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(54) Titre : PROCEDE DE PRODUCTION D'ACIDE GRAS POLYINSATURE UTILISANT UNE NOUVELLE TECHNIQUE  
DE CONSERVATION CELLULAIRE  
(54) Title: METHOD FOR POLYUNSATURATED FATTY ACID PRODUCTION USING NOVEL CELL PRESERVATION  
TECHNIQUE

(57) **Abrégé/Abstract:**

A method for preservation of a microorganism capable of microbial production of a polyunsaturated fatty acid or a compound comprising a polyunsaturated fatty acid as a constituent fatty acid, which method comprises: (a) forming spores in a spore-forming medium at pH 4-7 containing a nutrient source comprising an inorganic salt and a saccharide; (b) suspending the spores obtained in (a) in sterilized water, or sterilized water containing a surfactant and/or an inorganic salt to prepare a spore suspension, and adding a cryoprotectant at 5-15% to prepare a cryopreserving spore suspension; and (c) preserving the cryopreserving spore suspension obtained in (b) at between -100°C and -20°C.



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(54) Title: METHOD FOR POLYUNSATURATED FATTY ACID PRODUCTION USING NOVEL CELL PRESERVATION TECHNIQUE

(57) Abstract: A method for preservation of a microorganism capable of microbial production of a polyunsaturated fatty acid or a compound comprising a polyunsaturated fatty acid as a constituent fatty acid, which method comprises: (a) forming spores in a spore-forming medium at pH 4-7 containing a nutrient source comprising an inorganic salt and a saccharide; (b) suspending the spores obtained in (a) in sterilized water, or sterilized water containing a surfactant and/or an inorganic salt to prepare a spore suspension, and adding a cryoprotectant at 5-15% to prepare a cryopreserving spore suspension; and (c) preserving the cryopreserving spore suspension obtained in (b) at between -100°C and -20°C.

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## DESCRIPTION

METHOD FOR POLYUNSATURATED FATTY ACID PRODUCTION USING  
NOVEL CELL PRESERVATION TECHNIQUE

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Technical Field

The present invention relates to a method for production of a microbial biomass which includes microorganisms that produce compounds comprising polyunsaturated fatty acids as constituent fatty acids, crude oils and/or crude phospholipids obtained by extraction from the biomass, and refined fats and oils and/or refined phospholipids obtained by refining of the crude oils and/or crude phospholipids, as well as to foods and beverages, therapeutic nutritional supplements, animal feeds and pharmaceuticals which incorporate the biomass and fats or oils (crude oils and/or refined oils) and/or phospholipids (crude phospholipids and/or refined phospholipids).

20

Background Art

Biosynthesis of polyunsaturated fatty acids (hereinafter abbreviated as "PUFA") in humans occurs for two representative series, the  $\omega$ 3 and  $\omega$ 6 series (where  $\omega$  represents the number of the carbon atom having the first double bond, counting from the methyl group end of the fatty acid), and in the case of  $\omega$ 6 fatty acids, for example, linoleic acid (18:2  $\omega$ 6) is converted to  $\gamma$ -linolenic acid (18:3  $\omega$ 6), dihomo- $\gamma$ -linolenic acid (20:3  $\omega$ 6), arachidonic acid (20:4  $\omega$ 6) and 4,7,10,13,16-docosapentaenoic acid (22:5  $\omega$ 6), by repeated desaturation and carbon chain elongation.

30

Similarly, in the case of  $\omega$ 3 fatty acids,  $\alpha$ -linolenic acid (18:3  $\omega$ 3) is converted to eicosapentaenoic acid (20:5  $\omega$ 3), 7,10,13,16,19-docosapentaenoic acid (22:5

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$\omega$ 3) and 4,7,10,13,16,19-docosapentaenoic acid (22:6  $\omega$ 3), by repeated desaturation and carbon chain lengthening. The  $\omega$ 3 PUFAs eicosapentaenoic acid (hereinafter, "EPA") and docosapentaenoic acid (hereinafter, "DHA") in particular are known to have numerous physiological functions including prophylactic effects against adult diseases such as atherosclerosis and thrombosis or anticancer effects, as well as learning reinforcement effects, and various attempts have been made to utilize them in pharmaceuticals and specific health foods. However, the physiological functions of PUFAs other than  $\omega$ 3 types (such as  $\omega$ 6 and  $\omega$ 9) have recently also been the subject of attention.

Arachidonic acid constitutes approximately 10% of the fatty acid components of vital organs such as the blood and liver (for example, the fatty acid compositional ratio of the phospholipids in human blood is 11% arachidonic acid, 1% eicosapentaenoic acid, 3% docosapentaenoic acid), and as a major structural component of cell membranes, it contributes to modulating membrane fluidity and performs various metabolic functions in the body, while also playing an important role as a direct precursor of prostaglandins. Recently the roles of arachidonic acid as a nursing infant nutrient and as a constituent fatty acid of endogenous cannabinoids which exhibit neurergic effects (2-arachidonoyl monoglycerol and anandamide) have been noted. Normally, ingestion of linoleic acid-rich foods leads to their conversion to arachidonic acid, but since the functions of the enzymes involved in its biosynthesis are reduced in patients with adult diseases or preliminary conditions as well as in infants and the elderly, such individuals tend to be deficient in arachidonic acid; it has therefore been desirable to provide means for its direct ingestion in the form of a constituent fatty acid of fats or oils (triglycerides).

Although fish oils are abundant sources of  $\omega$ 3 PUFAs such as EPA and DHA,  $\omega$ 6 PUFAs such as  $\gamma$ -linolenic acid, dihomogamma-linolenic acid, arachidonic acid and 4,7,10,13,16-docosapentaenoic acid (22:5  $\omega$ 6) are  
5 virtually unobtainable from traditional fat or oil sources, and therefore fats and oils comprising PUFAs as constituent fatty acids (hereinafter referred to as "PUFA-containing fats and oils") obtained by fermentation of microorganisms are most commonly used at the current  
10 time. For example, methods have been proposed for obtaining fats and oils comprising arachidonic acid as a constituent fatty acid (hereinafter referred to as "arachidonic acid-containing fats and oils") by culturing of various microorganisms capable of producing  
15 arachidonic acid-containing fats and oils.

It is known that fats and oils having a high proportion of arachidonic acid constituting the fatty acid portion (hereinafter referred to as "arachidonic acid-rich fats and oils") can be obtained by using  
20 microorganisms belonging to the genus *Mortierella* (Japanese Unexamined Patent Publication SHO No. 63-44891, Japanese Unexamined Patent Publication SHO No. 63-12290). In recent years, one of the essential uses of arachidonic acid is in the field of nursing infant nutrition, for  
25 example, and specifically the use of arachidonic acid-containing fats and oils obtained by fermentation in modified milk has been introduced. New effects of arachidonic acid-containing fats and oils have also been demonstrated (Japanese Unexamined Patent Publication No.  
30 2003-48831: Composition with prophylactic or ameliorative effect on symptoms and conditions associated with brain function impairment), and these are expected to be in high demand in the future.

Fats and oils obtained by culturing of *Mortierella*  
35 microorganisms largely consist of triglycerides (approximately 70% or greater) and phospholipids. The

edible fats and oils are in the form of triglycerides, and for the purpose of the use described above, the original fats and oils produced by the cells (fats and oils obtained by extraction from cells, known as "crude oils") are extracted from the cell biomass produced by culturing of the microorganisms, and then the crude oils are subjected to edible fat/oil refining steps (degumming, deoxidation, deodorization, decolorizing) to obtain refined fats and oils minus the phospholipids.

10           Since PUFA-containing fats and oils obtained by culturing of *Mortierella* microorganisms accumulate in hyphae, culturing must be carried out to a higher concentration to increase the yield of the PUFA-containing fats and oils per culture, for increased economy of the fat/oil production. The PUFA-containing fat and oil yield per culture is the product of the cell concentration and the PUFA-containing fat/oil content per cell, and it is therefore necessary to increase both the cell concentration and the PUFA-containing fat/oil content per cell. The cell concentration can be increased by raising the concentration of the medium nitrogen source, which is normally converted to cell components.

25           The PUFA-containing fat/oil content per cell can only be increased by satisfactorily controlling the cell morphology and by carrying out the culturing with an adequate oxygen supply. Methods reported for controlling the cell morphology include optimization of the medium salt composition (Japanese Domestic Re-publication No. 30 98/029558), while methods of supplying oxygen include pressurized culturing methods and oxygen enriched aerobic culturing methods (Japanese Unexamined Patent Publication HEI No. 06-153970). However, since these methods are affected by slight differences in the culturing conditions, it is not easy to ensure reproducibility of 35 culturing and as a result, stable production output has not been possible to achieve.

Patent document 1: Japanese Unexamined Patent  
Publication SHO No. 63-44891

Patent document 2: Japanese Unexamined Patent  
Publication SHO No. 63-12290

5 Patent document 3: Japanese Unexamined Patent  
Publication No. 2003-48831

Patent document 4: Japanese Domestic Re-publication  
No. 98/029558

10 Patent document 5: Japanese Unexamined Patent  
Publication HEI No. 06-153970

#### Disclosure of the Invention

15 Thus, in order to ensure stable production of PUFA-  
containing fats and oils by microorganisms, it is highly  
desirable to develop a method for ensuring  
reproducibility of culturing.

20 The present inventors conducted diligent research on  
the initial culturing stage conditions affecting the  
cultured cell growth phase during production of PUFA-  
containing fats and oils (triglycerides) and/or PUFA-  
containing phospholipids by culturing of microorganisms,  
and as a result discovered that by improving the  
transplanting conditions for hyphae or spores from  
previous steps, it is possible to increase the  
25 reproducibility of culturing and to achieve stable  
production of PUFA-containing fats and oils  
(triglycerides) and/or PUFA-containing phospholipids.

30 According to the invention, therefore, there is  
provided a method for production of PUFA-containing fats  
and oils (triglycerides) and/or PUFA-containing  
phospholipids and/or PUFA-containing cells is based on  
improved reproducibility of culturing and stable  
production of PUFA-containing fats and oils  
(triglycerides) and/or PUFA-containing phospholipids, the  
35 method being characterized by improving the transplanting  
conditions for hyphae or spores from previous steps.

Specifically, the present invention provides a

method for preservation of microorganisms capable of microbial production of polyunsaturated fatty acids or compounds comprising polyunsaturated fatty acids as constituent fatty acids, which method comprises:

5 (a) forming spores in a spore-forming medium at pH 4-7 containing a nutrient source comprising inorganic salts and saccharides;

(b) suspending the spores obtained in (a) above in sterilized water, or sterilized water containing a  
10 surfactant and/or inorganic salts to prepare a spore suspension, and adding a cryoprotectant at 5-15% to prepare a cryopreserving spore suspension; and

(c) preserving the cryopreserving spore suspension obtained in (b) above at between -100°C and -20°C.

15 In this method, the inorganic salts are preferably one or more inorganic salts selected from the group consisting of sodium nitrate, dipotassium hydrogen phosphate, magnesium sulfate, potassium chloride and iron (I) sulfate, and the spore-forming medium is preferably a  
20 Czapek agar medium or Czapek-Dox agar medium adjusted to a pH of 4-7.

The cryoprotectant used for this method is preferably glycerin.

25 Examples of polyunsaturated fatty acids or compounds comprising polyunsaturated fatty acids as constituent fatty acids include triglycerides comprising polyunsaturated fatty acids as constituent fatty acids and phospholipids comprising polyunsaturated fatty acids as constituent fatty acids, where the polyunsaturated  
30 fatty acids are preferably  $\omega$ 6 unsaturated fatty acids,  $\omega$ 3 polyunsaturated fatty acids or  $\omega$ 9 polyunsaturated fatty acids, or combinations thereof.

The aforementioned  $\omega$ 6 unsaturated fatty acids are preferably 9,12-octadecadienoic acid (linoleic acid)  
35 18:2 $\omega$ 6, 6,9,12-octadecatrienoic acid ( $\gamma$ -linolenic acid) 18:3 $\omega$ 6, 8,11,14-eicosatrienoic acid (dihomo- $\gamma$ -linolenic



acid) 20:3 $\omega$ 6, 5,8,11,14-eicosatetraenoic acid  
(arachidonic acid) 20:4 $\omega$ 6, 7,10,13,16-docosatetraenoic  
acid 22:4 $\omega$ 6 or 4,7,10,13,16-docosapentaenoic acid 22:5 $\omega$ 6.

The aforementioned  $\omega$ 3 unsaturated fatty acids are  
5 preferably 9,12,15-octadecatrienoic acid ( $\alpha$ -linolenic  
acid) 18:3 $\omega$ 3, 6,9,12,15-octadecatetraenoic acid  
(stearidonic acid) 18:4 $\omega$ 3, 11,14,17-eicosatrienoic acid  
(dihomo- $\alpha$ -linolenic acid) 20:3 $\omega$ 3, 8,11,14,17-  
eicosatetraenoic acid 20:4 $\omega$ 3, 5,8,11,14,17-  
10 eicosapentaenoic acid 20:5 $\omega$ 3, 7,10,13,16,19-  
docosapentaenoic acid 22:5 $\omega$ 3 or 4,7,10,13,16,19-  
docosahexaenoic acid 22:6 $\omega$ 3.

The aforementioned  $\omega$ 9 unsaturated fatty acids are  
preferably 6,9-octadecadienoic acid 18:2 $\omega$ 9, 8,11-  
15 eicosadienoic acid 20:2 $\omega$ 9 or 5,8,11-eicosatrienoic acid  
(Mead acid) 20:3 $\omega$ 9.

The microorganism used for the method described  
above is preferably one belonging to the genus  
*Mortierella*, such as *Mortierella alpina*.

20 The present invention relates to a method for  
production of polyunsaturated fatty acids or compounds  
comprising polyunsaturated fatty acids as constituent  
fatty acids, characterized by using microorganisms  
preserved by the method described above.

25

#### Best Mode for Carrying Out the Invention

More specifically, one of the features of time-  
dependent change of *Mortierella* filamentous fungi in  
liquid culture is increase in cellular mass by cell  
30 proliferation (cell growth stage). Intracellular  
accumulation of PUFA-containing fats and oils increases  
after the increase in cellular mass has essentially  
ceased (fat/oil accumulation stage), eventually resulting  
in abundant intracellular accumulation of PUFA-containing  
35 fats and oils. The present inventors have already

reported culturing with a cell growth phase of approximately two days followed by six days of the fat/oil accumulation stage (J. Biosci. Bioeng., 87:489-494 (1999)).

5           It was also reported that the cell morphology is essentially determined during the cell growth phase, suggesting the extreme importance of setting and managing the initial culturing conditions. However, even though it appears to be important to set and manage the initial  
10           culturing conditions, no published reports can be found with regard to setting of the initial culturing conditions, and particularly the transplanting conditions for hyphae or spores. Focusing on this aspect, the present inventors conducted diligent research which led  
15           to the discovery that the transplanting method has a major effect on the results of culturing and that improving the transplanting method contributes significantly to improving the productivity of PUFA-containing fats and oils.

20           In order to obtain compounds comprising polyunsaturated fatty acids as constituent fatty acids (PUFA-containing fats and oils and/or PUFA-containing phospholipids) by liquid culturing of microorganisms, a small amount of cells of the preserved strain are first  
25           seeded in the culture solution and allowed to proliferate (first seed culturing stage). Next, scaling-up is accomplished by successive transfer to large-volume media, and the main culturing is the final step of culturing in which the microbial mass is recovered to  
30           obtain the PUFA-containing fats and oils. The seed culturing is the culturing at each stage during scaling-up by successive subculturing.

          Known methods of preserving cells include methods wherein cells cultured on agar slant medium are preserved  
35           in a 5°C refrigerator or a -20°C freezer, methods wherein a filamentous fungal spore suspension is preserved in a 5°C refrigerator, methods wherein a cryoprotectant is

added and the cells are preserved in a liquid nitrogen freezer or at an ultra low temperature of between -150°C and -196°C created with liquid nitrogen, methods wherein cells are soil cultured and dried, and methods wherein  
5 cells are freeze-dried and refrigerated ("Hakko Kogaku no Kiso [Fundamentals of Fermentation Engineering]" (1988), translated by Ishizaki, F., Center for Academic Publications, Japan).

Also, "Maintaining cultures for Biotechnology and  
10 Industry (1996)", edited by J.C. Hunter-Cevera & A. Belt, Academic Press, compares -20°C preservation at a slant, liquid nitrogen preservation and freeze-dried preservation and summarizes the effects on survival rates and productivity maintenance. According to this report,  
15 methods of slant preservation at -20°C provide the most satisfactory survival rates and productivity maintenance (p.25).

For filamentous fungi, the report teaches that it is difficult to stably maintain the cells in a live, pure  
20 state, and that although no method exists which can be broadly applied to all filamentous fungi, preservation in liquid nitrogen may be considered ideal (ibid, p.105). A list of examples of using various cryoprotectants is also given, and all of the prior art techniques are  
25 combinations of liquid nitrogen preservation or freeze-drying (ibid, p.118, Table 4). Since the boiling point of liquid nitrogen at 1 atmospheric pressure is -195.8°C., the temperature in the liquid nitrogen storage tube is kept at about -196°C.

30 For filamentous fungi of the genus *Mortierella*, the preservation method and method of transfer to seed culture used is slant refrigerating preservation on Czapek agar medium, in an article by the present inventors (J. Am. Oil Chem. Soc., 75:1501-1505(1998)).  
35 In an article by Park et al. (Biotechnol. Bioprocess Eng., 6:161-166(2001)), the method employed involves

preparation of a spore suspension at  $10^3$  spores/mL by agar medium slant and transfer thereof to medium.

In light of the major importance of setting and managing the conditions for the initial culturing, the present inventors also focused on and diligently  
5 researched the importance of cell preservation which likely affects the initial culturing. As a result, it was discovered that a method of adding a cryoprotectant and preserving the mixture at between  $-100^{\circ}\text{C}$  and  $-20^{\circ}\text{C}$  is  
10 effective as a new method distinct from slant preservation, liquid nitrogen preservation or freeze-drying which have been reported in prior literature, while a method for preparing excellent spore suspensions was also discovered.

It is an object of the present invention to provide a method for production of PUFA-containing fats and oils (triglycerides) and/or PUFA-containing phospholipids and/or PUFA-containing cells, whereby PUFA-containing  
15 fats and oils (triglycerides) and/or PUFA-containing phospholipids are produced in a stable manner with improved reproducibility of culturing, the method being characterized by transferring and culturing cells preserved by a novel preserved cell preparation method and preservation method.  
20

The present invention relates to production of compounds comprising polyunsaturated fatty acids as constituent fatty acids (fats and oils (triglycerides) and/or phospholipids) and to a method for production of microbial cells which produce the compounds (fats and  
25 oils (triglycerides) and/or phospholipids), by transfer and culturing of cells preserved by a novel preserved cell preparation method and preservation method.  
30

Thus, culturing of a microorganism capable of producing compounds comprising polyunsaturated fatty acids as constituent fatty acids (fats/oils  
35 (triglycerides) and/or phospholipids) is essential. The microorganism referred to here is preferably a

microorganism which produces at least one type of polyunsaturated fatty acid from among  $\omega$ 6 polyunsaturated fatty acids having 18 or greater number of carbon and three or more double bonds,  $\omega$ 9 polyunsaturated fatty acids having 18 or greater number of carbon and two or more double bonds and  $\omega$ 3 polyunsaturated fatty acids having 18 or greater number of carbon and three or more double bonds, as the major constituent fatty acid of the triglycerides and/or phospholipids.

As  $\omega$ 6 polyunsaturated fatty acids having 18 or greater number of carbon and three or more double bonds there may be mentioned  $\gamma$ -linolenic acid (6,9,12-octadecatrienoic acid), dihomo- $\gamma$ -linolenic acid (8,11,14-eicosatrienoic acid), arachidonic acid (5,8,11,14-eicosatetraenoic acid), 7,10,13,16-docosatetraenoic acid (22:4  $\omega$ 6) and DPA $\omega$ 6 (4,7,10,13,16-docosapentaenoic acid), as  $\omega$ 9 polyunsaturated fatty acids having 18 or greater number of carbon and two or more double bonds there may be mentioned 6,9-octadecadienoic acid, 8,11-eicosadienoic acid and Mead acid (5,8,11-eicosatrienoic acid), and as  $\omega$ 3 polyunsaturated fatty acids having 18 or greater number of carbon and three or more double bonds there may be mentioned  $\alpha$ -linolenic acid (9,12,15-octadecatrienoic acid), 6,9,12,15-octadecatetraenoic acid (18:4 $\omega$ 3), 8,11,14,17-eicosatetraenoic acid (20:4 $\omega$ 3), EPA (5,8,11,14,17-eicosapentaenoic acid), DPA $\omega$ 3 (7,10,13,16,19-docosapentaenoic acid) and DHA (4,7,10,13,16,19-docosahexaenoic acid).

According to the invention, therefore, any microorganism may be used which can produce a compound comprising a polyunsaturated fatty acid as a constituent fatty acid (fat/oil (triglyceride) and/or phospholipid). As examples of microorganisms capable of producing oils and fats (triglycerides) containing arachidonic acid as a constituent fatty acid there may be mentioned fungi

belonging to the genera *Mortierella*, *Conidiobolus*, *Pythium*, *Phytophthora*, *Penicillium*, *Cladosporium*, *Mucor*, *Fusarium*, *Aspergillus*, *Rhodotorula*, *Entomophthora*, *Echinosporangium* and *Saprolegnia*.

5 As examples of fungi belonging to the genus *Mortierella*, subgenus *Mortierella*, there may be mentioned *Mortierella elongata*, *Mortierella exigua*, *Mortierella hygrophila* and *Mortierella alpina*. More specifically, there may be mentioned the strains *Mortierella elongata*  
10 IFO8570, *Mortierella exigua* IFO8571, *Mortierella hygrophila* IFO5941, and *Mortierella alpina* IFO8568, ATCC16266, ATCC32221, ATCC42430, CBS219.35, CBS224.37, CBS250.53, CBS343.66, CBS527.72, CBS529.72, CBS608.70, CBS754.68, etc.

15 As examples of microorganisms capable of producing DHA there may be mentioned microorganisms belonging to the genera *Cryptocodenum*, *Thrautochytrium*, *Schizochytrium*, *Ulkenia*, *Japonochytrium* and *Haliphthoros*.

All of these strains may be acquired without any  
20 special restrictions from the Institute for Fermentation, Osaka (IFO), American Type Culture Collection (ATCC) or Centralbureau voor Schimmelcultures (CBS). There may also be used the strains *Mortierella alpina* 1S-4 and *Mortierella elongata* SAM0219 (FERM-P 8703) (FERM-BP  
25 1239), isolated from soil by the research group for the present invention.

For culturing of a strain to be used for the invention, it is necessary to first prepare preserved cells of the obtained strain. The method of preparing  
30 the preserved cells involves first preparing a spore-forming medium. The spore-forming medium is produced by preparing a medium comprising some or all of the following components: sodium nitrate, dipotassium hydrogen phosphate, magnesium sulfate, potassium chloride  
35 and iron (I) sulfate, with saccharides, and adjusting the pH to the range of 4-7, and preferably 5-6.5. After adding agar to the prepared medium and conducting heat

sterilization, the cooled solid is used as the spore-forming medium. The spore-forming medium is not particularly restricted so long as it allows hyphal growth and spore formation, but it is generally characterized by having a pH range of 4-7 which is suitable for spore formation.

As a specific example, there may be mentioned a medium obtained by adding hydrochloric acid or sulfuric acid to Czapek agar medium (2 g/L sodium nitrate, 1 g/L dipotassium hydrogen phosphate, 0.5 g/L magnesium sulfate heptahydrate, 0.5 g/L potassium chloride, 0.01 g/L iron (I) sulfate heptahydrate, 30 g/L saccharose, 13 g/L agar), to adjust the pH to 6.0. As another example there may be mentioned a medium obtained by adding hydrochloric acid or sulfuric acid to Czapek-Dox agar medium (2 g/L sodium nitrate, 1 g/L dipotassium hydrogen phosphate, 0.5 g/L magnesium sulfate heptahydrate, 0.5 g/L potassium chloride, 0.01 g/L iron (I) sulfate heptahydrate, 30 g/L glucose, 13 g/L agar), to adjust the pH to 6.0.

This method is used to prepare a slant medium or a plate medium, and the hyphae or spores are inoculated into the medium for solid culturing under aerobic conditions. The culturing temperature is kept at 0-40°C, preferably 10-35°C and more preferably 15-30°C for cell growth and spore formation. The culturing temperature may be changed during the course of culturing, and for example, growth at 25°C may be followed by culturing at 5°C.

After confirming spore formation, sterilized water is added to the solid cultured hyphae and the mixture is agitated by an ordinary method to obtain a spore suspension. There are no particular restrictions on the additives in the added sterilized water, and instead of purified water there may be used water containing added surfactants, inorganic salts or the like, or prepared saline may be used. The agitating method may involve

simple application of external force to the solid culturing vessel, or force may be applied directly to the hyphae with a sterilized brush or the like.

5 The spore suspension or suspension of spores and hyphae which is obtained in this manner is used as the preservation stock solution. The preservation stock solution may also be diluted with sterilized water or with a solution containing surfactants or inorganic salts for preparation of the final preservation stock solution.  
10 Next, a cryoprotectant is added to the preservation stock solution. The cryoprotectant is not particularly restricted so long as it is a commonly used one, and there may be added one or more selected from among agar powder, fetal bovine serum, DMSO, glycerin, inositol,  
15 polyvinyl alcohol, skim milk and the like. As a specific example, glycerin may be added to the cryoprotectant to a glycerin concentration of 10% in the preservation solution.

After adding the cryoprotectant, the preservation  
20 solution is dispensed into a preservation container. The container is suitably a sterilized plastic tube or the like. As a specific example, the preservation solution is dispensed into a 1.2 mL volume cryogenic vial by a sterile procedure. Next, the preservation solution is  
25 stored in an ultra low temperature freezer. The temperature inside the ultra low temperature freezer is controlled to a range of between  $-100^{\circ}\text{C}$  and  $-20^{\circ}\text{C}$ , preferably between  $-90^{\circ}\text{C}$  and  $-30^{\circ}\text{C}$  and more preferably between  $-85^{\circ}\text{C}$  and  $-50^{\circ}\text{C}$ .

30 The cells stored in the ultra low temperature freezer in this manner can be stably preserved for long periods.

When the preserved cells are to be used for culturing, the preservation solution is first thawed.  
35 The thawing should be carried out as rapidly as possible, preferably within 30 minutes at a temperature of no



higher than 40°C, more preferably within 30 minutes at a temperature of no higher than 30°C and even more preferably within 10 minutes at a temperature of no higher than 30°C.

5           The preservation solution thawed in this manner is transferred to liquid medium for culturing. The carbon source used may be a common one such as glucose, fructose, xylose, saccharose, maltose, soluble starch, molasses, glycerol, mannitol or saccharified starch,  
10 although there is no limitation to these.

          As nitrogen sources there may be used natural nitrogen sources such as peptone, yeast extract, malt extract, meat extract, casamino acid, corn steep liquor, soybean protein, defatted soybean and cotton seed meal,  
15 as well as organic nitrogen sources including urea or inorganic nitrogen sources such as sodium nitrate, ammonium nitrate and ammonium sulfate, among which there may be particularly mentioned nitrogen sources obtained from soybean, and specifically soybean, defatted soybean,  
20 soybean flakes, edible soybean protein, okara, soy milk, soy flour and the like. Especially preferred for use is heat denatured defatted soybean, and more preferably one or more different types of defatted soybean heat treated at about 70-90°C and depleted of the ethanol-soluble  
25 components, optionally in combination with any of the nitrogen sources mentioned above.

          If necessary, trace nutrients including phosphate ion, potassium ion, sodium ion, magnesium ion or calcium ion, metal ions such as iron, copper, zinc, manganese,  
30 nickel or cobalt, or vitamins may also be added. Such medium components are not particularly restricted so long as they are in concentrations which do no interfere with growth of the microorganism. For practical applications, the carbon source is generally added at a total  
35 concentration of 0.1-40 wt% and preferably 1-25 wt% and the nitrogen source at a total concentration of 2-15 wt%

and preferably 2-10 wt%, and especially an initial carbon source addition of 1-5 wt% and an initial nitrogen source addition of 3-8 wt%, with further feeding of the carbon and nitrogen sources (more preferably the carbon source  
5 alone) during culturing.

The yield of the PUFA-containing fat or oil can be increased by using an unsaturated fatty acid precursor, for example, a hydrocarbon such as hexadecane or octadecane; a fatty acid such as oleic acid or linoleic  
10 acid or a salt thereof, a fatty acid ester such as an ethyl ester, glycerin fatty acid ester or sorbitan fatty acid ester, or a fat or oil such as olive oil, soybean oil, rapeseed oil, cottonseed oil or coconut oil, either alone or in combinations. Addition of the substrate may  
15 be at 0.001-10% and preferably 0.05-10% with respect to the medium. Such substrates may also be used as the sole carbon source for culturing.

The culturing temperature for the microorganism which produces the PUFA-containing fat or oil will differ  
20 depending on the microorganism used, and may be 5-40°C and preferably 20-30°C, or cells grown by culturing at 20-30°C may be subsequently cultured at 5-20°C to produce PUFA-containing fats and oils. Such temperature control can also increase the proportion of PUFAs of the constituent  
25 fatty acids in the PUFA-containing fats and oils. Seed culturing may be carried out by jar fermenter culturing, shake culturing or stationary liquid culturing, and jar fermenter culturing is carried out for the main culturing. The medium pH at the start of the main  
30 culturing (upon transfer of the seed culture) is adjusted to 5-7, and preferably 5.5-6.5. The culturing period for each stage of seed culturing will normally be 1-10 days, preferably 1-5 days and more preferably 1-3 days. The culturing period for the main culturing will normally be  
35 2-30 days, preferably 5-20 days and more preferably 5-15 days.

Microorganisms belonging to the genus *Mortierella* subgenus *Mortierella* are known to produce compounds comprising arachidonic acid as the main constituent fatty acid (fats and oils (arachidonic acid-containing triglycerides) and/or arachidonic acid-containing phospholipids), but through mutagenesis of the  
5 aforementioned strain, the present inventors have succeeded in obtaining a microorganism capable of producing fats and oils comprising dihomo- $\gamma$ -linolenic acid  
10 as the main constituent fatty acid (Japanese Unexamined Patent Publication HEI No. 5-91887), and microorganisms capable of producing fats and oils comprising  $\omega$ 9 polyunsaturated fatty acids as the main constituent fatty acids (Japanese Unexamined Patent Publication HEI No. 5-  
15 91888).

In addition, we have obtained microorganisms having resistance to high-concentration carbon sources (Japanese Unexamined Patent Publication HEI No. 5-9188, Japanese Unexamined Patent Publication HEI No. 10-57085, Japanese  
20 Unexamined Patent Publication HEI No. 5-91886), which are fungi belonging to the genus *Mortierella* subgenus *Mortierella* and capable of producing PUFA-containing fats and oils and/or PUFA-containing phospholipids by culturing using preserved strains which have been stored  
25 by the culturing method, and specifically the novel preserved cell preparation method and storage method, according to the invention. However, the present invention is not limited to fungi belonging to the genus *Mortierella* subgenus *Mortierella*, and the culturing  
30 method of the invention may be applied to microorganisms capable of producing compounds comprising polyunsaturated fatty acids as constituent fatty acids (fats and oils (triglycerides) and/or phospholipids), to obtain the intended microbial biomass, crude oils and/or crude  
35 phospholipids, and refined fats and oils and/or refined phospholipids obtained by refining of the crude oils and/or crude phospholipids.

The method for obtaining the crude oil and/or crude phospholipid from microorganisms having the fat or oil accumulated in the cells may involve treating the fully cultured solution directly or after sterilization, concentration and acidification, and then recovering the cultured cells by ordinary solid/liquid separation means such as natural precipitation, centrifugal separation and/or filtration. The solid/liquid separation can be aided by addition of an aggregating agent or filtering aid. Examples of aggregating agents include aluminum chloride, calcium chloride, algin and chitosan. Diatomaceous earth may be mentioned as a filtering aid. The cultured cells are preferably rinsed, ruptured and dried. The drying may be carried out by freeze drying, blow drying, fluidized bed drying or the like.

The means for recovering the crude oil and/or crude phospholipids from the dried cells may be an organic solvent extraction method or a pressing method, but extraction with an organic solvent under a nitrogen stream is preferred. As organic solvents there may be used ethanol, hexane, methanol, chloroform, dichloromethane, petroleum ether, acetone and the like, or there may be employed alternating extraction with methanol and petroleum ether, or a single-layer solvent system of chloroform-methanol-water. However, the extraction method used to obtain the crude oil and/or crude phospholipid is not limited to the method described above and may instead be any method which accomplishes efficient extraction of cellular fats and oils (triglycerides) and/or phospholipids. For example, extraction with a supercritical CO<sub>2</sub> flow may be employed as an effective means.

By reduced pressure removal of the organic solvent or the supercritical flow components from the extract obtained by extraction using the organic solvent or supercritical flow, it is possible to obtain the target crude oils and/or crude phospholipids. In this case as

well, the crude oils and/or crude phospholipids may be extracted by the same method as from dried cells, but the extraction efficiency may be greater by using a water-compatible solvent such as methanol, ethanol or acetone, or a water-compatible mixture comprising any of these with water and/or another solvent.

The microbial biomass containing polyunsaturated fatty acids as constituent fatty acids obtained according to the invention, or the crude oils and/or crude phospholipids, may be used directly by incorporation into animal feeds. For applications to foods, however, a common fat/oil refining process is preferably used. The fat/oil refining process used may be an ordinary process such as degumming, deoxidation, deodorization, decolorizing, column treatment, molecular distillation, wintering or the like.

An unlimited number of uses exist for the microbial biomass, crude oils, refined fats and oils (triglycerides), crude phospholipids and refined phospholipids of the invention: for example, they may be used as starting materials and additives for foods, beverages, cosmetics and pharmaceuticals. The purposes of use and amounts of use are also completely unrestricted.

As examples of food compositions there may be mentioned ordinary foods, as well as functional foods, nutritional supplements, premature infant modified milk, mature infant modified milk, nursing infant modified milk, infant foods, maternal foods or geriatric foods. As examples of fat/oil-containing foods there may be mentioned natural fat/oil-containing foods such as meat, fish and nuts, foods to which fats/oils are added during preparation, such as soups, foods employing fats/oils as heating media, such as donuts, fat and oil foods such as butter, processed foods to which fats/oils are added during processing, such as cookies, or foods which are sprayed or coated with fats/oils upon finishing, such as

hard biscuits. Such compositions may also be added to agricultural foods, fermented foods, livestock feeds, marine foods and beverages which contain no fats or oils. They may also be in the form of functional foods or pharmaceuticals, and for example, in processed forms such as enteral nutrients, powders, granules, lozenges, oral solutions, suspensions, emulsions, syrups and the like.

#### Examples

10 The present invention will now be explained in greater detail by the following examples, with the understanding that the invention is not limited thereto.

#### Example 1 Method for cryopreservation of spore suspension

15 *Mortierella alpina* 1S-4 was used as an arachidonic acid-producing strain. Stationary culturing was carried out for 7 days at 25°C at a slant in Czapek agar medium (adjusted to pH 6.0 and sterilized) provided in a test tube, and after confirming hyphal growth, the test tube was stored in a refrigerator (4°C) for 10 days.

20 Sterilized water was added to the test tube and the mixture was well agitated to prepare a spore suspension. The spore suspension was appropriately diluted and coated onto a potato dextrose agar medium plate, and a colony counting method was used to count the spores in the spore suspension, giving a result of  $1 \times 10^6$  spores/mL. Next, the spore suspension was diluted 100-fold with sterilized water. The diluted spore suspension, glycerin and water were mixed in the following proportion: diluted spore suspension:glycerin:water = 1:1:8 (by volume) (the water and glycerin were premixed and sterilized). A 1 mL portion of the mixture was placed in a 1.2 mL volume sterilized cryogenic vial and cryopreserved in an ultra low temperature freezer at -80°C.

35 For use of the *M. alpina* in liquid culture, the cryopreserved strain was rapidly thawed in a 25°C

incubator and transferred to the liquid culture.

Comparative Example 1 Method for preparation of spore suspension

5 *Mortierella alpina* 1S-4 was used as an arachidonic acid-producing strain. Stationary culturing was carried out for 7 days at 25°C at a slant in Czapek agar medium (adjusted to pH 6.0 and sterilized) provided in a test tube, and after confirming hyphal growth, the test tube was stored in a refrigerator.

10 For use of the *M. alpina* 1S-4 in liquid culture, sterilized water was added to the test tube and the mixture was agitated well to prepare a spore suspension. The spore suspension was transferred to liquid medium.

15 Example 2 Culturing experiment, difference in reproducibility by preservation method

Culturing was carried out from a seed strain prepared by the method of Example 1 and Comparative Example 1 using *M. alpina* 1S-4.

20 After transferring 0.1 vol% of the preserved seed strain to medium at pH 6.3 containing 1.0% yeast extract and 2.0% glucose, seed culturing was initiated under conditions of 100 rpm reciprocal shaking, 28°C temperature, and culturing was continued for 3 days.

25 Next, 25 L of medium at pH 6.3 containing 5.0% defatted soybean powder, 0.3%  $\text{KH}_2\text{PO}_4$ , 0.1%  $\text{Na}_2\text{SO}_4$ , 0.05%  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.05%  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 1.8% glucose and 0.1% soybean oil was prepared in a 50 L volume jar fermenter, and then 100 mL of the seed culture solution was transferred and culturing was initiated under conditions of 92 rpm  
30 agitation, 26°C temperature, 200 kPa internal pressure and 12.5 L/min airflow. During the culturing, glucose was added at the concentration shown in Table 1, and culturing was continued for 10 days.

Table 1

Time and concentration of glucose feeding (concentration with respect to culture solution)

Feeding time	Feeding concentration
1st day	4.5%
2nd day	4.5%
3rd day	4%
4th day	3%
5th day	3%
6th day	1%

5           Culturing was carried out several times by the same method, at different days after initiating seed strain preservation. The yields of arachidonic acid obtained on the 10th day of culturing for each culture are shown in Table 2.

10           In the case of the seed strain prepared and preserved by the method of Example 1, the arachidonic acid yield reproducibility was satisfactory for each culture. However, poor reproducibility for each culture resulted when using the seed strain prepared and  
15           preserved by the method of Comparative Example 1.



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Table 2  
Results

Seed strain preparation method	Spore suspension cryopreservation (Method of Example 1)	Preparation of spore suspension by slant (Method of Comp. Example 1)
Seed strain preservation method	Cryopreservation of spore suspension	Refrigeration of Czapek agar medium slant solid-cultured hyphae
Days of preservation	1 day	1 day
Arachidonic acid yield	13.9 g/L	13.8 g/L
Days of preservation	30 days	30 days
Arachidonic acid yield	13.0 g/L	13.5 g/L
Days of preservation	30 days	30 days
Arachidonic acid yield	14.2 g/L	10.5 g/L
Days of preservation	60 days	60 days
Arachidonic acid yield	12.9 g/L	14.2 g/L
Days of preservation	60 days	60 days
Arachidonic acid yield	13.6 g/L	10.5 g/L
Days of preservation	90 days	90 days
Arachidonic acid yield	13.5 g/L	13.5 g/L
Days of preservation	90 days	90 days
Arachidonic acid yield	14.1 g/L	10.1 g/L

Example 3 Culturing experiment, change in productivity with prolonged preservation

5

Culturing was carried out from a seed strain prepared by the method of Example 1 and Comparative Example 1 using *M. alpina* 1S-4.

10 After transferring 0.1 vol% of the preserved seed strain to medium at pH 6.3 containing 1.0% yeast extract and 2.0% glucose, seed culturing was initiated under conditions of 100 rpm reciprocal shaking, 28°C temperature, and culturing was continued for 3 days.

15 Next, 25 L of medium at pH 6.3 containing 1.0% yeast extract, 1.8% glucose and 0.1% soybean oil was prepared in a 50 L volume jar fermenter, and then 100 mL of the seed culture solution was transferred and culturing was initiated under conditions of 200 rpm agitation, 28°C temperature, 150 kPa internal pressure and 25 L/min  
20 airflow. During the culturing, glucose was added at the concentration shown in Table 3, and culturing was

continued for 7 days.

Table 3

Time and concentration of glucose feeding (concentration  
with respect to culture solution)

Feeding time	Feeding concentration
1st day	1.5%
2nd day	1.5%
3rd day	1%
4th day	1%

Culturing was carried out several times by the same method, at different days after initiating seed strain preservation. The yields of arachidonic acid obtained on the 7th day of culturing for each culture are shown in Table 4.

In the case of the seed strain prepared and preserved by the method of Example 1, the arachidonic acid productivity was satisfactorily reproduced even with prolonged preservation. In the case of the seed strain prepared and preserved by the method of Comparative Example 1, however, the arachidonic acid productivity tended to fall during prolonged preservation.

Table 4

Results

Seed strain preparation method	Spore suspension cryopreservation (Method of Example 1)	Preparation of spore suspension by slant (Method of Comp. Example 1)
Seed strain preservation method	Cryopreservation of spore suspension	Refrigeration of Czapek agar medium slant solid-cultured hyphae
Days of preservation	1 day	1 day
Arachidonic acid yield	3.7 g/L	3.6 g/L
Days of preservation	4 years	4 years
Arachidonic acid yield	4.0 g/L	2.5 g/L
Days of preservation	8 years	8 years
Arachidonic acid yield	3.9 g/L	2.2 g/L

Example 4 Effect of spore-forming medium pH

*M. alpina* 1S-4 was used as an arachidonic acid-producing strain. Two different Czapek agar media with different pH, having the compositions shown in Table 5, were prepared in test tubes. The pH of the Czapek medium without pH adjustment was measured to be 8.5.

Table 5

	pH adjusted (6.0)	No pH adjustment (*2)
Sodium nitrate (NaNO <sub>3</sub> )	2 g/L	2 g/L
Dipotassium hydrogen phosphate (K <sub>2</sub> HPO <sub>4</sub> )	1 g/L	1 g/L
Magnesium sulfate heptahydrate (MgSO <sub>4</sub> ·7H <sub>2</sub> O)	0.5 g/L	0.5 g/L
Potassium chloride (KCl)	0.5 g/L	0.5 g/L
Iron (I) sulfate heptahydrate (FeSO <sub>4</sub> ·7H <sub>2</sub> O)	0.01 g/L	0.01 g/L
Saccharose	30 g/L	30 g/L
Agar powder	13 g/L	13 g/L
HCl solution	Added (*1)	Not added

\*1: Added for pH adjustment to 6.0

10 \*2: Measured pH: 8.5.

One loop each of the hyphae was transferred to two agar media of different pH and stationary culturing was carried out at 25°C for 7 days, and after confirming hyphal growth, the test tubes were stored in a refrigerator (4°C) for 10 days.

20 Sterilized water was added to each of the test tubes and the mixture was well agitated to prepare a spore suspension. The spore suspension was appropriately diluted and coated onto a potato dextrose agar medium plate, and a colony counting method was used to count the spores in the spore suspension, giving a result of  $1 \times 10^6$  spores/mL obtained in the agar medium at pH 6.0, and  $5 \times 10^4$  spores/mL obtained in the medium without pH adjustment

(measured pH: 8.5).

Spore suspensions prepared by both of these methods were used for culturing by the method of Example 3, and the arachidonic acid yields were compared. As a result, an arachidonic acid yield of 3.5 g/L was obtained from the spore suspension produced in the medium adjusted to pH 6.0, and an arachidonic acid yield of 2.9 g/L was obtained from the medium without pH adjustment (measured pH: 8.5).

#### 10 Example 5 Cryopreservation system

Example 1 was repeated but with the preserved spore suspension prepared in a 1.2 mL volume cryogenic vial stored in a -20°C freezer for condition (5-1), in a -80°C ultra low temperature freezer for condition (5-2) and in liquid nitrogen (approximately -196°C) for condition (5-3). After 30 days of storage under these three conditions, culturing was carried out in the same manner as Example 2. As a result, both the spore survival rate and arachidonic acid productivity were superior under the condition of storage in a -80°C ultra low temperature freezer.

Table 6

Condition	5-1 -20°C storage	5-2 -80°C storage	5-3 Liquid nitrogen storage
Number of spores after thawing	2 x 10 <sup>2</sup> spores/mL	9 x 10 <sup>3</sup> spores/mL	1 x 10 <sup>2</sup> spores/mL
Arachidonic acid yield	9.1 g/L	13.0 g/L	8.7 g/L

#### 25 Example 6 Corroboration of culturing reproducibility of frozen strain by large tank culturing

Culturing was carried out from a seed strain prepared by the method of Example 1 using *M. alpina* 1S-4.

After transferring 0.1 vol% of the preserved strain to medium at pH 6.3 containing 1.0% yeast extract and

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2.0% glucose, seed culturing was initiated under conditions of 100 rpm reciprocal shaking, 28°C temperature (1st stage), and culturing was continued for 3 days.

5 Next, 30 L of medium at pH 6.3 containing 1% yeast extract, 2% glucose and 0.1% soybean oil was prepared in a 50 L volume jar fermenter, and then the seed culture (1st stage) solution was transferred and seed culturing (2nd stage) was initiated under conditions of 200 rpm agitation, 28°C temperature, 150 kPa internal pressure and  
10 12.5 L/min airflow, for 2 days of culturing.

Next, 4500 L of medium (Medium A: 336 kg soybean powder, 16.8 kg  $\text{KH}_2\text{PO}_4$ , 2.8 kg  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 2.8 kg  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 5.6 kg soybean oil) was adjusted to a pH of 4.5 and sterilized under conditions of 121°C, 20 minutes.  
15 As a separate medium there was prepared Medium C by sterilizing 1000 L of medium (Medium B: 112 kg hydrous glucose) at 140°C for 40 seconds and adding it to the previous Medium A. After adjusting Medium C to pH 6.3, a 28 L volume of the seed culture (2nd stage) was  
20 transferred, for a combined total of 5600 L of initial culture solution (10 kL volume culturing tank).

For transfer of the seed culture solution (2nd stage) to the main culturing medium, vapor was passed through the tube connecting the seed culturing tank and  
25 the main culturing tank for sterilization ( $\geq 30$  min at 121-126°C), and then sterile air was introduced through the tube for cooling to a tube surface temperature of below 60°C. After cooling, the seed culture (2nd stage) was allowed passage and transported in a prescribed  
30 volume to the main culturing tank. When transfer of the seed culture to the main culturing medium was completed, culturing was initiated at a temperature of 26°C, an airflow quantity of 49  $\text{Nm}^3/\text{hr}$  and an internal pressure of 200 kPa. The medium was fed during the culturing  
35 according to the schedule shown in the following table, for 306 hours of main culturing. When the culturing was

complete, the culture volume was 7750 L as a result of the increase by medium feeding and loss by evaporation. The arachidonic acid yield per culture upon completion of culturing was 18.2 g/L.

5

Main culturing time	Feeding medium
After 19 hrs	280 kg hydrous glucose/460 L
After 43 hrs	280 kg hydrous glucose/450 L
After 67 hrs	252 kg hydrous glucose/390 L
After 91 hrs	252 kg hydrous glucose/410 L
After 120 hrs	224 kg hydrous glucose/370 L
After 140 hrs	168 kg hydrous glucose/280 L
After 163 hrs	168 kg hydrous glucose/270 L

After completion of culturing, sterilization was carried out under conditions of 120°C, 20 minutes, and then the wet cell mass was recovered with a continuous dehydrator and dried to a moisture content of 1 wt% with an oscillating fluidized bed drier, and the dried cells were transported to a packing location using an air conveyor. The obtained dry cell mass was packed into an approximately 1 m<sup>3</sup> volume aluminum pouch container bag together with nitrogen gas, and the mouth of the bag was heat sealed prior to storage in a cold storage room at below 10°C.

After removal from the container bag, the dry cell mass was subjected to hexane extraction and the hexane solution was filtered to remove the solid portion, after which it was heated under reduced pressure to remove the hexane and obtain a crude oil comprising arachidonic acid as a constituent fatty acid.

The same culturing was repeated three times. The results for the arachidonic yields upon completion of culturing are summarized in Table 7. The reproducibility of the preserved strain was satisfactory, thus confirming that more stable arachidonic acid productivity had been achieved.

30

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Table 7

Summary of arachidonic acid yields upon completion of culturing

Culturing	
1	18.2 g/L
2 (1 year later)	17.8 g/L
3 (5 years later)	17.9 g/L

5 Example 7 Corroboration of culturing reproducibility of frozen dihomono- $\gamma$ -linolenic acid-producing strain

*Mortierella alpina* SAM1860 was used as a dihomono- $\gamma$ -linolenic acid-producing strain. Stationary culturing was carried out for 12 days at 25°C at a slant in Czapek agar medium (adjusted to pH 6.0 and sterilized) provided in a test tube, and after confirming hyphal growth, the test tube was stored in a refrigerator (4°C) for 20 days.

10 Sterilized water was added to the test tube and the mixture was well agitated to prepare a spore suspension. The spore suspension was appropriately diluted and coated onto a potato dextrose agar medium plate, and a colony counting method was used to count the spores in the spore suspension, giving a result of  $5 \times 10^6$  spores/mL.

15 Next, the spore suspension was diluted 100-fold with sterilized water. The diluted spore suspension, glycerin and sterilized water were mixed in the following proportion: diluted spore suspension:glycerin:sterilized water = 1:1:8 (by volume). A 1 mL portion of the mixture was placed in a 1.2 mL volume sterilized cryogenic vial and cryopreserved for one month in an ultra low temperature freezer at -80°C.

20 As a comparative example, a spore suspension prepared by the same method was stored for one month in a refrigerator (5°C).

30 The two preserved strains stored for one month were used for culturing by the same method as in Example 3. As a result, the DGLA yields were as shown in Table 8, indicating the effectiveness of the cryopreservation

method.

Table 8  
Results

Seed strain preparation method	Cryopreservation of spore suspension	Refrigeration of spore suspension
DGLA yield	3.1 g/L	2.7 g/L

5

Example 8 Corroboration of culturing reproducibility of other frozen arachidonic acid-product strains

*Mortierella elongata* IF08570 and *Mortierella alpina* CBS754.68 were used as arachidonic acid-producing strains.

10

Stationary culturing was carried out for 10 days at 25°C at a slant in Czapek agar medium (adjusted to pH 6.0 and sterilized) provided in a test tube, and after confirming hyphal growth, the test tube was stored in a refrigerator (4°C) for 20 days.

15

Sterilized water was added to the test tube and the mixture was well agitated to prepare a spore suspension. The spore suspension was diluted 10-fold with sterilized water. The diluted spore suspension, glycerin and sterilized water were mixed in the following proportion: diluted spore suspension:glycerin:sterilized water = 1:1.5:7.5 (by volume). A 1 mL portion of the mixture was placed in a 1.2 mL volume sterilized cryogenic vial and cryopreserved for one month in an ultra low temperature freezer at -80°C.

25

As a comparative example, a spore suspension prepared by the same method was stored for one month in a refrigerator (5°C).

30

Each of the four preserved strains stored for one month (two different strains x two different preservation methods) was transferred to a medium at pH 6.3 containing 1% yeast extract and 2% glucose, and used for 3 days of seed culturing under conditions of 100 rpm reciprocal shaking, 28°C temperature.



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Next, 25 L of medium (500 g glucose, 775 g defatted soybean powder, 50 g  $\text{KH}_2\text{PO}_4$ , 7.5 g/L  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 7.5 g/L  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 25 g soybean oil, pH 6.0) was prepared in a 50 L volume jar fermenter, and then the seed culture solution was transferred and main culturing was initiated under conditions of 200 rpm agitation, 28°C temperature and 150 kPa internal pressure. The culturing was continued for 186 hours, while adding a 50% glucose solution approximately every 24 hours for a glucose concentration of about 1.2%. As a result, the arachidonic acid yields shown in Table 9 were obtained, indicating the effectiveness of the cryopreservation method.

15

Table 9  
Results

Seed strain preparation method	Cryopreservation of spore suspension	Refrigeration of spore suspension
Arachidonic acid yield		
<i>M. elongata</i> IFO8570	3.4 g/L	2.0 g/L
<i>M. alpina</i> CBS754.68	7.2 g/L	5.1 g/L

Example 9 Comparison of L-drying method and cryopreservation method for 9-year stored samples

20

A spore suspension was prepared, and dry spore ampules were created and preserved by the conventionally known L-drying method ("Maintaining cultures for Biotechnology and Industry (1996)", edited by J.C. Hunter-Cevera & A. Belt, Academic Press, p.115). After creation and storage for 9 years, the six L-dried ampules were opened and their contents restored and coated onto agar medium, and the number of surviving spores were counted. Likewise, the six 1.2 mL volume cryogenic vials prepared by the method of Example 1 (and cryopreserved for 9 years) were opened, and the number of surviving spores was counted.

25

30

The results shown in the table below confirmed that

the preservation method according to Example 1 resulted in a notably superior survival rate compared to the L-drying method of the prior art. It was concluded that the method of the invention is a highly effective method for prolonged preservation of microbial strains.

Table 10

Condition	L-drying method	Cryopreservation
Number of surviving ampoules	3 ampoules survived 3 ampoules died	All 6 tubes survived
Number of surviving spores	2 in surviving ampoules 0 in dead ampoules	200 in tube

## CLAIMS

1. A method for preservation of a microorganism belonging to the genus *Mortierella* capable of microbial production of a polyunsaturated fatty acid or a compound comprising a polyunsaturated fatty acid as a constituent fatty acid, and for producing the polyunsaturated fatty acid or the compound comprising a polyunsaturated fatty acid as a constituent fatty acid by using the microorganism which has been preserved, wherein the method for preservation of the microorganism comprises:

(a) forming spores in a spore-forming medium at pH 4-7 containing a nutrient source comprising an inorganic salt and a saccharide;

(b) suspending the spores obtained in (a) in sterilized water, or sterilized water containing a surfactant and/or an inorganic salt to prepare a spore suspension, and adding a cryopreservant at 5-15% to prepare a cryopreserving spore suspension; and

(c) preserving the cryopreserving spore suspension obtained in (b) at between -100°C and -20°C.

2. A method according to claim 1, wherein said inorganic salt is at least one type of inorganic salt selected from the group consisting of sodium nitrate, dipotassium hydrogen phosphate, magnesium sulfate, potassium chloride and iron (I) sulfate.

3. A method according to claim 1 or 2, characterized in that said spore-forming medium is Czapek agar medium or Czapek-Dox agar medium adjusted to a pH of 4-7.

4. A method according to any one of claims 1 to 3, characterized in that said cryopreservant is glycerin.

5. A method according to any one of claims 1 to 4, characterized in that said polyunsaturated fatty acid or compound comprising a polyunsaturated fatty acid as a constituent fatty acid is a triglyceride comprising a polyunsaturated fatty acid as a constituent fatty acid or a phospholipid comprising a polyunsaturated fatty acid as a constituent fatty acid.

6. A method according to any one of claims 1 to 5, characterized in that said polyunsaturated fatty acid is an  $\omega 6$  unsaturated fatty acid,  $\omega 3$  polyunsaturated fatty acid or  $\omega 9$  polyunsaturated fatty acid, or a combination thereof.

7. A method according to claim 6, characterized in that said  $\omega 6$  unsaturated fatty acid is 9,12-octadecadienoic acid (linoleic acid) 18:2 $\omega 6$ , 6,9,12-octadecatrienoic acid ( $\gamma$ -linolenic acid) 18:3 $\omega 6$ , 8,11,14-eicosatrienoic acid (dihomo- $\gamma$ -linolenic acid) 20:3 $\omega 6$ , 5,8,11,14-eicosatetraenoic acid (arachidonic acid) 20:4 $\omega 6$ , 7,10,13,16-docosatetraenoic acid 22:4 $\omega 6$  or 4,7,10,13,16-docosapentaenoic acid 22:5 $\omega 6$ .

8. A method according to claim 6, characterized in that said  $\omega 3$  unsaturated fatty acid is 9,12,15-octadecatrienoic acid ( $\alpha$ -linolenic acid) 18:3 $\omega 3$ , 6,9,12,15-octadecatetraenoic acid (stearidonic acid) 18:4 $\omega 3$ , 11,14,17-eicosatrienoic acid (dihomo- $\alpha$ -linolenic acid) 20:3 $\omega 3$ , 8,11,14,17-eicosatetraenoic acid 20:4 $\omega 3$ , 5,8,11,14,17-eicosapentaenoic acid 20:5 $\omega 3$ , 7,10,13,16,19-docosapentaenoic acid 22:5 $\omega 3$  or 4,7,10,13,16,19-docosahexaenoic acid 22:6 $\omega 3$ .

9. A method according to claim 6, characterized in that said  $\omega$ 9 unsaturated fatty acid is 6,9-octadecadienoic acid 18:2 $\omega$ 9, 8,11-eicosadienoic acid 20:2 $\omega$ 9 or 5,8,11-eicosatrienoic acid (Mead acid) 20:3 $\omega$ 9.

10. A method according to any one of claims 1 to 9, characterized in that said microorganism belonging to the genus *Mortierella* is *Mortierella alpina*.