), suitably from algae or fungi, such as the (dinoflagellate) *Crypthecodinium* or the (fungus) *Thraustochytrium*;

γ-linolenic acid (GLA, 18:3 Ω6);

α-linolenic acid (ALA, 18:3 Ω3);

conjugated linoleic acid (octadecadienoic acid ,CLA);

dihomo-γ-linolenic acid (DGLA, 20:3 Ω6);

arachidonic acid (ARA, 20:4 Ω6); and

eicosapentaenoic acid (EPA, 20:5 Ω3).

Preferred PUFAs include arachidonic acid (ARA), docosohexaenoic acid (DHA), eicosapentaenoic acid (EPA) and/or γ-linoleic acid (GLA). In particular, ARA is preferred.

The PUFAs may be from a natural (e.g. vegetable or marine) source or may be derived from a single cell or microbial source. Thus the PUFA may be of (or from) microbial, algal or plant origin (or source). In particular, the PUFA may be produced by a bacteria, fungus or yeast. Fungi are preferred, preferably of the order *Mucorales*, for example *Mortierella, Phycomyces, Blakeslea, Aspergillus, Thraustochytrium, Pythium* or *Entomophthora.* The preferred source of ARA is from *Mortierella alpina, Blakeslea trispora, Aspergillus terreus* or *Pythium insidiosum.* Algae can be dinoflagellate and/or include *Porphyridium, Nitszchia,* or *Crypthecodinium* (e.g. *Crypthecodinium cohnii).* Yeasts include those of the genus *Pichia* or *Saccharomyces,* such as *Pichia ciferii.* Bacteria can be of the genus *Propionibacterium.*

In the process of the invention the microbial cells (or microorganisms) can first be suitably cultured or fermented, such as in a fermenter vessel containing an (e.g. aqueous) culture medium. The fermentation conditions may be optimised for a high oil and/or PUFA content in the resulting biomass. If desirable, and for example after fermentation is finished, the microorganisms may be killed and/or pasteurised.

This may be to inactivate any undesirable enzymes, for example enzymes that might degrade the oil or reduce the yield of the PUFAs.

The fermentation broth (biomass and culture medium) may then be removed (e.g. let out) from the fermenter, and may be passed to cell-wall disrupting equipment (e.g. a homogeniser). If necessary liquid (usually water) can (firstly) be removed therefrom. Any suitable solid liquid separation technique can be used. This (dewatering) may be by centrifugation and/or filtration. The cells may be washed, for example using an aqueous solution (such as water) for example to remove any extracellular water-soluble or water-dispersible compounds. The cells may then be ready for disruption or lysis.

Cell lysis (stage (a))

The cell walls of the microbial cells can then be disrupted (or lysed). This can be achieved using one or more enzymatic, physical or mechanical methods or techniques, for example at high shear conditions. Physical techniques include heating and/or drying the cells to a sufficient temperature whereby the cell walls are ruptured. This may comprise boiling.

Enzymatic methods include lysis by one or more enzymes, e.g. cell wall degrading enzymes. The cell wall degrading enzyme may be a lytic enzyme. Other enzymes include (e.g. alkaline) proteases, cellulases, hemicellulases, chitinases and/or pectinases. Other cell wall degrading substances may be used instead of or in combination with one or more enzymes, e.g. salts, alkali, and/or one or more surfactants or detergents. A combination of physical, mechanical and/or enzymatic methods is also contemplated.

If a mechanical technique is employed this may comprise homogenisation, for example using a homogeniser. This may be a ball mill or any other machine able to disrupt the cell walls. Suitable homogenizers include high pressure homogenizers (for example at a pressure of 300 to 500kg/cm^2 or bar) such as a polytron homogenizer. Other homogenization techniques may involve mixing with particles, e.g. sand and/or glass beads (e.g. use of a bead mill). Alternative mechanical techniques include the use of milling apparatus, for example homoblenders. Other methods of disrupting the cell walls include ultrasound, spray drying and/or pressing

or appliance of high pressure. This last technique is called cold-pressing: it may be performed at pressures of 100 to 600 or 700 bar (Atm or kg/cm^2), such as 150-500 bar, optimally from 200-400 bar.

Homogenization is the preferred method of disrupting the cell walls. There may be from 1 to 3 passes through the homogeniser, either at high and/or low during disruption (e.g. homogenisation) pressures. For example one may use a Gaulin™ homogenizer. The pressure during disruption (e.g. homogenisation) may be from 300 to 900, such as 400 to 800, and optimally 500 to 600 or 700 bar (Atm or kg/m^2). Lower pressures may be employed if required, e.g. from 150 to 300 bar. Hence working pressures can vary from 150 to 900 bar depending on the type of homogeniser, number of passes, etc.

Although cell lysis can be performed chemically this is preferably not employed as (this stage in) the process is desireably solvent-free.

The disruption of the cell walls may be performed either on the broth resulting from fermentation, for example the cells may still be contained in culture medium or such medium may be present. One or more additives my be added or present (such as an alkali metal salt, e.g. NaCl) during disruption or may be added after disruption (e.g. to a homogenised broth). During disruption an organic solvent (e.g. MeOH, chloroform) is preferably not present. The disruption may be performed on the (optionally washed and/or concentrated) biomass (e.g following solid liquid separation). Disruption is therefore performed on an (e.g. aqueous) composition comprising the cells and water but not containing a solvent.

In order to improve cell wall disruption, disruption may be performed at a dry matter content of about 10 to 200g/l. This may be on the fermentation broth, for example after fermentation, or it may be derived from the broth, for example after the broth has been subjected to de-watering and/or solid/liquid separation.

If necessary a separation inducer, to encourage separation of the oil from the debris, may be added at this stage, such as to the homogenised material.

Separation of oil from cell debris (stage (b))

The microbial oil is then separated from at least part of the cell wall debris formed. At this stage there may be in an oily or lipid phase or layer (and this may comprise the PUFA). This may be a top or upper layer. This layer can be above a (lower) aqueous layer, e.g. containing cell wall debris. The oily layer (comprising the PUFA) can then be separated from the aqueous layer (or phase). One or more surfactants or detergents may be present or added to assist this process.

The separation of the oil from at least some of the cell wall debris is preferably achieved or assisted by using a mechanical method, in particular by centrifugation. Suitable centrifuges can be obtained from Westfalia™ (semi- and industrial scale) or Beckman™ (e.g. laboratory centrifuges). Centrifugation (e.g. for a laboratory scale centrifuge) may last for from 2 or 4 to 8 or 15, such as from 3 or 5 to 7 or 12, optimally from 4 or 5 to 6 or 10 minutes (residence time).

The centrifugal force (g) may be from 1,000 or 2,000 to 10,000 or 25,000, such as from 3,000 or 5,000 to 8,000 or 20,000, optimally from 4,000 to 6,000g, or from 7,000 to 9,000g, although centrifugation can be employed at g-forces up to 12,000g, 15,000g, 20,000g or 25,000g. Centrifugation may be at 4,000 to 14,000 rpm such as 6,000 to 12,000rpm, optimally at from 8,000 to 10,000rpm. One or more centrifugations may be necessary. The maximum g force may be lower if using certain centrifuges, for example this may be 6000g if using a Westfalia™ centrifuge (e.g. model NA-7). The flow rate may be from 100-500 litres/hour, such as 150 to 450 l/hr, optimally from 200 to 400 l/hr. Centrifugation may result in either a 2-phase system (a fatty or oily top layer and a lower aqueous layer) or a 3-phase system (a fatty or oily top layer, a middle aqueous layer and a bottom layer, usually containing the cell debris).

A separation inducer, or agent that aids separation, may be added. This may be present or supplemented during (a), after (a) but before (b), or during (b). This may aid the formation of separate oil and aqueous phases. The inducer may increase the density of the aqueous phase, which may then become even more dense than the oily phase. Suitable inducers include alkali metal salts, e.g. NaCl. The inducer may

be added at a concentration of 10-150g/l, such as 30-130 g/l, optimally from 50-100g/l.

The oil may be free of any carotenoids, e.g. β-carotene. Following disruption and separation the process of the invention may further comprise extracting, purifying or isolating the oil or one more PUFAs.

Solvent avoidance

One advantage of the process of the invention is that one can avoid the need for a solvent. (In this context solvent excludes water, since the culture medium is usually aqueous and the cells may be washed with water). Thus, no (e.g. organic) solvent(s) may be employed either during disruption of the cell walls in (a), or in the separation of the PUFA from at least part of the cell wall debris, in (b). Preferably, no (e.g. organic) solvent is used either in the extraction, purification or isolation of the oil or one or more PUFAs. Thus, in essence, the process can be solvent-free. Thus stages (a), (b) and optionally also (c) can be performed without an (e.g. organic) solvent, for example without the need of a solvent for the oil (or PUFA), e.g. an alkane such as hexane, an alcohol (e.g. methanol) or a haloalkane (e.g. chloroform).

Preferably, the use of a surfactant can also be avoided, and each or both of the disruption and separation stages (a) and (b) can also be performed without the need of a surfactant, for example in the absence of any detergents.

A second aspect of the invention relates to an oil preparable (or prepared) by a process of the first aspect.

If the oil comprises a PUFA, then the PUFA is preferably predominantly (such as greater than 50%, 70% or even 90% or 95%) in the form of triglycerides.

The oil may have one or more of the following characteristics (or components):

(a) sterols, e.g. desmosterol, or cell debris, such as from 0.01 to 1.0%, e.g. 0.05 to 0.5%, preferably from 0.1 to 0.2%;

(b) phospholipids or triglycerides, such as from 0.1 to 2.0%, e.g. from 0.3 to 1.5%, preferably from 0.5 to 1.0%; and/or

(c) diglycerides at no more than 0.1, 0.05 or 0.001%.

The oil may be refined and/or treated with an acid and/or alkali if required.

The PUFA (or oil containing a PUFA) may be subjected to further downstream processing, for example degumming, neutralisation, bleaching, deodorization, or winterization.

Overall protocol

A preferred process of the present invention therefore comprises:

(a) culturing microbial cells, for example under conditions whereby they produce a microbial oil or at least one PUFA;

(b) optionally heating or pasteurising the cells, for example to kill the cells and/or to inactivate any undesirable enzymes;

(c) optionally removing an (aqueous) liquid (such as dewatering), for example by centrifugation, filtration or a suitable solid-liquid separation technique;

(d) optionally, washing the microbial cells, for example with water, preferably to remove extracellular water-soluble or water-dispersible compounds;

(e) disrupting or lysing the cell walls of the microbial cells, for example by a physical, enzymatic or mechanical technique (such as homogenisation, e.g. with an homogeniser or a ball mill). This can release some of the oil and/or PUFA present in the microbial cells. The (mechanical) disruption may be supplemented with or substituted by chemical and/or enzymatic disruption. A separation inducer (for example to aid formation of two layers, in the next stage, may be added);

(f) separation of the microbial oil (or PUFA) from the cell wall debris, for example formation and then separation of an oil phase from the resultant cell wall debris and/or aqueous phase. This may comprise centrifugation, optionally with the addition of one or more salts, a pH shift (towards alkaline), and may involve the presence of one or more cell degrading enzymes, surfactants or emulsifiers. One can obtain an (e.g. upper) oil phase and an (e.g. lower) aqueous phase. The oil phase may contain the PUFA. The aqueous phase may contain cell debris;

(g) extraction, purification or isolation of the oil (or of the PUFA from the oil phase), for example resulting in a PUFA-containing oil; and

(h) optionally acid treatment (or degumming), alkali treatment (or neutralisation), bleaching, deodorising, cooling (or winterisation). This may remove undesirable substances such as free fatty acids (FFAs), proteins, phospholipids, trace metals, pigments, carbohydrates, soaps, oxidation products, sulphur, pigment decomposition products, sterols, saturated triglycerides and/or mono- or di-glycerides.

The heat treatment or pasteurization preferably inactivates or denatures one or more oil (or PUFA) degrading enzymes. The temperature of heating may be from 70 to 90°C, such as about 80°C. It may inactivate or denature enzymes such as lipases and/or lipoxygenases.

One may add one or more (e.g. water and/or oil-soluble) antioxidants, for example vitamin C, ascorbyl palmitate and/or tocopherol, and this may be done after stage (b), or at a later stage for example after extraction, such as before or after any refining (step (h) above).

There may be one or more additional heating steps, for example to remove other undesirable compounds or components. For example, heating may take place at an acid pH, for example to remove components such as phospholipids, trace metals, pigments, carbohydrates and/or proteins. Here the temperature may be from 50 to 80°C, such as 55 to 75°C, optimally from 60 to 70°C. The pH may be from 1 to 6, such as 2 to 5, optimally at a pH from 3 to 4. This can result in degumming and/or removal of proteins and/or water-soluble or water-dispersible compounds.

Alternatively or in addition a further heating step, this time at alkaline pH, may employed. The pH may be from 8 to 13, such as from 9 to 12, optimally at a pH of from 10 to 11. The temperature may be the same as that described in the previous paragraph.

Equipment (industrial process plant)

A third aspect of the invention relates to apparatus for conducting the process of the first aspect. The third aspect may thus comprise:

(a) means for culturing (or fermenting) microbial cells (e.g. a fermenter), optionally (e.g. directly) linked to;

(b) means for disrupting (or lysing) cell walls of the microbial cells (e.g. a homogeniser), optionally linked to;

(c) means for separating a (resulting) oil from (resulting) cell debris

The cells and culture medium (e.g. broth) may be passed directly to the means in (b). Each of the means can be positioned in the order specified, so following the order of the stages of the process of the first aspect. Means for performing any or all of the disruption and separation steps as described earlier may be provided, for example means to add a separation inducer (e.g. to homogenised material), or for performing any of the steps described in the overall protocol (e.g. heating/pasteurising means, solid-liquid separation means, etc).

Features or characteristics of one aspect of the invention are applicable to another aspect *mutatis mutandis*.

The invention will now be described, by way of example, with reference to the following Examples which are provided by way of illustration only.

Example 1: Preparation of crude PUFA (ARA) oil from a fermentation broth of *Mortierella alpina*.

A fermentation broth of *Mortierella alpina* (previously pasteurized at 65°C for one hour) containing arachidonic acid (ARA) was homogenized once by means of an MC-4 APV Gaulin™ homogenizer at 600 bar (600 Atm) to disrupt the cell walls. NaCl was added to the homogenized broth to a final concentration of 100g/l. Subsequently the homogenized broth was centrifuged by means of a Sorval RC 5B centrifuge for 10 minutes at 9,000rpm (equivalent to about 20,000g) resulting in an arachidonic acid-enriched oily top layer and a lower aqueous layer containing the cell debris. Crude PUFA oil was recovered.

The yield of oil was 9% (based on the oil in the cell). The (oil) layer had the following approximate composition: 0.1% desmosterols; 0.7% phospholipids; 6.7% triglycerides; 0.1% diglycerides, 70% water and 20% medium components and cell debris.

Example 2: Preparation of crude PUFA (ARA) oil from a fermentation broth of *Mortierella alpina.*

A fermentation broth of *Mortierella alpina* (previously pasteurized at 65°C for 1 hour) containing arachidonic acid (ARA) was homogenized once by means of an MC-4 APV Gaulin™ homogenizer at 600 bar (600 Atm) to disrupt the cell walls. Subsequently the homogenized broth was centrifuged by means of a Westfalia™ NA-7 disc centrifuge at maximum speed (about 8,000 rpm, equivalent to about 8,000g at the disc stack) resulting in an arachidonic acid-enriched oily top layer (that was recovered from the centrifuge) and a lower aqueous layer containing the cell debris. A crude PUFA oil was recovered: the yield of oil was 95% (based on the oil in the cell). The crude oil had the following approximate composition: 1 to 2% sterols and cell debris; 3 to 4% phospholipids; 4% monoglycerides; 6% diglycerides; and the remainder being triglycerides.

Example 3: Preparation of crude PUFA (DHA) oil from a fermentation broth of *Crypthecodinium cohnii*

Following fermentation 20 litres of fermentation broth (pasteurised at 65°C for one hour) of the algae *Crypthecodinium cohnii* was homogenized three times by means of an APV Gaulin™ homogenizer (type: Lab 60/60-10 TB SX), each time at 600 bar (Atm), to lyse the algal cell walls. Subsequently NaCl was added to the homogenized broth to a final concentration of 50g/l. Oil was recovered using a labscale centrifuge (Beckman ™ JM/6E) by centrifuging the broth in 800ml portion s each for 5 mintues at 5,000g. This resulted in a DHA-enriched fatty top layer (crude oil) and a lower aqueous layer. Crude oil was recovered from the fatty top layer.

Example 4: Preparation of crude PUFA (DHA) oil from a fermentation broth of *Crypthecodinium cohnii*

Following fermentation 20 litres of a fermentation broth (pasteurised at 65°C for 1 hour) of the algae *Crypthecodinium cohnii* was homogenized three times by means of an of APV Gaulin™ homogenizer (type: Lab 60/60-10 TB SX), each time at 600 bar (600 Atm), to lyse the algal cell walls. Subsequently a crude oil was

recovered using a labscale centrifuge (Beckman™ JM/6E) by centrifuging the broth in 800ml portions each for 5 minutes at 5000g. This resulted in a DHA-enriched fatty top layer (crude oil) and a lower aqueous layer. A crude PUFA oil was recovered from the fatty top layer.

CLAIMS

1. A process for obtaining an oil from microbial cells, the oil comprising one or more polyunsaturated fatty acids (PUFAs), the process comprising:

(a) disrupting the cell walls of the microbial cells to release the oil; and

(b) separating, by centrifugation, the oil from at least part of the cell wall debris formed in (a).

2. A process according to claim 1 wherein the cells are physically, enzymatically or mechanically disrupted.

3. A process according to claim 2 wherein the disrupting comprises homogenisation.

4. A process according to claim 3, wherein the disrupting comprises homogenisation at a pressure from 150 to 900 bar.

5. A process according to any preceding claim which further comprises:

(c) extracting, purifying or isolating the microbial oil or one or more PUFAs.

6. A process according to any preceding claim wherein the separation results in the formation of an oily layer and an aqueous layer.

7. A process according to claim 6 wherein the oily layer is an upper layer above the aqueous layer.

8. A process according to any preceding claim wherein the oil comprises a PUFA which is a C_{18}, C_{20} or C_{22} Ω-3 or Ω-6 PUFA (optionally ARA, EPA, DHA and/or GLA).

9. A process according to any preceding claim wherein the microbial cells are yeast, bacterial, fungal or algal cells.

10. A process according to any preceding claim wherein the source of the PUFA is from *Mortierella alpina. Phycomyces, Blakeslea, Aspergillus, Phythium, Entomophthora, Porphyridium, Nitszchia, Pichia, Saccharomyces or Propionibacterium.*

11. A process according to any preceding claim wherein stages (a) and/or (b) are free of any organic solvent(s).

12. A process according to any of claims 1 to 7 wherein no solvent (such as for the oil or PUFA) is employed in stages (a) and (b), and optionally also in (c).

13. A process according to any preceding claim which comprises, before (a), culturing or fermenting microbial cells under conditions that allow production of the oil, and if necessary pasteurising and/or heating the cells.

14. A process according to any preceding claim, wherein the process comprises, before (a),pasteurizing and/or heating the cells.

15. A process according to any preceding claims wherein the disruption of the cell walls is assisted by one or more cell wall degrading enzymes or surfactants.

16. A process according to any preceding claim wherein a separation inducer is present during (b).

17. A process according to any preceding claim wherein one or more surfactants are present during stage (b)

18. A microbial or single cell oil comprising a PUFA, or a PUFA, obtained by a process according to any preceding claim.