(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
09 November 2023 (09.11.2023)

WIPO | PCT

(10) International Publication Number
WO 2023/214072 A2

(51) International Patent Classification:
Not classified

(21) International Application Number:
PCT/EP2023/062052

(22) International Filing Date:
05 May 2023 (05.05.2023)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
63/338,809 05 May 2022 (05.05.2022) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CV, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, MG, MK, MN, MU, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, CV, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SC, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*
— *of inventorship (Rule 4.17(iv))*

Published:
— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

(54) Title: NOVEL PROCESS

(57) Abstract: The present invention is related to fermentative production of isoprenoids, lipids, and sterols, particularly various forms of vitamin E and vitamin D3, as well as various polyunsaturated fatty acids (PUFAs), including derivatives or intermediates, comprising cultivation of a suitable host cell, such as fungal host cell, particularly oleaginous host cell, in an improved two-phase culture system.

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Novel process

The present invention is related to fermentative production of isoprenoids, lipids, and sterols, particularly various forms of vitamin E and vitamin D3, as well as various polyunsaturated fatty acids (PUFAs), including derivatives or intermediates, comprising cultivation of a suitable host cell, such as fungal host cell, particularly oleaginous host cell, in an improved two-phase culture system.

Vitamin D3 (also known as cholecalciferol or calciol) can be synthesized in the skin of mammals from provitamin D3 (also known as 7-dehydrocholesterol or 7-DHC) which is product of cholesterol biosynthesis upon exposure to UV light, whereby 7-DHC is photochemically converted into provitamin D3, which isomerizes at body temperature to the biologically active form vitamin D3. In the liver, vitamin D3 is converted to the biologically inactive 25-hydroxyvitamin D3 (also known as calcidiol, calcifediol, 25-hydroxycholecalciferol, 25-OH-D3 or HyD), which is the major circulating form of vitamin D3. Further hydroxylation occurs in the kidney.

For industrial production of vitamin D3, both chemical and biotechnological synthesis is (in principle) available. Chemical synthesis starts with cholesterol isolated from e.g. wool fat which is dehydrogenated into 7-DHC, followed by UV-light exposure and further purification/extraction steps leading to vitamin D3. Alternatively, modified yeast strains can be used for producing vitamin D3 precursors, which can be isolated and further converted into vitamin D3. Excessive amounts of sterols such as the vitamin D3 precursors, including 7-DHC, are stored in intracellular organelles (so-called lipid droplets) from which they can be further isolated. The equilibrium between free sterols including the vitamin D3 precursors and those stored in the lipid droplets (in the form of sterol or steryl esters) is triggered via the action of several proteins, in particular enzymes, including sterol acyltransferases.

Due to the unspecific action of said sterol acyltransferase enzymes, the steryl ester pool which is stored within the lipid bodies is relatively diverse, including but not limited to e.g. esters of ergosterol, zymosterol, lanosterol, lathosterol, cholesta-5,7,24(25)-trienol, cholesta-8-enol, or 7-DHC. Only 7-DHC can be further processed into vitamin D3.

Vitamin E, an isoprenoid containing a hydrophobic hydrocarbon tail and a polar aromatic head, is mainly used as antioxidant or in treatment of cancer. Vitamin E forms include tocotrienol and tocopherol, each form comprising 4 isoforms named alpha, beta, gamma, and delta. Since the chemical synthesis of vitamin E is a non-environmental-friendly multi-step process, efforts have been made towards fermentative production of vitamin E forms, such as e.g. delta-tocotrienol (Sun et al., J. Agric. Food Chem. 2020, 68, 7710-7717).

Fatty acids are classified based on the length and saturation characteristics of the carbon chain. Fatty acids are termed short chain, medium chain, or long chain fatty acids based on the number of carbons present in the chain, are termed saturated fatty acids when no double bonds are present between the carbon atoms, and are termed unsaturated fatty acids when double bonds are present. Unsaturated long chain fatty acids are monounsaturated when only one double bond is present and are polyunsaturated when more than one double bond is present.

Polyunsaturated fatty acids (PUFAs) are classified based on the position of the first double bond from the methyl end of the fatty acid: omega-3 (n-3) fatty acids contain a first double bond at the third carbon, while omega-6 (n-6) fatty acids contain a first double bond at the sixth carbon. For example, docosahexaenoic acid ("DHA") is an omega-3 long chain polyunsaturated fatty acid (LC-PUFA) with a chain length of 22 carbons and 6 double bonds, often designated as "22:6 n-3." Other omega-3 LC-PUFAs include eicosapentaenoic acid ("EPA"), designated as "20:5 n-3," and omega-3 docosapentaenoic acid ("DPA n-3"), designated as "22:5 n-3." DHA and EPA have been termed "essential" fatty acids. Omega-6 LC-PUFAs include arachidonic acid ("ARA"), designated as "20:4 n-6," and omega-6 docosapentaenoic acid ("DPA n-6"), designated as "22:5 n-6."

Omega-3 fatty acids are biologically important molecules that affect cellular physiology due to their presence in cell membranes, regulate production and gene expression of biologically active compounds, and serve as biosynthetic substrates. (Roche, Proc. Nutr. Soc. 58: 397-401, 1999). DHA, for example, accounts for approximately 15%-20% of lipids in the human cerebral cortex,

30%-60% of lipids in the retina, is concentrated in the testes and sperm, and is an important component of breast milk (Bergé and Barnathan, G. Adv. Biochem. Eng. Biotechnol. 96:49-125,2005). DHA accounts for up to 97% of the omega-3 fatty acids in the brain and up to 93% of the omega-3 fatty acids in the retina. Moreover, DHA is essential for both fetal and infant development as well as maintenance of cognitive functions in adults. Id. Because omega-3 fatty acids are not synthesized de novo in the human body, these fatty acids must be derived from nutritional sources.

In general, the biological systems that produce the herein defined isoprenoids, sterols or lipids are industrially intractable, are increasingly under strict economic pressure and/or produce the compounds at such low levels that commercial scale isolation is not practical. The most limiting factors include instability of intermediates in such biological systems and the relatively high production of by-products, such as e.g. unwanted sterols in bio-production of vitamin D3, leading to complicated and costly purification steps. With regards to PUFAs, further productivity improvements are prevented due to biological titer limits.

For fermentation products that are secreted outside the cells, the so-called two-phase cultivation systems have been developed, wherein the fermentation products are collected outside the cell in the so-called second phase comprising lipophilic solvents such as e.g. Drakeol®, silicone oil or n-dodecane (see WO2020/141168 or Jang et al., Microbial Cell Factories 10:59, 2011). However, the yield as well as impurity profile with the known solvents is not satisfactory and/or the fermentation products are stored in lipid bodies that are not accessible by the second phase solvents.

Thus, it is an ongoing task to look for more efficient production processes using the two-phase culture system, in particular to look for solvents with improved properties which can be used in extraction of sterols, particularly vitamin D3, or isoprenoids, particularly vitamin E, or lipids, particularly PUFAs.

Surprisingly, we now identified an improved process for fermentative production of sterols and isoprenoids with less than 40 carbons as backbone, particularly vitamin D3 or vitamin E, as well as an improved process for fermentative production of certain lipids, particularly C20 or C22-PUFAs, including derivatives and intermediates thereof, using a two-phase culture system, wherein second phase solvents are not Drakeol®, silicone oil or n-dodecane, preferably wherein the second phase solvents are selected from isoparaffins collecting the

fermentation products, wherein the purity of said products could be specifically improved.

Particularly, the present invention is directed to a two-phase culture system including an in vitro extraction system for fermentative production of isoprenoids, lipids, and sterols, particularly various forms of vitamin E and vitamin D3, as well as various polyunsaturated fatty acids (PUFAs), including derivatives thereof, wherein a suitable host cell, particularly fungal host cell, such as e.g. an oleaginous yeast, e.g. Yarrowia or Saccharomyces, is grown on a suitable carbon source and in the presence of a lipophilic solvent as defined herein.

Particularly, the present invention allows for increased productivity of intracellular lipids, wherein the second phase allows for in-situ extraction of certain lipids as defined herein, particularly PUFAs, more particularly omega-3 fatty acids, which thereby allows for more PUFAs to be made intracellularly. This overcomes the titer and productivity limitations of a typical single-phase fermentation system.

A suitable lipophilic solvent, i.e. second phase solvent to be used for the present invention might be selected from isoparaffins including mixtures of alkanes, cycloparaffin, isoalkanes, cycloalkanes, or dodecanes. The solvents might be natural or synthetic ones. Examples of commercially available useful solvents might be selected from Total, e.g. Isane® solvents, Shell, e.g. ShellSolTD or ShellSolT, Exxon Mobile, e.g. Isopar™ fluids, particularly such as e.g. Isopar M, Isopar H, Isopar K, Isopar L, or mixtures with iso-dodecane isomers, as e.g. commercially available under the tradename AC365770010 (Acros Organics). Preferably, the second phase solvent is selected from isoparaffins, such as e.g. Isopar M, Isopar H, Isopar K, Isopar L, more preferably selected from Isopar L or Isopar M.

Further suitable lipophilic solvents, i.e. second phase solvents to be used for the present invention might be selected from lipophilic solvents comprising mixtures of n-alkanes, isoalkanes, hydrocarbons. The solvents might be natural or synthetic ones. Examples of commercially available useful solvents might be selected from Exxon Mobil, as e.g. commercially available under the tradenames Exxsol D60, D80, D95 or D110.

It is understood that useful solvents include the above listed commercially available solvents as well as the respective solvents with the same or equivalent properties but known/available from other suppliers.

As used herein, a solvent has equivalent or identical properties as Isopar fluids, including Isopar M, Isopar H, Isopar K, Isopar L, are defined as branched-chain isomers, preferably terminally methylated form of a straight-chain alkane, which contain six to twenty six carbons, perhaps chemically coupled from smaller alkane precursors in strong acid, hydrogenated with H2 and catalyst, such as nickel or platinum, to remove unsaturation and trace aromatics. These are known in current industrial suppliers as Isopar (Exxon Mobil Chemical), Soltrol (Chevron Phillips Chemical Company), Shellsol OMS (Royal Dutch Shell), iso-octane and iso-dodecane. Specifically, the use of Isopar M is preferred. The use of these in consumer products is outlined in a review by Johnson et al., Int J Toxicol. 2012 Nov-Dec;31(6 Suppl):269S-95S.

As used herein, a solvent has equivalent or identical properties as Exxsol D60, D80, D95, D110. These Exxsol Ds are narrow boiling distillation cuts from cracked hydrocarbons that have been reduced by catalytic hydrogenation to remove aromatics and unsaturation.

A suitable sterol that is produced in the two-phase culture system according to the present invention might be selected from vitamin D3, including but not limited to intermediates and derivatives thereof, e.g. 7-DHC, 25-hydroxycholecalciferol (HyD), 25-7-DHC (HyDHC), cholesta-5,7,24(25)-trienol, Zymosterol, lanosterol, lathosterol, cholesta-5,8,24(25)-trienol, i.e. fermentation products, that can pass through the cell wall of the host cell to be collected outside the cell in the second phase solvent as defined herein.

Using the two-phase culture system comprising a lipophilic solvent as defined herein results in a mix of sterols comprising 7-DHC, zymosterol, trienols and/or hydroxylated forms such as e.g. HyDHC, wherein the percentage of HyDHC based on total sterols in the mix is in the range of at least about 65%, such as e.g. 70, 75, 80, 85, 90, 95 or more HyDHC based on total sterols in the mix.

A suitable isoprenoid that is produced in the two-phase culture system according to the present invention might be selected from vitamin E, including but not limited to intermediates and derivatives thereof, e.g. isoforms of tocotrienol or tocopherols, i.e. fermentation products, that can pass through the

cell wall of the host cell to be collected outside the cell in the second phase solvent as defined herein.

Suitable lipids produced in the two-phase culture system according to the present invention might be selected from polyunsaturated fatty acids (PUFAs) and microbial oils. PUFAs might be selected from either a single PUFA or two or more different PUFAs. The PUFAs or each PUFA can be of the n-3 or n-6 family. Preferably it is a C18, C20 or C22 PUFA. It may be a PUFA with at least 18 carbon atoms and/or at least 3 or 4 double bonds. The PUFA 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 but are not limited to docosahexaenoic acid (DHA, 22:6 omega-3), suitably from algae or fungi, such as the (dinoflagellate) Crypthecodinium or the (fungus) Thraustochytrium; γ-linolenic acid (GLA, 18:3 omega-6); alpha-linolenic acid (ALA, 18:3 omega-3); conjugated linoleic acid (octadecadienoic acid, CLA); dihomo-gamma-linolenic acid (DGLA, 20:3 omega-6); arachidonic acid (ARA, 20:4 omega-6); and eicosapentaenoic acid (EPA, 20:5 omega-3).

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

The PUFA may be produced by the cells pasteurised in the process of the invention, such as a microbial cell. This may be a bacteria, algae, fungus or yeast cell. Fungi are preferred, preferably selected from Mucorales, such as e.g., 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 Yarrowia, Pichia or Saccharomyces, such as Yarrowia lypolitica or Pichia ciferii. Bacteria can be of the genus Propionibacterium. The microbial oil may be a liquid (at room temperature).

Preferably, the PUFAs are in the form of triglycerides, such as e.g. at least about 50%, 60, 70% of the PUFA is in triglyceride form. However, the percentage of triglycerides may be higher, such as at least about 85%, preferably at least about 90%, 93%, 95% of the oil. Of these triglycerides, preferably at least about 40%,

such as at least about 50% to 60% of the PUFA is present at the a-position of the glycerol (present in the triglyceride backbone), also known at the 1 or 3 position. It is preferred that at least about 20%, such as at least about 30 to 40% 40% of the PUFA is at the b(2) position.

The present invention is related to a process using a two-phase cultivation system for fermentative production of isoprenoids, lipids, and sterols, particularly various forms of vitamin E and vitamin D3, as well as various polyunsaturated fatty acids (PUFAs), including derivatives or intermediates thereof, wherein a lipophilic solvent is used as a second phase and as defined herein, wherein a suitable host cell, particularly fungal host cell, such as e.g. an oleaginous yeast, e.g. Yarrowia or Saccharomyces, is grown on a suitable carbon source and in the presence of said lipophilic solvent that is not disappearing during the fermentation.

As used herein, the term "not disappearing" in connection with the novel solvents as defined herein means that the same amount of solvent present at the start of the fermentation is still detectable at the end of the fermentation process. A loss or disappearance of solvent might be due to e.g., consumption by the host cell and/or evaporation during the fermentation. The terms "loss" or "disappearance" are used interchangeably herein.

In one embodiment of the present invention, the two-phase culture system as defined herein including the use of the solvents as defined herein results in a disappearance of solvents of about 20% or less, i.e., wherein at least about 80%, such as e.g. 85, 90, 92, 95, 97, 98, 99, 100% of solvent present at the start of the fermentation is still present at the end of the fermentation process. Particularly useful solvents that are still present at the end of the fermentation might be selected from solvents identical or equivalent to isopars, more particularly Isopar M.

Thus, the present invention is related to fermentative production of isoprenoids, lipids, and sterols, particularly various forms of vitamin E and vitamin D3, as well as various polyunsaturated fatty acids (PUFAs), including derivatives or intermediates as defined herein, comprising cultivating a suitable host cell as defined herein under suitable culture conditions in a two-phase culture system, in the presence of a solvent as defined herein with a loss of solvent of about 20% or lower during the fermentation.

As used herein, the term "solvent comprising isoparaffins" or "solvent comprising mixtures of alkanes" means that the percentage of isoparaffins and/or mixtures of alkanes is at least in the range of about less than about 30%, such as in the range of 5 to 30%, preferably in the range of about 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20% (v/v), including the solvents described herein, particularly solvents known under the tradename Isopar M, Isopar H, Isopar K, Isopar L, Exxsol D60, D80, D95, D110 or solvents with equivalent or identical properties but from other suppliers. The use of a solvent with a percentage of isoparaffins and/or mixtures of alkanes as defined herein is used interchangeably with the term "in the presence of a lipophilic solvent".

In one embodiment, the present invention is related to a process for reducing or abolishing the consumption of the second phase solvent, i.e., consumption of the second phase solvent by the host cell, as compared to consumption using n-dodecane as second phase. Particularly, the consumption of the second phase might be reduced by at least about 50%, such as e.g. 60, 65, 70, 75, 80, 85, 90, 95, 97, 99 or 100% compared to consumption using n-dodecane as second phase solvent via a process using a lipophilic solvent as described herein. Preferably, the lipophilic solvent is selected from solvents known under the tradename Isopar M, Isopar N, Isopar H, Isopar K, Isopar L, Exxsol D110 or solvents with equivalent or identical properties but from other suppliers.

In a further embodiment, the present invention is related to a two-phase culture system for fermentative production of isoprenoids, lipids, and sterols, particularly various forms of vitamin E and vitamin D3, as well as various PUFAs, including derivatives or intermediatesas defined herein in the presence of a lipophilic solvent as described herein, wherein the evaporation of the solvent is reduced or abolished, particularly reduced by at least about 50%, such as e.g. 60, 65, 70, 75, 80, 85, 90, 95, 97, 99 or 100%. Preferably, the lipophilic solvent is selected from solvents known under the tradename Isopar M, Isopar N, Isopar H, Isopar K, Isopar L, Exxsol D60, Exxsol D110, more preferably Isopar L, Isopar M, Exxsol D110, or solvents with equivalent or identical properties but from other suppliers.

Thus, a stable solvent according to the present invention means that the evaporation and/or consumption is reduced by at least about 50% compared to known solvents such as e.g. n-dodecane, silicone oil or hexadecane. Such solvents with increased evaporations and/or consumption during the fermentation in the range of about more than 50% compared to the stable

solvents as defined herein are defined as "non-stable solvents". Examples of such non-stable solvents are n-dodecane, silicone oil or hexadecane.

Evaporation of a solvent used in a two-phase culture system, including the measurement of mass of fermentation over time, and measurement of second phase volume at the beginning and end of the fermentation can be measured by mass balancing using LCMS. Further the measurement of incorporation of second phase into the product, such as e.g. forms of vitamin D3 including but not limited to 7-DHC, HyDHC, or vitamin E forms as defined herein, or forms of PUFAs as defined herein, indicates consumption. Measurement of isotopic ^{13}C ratios in product weighted towards the ratio in petrol vs the corn derived ethanol feed can be done by ^{13}C NMR or mass spectroscopy. Consumption of the solvent, such as e.g. isoparaffins, according to the present invention by the host organism, such as e.g. Saccharomyces or Yarrowia, is detection of oxygen consumption (measured by a dissolved oxygen probe) in the presence of the second phase in the absence of another carbon source, wherein consumption of oxygen above the background being the same as with no solvent correlates with consumption of the solvent. Finally, measurement/mass spec analysis of off-gas or condensation in a cold trap from fermentation can be used to measure evaporation.

In one embodiment the present invention features a fermentative process for production of isoprenoids, lipids and sterols, particularly various forms of vitamin E and vitamin D3, as well as various PUFAs as defined herein using a two-phase culture system, wherein the formation of by-products could be reduced, particularly reduced by at least about 10% in comparison to the use of a non-stable solvent, such as e.g. in the range of about 15, 20, 25, 30, 35, 40, 45, 50% or more. Particularly, such process for reducing the formation of by-products as defined herein is performed in the presence of a lipophilic solvent as defined herein, preferably wherein the solvent is selected from solvents known under the tradename Isopar M, Isopar N, Isopar L, Exxsol D60, Exxsol D95, Exxsol D110, more preferably Isopar M, Exxsol D60, Exxsol D95, Exxsol D110, or solvents with equivalent or identical properties but from other suppliers. Compared to the use of n-dodecane or Drakeol®, the reduction might be in the range of a least about 25%.

As used herein, the terms "by-products", "side-products" or "undesired fermentation products" in connection with fermentative production of target fermentation products as defined herein, i.e. isoprenoids lipids, and sterols,

according to the definition given in the present application, according to the present invention are used interchangeably herein and refer to undesired co-production of competing products, i.e. impurities, which have to be separated from the desired fermentation products and/or limit the yield and/or productivity of the desired fermentation products. Furthermore, it relates to undesired conversion processes, i.e., wherein the conversion of an intermediate into the desired fermentation product is competing with undesired conversion of the intermediate into a side-product or undesired by-product. The terms "isoprenoids or sterols according to the present invention" and (desired) fermentation or target fermentation products are used interchangeably herein.

With regards to production of sterols, particularly production of a sterol mix as defined herein comprising 7-DHC and HyDHC, undesired side-products include but are not limited to formation of zymosterol, trienols, ergosterol, lanosterol, lathosterol, squalene, sitosterol, cholesta-5,7,24(25)-trienol, cholesta-5,8,24(25)-trienol, cholesta-7,24(25)-dienol, which should be reduced or abolished.

With regards to lipids, particularly various PUFAs, undesired side-products include but are not limited to formation of saturated fatty acids, diacylglyceride and monoglyceride fatty acids as well as free fatty acids (FFAs).

Production of sterols, lipids or isoprenoids, particularly various forms of vitamin D3 and vitamin E, as well as various PUFAs including derivatives or intermediates as defined herein, using the two-phase culture system as defined herein in the presence of a lipophilic solvent as defined herein includes cultivation of a suitable host cell, particularly fungal host cell, such as e.g. an oleaginous yeast, e.g. Yarrowia or Saccharomyces, or host cells selected from Mortierella, Phycomyces, Blakeslea, Aspergillus, Thraustochytrium, Pythium or Entomophthora, Porphyridium, Nitszchia, or Crypthecodinium, particularly Mortierella alpina, Blakeslea trispora, Aspergillus terreus or Pythium insidiosum, Crypthecodinium cohnii said host cell being grown on a suitable carbon source.

Suitable host cells to be used for the present invention might be selected from any host cell capable of sterol/isoprenoid/lipid production including various forms of vitamin D3 and vitamin E as well as various PUFAs including derivatives or intermediatesas defined herein, such as e.g. fungal host cell, more particularly an oleaginous host cell, such as e.g. Yarrowia, Schizosaccharomyces spp., Pichia spp., Klyuveromyces spp., Hansenula spp., Saccharomyces or Yarrowia, preferably Yarrowia or Saccharomyces, more preferably Yarrowia lipolytica or Saccharomyces cerevisiae. Depending on the fermentation product,

the skilled person knows which host cell to select including the respective culture conditions (see e.g. WO2003064650, WO2011067144 or WO2017108799). Most preferred are fungal host cells wherein the endogenous genes encoding ERG5 and ERG6 are inactivated, the endogenous HMG1 gene has been truncated and overexpressed and wherein a heterologous gene encoding sterol delta24-reductase preferably from mammals or plant origin have been expressed and optionally, wherein a heterologous gene encoding cholesterol C25-hydroxylase is expressed, as e.g. exemplified in WO200023596 (see SEQ ID Nos: 1, 3 in said reference) WO2011067144 (see SEQ ID Nos:5, 8, 23, 25, 26, 27, 28, 30 and Table 15 in said reference). The fermentation may be conducted in batch, fed-batch, semi-continuous or continuous mode.

A list of suitable host cells for production of lipids, including various forms of PUFAs can be found in US10385289, including particularly cells from Stramenopiles, Hamatores, Proteromonads, Opalines, Develpayella, Diplophrys, Labrinthulids, Thraustochytrids, Biosecids, Oomycetes, Hypochytridiomycetes, Commotion, Reticulosphaera, Pelagomonas, Pelagococcus, Ollicola, Aureococcus, Parmales, Diatoms, Xanthophytes, Phaeophytes, Eustigmatophytes, Raphidophytes, Synurids, Axodines (including Rhizochromulinaales, Pedinellales, Dictyochales), Chrysomeridales, Sarcinochrysidales, Hydrurales, Hibberdiales, and Chromulinales.

In some embodiments, a microbial cell for use with the present invention is a microorganism of the phylum Labyrinthulomycota. In some embodiments, a microbial cell of the phylum Labyrinthulomycota is a thraustochytrid, such as a Schizochytrium or Thraustochytrium. According to the present invention, the term “thraustochytrid” refers to any member of the order Thraustochytriales, which includes the family Thraustochytriaceae, and the term “labyrinthulid” refers to any member of the order Labyrinthulales, which includes the family Labyrinthulaceae.

Members of the family Labyrinthulaceae were previously considered to be members of the order Thraustochytriales, but in more recent revisions of the taxonomic classification of such organisms, the family Labyrinthulaceae is now considered to be a member of the order Labyrinthulales. Both Labyrinthulales and Thraustochytriales are considered to be members of the phylum Labyrinthulomycota. Taxonomic theorists now generally place both of these groups of microorganisms with the algae or algae-like protists of the Stramenopile lineage. The current taxonomic placement of the thraustochytrids

and labyrinthulids can be summarized as follows: Realm: Stramenopila (Chromista); Phylum: Labyrinthulomycota (Heterokonta); Class: Labyrinthulomycetes (Labyrinthulae); Order: Labyrinthulales, Family: Labyrinthulaceae; Order: Thraustochytriales, Family: Thraustochytriaceae.

For purposes of the present invention, strains of microbial cells described as thraustochytrids include the following organisms: Order: Thraustochytriales; Family: Thraustochytriaceae; Genera: Thraustochytrium (Species: sp., arudimentale, aureum, benthicola, globosum, kinnei, motivum, multirudimentale, pachydermum, proliferum, roseum, and striatum), Ulkenia (Species: sp., amoeboidea, kerguelensis, minuta, profunda, radiata, sailens, sarkariana, schizochytrops, visurgensis, yorkensis, and sp. BP-5601), Schizochytrium (Species: sp., aggregatum, limnaceum, mangrovei, minutum, and octosporum), Japonochytrium (Species: sp., marinum), Aplanochytrium (Species: sp., haliotidis, kerguelensis, profunda, and stocchinoi), Althornia (Species: sp., crouchii), or Elina (Species: sp., marisalba, and sinorifica). For the purposes of this invention, species described within Ulkenia will be considered to be members of the genus Thraustochytrium. Aurantiacochytrium and Oblogospora are two additional genuses encompassed by the phylum Labyrinthulomycota in the present invention. In some embodiments, a microbial cell is of the genus Thraustochystrium, Schizochytrium, and mixtures thereof.

Microbial cells suitable for use with the present invention include, but are not limited to, Labyrinthulids selected from: Order: Labyrinthulales, Labyrinthulaceae, Genera: Labyrinthula (Species: sp., algeriensis, coenocystis, chattonii, macrocystis, macrocystis atlantica, macrocystis macrocystis, marina, minuta, roscoffensis, valkanovii, vitellina, vitellina pacifica, vitellina vitellina, and zopfii), Labyrinthuloides (Species: sp., haliotidis, and yorkensis), Labyrinthomyxa (Species: sp., marina), Diplophrys (Species: sp., archeri), Pyrrhosorus (Species: sp., marinus), Sorodiplophrys (Species: sp., stercorea), and Chlamydomyxa (Species: sp., labyrinthuloides, and montana) (although there is currently not a consensus on the exact taxonomic placement of Pyrrhosorus, Sorodiplophrys, and Chlamydomyxa).

Host cells of the phylum Labyrinthulomycota include, but are not limited to, deposited strains PTA-10212, PTA-10213, PTA-10214, PTA-10215, PTA-9695, PTA-9696, PTA-9697, PTA-9698, PTA-10208, PTA-10209, PTA-10210, PTA-10211, the microorganism deposited as SAM2179 (named “Ulkenia SAM2179” by the depositor), any Thraustochytrium species (including former Ulkenia species such

as U. visurgensis, U. amoeboida, U. sarkariana, U. profunda, U. radiata, U. minuta and Ulkenia sp. BP-5601), and including Thraustochytrium striatum, Thraustochytrium aureum, Thraustochytrium roseum; and any Japonochytrium species. Strains of Thraustochytriales include but are not limited to Thraustochytrium sp. (23B) (ATCC 20891); Thraustochytrium striatum (Schneider) (ATCC 24473); Thraustochytrium aureum (Goldstein) (ATCC 34304); Thraustochytrium roseum (Goldstein) (ATCC 28210); Japonochytrium sp. (L1) (ATCC 28207); ATCC 20890; ATCC 20892; a mutant strain derived from any of the aforementioned microorganisms; and mixtures thereof. Schizochytrium include, but are not limited to Schizochytrium aggregatum, Schizochytrium limacinum, Schizochytrium sp. (S31) (ATCC 20888), Schizochytrium sp. (58) (ATCC 20889), Schizochytrium sp. (LC-RM) (ATCC 18915), Schizochytrium sp, (SR 21), deposited strain ATCC 28209, deposited Schizochytrium limacinum strain IFO 32693, a mutant strain derived from any of the aforementioned microorganisms, and mixtures thereof. In some embodiments, the host cell is a Schizochytrium or a Thraustochytrium. Schizochytrium can replicate both by successive bipartition and by forming sporangia, which ultimately release zoospores. Thraustochytrium, however, replicate only by forming sporangia, which then release zoospores. In some embodiments, the host cell of the invention is a recombinant host cell.

Suitable carbon sources to be used for the present invention might be selected from linear alkanes, free fatty acids, including triglycerides, particularly vegetable oil, such as e.g., selected from the group consisting of oil originated from corn, soy, olive, sunflower, canola, cottonseed, rapeseed, sesame, safflower, grapeseed or mixtures thereof, including the respective free fatty acids, such as e.g., oleic acid, palmitic acid or linoleic acid. Suitable carbon sources might furthermore be selected from ethanol, glycerol or glucose and mixtures of one or more of the above-listed carbon sources. The use of suitable carbon sources as well as suitable culture conditions for production of isoprenoids, lipids, and sterols, particularly various forms of vitamin E and vitamin D3, as well as various PUFAs, including derivatives or intermediates particularly vitamin D3 as defined herein are known in the art. Particularly, vitamin D3 as defined herein is produced under suitable culture conditions in a two-phase culture system as described herein, preferably using lipophilic solvents selected from Isopar™ fluids, particular selected from Isopar M, Isopar K, Isopar L, Isopar H or solvents with equivalent or identical properties but from other suppliers.

In one embodiment, the present invention is directed to a process for the production of isoprenoids, lipids, and sterols, particularly various forms of vitamin E and vitamin D3, as well as various PUFAs, including derivatives or intermediates, i.e. a two-phase culture system in the presence of a lipophilic solvent as defined herein, wherein a respective host cell, particularly fungal host cell such as Saccharomyces or Yarrowia, is cultivated under suitable culture conditions, wherein the lipophilic solvent, preferably selected from solvents that are commercially available as Isopar™ fluids, particularly selected from Isopar M, Isopar N, Isopar K, Isopar L, Isopar H or solvents with equivalent or identical properties but from other suppliers, is not consumed or evaporated during the fermentation process.

In one embodiment, the present invention is directed to a process for the production of sterols, in particular production of vitamin D3, i.e. a two-phase culture system in the presence of a lipophilic solvent as defined herein, wherein a respective host cell, particularly fungal host cell such as Saccharomyces or Yarrowia, is cultivated under suitable culture conditions, wherein the percentage of 7-DHC could be increased by at least about 5%, such as e.g. about 8, 10, 12, 14, 15, 18, 20% or more as compared to a process without the addition of said lipophilic solvent as defined herein.

In one embodiment, the present invention is directed to a process for the production of sterols, in particular production of vitamin D3, i.e. a two-phase culture system in the presence of a lipophilic solvent as defined herein, wherein a respective host cell, particularly fungal host cell such as Saccharomyces or Yarrowia, is cultivated under suitable culture conditions, wherein the percentage of HyDHC could be increased by at least about 15%, such as e.g. about 20, 30, 40, 50, 60, 70, 80, 90, 100, 120, 130, 140, 150% or more as compared to a process without the addition of said lipophilic solvent as defined herein.

In another embodiment, the present invention is directed to a process for the production of sterols, in particular production of vitamin D3, i.e. a two-phase culture system in the presence of a lipophilic solvent as defined herein, wherein a respective host cell, particularly fungal host cell such as Saccharomyces or Yarrowia, is cultivated under suitable culture conditions, wherein the percentage of zymosterol could be reduced by at least about 5%, such as e.g. about 10, 12, 15, 20, 25, 30, 35, 40, 45, 50, 5, 60, 65, 70% or more as compared to a process without the addition of said lipophilic solvent as defined herein.

With regards to the present invention, it is understood that organisms, such as e.g. microorganisms, fungi, algae or plants also include synonyms or basonyms of such species having the same physiological properties, as defined by the International Code of Nomenclature of Prokaryotes or the International Code of Nomenclature for algae, fungi, and plants (Melbourne Code).

As used herein, the term "specific activity" or "activity" with regards to enzymes means its catalytic activity, i.e., its ability to catalyze formation of a product from a given substrate. The specific activity defines the amount of substrate consumed and/or product produced in a given time period and per defined amount of protein at a defined temperature. Typically, specific activity is expressed in µmol substrate consumed or product formed per min per mg of protein. Typically, µmol/min is abbreviated by U (= unit). Therefore, the unit definitions for specific activity of µmol/min/(mg of protein) or U/(mg of protein) are used interchangeably throughout this document. An enzyme is active, if it performs its catalytic activity in vivo, i.e. within the host cell as defined herein or within a suitable (cell-free) system in the presence of a suitable substrate. The skilled person knows how to measure enzyme activity. Analytical methods to evaluate the capability of enzymes involved in sterol biosynthesis as defined herein are known in the art and include measurement via HPLC and the like.

A host cell comprising the above-described modifications in endogenous gene activities, such as e.g. inactivation of endogenous genes and or expression of heterologous genes, such as e.g. ERG5 and ERG6 or cholesterol C25-hydroxylase, is also referred to as "modified host cell".

As used herein, a "wild-type host cell" means the respective host cell which is wild-type, i.e. non-modified, with respect to the above-mentioned genetic modifications. Thus, in a wild-type host cell the corresponding endogenous enzymes as defined herein are (still) expressed and active in vivo and/or no heterologous enzymes are expressed.

Conversion according to the present invention is defined as specific enzymatic activity, i.e. catalytic activity of enzymes described herein, including but not limited to the enzymatic activity of hydroxylases, such as e.g. cholesterol C25-hydroxylase involved in conversion of 7-DHC into HyDHC.

The terms "triglycerides" and "triglyceride oils" are used interchangeably herein.

The following examples are illustrative only and are not intended to limit the scope of the invention in any way. The contents of all references, patent

applications, patents, and published patent applications, cited throughout this application are hereby incorporated by reference, particularly WO2003064650, WO2011067144, WO2017108799, US10385289, and WO200023596.

The invention is particularly directed to the following embodiment (1) to (12):

(1) Fermentative production of isoprenoids or sterols as backbone in a two-phase culture system, comprising in situ extraction of said isoprenoids or sterols, wherein a suitable host cell is cultivated in the presence of a carbon source and a lipophilic solvent, wherein the lipophilic solvent is different from the carbon source and with a minimal loss of solvent during the fermentation process, and wherein the isoprenoid is selected from vitamin E and the sterol is selected from vitamin D3 including derivatives and intermediates of vitamin E or vitamin D3.

(2) Embodiment (1), wherein the lipophilic solvent is not selected from Drakeol, silicone oil or n-dodecane.

(3) Embodiment (1) or (2), wherein the lipophilic solvent present at the end of the fermentation is in the range of at least 80% of the solvent present at the start of the fermentation process.

(4) Embodiment (1), (2) or (3), wherein the lipophilic solvent comprises isoparaffins, preferably wherein the lipophilic solvent comprises a percentage of less than 30% (v/v) of isoparaffins.

(5) Embodiment (1), (2), (3) or (4), wherein the fermentation product is selected from the group consisting of 7-dehydrocholesterol (7-DHC), 25-hydroxycholecalciferol (HyD), 25-7-DHC (HyDHC), zymosterol, tocotrienols, and tocopherols.

(6) Embodiment (1), (2), (3), (4) or (5), wherein the carbon source is selected from linear alkanes, free fatty acids, ethanol, glucose, including triglycerides, particularly vegetable oil, such as e.g. selected from the group consisting of oil originated from corn, soy, olive, sunflower, canola, cottonseed, rapeseed, sesame, safflower, grapeseed or mixtures thereof, including the respective free fatty acids, such as e.g. oleic acid, palmitic acid or linoleic acid.

(7) Embodiment (1), (2), (3), (4), (5) or (6), wherein 20% or less of the solvent is lost during the fermentation, preferably wherein the evaporation of the solvent is reduced by at least about 50%.

(8) Embodiment (1), (2), (3), (4), (5), (6) or (7), wherein the consumption of the solvent by the host cell is reduced by at least about 50%.

(9) Embodiment (1), (2), (3), (4), (5), (6), (7) or (8) for production of a mix of sterols comprising 7-DHC and HyDHC, wherein the percentage of HyDHC based on total sterols in the mix is in the range of at least about 65%, such as e.g. 70, 75, 80, 85, 90, 95 or more HyDHC based on total sterols in the mix.

(10) Embodiment (1), (2), (3), (4), (5), (6), (7), (8) or (9), wherein the formation of by-products including zymosterol is reduced by at least about 5%, such as e.g. about 10, 12, 15, 20, 25, 30, 35, 40, 45, 50, 5, 60, 65, 70% or more as compared to a process without the addition of said lipophilic solvent as defined herein.

(11) Embodiment (1), (2), (3), (4), (5), (6), (7), (8) or (9), wherein the percentage of HyDHC could be increased by at least about 15%, such as e.g. about 20, 30, 40, 50, 60, 70, 80, 90, 100, 120, 130, 140, 150% or more as compared to a process without the addition of said lipophilic solvent as defined herein.

(12) Embodiment (1), (2), (3), (4), (5), (6), (7), (8), (9), (10) or (11), wherein the host cell is selected from fungal host cells, particularly oleaginous host cells, such as e.g. yeast, preferably selected from Yarrowia or Saccharomyces.

Examples

Example 1: General methods and analytics

All basic molecular biology and DNA manipulation procedures described herein are generally performed according to Sambrook et al. (eds.), Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press: New York (1989) or Ausubel et al. (eds). Current Protocols in Molecular Biology. Wiley: New York (1998).

Strains. A HyDHC producing Saccharomyces cerevisiae strain was constructed as described in WO2011067144, wherein the cholesterol C25-hydroxylase from S. scrofa has been integrated (see SEQ ID NO: 27 in WO2011067144). A HyDHC producing Yarrowia lipolytica was constructed accordingly (Table 1), i.e. wherein the Erg5 and Erg6 genes are knocked out, a heterologous cholesterol C25-hydroxylase is expressed and a heterologous sterol Δ24-reductase such as selected from *Danio rerio*, rat or human. The sequences expressing said sterol Δ24-reductase enzymes are publicly available, including but not limited to polypeptide sequences derived from UniProtKB/Swiss-Prot reference Q15392, Q60HC5, Q8VCH6, Q5BQE6, Q39085 or P93472 (see e.g. WO2003064650).

Yarrowia lipolytica PO1f strain (MATa leu2-270 ura3-302 xpr2-322 axp1) (ATCC MYA-2613) was used to produce PUFAs.

C18 UPLC chromatography. determination of discrete sterols were monitored at 282nm by UV absorbance as in Table 1, specifically the instrument was calibrated using pure standards that were measured at 282nm and adjusted using Beer's law and published extinction coefficients. Samples were processed by Precellys to extract total sterols.

Table 1: list of analytes using C18 UPLC chromatography. "RT" means retention time. For more details, see text.

Intermediates	**RT [min]**
HyDHC	2.830
trienol	3.252
ergosterol	3.298
7-DHC	3.348
sterol esters	3.6-3.9

Fermentation conditions (Saccharomyces): Fed-batch fermentations using Isopar M as a second phase were performed in a stirred tank in a bench top reactor with 0.5L initial batch volume. The batch medium carbon source composition and feed media are glucose and ethanol. Feeds were added using two different feed profiles between hours 2-60 for glucose and hours 5-120 for ethanol.

Briefly, the fermentations were run in 1.0L flood volume in glass Eppendorf fermentation systems. The fermentor was batched and autoclaved according to Table 5. After cooling, glucose and vitamin solution were added along with 50mL Isopar M as the second phase. The fermentation was inoculated with 45 ml of an overnight shake flask culture of Sterol seed flask media (Table 4) grown with 250RPM agitation at 29°C. Fermentation parameters were agitation at 1500RPM, airflow at 0.5LPM, pH controlled at 5.7 control with NH4OH, and the temperature set to 29°C. Feeds were added using two different feed profiles, 52mL of 65% glucose between hours 2-60 and 175g of pure ethanol between hours 5-120.

Table 2: Sterol trace metals solution. For more explanation, see text.

Component	**Concentration**

ZnSO4*7H2O	800 mg/L
Citric acid	7300 mg/L
MnSO4*H2O	160 mg/L
CuSO4*5H2O	40 mg/L
Na2MoO4*2H2O	640 mg/L
CoCl2*6H2O	80 mg/L
H3BO3	240 mg/L
KI	120 mg/L
FeSO4*7H2O	10400 mg/L

Table 3: Sterol vitamin solution. For more explanation, see text.

Component	**Concentration**
Biotin	1 mg/L
Folic Acid	10 mg/L
CaPantothenate	40 mg/L
Inositol	200 mg/L
Niacin	40 mg/L
Riboflavin	20 mg/L
Pyridoxine*HCl	40 mg/L
Thiamine*HCl	40 mg/L
L-Tryptophan	100 mg/L

Table 4: Sterol seed shake flask media – 200mL per flask. For more explanation, see text.

Component	**Concentration**
$(NH_4)_2SO_4$	4.5 g/L
KH_2PO_4	1.2 g/L
$MgSO_4*7H_2O$	0.7 g/L
NaCl	0.05 g/L

$CaCl_2$	0.05 g/L
Difco yeast extract	10 g/L
Sheftone EK	3 g/L
Sterol trace metals solution (Table 2)	2.5 mL/L
After autoclave add: Sterol vitamin solution (Table 3) Glucose	 10 ml/L 40 g/L

Table 5: Fermentor batch medium. For more explanation, see text.

Component	**Without 2nd phase**	**10% Isopar M**
Water	428 g	378g
$(NH_4)_2SO_4$	1.35 g	1.35g
KH_2PO_4	1.58 g	1.58g
$MgSO_4*7H_2O$	0.315 g	0.315g
NaCl	0.045 g	0.045g
$CaCl_2*H_2O$	0.042 g	0.042g
Difco yeast extract	3.6 g	3.6g
Difco Soytone	9.0 g	9.0g
Sterol trace metals solution (Table 2)	1.13 mL	1.13mL
DF204	0.5 mL	0.1mL
After autoclave add: Sterol vitamin solution (Table 3) 40% Glucose 2nd phase (Isopar M)	 1.8 mL 8.5 mL ---	 1.8 mL 8.5 mL 50 mL

Fermentation conditions (Yarrowia): Fed-batch fermentations were similar to the previously described conditions except using Isopar M as a second phase in a stirred tank bench top reactor with 0.6L initial batch volume (see WO2016172282). The batch medium carbon source and feed medium are glucose. Feeding was

initiated after the initial batch carbon had been consumed, with feed added in a controlled manner to maintain a dissolved oxygen level (DO) setpoint.

Briefly, the fermentations were run in a 1.0L flood volume glass Eppendorf fermentation systems. The fermentor was batched with the following components: 385mL of deionized water (445mL if no Isopar M present), 0.94g $MgSO_4 \cdot 7H_2O$, 0.095g NaCl, 0.16g $CaCl_2 \cdot 2H_2O$, 3.93g $(NH_4)_2SO_4$, 4.07g KH_2PO_4, 2.94g Tastone yeast extract (Sensient), 3.9mL DF204 antifoam, 0.098µL thiamine HCl at 4 mg/ml, trace elements stock solution 1.5 7mL containing: 200g/kg citric acid, 27.3 g/kg $FeSO_4 \cdot 7H_2O$, 19.6 g/kg $Na_2MoO_4 \cdot 2H_2O$, 18.7 g/kg $CuSO_4 \cdot 5H_2O$, 4.9 g/kg H_3BO_3, 21.9 g/kg $MnSO_4 \cdot H2O$, 30.2 g/kg $ZnSO_4 \cdot 7H_2O$, and autoclaved. After cooling, glucose was added (5% w/w final concentration) along with 60mL Isopar M as the second phase if used. The fermentation was inoculated with 60 ml of an overnight shake flask culture (YPD medium grown with 250RPM agitation at 30°C). Fermentation parameters were agitation at 1300RPM, airflow at 0.7LPM, pH controlled at 5.5 control with NH_4OH, and the temperature set to 30°C. At feed start, feed (65% glucose) was added to maintain the DO setpoint at 60%. The DO setpoint was ramped down to 20% in a linear fashion over the following 24 hours by increased feed rate. The DO was then maintained at 20% via feed addition for the remainder of the fermentation.

Example 2: Vitamin D3 production using a two-phase culture system in Saccharomyces and Yarrowia

HyDHC-producing strains of Sscr opt 2.0 or the corresponding strain from Yarrowia lipolytica (see Ex. 1 for construction) were cultivated as described above, using glucose as carbon source and 10% Isopar M as second phase solvent. Titers of HyDHC could be increased by 30 to more than 150%, titers of 7-DHC increased in a range of about at least 10%, depending on the host strain (Table 6).

Table 6: effect of Isopar M (10%) on formation of HyDHC and 7-DHC, wherein the increase in % is shown as compared to the numbers without the use of Isopar M in the second phase (i.e. without the use of a two-phase culture system). For more details, see text.

Strain	HyDHC increase	7-DHC increase
Sscr opt 2.0	154%	14%

Yarrowia strain	23%	12%

Example 3: PUFA production using a two-phase culture system in Yarrowia

Yarrowia lipolytica strain PO1f was grown with and without Isopar M in YPD medium containing 100 g/l glucose. Cells were grown at 225 r.p.m. and 30° C for 96 hrs. Isopar M phase was separated from aqueous phase by centrifuging fermentation broth at 4,500 xg for 10 min. Total lipid in the isopar M phase and intracellular lipid were analyzed. As can be seen in Table 7, Isopar M culture (976 mg/L) contains 10% more total lipid than non-Isopar M culture (control: 887 mg/L). These data indicate that the additional Isopar M helps increase total lipid production in Yarrowia lipolytica. (Table 7).

Table 7: effect of Isopar M (10%) on formation of PUFAs, wherein the increase in % is shown as compared to the numbers without the use of Isopar M in the second phase (i.e. without the use of a two-phase culture system: "control"), which is set as 100%For more details, see text.

Strain	**PUFA increase**
control	100
with Isopar M	10%

Claims

1. Fermentative production of isoprenoids, lipids or sterols as backbone in a two-phase culture system, comprising in situ extraction of said isoprenoids, lipids or sterols, or a partial in situ extraction of said lipids, wherein a suitable host cell is cultivated in the presence of a carbon source and a lipophilic solvent, wherein the lipophilic solvent is different from the carbon source and with a minimal loss of solvent during the fermentation process, and wherein the isoprenoid is selected from vitamin E and the sterol is selected from vitamin D3 and the lipid is selected from polyunsaturated fatty acids (PUFAs) including derivatives and intermediates of vitamin E, PUFAs or vitamin D3.

2. The process according to claim 1, wherein the lipophilic solvent is not selected from Drakeol, silicone oil or n-dodecane.

3. The process according to claim 1 or 2, wherein the lipophilic solvent present at the end of the fermentation is in the range of at least 80% of the solvent present at the start of the fermentation process.

4. The process according to any one of claims 1 or 3, wherein the lipophilic solvent comprises isoparaffins.

5. The process according to claim 4, wherein the lipophilic solvent comprises a percentage of less than 30% (v/v) of isoparaffins.

6. The process according to any one of claims 1 or 5, wherein the fermentation product is selected from the group consisting of n-3 or n-6 PUFAs, 7-dehydrocholesterol (7-DHC), 25-hydroxycholecalciferol (HyD), 25-7-DHC (HyDHC), zymosterol, tocotrienols, and tocopherols.

6.2 The process of the previous claim, wherein the PUFA is selected from arachidonic acid (ARA), docosohexaenoic acid (DHA), eicosapentaenoic acid (EPA), or combinations thereof, preferably a combination of DHA and EPA.

7. The process according to any one of claims 1 to 5, wherein the carbon source is selected from linear alkanes, free fatty acids, ethanol, glucose, including triglycerides, particularly vegetable oil, such as e.g. selected from the group consisting of oil originated from corn, soy, olive, sunflower, canola, cottonseed, rapeseed, sesame, safflower, grapeseed or mixtures thereof, including the respective free fatty acids, such as e.g. oleic acid, palmitic acid or linoleic acid.

8. The process according to any one of claims 1 to 7, wherein 20% or less of the solvent is lost during the fermentation.

9. The process according to any one of claims 1 to 8, wherein the consumption of the solvent by the host cell is reduced by at least about 50%.

10. The process according to claim 8, wherein the evaporation of the solvent is reduced by at least about 50%.

11. The process according to any one of claims 1 to 10 for production of a mix of sterols comprising 7-DHC and HyDHC, wherein the percentage of HyDHC based on total sterols in the mix is in the range of at least about 65%, such as e.g. 70, 75, 80, 85, 90, 95 or more HyDHC based on total sterols in the mix.

12. The process according to any one of claims 1 to 11, wherein the formation of by-products including zymosterol is reduced by at least about 5%, such as e.g. about 10, 12, 15, 20, 25, 30, 35, 40, 45, 50, 5, 60, 65, 70% or more as compared to a process without the addition of said lipophilic solvent as defined herein.

13. The process according to any one of claims 1 to 11, wherein the wherein the percentage of HyDHC could be increased by at least about 15%, such as e.g. about 20, 30, 40, 50, 60, 70, 80, 90, 100, 120, 130, 140, 150% or more as compared to a process without the addition of said lipophilic solvent as defined herein.

14. The process according to any one of claims 1 to 13, wherein the host cell is selected from fungal host cells, particularly oleaginous host cells, such as e.g. yeast, preferably selected from Yarrowia or Saccharomyces.