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(54) Title: METHOD FOR PRODUCING SPHINGOID BASE OR SPHINGOLIPID

(57) Abstract: A method for producing an objective substance such as sphingoid bases and sphingolipids using yeast is provided. An objective substance is produced by cultivating yeast having an ability to produce the objective substance in a culture medium containing an additive that is able to associate with, bind to, solubilize, and/or capture the objective substance, and collecting the objective substance from cells of the yeast and/or the culture medium.



WO 2017/033463 A1

Description

Title of Invention: METHOD FOR PRODUCING SPHINGOID BASE OR SPHINGOLIPID

Technical Field

[0001] The present invention relates to a method for producing an objective substance such as sphingoid bases and sphingolipids using yeast. Sphingoid bases and sphingolipids are industrially useful as ingredients for pharmaceuticals, cosmetics, and so forth.

Background Art

[0002] There has been attempted to produce sphingoid bases and sphingolipids with bio-engineering techniques. As methods for producing sphingoid bases and sphingolipids with bioengineering techniques, there have been reported methods of using yeast (JP2014-529400).

[0003] Cyclodextrins are used to improve the solubilities of non-polar molecules such as lipid-soluble vitamins and hormones into aqueous media. Also, zeolites are microporous, aluminosilicate minerals, and are commonly used as commercial adsorbents and catalysts.

Summary of Invention

Object to be Achieved by the Invention

[0004] An object of the present invention is to develop a novel technique for improving production of an objective substance such as sphingoid bases and sphingolipids by yeast, and thereby to provide a method for efficiently producing the objective substance.

Means for Achieving the Object

[0005] The inventor of the present invention conducted research in order to achieve the aforementioned object. As a result, the inventor found that production of an objective substance such as sphingoid bases and sphingolipids by yeast could be improved by using a culture medium containing an additive that is able to associate with, bind to, solubilize, and/or capture the objective substance upon cultivation of the yeast, and thereby accomplished the present invention.

[0006] That is, the present invention can be embodied, for example, as follows.

[1] A method for producing an objective substance, the method comprising:
cultivating yeast having an ability to produce the objective substance in a culture medium containing an additive that is able to associate with, bind to, solubilize, and/or capture the objective substance; and
collecting the objective substance from cells of the yeast and/or the culture medium, wherein the objective substance is selected from the group consisting of sphingoid

bases and sphingolipids.

[2] The aforementioned method, wherein the additive is selected from the group consisting of cyclodextrin and zeolite.

[3] The aforementioned method, wherein the cyclodextrin is selected from the group consisting of alpha-cyclodextrin, beta-cyclodextrin, gamma-cyclodextrin, and derivatives thereof.

[4] The aforementioned method, wherein the derivatives are selected from the group consisting of methyl-alpha-cyclodextrin, methyl-beta-cyclodextrin, hydroxypropyl-alpha-cyclodextrin, and hydroxypropyl-beta-cyclodextrin.

[5] The aforementioned method, wherein the objective substance is selected from the group consisting of phytosphingosine (PHS), sphingosine, 6-hydroxysphingosine, sphinganine (DHS), tetraacetylphytosphingosine (TAPS), triacetylphytosphingosine, diacetylphytosphingosine, phytoceramides, dihydroceramides, 6-hydroxyceramides, and glucosylceramides.

[6] The aforementioned method, wherein the objective substance is selected from the group consisting of phytosphingosine (PHS), sphinganine (DHS), tetraacetylphytosphingosine (TAPS), phytoceramides, and glucosylceramides.

[7] The aforementioned method, wherein the phytosphingosine is selected from the group consisting of C16 PHS, C18 PHS, C20 PHS, C18:1 PHS, C20:1 PHS, 4-(hydroxymethyl)-2-methyl-6-tetradecanyl-1,3-oxazinan-5-ol, and 4-(hydroxymethyl)-2-methyl-6-hexadecanyl-1,3-oxazinan-5-ol.

[8] The aforementioned method, wherein the yeast has been modified so that the expression and/or activity or activities of one or more proteins selected from proteins encoded by LCB1, LCB2, TSC10, SUR2, SLI1, ATF2, LAG1, LAC1, LIP1, and UGCG genes are increased.

[9] The aforementioned method, wherein the activity or activities of the one or more proteins are increased by increasing the expression of the respective genes encoding the one or more proteins.

[10] The aforementioned method, wherein the yeast has been modified so that the expression and/or activity or activities of one or more proteins selected from proteins encoded by LCB4, LCB5, ELO3, CKA2, ORM2, CHA1, and YPC1 genes are reduced.

[11] The aforementioned method, wherein the activity or activities of the one or more proteins are reduced by attenuating the expression of the respective genes encoding the one or more proteins, or by disrupting the respective genes encoding the one or more proteins.

[12] The aforementioned method, wherein the yeast belongs to the genus *Saccharomyces* or *Pichia*.

[13] The aforementioned method, wherein the yeast is *Saccharomyces cerevisiae* or

Pichia ciferrii (Wickerhamomyces ciferrii).

[14] The aforementioned method, wherein the amount produced of the objective substance by the yeast is increased in the presence of the additive as compared with in the absence of the additive.

Brief Description of Drawings

[0007] [fig.1]The Figure shows data regarding phytosphingosine solubility with various cyclodextrins. Phytosphingosine amounts (mg/l) measured in supernatants after incubation of an excess of pure phytosphingosine (20 g/l) with each of the three types of cyclodextrin at various concentrations.

[fig.2]The Figure shows data regarding excretion of sphingoid bases in shake flask cultures with or without α -cyclodextrin.

[fig.3]The Figure shows data regarding excretion of sphingoid bases in shake flask cultures with or without α -cyclodextrin and with or without overexpression of Wickerhamomyces ciferrii acetyltransferase genes ATF2 and SLI1 in *S. cerevisiae*. Control strain EYS4299 and acetyltransferase genes expression strain EYS4300 were grown under standard conditions with or without α -cyclodextrin (α CD), pellets were extracted, and supernatants were analyzed directly. Sphinganine, phytosphingosine, and tetra-acetylated-phytosphingosine were quantified. Mono- to tri-acetylated sphinganine and phytosphingosine were detected, but were not quantified.

[fig.4]The Figure shows data regarding Biomass in shake flask cultures. The left panel shows biomass production of *S. cerevisiae* strains EYS2958 (basic strain) and EYS4423 (Δ cha1 Δ lcb4 Δ orm2 Δ cka2 [ScLCB1 ScLCB2][ScTSC10][PcSUR2]) in the presence or absence of 10 g/L α -cyclodextrin. The right panel shows biomass of Wickerhamomyces ciferrii (EYS3062) and Wickerhamomyces sydowiorum (EYS3063) strains.

[fig.5]The Figure shows data regarding long-chain base production in *S. cerevisiae*, *W. ciferrii*, and *W. sydowiorum* strains. Long-chain bases phytosphingosine and tetraacetyl-phytosphingosine (TAPS) in the supernatants of cultures with strains *S. cerevisiae* strains EYS2958 (basic strain), EYS4423 (Δ cha1 Δ lcb4 Δ orm2 Δ cka2 [ScLCB1 ScLCB2][ScTSC10][PcSUR2]), Wickerhamomyces ciferrii (EYS3062), and Wickerhamomyces sydowiorum (EYS3063) are presented in the graphs.

[fig.6]The Figure shows data regarding Ceramide and PHS amounts in supernatants. EYS2958 (basic strain), EYS4022 (*cha1* Δ *ypc1* Δ *lcb4* Δ *LAG1* *LAC1* *LIP1*), and EYS4798 (*cha1* Δ *ypc1* Δ *lcb4* Δ *LAG1* *LAC1* *LIP1* [*Xenopus laevis* UGT21-M]) were grown for 48h in SC medium containing 200 g/l methyl- β -cyclodextrin (Met-bCD). The ceramide content of the supernatants was quantified by LC/MS according to a calibration curve established in SC medium containing 200 g/l methyl- β -cyclodextrin.

Modes for Carrying out the Invention

[0008] Hereafter, the present invention will be explained in detail.

[0009] The method of the present invention is a method for producing an objective substance comprising cultivating yeast having an ability to produce the objective substance in a culture medium containing an additive that is able to associate with, bind to, solubilize, and/or capture the objective substance, and collecting the objective substance from cells of the yeast and/or the culture medium. The yeast used for method of the present invention is also referred to as "the yeast of the present invention".

[0010] <1> Yeast of the present invention

The yeast of the present invention is yeast having an ability to produce an objective substance. The "ability to produce an objective substance" may also be referred to as "objective substance-producing ability".

[0011] <1-1> Yeast having objective substance-producing ability

In the present invention, the term "yeast having an objective substance-producing ability" refers to yeast that is able to produce and accumulate an objective substance in a culture medium or cells of the yeast in such a degree that the objective substance can be collected, when the yeast is cultivated in the culture medium. The culture medium may be a medium that can be used in the method of the present invention, and may specifically be a medium containing an additive that is able to associate with, bind to, solubilize, and/or capture the objective substance. The yeast having an objective substance-producing ability may also be yeast that is able to produce and accumulate an objective substance in a culture medium or cells of the yeast in an amount larger than that obtainable with a non-modified strain. The term "non-modified strain" may refer to a reference strain that has not been modified so that an objective substance-producing ability is imparted or enhanced. Examples of the non-modified strain include a wild-type strain and parent strain, such as *Saccharomyces cerevisiae* strains BY4742 (ATCC 201389; EUROSCARF Y10000), S288C (ATCC 26108), and NCYC 3608. The yeast having an objective substance-producing ability may also be yeast that is able to produce and accumulate an objective substance in a culture medium in an amount of preferably 5 mg/L or more, more preferably 10 mg/L or more.

[0012] In the present invention, the objective substance is selected from the group consisting of sphingoid bases and sphingolipids.

[0013] The term "sphingoid base" refers to a compound comprising a long alkyl chain having an amino group at C2 and one or more hydroxyl groups usually at C1 and/or C3, wherein one or more of the amino group and hydroxyl groups may be acetylated. Examples of sphingoid bases include phytosphingosine (PHS), sphingosine, sphingadinenine, 6-hydroxysphingosine, sphinganine (dihydrosphingosine; DHS), and acetylated derivatives thereof. Examples of acetylated derivatives include tetraacetyl-

phytosphingosine (TAPS), triacetylphytosphingosine, diacetylphytosphingosine, O-acetylphytosphingosine, N-acetylphytosphingosine, triacetylsphinganine, diacetylsphinganine, O-acetylsphinganine, N-acetylsphinganine, triacetylsphingosine, diacetylsphingosine, O-acetylsphingosine, N-acetylsphingosine, tetraacetyl-6-hydroxysphingosine, triacetyl-6-hydroxysphingosine, diacetyl-6-hydroxysphingosine, O-acetyl-6-hydroxysphingosine, N-acetyl-6-hydroxysphingosine, triacetylsphingadienine, diacetylsphingadienine, and O-acetylsphingadienine. The length and the unsaturation degree of the alkyl chain constituting the sphingoid base may vary. The alkyl chain may have a length of, for example, C16, C18, or C20. The alkyl chain may have one or more unsaturated double bonds. That is, examples of sphingoid bases also include such variant species of the sphingoid bases exemplified above, which variant species have different lengths and/or different unsaturation degrees. The term “phytosphingosine (PHS)” may refer to C18 PHS, which is a typical species of PHS, or may collectively refer to such variant species of PHS, such as C16 PHS, which has a saturated C16 alkyl chain; C18 PHS, which has a saturated C18 alkyl chain; C20 PHS, which has a saturated C20 alkyl chain; C18:1 PHS, which has a C18 alkyl chain having one unsaturated double bond; and C20:1 PHS, which has a C20 alkyl chain having one unsaturated double bond. The term “phytosphingosine (PHS)” may also include adducts of PHS, such as 4-(hydroxymethyl)-2-methyl-6-tetradecanyl-1,3-oxazinan-5-ol and 4-(hydroxymethyl)-2-methyl-6-hexadecanyl-1,3-oxazinan-5-ol, which may be generated via reaction of C18 PHS and C20 PHS with acetaldehyde, respectively. Similarly, the term “sphinganine (DHS)” may refer to C18 DHS, which is a typical species of DHS and has a saturated C18 alkyl chain, or may collectively refer to such variant species of DHS. Such description regarding variant species may also be applied to other sphingoid bases.

- [0014] The term “sphingolipid” refers to a compound that comprises an N-acyl sphingoid base moiety, i.e. a compound that comprises a sphingoid base moiety covalently linked via an amide bond to a fatty acid. Examples of sphingolipids include ceramides, glucosylceramides (cerebrosides), inositol phosphorylceramides, mannosylinositol phosphorylceramides, and mannosyldiinositol phosphorylceramides. Examples of ceramides include phytoceramides, which correspond to ceramides of PHS; dihydroceramides, which correspond to ceramides of DHS; and 6-hydroxyceramides, which correspond to ceramides of 6-hydroxysphingosine. The length and the unsaturation degree of the acyl chain constituting the sphingolipid may vary. The acyl chain may have a length of, for example, C14 to C26, such as C14, C16, C18, C20, C22, C24, and C26. The acyl chain may have one or more unsaturated double bonds. The acyl chain may have one or more functional groups such as hydroxy group.

- [0015] In cases where the objective substance is a compound that can form a salt, the objective substance to be produced may be a free compound, a salt thereof, or a mixture thereof. That is, in the present invention, the term “objective substance” may refer to an objective substance in a free form, a salt thereof, or a mixture thereof. Examples of the salt include, for example, inorganic acid salts such as sulfate salt, hydrochloride salt, and carbonate salt, and organic acid salts such as lactic acid salt and glycolic acid salt (Acta Derm Venereol. 2002;82(3):170-3.). As the salt of the objective substance, one kind of salt may be employed, or two or more kinds of salts may be employed.
- [0016] The yeast is not particularly limited so long as it can be used for the method of the present invention. The yeast may be budding yeast, or may be fission yeast. The yeast may be haploid yeast, or may be diploid or more polyploid yeast.
- [0017] Examples of the yeast include yeast belonging to the genus *Saccharomyces* such as *Saccharomyces cerevisiae*, the genus *Pichia* (also referred to as the genus *Wickerhamomyces*) such as *Pichia ciferrii*, *Pichia sydowiorum*, and *Pichia pastoris*, the genus *Candida* such as *Candida utilis*, the genus *Hansenula* such as *Hansenula polymorpha*, the genus *Schizosaccharomyces* such as *Schizosaccharomyces pombe*. Some species of the genus *Pichia* has been reclassified into the genus *Wickerhamomyces* (Int J Syst Evol Microbiol. 2014 Mar;64(Pt 3):1057-61). Therefore, for example, *Pichia ciferrii* and *Pichia sydowiorum* are also called *Wickerhamomyces ciferrii* and *Wickerhamomyces sydowiorum*, respectively. In the present invention, the term “*Pichia*” should include such species that had been classified into the genus *Pichia* but have been reclassified into another genus such as *Wickerhamomyces*.
- [0018] Specific examples of *Saccharomyces cerevisiae* include strains BY4742 (ATCC 201389; EUROSCARF Y10000), S288C (ATCC 26108), Y006 (FERM BP-11299), NCYC 3608, and derivative strains thereof. Specific examples of *Pichia ciferrii* (*Wickerhamomyces ciferrii*) include strain NRRL Y-1031 (ATCC 14091), strain CS.PCΔPro2 (Schorsch et al., 2009, Curr Genet. 55, 381-9.), strains disclosed in WO 95/12683, and derivative strains thereof. Specific examples of *Pichia sydowiorum* (*Wickerhamomyces sydowiorum*) include strain NRRL Y-7130 (ATCC 58369) and derivative strains thereof.
- [0019] These strains are available from, for example, the American Type Culture Collection (ATCC, Address: 12301 Parklawn Drive, Rockville, Maryland 20852, P.O. Box 1549, Manassas, VA 20108, United States of America), EUROpean *Saccharomyces Cerevisiae* ARchive for Functional Analysis (EUROSCARF, Address: Institute for Molecular Biosciences, Johann Wolfgang Goethe-University Frankfurt, Max-von-Laue Str. 9; Building N250, D-60438 Frankfurt, Germany), the National Collection of Yeast Cultures (NCYC, Address: Institute of Food Research, Norwich Research Park,

Norwich, NR4 7UA, UK), or depositary institutions corresponding to deposited strains. That is, for example, in cases of ATCC strains, registration numbers are assigned to the respective strains, and the strains can be ordered by using these registration numbers (refer to <http://www.atcc.org/>). The registration numbers of the strains are listed in the catalogue of the American Type Culture Collection (ATCC).

[0020] The yeast of the present invention may be yeast inherently having an objective substance-producing ability, or may be yeast modified so that it has an objective substance-producing ability. The yeast having an objective substance-producing ability can be obtained by imparting an objective substance-producing ability to yeast such as those mentioned above, or by enhancing an objective substance-producing ability of yeast such as those mentioned above.

[0021] Hereafter, methods for imparting or enhancing an objective substance-producing ability are specifically exemplified. All the modifications for imparting or enhancing an objective substance-producing ability may be used independently or in any appropriate combination. Modifications for constructing the yeast of the present invention can be performed in an arbitrary order.

[0022] An objective substance-producing ability may be imparted or enhanced by modifying yeast so that the expression and/or activity or activities of one or more kinds of proteins involved in production of the objective substance are increased or reduced. That is, the yeast of the present invention may have been modified so that the expression and/or activity or activities of one or more kinds of proteins involved in production of the objective substance are increased or reduced. The term “protein” also includes so-called peptides such as polypeptides. Examples of the proteins involved in production of the objective substance include enzymes that catalyze the synthesis of the objective substance (also referred to as “biosynthetic enzyme of objective substance”), enzymes that catalyze a reaction branching away from the biosynthetic pathway of the objective substance to generate a compound other than the objective substance (also referred to as “biosynthetic enzyme of byproduct”), enzymes that catalyze decomposition of the objective substance (also referred to as “decomposition enzyme of objective substance”), and proteins that affect, e.g. increase or reduce, the activity of an enzyme such as those described above.

[0023] The protein of which the expression and/or activity is to be increased or reduced can be appropriately chosen depending on the type of the objective substance and on the types and activities of the proteins involved in production of the objective substance and inherently possessed by the yeast of the present invention. For example, the expression and/or activity or activities of one or more kinds of proteins selected from biosynthetic enzymes of the objective substance may preferably be increased. Also, for example, the expression and/or activity or activities of one or more kinds of proteins

selected from biosynthetic enzymes of a byproduct and decomposition enzymes of the objective substance may preferably be reduced.

[0024] Methods for increasing or reducing the expression and/or activity of a protein will be described in detail later. The activity of a protein can be increased by, for example, increasing the expression of a gene encoding the protein. The activity of a protein can be reduced by, for example, attenuating the expression of a gene encoding the protein or disrupting a gene encoding the protein. The expression of a gene is also referred to as “the expression of a protein (i.e. the protein encoded by the gene)”. Such methods of increasing or reducing the expression and/or activity of a protein are well known in the art.

[0025] Specific examples of the proteins involved in production of the objective substance include proteins encoded by LCB1, LCB2, TSC10, SUR2, SLI1, ATF2, LAG1, LAC1, LIP1, UGCG, LCB4, LCB5, ELO3, CKA2, ORM2, CHA1, and YPC1 genes. These genes may be collectively referred to as “target gene”, and proteins encoded thereby may be collectively referred to as “target protein”.

[0026] For example, the yeast of the present invention may have been modified so that the expression and/or activity or activities of one or more proteins selected from proteins encoded by LCB1, LCB2, TSC10, SUR2, SLI1, ATF2, LAG1, LAC1, LIP1, and UGCG genes are increased, and/or that the expression and/or activity or activities of one or more proteins selected from proteins encoded by LCB4, LCB5, ELO3, CKA2, ORM2, CHA1, and YPC1 genes are reduced. The expression “the activity or activities of one or more proteins selected from proteins encoded by LCB1, LCB2, TSC10, SUR2, SLI1, ATF2, LAG1, LAC1, LIP1, and UGCG genes are increased” may specifically mean that the expression of one or more genes selected from LCB1, LCB2, TSC10, SUR2, SLI1, ATF2, LAG1, LAC1, LIP1, and UGCG genes is increased. The expression “the activity or activities of one or more proteins selected from proteins encoded by LCB4, LCB5, ELO3, CKA2, ORM2, CHA1, and YPC1 genes are reduced” may specifically mean that the expression of one or more genes selected from LCB4, LCB5, ELO3, CKA2, ORM2, CHA1, and YPC1 genes is attenuated, or one or more genes selected from LCB4, LCB5, ELO3, CKA2, ORM2, CHA1, and YPC1 genes are disrupted.

[0027] LCB1 and LCB2 genes encode serine palmitoyltransferase. The term “serine palmitoyltransferase” refers to a protein having an activity of catalyzing the synthesis of 3-ketosphinganine from serine and palmitoyl-CoA (EC 2.3.1.50). This activity may be referred to as “serine palmitoyltransferase activity”. Proteins encoded by LCB1 and LCB2 genes may be referred to as “Lcb1p” and “Lcb2p”, respectively. Examples of LCB1 and LCB2 genes include those of yeast such as *S. cerevisiae* and *Pichia ciferrii*. The nucleotide sequences of LCB1 and LCB2 genes of *S. cerevisiae* S288C are shown

as SEQ ID NOS: 1 and 3, and the amino acid sequences of Lcb1p and Lcb2p encoded thereby are shown as SEQ ID NOS: 2 and 4. Lcb1p and Lcb2p may form a heterodimer to function as serine palmitoyltransferase (Plant Cell. 2006 Dec;18(12):3576-93.). The activity or activities of either one or both of Lcb1p and Lcb2p may be increased, for example, in cases of producing any objective substance, and specifically in cases of producing sphingoid bases such as PHS and TAPS. An increased activity or activities of either one or both of Lcb1p and Lcb2p may specifically mean an increased serine palmitoyltransferase activity. Serine palmitoyltransferase activity can be measured by, for example, a known method (J Biol Chem. 2000 Mar 17;275(11):7597-603.).

[0028] TSC10 gene encodes 3-dehydrosphinganine reductase. The term “3-dehydrosphinganine reductase” refers to a protein having an activity of catalyzing the conversion of 3-ketosphinganine to dihydrosphingosine (sphinganine) in the presence of an electron donor such as NADPH (EC 1.1.1.102). This activity may be referred to as “3-dehydrosphinganine reductase activity”. A protein encoded by TSC10 gene may be referred to as “Tsc10p”. Examples of TSC10 gene include those of yeast such as *S. cerevisiae* and *Pichia ciferrii*. The nucleotide sequence of TSC10 gene of *S. cerevisiae* S288C is shown as SEQ ID NO: 5, and the amino acid sequence of Tsc10p encoded thereby is shown as SEQ ID NO: 6. The activity of Tsc10p may be increased, for example, in cases of producing any objective substance, and specifically in cases of producing sphingoid bases such as PHS and TAPS. An increased activity of Tsc10p may specifically mean an increased 3-dehydrosphinganine reductase activity. 3-dehydrosphinganine reductase activity can be measured by, for example, a known method (Biochim Biophys Acta. 2006 Jan;1761(1):52-63.).

[0029] SUR2 (SYR2) gene encodes sphingosine hydroxylase. The term “sphingosine hydroxylase” refers to a protein having an activity of catalyzing the hydroxylation of a sphingoid base or the hydroxylation of sphingoid base moiety of a ceramide (EC 1.-.-.-). This activity may be referred to as “sphingosine hydroxylase activity”. Sphingosine hydroxylase may catalyze, for example, the hydroxylation of dihydrosphingosine (DHS; sphinganine) to form phytosphingosine (PHS), or the hydroxylation of a ceramide containing DHS (dihydroceramide) to form a ceramide containing PHS (phytoceramide). A protein encoded by SUR2 gene may be referred to as “Sur2p”. Examples of SUR2 gene include those of yeast such as *S. cerevisiae* and *Pichia ciferrii*. The nucleotide sequence of SUR2 gene of *S. cerevisiae* S288C is shown as SEQ ID NO: 7, and the amino acid sequence of Sur2p encoded thereby is shown as SEQ ID NO: 8. The nucleotide sequence of SUR2 gene of *Pichia ciferrii* is shown as SEQ ID NO: 37, and the amino acid sequence of Sur2p encoded thereby is shown as SEQ ID NO: 38. The activity of Sur2p may be increased, for example, in cases of producing

PHS and phytoceramides. An increased activity of Sur2p may specifically mean an increased sphingosine hydroxylase activity. Sphingosine hydroxylase activity can be measured by, for example, incubating the enzyme with DHS or a dihydroceramide and determining an enzyme-dependent production of PHS or a phytoceramide.

[0030] SLI1 and ATF2 genes encode acetyltransferases. The term “acetyltransferase” refers to a protein having an activity of catalyzing the acetylation of a sphingoid base in the presence of an acetyl donor such as acetyl-CoA (EC 2.3.1.-). This activity may be referred to as “acetyltransferase activity”. Proteins encoded by SLI1 and ATF2 genes may be referred to as “Sli1p” and “Atf2p”, respectively. Examples of SLI1 and ATF2 genes include those of yeast such as *S. cerevisiae* and *Pichia ciferrii*. The nucleotide sequences of SLI1 and ATF2 genes of *S. cerevisiae* S288C are shown as SEQ ID NOS: 9 and 11, and the amino acid sequences of Sli1p and Atf2p encoded thereby are shown as SEQ ID NOS: 10 and 12. The nucleotide sequences of SLI1 (partial) and ATF2 genes of *Pichia ciferrii* are shown as SEQ ID NOS: 39 and 42, the nucleotide sequences of SLI1 and ATF2 genes of *Pichia ciferrii* used in the Examples section, which are optimized for *S. cerevisiae* codon usage, are shown as SEQ ID NOS: 40 and 43, and the amino acid sequences of Sli1p and Atf2p encoded thereby are shown as SEQ ID NOS: 41 and 44. The activity or activities of either one or both of Sli1p and Atf2p may be increased, for example, in cases of producing an acetylated derivative according to the type of acetylation. For example, tetraacetyl phytosphingosine (TAPS) can be synthesized from PHS by combination of Sli1p and Atf2p (Appl Microbiol Biotechnol. 2013 Oct;97(19):8537-46.). Specifically, Sli1p may catalyze initial O- and N-acetylation of PHS to yield triacetyl phytosphingosine, and Atf2p may catalyze final O-acetylation to yield TAPS (Appl Microbiol Biotechnol. 2013 Oct;97(19):8537-46.). Hence, for example, when the objective substance is TAPS, the activities of both of Sli1p and Atf2p may preferably be increased. By contrast, for example, when the objective substance is triacetyl phytosphingosine, the activity of Sli1p may preferably be increased. An increased activity or activities of either one or both of Sli1p and Atf2p may specifically mean an increased acetyltransferase activity. Acetyltransferase activity can be measured by, for example, incubating the enzyme with a sphingoid base and determining an enzyme-dependent production of an acetylated sphingoid base.

[0031] LAG1, LAC1, and LIP1 genes encode ceramide synthase. The term “ceramide synthase” refers to a protein having an activity of catalyzing the synthesis of a ceramide from a sphingoid base and an acyl-coenzyme A (EC 2.3.1.24). This activity may be referred to as “ceramide synthase activity”. Proteins encoded by LAG1, LAC1, and LIP1 genes may be referred to as “Lag1p”, “Lac1p”, and “Lip1p”, respectively. Examples of LAG1, LAC1, and LIP1 genes include those of yeast such as *S. cerevisiae* and *Pichia ciferrii*. The nucleotide sequences of LAG1, LAC1, and LIP1 genes of *S.*

cerevisiae S288C are shown as SEQ ID NOS: 13, 15, and 17, and the amino acid sequences of Lag1p, Lac1p, and Lip1p encoded thereby are shown as SEQ ID NOS: 14, 16, and 18. LAG1 and LAC1 genes specifically encode functionally equivalent catalytic subunits of ceramide synthase. LIP1 gene specifically encodes a non-catalytic subunit of ceramide synthase. The non-catalytic subunit Lip1p is associated with each of the catalytic subunits Lag1p and Lac1p, and is required for ceramide synthase activity. The activity of any one of Lag1p, Lac1p, and Lip1p may be increased alone, the activity of either one of Lag1p and Lac1p may be increased in combination with Lip1p, the activities of both of Lag1p and Lac1p may be increased, or the activities of all of Lag1p, Lac1p, and Lip1p may be increased. The activity or activities of one or more of Lag1p, Lac1p, and Lip1p may be increased, for example, in cases of producing sphingolipids such as phytoceramides and glucosylceramides. An increased activity or activities of one or more of Lag1p, Lac1p, and Lip1p may specifically mean an increased ceramide synthase activity. Ceramide synthase activity can be measured by, for example, a known method (Guillas, Kirchman, Chuard, Pfefferli, Jiang, Jazwinski and Conzelman (2001) EMBO J. 20, 2655-2665; Schorling, Vallee, Barz, Reizman and Oesterhelt (2001) Mol. Biol. Cell 12, 3417-3427; Vallee and Riezman (2005) EMBO J. 24, 730-741).

[0032] UGCG gene encodes ceramide UDP-glucose ceramide glucosyltransferase. The term “UDP-glucose ceramide glucosyltransferase” refers to a protein having an activity of catalyzing the reaction of glycosylating a ceramide to form a glucosylceramide (EC 2.4.1.80). This activity may be referred to as “UDP-glucose ceramide glucosyltransferase activity”. A protein encoded by UGCG gene may be referred to as “Ugcg protein”. Examples of UGCG gene include ugcg-a and ugcg-b genes. Proteins encoded by ugcg-a and ugcg-b genes may be referred to as “Ugcg-a protein” and “Ugcg-b protein”, respectively. Examples of the ugcg-a and ugcg-b genes include those of *Xenopus laevis* (African clawed frog) (Dev Dyn. 2008 Jan;237(1):112-23.). The nucleotide sequences of ugcg-a and ugcg-b mRNAs of *Xenopus laevis* are shown as SEQ ID NOS: 19 and 21, and the amino acid sequences of Ugcg-a and Ugcg-b proteins encoded thereby are shown as SEQ ID NOS: 20 and 22. The activity or activities of either one or both of Ugcg-a and Ugcg-b proteins may be increased, for example, in cases of producing glucosylceramides. An increased activity or activities of either one or both of Ugcg-a and Ugcg-b proteins may specifically mean an increased UDP-glucose ceramide glucosyltransferase activity. UDP-glucose ceramide glucosyltransferase activity can be measured by, for example, a known method (Dev Dyn. 2008 Jan;237(1):112-23.).

[0033] LCB4 and LCB5 genes encode sphingoid base kinases. The term “sphingoid base kinase” refers to a protein having an activity of catalyzing the phosphorylation a

sphingoid base to form a sphingoid base phosphate (EC 2.7.1.91). This activity may be referred to as “sphingoid base kinase activity”. Proteins encoded by LCB4 and LCB5 genes may be referred to as “Lcb4p” and “Lcb5p”, respectively. The nucleotide sequences of LCB4 and LCB5 genes of *S. cerevisiae* S288C are shown as SEQ ID NOS: 23 and 25, and the amino acid sequences of Lcb4p and Lcb5p encoded thereby are shown as SEQ ID NOS: 24 and 26. Of these, Lcb4p is the major sphingoid base kinase in *S. cerevisiae* (J Biol Chem. 2003 Feb 28;278(9):7325-34.). At least the activity of Lcb4p may preferably be reduced. The activity or activities of either one or both of Lcb4p and Lcb5p may be reduced, for example, in cases of producing any objective substance, and specifically in cases of producing sphingoid bases such as PHS and TAPS and sphingolipids such as phytoceramides and glucosylceramides. A reduced activity or activities of either one or both of Lcb4p and Lcb5p may specifically mean a reduced sphingoid base kinase activity. Sphingoid base kinase activity can be measured by, for example, a known method (Plant Physiol. 2005 Feb;137(2):724-37.).

[0034] ELO3 gene encodes fatty acid elongase III. The term “fatty acid elongase III” refers to a protein having an activity of catalyzing the elongation of C18-CoA to form C20-C26-CoA (EC 2.3.1.199). This activity may be referred to as “fatty acid elongase III activity”. C26-CoA may preferably be used for the synthesis of ceramides catalyzed by ceramide synthase. A protein encoded by ELO3 gene may be referred to as “Elo3p”. The nucleotide sequence of ELO3 gene of *S. cerevisiae* S288C is shown as SEQ ID NO: 27, and the amino acid sequence of Elo3p encoded thereby is shown as SEQ ID NO: 28. The activity of Elo3p may be reduced, for example, in cases of producing sphingoid bases such as PHS. A reduced activity of Elo3p may specifically mean a reduced fatty acid elongase III activity. Fatty acid elongase III activity can be measured by, for example, a known method (J Biol Chem. 1997 Jul 11;272(28):17376-84.).

[0035] CKA2 gene encodes an alpha' subunit of casein kinase 2. The term “casein kinase 2” refers to a protein having an activity of catalyzing the serine/threonine-selective phosphorylation of proteins (EC 2.7.11.1). This activity may be referred to as “casein kinase 2 activity”. A protein encoded by CKA2 gene may be referred to as “Cka2p”. The nucleotide sequence of CKA2 gene of *S. cerevisiae* S288C is shown as SEQ ID NO: 29, and the amino acid sequence of Cka2p encoded thereby is shown as SEQ ID NO: 30. Cka2p may form a heterotetramer in combination with CKA1, CKB1, and CKB2 gene products, i.e. Cka1p, Ckb1p, and Ckb2p, to function as casein kinase 2. Cka2p may be required for full activation of ceramide synthase (Eukaryot Cell. 2003 Apr;2(2):284-94.). The activity of Cka2p may be reduced, for example, in cases of producing sphingoid bases such as PHS. A reduced activity of Cka2p may specifically mean a reduced casein kinase 2 activity. Also, a reduced activity of Cka2p may

specifically mean a reduced ceramide synthase activity. Casein kinase 2 activity can be measured by, for example, a known method (Gene. 1997 Jun 19;192(2):245-50.).

- [0036] ORM2 gene encodes a membrane protein that regulates serine palmitoyltransferase activity. A protein encoded by ORM2 gene may be referred to as “Orm2p”. The nucleotide sequence of ORM2 gene of *S. cerevisiae* S288C is shown as SEQ ID NO: 31, and the amino acid sequence of Orm2p encoded thereby is shown as SEQ ID NO: 32. The activity of Orm2p may be reduced, for example, in cases of producing any objective substance, and specifically in cases of producing sphingoid bases such as PHS. A reduced activity of Orm2p may specifically mean an increased serine palmitoyltransferase activity.
- [0037] CHA1 gene encodes L-serine/L-threonine ammonia-lyase. The term “L-serine/L-threonine ammonia-lyase” refers to a protein having an activity of catalyzing the reaction of degrading of L-serine and L-threonine (EC 4.3.1.17 and EC 4.3.1.19). This activity may be referred to as “L-serine/L-threonine ammonia-lyase activity”. A protein encoded by CHA1 gene may be referred to as “Cha1p”. The nucleotide sequence of CHA1 gene of *S. cerevisiae* S288C is shown as SEQ ID NO: 33, and the amino acid sequence of Cha1p encoded thereby is shown as SEQ ID NO: 34. The activity of Cha1p may be reduced, for example, in cases of producing any objective substance, and specifically in cases of producing sphingoid bases such as PHS and sphingolipids such as phytoceramides and glucosylceramides. A reduced activity of Cha1p may specifically mean a reduced L-serine/L-threonine ammonia-lyase activity. L-serine/L-threonine ammonia-lyase activity can be measured by, for example, a known method (Eur J Biochem. 1982 Apr;123(3):571-6.).
- [0038] YPC1 gene encodes phytoceramidase. The term “phytoceramidase” refers to a protein having an activity of catalyzing the decomposition of phytoceramides (EC 3.5.1.-). This activity may be referred to as “phytoceramidase activity”. A protein encoded by YPC1 gene may be referred to as “Ypc1p”. The nucleotide sequence of YPC1 gene of *S. cerevisiae* S288C is shown as SEQ ID NO: 35, and the amino acid sequence of Ypc1p encoded thereby is shown as SEQ ID NO: 36. The activity of Ypc1p may be reduced, for example, in cases of producing sphingolipids such as phytoceramides and glucosylceramides. A reduced activity of Ypc1p may specifically mean a reduced phytoceramidase activity. Phytoceramidase activity can be measured by, for example, a known method (J Biol Chem. 2000 Mar 10;275(10):6876-84.).
- [0039] The target genes and proteins, i.e. LCB1, LCB2, TSC10, SUR2, SLI1, ATF2, LAG1, LAC1, LIP1, UGCG, LCB4, LCB5, ELO3, CKA2, ORM2, CHA1, and YPC1 genes, and proteins encoded thereby, may have the aforementioned nucleotide and amino acid sequences. The expression “a gene or protein has a nucleotide or amino acid sequence” encompasses cases where a gene or protein comprises the nucleotide or amino acid

sequence and cases where a gene or protein consists of the nucleotide or amino acid sequence.

[0040] The target genes may be variants of the respective genes exemplified above, so long as the original function thereof is maintained. Similarly, the target proteins may be variants of the respective proteins exemplified above, so long as the original function thereof is maintained. Such variants that maintain the original function thereof may also be referred to as "conservative variant". The term "LCB1", "LCB2", "TSC10", "SUR2", "SLI1", "ATF2", "LAG1", "LAC1", "LIP1", "UGCG", "LCB4", "LCB5", "ELO3", "CKA2", "ORM2", "CHA1", and "YPC1" genes include, in addition to the respective genes exemplified above, conservative variants thereof. Similarly, the term "Lcb1p", "Lcb2p", "Tsc10p", "Sur2p", "Sli1p", "Atf2p", "Lag1p", "Lac1p", "Lip1p", "Ugcg protein", "Lcb4p", "Lcb5p", "Elo3p", "Cka2p", "Orm2p", "Cha1p", and "Ypc1p" include, in addition to the respective proteins exemplified above, conservative variants thereof. That is, for example, the term "LCB1 gene" includes the LCB1 gene exemplified above, e.g. LCB1 gene of *S. cerevisiae*, and further includes variants thereof. Similarly, for example, the term "Lcb1 protein" includes the Lcb1 protein exemplified above, e.g. the protein encoded by LCB1 gene of *S. cerevisiae*, and further includes variants thereof. Examples of the conservative variants include, for example, homologues and artificially modified versions of the target genes and proteins exemplified above. Methods of generating variants of a gene or a protein are well known in the art.

[0041] The expression "the original function is maintained" means that a variant of a gene or protein has a function (such as activity and property) corresponding to the function (such as activity and property) of the original gene or protein. The expression "the original function is maintained" regarding a gene means that a variant of the gene encodes a protein of which the original function is maintained. The expression "the original function is maintained" regarding a protein means that a variant of the protein has the corresponding function such as activity and property exemplified above. That is, the expression "the original function is maintained" regarding the target proteins may mean that a variant protein has serine palmitoyltransferase activity as for Lcb1p and Lcb2p; 3-dehydrosphinganine reductase activity as for Tsc10p; sphingosine hydroxylase activity as for Sur2p; acetyltransferase activity as for Atf2p and Sli1p; ceramide synthase activity as for Lag1p, Lac1p, and Lip1p; UDP-glucose ceramide glucosyltransferase as for Ugcg proteins; sphingoid base kinase activity as for Lcb4p and Lcb5p; fatty acid elongase III activity as for Elo3p; casein kinase 2 activity as for Cka2p; property of regulating serine palmitoyltransferase activity as for Orm2p; L-serine/L-threonine ammonia-lyase activity as for Cha1p; and phytoceramidase activity as for Ypc1p. In addition, the expression "the original function is maintained"

regarding Cka2p may also mean that a variant of the protein has a property that a reduced activity thereof results in a reduced ceramide synthase activity. In addition, the expression “the original function is maintained” regarding Orm2p may also mean that a variant of the protein has a property that a reduced activity thereof results in an increased serine palmitoyltransferase activity. In cases where a target protein functions as a complex consisting of a plurality of subunits, the expression “the original function is maintained” regarding the target protein may also mean that a variant of the protein exhibits the corresponding function such as activity and property exemplified above in combination with other appropriate subunit(s). That is, for example, the expression “the original function is maintained” regarding Lcb1p may also mean that a variant protein has serine palmitoyltransferase activity in combination with an appropriate Lcb2p, and the expression “the original function is maintained” regarding Lcb2p may also mean that a variant protein has serine palmitoyltransferase activity in combination with an appropriate Lcb1p. Also, the expression “the original function is maintained” regarding Lag1p or Lac1p may also mean that a variant protein has ceramide synthase activity in combination with an appropriate Lip1p, and the expression “the original function is maintained” regarding Lip1p may also mean that a variant protein has ceramide synthase activity in combination with an appropriate Lag1p or Lac1p.

[0042] Hereafter, conservative variants will be exemplified.

[0043] Homologues of the genes exemplified above or homologues of the proteins exemplified above can easily be obtained from a public database by, for example, BLAST search or FASTA search using the nucleotide sequence of any of the genes exemplified above or the amino acid sequence of any of the proteins exemplified above as a query sequence. Furthermore, homologues of the genes exemplified above can be obtained by, for example, PCR using the chromosome of an organism such as yeast as the template, and oligonucleotides prepared on the basis of the nucleotide sequence of any of the genes exemplified above as primers.

[0044] The target genes each may be a gene encoding a protein having any of the aforementioned amino acid sequences but including substitution, deletion, insertion, and/or addition of one or several amino acid residues at one or several positions, so long as the original function is maintained. For example, the encoded protein may have an extended or deleted N-terminus and/or C-terminus. Although the number meant by the term “one or several” used above may differ depending on the positions of amino acid residues in the three-dimensional structure of the protein or the types of amino acid residues, specifically, it is, for example, 1 to 50, 1 to 40, or 1 to 30, preferably 1 to 20, more preferably 1 to 10, still more preferably 1 to 5, particularly preferably 1 to 3.

[0045] The aforementioned substitution, deletion, insertion, and/or addition of one or several amino acid residues is a conservative mutation that maintains normal function of the

protein. Typical examples of the conservative mutation are conservative substitutions. The conservative substitution is a mutation wherein substitution takes place mutually among Phe, Trp, and Tyr, if the substitution site is an aromatic amino acid; among Leu, Ile, and Val, if it is a hydrophobic amino acid; between Gln and Asn, if it is a polar amino acid; among Lys, Arg, and His, if it is a basic amino acid; between Asp and Glu, if it is an acidic amino acid; and between Ser and Thr, if it is an amino acid having a hydroxyl group. Examples of substitutions considered as conservative substitutions include, specifically, substitution of Ser or Thr for Ala, substitution of Gln, His, or Lys for Arg, substitution of Glu, Gln, Lys, His, or Asp for Asn, substitution of Asn, Glu, or Gln for Asp, substitution of Ser or Ala for Cys, substitution of Asn, Glu, Lys, His, Asp, or Arg for Gln, substitution of Gly, Asn, Gln, Lys, or Asp for Glu, substitution of Pro for Gly, substitution of Asn, Lys, Gln, Arg, or Tyr for His, substitution of Leu, Met, Val, or Phe for Ile, substitution of Ile, Met, Val, or Phe for Leu, substitution of Asn, Glu, Gln, His, or Arg for Lys, substitution of Ile, Leu, Val, or Phe for Met, substitution of Trp, Tyr, Met, Ile, or Leu for Phe, substitution of Thr or Ala for Ser, substitution of Ser or Ala for Thr, substitution of Phe or Tyr for Trp, substitution of His, Phe, or Trp for Tyr, and substitution of Met, Ile, or Leu for Val. Further, such substitution, deletion, insertion, addition, inversion, or the like of amino acid residues as mentioned above includes a naturally occurring mutation due to an individual difference, or a difference of species of the organism from which the gene is derived (mutant or variant).

[0046] Furthermore, the target genes each may be a gene encoding a protein showing a homology of 80% or more, preferably 90% or more, more preferably 95% or more, still more preferably 97% or more, particularly preferably 99% or more, to any of the total amino acid sequence mentioned above, so long as the original function is maintained. In addition, in this specification, "homology" means "identity".

[0047] Furthermore, the target genes each may be a DNA that is able to hybridize under stringent conditions with a probe that can be prepared from any of the aforementioned nucleotide sequences, such as a sequence complementary to the whole sequence or a partial sequence of any of the aforementioned nucleotide sequences, so long as the original function is maintained. The "stringent conditions" refer to conditions under which a so-called specific hybrid is formed, and a non-specific hybrid is not formed. Examples of the stringent conditions include those under which highly homologous DNAs hybridize to each other, for example, DNAs not less than 80% homologous, preferably not less than 90% homologous, more preferably not less than 95% homologous, still more preferably not less than 97% homologous, particularly preferably not less than 99% homologous, hybridize to each other, and DNAs less homologous than the above do not hybridize to each other, or conditions of washing of typical

Southern hybridization, i.e., conditions of washing once, preferably 2 or 3 times, at a salt concentration and temperature corresponding to 1 x SSC, 0.1% SDS at 60°C, preferably 0.1 x SSC, 0.1% SDS at 60°C, more preferably 0.1 x SSC, 0.1% SDS at 68°C.

- [0048] The probe used for the aforementioned hybridization may be a part of a sequence that is complementary to the gene as described above. Such a probe can be prepared by PCR using oligonucleotides prepared on the basis of a known gene sequence as primers and a DNA fragment containing the nucleotide sequence as a template. As the probe, for example, a DNA fragment having a length of about 300 bp can be used. When a DNA fragment having a length of about 300 bp is used as the probe, in particular, the washing conditions of the hybridization may be, for example, 50°C, 2 x SSC and 0.1% SDS.
- [0049] Further, the target genes each may be a gene having any of the aforementioned nucleotide sequences in which an arbitrary codon is replaced with an equivalent codon. For example, the target genes each may be a gene modified so that it has optimal codons according to codon frequencies in a host to be used.
- [0050] The percentage of the sequence identity between two sequences can be determined by, for example, using a mathematical algorithm. Non-limiting examples of such a mathematical algorithm include the algorithm of Myers and Miller (1988) CABIOS 4:11-17, the local homology algorithm of Smith et al (1981) Adv. Appl. Math. 2:482, the homology alignment algorithm of Needleman and Wunsch (1970) J. Mol. Biol. 48:443-453, the method for searching homology of Pearson and Lipman (1988) Proc. Natl. Acad. Sci. 85:2444-2448, and an modified version of the algorithm of Karlin and Altschul (1990) Proc. Natl. Acad. Sci. USA 87:2264, such as that described in Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90:5873-5877.
- [0051] By using a program based on such a mathematical algorithm, sequence comparison (i.e. alignment) for determining the sequence identity can be performed. The program can be appropriately executed by a computer. Examples of such a program include, but not limited to, CLUSTAL of PC/Gene program (available from Intelligenetics, Mountain View, Calif.), ALIGN program (Version 2.0), and GAP, BESTFIT, BLAST, FASTA, and TFASTA of Wisconsin Genetics Software Package, Version 8 (available from Genetics Computer Group (GCG), 575 Science Drive, Madison, Wis., USA). Alignment using these programs can be performed by using, for example, initial parameters. The CLUSTAL program is well described in Higgins et al. (1988) Gene 73:237-244, Higgins et al. (1989) CABIOS 5:151-153, Corpet et al. (1988) Nucleic Acids Res. 16:10881-90, Huang et al. (1992) CABIOS 8:155-65, and Pearson et al. (1994) Meth. Mol. Biol. 24:307-331.
- [0052] In order to obtain a nucleotide sequence homologous to a target nucleotide sequence,

in particular, for example, BLAST nucleotide search can be performed by using BLASTN program with score of 100 and word length of 12. In order to obtain an amino acid sequence homologous to a target protein, in particular, for example, BLAST protein search can be performed by using BLASTX program with score of 50 and word length of 3. See <http://www.ncbi.nlm.nih.gov> for BLAST nucleotide search and BLAST protein search. In addition, Gapped BLAST (BLAST 2.0) can be used in order to obtain an alignment including gap(s) for the purpose of comparison. In addition, PSI-BLAST can be used in order to perform repetitive search for detecting distant relationships between sequences. See Altschul et al. (1997) *Nucleic Acids Res.* 25:3389 for Gapped BLAST and PSI-BLAST. When using BLAST, Gapped BLAST, or PSI-BLAST, initial parameters of each program (e.g. BLASTN for nucleotide sequences, and BLASTX for amino acid sequences) can be used. Alignment can also be manually performed.

[0053] The sequence identity between two sequences is calculated as the ratio of residues matching in the two sequences when aligning the two sequences so as to fit maximally with each other.

[0054] <1-2> Methods for increasing activity of protein

Hereafter, methods for increasing the activity of a protein will be explained.

[0055] The expression "the activity of a protein is increased" means that the activity of the protein per cell is increased as compared with that of a non-modified strain. The term "non-modified strain" may refer to a reference strain that has not been modified so that the activity of an objective protein is increased. Examples of the non-modified strain include a wild-type strain and parent strain. The state that "the activity of a protein is increased" may also be expressed as "the activity of a protein is enhanced". Specifically, the expression "the activity of a protein is increased" means that the number of molecules of the protein per cell is increased, and/or the function of each molecule of the protein is increased as compared with those of a non-modified strain. That is, the term "activity" in the expression "the activity of a protein is increased" is not limited to the catalytic activity of the protein, but may also mean the transcription amount of a gene (i.e. the amount of mRNA) coding for the protein, or the translation amount of the protein (i.e. the amount of the protein). Although the degree of the increase in the activity of a protein is not particularly limited so long as the activity of the protein is increased as compared with that of a non-modified strain, the activity of the protein may be increased 1.5 times or more, 2 times or more, or 3 times or more, as compared with that of a non-modified strain. Further, the state that "the activity of a protein is increased" includes not only a state that the activity of an objective protein is increased in a strain inherently having the activity of the objective protein, but also a state that the activity of an objective protein is imparted to a strain not inherently

having the activity of the objective protein. Further, so long as the activity of the protein is eventually increased, the activity of an objective protein inherently contained in a host may be attenuated and/or eliminated, and then an appropriate type of the objective protein may be introduced thereto.

[0056] The modification for increasing the activity of a protein is attained by, for example, increasing the expression of a gene coding for the protein. The expression "the expression of a gene is increased" means that the expression amount of the gene per cell is increased as compared with that of a non-modified strain such as a wild-type strain and parent strain. The expression "the expression of a gene is increased" may specifically mean that the transcription amount of the gene (i.e. the amount of mRNA) is increased, and/or the translation amount of the gene (i.e. the amount of the protein expressed from the gene) is increased. The state that "the expression of a gene is increased" may also be referred to as "the expression of a gene is enhanced". The expression of a gene may be increased 1.5 times or more, 2 times or more, or 3 times or more, as compared with that observed in a non-modified strain. Further, the state that "the expression of a gene is increased" includes not only a state that the expression amount of an objective gene is increased in a strain that inherently expresses the objective gene, but also a state that the gene is introduced into a strain that does not inherently express the objective gene, and expressed therein. That is, the phrase "the expression of a gene is increased" may also mean, for example, that an objective gene is introduced into a strain that does not possess the gene, and is expressed therein.

[0057] The expression of a gene can be increased by, for example, increasing the copy number of the gene.

[0058] The copy number of a gene can be increased by introducing the gene into the chromosome of a host. A gene can be introduced into a chromosome by, for example, using homologous recombination (Miller, J.H., Experiments in Molecular Genetics, 1972, Cold Spring Harbor Laboratory). Only one copy, or two or more copies of a gene may be introduced. For example, by performing homologous recombination using a sequence which is present in multiple copies on a chromosome as a target, multiple copies of a gene can be introduced into the chromosome. Examples of such a sequence which is present in multiple copies on a chromosome include autonomously replicating sequences (ARS) consisting of a specific short repeated sequence, and rDNA sequences present in about 150 copies on the chromosome. WO95/32289 discloses an example where gene recombination was performed in yeast by using homologous recombination. In addition, a gene can also be introduced into a chromosome by, for example, integrating the gene into a transposon and transferring the transposon to the chromosome.

[0059] Introduction of an objective gene into a chromosome can be confirmed by Southern

hybridization using a probe having a sequence complementary to the whole or a part of the gene, PCR using primers prepared on the basis of the sequence of the gene, or the like.

- [0060] Further, the copy number of an objective gene can also be increased by introducing a vector including the gene into a host. For example, the copy number of an objective gene can be increased by ligating a DNA fragment including the objective gene with a vector that functions in a host to construct an expression vector of the gene, and by transforming the host with the expression vector. The DNA fragment including the objective gene can be obtained by, for example, PCR using the genomic DNA of a microorganism having the objective gene as the template. As the vector, a vector autonomously replicable in the cell of the host can be used. The vector may be a single copy vector or may be a multi-copy vector. Further, the vector preferably includes a marker for selection of transformant. Examples of the marker include antibiotic resistance genes such as KanMX, NatMX (nat1), and HygMX (hph) genes, and genes complementing auxotrophy such as LEU2, HIS3, and URA3 genes. Examples of vector autonomously replicable in yeast include plasmids having a CEN4 replication origin and plasmids having a 2 μ m DNA replication origin. Specific examples of vector autonomously replicable in yeast include pAUR123 (TAKARA BIO) and pYES2 (Invitrogen).
- [0061] When a gene is introduced, it is sufficient that the gene is expressibly harbored by the yeast of the present invention. Specifically, it is sufficient that the gene is introduced so that it is expressed under the control of a promoter sequence that functions in the yeast of the present invention. The promoter may be a promoter derived from the host, or a heterogenous promoter. The promoter may be the native promoter of the gene to be introduced, or a promoter of another gene. As the promoter, for example, such a stronger promoter as mentioned later may also be used.
- [0062] A terminator can be located downstream the gene. The terminator is not particularly limited as long as a terminator that functions in the yeast of the present invention is chosen. The terminator may be a terminator derived from the host, or a heterogenous terminator. The terminator may be the native terminator of the gene to be introduced, or a terminator of another gene. Examples of the terminator that functions in the yeast of the present invention include CYC1, ADH1, ADH2, ENO2, PGI1, and TDH1 terminators.
- [0063] Vectors, promoters, and terminators available in various microorganisms are disclosed in detail in "Fundamental Microbiology Vol. 8, Genetic Engineering, KYORITSU SHUPPAN CO., LTD, 1987", and those can be used.
- [0064] Further, when two or more kinds of genes are introduced, it is sufficient that the genes each are expressibly harbored by the yeast of the present invention. For example,

all the genes may be carried by a single expression vector or a chromosome. Alternatively, the genes may be separately carried by two or more expression vectors, or separately carried by a single or two or more expression vectors and a chromosome. An operon constituted by two or more genes may also be introduced.

- [0065] The gene to be introduced is not particularly limited so long as it codes for a protein that functions in the host. The gene to be introduced may be a gene derived from the host, or may be a heterogenous gene. The gene to be introduced can be obtained by, for example, PCR using primers designed on the basis of the nucleotide sequence of the gene and the genomic DNA of an organism having the gene or a plasmid carrying the gene as a template. The gene to be introduced may also be totally synthesized, for example, on the basis of the nucleotide sequence of the gene (Gene, 60(1), 115-127 (1987)). The obtained gene can be used as it is, or after being modified as required.
- [0066] Further, the expression of a gene can be increased by improving the transcription efficiency of the gene. In addition, the expression of a gene can also be increased by improving the translation efficiency of the gene. The transcription efficiency of the gene and the translation efficiency of the gene can be improved by, for example, modifying an expression control sequence of the gene. The term "expression control sequence" collectively refers to sites that affect the expression of a gene, such as a promoter. Expression control sequences can be identified by using a promoter search vector or gene analysis software such as GENETYX.
- [0067] The transcription efficiency of a gene can be improved by, for example, replacing the promoter of the gene on a chromosome with a stronger promoter. The "stronger promoter" means a promoter providing an improved transcription of a gene as compared with an inherently existing wild-type promoter of the gene. Examples of stronger promoters usable in yeast include PGK1, PGK2, PDC1, TDH3, TEF1, TEF2, TPI1, HXT7, ADH1, GPD1, and KEX2 promoters. Further, as the stronger promoter, a highly-active type of an existing promoter may also be obtained by using various reporter genes.
- [0068] The translation efficiency of a gene can also be improved by, for example, modifying codons. For example, in the case of heterogenous expression of a gene or the like, the translation efficiency of the gene can be improved by replacing a rare codon present in the gene with a more frequently used synonymous codon. That is, a gene to be introduced may have been modified, for example, so that it has optimal codons according to codon frequencies observed in the host to be used. Codons can be replaced by, for example, the site-specific mutation method for introducing an objective mutation into an objective site of DNA. Alternatively, a gene fragment in which objective codons are replaced may be totally synthesized. Frequencies of codons in various organisms are disclosed in the "Codon Usage Database"

(<http://www.kazusa.or.jp/codon>; Nakamura, Y. et al, Nucl. Acids Res., 28, 292 (2000)).

- [0069] Further, the expression of a gene can also be increased by amplifying a regulator that increases the expression of the gene, or deleting or attenuating a regulator that reduces the expression of the gene.
- [0070] Such methods for increasing the gene expression as mentioned above may be used independently or in an arbitrary combination.
- [0071] Further, the modification that increases the activity of an enzyme can also be attained by, for example, enhancing the specific activity of the enzyme. An enzyme showing an enhanced specific activity can be obtained by, for example, searching various organisms. Further, a highly-active type of an existing enzyme may also be obtained by introducing a mutation into the existing enzyme. Enhancement of the specific activity may be independently used, or may be used in an arbitrary combination with such methods for enhancing the gene expression as mentioned above.
- [0072] The method for transformation is not particularly limited, and methods conventionally used for transformation of yeast can be used. Examples of such methods include protoplast method, KU method (H.Ito et al., J. Bacteriol., 153-163 (1983)), KUR method (Fermentation and industry, vol.43, p.630-637 (1985)), electroporation method (Luis et al., FEMS Micro biology Letters 165 (1998) 335-340), and a method using a carrier DNA (Gietz R.D. and Schiestl R.H., Methods Mol.Cell. Biol. 5:255-269 (1995)). Methods for manipulating yeast such as methods for spore-forming and methods for isolating haploid yeast are disclosed in Chemistry and Biology, Experimental Line 31, Experimental Techniques for Yeast, 1st Edition, Hirokawa-Shoten; Bio-Manual Series 10, Genetic Experimental Methods for Yeast, 1st Edition, Yodosha; and so forth.
- [0073] An increase in the activity of a protein can be confirmed by measuring the activity of the protein.
- [0074] An increase in the activity of a protein can also be confirmed by confirming an increase in the expression of a gene coding for the protein. An increase in the expression of a gene can be confirmed by confirming an increase in the transcription amount of the gene, or by confirming an increase in the amount of a protein expressed from the gene.
- [0075] An increase of the transcription amount of a gene can be confirmed by comparing the amount of mRNA transcribed from the gene with that observed in a non-modified strain such as a wild-type strain or parent strain. Examples of the method for evaluating the amount of mRNA include Northern hybridization, RT-PCR, and so forth (Sambrook, J., et al., Molecular Cloning A Laboratory Manual/Third Edition, Cold spring Harbor Laboratory Press, Cold Spring Harbor (USA), 2001). The amount

of mRNA may increase, for example, 1.5 times or more, 2 times or more, or 3 times or more, as compared with that of a non-modified strain.

[0076] An increase in the amount of a protein can be confirmed by Western blotting using antibodies (Molecular Cloning, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (USA), 2001). The amount of the protein may increase, for example, 1.5 times or more, 2 times or more, or 3 times or more, as compared with that of a non-modified strain.

[0077] <1-3> Method for reducing activity of protein

Hereafter, methods for reducing the activity of a protein will be explained.

[0078] The expression "the activity of a protein is reduced" means that the activity of the protein per cell is reduced as compared with that of a non-modified strain. The term "non-modified strain" may refer to a reference strain that has not been modified so that the activity of an objective protein is reduced. Examples of the non-modified strain include a wild-type strain or parent strain. The state that "the activity of a protein is reduced" also includes a state that the activity of the protein has completely disappeared. Specifically, the expression "the activity of a protein is reduced" means that the number of molecules of the protein per cell is reduced, and/or the function of each molecule of the protein is reduced as compared with those of a non-modified strain. That is, the term "activity" in the expression "the activity of a protein is reduced" is not limited to the catalytic activity of the protein, but may also mean the transcription amount of a gene (i.e. the amount of mRNA) coding for the protein or the translation amount of the protein (i.e. the amount of the protein). The state that "the number of molecules of the protein per cell is reduced" also includes a state that the protein does not exist at all. The state that "the function of each molecule of the protein is reduced" also includes a state that the function of each protein molecule completely disappears. Although the degree of the reduction in the activity of a protein is not particularly limited so long as the activity is reduced as compared with that of a non-modified strain, it may be reduced to, for example, 50% or less, 20% or less, 10% or less, 5% or less, or 0% of that of a non-modified strain.

[0079] The modification for reducing the activity of a protein can be attained by, for example, reducing the expression of a gene coding for the protein. The expression "the expression of a gene is reduced" means that the expression of the gene per cell is reduced as compared with that of a non-modified strain such as a wild-type strain and parent strain. The expression "the expression of a gene is reduced" may specifically mean that the transcription amount of the gene (i.e. the amount of mRNA) is reduced, and/or the translation amount of the gene (i.e. the amount of the protein expressed from the gene) is reduced. The state that "the expression of a gene is reduced" also includes a state that the gene is not expressed at all. The state that "the expression of a gene is

reduced" is also referred to as "the expression of a gene is attenuated". The expression of a gene may be reduced to 50% or less, 20% or less, 10% or less, 5% or less, or 0% of that of a non-modified strain.

[0080] The reduction in gene expression may be due to, for example, a reduction in the transcription efficiency, a reduction in the translation efficiency, or a combination of them. The expression of a gene can be reduced by modifying an expression control sequence of the gene such as a promoter. When an expression control sequence is modified, preferably one or more nucleotides, more preferably two or more nucleotides, particularly preferably three or more nucleotides, of the expression control sequence are modified. Further, a part or the whole of an expression control sequence may be deleted. The expression of a gene can also be reduced by, for example, manipulating a factor responsible for expression control. Examples of the factor responsible for expression control include low molecules responsible for transcription or translation control (inducers, inhibitors, etc.), proteins responsible for transcription or translation control (transcription factors etc.), nucleic acids responsible for transcription or translation control (siRNA etc.), and so forth. Further, the expression of a gene can also be reduced by, for example, introducing a mutation that reduces the expression of the gene into the coding region of the gene. For example, the expression of a gene can be reduced by replacing a codon in the coding region of the gene with a synonymous codon used less frequently in a host. Further, for example, the gene expression may be reduced due to disruption of a gene as described later.

[0081] The modification for reducing the activity of a protein can also be attained by, for example, disrupting a gene coding for the protein. The expression "a gene is disrupted" means that a gene is modified so that a protein that can normally function is not produced. The expression "a protein that can normally function is not produced" encompasses cases where no protein is expressed from the gene at all and cases where a protein of which the function (such as activity and property) has been reduced or completely eliminated is expressed from the gene.

[0082] Disruption of a gene can be attained by, for example, deleting a part or the whole of the coding region of the gene on a chromosome. Furthermore, the whole of a gene including sequences upstream and downstream from the gene on a chromosome may be deleted. The region to be deleted may be any region such as an N-terminus region, an internal region, or a C-terminus region, so long as the activity of the protein can be reduced. Deletion of a longer region can usually more surely inactivate the gene. Further, it is preferred that reading frames of the sequences upstream and downstream from the region to be deleted are not the same.

[0083] Disruption of a gene can also be attained by, for example, introducing a mutation for an amino acid substitution (missense mutation), a stop codon (nonsense mutation), a

frame shift mutation which adds or deletes one or two nucleotide residues, or the like into the coding region of the gene on a chromosome (Journal of Biological Chemistry, 272:8611-8617 (1997); Proceedings of the National Academy of Sciences, USA, 95 5511-5515 (1998); Journal of Biological Chemistry, 26 116, 20833-20839 (1991)).

[0084] Disruption of a gene can also be attained by, for example, inserting another sequence into a coding region of the gene on a chromosome. Site of the insertion may be in any region of the gene, and insertion of a longer region can usually more surely inactivate the gene. It is preferred that reading frames of the sequences upstream and downstream from the insertion site are not the same. The other sequence is not particularly limited so long as a sequence that reduces or eliminates the activity of the encoded protein is chosen, and examples thereof include, for example, a marker gene such as antibiotic resistance genes, and a gene useful for production of an objective substance.

[0085] Such modification of a gene on a chromosome as described above can be attained by, for example, using a recombinant DNA. The structure of the recombinant DNA to be used for homologous recombination is not particularly limited as long as it causes homologous recombination in a desired manner. For example, a host can be transformed with a linear DNA comprising an arbitrary sequence such as a deficient type gene or any appropriate insertion sequence, which arbitrary sequence is flanked with upstream and downstream sequences of the homologous recombination target region on the chromosome, so that homologous recombination can occur at upstream and downstream sides of the target region, to thereby replace the target region with the arbitrary sequence. Specifically, such modification of a gene on a chromosome as described above can be attained by, for example, preparing a deficient type gene modified so that it cannot produce a protein that can normally function, and transforming a host with a recombinant DNA including the deficient type gene to cause homologous recombination between the deficient type gene and the wild-type gene on a chromosome and thereby substitute the deficient type gene for the wild-type gene on the chromosome. In this procedure, if a marker gene selected according to the characteristics of the host such as auxotrophy is included in the recombinant DNA, the operation becomes easy. Examples of the deficient type gene include a gene in which a part or whole of the gene is deleted, a gene introduced with missense mutation, a gene introduced with an insertion sequence such as a transposon and a marker gene, a gene introduced with nonsense mutation, and a gene introduced with frameshift mutation. The protein encoded by the deficient type gene has a conformation different from that of the wild-type protein, even if it is produced, and thus the function thereof is reduced or eliminated.

[0086] The modification for reducing the activity of a protein can also be attained by, for example, a mutagenesis treatment. Examples of the mutagenesis treatment include

usual mutation treatments such as irradiation of X-ray or ultraviolet and treatment with a mutation agent such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), ethyl methanesulfonate (EMS), and methyl methanesulfonate (MMS).

- [0087] A reduction in the activity of a protein can be confirmed by measuring the activity of the protein.
- [0088] A reduction in the expression of a gene can be confirmed by confirming a reduction in the transcription amount of the gene or a reduction in the amount of the protein expressed from the gene.
- [0089] A reduction in the transcription amount of a gene can be confirmed by comparing the amount of mRNA transcribed from the gene with that observed in a non-modified strain. Examples of the method for evaluating the amount of mRNA include Northern hybridization, RT-PCR, and so forth (Molecular Cloning, Cold spring Harbor Laboratory Press, Cold Spring Harbor (USA), 2001). The amount of mRNA is preferably reduced to, for example, 50% or less, 20% or less, 10% or less, 5% or less, or 0%, of that observed in a non-modified strain.
- [0090] A reduction in the amount of a protein can be confirmed by Western blotting using antibodies (Molecular Cloning, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (USA) 2001). The amount of the protein is preferably reduced to, for example, 50% or less, 20% or less, 10% or less, 5% or less, or 0%, of that observed in a non-modified strain.
- [0091] Disruption of a gene can be confirmed by determining nucleotide sequence of a part or the whole of the gene, restriction enzyme map, full length, or the like of the gene depending on the means used for the disruption.
- [0092] <2> Method for producing objective substance of the present invention
The method of the present invention is a method for producing an objective substance comprising cultivating the yeast of the present invention in a culture medium containing an additive that is able to associate with, bind to, solubilize, and/or capture the objective substance, and collecting the objective substance from cells of the yeast and/or the culture medium. In the method of the present invention, a single kind of objective substance may be produced, or two or more kinds of objective substances may be produced.
- [0093] The medium to be used is not particularly limited, so long as it contains the additive that is able to associate with, bind to, solubilize, and/or capture the objective substance, the yeast of the present invention can proliferate in it, and an objective substance can be produced. As the medium, for example, a usual medium used for cultivating yeast can be used, except that it contains the additive. Examples of such a medium include SD medium, SG medium, SDTE medium, and YPD medium, supplemented with the additive. The medium may contain carbon source, nitrogen source, phosphorus source,

and sulfur source, as well as components selected from other various organic components and inorganic components as required, in addition to the additive. The types and concentrations of the medium components can be appropriately determined according to various conditions such as the type of the yeast to be used and the type of the objective substance to be produced.

[0094] The culture medium contains an additive that is able to associate with, bind to, solubilize, and/or capture the objective substance. Use of the additive results in an increased production of the objective substance. That is, the amount produced of the objective substance by the yeast of the present invention is increased in the presence of the additive as compared with in the absence of the additive. Use of the additive may specifically result in an increased production of the objective substance in the culture medium. The production of the objective substance in the culture medium may also be referred to as “excretion of the objective substance”. The expression “associating with, binding to, solubilizing, and/or capturing an objective substance” may specifically mean increasing the solubility of the objective substance into the culture medium. Examples of the additive include cyclodextrins and zeolites. The number of glucose residues constituting cyclodextrins is not particularly limited, and it may be, for example, 5, 6, 7, or 8. That is, examples of cyclodextrins include cyclodextrin consisting of 5 glucose residues, alpha-cyclodextrin, beta-cyclodextrin, gamma-cyclodextrin, and derivatives thereof. Examples of cyclodextrin derivatives include cyclodextrins into which one or more functional groups have been introduced. The type, number, and amount of the functional group, and the position to which the functional group is introduced are not particularly limited as long as the derivative is able to associate with, bind to, solubilize, and/or capture the objective substance. The functional group may be introduced to, for example, hydroxyl group of C2, C3, C6, or a combination thereof, which may result in an increased solubility of cyclodextrin itself. Examples of the functional group include alkyl groups and hydroxyalkyl groups. The alkyl groups and hydroxyalkyl groups each may have a linear alkyl chain or may have a branched alkyl chain. The alkyl groups and hydroxyalkyl groups each may have a carbon number of, for example, 1, 2, 3, 4, or 5. Specific examples of the alkyl groups include methyl, ethyl, propyl, butyl, pentyl, isopropyl, and isobutyl groups. Specific examples of the hydroxyalkyl groups include hydroxymethyl, hydroxyethyl, hydroxypropyl, hydroxybutyl, hydroxypentyl, hydroxyisopropyl, and hydroxyisobutyl groups. Specific examples of cyclodextrin derivatives include methyl-alpha-cyclodextrin, methyl-beta-cyclodextrin, hydroxypropyl-alpha-cyclodextrin such as 2-hydroxypropyl-alpha-cyclodextrin, and hydroxypropyl-beta-cyclodextrin such as 2-hydroxypropyl-beta-cyclodextrin. The types of zeolites are not particularly limited. As the additive, a single kind of additive may be used, or two or more kinds of

additives may be used in combination.

- [0095] The additive may be contained in the medium during the whole period of the culture, or may be contained in the medium during only a part of the culture period. For example, the additive may be or may not be contained in the medium from the start of the culture. When the additive is not contained in the medium at the time of the start of the culture, the additive is supplied to the medium after the start of the culture. Timing of the supply can be appropriately determined according to various conditions such as the length of culture period. For example, the additive may be supplied to the medium after the yeast of the present invention fully grows. Further, in any case, the additive may be additionally supplied to the medium as required. Means for supplying the additive to the medium is not particularly limited. For example, the additive can be supplied to the medium by feeding a feed medium containing the additive to the medium. The concentration of the additive in the medium is not particularly limited so long as production of the objective substance can be improved. For example, the concentration of the additive in the medium may be 0.1 g/L or higher, 1 g/L or higher, 2 g/L or higher, 5 g/L or higher, or 10 g/L or higher, may be 200 g/L or lower, 100 g/L or lower, 50 g/L or lower, or 20 g/L or lower, or may be within a range defined with a combination thereof. The concentration of the additive in the medium may be, for example, 0.1 g/L to 200 g/L, 1 g/L to 100 g/L, or 5 g/L to 50 g/L. The additive may be or may not be contained in the medium at a concentration within the range exemplified above during the whole period of the culture. For example, the additive may be contained in the medium at a concentration within the range exemplified above at the start of the culture, or it may be supplied to the medium so that a concentration within the range exemplified above is attained after the start of the culture.
- [0096] Specific examples of the carbon source include, for example, saccharides such as glucose, fructose, sucrose, lactose, galactose, xylose, arabinose, blackstrap molasses, starch hydrolysates, and hydrolysates of biomass, organic acids such as acetic acid, fumaric acid, citric acid, and succinic acid, alcohols such as glycerol, crude glycerol, and ethanol, and aliphatic acids. As the carbon source, a single kind of carbon source may be used, or two or more kinds of carbon sources may be used in combination.
- [0097] Specific examples of the nitrogen source include, for example, ammonium salts such as ammonium sulfate, ammonium chloride, and ammonium phosphate, organic nitrogen sources such as peptone, yeast extract, meat extract, and soybean protein decomposition products, ammonia, and urea. Ammonia gas or aqueous ammonia used for adjusting pH may also be used as the nitrogen source. As the nitrogen source, a single kind of nitrogen source may be used, or two or more kinds of nitrogen sources may be used in combination.
- [0098] Specific examples of the phosphate source include, for example, phosphoric acid

salts such as potassium dihydrogenphosphate and dipotassium hydrogenphosphate, and phosphoric acid polymers such as pyrophosphoric acid. As the phosphate source, a single kind of phosphate source may be used, or two or more kinds of phosphate sources may be used in combination.

[0099] Specific examples of the sulfur source include, for example, inorganic sulfur compounds such as sulfates, thiosulfates, and sulfites, and sulfur-containing amino acids such as cysteine, cystine, and glutathione. As the sulfur source, a single kind of sulfur source may be used, or two or more kinds of sulfur sources may be used in combination.

[0100] Specific examples of other various organic components and inorganic components include, for example, inorganic salts such as sodium chloride and potassium chloride; trace metals such as iron, manganese, magnesium, and calcium; vitamins such as vitamin B1, vitamin B2, vitamin B6, nicotinic acid, nicotinamide, and vitamin B12; amino acids; nucleic acids; and organic components containing those such as peptone, casamino acid, yeast extract, and soybean protein decomposition product. As other various organic components and inorganic components, a single kind of component may be used, or two or more kinds of components may be used in combination.

[0101] Further, when an auxotrophic mutant that requires an amino acid, a nucleic acid, or the like for growth thereof is used, it is preferable to supplement a required nutrient to the medium.

[0102] The culture conditions are not particularly limited so long as the yeast of the present invention can proliferate, and the objective substance can be produced. The culture can be performed, for example, under usual conditions used for cultivating yeast. The culture conditions can be appropriately determined according to various conditions such as the type of yeast to be used and the type of objective substance to be produced.

[0103] The culture can be performed by using a liquid medium under an aerobic condition, a microaerobic condition, or an anaerobic condition. The culture can preferably be performed under an aerobic condition. The term "aerobic condition" may refer to a condition where the dissolved oxygen concentration in the liquid medium is 0.33 ppm or higher, or preferably 1.5 ppm or higher. In cases of the aerobic condition, the oxygen concentration can be controlled to be, for example, 5 to 50%, preferably about 10 to 20%, of the saturated oxygen concentration. Specifically, the aerobic culture can be performed with aeration or shaking. The term "microaerobic condition" may refer to a condition where oxygen is supplied to the culture system but the dissolved oxygen concentration in the liquid medium is lower than 0.33 ppm. The term "anaerobic condition" may refer to a condition where oxygen is not supplied to the culture system. The culture temperature may be, for example, 25 to 35°C, preferably 27 to 33°C, more preferably 28 to 32°C. pH of the medium may be, for example, 3 to 10, or 4 to 8. pH of

the medium may be adjusted as required during the culture. For adjusting pH, inorganic or organic acidic or alkaline substances, such as ammonia gas and so forth, can be used. The culture period may be, for example, 10 to 200 hours, or 15 to 120 hours. The culture condition may be constant during the whole period of the culture, or may be changed during the culture. The culture can be performed as batch culture, fed-batch culture, continuous culture, or a combination of these. Further, the culture may be performed as two steps of a seed culture and a main culture. In such a case, the culture conditions of the seed culture and the main culture may or may not be the same. For example, both the seed culture and the main culture may be performed as batch culture. Alternatively, for example, the seed culture may be performed as batch culture, and the main culture may be performed as fed-batch culture or continuous culture.

- [0104] By culturing the yeast of the present invention under such conditions, the objective substance is accumulated in the medium and/or cells of the yeast.
- [0105] Production of the objective substance can be confirmed by known methods used for detection or identification of compounds. Examples of such methods include, for example, HPLC, UPLC, LC/MS, GC/MS, and NMR. These methods may be used independently or in any appropriate combination.
- [0106] The produced objective substance can be collected by known methods used for separation and purification of compounds. Examples of such methods include, for example, ion-exchange resin method, membrane treatment, precipitation, and crystallization. These methods may be used independently or in any appropriate combination. When the objective substance accumulates in cells, the cells can be disrupted with, for example, ultrasonic waves or the like, and then the objective substance can be collected from the supernatant obtained by removing the cells from the cell-disrupted suspension by centrifugation. The objective substance to be collected may be a free compound, a salt thereof, or a mixture thereof.
- [0107] Further, when the objective substance deposits in the medium, it can be collected by centrifugation, filtration, or the like. The objective substance deposited in the medium may also be isolated together with the objective substance dissolved in the medium after the objective substance dissolved in the medium is crystallized.
- [0108] The objective substance collected may contain yeast cells, medium components, moisture, and by-product metabolites of the yeast, in addition to the objective substance. The purity of the objective substance collected may be, for example, 50%(w/w) or higher, preferably 85%(w/w) or higher, particularly preferably 95%(w/w) or higher.
- [0109] When a sphingoid base such as phytosphingosine (PHS) and sphinganine (DHS) is obtained as the objective substance, the sphingoid base obtained may be converted to a

corresponding sphingolipid such as phytoceramides (PHC) and dihydroceramides (DHC) by chemical reaction of mixture of the sphingoid base and a fatty acid (J. Biol. Chem. July 2002 277 (29): 25847-5).

Examples

- [0110] The present invention will be more specifically explained with reference to the following examples. However, these examples should not be construed to limit the present invention in any meanings.
- [0111] Example 1: Construction of strains
- S. cerevisiae* strain EYS3762 was constructed from strain BY4742 (MAT α his3 Δ 1 leu2 Δ 0 lys2 Δ 0 ura3 Δ 0, EUROSCARF Y10000) by sequential deletion of the LCB4 and LCB5 genes. This was done by first transforming the strain with a deletion construct containing the hygromycin resistance gene HygMX (hph) flanked by loxP sites that was PCR amplified from a plasmid template pEVE698 using primers EV4024 and EV4025, which introduced in the deletion construct sequences homologous to the native promoter and terminator of the LCB4 gene. Upon transformation, the recombination fragment replaced the complete native LCB4 open reading frame. Transformants were selected on YPD-agarose plates (10 g/l yeast extract, 20 g/L bacto-peptone, 20 g/L glucose, 20 g/L agar) containing 300 mg/L hygromycin. Clones were verified by PCR testing for proper insertion of the deletion construct.
- [0112] In a second step, the LCB4 gene deletion strain was transformed with a deletion construct containing the KanMX gene that confers resistance to the aminoglycoside antibiotic G418 flanked by loxP sites, that was PCR amplified from a plasmid template pEVE73 using primers EV4030 and EV4031, which introduced in the deletion construct sequences homologous to the native promoter and terminator of the LCB5 gene. Upon transformation, the recombination fragment replaced the complete native LCB5 open reading frame. Transformants were selected on YPD-agarose plates containing 100 mg/L G418. Clones were verified by PCR testing for proper insertion of the deletion construct.
- [0113] *S. cerevisiae* strain EYS4061 was constructed from the previously described strain EYS3762 by insertion into the YNRC Δ 9 locus of a triple expression construct consisting of the GPD1 promoter followed by the open reading frame of the LCB1 gene and a CYC1 terminator, the PGK1 promoter followed by the open reading frame of the LCB2 gene and the ADH2 terminator and the PGK1 promoter followed by the open reading frame of the TSC10 gene and the ADH2 terminator. All promoters, open reading frames and terminators were from *S. cerevisiae*. The integration construct also contained an expression cassette for the NatMX (nat1) gene that confers resistance to the antibiotic nourseothricin flanked by loxP sequences.

- [0114] *S. cerevisiae* strain EYS4299 was constructed from the previously described strain EYS4061 by transformation with 2 plasmids (pEVE2785 and pEVE2120) to render the strain prototrophic for histidine and uracil. Plasmid pEVE2785 expressed a HIS3 selection marker and contained a double expression cassette with TEF1 promoter and ADH1 terminator, and PGK1 promoter and CYC1 terminator. Both expression cassettes lacked an open reading frame. Plasmid pEVE2120 expressed a URA3 selection marker contained an expression cassette with PGK1 promoter and ADH2 terminator lacking an open reading frame.
- [0115] *S. cerevisiae* strain EYS4300 was constructed from the previously described strain EYS4061 by transformation with 2 plasmids, pEVE3910 and pEVE3908. Plasmid pEVE3910 contained an expression cassette for expression of *Pichia ciferrii* acetyltransferase gene ATF2 under the *S. cerevisiae* GPD1 promoter and CYC1 terminator. The plasmid also had a HIS3 selection marker. Plasmid pEVE3908 contained an expression cassette for expression of *Pichia ciferrii* acetyltransferase gene SLI1 under the *S. cerevisiae* GPD1 promoter and CYC1 terminator. The nucleotide sequences of SLI1 and ATF2 genes used were those optimized for *S. cerevisiae* codon usage. The plasmid also had a LEU2 selection marker. The presence of the two plasmids was verified by growth on EYS4300 on selective SC plates lacking the amino acids histidine and leucine (1.546 g/L SC-mix without histidine and leucine, 6.7 g/L yeast nitrogen base, 20 g/L glucose, pH 5.8) (for SC mix see Table 11).
- [0116] *Saccharomyces cerevisiae* strain EYS3805 was constructed from the previously described strain BY4742 by deletion of the YPC1 gene and by insertion of the LAG1, LAC1, and LIP1 genes. Lag1p and Lac1p are functionally equivalent catalytic ceramide synthase component, involved in synthesis of ceramide from C26(acyl)-coenzyme A and dihydrosphingosine or phytosphingosine. Lip1p is a non-catalytic ceramide synthase subunit associated with Lag1p and Lac1p and required for ceramide synthase activity. Deletion of the YPC1 gene was done through a PCR-based gene deletion strategy which generated a start-to-stop-codon deletion of the open reading frame. A deletion construct containing the hygromycin resistance gene HygMX (hph) flanked by loxP sites was PCR amplified from a plasmid template pEVE698 using primers EV4018 and EV4019, which introduced in the deletion construct sequences homologous to the native promoter and terminator of the YPC1 gene. Upon transformation the recombination fragment replaced the complete native YPC1 open reading frame. Transformants were selected on YPD-agarose plates containing 300 mg/L hygromycin. Clones were verified by PCR testing for correct insertion of the deletion construct. Insertion of the LAG1, LAC1, and LIP1 genes was done by insertion of a triple expression construct into the integration locus YORW22Δ (chrXV:969221-969906). The construct consisted of (1) Nourseothricin resistance

gene NatMX (nat1) flanked by loxP sites; (2) *S. cerevisiae* GPD1 promoter, followed by the *S. cerevisiae* LAG1 open reading frame and *S. cerevisiae* CYC1 terminator; (3) *S. cerevisiae* PGK1 promoter followed by *S. cerevisiae* LAC1 open reading frame and *S. cerevisiae* ADH2 terminator; (4) *S. cerevisiae* TEF1 promoter followed by *S. cerevisiae* LIP1 open reading frame and *S. cerevisiae* ENO2 terminator; (5) sequences homologous to the integration locus YORWΔ22 upstream of the selection marker and downstream of the LIP1 expression cassette. Transformants were selected on SC-agar plates (6.7 g/L yeast nitrogen base w/o amino acids, 2.0 g/L complete SC mixture, 20 g/L glucose, 20 g/L agar) containing 100 mg/L nourseothricin. Clones were verified by PCR for correct insertion of the expression construct. Strain EYS3805 was constructed by removal of the previously inserted selection markers for nourseothricin and hygromycin, nat1 and hph. This was done by transformation with a URA3 selectable plasmid pEVE0078 containing an expression cassette for the Cre recombinase. Cre recombinase catalyzed site specific recombination between two loxP sites which led to removal of the selection marker flanked by loxP sites. Clones expressing the Cre recombinase were picked and tested for the loss of the loxP flanked selection markers by plating on the respective selective plates. The Cre recombinase bearing plasmid pEVE0078 that was under URA3 selection was removed by growing strains in presence of 5'-fluoroorotic acid which is turned into a toxic compound by the activity of the URA3 gene encoded enzyme. Only clones that have lost the plasmid were able to grow on selective medium.

- [0117] *S. cerevisiae* strain EYS4423 was constructed from the previously described strain BY4742 by deletion of CHA1, LCB4, ORM2, and CKA2 genes and by overexpression of LCB1, LCB2 TSC10 and SUR2 genes. Deletion of the CHA1 gene was done by a PCR-based gene deletion strategy generating a start-to-stop-codon deletion of the open reading frame. CHA1 gene was replaced by a deletion construct of SEQ ID NO: 76, which was comprised of the KanMX gene flanked by loxP sites, and sequences homologous to the native promoter and terminator of the CHA1 gene that were added by PCR using primers EV3782 and EV3783. Transformants were selected on YPD-agar plates containing 100 mg/L G418. Clones were verified by PCR testing for proper insertion of the deletion construct. Deletion of the LCB4 gene was done by a PCR-based gene deletion strategy generating a start-to-stop-codon deletion of the open reading frame. LCB4 gene was replaced by a deletion construct of SEQ ID NO: 77, which was comprised of the NatMX gene flanked by loxP sites, and sequences homologous to the native promoter and terminator of the LCB4 gene that were added by PCR using primers EV4024 and EV4025. Transformants were selected on SC-agar plates containing 100 mg/L nourseothricin. Clones were verified by PCR testing for proper insertion of the deletion construct. Deletion of the ORM2 gene was done by a

PCR-based gene deletion strategy generating a start-to-stop-codon deletion of the open reading frame. ORM2 gene was replaced by a deletion construct of SEQ ID NO: 78, which was comprised of the NatMX gene flanked by loxP sites, and sequences homologous to the native promoter and terminator of the ORM2 gene that were added by PCR using primers EV4215 and EV4216. Transformants were selected on SC-agar plates containing 100 mg/L nourseothricin. Clones were verified by PCR testing for proper insertion of the deletion construct. Deletion of the CKA2 gene was done by a PCR-based gene deletion strategy generating a start-to-stop-codon deletion of the open reading frame. CKA2 gene was replaced by a deletion construct of SEQ ID NO: 79, which was comprised of the KanMX gene flanked by loxP sites, and sequences homologous to the native promoter and terminator of the CKA2 gene that were added by PCR using primers EV4740 and EV4741. Transformants were selected on SC-agar plates containing 100 mg/L G418. Clones were verified by PCR testing for proper insertion of the deletion construct. Overexpression of LCB1, LCB2, TSC10, and SUR2 genes were from plasmids pEVE3105, pEVE2932 and pEVE4321. Plasmid pEVE3105 contained the HIS3 selection marker and cassettes for expression of LCB1 gene under the GPD1 promoter and a CYC1 terminator, as well as cassette for expression of LCB2 gene under the PGK1 promoter and the ADH2 terminator. Plasmid pEVE2932 contained an expression cassette for expression of *S. cerevisiae* TSC10 gene under the KEX2 promoter and ADH1 terminator. The plasmid also had a URA3 selection marker. Plasmid pEVE4321 contained an expression cassette for expression of *Pichia ciferrii* SUR2 gene under the GPD1 promoter and a CYC1 terminator. The plasmid also contained a LEU2 selection marker. The presence of the three plasmids was verified by growth of EYS4423 on selective plates lacking the amino acids histidine and leucine and the nucleobase uracil (1.47 g/L SC-mix without histidine and leucine, 6.7 g/L yeast nitrogen base, 20 g/L glucose, pH 5.8).

- [0118] *S. cerevisiae* strain EYS4022 was constructed from the previously described strain BY4742 by deletion of the LCB4 gene, CHA1 gene, and YPC1 gene and by insertion of LAG1, LAC1, and LIP1 genes. Deletion of the LCB4 gene was done by a PCR-based gene deletion strategy generating a start-to-stop-codon deletion of the open reading frame. LCB4 gene was replaced by a deletion construct of SEQ ID NO: 77, which was comprised of the Nourseothricin resistance gene NatMX (*nat1*) flanked by loxP sites, and sequences homologous to the native promoter and terminator of the LCB4 gene that were added by PCR using primers EV4024 and EV4025. Transformants were selected on SC-agar plates containing nourseothricin. Clones were verified by PCR testing for proper insertion of the deletion construct. Deletion of the CHA1 gene was done by a PCR-based gene deletion strategy generating a start-to-stop-codon deletion of the open reading frame. CHA1 gene was replaced by a

deletion construct of SEQ ID NO: 76, which was comprised of the KanMX gene flanked by loxP sites, and sequences homologous to the native promoter and terminator of the CHA1 gene that were added by PCR using primers EV3782 and EV3783. Transformants were selected on YPD-agar plates containing 100 mg/L G418. Deletion of the YPC1 gene was done by a PCR-based gene deletion strategy generating a start-to-stop-codon deletion of the open reading frame. YPC1 gene was replaced by a deletion construct PCR amplified from a plasmid template pEVE698, which construct was comprised of the hygromycin resistance gene HygMX (hph) flanked by loxP sites, and sequences homologous to the native promoter and terminator of the YPC1 gene that were added by PCR using primers EV4018 and EV4019. Transformants were selected on SC-agar plates containing hygromycin. Clones were verified by PCR testing for proper insertion of the deletion construct. Insertion of LAG1, LAC1, and LIP1 genes was done by insertion of a triple expression construct into the integration locus YORW22Δ. The construct consisted of (1) Nourseothricin resistance gene NatMX (nat1) flanked by loxP sites; (2) *S. cerevisiae* GPD1 promoter, followed by the *S. cerevisiae* LAG1 open reading frame and *S. cerevisiae* CYC1 terminator; (3) *S. cerevisiae* PGK1 promoter followed by *S. cerevisiae* LAC1 open reading frame and *S. cerevisiae* ADH2 terminator; (4) *S. cerevisiae* TEF1 promoter followed by *S. cerevisiae* LIP1 open reading frame and *S. cerevisiae* ENO2 terminator; (5) sequences homologous to the integration locus YORWΔ22 upstream of the selection marker and downstream of the LIP1 expression cassette. Transformants were selected on SC-agar plates containing nourseothricin. Clones were verified by PCR for correct insertion of the expression construct. In a second step, the previously inserted selection markers were removed by transformation with a URA3 selectable plasmid pEVE0078 containing an expression cassette for the Cre recombinase enzyme. Cre recombinase catalyzed site specific recombination between two loxP sites flanking the hph selection marker with concomitant removal of the same. Clones expressing the Cre recombinase were selected on SC-agar plates without uracil. A few clones were picked and tested for the loss of the selection marker by plating on the respective selective plates. The Cre recombinase bearing plasmid pEVE0078 was removed by growing strains in the presence of 5'-fluoroorotic acid which is converted into a toxic compound by the activity of the URA3 gene encoded enzyme. Only clones that have lost the plasmid were able to grow on selective medium.

- [0119] *S. cerevisiae* strain EYS4798 was constructed from the previously described EYS4022 by transformation with plasmid pEVE4782 containing an expression cassette for *Xenopus laevis* UDP-glucose ceramide glucosyltransferase gene Ugcg-a (GenBank Accession No. AY112732), which encodes a protein belonging to UGT family 21,

under the *S. cerevisiae* GPD1 promoter and CYC1 terminator. The *Xenopus laevis* Ugcg-a gene used is also referred to as “*Xenopus laevis* UGT21-M”. The plasmid also had a LEU2 selection maker. The presence of the plasmid was verified by growth of EYS4798 on selective plates lacking the amino acids leucine.

[0120] Example 2: Cyclodextrins solubilize sphingoid bases in aqueous solution

These experiments were conducted to determine if and to what extent cyclodextrins solubilized sphingoid bases. To that end, an excess of phytosphingosine was incubated with increasing concentrations of solutions of three different types of cyclodextrin species -- α -cyclodextrin, 2-hydroxypropyl- α -cyclodextrin, 2-hydroxypropyl- β -cyclodextrin. Solubilized phytosphingosine in the supernatants were quantified by liquid chromatography-mass spectrometry (LC-MS) using standards prepared in the same cyclodextrin solutions. The results showed that the tested cyclodextrins solubilized phytosphingosine to different concentrations depending on the cyclodextrin concentration, in a dose-dependent fashion (Figure 1). Impressively, more than 9 g/L phytosphingosine were shown to be solubilized with 2-hydroxypropyl- α -cyclodextrin.

[0121] Example 3: Cyclodextrin enhances excretion of sphingoid bases in shake flask cultures

The *S. cerevisiae* strain EYS3399 was used as basic strain to improve sphingoid base production. Strain EYS3399 is the Applicant's nomenclature identical to strain NCYC 3608 of the National Collection of Yeast Cultures (genotype MAT α gal2 ho::HygMX ura3::KanMX), which is a Mat α derivative of S288C. As described previously, deletion of LCB4 and CKA2 genes encoding long-chain base kinase and the alpha subunit of casein kinase 2 in the strain EYS3399, resulted in the generation of another strain, EYS5009. Strain EYS5009 showed increased sphingoid base biosynthesis as compared with the strain EYS3399 (Figure 2). Both strains, EYS3399 and EYS5009, were grown in shake flask cultures in synthetic complete (SC)-medium (2.0 g/L SC-mix, 6.7 g/L YNB, 20 g/L glucose, pH 5.8) with or without 20 g/L α -cyclodextrin for 24h at 30°C. Supernatants were analyzed by LC-MS and the 3-keto sphinganine, sphinganine and phytosphingosine were quantified (Figure 2). Samples without cyclodextrin (CD) were not diluted, samples with CD were diluted 50-fold in methanol. These results showed that in both strains EYS3399 and EYS5009, cyclodextrin exposure resulted in enhanced production of sphingoid bases. This effect of cyclodextrin was more pronounced for phytosphingosine than for 3-keto sphinganine or for sphinganine. Further, this effect of cyclodextrin was more pronounced in strain EYS5009 as compared to strain EYS3399. Similar effects were observed when other cyclodextrin species were examined on these and other yeast strains.

[0122] Example 4: Cyclodextrin enhances excretion of sphingoid base in bioreactors

Strain EYS4928 was constructed in several steps from EYS3399, identical to NCYC 3608 (genotype MAT α gal2 ho::HygMX ura3::KanMX), which is a Mat α derivative of S288C. The selection marker HygMX was removed by initially inserting a loxP flanked URA3 gene into the ho locus (Chromosome IV 46271..48031) of EYS3399, followed by removal of the URA3 gene via expression of Cre recombinase using pEVE0078. Then, the selection marker KanMX was removed from the URA3 locus by a similar approach. In order to allow further genetic manipulations, the thus-obtained strain was made auxotrophic for LEU2 and HIS3 by removal of the whole open reading frames of these genes. For growth in a bioreactor, the thus-obtained auxotrophic strain was rendered prototrophic by transformation with two expression plasmids: the first plasmid carrying the HIS3 and LEU2 genes, and the second plasmid carrying the URA3 gene, to thereby obtain strain EYS4928.

- [0123] A fed-batch fermentation was performed with the following parameters: temperature kept at 30°C, pH controlled at 5.85 (regulated by 0.5 M HCl and 5 M NH₄OH), and pO₂ was maintained above 20% of the maximum oxygen saturation by a cascade on stirrer and aeration. As for the fermentation media, selective SC medium was used in the batch phase and a 30-fold concentrated selective SC medium was added to the fed-batch phase, both supplemented with 15 g/L α -cyclodextrin (Sigma-Aldrich). The batch phase ended latest after 11 hours, after which the feeding was initiated following an exponential profile calculated on an hourly rate. Over a period of about 100 h, samples were taken and analyzed either for both, biomass production and production of phytosphingosine. Phytosphingosine was quantified by LC-MS. LC-MS analysis was performed using Waters Ultra Performance Liquid Chromatography (UPLC) coupled with a Bruker Micro Q-TOF II mass spectrometer. Typically 5 μ l samples were injected into an Acquity BEH UPLC C8 2.1 x 100 mm 1.7 μ m column (Waters). 2 mM ammonium formate in water with 0.2% formic acid (mobile phase A) and a mixture of acetonitrile and methanol 1:1 containing 1 mM ammonium formate and 0.2% formic acid (mobile phase B) were used as mobile phases. The gradient was from 50% mobile phase B to 85% mobile phase B in 1 minute and then went to 100% B in 3 minutes. Mobile phase B was kept at 100% for 1 minute followed by a reconditioning step of 50% mobile phase B for 1 minute. The column temperature was kept at 50°C and the flow was 0.4 ml/min. The mass spectrometry analysis was performed in electrospray positive mode with a capillary voltage of 4.5 kV, a source temperature of 180°C and nebulizer pressure of 1.6 bar. The mass spectrum was acquired from a mass-to-charge ratio (m/z) of 100-1400. Concentration of phytosphingosine was determined according to a calibration curve of phytosphingosine (Santa Cruz Biotechnology) (4 mg/L, 2 mg/L, 1 mg/L, 500 μ g/L, 250 μ g/L, 125 μ g/L, 62.5 μ g/L, 31.25 μ g/L) in the matrix corresponding to the samples (for example medium

containing cyclodextrin diluted 10-fold in methanol).

[0124] Results are shown in Tables 1 and 2. In presence of α -cyclodextrin, phytosphingosine was detected in cell culture supernatants in concentrations of up to 2.03 mg/L, whereas in absence of α -cyclodextrin the concentration was below limit of quantification. Total phytosphingosine concentration in biomass and supernatant reached as high as 1.54 mg/L in presence of α -cyclodextrin, and 0.35 mg/L in the absence of α -cyclodextrin, representing a more than 4-fold increase in phytosphingosine in the presence of cyclodextrin. These studies provide further support that cyclodextrin enhances production and excretion of sphingoid bases.

[0125] [Table 1]

Table 1: Fermentation with strain EYS4928 in absence of cyclodextrin

WITHOUT CYCLODEXTRIN				
time (h)	Biomass	PHS supernatant	PHS broth	PHS broth std deviation
	g/L	mg/L	mg/L	mg/L
0	0.112			
69.75	63	none detectable	0.42	0.15
73.83	77	none detectable	0.42	0.18
75.75	63	none detectable	0.37	0.18
94	117.25	none detectable	0.46	0.14
98.25	120.75	none detectable	0.35	0.07

Genotype: *MATalpha gal2 ho his3Δ0 leu2Δ0 ura3Δ0 CAT5-I91M MIP1-A661T SAL1-1*

[*HIS3*][*LEU2 URA3*]

Media: Synthetic complete dropout minus His, Leu, Ura; fermentation mode: fed batch

[0126] [Table 2]

Table 2: Fermentation with strain EYS4928 in presence of cyclodextrin

WITH CYCLODEXTRIN (15 g/L alpha-cyclodextrin)							
time (h)	Biomass	PHS supernatant	PHS Supernatant std deviation	PHS broth	PHS broth std deviation	PHS pellet	PHS wash with 15 g/L alpha-CD medium containing azide
	g/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
0	0.0735						
69.75	112	1.25	0.12	1.15	0.10		
73.83	147	2.03	0.22	1.54	0.10	0.57	0.36

Genotype: *MATalpha gal2 ho his3Δ0 leu2Δ0 ura3Δ0 CAT5-I91M MIP1-A661T SAL1-1*

[*HIS3*][*LEU2 URA3*]

Media: Synthetic complete dropout minus His, Leu, Ura; fermentation mode: fed batch

[0127] Example 5: Cyclodextrin improves excretion of tetra-acetyl phytosphingosine

(TAPS)

The *S. cerevisiae* strain EYS4061, which overexpresses acetyltransferase genes ATF2 and SLI1 from *Wickerhamomyces ciferrii* (*Pichia ciferrii*), was subject to further genetic modifications: deletions of the long-chain base kinase genes, LCB4 and LCB5, and overexpression of the *S. cerevisiae* LCB1, LCB2 and TSC10 genes, thereby giving rise to strain EYS4300. A similar control strain EYS4299 was also generated that contained plasmids pEVE2152 and pEVE2159 with the selection markers HIS3 and LEU2, respectively, but no acetyltransferase genes. Both strains were grown in shake flask cultures with selective SC-media for 48h at 30°C, and biomass and culture supernatant was subject to analysis and quantification for long-chain bases phytosphingosine, sphinganine, and tetra-acetylated-phytosphingosine levels. LC-MS analysis was performed as described in Example 4 with the exception that the samples were not diluted. Concentration of tetraacetyl-phytosphingosine (TAPS) was determined according to a calibration curve with purified TAPS in the matrix corresponding to the samples (for example medium containing cyclodextrin diluted in methanol).

[0128] Results are shown in Figure 3. Upon expression of *Wickerhamomyces ciferrii* ATF2 and SLI1 genes, phytosphingosine was quantitatively converted to tetra-acetylated-phytosphingosine (i.e., TAPS). Addition of α -cyclodextrin resulted in increased acetylated long-chain base concentrations in the media. This demonstrates that cyclodextrin enhances the secretion of TAPS.

[0129] Example 6: Cyclodextrin improves excretion of sphingoid bases produced in different yeast strains in shake flask cultures

Stimulation of production of phytosphingosine and acetylated phytosphingosine and acetyl-phytosphingosine was evaluated in culture supernatants of two *S. cerevisiae* and two *Wickerhamomyces ciferrii* strains as indicated below:

EYS2958 (wild type strain)

EYS4423 (phytosphingosine producer strain)

EYS3062 (*Wickerhamomyces ciferrii*)

EYS3063 (*Wickerhamomyces sydowiorum*)

[0130] Strain EYS2958 is the Applicant's nomenclature for the previously described strain BY4742 (ATCC 201389; EUROSCARF Y10000) and represents a wild type strain. Strain EYS4423 (Δ cha1 Δ lcb4 Δ orm2 Δ cka2 [ScLCB1 ScLCB2][ScTSC10][PcSUR2]) is a phytosphingosine producer strain, which was also described previously. Strain EYS3062 is a *Wickerhamomyces ciferrii* (*Pichia ciferrii*) strain (EXT. PRODUCER: ATCC, NOTES: *Wickerhamomyces ciferrii* Y-1031 (ATCC 14091)). Strain EYS3063 is a *Wickerhamomyces sydowiorum* (*Pichia sydowiorum*) strain (XT. PRODUCER: ATCC, NOTES: *Wickerhamomyces sydowiorum* Y-7130 (ATCC 58369)). All four different strains were grown in shake

flask cultures in SC medium in presence or in absence of 10 g/L α -cyclodextrin for 48h at 30°C, in duplicate. For strain EYS4423 histidine, leucine and uracil were not added to the SC medium. The amount of biomass is shown in Figure 4. The broth (media + cells) was diluted in methanol and centrifuged prior to analysis for presence of phytosphingosine or mono-, di-, tri-, and tetra-acetylated phytosphingosine. LC-MS analysis was performed as described in Example 4 with the exception that samples were diluted 20-fold in methanol. Concentration of tetraacetyl-phytosphingosine (TAPS) was determined according to a calibration curve with purified TAPS in the matrix corresponding to the samples (for example medium containing cyclodextrin diluted in methanol). Monoacetyl-, diacetyl-, triacetyl-phytosphingosine peak areas were measured based on their respective extracted mass chromatograms. Due to an absence of standards, absolute quantification could not be made for mono-, di-, tri-acetylated phytosphingosine. However, tetra-acetylated phytosphingosine production could be quantified, owing to the presence of appropriate standards.

[0131] Results are shown in Figure 5 and Table 3. The presence α -cyclodextrin increased production of phytosphingosine in the engineered strain EYS4423 (Δ cha1 Δ lcb4 Δ orm2 Δ cka2 [ScLCB1 ScLCB2][ScTSC10][PcSUR2]) by more than 2-fold (Figure 5). On the contrary, tetra-acetylated phytosphingosine (TAPS) production in either of the *Wickerhamomyces* strains was reduced in the presence of α -cyclodextrin (Figure 5, Table 3). However, production of the lesser acetylated forms --mono-, di-, tri-acetylated phytosphingosine-- in either of the *Wickerhamomyces* strains was clearly increased in presence of α -cyclodextrin (Figure 5, Table 3). This example shows that cyclodextrin improves the excretion of sphingoid bases in different yeast strains in shake flask cultures.

[0132] [Table 3]

Table 3: Phytosphingosine species produced in *Wickerhamomyces ciferrii* and *Wickerhamomyces sydowiorum*.

	Tetra-acetyl-PHS mg/g CDW	Tetra-acetyl-PHS mg/g CDW	Peak Area Triacetyl PHS	Peak Area Triacetyl PHS	Peak Area Diacetyl-PHS	Peak Area Diacetyl-PHS	Peak Area mono-acetyl-PHS	Peak Area mono-acetyl-PHS
Cyclodextrin	-	+	-	+	-	+	-	+
<i>W. ciferrii</i>	10.8	0.9	2011710	4323410	125430	312810	5880	28240
<i>W. sydowiorum</i>	6.9	4.5	3284160	3670530	252830	367450	21620	52370

For tetraacetyl-phytosphingosine, absolute concentrations in mg/g CDW are given.

For monoacetyl-, diacetyl-, triacetyl-phytosphingosine peak areas are reported.

[0133] Example 7: Cyclodextrin improves excretion of sphingoid bases produced in *Wickerhamomyces ciferrii* in bioreactor

Fed-batch fermentation with *Wickerhamomyces ciferrii* strain EYS3062 was performed in SC media supplemented with or without 15 g/L α -cyclodextrin in batch and feed media at 30°C. Samples were taken at various time points during fermentation and analyzed by LC-MS for the presence of monoacetyl-, diacetyl-, triacetyl- and tetraacetyl-phytosphingosine. LC-MS analysis was performed as described in Example 4 except that samples were diluted 2000-fold in methanol. Concentration of tetraacetyl-phytosphingosine (TAPS) was determined according to a calibration curve with purified TAPS in the matrix corresponding to the samples (for example medium containing cyclodextrin diluted in methanol). Monoacetyl-, diacetyl-, triacetyl-phytosphingosine peak areas were measured on their respective extracted mass chromatograms.

[0134] Results are shown in Table 4. Cyclodextrin exposure resulted in increased production of the intermediates diacetyl- and triacetyl- phytosphingosine (Table 4). For triacetyl-phytosphingosine and diacetyl-phytosphingosine, chromatogram peak areas increased by 64 % and 126%, respectively (Table 4). Addition of cyclodextrin to the medium decreased TAPS production by of 43% (Table 4). This decrease may be due to capturing of intermediates before being further metabolized to TAPS. Phytosphingosine and sphinganine were not found in these samples (data not shown). This example demonstrates that cyclodextrin enhances the excretion of sphingoid bases in *Wickerhamomyces ciferrii* in bioreactor.

[0135] [Table 4]

Table 4: Analysis of *Wickerhamomyces ciferrii* fermentations in the presence or absence of α -cyclodextrin.

Molecules	Tetra-acetyl PHS (mg/L)	Tetra-acetyl PHS (mg/L)	Peak area Triacetyl PHS	Peak area Triacetyl PHS	Peak area Diacetyl PHS	Peak area Diacetyl PHS	Peak area acetyl PHS	Peak area acetyl PHS
Cyclodextrin	-	+	-	+	-	+	-	+
25.6 h	235	0	13190	6075	366	1	0	0
27.6 h	442	0	32094	21327	1	1	29	0
45 h	1124	0	52199	56337	2687	2584	62	0
47.1 h	1251	0	57284	73690	3097	6156	111	0
49.9 h	1332	694	49776	95277	1	7861	219	0
51.8 h	1414	800	65261	107192	4061	9198	114	0

Total broth, which included biomass and supernatant, was analyzed. Absolute quantification is shown for tetraacetyl-phytosphingosine. Peak area numbers are given for tri-, di-, and monoacetyl-phytosphingosine (PHS).

[0136] Example 8: Cyclodextrin improves the excretion of phytoceramide and glycosyl-

phytoceramide

Strains BY4742 (wt), EYS4022 (*cha1Δ ypc1Δ lcb4Δ LAG1 LAC1 LIP1*), and EYS4798 (*cha1Δ ypc1Δ lcb4Δ LAG1 LAC1 LIP1* and *Xenopus laevis* UGT21-M expressed from plasmid pEVE4782) were grown in shake flask cultures in selective SC medium with 200 g/l methyl- β -cyclodextrin for 48h at 30°C. Supernatants were analyzed by LC-MS for the presence of phytoceramide and glucosyl-phytoceramide. LC-MS analysis was performed using Waters Ultra Performance Liquid Chromatography (UPLC) coupled with a Bruker Micro Q-TOF II mass spectrometer. Typically 5 μ l samples were injected into an Acquity BEH UPLC C8 2.1 x 100 mm 1.7 μ m column (Waters). 2 mM ammonium formate in water with 0.2% formic acid (mobile phase A) and a mixture of acetonitrile and methanol 1:1 containing 1 mM ammonium formate and 0.2% formic acid (mobile phase B) were used as mobile phases. The gradient was from 50% mobile phase B to 85% mobile phase B in 1 minute and then went to 100% B in 3 minutes. Mobile phase B was kept at 100% for 3 minutes followed by a reconditioning step of 50% mobile phase B during 2 minutes. The column temperature was kept at 50°C and the flow was 0.4 ml/min. The mass spectrometry analysis was performed in electrospray positive mode with a capillary voltage of 4.5 kV, a source temperature of 180°C and nebulizer pressure of 1.6 bar. The mass spectrum was acquired from a mass-to-charge ratio (*m/z*) of 100-1400. Concentration of phytosphingosine, C24-phytoceramide and glycosyl-C26-phytoceramide were determined according to the calibration curve of phytosphingosine standard (Santa Cruz Biotechnology), C24-phytoceramide standard (Avanti Polar Lipids Inc.) and α -Galactosyl-C26-phytoceramide (Larodan Fine Chemicals AB) in a matrix corresponding to the samples (for example medium containing cyclodextrin diluted in methanol). Concentration of C26-phytoceramide was estimated using C24-phytosphingosine calibration curve.

[0137] Results are shown in Figure 6. Up to 2.5 mg/l phytoceramide was detected in the supernatants of EYS4022 cultures (Figure 6). Glycosylated phytoceramide was detected in the supernatants of strain EYS4798 (Figure 6). These studies demonstrate that cyclodextrin improves the excretion of both phytoceramide and glycosyl-phytoceramide.

[0138] Materials

Materials used in the Examples are shown in Tables 5-11.

[0139]

[Table 5]

Table 5: Primer sequences

Target Gene	Forward primer #	Forward Primer Sequence	SEQ ID	Reverse Primer #	Reverse primer Sequence	SEQ ID
<i>LCB4</i>	EV4024	AAGTCTAGCAGCGAAAAGTA CGCGAAGAATCTACTATAGA TAATGCCAGCTGAAGCTTCG TACGC	45	EV4025	TTTACAAAAAATCATT TGAAGGAAAATATAACGTTA ATCTAGCATAGGCCACTAGT GGATCTG	46
<i>LCB5</i>	EV4030	AAACCACAAATAGTGTAAAG TTTAAACAGTAAGCCAAAAG AGATGCCAGCTGAAGCTTCG TACGC	47	EV4031	TTGATTAATTGTTCACTAGC AAGGAAAAGATTAAGTAAAG TGTCAGCATAGGCCACTAGT GGATCTG	48
<i>YPC1</i>	EV4018	TAAATAAAAGCAGAAATCTT TATTTAAGAAAATAGAAGAT TGATGCCAGCTGAAGCTTCG TACGC	49	EV4019	AAATATGCCTATACATTATG AATATATATTATGCTATAAT TATTAGCATAGGCCACTAGT GGATCTG	50
<i>CHA1</i>	EV3782	TAAGTGCTGGATAGACAAGA GACAGGAAAATTAACCAGCG AGATGCCAGCTGAAGCTTCG TACGC	51	EV3783	TCAAGGGCAAATTGATGCTT CAACGAAAAGTTATTGGAT TTTCAGCATAGGCCACTAGT GGATCTG	52
<i>ORM2</i>	EV4215	AAGACTATACCATTATAAAA ACGCATAAGAAACAGTTTCA TCATGCCAGCTGAAGCTTCG TACGC	53	EV4216	ATATATATATATATACAT ATATGCGTATAGGCAGAGCC AACTAGCATAGGCCACTAGT GGATCTG	54
<i>CKA2</i>	EV4740	AAATAGAAGGAACAATAAAC CTAAAAGAATAGAAGAAACA GAATGCCAGCTGAAGCTTCG TACGCTGC	55	EV4741	TGGTGGAAGAATTGCCT TGCTAAGAGTATTGTTGTCC AATTACCGCATAGGCCACTA GTGGATCTG	56

[0140]

[Table 6]

Table 6: Plasmid sequences

Plasmid	SEQ ID
pEVE698	57
pEVE73	58
pEVE2785	59
pEVE2120	60
pEVE3908	61
pEVE3910	62
pEVE3105	63
pEVE2932	64
pEVE4321	65
pEVE2152	66
pEVE2159	67
pEVE4782	68
pEVE0078	69

[0141] [Table 7]

Table 7: Promoter sequences

Promoter	SEQ ID
<i>GPD1</i>	70
<i>PGK1</i>	71
<i>TEF1</i>	80
<i>KEX2</i>	81

[0142] [Table 8]

Table 8: Terminator sequences

Terminator	SEQ ID
<i>CYC1</i>	72
<i>ADH2</i>	73
<i>ADH1</i>	82
<i>ENO2</i>	83

[0143]

[Table 9]

Table 9: Additional sequences

LoxP	Sequence	SEQ ID
loxP used	ATAACTTCGTATA GCATACAT TATACGAAGTTAT	74
loxP consensus	ATAACTTCGTATA NNNTANNN TATACGAAGTTAT	75

[0144] [Table 10]

Table 10: Gene deletion constructs

Deletion construct	SEQ ID
<i>CHA1</i>	76
<i>LCB4</i>	77
<i>ORM2</i>	78
<i>CKA2</i>	79

[0145]

[Table 11]

Table 11: Complete SC mixture

Component	Concentration mg/l
Adenine	18
L-Alanine	76
L-Arginine HCl	76
L-Asparagine	76
Aspartic Acid	76
L-Cysteine	76
L-Glutamine	76
L-Glutamic Acid	76
Glycine	76
L-Histidine	76
<i>myo</i> -Inositol	76
L-Isoleucine	76
L-Leucine	380
L-Lysine	76
L-Methionine	76
<i>para</i> -Aminobenzoic Acid	8
L-Phenylalanine	76
L-Proline	76
L-Serine	76
L-Threonine	76
L-Tryptophan	76
L-Tyrosine	76
Uracil	76
L-Valine	76

Industrial Applicability

[0146] According to the present invention, production of an objective substance such as sphingoid bases and sphingolipids by yeast can be improved, and an objective substance can be efficiently produced.

[0147] <Explanation of Sequence Listing>

SEQ ID NO: 1, Nucleotide sequence of LCB1 gene of *Saccharomyces cerevisiae*

SEQ ID NO: 2, Amino acid sequence of Lcb1 protein of *Saccharomyces cerevisiae*

SEQ ID NO: 3, Nucleotide sequence of LCB2 gene of *Saccharomyces cerevisiae*

SEQ ID NO: 4, Amino acid sequence of Lcb2 protein of *Saccharomyces cerevisiae*
SEQ ID NO: 5, Nucleotide sequence of TSC10 gene of *Saccharomyces cerevisiae*
SEQ ID NO: 6, Amino acid sequence of Tsc10 protein of *Saccharomyces cerevisiae*
SEQ ID NO: 7, Nucleotide sequence of SUR2 gene of *Saccharomyces cerevisiae*
SEQ ID NO: 8, Amino acid sequence of Sur2 protein of *Saccharomyces cerevisiae*
SEQ ID NO: 9, Nucleotide sequence of SLI1 gene of *Saccharomyces cerevisiae*
SEQ ID NO: 10, Amino acid sequence of Sli1 protein of *Saccharomyces cerevisiae*
SEQ ID NO: 11, Nucleotide sequence of ATF2 gene of *Saccharomyces cerevisiae*
SEQ ID NO: 12, Amino acid sequence of Atf2 protein of *Saccharomyces cerevisiae*
SEQ ID NO: 13, Nucleotide sequence of LAG1 gene of *Saccharomyces cerevisiae*
SEQ ID NO: 14, Amino acid sequence of Lag1 protein of *Saccharomyces cerevisiae*
SEQ ID NO: 15, Nucleotide sequence of LAC1 gene of *Saccharomyces cerevisiae*
SEQ ID NO: 16, Amino acid sequence of Lac1 protein of *Saccharomyces cerevisiae*
SEQ ID NO: 17, Nucleotide sequence of LIP1 gene of *Saccharomyces cerevisiae*
SEQ ID NO: 18, Amino acid sequence of Lip1 protein of *Saccharomyces cerevisiae*
SEQ ID NO: 19, Nucleotide sequence of *ugcg-a* gene of *Xenopus laevis* (mRNA)
SEQ ID NO: 20, Amino acid sequence of Ugcg-a protein of *Xenopus laevis*
SEQ ID NO: 21, Nucleotide sequence of *ugcg-b* gene of *Xenopus laevis* (mRNA)
SEQ ID NO: 22, Amino acid sequence of Ugcg-b protein of *Xenopus laevis*
SEQ ID NO: 23, Nucleotide sequence of LCB4 gene of *Saccharomyces cerevisiae*
SEQ ID NO: 24, Amino acid sequence of Lcb4 protein of *Saccharomyces cerevisiae*
SEQ ID NO: 25, Nucleotide sequence of LCB5 gene of *Saccharomyces cerevisiae*
SEQ ID NO: 26, Amino acid sequence of Lcb5 protein of *Saccharomyces cerevisiae*
SEQ ID NO: 27, Nucleotide sequence of ELO3 gene of *Saccharomyces cerevisiae*
SEQ ID NO: 28, Amino acid sequence of Elo3 protein of *Saccharomyces cerevisiae*
SEQ ID NO: 29, Nucleotide sequence of CKA2 gene of *Saccharomyces cerevisiae*
SEQ ID NO: 30, Amino acid sequence of Cka2 protein of *Saccharomyces cerevisiae*
SEQ ID NO: 31, Nucleotide sequence of ORM2 gene of *Saccharomyces cerevisiae*
SEQ ID NO: 32, Amino acid sequence of Orm2 protein of *Saccharomyces cerevisiae*
SEQ ID NO: 33, Nucleotide sequence of CHA1 gene of *Saccharomyces cerevisiae*
SEQ ID NO: 34, Amino acid sequence of Cha1 protein of *Saccharomyces cerevisiae*
SEQ ID NO: 35, Nucleotide sequence of YPC1 gene of *Saccharomyces cerevisiae*
SEQ ID NO: 36, Amino acid sequence of Ypc1 protein of *Saccharomyces cerevisiae*
SEQ ID NO: 37, Nucleotide sequence of SUR2 gene of *Pichia ciferrii*
SEQ ID NO: 38, Amino acid sequence of Sur2 protein of *Pichia ciferrii*
SEQ ID NO: 39, Nucleotide sequence of SLI1 gene of *Pichia ciferrii* (partial)
SEQ ID NO: 40, Nucleotide sequence of SLI1 gene of *Pichia ciferrii*, optimized for *S. cerevisiae* codon usage

SEQ ID NO: 41, Amino acid sequence of Sli1 protein of *Pichia ciferrii*

SEQ ID NO: 42, Nucleotide sequence of ATF2 gene of *Pichia ciferrii*

SEQ ID NO: 43, Nucleotide sequence of ATF2 gene of *Pichia ciferrii*, optimized for *S. cerevisiae* codon usage

SEQ ID NO: 44, Amino acid sequence of Atf2 protein of *Pichia ciferrii*

SEQ ID NOS: 45-56, Primers

SEQ ID NOS: 57-69, Plasmids

SEQ ID NOS: 70, 71, Promoters

SEQ ID NOS: 72, 73, Terminators

SEQ ID NOS: 74, 75, LoxP sequences

SEQ ID NOS: 76-79, Gene deletion constructs

SEQ ID NOS: 80, 81, Promoters

SEQ ID NOS: 82, 83, Terminators

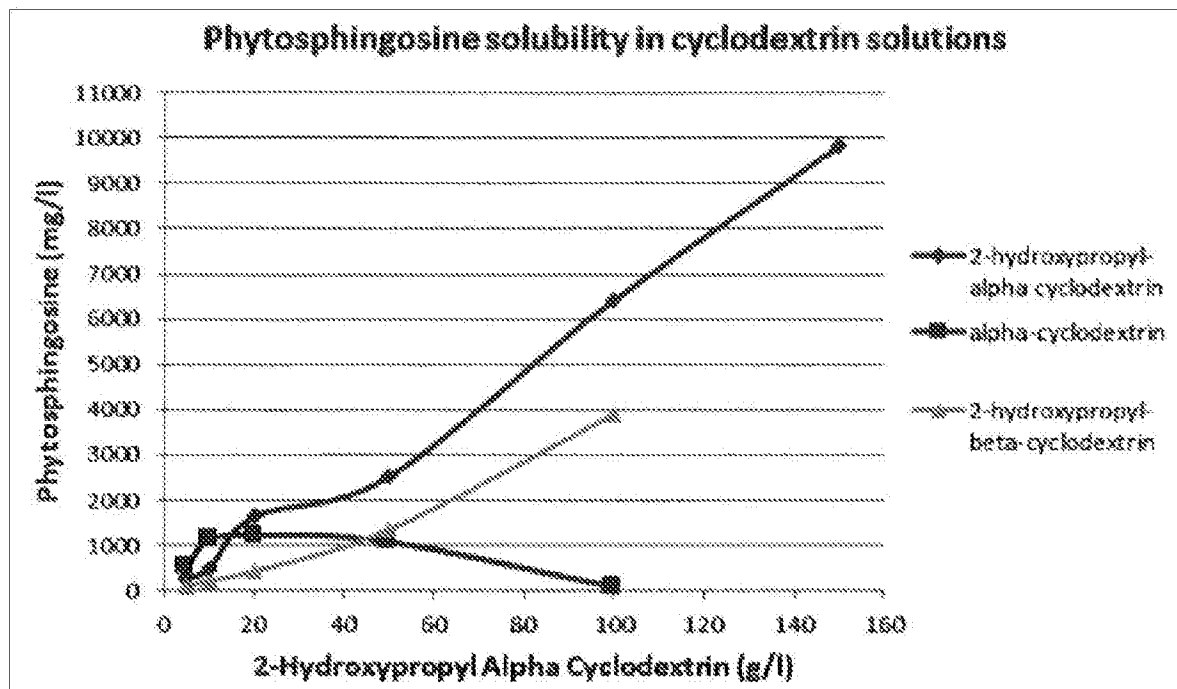
Claims

- [Claim 1] A method for producing an objective substance, the method comprising:
cultivating yeast having an ability to produce the objective substance in a culture medium containing an additive that is able to associate with, bind to, solubilize, and/or capture the objective substance; and
collecting the objective substance from cells of the yeast and/or the culture medium,
wherein the objective substance is selected from the group consisting of sphingoid bases and sphingolipids.
- [Claim 2] The method according to claim 1, wherein the additive is selected from the group consisting of cyclodextrin and zeolite.
- [Claim 3] The method according to claim 2, wherein the cyclodextrin is selected from the group consisting of alpha-cyclodextrin, beta-cyclodextrin, gamma-cyclodextrin, and derivatives thereof.
- [Claim 4] The method according to claim 3, wherein the derivatives are selected from the group consisting of methyl-alpha-cyclodextrin, methyl-beta-cyclodextrin, hydroxypropyl-alpha-cyclodextrin, and hydroxypropyl-beta-cyclodextrin.
- [Claim 5] The method according to any one of claims 1 to 4, wherein the objective substance is selected from the group consisting of phytosphingosine (PHS), sphingosine, 6-hydroxysphingosine, sphinganine (DHS), tetraacetylphytosphingosine (TAPS), triacetylphytosphingosine, diacetylphytosphingosine, phytoceramides, dihydroceramides, 6-hydroxyceramides, and glucosylceramides.
- [Claim 6] The method according to any one of claims 1 to 5, wherein the objective substance is selected from the group consisting of phytosphingosine (PHS), sphinganine (DHS), tetraacetylphytosphingosine (TAPS), phytoceramides, and glucosylceramides.
- [Claim 7] The method according to claim 6, wherein the phytosphingosine is selected from the group consisting of C16 PHS, C18 PHS, C20 PHS, C18:1 PHS, C20:1 PHS,
4-(hydroxymethyl)-2-methyl-6-tetradecanyl-1,3-oxazinan-5-ol, and
4-(hydroxymethyl)-2-methyl-6-hexadecanyl-1,3-oxazinan-5-ol.
- [Claim 8] The method according to any one of claims 1 to 7, wherein the yeast has been modified so that the expression and/or activity or activities of one or more proteins selected from proteins encoded by LCB1, LCB2,

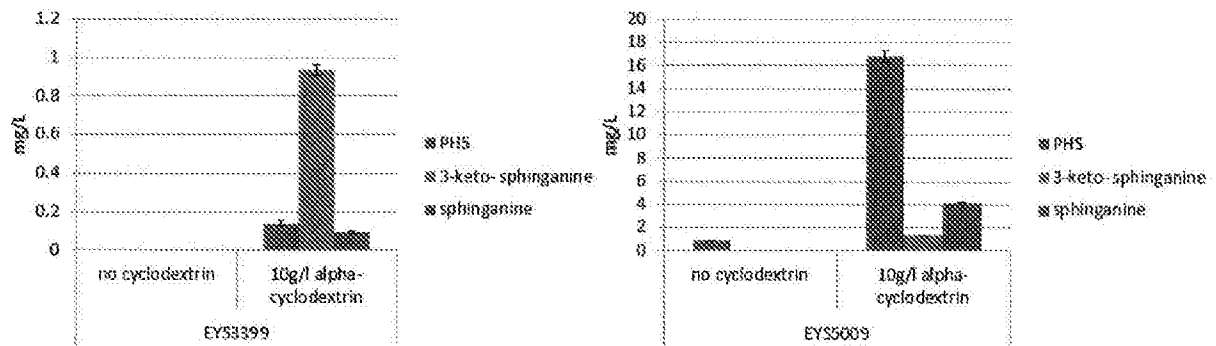
TSC10, SUR2, SLI1, ATF2, LAG1, LAC1, LIP1, and UGCG genes are increased.

- [Claim 9] The method according to claim 8, wherein the activity or activities of the one or more proteins are increased by increasing the expression of the respective genes encoding the one or more proteins.
- [Claim 10] The method according to any one of claims 1 to 9, wherein the yeast has been modified so that the expression and/or activity or activities of one or more proteins selected from proteins encoded by LCB4, LCB5, ELO3, CKA2, ORM2, CHA1, and YPC1 genes are reduced.
- [Claim 11] The method according to claim 10, wherein the activity or activities of the one or more proteins are reduced by attenuating the expression of the respective genes encoding the one or more proteins, or by disrupting the respective genes encoding the one or more proteins.
- [Claim 12] The method according to any one of claims 1 to 11, wherein the yeast belongs to the genus *Saccharomyces* or *Pichia*.
- [Claim 13] The method according to any one of claims 1 to 12, wherein the yeast is *Saccharomyces cerevisiae* or *Pichia ciferrii* (*Wickerhamomyces ciferrii*).
- [Claim 14] The method according to any one of claims 1 to 13, wherein the amount produced of the objective substance by the yeast is increased in the presence of the additive as compared with in the absence of the additive.

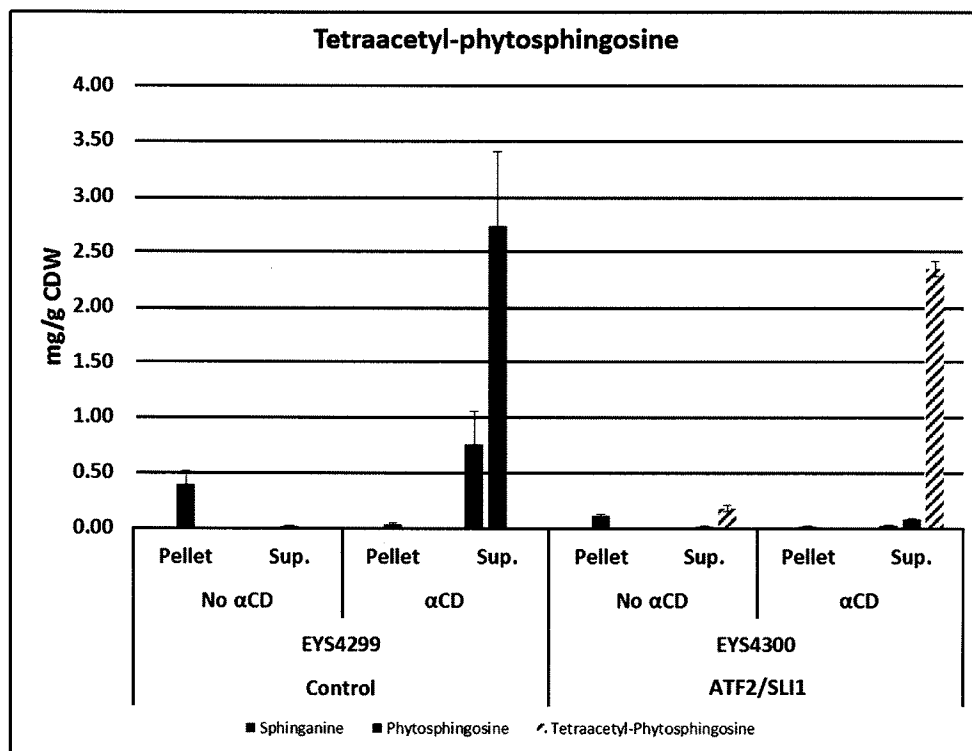
[Fig. 1]



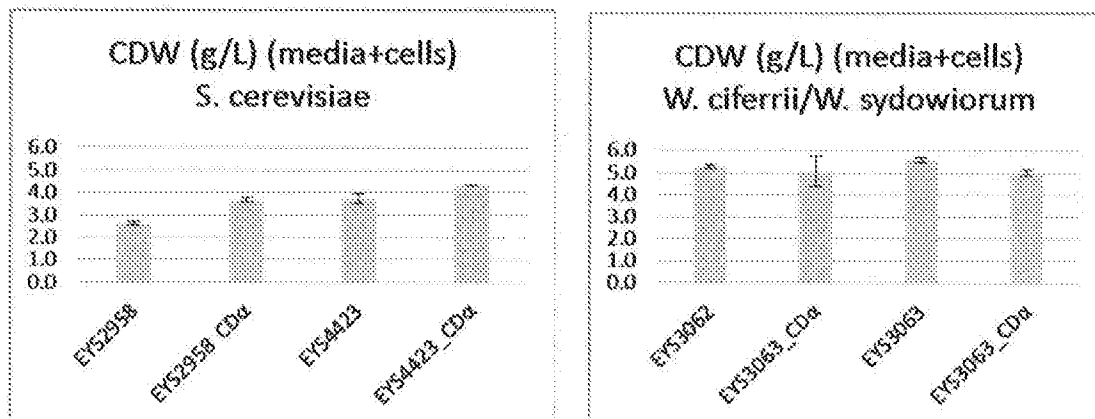
[Fig. 2]



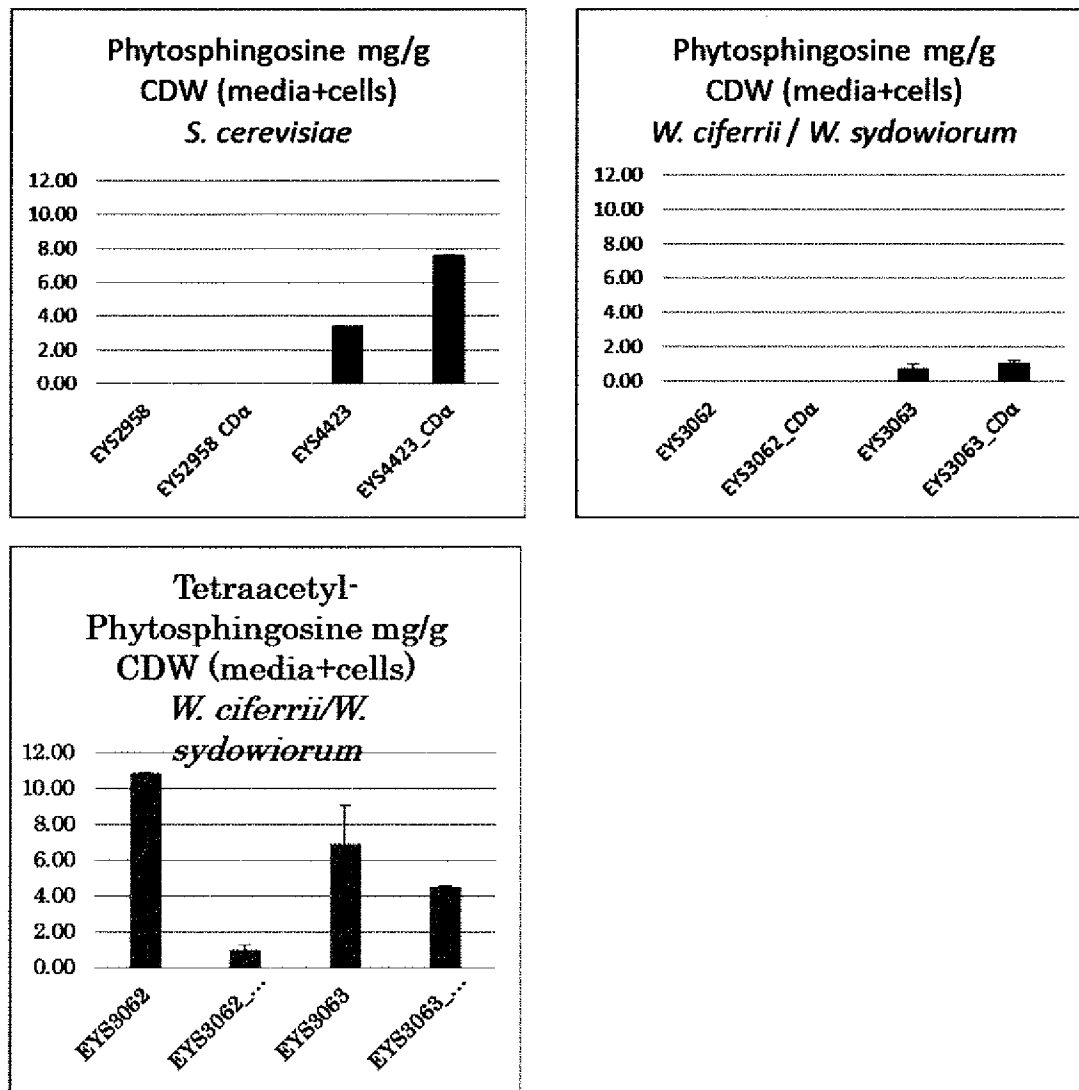
[Fig. 3]



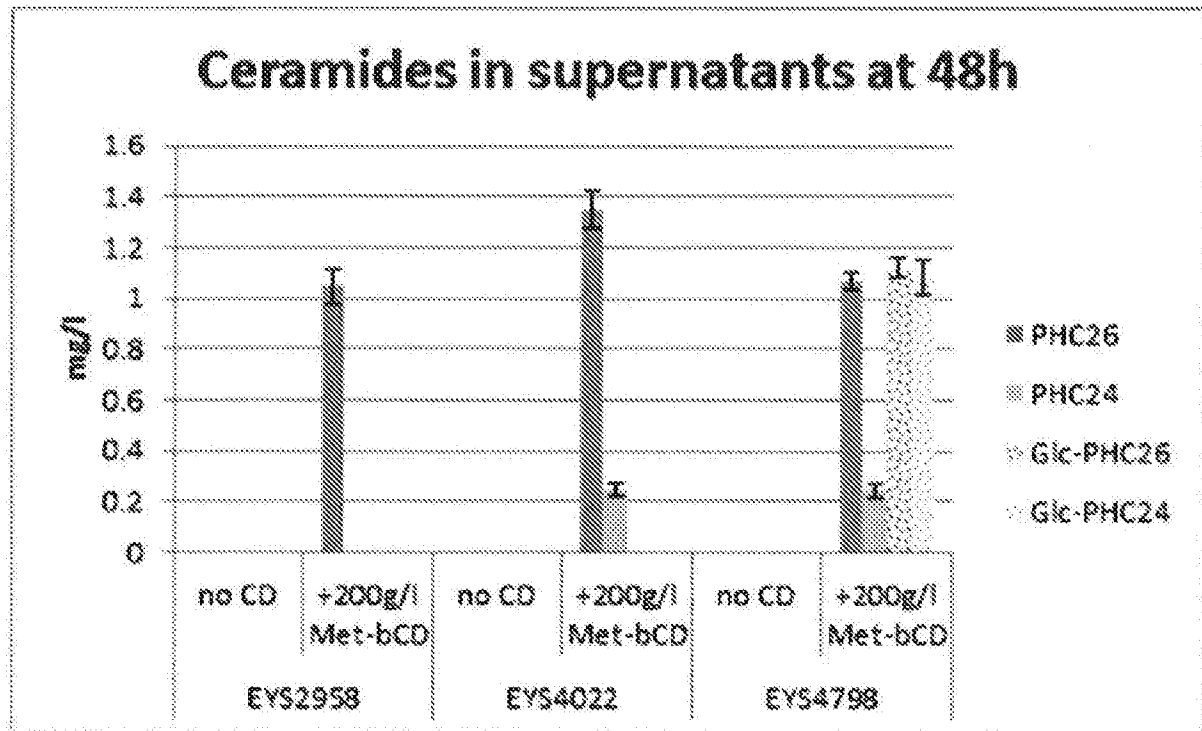
[Fig. 4]



[Fig. 5]



[Fig. 6]



INTERNATIONAL SEARCH REPORT

International application No
PCT/JP2016/003865

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P13/02
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12P

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

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

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	W J Pinto ET AL: "Sphingolipid long-chain-base auxotrophs of Saccharomyces cerevisiae: genetics, physiology, and a method for their selection", Journal of Bacteriology, April 1992 (1992-04), pages 2565-2574, XP55315146, UNITED STATES Retrieved from the Internet: URL:http://jb.asm.org/content/174/8/2565.full.pdf [retrieved on 2016-11-01]	1-7,12,13
Y	right-hand column; table 1 abstract ----- -/-	8-11,14



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Date of the actual completion of the international search

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Date of mailing of the international search report

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Name and mailing address of the ISA/

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Authorized officer

Oderwald, Harald

INTERNATIONAL SEARCH REPORT

International application No

PCT/JP2016/003865

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SINGH I ET AL: "EFFECT OF CYCLODEXTRINS ON THE SOLUBILIZATION OF LIGNOCERIC-ACID CERAMIDE AND CEREBROSIDE AND ON THE ENZYMATIC REACTIONS INVOLVING THESE COMPOUNDS", JOURNAL OF LIPID RESEARCH, vol. 24, no. 5, 1983, pages 662-665, XP002763793, ISSN: 0022-2275 the whole document	14
Y	----- WO 2013/023878 A1 (EVONIK DEGUSSA GMBH [DE]; KOEHLER TIM [DE]; SCHORSCH CHRISTOPH [DE]; B) 21 February 2013 (2013-02-21) the whole document	8-11
Y	----- COWART ET AL: "Yeast sphingolipids: Recent developments in understanding biosynthesis, regulation, and function", BIOCHIMICA AND BIOPHYSICA ACTA. MOLECULAR AND CELL BIOLOGY OF LIPIDS, ELSEVIER, AMSTERDAM, NL, vol. 1771, no. 3, 21 March 2007 (2007-03-21), pages 421-431, XP005930176, ISSN: 1388-1981, DOI: 10.1016/J.BBALIP.2006.08.005 the whole document	8-11
Y	----- WO 2007/131720 A1 (COSMOFORM BV [NL]; SCHAFFER STEFFEN [DE]; VAN DEN BERG MARCO ALEXANDER) 22 November 2007 (2007-11-22) the whole document	8,9
Y	----- WO 2015/076423 A1 (KAO CORP [JP]) 28 May 2015 (2015-05-28) the whole document	8,9
Y	----- VELD FRANK TER ET AL: "Production of tetraacetyl phytosphingosine (TAPS) in <i>Wickerhamomyces ciferrii</i> catalyzed by acetyltransferases Slip and Atf2p", APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, SPRINGER, DE, vol. 97, no. 19, 15 January 2013 (2013-01-15), pages 8537-8546, XP035328617, ISSN: 0175-7598, DOI: 10.1007/S00253-012-4670-3 [retrieved on 2013-01-15] cited in the application the whole document ----- -/--	8,9

INTERNATIONAL SEARCH REPORT

International application No

PCT/JP2016/003865

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHRISTOPH SCHORSCH ET AL: "Knockout of the DNA ligase IV homolog gene in the sphingoid base producing yeast <i>Pichia ciferrii</i> significantly increases gene targeting efficiency", CURRENT GENETICS ; EUKARYOTES WITH EMPHASIS ON YEASTS, FUNGI, PROTISTS, CELL ORGANELLES, SPRINGER, BERLIN, DE, vol. 55, no. 4, 26 May 2009 (2009-05-26), pages 381-389, XP019738673, ISSN: 1432-0983, DOI: 10.1007/S00294-009-0252-Z cited in the application the whole document -----	1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/JP2016/003865

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2013023878 A1	21-02-2013	CN 103748218 A DE 102011110959 A1 EP 2744896 A1 JP 2014529400 A KR 20140063599 A US 2014199736 A1 US 2016304916 A1 WO 2013023878 A1	23-04-2014 21-02-2013 25-06-2014 13-11-2014 27-05-2014 17-07-2014 20-10-2016 21-02-2013
WO 2007131720 A1	22-11-2007	CN 101490260 A EP 2024501 A1 JP 5357011 B2 JP 2009536521 A US 2010190219 A1 WO 2007131720 A1	22-07-2009 18-02-2009 04-12-2013 15-10-2009 29-07-2010 22-11-2007
WO 2015076423 A1	28-05-2015	CN 105765072 A EP 3071700 A1 JP 2015119698 A KR 20160086846 A US 2016298150 A1 WO 2015076423 A1	13-07-2016 28-09-2016 02-07-2015 20-07-2016 13-10-2016 28-05-2015