(19) DANMARK

(10) DK/EP 3760705 T3

(12) Oversættelse af europæisk patentskrift

Patent- og Varemærkestyrelsen

(51) Int.Cl.: *C 12 N 1/12 (2006.01)* *C 12 P 7/64 (2022.01)* *C 12 R 1/89 (2006.01)*

(45) Oversættelsen bekendtgjort den: **2023-01-09**

(80) Dato for Den Europæiske Patentmyndigheds bekendtgørelse om meddelelse af patentet: **2022-11-09**

(86) Europæisk ansøgning nr.: **19825193.6**

(86) Europæisk indleveringsdag: **2019-06-26**

(87) Den europæiske ansøgnings publiceringsdag: **2021-01-06**

(86) International ansøgning nr.: **KR2019007697**

(87) Internationalt publikationsnr.: **WO2020004924**

(30) Prioritet: **2018-06-29 KR 20180075945**

(84) Designerede stater: **AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR**

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(54) Benævnelse: **Hidtil ukendte mikroalgestammer af Thraustochytrium-slægten og fremgangsmåde til fremstilling af flerumættede fedtsyrer ved hjælp af samme**

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DESCRIPTION

Technical Field

[0001] The present disclosure relates to strains of *Thraustochytrium* genus, including a high content of polyunsaturated fatty acids, a biomass produced from the strains, a lipid including the strains, and a method of producing polyunsaturated fatty acids.

Background Art

[0002] Docosahexaenoic acid (DHA), which is a polyunsaturated fatty acid, is a fatty acid essential for brain, eye tissues and nervous systems, and is known to play an important role in the development of visual acuity and motor neuron ability of infants. It was reported that the amount of DHA is significantly reduced in the brain of a dementia patient, and it is newly discovered that DHA has various anti-aging functions such as suppression of macular degeneration in presbyopia Further, it was reported that DHA can also be used as a feed additive for fish (Korean Patent Application Publication No. 10-2007-0040751). Since most higher animals, including humans, cannot smoothly synthesize polyunsaturated fatty acids required for normal biological functions, they must ingest polyunsaturated fatty acids as essential nutrients, and the World Health Organization recommends a steady consumption of DHA-containing polyunsaturated fatty acids at least 1 g/day. Traditionally, the supply sources of DHA polyunsaturated fatty acids are deep sea fish such as tuna and salmon which occupy the top level of the marine ecosystem However, as the pollution of the marine environment becomes worse, the risk of ingestion of deep sea fish is increasing due to the accumulation of pollutants such as mercury, heavy metals, environmental hormones and radioactive substances in the body of deep sea fish. Therefore, as new means to safely and reliably supply DHA polyunsaturated fatty acid oil, microalgae of *Thraustochytrium* genus have very important industrial values.

[0003] Various methods for gene overexpression have been suggested in microalgae of *Thraustochytrium* genus. Transformation technologies of microalgae of *Thraustochytrium* genus using various antibiotic resistance genes as selection markers were reported since genetic transformation methods of microalgae of *Thraustochytrium* genus using acetolactate synthase as a selection marker was first introduced by Martec Corporation. Specifically, Korean Patent Application Publication No. 2015-0084148 discloses "a recombinant vector for increasing the productivity of microalgal biomass and lipid and a use thereof".

[0004] However, up to now, a genetic transformation technology developed from microalgae of *Thraustochytrium* genus is a chromosomal integration method in which genes introduced in common are inserted into chromosomal DNA, and has an advantage of the inserted genes being stably maintained, but has limitations in gene copy number and expression control as

compared with a gene expression method using centromeric or episomal plasmid with self-replication ability.

Disclosure

Technical Problem

[0005] Accordingly, the present inventors have developed microalgae having improved the content and productivity of docosahexaenoic acid by mutating KC01 microalgae of *Thraustochytrium* genus, and have established a biomass including a docosahexaenoic acid-containing lipid and a method of producing a bio-oil by culturing these microalgae. Based on these development and establishment, the present disclosure has been completed.

Technical Solution

[0006] One object of the present disclosure is to provide CJM01 microalgae (deposit number: KCTC 13538BP) of *Thraustochytrium* genus by which the production of docosahexaenoic acid (DHA) increases and the production of amino acid decreases as compared with wild microalgae.

[0007] Another object of the present disclosure is to provide a method of producing a biomass, including steps of: culturing CJM01 microalgae of *Thraustochytrium* genus; and recovering a biomass containing docosahexaenoic acid (DHA) from the microalgae, a cultured product thereof, a dried product thereof, or a pulverized product thereof

[0008] Still another object of the present disclosure is to provide a method of producing a bio-oil, including steps of: culturing CJM01 microalgae of *Thraustochytrium* genus; and recovering a lipid containing docosahexaenoic acid (DHA) from the microalgae, a cultured product thereof, a dried product thereof, or a pulverized product thereof.

Advantageous Effects

[0009] According to the novel CJM01 microalgae of *Thraustochytrium* genus of the present disclosure, the production of an amino acid is remarkably reduced, and the content of a fat in biomass and the content of an unsaturated fatty acid such as docosahexaenoic acid are high, so that the microalgae itself, biomass produced by the culturing and fermentation of microalgae, a condensate of the biomass, and a dried product of the biomass are very useful as a feed composition.

Brief Description of Drawings

[0010]

FIG. 1 is a photograph showing KC01 strains of *Thraustochytrium* genus observed by an optical microscope.

FIG. 2 shows a phylogenetic tree among KC01 strains of *Thraustochytrium* genus, strains of *Thraustochytrium* genus, strains of *Aurantiochytrium* genus, and strains of *Schizochytrium* genus.

Best Mode for Invention

[0011] Hereinafter, the present disclosure will be described in detail.

[0012] Meanwhile, specific structural and functional descriptions of embodiments disclosed herein are only for illustrative purposes of other embodiments. The present disclosure may be embodied in many different forms without departing from the spirit and significant characteristics of the present disclosure. Therefore, the embodiments of the present disclosure are disclosed only for illustrative purposes and should not be construed as limiting the present disclosure.

[0013] In order to accomplish the above objects, an aspect of the present disclosure provides CJM01 microalgae of *Thraustochytrium* genus by which the production of docosahexaenoic acid (DHA) increases and the production of amino acid decreases, as compared with wild microalgae.

[0014] As used herein, the term "strains of *Thraustochytrium* genus" refers to organic heterotrophic microalgae, which play an important role as supply sources of triacylglycerol containing various polyunsaturated fatty acids including docosahexaenoic acid (DHA) at a high concentration. Further, the "microalgae" refer to living organisms that can be seen only through a microscope because it cannot be seen by the naked eye and that floats freely in water, and are also called phytoplankton.

[0015] As used herein, for example, wild KC01 strains of *Thraustochytrium* genus are irradiated with gamma rays to generate mutant strains, strains having improved productivity of oil containing polyunsaturated acids are selected from the mutant strains, and these strains were named as CJM01 strains of *Thraustochytrium* genus, deposited on May 30, 2018 with the Korean Collection for Type Cultures (KCTC), an international depository organization under the Budapest Treaty, and granted the deposit number KCTC 13538BP.

[0016] Further, the CJM01 microalgae of *Thraustochytrium* genus of the present disclosure may have a 18s rRNA of SEQ ID NO. 1, but the present disclosure is not limited thereto.

[0017] As used herein, the term "docosahexaenoic acid (DHA)" is one of polyunsaturated fatty acids represented by Formula $C_{22}H_{32}O_2$, and is a material extracted extensively from blue fish such as tuna or sardine. Further, docosahexaenoic acid (DHA) belongs to omega 3 together with eicosapentaenoic acid (EPA) and α-linolenic acid (ALA).

[0018] The CJM01 microalgae of *Thraustochytrium* genus of the present disclosure may include a large amount of docosahexaenoic acid (DHA), as compared with the KC01 strains of *Thraustochytrium* genus which are parent strains. Specifically, the CJM01 microalgae may include docosahexaenoic acid (DHA) in an amount of 30 wt% to 65 wt%, 30 wt% to 60 wt%, 40 wt% to 65 wt%, or 40 wt% to 60 wt%, based on the total weight of fatty acids included in the microalgae, but the present disclosure is not limited thereto.

[0019] Additionally, the CJM01 microalgae of *Thraustochytrium* genus of the present disclosure may have improved docosahexaenoic acid (DHA) productivity, as compared with the KC01 strains of *Thraustochytrium* genus which are parent strains. The docosahexaenoic acid (DHA) productivity may be measured by the concentration (g/L) of docosahexaenoic acid (DHA) produced for 1 hour. The microalgae of the present disclosure may have a docosahexaenoic acid (DHA) productivity of 0.4 to 0.8 (g/l/h), 0.4 to 0.7 (g/l/h), 0.5 to 0.8 (g/l/h), or 0.5 to 0.7 (g/l/h), but the present disclosure is not limited thereto.

[0020] Meanwhile, in the CJM01 microalgae of *Thraustochytrium* genus of the present disclosure, the production of an amino acid may be reduced, as compared with the KC01 strains of *Thraustochytrium* genus which are parent strains. Specifically, the CJM01 microalgae of *Thraustochytrium* genus of the present disclosure or a culture solution thereof may not include at least one amino acid selected from the group consisting of aspartate, serine, glutamate, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, and arginine. For example, as can be seen in Example 2, aspartate, serine, glutamate, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, and arginine may not be detected from the culture solution of the CJM01 microalgae of *Thraustochytrium* genus of the present disclosure.

[0021] For example, the total production of an amino acid by the CJM01 microalgae of *Thraustochytrium* genus of the present disclosure may be reduced by 90% or more, 95% or more, 97% or more, or 99% or more, as compared with the KC01 strains of *Thraustochytrium* genus which are parent strains. This indicates that the CJM01 strains are more effectively used in the pathway of docosahexaenoic acid biosynthesis as compared with the KC01 parent strains. Specifically, since the CJM01 microalgae of *Thraustochytrium* genus of the present disclosure rarely produce amino acids, the culture solution of the strains may include amino acids only in an amount of 0.1 to 20 mg/L, 0.1 to 15 mg/L, 0.1 to 10 mg/L, 0.1 to 7 mg/L, or 0.1 to 5 mg/L.

[0022] Another aspect of the present disclosure provides a method of producing a biomass, including: culturing CJM01 microalgae of *Thraustochytrium* genus; and recovering a biomass containing docosahexaenoic acid (DHA) from the microalgae, a cultured product thereof, a dried product thereof, or a pulverized product thereof. Further, the biomass may be made in the form of a dry fungus body, but the present disclosure is not limited thereto.

[0023] The present disclosure provides a biomass produced by the method. The biomass may include docosahexaenoic acid (DHA) in an amount of 15 to 40 wt%, 20 to 35 wt%, or 25 to 30 wt%, based on the total weight thereof, but the present disclosure is not limited thereto.

[0024] A still another aspect of the present disclosure provides a method of producing a bio-oil, including: culturing CJM01 microalgae of *Thraustochytrium* genus; and recovering a lipid containing docosahexaenoic acid (DHA) from the microalgae, a cultured product thereof, a dried product thereof, or a pulverized product thereof.

[0025] The present disclosure provides a bio-oil produced by the method. The bio-oil may include docosahexaenoic acid (DHA) in an amount of 30 to 65 wt%, 30 to 60 wt%, 40 to 65 wt%, or 40 to 60 wt%, based on the total weight of fatty acids, but the present disclosure is not limited thereto.

[0026] Specifically, the method of producing a bio-oil according to the present disclosure may include steps of: culturing the CJM01 microalgae of *Thraustochytrium* genus; producing a biomass containing docosahexaenoic acid (DHA) from the microalgae, a cultured product thereof, a dried product thereof, or a pulverized product thereof; and recovering a lipid containing docosahexaenoic acid (DHA) from the produced biomass. However, the present disclosure is not limited thereto.

[0027] The "*Thraustochytrium* genus" and docosahexaenoic acid" are as described above.

[0028] As used herein, the "bio-oil" is obtained from a biomass by a biological, thermochemical, or physiochemical extraction process. The bio-oil produced according to the present disclosure may include polyunsaturated fatty acid, specifically, docosahexanoic acid, but the present disclosure is not limited thereto.

[0029] Additionally, the "biomass" refers to organisms such as plants, animals and microorganisms that can be used as chemical energy, that is, energy sources of bio-energy. In addition, the biomass ecologically also refers to the weight or energy amount of a specific organism exiting within unit time and space. Further, although the biomass includes compounds secreted by cells, it may also include extracellular materials as well as cells and/or intracellular contents. As used herein, the biomass may be the CJM01 microalgae itself of *Thraustochytrium* genus, a cultured product thereof, a dried product thereof, a pulverized product thereof, a product produced by culturing or fermenting the microalgae, or may be a condensate of the biomass or a dried product of the biomass. However, the biomass is not

limited thereto.

[0030] As used herein, the cultured product of the CJM01 microalgae of *Thraustochytrium* genus refers to a product obtained by culturing the microalgae, and specifically, may be a culture solution including the microalgae or a culture solution not including the microalgae, but the present disclosure is not limited thereto. As used herein, the cultured product of the CJM01 microalgae of *Thraustochytrium* genus refers to a product obtained by removing moisture from the microalgae, and specifically, may be made in the form of a dry fungus body, but the present disclosure is not limited thereto. As used herein, the pulverized product of the CJM01 microalgae of *Thraustochytrium* genus collectively refers to a product obtained by pulverizing the microalgae, and may be made in the form of supernatant or pellet, but the present disclosure is not limited thereto.

[0031] The CJM01 microalgae itself of *Thraustochytrium* genus, the cultured product thereof, the dried product thereof, or the pulverized product thereof includes docosahexaenoic acid, and may be used to produce a biomass or a bio-oil.

[0032] As used herein, the term "culturing" means that the microalgae are grown under moderately controlled environmental conditions. The culturing process according to the present disclosure may be performed depending on appropriate culture medium and culturing conditions. Such a culturing process may be easily adjusted by those skilled in the art according to the selected microalgae.

[0033] Specifically, the culturing of the CJM01 microalgae of *Thraustochytrium* genus according to the present disclosure may be performed under heterotrophic conditions, but the present disclosure is not limited thereto.

[0034] As used herein, the "heterotrophic nutrition" is a nutritional form that depends on organic matter obtained from *in-vitro* energy (nutrition) source, and is a term corresponding to independent nutrition. The CJM01 microalgae of *Thraustochytrium* genus according to the present disclosure can improve the amount and productivity of docosahexaenoic acid by optimizing the composition of a culture medium of a carbon source or a nitrogen source under heterotrophic conditions. Further, as used herein, the term "heterotrophic nutrition" may be used interchangeably with "dark culture".

[0035] Additionally, the step of culturing the microalgae is not particularly limited, and may be performed by a known batch culture method, a known continuous culture method, a fedbatch culture method, or the like. The culture medium and other culture conditions used in culturing the microalgae of the present disclosure may be used without limitations as long as they can be generally used to culture the microalgae. Specifically, the microalgae of the present disclosure may be cultured in a general culture medium including a carbon source, a nitrogen source, a phosphorus source, an inorganic compound, amino acids, and/or vitamins while adjusting temperature, pH, and the like under aerobic conditions.

[0036] Specifically, an optimum pH (for example, a pH of 5 to 9, specifically a pH of 6 to 8, and most specifically a pH of 6.8) may be adjusted by using a basic compound (for example, sodium hydroxide, potassium hydroxide, or ammonia) or an acidic compound (for example, a phosphoric acid or a sulfuric acid), but the present disclosure is not limited thereto.

[0037] Further, oxygen or oxygen-containing gas may be injected into a culture to maintain the aerobic state of the culture, or nitrogen gas, hydrogen gas or carbon dioxide gas may be injected into the culture without injecting oxygen or oxygen-containing gas to maintain the anaerobic or non-aerobic state of the culture, but the present disclosure is not limited thereto.

[0038] Further, the culturing temperature may be maintained at 20°C to 45°C, specifically, 25°C to 40°C, and the culturing may be performed for about 10 to 160 hours, but the present disclosure is not limited thereto. In addition, during the culturing, the formation of bubbles may be inhibited by using a deforming agent such as fatty acid polyglycol ester, but the present disclosure is not limited thereto.

[0039] The culturing of the CJM01 microalgae of *Thraustochytrium* genus according to the present disclosure may be performed by using a culture medium including a carbon source and a nitrogen source.

[0040] As used herein, the term "culture medium" refers to a medium for culturing the microalgae of the present disclosure and/or a product obtained after culturing the microalgae. The culture medium may have both a form including the microalgae and a form obtained by removing the microalgae from a culture solution including the microalgae through centrifugation, filtration, or the like.

[0041] Additionally, in the culture medium used in the present disclosure, as carbon sources, sugars and carbohydrates (for example, glucose, sucrose, lactose, fructose, galactose, mannose, maltose, arabinose, xylose, molasses, starch, and cellulose), fats and oils (soybean oil, sunflower seed oil, peanut oil, and coconut oil), fatty acids (for example, palmitic acid, stearic acid, and linoleic acid), alcohols (for example, glycerol and ethanol), and organic acids (for example, acetic acid) may be used individually or in combination. Specifically, the carbon source may be at least one selected from the group consisting of glucose, fructose, maltose, galactose, mannose, sucrose, arabinose, xylose, and glycerol, but is not limited as long as it can be used to culture microalgae. Further, in the culture medium used in the present disclosure, as the carbon source, glucose having a concentration of 10 to 50 g/L, 10 to 40 g/L, 20 to 50 g/L, 20 to 40 g/L, or 25 to 35 g/L may be used, but the present disclosure is not limited thereto.

[0042] The nitrogen sources of the culture medium used in the present disclosure may be classified into organic nitrogen sources and inorganic nitrogen sources, but these organic nitrogen sources and inorganic nitrogen sources may used individually or in combination. Specifically, the nitrogen source may be an organic nitrogen source selected from the group consisting of a yeast extract, a beef extract, peptone, and tryptone, or may be an inorganic

nitrogen source selected from the group consisting of ammonium acetate, ammonium nitrate, ammonium chloride, ammonium sulfate, sodium nitrate, urea, and monosodium glutamate (MSG).

[0043] Further, in the culture medium used in the present disclosure, examples of the nitrogen source may include a yeast extract, ammonium sulfate, sodium nitrate, and MSG, but are not limited thereto as long as it can be used to culture microalgae.

[0044] Specifically, the yeast extract may be included in the culture medium in a concentration of 0.1 to 10 g/L, 0.5 to 10 g/L, 0.5 to 7 g/L, 0.5 to 5 g/L, 0.5 to 3 g/L, 0.5 to 2 g/L, or 0.5 to 1.5 g/L, the ammonium sulfate may be included in the culture medium in a concentration of 1 to 5 g/L, 1 to 4 g/L, 2 to 5 g/L, or 2 to 4 g/L, the sodium nitrate may be included in the culture medium in a concentration of 0.1 to 10 g/L, 0.5 to 9 g/L, 1 to 9 g/L, 2 to 9 g/L, 3 to 9 g/L, 5 to 9 g/L, or 7 to 9 g/L, and the MSG may be included in the culture medium in a concentration of 0.1 to 2 g/L, 0.1 to 1.5 g/L, 0.5 to 2 g/L, or 0.5 to 1.5 g/L. However, the present disclosure is not limited thereto.

[0045] For the purpose of the present disclosure, since the CJM01 strains are characterized in that they have no ammonia inhibition and can grow in a wide salt concentration, carbon sources and nitrogen sources may be appropriately adjusted in consideration of these characteristics.

[0046] In the culture medium used in the present disclosure, as the phosphorus source, potassium dihydrogenphosphate, dipotassium hydrogenphosphate, and sodium-containing salts corresponding thereto may be used individually or in combination, but the present disclosure is not limited thereto. The culture medium may include other metal salts (for example, magnesium sulfate and iron sulfate), amino acid, and an essential growth-promoting material such as vitamin

[0047] In the step of recovering a biomass from the microalgae cultured in the culturing step, the cultured product thereof, the dried product, or the pulverized product thereof, a desired biomass may be collected by using a suitable method known in the art.

[0048] In the step of recovering docosahexaenoic acid produced in the culturing step, desired docosahexaenoic acid may be collected from the microalgae itself or the cultured product thereof by using a suitable method known in the art. For example, a desired biomass or desired docosahexaenoic acid may be recovered from the microalgae cultured by a suitable method known in the art, the cultured product thereof, the dried product thereof, or the pulverized product thereof, and, in this case, centrifugation, filtration, anion exchange chromatography, crystallization, HPLC, or the like may be used. The step of recovering the biomass or docosahexaenoic may additionally include a separation step and/or a purification step.

[0049] For example, lipids and lipid derivatives such as fatty aldehydes, fatty alcohols and

hydrocarbons (for example, alkanes) may be extracted by a hydrophobic solvent such as hexane (Frenz et al. 1989, Enzyme Microb. Technol., 11:717). Lipids and lipid derivatives may also be extracted by using liquefaction (Sawayama et al. 1999, Biomass and Bioenergy 17: 33-39 and Inoue et al. 1993, Biomass Bioenergy 6 (4): 269-274); oil liquefaction (Minowa et al. 1995, Fuel 74 (12): 1735-1738); and supercritical CO_2 extraction (Mendes et al. 2003, Inorganica Chimica Acta 356: 328-334). Further, the protocol of known microalgae lipid recovery discloses a method including the steps of i) collecting cells using centrifugation, washing the collected cells with distilled water and then freeze-drying the washed cells to obtain cell powder, and ii) pulverizing the obtained cell powder in a mortar and then extracting lipids using n-hexane [Miao and Wu, Biosource Technology (2006) 97:841-846].

[0050] Still another aspect of the present disclosure provides a composition including CJM01 microalgae of *Thraustochytrium* genus, a cultured product thereof, a dried product thereof, or a pulverized product thereof The composition may include a biomass or bio-oil produced using the microalgae.

[0051] The CJM01 microalgae of *Thraustochytrium* genus, the cultured product thereof, the dried product thereof, and the pulverized product thereof are as described above. The biomass or bio-oil produced using the microalgae are also as described above. For the purpose of preparing a composition including a high content of docosahexaenoic acid, the microalgae of the present disclosure may be used. The composition may be made in the form of a solution, a powder, or a suspension, but the present disclosure is not limited thereto. More specifically, a food composition, a feed composition, or a feed additive, which includes CJM01 microalgae of *Thraustochytrium* genus, the cultured product thereof, the dried product thereof, or the pulverized product thereof, may be provided.

[0052] As used herein, the term "feed" refers to an animal's food for eating, ingesting and digesting, or refers to any suitable natural or artificial diet, one meal, or an ingredient of the one meal. The feed according to the present disclosure, which includes a composition for preventing or treating metabolic diseases as an active ingredient, can be made into various types of feeds known in the art, and specific examples thereof may include concentrated feed, coarse feed, and/or special feed.

[0053] As used herein, the term "feed additive" refers to a material that is added to a feed for the purpose of various effects such as nutrient replenishment, weight loss prevention, improvement in digestive utilization of cellulose in feed, oil quality improvement, reproductive disorder prevention, conception rate improvement, and prevention of high-temperature stress in summer. The feed additive of the present disclosure corresponds to a supplementary feed under the feed management law, and may further include mineral preparation such as sodium hydrogencarbonate, bentonite, magnesium oxide, or complex mineral; mineral preparation that is trace mineral such as zinc, copper, cobalt, or selenium; vitamin preparation such as carotene, vitamin E, vitamins A, vitamin D, vitamin E, nicotinic acid, or vitamin B complex; protective amino acid preparation such as methionine or lysine; protective fatty acid preparation such as fatty acid calcium salt; live bacteria preparation such as probiotic bacteria

(lactic acid bacteria), yeast cultures, or mold fermentation products; and yeast preparation.

[0054] As used herein, the term "food composition" includes all types of foods, such as functional food, nutritional supplement, health food, and food additives. The above food composition may be produced in various forms according to methods commonly known in the art.

[0055] The present disclosure provides a method of producing a composition including the biomass or bio-oil. The biomass, bio-oil, and composition are as described above.

[0056] In the above method of producing a bio-oil, a bio-oil including a high content of docosahexaenoic acid may be produced by the step of culturing the CJM01 microalgae of *Thraustochytrium* genus, having high productivity of docosahexaenoic acid, in a culture medium including a carbon source of a specific composition and a nitrogen source of a specific composition under heterotrophic conditions.

Mode for Invention

[0057] Hereinafter, the present disclosure will be described in more detail with reference to Examples. However, these Examples are only illustrative the present disclosure, and the scope of the present disclosure is not limited to these Examples.

[0058] The CJM01 microalgae of the present disclosure are microalgae belonging to *Thraustochytrid* family, and have an ability to produce polyunsaturated fatty acids including a high content of docosahexaenoic acid. The CJM01 microalgae of the present disclosure have a DNA nucleotide sequence of the 18S rRNA gene represented by SEQ ID NO. 1, and have a high content of a biomass under heterotrophic conditions, not under growth conditions in light culture.

[0059] In the following examples, experimental methods will be described in more detail.

Example 1: Separation of KC01 microalgae of *Thraustochytrid* family

[0060] In order to separate the microalgal strains of *Thraustochytrid* family, the following experiments were carried out.

[0061] Specifically, seawater, soil, and leaf environmental samples were collected from 20 coastal areas of Geoje and Tongyeong areas of Gyeongsangnam-do, Korea Then, the samples were stored in an ice box at 10°C and carried to a laboratory. The samples were used in a bacteria separation work within 2 to 3 days. These samples were directly smeared on an agar medium, and then *Thraustochytrid* strains were separated using a liquid pine powder

application method. Samples each including a microalgae-like form observed by a microscope were smeared on an IYP culture medium for microbial separation (1 g/L of yeast extract, 1g/L of peptone, 2g/L of $MgSO_4 \cdot 7H_2O$, 20g/L of sea salt, 5.0 mg/L of H_3BO_3, 3.0 mg/L of $MnCl_2$, 0.2 mg/L of $CuSO_4$, 0.05 mg/L of $NaMo_4 \cdot 2H_2O$, 0.05 mg/L of $CoSO_4$, 0.7 mg/L of $ZnSO_4 \cdot 7H_2O$, and 15 g/L of agar) to obtain colonies. The obtained colonies were subjected to subculture several times to be purified and separated, and then only strains forming zoosporangia that are typical characteristics of *Thraustochytrid* microalgae were selected and separated. Environmental samples that cannot be confirmed through microscopic observation were diluted and washed using sterilized sea water having a salinity of 1.5%, and then these samples were sprayed with pine powder and cultured. Microbial communities obtained by culturing under temperature and pH conditions similar to each collection environment were smeared on an IYP culture medium for microbial separation and subcultured to be purified and separated. In this case, an antibiotic cocktail mix solution (0 to 500 mg/L of streptomycin sulfate, 0 to 500 mg/L of ampicillin, 0 to 500 mg/L of penicillin G, and 0 to 500 mg/L of kanamycin sulfate) was introduced while adjusting the concentration thereof, and thus the growth and pollution of other microbes were controlled.

[0062] The separated colonies were cultured in a 500 mL flask at a temperature of 15°C to 28°C and a rotation speed of 50 to 200 rpm for about 7 days using an IGGYP culture medium (glycerol 10 g/L, glucose 10 g/L, yeast extract 1 g/L, peptone 1 g/L, $MgSO_4 \cdot 7H_2O$ 2 g/L, solar salt 20 g/L, H_3BO_3 5.0 mg/L, $MnCl_2$ 3.0 mg/L, $CuSO_4$ 0.2 mg/L, $NaMo_4 \cdot 2H_2O$ 0.05 mg/L, $CoSO_4$ 0.05 mg/L, $ZnSO_4 \cdot 7H_2O$ 0.7 mg/L, and vitamin mixed solution 10 ml/L). One kind of the microalgae, whose growth rate was fast and whose culture condition was not complicated, was finally selected, and fungus bodies were recovered. The forms of the selected strains were observed using an optical microscope (shown in FIG. 1). The selected fungus bodies were washed with a phosphate buffered solution (PBS, pH 7.5), and then dried in a dry oven at 55°C for 16 hours to obtain dry fungus bodies.

[0063] The 18s rRNA gene sequence was analyzed for the molecular identification of strains of the finally selected microalgae. DNA is separated from the purely separated colonies of the selected species, and then 18s rRNA was amplified by polymerase chain reaction (PCR) using primers for gene amplification in a 18s rRNA region. The primers for gene amplification are summarized in Table 1 below.

[Table 1]

Primer	**Sequence (5'-3')**	**SEQ ID NO.**
18s-001F	AACCTGGTTGATCCTGCCAGTA	2
18s-013R	CCTTGTTACGACTTCACCTTCCTCT	3

[0064] In this case, after denaturation at 95°C for 5 minutes, the PCR was carried out for a total of 25 cycles under the following conditions: denaturation at 95°C for 30 seconds; annealing at 52°C for 30 seconds; and polymerization at 72°C for 1 minute and 30 seconds. Thereafter, the polymerization reaction was carried out at 72°C for 7 minutes.

[0065] As a result of analyzing the nucleotide sequence using the amplified reaction solution, nucleotide sequence 1 (SEQ ID NO. 1) having a size of about 1792 bp was obtained. As a result of NCBI BLAST search, it was found that the nucleotide sequence 1 had about 99% homology with the previously reported strains of *Thraustochytrium* genus and had about 84% homology with strains of *Aurantiochytrium* genus and *Schizochytrium* genus.

[0066] Thus, phylogenetic tree among strains are expressed, and the results thereof are shown in FIG. 2. The corresponding strains were identified as microalgae of *Thraustochytrium* genus of *Thraustochytrid* family, and were thus named as KC01 of *Thraustochytrium* genus.

Example 2: Development of mutant microaglae

Example 2-1: Selection of mutant microalgae through an artificial mutation method

[0067] In the present disclosure, the following experiment was carried out so as to separate strains having improved productivity of docosahexaenoic acid (DHA) by gamma ray irradiation mutation of the KC01 strains of *Thraustochytrium* genus, which were separated in Example 1.

[0068] Specifically, the KC01 strains of *Thraustochytrium* genus were cultured in a GYEP culture medium (glucose 2%, peptone 1%, yeast extract 0.5%, and solar salt 2%) for 24 hours to activate the strains. The activated strains were inoculated into a subculture medium (glucose 5%, peptone 1%, yeast extract 0.5%, and solar salt 2%) having been sterilized at 121°C for 15 minutes and cultured for 14 hours, and then fungus bodies were recovered. The recovered fungus bodies were suspended in 50 mL of a PBS buffer, irradiated with gamma rays at a dose of 1 to 5 kGy for 1 hour, and then cultured in 50 mL of a basic culture medium (glucose 5%, peptone 1%, yeast extract 0.5%, and solar salt 2%) using a 500 mL flask at a temperature of 28°C and a revolution of 120 rpm for 2 days. Then, the suspended fungus bodies were appropriately diluted when the death rate is 99%, and subcultured in a GYEP flat plate culture medium (glucose 2%, peptone 1%, yeast extract 0.5%, and solar salt 2%, agar 2%, pH 7.0) two times.

[0069] Primarily, colonies, whose color becomes white, were selected in order to select strains predicted to have reduced sulfated pigments produced in addition to oils containing polyunsaturated fatty acids. The mutant strains obtained by the above method were named as CJM01 strains of *Thraustochytrium* genus, deposited on May 30, 2018 with the Korean Collection for Type Cultures (KCTC), an international depository organization under the Budapest Treaty, and granted the deposit number KCTC 13538BP.

Example 2-2: Analysis of abilities of newly separated microalgae and mutant strains to produce oil containing polyunsaturated fatty acids.

[0070] The CJM01 and KC01 strains selected from Example 2-1 were cultured as follows in order to compare the ability of CJM01 strains to produce oil containing polyunsaturated fatty acids with the ability of KC01 strains to produce oil containing polyunsaturated fatty acids.

[0071] Specifically, in order to culture the CJM01 and KC01 strains, which are microalgae of *Thraustochytrium* genus according to the present disclosure, the CJM01 and KC01 strains were cultured in MJW01 culture medium (glucose 30g/L, $MgSO_4 \cdot 7H_2O$ 3.0g/L, Na_2SO_4 10g/L, NaCl 1.0g/L, yeast extract 9.0g/L, MSG·$1H_2O$ 1.0g/L, $NaNO_3$ 1.0g/L, KH_2PO_4 0.1g/L, K_2HPO_4 0.5g/L, $CaCl_2$ 0.5g/L, and vitamin mixed solution 10 ml/L) under basic culture medium conditions of 28°C, 300rpm, 1vvm, and pH 7.5 for 4 days. Fungus bodies were recovered by centrifugation, washed with a PBS buffer three times, and then dried at 55°C for 12 hours to measure the weight of the fungus bodies.

[0072] The content of docosahexaenoic acid-containing oil utilizing the dried fungus bodies was measured as follows. Specifically, 8.3 M of hydrochloric acid solution was applied to 2 g of the dried fungus bodies to hydrolyze the cell walls of fungus bodies of microalgae at 80°C, 30 mL of ethyl ether and 20 mL of petroleum ether were added to the dried fungus bodies and stirred for 30 seconds, and then the mixture was centrifugally separated 3. This procedure was repeated three times or more. Then, the separated solvent layer was recovered, put into a round flask whose weight had been previously measured, purged with nitrogen to remove a solvent, and then the resultant was put into a container, whose moisture had been removed, and dried. The weight of the dried oil was measured to calculate the total oil content. The content of DHA in the oil was measured by chromatography after pretreating the oil with 0.5 N methanolic NaOH and 14% trifluoroborane methanol (BF_3).

[0073] The culture performance of the KC01 strains of *Thraustochytrium* genus, cultured by the above method, and the culture performance of the CJM01 mutant strains selected by gamma ray irradiation are given in Tables 2 and 3. It was found from Tables 2 and 3 that DHA, which is highly functional omega-3 oil, was produced.

[0074] The "biomass" in Tables 2 and 3 refers to the concentration of the fungus bodies in a culture solution, and may be used in combination with dry cell weight (DCW). The content of DHA is expressed as the content thereof with respect to biomass or total fatty acid (TFA).

[0075] As shown in the following results, the production of DHA by the CJM01 mutant strains was found to be improved as compared with that by parent strains (KC01 strains of *Thraustochytrium* genus) (see Tables 2 and 3). Specifically, the production of DHA by the CJM01 mutant strains was increased by about 1.3 times as compared with that by the KC01 strains. Further, it was found that the productivity of DHA by the CJM01 mutant strains was increased by about 1.7 times as compared with that by the KC01 strains because culturing time was significantly shortened without a decrease in the obtained fungus bodies.

[Table 2]

Content and productivity of docosahexaenoic acid (DHA) according to culturing of KC01 parent strains *Thraustochytrium* genus						
Entry	**Time**	**Biomass**	**DHA**		**Lipid**	**DHA Productivity**
	(hr)	**g/L**	**(%/Biomass)**	**(%/TFA)**	**%**	**(g/l/h)**
1	71.5	115.2	22.7	35.6	63.7	0.365
2	73.0	127.5	21.6	33.6	64.3	0.377
3	78.5	141.8	22.3	36.8	60.5	0.402
Avg.	**74.3**	**128.2**	**22.2**	**35.3**	**62.8**	**0.381**

[Table 3]

Content and productivity of docosahexaenoic acid (DHA) according to culturing of CJM01 mutant strains *Thraustochytrium* genus						
Entry	**Time**	**Biomass**	**DHA**		**Lipid**	**DHA Productivity**
	(hr)	**g/L**	**(%/Biomass)**	**(%/TFA)**	**%**	**(g/l/h)**
1	57.3	123.0	29.3	47.6	61.5	0.629
2	55.5	133.5	27.5	45.5	60.4	0.661
3	54.8	133.5	28.0	57.1	49.0	0.682
Avg.	**55.9**	**130.0**	**28.3**	**50.1**	**57.0**	**0.657**

[0076] From the above results, it was found that the CJM01 strains, which are mutant strains, had an increased DHA content and improved DHA conductivity as compared with the KC01 strains, which are parent strains.

Example 2-3: Analysis of abilities of newly separated microalgae and mutant strains to produce amino acids.

[0077] In order to evaluate the culture characteristics of CJM01 strains, the total content of amino acids in the above culture solution was determined.

[0078] Specifically, 10 mL of sample was collected from each of the above culture solutions, diluted with distilled water by 20 times, and filtered, and then the total content of amino acids was analyzed using liquid chromatography. As the result of analyzing the total concentration of amino acids, aspartate, serine, glutamate, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, and arginine were respectively detected in the culture solution of the KC01 parent strains in an amount of 10 mg/L, whereas most of amino acids were not detected in the culture solution of the CJM01 strains having an improved DHA content.

[0079] Specifically, it was found that, in the case of the CJM01 mutant strains, the total concentration of amino acids as side products other than DHA is reduced by about 99% or more, as compared with the KC01 parent strains (see Table 4).

[0080] Thus, it was found that, in the case of the CJM01 mutant strains, the growth of fungus bodies is at an equivalent level, as compared with the KC01 parent strains, and the total content of lipids is not greatly reduced as compared with the content of fungus bodies, and thus the CJM01 mutant strains have used the supplied carbon source for a DHA biosynthetic mechanism more effectively as compared with the KC01 parent strains.

[Table 4]

Analysis of total amino acids in culture solution according to the culture of KC01 parent strains of *Thraustochytrium* genus and CJM01 mutant strains of *Thraustochytrium* genus

Amino acid	T.KC01 parent strains				T.CJM01 mutant strains			
(mg/L)	1	2	3	Avg	1	2	3	Avg
Asp	20.3	28.9	12.3	20.5	0.0	0.0	0.0	0.0
Ser	49.5	85.2	28.6	54.4	0.0	0.0	0.0	0.0
Glu	91.6	108.4	193.0	131.0	0.0	0.0	0.0	0.0
Gly	32.4	52.8	28.7	38.0	0.0	0.0	0.0	0.0
Ala	72.6	104.4	41.1	72.7	0.0	0.0	0.0	0.0
Val	83.0	130.8	59.5	91.1	10.1	0.0	0.0	3.4
Met	32.1	58.2	16.0	35.4	0.0	0.0	0.0	0.0
Ile	42.1	77.0	18.8	46.0	0.0	0.0	0.0	0.0
Leu	93.0	166.9	41.8	100.6	0.0	0.0	0.0	0.0
Tyr	51.7	86.5	0.0	46.1	0.0	0.0	0.0	0.0
Phe	83.4	129.9	58.4	90.5	0.0	0.0	0.0	0.0
Lys	45.9	83.3	20.2	49.8	0.0	0.0	0.0	0.0
His	15.9	25.0	0.0	13.6	0.0	0.0	0.0	0.0
Arg	57.0	93.7	24.0	58.2	0.0	0.0	0.0	0.0
Sum	770.5	1231.1	542.5	848.0	10.1	0.0	0.0	3.4

Example 3: Optimization of culture medium conditions

[0081] In order to further improve the content and productivity of DHA using the CJM01 strains having a reduced amino acid content and an improved DHA content, the strains having been selected from Example 2, culture medium conditions were optimized.

[0082] Specifically, based on the MJW01 culture medium used in Example 2-2, in order to increase the content of nitrogen sources in the culture medium and reduce production costs, the concentration of yeast extract as an organic nitrogen source was decreased, $(NH_4)_2SO_4$ as an inorganic nitrogen source was added, and the concentration of $NaNO_3$ was increased to convert the MJW01 culture medium into an MJW02 culture medium (glucose 30g/L, $MgSO_4 \cdot 7H_2O$ 5.0g/L, Na_2SO_4 3g/L, NaCl 0.5g/L, yeast extract 1.0g/L, $MSG \cdot 1H_2O$ 1.0g/L, $NaNO_3$ 8.0g/L., $(NH_4)_2SO_4$ 3.0g/L, KH_2PO_4 0.1g/L, K_2HPO_4 0.5g/L, $CaCl_2$ 0.1g/L, and vitamin mixed solution 10ml/L).

[0083] In order to compare the conditions of the MJW01 culture medium with the conditions of the MJW02culture medium, CJM01 strains were respectively cultured in the above culture mediums. As a result, as shown in Table 5 below, the amount of fungus bodies was decreased as the concentration of yeast extract was decreased, but culturing time was significantly shortened with an increase of an inorganic nitrogen source. Consequently, it was found that the content of DHA in the MJW02culture medium is equal to or more than the content of DHA in the MJW01culture medium, and the productivity of DHA in the MJW02culture medium was increased by 1.13 times with respect to the productivity of DHA in the MJW01 culture medium.

[Table 5]

Content and productivity of docosahexaenoic acid (DHA) according to culture medium conditions

Culture medium conditions	Time	Biomass	DHA		Lipid	Productivity
	(hr)	g/L	(%/Biomass)	(%/TFA)	%	(g/l/h)
MJW01	593	133.4	26.0	42.6	61.0	0.584
MJW02	52.1	1243	27.7	49.0	56.6	0.661

REFERENCES CITED IN THE DESCRIPTION

Cited references

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PATENTKRAV

1. CJM01-mikroalger med deponeringsnummer KCTC 13538BP af *Thraustochytrium*-slægten, hvorved produktionen af docosahexaensyre (DHA) øges, og produktionen af aminosyrer falder sammenlignet med moderstammen.

2. Mikroalger ifølge krav 1, hvor CJM01-mikroalgen af *Thraustochytrium*-slægten omfatter docosahexaensyre i en mængde på 40 vægtprocent til 60 vægtprocent baseret på den samlede vægt af fedtsyrer.

3. Mikroalger ifølge krav 1, hvor CJM01-mikroalgen af *Thraustochytrium*-slægten har en docosahexaensyre-produktivitet på 0,5 til 0,7 g/l/time.

4. Fremgangsmåde til fremstilling af en biomasse, omfattende:
dyrkning af CJM01-mikroalger af *Thraustochytrium*-slægten ifølge krav 1; og udvinding af en biomasse indeholdende docosahexaensyre fra mikroalgerne, et dyrket produkt deraf, et tørret produkt deraf eller et pulveriseret produkt deraf.

5. Fremgangsmåde ifølge krav 4, hvor dyrkningen udføres under heterotrofe betingelser.

6. Fremgangsmåde ifølge krav 4, hvor dyrkningen udføres under anvendelse af et dyrkningsmedium, der omfatter en carbonkilde og en nitrogenkilde.

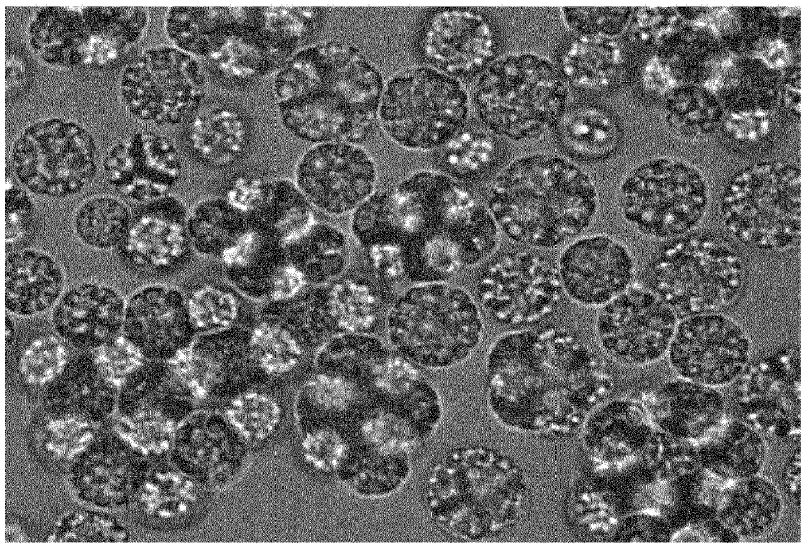
7. Fremgangsmåde ifølge krav 6, hvor carbonkilden er mindst én valgt fra gruppen bestående af glukose, fruktose, maltose, galaktose, mannose, sakkarose, arabinose, xylose og glycerol

8. Fremgangsmåde ifølge krav 6, hvor nitrogenkilden er i) en organisk nitrogenkilde valgt fra gruppen bestående af en gærekstrakt, en oksekødekstrakt, pepton og trypton, eller ii) en uorganisk nitrogenkilde valgt fra gruppen bestående af ammoniumacetat, ammoniumnitrat, ammoniumklorid, ammoniumsulfat, natriumnitrat, urinstof og mononatriumglutamat (MSG).

9. Fremgangsmåde til fremstilling af en bioolie, omfattende: dyrkning af CJM01-mikroalger af *Thraustochytrium*-slægten ifølge krav 1; og udvinding af et lipid indeholdende docosahexaensyre (DHA) fra mikroalgerne, et dyrket produkt deraf, et tørret produkt deraf eller et pulveriseret produkt deraf.

DRAWINGS

[FIG. 1]



[FIG. 2]