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(71) Applicant: **AJINOMOTO CO., INC.** [JP/JP]; 15-1, Kyobashi 1-chome, Chuo-ku, Tokyo, 1048315 (JP).

(72) Inventors: **TOYAZAKI, Miku**; c/o AJINOMOTO CO., INC., 1-1, Suzuki-cho, Kawasaki-ku, Kawasaki-shi, Kanagawa, 2108681 (JP). **FUKUI, Keita**; c/o AJINOMOTO CO., INC., 1-1, Suzuki-cho, Kawasaki-ku, Kawasaki-shi, Kanagawa, 2108681 (JP).

(74) Agent: **KAWAGUCHI, Yoshiyuki** et al.; Acropolis 21 Building 8th floor, 4-10, Higashi Nihonbashi 3-chome, Chuo-ku, Tokyo, 1030004 (JP).

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(54) Title: METHOD FOR PRODUCING ALDEHYDE

(57) Abstract: A method is described for producing an objective substance, for example, an aldehyde such as vanillin. The objective substance is produced from a carbon source or a precursor of the objective substance by using a microorganism having an ability to produce the objective substance, wherein the microorganism has been modified to have a specific carboxylic acid reductase (CAR) gene, such as a *Gordonia* CAR gene, *Novosphingobium* CAR gene, or *Coccomyxa* CAR gene.

Description

Title of Invention: METHOD FOR PRODUCING ALDEHYDE

Technical Field

[0001] The present invention relates to a method for producing an objective substance, for example, an aldehyde such as vanillin, by using a microorganism.

Background Art

[0002] Vanillin is the major ingredient responsible for the smell of vanilla, and is used as an aromatic by being blended in foods, drinks, perfumes, and so forth. Vanillin is mainly produced by extraction from natural products or by chemical synthesis.

[0003] Bioengineering techniques have also been attempted to produce vanillin, such as by using various microorganisms with eugenol, isoeugenol, ferulic acid, glucose, vanillic acid, coconut husk, or the like as a raw material (NPL 1). Other bioengineering methods include a method of producing vanillin as a glycoside (PTL 1 and 2), a method of producing vanillin from ferulic acid by using vanillin synthase (PTL 3), and a method of producing vanillic acid by fermentation of *Escherichia coli* and then enzymatically converting vanillic acid into vanillin (PTL 4).

[0004] Vanillin can be produced via the intermediate protocatechuic acid. Specifically, protocatechuic acid can be converted to vanillic acid or protocatechualdehyde by the action of O-methyltransferase (OMT) or aldehyde oxidoreductase (carboxylic acid reductase; CAR), respectively; and vanillic acid or protocatechualdehyde can be converted to vanillin by the action of CAR or OMT, respectively. In addition, isovanillin can be produced as a by-product via the intermediate protocatechuic acid. Specifically, protocatechuic acid can be converted to isovanillic acid by the action of OMT; and isovanillic acid can be converted to isovanillin by the action of CAR.

Citation List

Patent Literature

[0005] PTL 1: WO2013/022881
PTL 2: WO2004/111254
PTL 3: JP2015-535181
PTL 4: US6372461

Non Patent Literature

[0006] NPL 1: Kaur B. and Chakraborty D., Biotechnological and molecular approaches for vanillin production: a review. *Appl Biochem Biotechnol.* 2013 Feb;169(4):1353-72.

Summary of the Invention

[0007] It is difficult to separate an aldehyde such as vanillin from another aldehyde such as protocatechualdehyde and isovanillin during the purification process. Hence, selective

generation of vanillin can be useful for, for example, reducing the purification cost. However, a CAR suitable for selective generation of vanillin has not been previously reported.

[0008] It is an aspect of the present invention is to develop a novel technique for improving production of an objective substance, for example, an aldehyde such as vanillin, such as a novel technique useful for selective generation of the objective substance, and thereby provide a method for efficiently producing the objective substance.

[0009] It is described herein that an ability of a microorganism for producing an objective substance, for example, an aldehyde such as vanillin, could be significantly improved by using a microorganism that is able to express the carboxylic acid reductase (CAR) gene of *Gordonia effusa*, *Novosphingobium malaysiense*, or *Coccomyxa subellipsoidea*.

[0010] It is an aspect of the present invention to provide a method for producing an objective substance, the method comprising:
producing the objective substance by using a microorganism having an ability to produce the objective substance,
wherein the objective substance is an aldehyde,
wherein the microorganism has been modified to have an aldehyde oxidoreductase gene, and
wherein the aldehyde oxidoreductase gene encodes a protein selected from the group consisting of:
(a) a protein comprising the amino acid sequence of SEQ ID NO: 18, 79, or 83;
(b) a protein comprising the amino acid sequence of SEQ ID NO: 18, 79, or 83 but which includes substitution, deletion, insertion, and/or addition of 1 to 10 amino acid residues, and wherein said protein has aldehyde oxidoreductase activity;
(c) a protein comprising an amino acid sequence having an identity of 90% or higher to the amino acid sequence of SEQ ID NO: 18, 79, or 83, and wherein said protein has aldehyde oxidoreductase activity.

[0011] It is a further aspect of the invention to provide the method as described above, wherein said producing comprises:
cultivating the microorganism in a culture medium containing a carbon source to produce and accumulate the objective substance in the culture medium.

[0012] It is a further aspect of the invention to provide the method as described above, wherein said producing comprises:
converting a precursor of the objective substance into the objective substance by using the microorganism.

[0013] It is a further aspect of the invention to provide the method as described above, wherein said converting comprises:

cultivating the microorganism in a culture medium containing the precursor to produce and accumulate the objective substance in the culture medium.

[0014] It is a further aspect of the invention to provide the method as described above, wherein said converting comprises:

allowing cells of the microorganism to act on the precursor in a reaction mixture to produce and accumulate the objective substance in the reaction mixture.

[0015] It is a further aspect of the invention to provide the method as described above, wherein the cells are selected from the group consisting of:

- cells present in a culture broth of the microorganism,
- cells collected from the culture broth,
- cells present in a processed product of the culture broth,
- cells present in a processed product of the collected cells, and
- a combination of these.

[0016] It is a further aspect of the invention to provide the method as described above, wherein the precursor is a substance selected from the group consisting of proto-catechuic acid, vanillic acid, benzoic acid, L-phenylalanine, cinnamic acid, and combinations thereof.

[0017] It is a further aspect of the invention to provide the method as described above, the method further comprising collecting the objective substance from the culture medium or reaction mixture.

[0018] It is a further aspect of the invention to provide the method as described above, wherein the microorganism is a bacterium belonging to the family Enterobacteriaceae, a coryneform bacterium, or yeast.

[0019] It is a further aspect of the invention to provide the method as described above, wherein the microorganism is a bacterium belonging to the genus *Corynebacterium*.

[0020] It is a further aspect of the invention to provide the method as described above, wherein the microorganism is *Corynebacterium glutamicum*.

[0021] It is a further aspect of the invention to provide the method as described above, wherein the microorganism is a bacterium belonging to the genus *Escherichia*.

[0022] It is a further aspect of the invention to provide the method as described above, wherein the microorganism is *Escherichia coli*.

[0023] It is a further aspect of the invention to provide the method as described above, wherein the objective substance is an aromatic aldehyde.

[0024] It is a further aspect of the invention to provide the method as described above, wherein the objective substance is an aromatic aldehyde selected from the group consisting of vanillin, benzaldehyde, cinnamaldehyde, and combinations thereof.

[0025] It is a further aspect of the invention to provide the method as described above, wherein the microorganism has been further modified so that the activity of an enzyme

that is involved in the biosynthesis of the objective substance is increased as compared with a non-modified strain.

[0026] It is a further aspect of the invention to provide the method as described above, wherein the enzyme that is involved in the biosynthesis of the objective substance is able to catalyze the conversion from a precursor of the objective substance into the objective substance.

[0027] It is a further aspect of the invention to provide the method as described above, wherein the enzyme that is involved in the biosynthesis of the objective substance is selected from the group consisting of 3-deoxy-D-arabino-heptulosonic acid 7-phosphate synthase, 3-dehydroquinate synthase, 3-dehydroquinate dehydratase, 3-dehydroshikimate dehydratase, O-methyltransferase, phenylalanine ammonia lyase, and combinations thereof.

[0028] It is a further aspect of the invention to provide the method as described above, wherein the microorganism has been further modified so that the activity of phosphopantetheinyl transferase is increased as compared with a non-modified strain.

[0029] It is a further aspect of the invention to provide the method as described above, wherein the microorganism has been further modified so that the activity of an uptake system of a substance other than the objective substance is increased as compared with a non-modified strain.

[0030] It is a further aspect of the invention to provide the method as described above, wherein the uptake system is selected from the group consisting of a vanillic acid uptake system, a protocatechuic acid uptake system, and combinations thereof.

[0031] It is a further aspect of the invention to provide the method as described above, wherein the microorganism has been further modified so that the activity of an enzyme that is involved in the production of a byproduct during the production of the objective substance is reduced as compared with a non-modified strain.

[0032] It is a further aspect of the invention to provide the method as described above, wherein the enzyme that is involved in the production of a byproduct during the production of the objective substance is selected from the group consisting of vanillate demethylase, protocatechuate 3,4-dioxygenase, alcohol dehydrogenase, shikimate dehydrogenase, and combinations thereof.

Modes for Carrying out the Invention

[0033] Hereafter, the present invention will be explained in detail.

[0034] <1> Microorganism of the present invention

The microorganism as described herein is a microorganism having an ability to produce an objective substance, which microorganism has been modified to have (i.e. to harbor) a specific carboxylic acid reductase (CAR) gene such as *Gordonia* CAR gene, *Novosphingobium* CAR gene, or *Coccomyxa* CAR gene. The ability to produce

an objective substance can also be referred to as "objective substance-producing ability".

[0035] <1-1> Microorganism having objective substance-producing ability

The term "microorganism having an objective substance-producing ability" can refer to a microorganism that is able to produce an objective substance.

[0036] The term "microorganism having an objective substance-producing ability" can refer to a microorganism that is able to produce an objective substance by fermentation, if the microorganism is used in a fermentation method. That is, the term "microorganism having an objective substance-producing ability" may refer to a microorganism that is able to produce an objective substance from a carbon source. Specifically, the term "microorganism having an objective substance-producing ability" may refer to a microorganism that is able to, upon being cultured in a culture medium, such as a culture medium containing a carbon source, produce and accumulate the objective substance in the culture medium to such a degree that the objective substance can be collected therefrom.

[0037] Also, the term "microorganism having an objective substance-producing ability" can refer to a microorganism that is able to produce an objective substance by bio-conversion, if the microorganism is used in a bioconversion method. That is, the term "microorganism having an objective substance-producing ability" may refer to a microorganism that is able to produce an objective substance from a precursor of the objective substance. Specifically, the term "microorganism having an objective substance-producing ability" may refer to a microorganism that is able to, upon being cultured in a culture medium containing a precursor of an objective substance, produce and accumulate the objective substance in the culture medium in such a degree that the objective substance can be collected therefrom. Also, specifically, the term "microorganism having an objective substance-producing ability" may refer to a microorganism that is able to, upon being allowed to act on a precursor of an objective substance in a reaction mixture, produce and accumulate the objective substance in the reaction mixture to such a degree that the objective substance can be collected from the reaction mixture.

[0038] The microorganism having an objective substance-producing ability may be a microorganism that is able to accumulate the objective substance in the culture medium or reaction mixture in an amount larger than that is obtained with a non-modified strain. A non-modified strain can also be referred to as a "strain of a non-modified microorganism". The term "non-modified strain" can refer to a control strain that has not been modified to have a specific CAR gene. Examples of the non-modified strain can include a strain having a CAR gene other than the specific CAR gene, such as the CAR gene of *Nocardia brasiliensis*, instead of the specific CAR gene. The microorganism

having an objective substance-producing ability may be a microorganism that is able to accumulate the objective substance in the culture medium or reaction mixture in an amount of, for example, 0.01 g/L or more, 0.05 g/L or more, or 0.09 g/L or more.

[0039] The term "objective substance" can refer to an aldehyde. Examples of the aldehyde can include aromatic aldehydes. Examples of the aromatic aldehydes can include vanillin, benzaldehyde, and cinnamaldehyde. The microorganism may have an ability to produce only one kind of objective substance, or may have an ability to produce two or more kinds of objective substances. Also, the microorganism may have an ability to produce an objective substance from one kind of precursor of the objective substance or from two or more kinds of precursors of the objective substance.

[0040] A parent strain that is used to construct the microorganism as described herein is not particularly limited. Examples of the microorganism can include bacteria and yeast.

[0041] Examples of the bacteria can include bacteria belonging to the family Enterobacteriaceae and coryneform bacteria.

[0042] Examples of bacteria belonging to the family Enterobacteriaceae can include bacteria belonging to the genus *Escherichia*, *Enterobacter*, *Pantoea*, *Klebsiella*, *Serratia*, *Erwinia*, *Photobacterium*, *Providencia*, *Salmonella*, *Morganella*, or the like. Specifically, bacteria classified into the family Enterobacteriaceae according to the taxonomy used in the NCBI (National Center for Biotechnology Information) database (www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=91347) can be used.

[0043] The *Escherichia* bacteria are not particularly limited, and examples thereof can include those classified into the genus *Escherichia* according to the taxonomy known to those skilled in the field of microbiology. Examples of the *Escherichia* bacteria can include, for example, those described in the work of Neidhardt et al. (Backmann B.J., 1996, Derivations and Genotypes of some mutant derivatives of *Escherichia coli* K-12, pp.2460-2488, Table 1, In F.D. Neidhardt (ed.), *Escherichia coli* and *Salmonella* Cellular and Molecular Biology/Second Edition, American Society for Microbiology Press, Washington, D.C.). Examples of the *Escherichia* bacteria can include, for example, *Escherichia coli*. Specific examples of *Escherichia coli* can include, for example, *Escherichia coli* K-12 strains such as W3110 strain (ATCC 27325) and MG1655 strain (ATCC 47076); *Escherichia coli* K5 strain (ATCC 23506); *Escherichia coli* B strains such as BL21 (DE3) strain; and derivative strains thereof.

[0044] The *Enterobacter* bacteria are not particularly limited, and examples can include those classified into the genus *Enterobacter* according to the taxonomy known to those skilled in the field of microbiology. Examples the *Enterobacter* bacterium can include, for example, *Enterobacter agglomerans* and *Enterobacter aerogenes*. Specific examples of *Enterobacter agglomerans* can include, for example, the *Enterobacter agglomerans* ATCC 12287 strain. Specific examples of *Enterobacter aerogenes* can include, for

example, the *Enterobacter aerogenes* ATCC 13048 strain, NBRC 12010 strain (Biotechnol. Bioeng., 2007, Mar. 27;98(2):340-348), and AJ110637 strain (FERM BP-10955). Examples the *Enterobacter* bacteria can also include, for example, the strains described in European Patent Application Laid-open (EP-A) No. 0952221. In addition, *Enterobacter* agglomerans can also include some strains classified as *Pantoea* agglomerans.

- [0045] The *Pantoea* bacteria are not particularly limited, and examples can include those classified into the genus *Pantoea* according to the taxonomy known to those skilled in the field of microbiology. Examples the *Pantoea* bacteria can include, for example, *Pantoea ananatis*, *Pantoea stewartii*, *Pantoea agglomerans*, and *Pantoea citrea*. Specific examples of *Pantoea ananatis* can include, for example, the *Pantoea ananatis* LMG20103 strain, AJ13355 strain (FERM BP-6614), AJ13356 strain (FERM BP-6615), AJ13601 strain (FERM BP-7207), SC17 strain (FERM BP-11091), SC17(0) strain (VKPM B-9246), and SC17sucA strain (FERM BP-8646). Some of *Enterobacter* bacteria and *Erwinia* bacteria were reclassified into the genus *Pantoea* (Int. J. Syst. Bacteriol., 39, 337-345 (1989); Int. J. Syst. Bacteriol., 43, 162-173 (1993)). For example, some strains of *Enterobacter* agglomerans were recently reclassified into *Pantoea agglomerans*, *Pantoea ananatis*, *Pantoea stewartii*, or the like on the basis of nucleotide sequence analysis of 16S rRNA etc. (Int. J. Syst. Bacteriol., 39, 337-345 (1989)). The *Pantoea* bacteria can include those reclassified into the genus *Pantoea* as described above.
- [0046] Examples of the *Erwinia* bacteria can include *Erwinia amylovora* and *Erwinia carotovora*. Examples of the *Klebsiella* bacteria can include *Klebsiella planticola*.
- [0047] Examples of coryneform bacteria can include bacteria belonging to the genus *Corynebacterium*, *Brevibacterium*, *Microbacterium*, or the like.
- [0048] Specific examples of such coryneform bacteria can include the following species.
- Corynebacterium acetoacidophilum*
 - Corynebacterium acetoglutamicum*
 - Corynebacterium alkanolyticum*
 - Corynebacterium callunae*
 - Corynebacterium crenatum*
 - Corynebacterium glutamicum*
 - Corynebacterium lilium*
 - Corynebacterium melassecola*
 - Corynebacterium thermoaminogenes* (*Corynebacterium efficiens*)
 - Corynebacterium herculis*
 - Brevibacterium divaricatum* (*Corynebacterium glutamicum*)
 - Brevibacterium flavum* (*Corynebacterium glutamicum*)

Brevibacterium immariophilum
Brevibacterium lactofermentum (*Corynebacterium glutamicum*)
Brevibacterium roseum
Brevibacterium saccharolyticum
Brevibacterium thiogenitalis
Corynebacterium ammoniagenes (*Corynebacterium stationis*)
Brevibacterium album
Brevibacterium cerinum
Microbacterium ammoniaphilum

- [0049] Specific examples of the coryneform bacteria can include the following strains.
- Corynebacterium acetoacidophilum* ATCC 13870
 - Corynebacterium acetoglutamicum* ATCC 15806
 - Corynebacterium alkanolyticum* ATCC 21511
 - Corynebacterium callunae* ATCC 15991
 - Corynebacterium crenatum* AS1.542
 - Corynebacterium glutamicum* ATCC 13020, ATCC 13032, ATCC 13060, ATCC 13869, FERM BP-734
 - Corynebacterium lilium* ATCC 15990
 - Corynebacterium melassecola* ATCC 17965
 - Corynebacterium efficiens* (*Corynebacterium thermoaminogenes*) AJ12340 (FERM BP-1539)
 - Corynebacterium herculis* ATCC 13868
 - Brevibacterium divaricatum* (*Corynebacterium glutamicum*) ATCC 14020
 - Brevibacterium flavum* (*Corynebacterium glutamicum*) ATCC 13826, ATCC 14067, AJ12418 (FERM BP-2205)
 - Brevibacterium immariophilum* ATCC 14068
 - Brevibacterium lactofermentum* (*Corynebacterium glutamicum*) ATCC 13869
 - Brevibacterium roseum* ATCC 13825
 - Brevibacterium saccharolyticum* ATCC 14066
 - Brevibacterium thiogenitalis* ATCC 19240
 - Corynebacterium ammoniagenes* (*Corynebacterium stationis*) ATCC 6871, ATCC 6872
 - Brevibacterium album* ATCC 15111
 - Brevibacterium cerinum* ATCC 15112
 - Microbacterium ammoniaphilum* ATCC 15354
- [0050] The coryneform bacteria can include bacteria that had previously been classified into the genus *Brevibacterium*, but are presently united into the genus *Corynebacterium* (Int. J. Syst. Bacteriol., 41, 255 (1991)). Moreover, *Corynebacterium stationis* can

include bacteria that had previously been classified as *Corynebacterium ammoniagenes*, but are presently re-classified into *Corynebacterium stationis* on the basis of nucleotide sequence analysis of 16S rRNA etc. (Int. J. Syst. Evol. Microbiol., 60, 874-879 (2010)).

- [0051] 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. Examples of the yeast can 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*, and the genus *Schizosaccharomyces* such as *Schizosaccharomyces pombe*.
- [0052] These strains are available from, for example, the American Type Culture Collection (Address: P.O. Box 1549, Manassas, VA 20108, United States of America). That is, registration numbers are given to the respective strains, and the strains can be ordered by using these registration numbers (refer to www.atcc.org/). The registration numbers of the strains are listed in the catalogue of the American Type Culture Collection. These strains can also be obtained from, for example, the depositories at which the strains were deposited.
- [0053] The microorganism may inherently have an objective substance-producing ability, or may be modified so that it has an objective substance-producing ability. The microorganism having an objective substance-producing ability can be obtained by imparting an objective substance-producing ability to such a microorganism as described above, or enhancing an objective substance-producing ability of such a microorganism as described above.
- [0054] Hereafter, specific examples of the method for imparting or enhancing an objective substance-producing ability will be explained. Such modifications as exemplified below for imparting or enhancing an objective substance-producing ability may be independently used, or may be used in an appropriate combination.
- [0055] An objective substance can be generated by the action of an enzyme that is involved in the biosynthesis of the objective substance. Such an enzyme can also be referred to as "objective substance biosynthesis enzyme". Therefore, the microorganism may have an objective substance biosynthesis enzyme. In other words, the microorganism may have a gene encoding an objective substance biosynthesis enzyme. Such a gene can also be referred to as "objective substance biosynthesis gene". The microorganism may inherently have an objective substance biosynthesis gene, or may be introduced with an objective substance biosynthesis gene. The methods for introducing a gene are described herein.
- [0056] Also, an objective substance-producing ability of a microorganism can be improved

by increasing the activity of an objective substance biosynthesis enzyme. That is, examples of the method for imparting or enhancing an objective substance-producing ability can include a method of increasing the activity of an objective substance biosynthesis enzyme. That is, the microorganism may be modified so that the activity of an objective substance biosynthesis enzyme is increased. The activity of one kind of objective substance biosynthesis enzyme may be increased, or the activities of two or more kinds of objective substance biosynthesis enzymes may be increased. The method for increasing the activity of a protein (enzyme etc.) is described herein. The activity of a protein (enzyme etc.) can be increased by, for example, increasing the expression of a gene encoding the protein.

[0057] An objective substance can be generated from, for example, a carbon source and/or a precursor of the objective substance. Hence, examples of the objective substance biosynthesis enzyme can include, for example, enzymes that catalyze the conversion from the carbon source and/or the precursor into the objective substance. For example, 3-dehydroshikimic acid can be produced via a part of the shikimate pathway, which may include steps catalyzed by 3-deoxy-D-arabino-heptulosonic acid 7-phosphate synthase (DAHP synthase), 3-dehydroquinate synthase, and 3-dehydroquinate dehydratase; 3-dehydroshikimic acid can be converted to protocatechuic acid by the action of 3-dehydroshikimate dehydratase (DHSD); protocatechuic acid can be converted to vanillic acid or protocatechualdehyde by the action of O-methyltransferase (OMT) or aldehyde oxidoreductase (carboxylic acid reductase; CAR), respectively; and vanillic acid or protocatechualdehyde can be converted to vanillin by the action of CAR or OMT, respectively. Also, benzaldehyde and cinnamaldehyde can be generated from, for example, benzoic acid and cinnamic acid, respectively, by the action of CAR. That is, specific examples of the objective substance biosynthesis enzyme can include, for example, DAHP synthase, 3-dehydroquinate synthase, 3-dehydroquinate dehydratase, DHSD, OMT, and CAR. Particularly, use of the specific CAR may be effective for vanillin production via vanillic acid as an intermediate. Hence, vanillin production and related matters thereof are described on the premise that vanillin is generated partially or wholly via vanillic acid as an intermediate.

[0058] The term "3-deoxy-D-arabino-heptulosonic acid 7-phosphate synthase (DAHP synthase)" can refer to a protein that has the activity of catalyzing the reaction of converting D-erythrose 4-phosphate and phosphoenolpyruvic acid into 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) and phosphate (EC 2.5.1.54). A gene encoding DAHP synthase can also be referred to as "DAHP synthase gene". Examples of DAHP synthase can include AroF, AroG, and AroH proteins, which are encoded by *aroF*, *aroG*, and *aroH* genes, respectively. Among them, AroG may

function as the major DAHP synthase. Examples of DAHP synthase such as AroF, AroG, and AroH proteins can include those of various organisms such as Enterobacteriaceae bacteria and coryneform bacteria. Specific examples of DAHP synthase can include AroF, AroG, and AroH proteins of *E. coli*. The nucleotide sequence of the *aroG* gene of the *E. coli* K-12 MG1655 strain is shown as SEQ ID NO: 1, and the amino acid sequence of the AroG protein encoded by this gene is shown as SEQ ID NO: 2.

[0059] The DAHP synthase activity can be measured by, for example, incubating the enzyme with substrates (i.e. D-erythrose 4-phosphate and phosphoenolpyruvic acid), and measuring the enzyme- and substrate-dependent generation of DAHP.

[0060] The term "3-dehydroquinate synthase" can refer to a protein that has the activity of catalyzing the reaction of dephosphorylating DAHP to generate 3-dehydroquinic acid (EC 4.2.3.4). A gene encoding 3-dehydroquinate synthase can also be referred to as "3-dehydroquinate synthase gene". Examples of 3-dehydroquinate synthase can include AroB protein, which is encoded by *aroB* gene. Examples of 3-dehydroquinate synthase such as AroB protein can include those of various organisms such as Enterobacteriaceae bacteria and coryneform bacteria. Specific examples of 3-dehydroquinate synthase can include AroB protein of *E. coli*. The nucleotide sequence of the *aroB* gene of the *E. coli* K-12 MG1655 strain is shown as SEQ ID NO: 3, and the amino acid sequence of the AroB protein encoded by this gene is shown as SEQ ID NO: 4.

[0061] The 3-dehydroquinate synthase activity can be measured by, for example, incubating the enzyme with a substrate (i.e. DAHP), and measuring the enzyme- and substrate-dependent generation of 3-dehydroquinic acid.

[0062] The term "3-dehydroquinate dehydratase" can refer to a protein that has the activity of catalyzing the reaction of dehydrating 3-dehydroquinic acid to generate 3-dehydroshikimic acid (EC 4.2.1.10). A gene encoding 3-dehydroquinate dehydratase can also be referred to as "3-dehydroquinate dehydratase gene". Examples of 3-dehydroquinate dehydratase can include AroD protein, which is encoded by *aroD* gene. Examples of 3-dehydroquinate dehydratase such as AroD protein can include those of various organisms such as Enterobacteriaceae bacteria and coryneform bacteria. Specific examples of 3-dehydroquinate dehydratase can include AroD protein of *E. coli*. The nucleotide sequence of the *aroD* gene of the *E. coli* K-12 MG1655 strain is shown as SEQ ID NO: 5, and the amino acid sequence of the AroD protein encoded by this gene is shown as SEQ ID NO: 6.

[0063] The 3-dehydroquinate dehydratase activity can be measured by, for example, incubating the enzyme with a substrate (i.e. 3-dehydroquinic acid), and measuring the enzyme- and substrate-dependent generation of 3-dehydroshikimic acid.

- [0064] The term "3-dehydroshikimate dehydratase (DHSD)" can refer to a protein that has the activity of catalyzing the reaction of dehydrating 3-dehydroshikimic acid to generate protocatechuic acid (EC 4.2.1.118). A gene encoding DHSD can also be referred to as "DHSD gene". Examples of DHSD can include AsbF protein, which is encoded by asbF gene. Examples of DHSD such as AsbF protein can include those of various organisms such as *Bacillus thuringiensis*, *Neurospora crassa*, and *Podospira pauciseta*. The nucleotide sequence of the asbF gene of the *Bacillus thuringiensis* BMB171 strain is shown as SEQ ID NO: 7, and the amino acid sequence of the AsbF protein encoded by this gene is shown as SEQ ID NO: 8.
- [0065] The DHSD activity can be measured by, for example, incubating the enzyme with a substrate (i.e. 3-dehydroshikimic acid), and measuring the enzyme- and substrate-dependent generation of protocatechuic acid.
- [0066] The expression of a gene encoding an enzyme of shikimate pathway, such as DAHP synthase, 3-dehydroquinate synthase, and 3-dehydroquinate dehydratase, is repressed by a tyrosine repressor TyrR, which is encoded by tyrR gene. Therefore, the activity of an enzyme of shikimate pathway can also be increased by reducing the activity of the tyrosine repressor TyrR. The nucleotide sequence of the tyrR gene of the *E. coli* K-12 MG1655 strain is shown as SEQ ID NO: 9, and the amino acid sequence of the TyrR protein encoded by this gene is shown as SEQ ID NO: 10.
- [0067] The term "O-methyltransferase (OMT)" can refer to a protein that has the activity of catalyzing the reaction of methylating protocatechuic acid in the presence of a methyl group donor to generate vanillic acid (i.e. methylation of hydroxyl group at the meta-position of protocatechuic acid, EC 2.1.1.68 etc.). This activity can also be referred to as "OMT activity". A gene encoding OMT can also be referred to as "OMT gene". OMT may also catalyze the reaction of methylating protocatechualdehyde in the presence of a methyl group donor to generate vanillin (i.e. methylation of hydroxyl group at the meta-position of protocatechualdehyde). OMT may generally use both protocatechuic acid and protocatechualdehyde as the substrate, but is not necessarily limited thereto. Examples of the methyl group donor can include S-adenosylmethionine (SAM). Examples of OMT can include OMTs of various organisms, such as OMT of *Homo sapiens* (Hs) (GenBank Accession No. NP_000745 and NP_009294), OMT of *Arabidopsis thaliana* (GenBank Accession Nos. NP_200227 and NP_009294), OMT of *Fragaria x ananassa* (GenBank Accession No. AAF28353), and other various OMTs of mammals, plants, and microorganisms exemplified in WO2013/022881A1. Four kinds of transcript variants and two kinds of OMT isoforms are known for the OMT gene of *Homo sapiens*. The nucleotide sequences of these four transcript variants (transcript variant 1-4, GenBank Accession No. NM_000754.3, NM_001135161.1, NM_001135162.1, and NM_007310.2) are shown as SEQ ID

NOS: 11 to 14, the amino acid sequence of the longer OMT isoform (MB-COMT, GenBank Accession No. NP_000745.1) is shown as SEQ ID NO: 15, and the amino acid sequence of the shorter OMT isoform (S-COMT, GenBank Accession No. NP_009294.1) is shown as SEQ ID NO: 16. SEQ ID NO: 16 corresponds to SEQ ID NO: 15 of which the N-terminal 50 amino acid residues are truncated.

- [0068] OMT may also catalyze the reaction of methylating protocatechuic acid and/or protocatechualdehyde to generate isovanillic acid and/or isovanillin (i.e. methylation of hydroxyl group at the para-position) as a side reaction. OMT may selectively catalyze the methylation of a hydroxyl group at the meta-position. The expression "selectively catalyzing the methylation of hydroxyl group at the meta-position" may mean that OMT selectively generates vanillic acid from protocatechuic acid and/or that OMT selectively generates vanillin from protocatechualdehyde. The expression "selectively generating vanillic acid from protocatechuic acid" may mean that OMT generates vanillic acid in an amount of, for example, 3 times or more, 5 times or more, 10 times or more, 15 times or more, 20 times or more, 25 times or more, or 30 times or more of that of isovanillic acid in terms of molar ratio, when OMT is allowed to act on protocatechuic acid. Also, the expression "selectively generating vanillic acid from protocatechualdehyde" may mean that OMT generates vanillin in an amount of, for example, 3 times or more, 5 times or more, 10 times or more, 15 times or more, 20 times or more, 25 times or more, or 30 times or more of that of isovanillin in terms of molar ratio, when OMT is allowed to act on protocatechualdehyde. Examples of OMT that selectively catalyzes the methylation of hydroxyl group at the meta-position can include OMT having a "specific mutation" described herein.
- [0069] OMT having the "specific mutation" can also be referred to as "mutant OMT". A gene encoding a mutant OMT can also be referred to as "mutant OMT gene".
- [0070] OMT not having the "specific mutation" can also be referred to as "wild-type OMT". A gene encoding a wild-type OMT can also be referred to as "wild-type OMT gene". The term "wild-type" is used for convenience to distinguish the "wild-type" OMT from the "mutant" OMT, and the "wild-type" OMT is not limited to those obtained as natural substances, and can include any OMT not having the "specific mutation". Examples of the wild-type OMT can include, for example, OMTs exemplified above. In addition, all conservative variants of OMTs exemplified above can be considered as wild-type OMTs, provided that such conservative variants do not have the "specific mutation".
- [0071] Examples of the "specific mutation" can include the mutations contained in the mutant OMTs described in WO2013/022881A1. That is, examples of the "specific mutation" can include the mutation that the leucine residue at position 198 of the wild-type OMT (L198) is replaced with an amino acid residue showing a hydrophobic index

(hydropathy index) lower than that of leucine residue, and the mutation that the glutamate residue at position 199 of the wild-type OMT (E199) is replaced with an amino acid residue having either a neutral or positive side-chain charge at pH 7.4. The mutant OMT may have either one or both of these mutations.

- [0072] Examples of the "amino acid residue having a hydrophobic index (hydropathy index) lower than that of leucine residue" can include Ala, Arg, Asn, Asp, Cys, Glu, Gln, Gly, His, Lys, Met, Phe, Pro, Ser, Thr, Trp, and Tyr. As the "amino acid residue having a hydrophobic index (hydropathy index) lower than that of leucine residue", especially, an amino acid residue such as Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Lys, Met, Pro, Ser, Thr, Trp, and Tyr is preferred, and Tyr is more preferred.
- [0073] The "amino acid residue having either a neutral or positive side-chain charge at pH 7.4" can include Ala, Arg, Asn, Cys, Gln, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val. As the "amino acid residue having either a neutral or positive side-chain charge at pH 7.4", Ala or Gln is especially preferred.
- [0074] The terms "L198" and "E199" in an arbitrary wild-type OMT can refer to "an amino acid residue corresponding to the leucine residue at position 198 of the amino acid sequence shown as SEQ ID NO: 16" and "an amino acid residue corresponding to the glutamate residue at position 199 of the amino acid sequence shown as SEQ ID NO: 16", respectively. The positions of these amino acid residues represent the relative positions, and the absolute positions thereof may shift due to deletion, insertion, addition, and so forth of amino acid residue(s). For example, if one amino acid residue is deleted or inserted at a position on the N-terminus side of position X in the amino acid sequence shown as SEQ ID NO: 16, the amino acid residue originally at position X is relocated at position X-1 or X+1, however, it is still regarded as the "amino acid residue corresponding to the amino acid residue at position X of the amino acid sequence shown as SEQ ID NO: 16". Furthermore, although "L198" and "E199" are usually leucine residue and glutamate residue, respectively, they may not be leucine residue and glutamate residue, respectively. That is, when "L198" and "E199" are not leucine residue and glutamate residue, respectively, the "specific mutation" can include a mutation in which those amino acid residues each are replaced with any of the aforementioned amino acid residues.
- [0075] In the amino acid sequence of an arbitrary OMT, which amino acid residue is the amino acid residue corresponding to "L198" or "E199" can be determined by aligning the amino acid sequence of the arbitrary OMT and the amino acid sequence of SEQ ID NO: 16. The alignment can be performed by, for example, using known gene analysis software. Specific examples of such software can include DNASIS produced by Hitachi Solutions, GENETYX produced by Genetyx, and so forth (Elizabeth C. Tyler et al., Computers and Biomedical Research, 24 (1) 72-96, 1991; Barton GJ et al.,

Journal of Molecular Biology, 198 (2), 327-37, 1987).

- [0076] A mutant OMT gene can be obtained by, for example, modifying a wild-type OMT gene so that OMT encoded thereby has the "specific mutation". The wild-type OMT gene to be modified can be obtained by, for example, cloning from an organism having the wild-type OMT gene, or chemical synthesis. Furthermore, a mutant OMT gene can also be obtained without using a wild-type OMT gene. For example, a mutant OMT gene may be directly obtained by chemical synthesis. The obtained mutant OMT gene may be used as it is, or may be further modified before use.
- [0077] Genes can be modified by using a known method. For example, an objective mutation can be introduced into a target site of DNA by the site-specific mutagenesis method. Examples of the site-specific mutagenesis method can include a method using PCR (Higuchi, R., 61, in PCR Technology, Erlich, H.A. Eds., Stockton Press (1989); Carter P., Meth. In Enzymol., 154, 382 (1987)), and a method of using a phage (Kramer, W. and Frits, H.J., Meth. in Enzymol., 154, 350 (1987); Kunkel, T.A. et al., Meth. in Enzymol., 154, 367 (1987)).
- [0078] The activity of methylating hydroxyl group at the meta-position, such as the OMT activity, can be measured by, for example, incubating the enzyme with a substrate (i.e. protocatechuic acid or protocatechualdehyde) in the presence of SAM, and measuring the enzyme- and substrate-dependent generation of the corresponding product (i.e. vanillic acid or vanillin) (WO2013/022881A1). Furthermore, by measuring the generation of the corresponding by-product (i.e. isovanillic acid or isovanillin) under the same conditions, and comparing the generation of the by-product with the generation of the product, it can be determined whether OMT selectively generates the product.
- [0079] Carboxylic acid reductase (CAR) is described below in "Introduction of specific carboxylic acid reductase gene".
- [0080] CAR can be made into an active enzyme by phosphopantetheinylation (J. Biol. Chem., 2007, Vol. 282, No. 1, pp.478-485). Therefore, CAR activity can also be increased by increasing the activity of an enzyme that catalyzes phosphopantetheinylation of a protein (also referred to as "phosphopantetheinylation enzyme"). That is, examples of the method for imparting or enhancing an objective substance-producing ability can include a method of increasing the activity of a phosphopantetheinylation enzyme. That is, the microorganism may be modified so that the activity of a phosphopantetheinylation enzyme is increased. Examples of the phosphopantetheinylation enzyme can include phosphopantetheinyl transferase (PPT).
- [0081] The term "phosphopantetheinyl transferase (PPT)" can refer to a protein that has an activity of catalyzing the reaction of phosphopantetheinylating CAR in the presence of a phosphopantetheinyl group donor. This activity can also be referred to as "PPT

activity". A gene encoding PPT can also be referred to as "PPT gene". Examples of the phosphopantetheinyl group donor can include coenzyme A (CoA). Examples of PPT can include EntD protein, which is encoded by entD gene. Examples of PPT such as EntD protein can include those of various organisms. Specific examples of PPT can include EntD protein of *E. coli*. The nucleotide sequence of the entD gene of the *E. coli* K-12 MG1655 strain is shown as SEQ ID NO: 21, and the amino acid sequence of the EntD protein encoded by this gene is shown as SEQ ID NO: 22. Specific examples of PPT can also include PPT of *Nocardia brasiliensis*, PPT of *Nocardia farcinica* IFM10152 (*J. Biol. Chem.*, 2007, Vol. 282, No. 1, pp.478-485), and PPT of *Corynebacterium glutamicum* (App. Env. Microbiol. 2009, Vol.75, No.9, p2765-2774). The nucleotide sequence of the PPT gene of the *C. glutamicum* ATCC 13032 strain is shown as SEQ ID NO: 23, and the amino acid sequence of PPT encoded by this gene is shown as SEQ ID NO: 24.

- [0082] The PPT activity can be measured on the basis of, for example, enhancement of the CAR activity observed when the enzyme is incubated with CAR in the presence of CoA (*J. Biol. Chem.*, 2007, Vol. 282, No. 1, pp.478-485).
- [0083] Also, as described above, benzaldehyde and cinnamaldehyde can be generated from, for example, benzoic acid and cinnamic acid, respectively. That is, examples of the objective substance biosynthesis enzyme can also include, for example, benzoic acid biosynthesis enzymes and cinnamic acid biosynthesis enzymes. Specifically, cinnamic acid can be generated from, for example, L-phenylalanine, by the action of phenylalanine ammonia lyase (PAL; EC 4.3.1.24). That is, examples of cinnamic acid biosynthesis enzymes can include, for example, L-phenylalanine biosynthesis enzymes and PAL. Examples of the L-phenylalanine biosynthesis enzymes can include common biosynthesis enzymes of aromatic amino acids, such as 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase (aroF, aroG, aroH), 3-dehydroquinate synthase (aroB), 3-dehydroquinate dehydratase (aroD), shikimate dehydrogenase (aroE), shikimate kinase (aroK, aroL), 5-enolpyruvylshikimate-3-phosphate synthase (aroA), and chorismate synthase (aroC); as well as chorismate mutase (pheA), prephenate dehydratase (pheA), and tyrosine amino transferase (tyrB). Chorismate mutase and prephenate dehydratase may be encoded by pheA gene as a bifunctional enzyme.
- [0084] Examples of the method for imparting or enhancing an objective substance-producing ability can also include a method of increasing the activity of an uptake system of a substance other than an objective substance, such as a substance generated as an intermediate during production of an objective substance and a substance used as a precursor of an objective substance. That is, the microorganism may have been modified so that the activity of such an uptake system is increased. The term "uptake

system of a substance" can refer to a protein having a function of incorporating the substance from the outside of a cell into the cell. This activity can also be referred to as "uptake activity of a substance". A gene encoding such an uptake system can also be referred to as "uptake system gene". Examples of such an uptake system can include a vanillic acid uptake system and a protocatechuic acid uptake system. Examples of the vanillic acid uptake system can include VanK protein, which is encoded by vanK gene (M.T. Chaudhry, et al., Microbiology, 2007, 153:857-865). The nucleotide sequence of the vanK gene (NCgl2302) of the *C. glutamicum* ATCC 13869 strain is shown as SEQ ID NO: 25, and the amino acid sequence of the VanK protein encoded by this gene is shown as SEQ ID NO: 26. Examples of the protocatechuic acid uptake system gene can include PcaK protein, which is encoded by pcaK gene (M.T. Chaudhry, et al., Microbiology, 2007, 153:857-865). The nucleotide sequence of the pcaK gene (NCgl1031) of the *C. glutamicum* ATCC 13869 strain is shown as SEQ ID NO: 27, and the amino acid sequence of the PcaK protein encoded by this gene is shown as SEQ ID NO: 28.

[0085] The uptake activity of a substance can be measured according to, for example, a known method (M. T. Chaudhry, et al., Microbiology, 2007. 153:857-865).

[0086] Examples of the method for imparting or enhancing an objective substance-producing ability further can include a method of reducing the activity of an enzyme that is involved in the by-production of a substance other than an objective substance. Such a substance other than an objective substance can also be referred to as "byproduct". Such an enzyme can also be referred to as "byproduct generation enzyme". Specifically, a byproduct generation enzyme can refer to an enzyme that is involved in the production of a byproduct during the production of the objective substance. Examples of the byproduct generation enzyme can include, for example, enzymes that are involved in the utilization of an objective substance, and enzymes that catalyze a reaction branching away from the biosynthetic pathway of an objective substance to generate a substance other than the objective substance. The method for reducing the activity of a protein (enzyme etc.) is described herein. The activity of a protein (enzyme etc.) can be reduced by, for example, disrupting a gene that encodes the protein. For example, it has been reported that, in coryneform bacteria, vanillin is metabolized in the order of vanillin → vanillic acid → protocatechuic acid, and utilized (Current Microbiology, 2005, Vol. 51, pp.59-65). That is, specific examples of the byproduct generation enzyme can include an enzyme that catalyzes the conversion from vanillin into protocatechuic acid and enzymes that catalyze further metabolization of protocatechuic acid. Examples of such enzymes can include vanillate demethylase, protocatechuate 3,4-dioxygenase, and various enzymes that further decompose the reaction product of protocatechuate 3,4-dioxygenase to succinyl-CoA and acetyl-CoA

(Appl. Microbiol. Biotechnol., 2012, Vol.95, p77-89). In addition, an aldehyde such as vanillin can be converted into the corresponding alcohol such as vanillyl alcohol by the action of alcohol dehydrogenase (Kunjapur AM. et al., J. Am. Chem. Soc., 2014, Vol.136, p11644-11654.; Hansen EH. et al., App. Env. Microbiol., 2009, Vol.75, p2765-2774.). That is, specific examples of the byproduct generation enzyme can also include alcohol dehydrogenase (ADH). In addition, 3-dehydroshikimic acid, which is an intermediate of the biosynthetic pathway of vanillic acid and vanillin, can also be converted into shikimic acid by the action of shikimate dehydrogenase. That is, specific examples of the byproduct generation enzyme for vanillin production can also include shikimate dehydrogenase.

[0087] The term "vanillate demethylase" can refer to a protein having an activity for catalyzing the reaction of demethylating vanillic acid to generate protocatechuic acid. This activity can also be referred to as "vanillate demethylase activity". A gene encoding vanillate demethylase can also be referred to as "vanillate demethylase gene". Examples of vanillate demethylase can include VanAB proteins, which are encoded by vanAB genes (Current Microbiology, 2005, Vol. 51, pp.59-65). The vanA gene and vanB gene encode the subunit A and subunit B of vanillate demethylase, respectively. For reducing the vanillate demethylase activity, both the vanAB genes may be disrupted or the like, or only one of the two may be disrupted or the like. The nucleotide sequences of the vanAB genes of the *C. glutamicum* ATCC 13869 strain are shown as SEQ ID NOS: 29 and 31, and the amino acid sequences of the VanAB proteins encoded by these genes are shown as SEQ ID NOS: 30 and 6032, respectively. The vanAB genes usually constitute the vanABK operon together with the vanK gene. Therefore, in order to reduce the vanillate demethylase activity, the vanABK operon may be totally disrupted or the like (for example, deleted). In such a case, the vanK gene may be introduced to a host again. For example, when vanillic acid present outside cells is used, and the vanABK operon is totally disrupted or the like (for example, deleted), it is preferable to introduce the vanK gene anew.

[0088] The vanillate demethylase activity can be measured by, for example, incubating the enzyme with a substrate (i.e. vanillic acid), and measuring the enzyme- and substrate-dependent generation of protocatechuic acid (J Bacteriol, 2001, Vol.183, p3276-3281).

[0089] The term "protocatechuate 3,4-dioxygenase" can refer to a protein having an activity for catalyzing the reaction of oxidizing protocatechuic acid to generate beta-Carboxy-cis,cis-muconic acid. This activity can also be referred to as "protocatechuate 3,4-dioxygenase activity". A gene encoding protocatechuate 3,4-dioxygenase can also be referred to as "protocatechuate 3,4-dioxygenase gene". Examples of protocatechuate 3,4-dioxygenase can include PcaGH proteins, which are encoded by pcaGH genes (Appl. Microbiol. Biotechnol., 2012, Vol.95, p77-89). The pcaG gene and pcaH gene

encode the alpha subunit and beta subunit of protocatechuate 3,4-dioxygenase, respectively. For reducing the protocatechuate 3,4-dioxygenase activity, both the *pcaGH* genes may be disrupted or the like, or only one of the two may be disrupted or the like. The nucleotide sequences of the *pcaGH* genes of the *C. glutamicum* ATCC 13032 strain are shown as SEQ ID NOS: 33 and 35, and the amino acid sequences of the PcaGH proteins encoded by these genes are shown as SEQ ID NOS: 34 and 36, respectively.

- [0090] The protocatechuate 3,4-dioxygenase activity can be measured by, for example, incubating the enzyme with a substrate (i.e. protocatechuic acid), and measuring the enzyme- and substrate-dependent oxygen consumption (Meth. Enz., 1970, Vol.17A, p526-529).
- [0091] The term "alcohol dehydrogenase (ADH)" can refer to a protein that has an activity for catalyzing the reaction of reducing an aldehyde in the presence of an electron donor to generate an alcohol (EC 1.1.1.1, EC 1.1.1.2, EC 1.1.1.71, etc.). This activity can also be referred to as "ADH activity". A gene encoding ADH is also referred to as "ADH gene". Examples of the aldehyde used as a substrate of ADH can include aldehydes exemplified as objective substances in the method as described herein, e.g. aromatic aldehydes such as vanillin, benzaldehyde, and cinnamaldehyde. That is, examples of combinations of the aldehyde and alcohol referred to in the definition of "ADH activity" can include a combination of an aromatic aldehyde and the corresponding aromatic alcohol, such as the combination of vanillin and vanillyl alcohol, the combination of benzaldehyde and benzyl alcohol, and the combination of cinnamaldehyde and cinnamyl alcohol. ADH that uses an aromatic aldehyde, vanillin, benzaldehyde, or cinnamaldehyde can also be referred to as "aromatic alcohol dehydrogenase", "vanillyl alcohol dehydrogenase", "benzyl alcohol dehydrogenase", or "cinnamyl alcohol dehydrogenase", respectively. Furthermore, the ADH activity wherein an aromatic aldehyde, vanillin, benzaldehyde, or cinnamaldehyde is used as a substrate can also be referred to as "aromatic alcohol dehydrogenase activity", "vanillyl alcohol dehydrogenase activity", "benzyl alcohol dehydrogenase activity", or "cinnamyl alcohol dehydrogenase activity", respectively. ADH may use one kind of alcohol, or may use two or more kinds of alcohols. Examples of the electron donor can include NADH and NADPH.
- [0092] Examples of ADH can include YqhD protein, NCgl0324 protein, NCgl0313 protein, NCgl2709 protein, NCgl0219 protein, and NCgl2382 protein, which are encoded by *yqhD* gene, NCgl0324 gene, NCgl0313 gene, NCgl2709 gene, NCgl0219 gene, and NCgl2382 gene, respectively. The *yqhD* gene can be found in, for example, bacteria belonging to the family Enterobacteriaceae such as *E. coli*. The NCgl0324 gene, NCgl0313 gene, NCgl2709 gene, NCgl0219 gene, and NCgl2382 gene can be found

in, for example, coryneform bacteria such as *C. glutamicum*. The nucleotide sequence of the *yqhD* gene of the *E. coli* K-12 MG1655 strain is shown as SEQ ID NO: 37, and the amino acid sequence of the YqhD protein encoded by this gene is shown as SEQ ID NO: 38. The nucleotide sequences of the NCgl0324 gene, NCgl0313 gene, and NCgl2709 gene of the *C. glutamicum* ATCC 13869 strain are shown as SEQ ID NOS: 39, 41, and 43, respectively, and the amino acid sequences of the proteins encoded by these genes are shown as SEQ ID NOS: 40, 42, and 44, respectively. The nucleotide sequences of the NCgl0219 gene and NCgl2382 gene of the *C. glutamicum* ATCC 13032 strain are shown as SEQ ID NOS: 45 and 47, respectively, and the amino acid sequences of the proteins encoded by these genes are shown as SEQ ID NOS: 46 and 48, respectively.

[0093] The activity of one kind of ADH may be reduced, or the activities of two or more kinds of ADHs may be reduced. For example, the activity or activities of one or more kinds of ADHs, e.g. all ADHs, such as NCgl0324 protein, NCgl2709 protein, and NCgl0313 protein may be reduced. Also, at least the activity or activities of either one or both of NCgl0324 protein and NCgl2709 protein may be reduced. That is, for example, at least the activity of NCgl0324 protein may be reduced, and the activity of NCgl2709 protein may further be reduced. Alternatively, at least the activity of NCgl2709 protein may be reduced, and the activity of NCgl0324 protein may further be reduced. Combination of ADH and the objective substance is not particularly limited, so long as a reduction in the activity of ADH in a coryneform bacterium provides an increased production of the objective substance. For example, the activity of ADH that uses at least an aldehyde to be produced as an objective substance may be reduced. That is, for example, the activity of an aromatic alcohol dehydrogenase such as vanillyl alcohol dehydrogenase, benzyl alcohol dehydrogenase, and cinnamyl alcohol dehydrogenase may be reduced for production of an aromatic aldehyde such as vanillin, benzaldehyde, and cinnamaldehyde, respectively. Specifically, for example, when vanillin is produced, the activity of YqhD protein may be reduced. Also, specifically, for example, when vanillin is produced, the activity or activities of either one or both of NCgl0324 protein and NCgl0313 protein may be reduced, or at least the activity of NCgl0324 protein may be reduced. Also, specifically, when benzaldehyde is produced, the activity or activities of either one or both of NCgl0324 protein and NCgl2709 protein may be reduced. Also, specifically, when cinnamaldehyde is produced, the activity or activities of either one or both of NCgl0324 protein and NCgl2709 protein may be reduced. YqhD protein may have the vanillyl alcohol dehydrogenase activity. NCgl0324 protein may have all of the vanillyl alcohol dehydrogenase activity, benzyl alcohol dehydrogenase activity, and cinnamyl alcohol dehydrogenase activity. NCgl2709 protein may have both the benzyl alcohol dehydrogenase activity and cinnamyl alcohol dehydrogenase activity.

drogenase activity and cinnamyl alcohol dehydrogenase activity.

- [0094] The ADH activity can be measured by, for example, incubating the enzyme with a substrate (i.e. an aldehyde such as vanillin) in the presence of NADPH or NADH, and measuring the enzyme- and substrate-dependent oxidation of NADPH or NADH. It is sufficient that the ADH activity is detected under at least one appropriate condition, e.g. in the presence of an appropriate electron donor such as NADPH or NADH.
- [0095] The term "shikimate dehydrogenase" can refer to a protein that has the activity of catalyzing the reaction of reducing 3-dehydroshikimic acid in the presence of an electron donor to generate shikimic acid (EC 1.1.1.25). This activity can also be referred to as "shikimate dehydrogenase activity". A gene encoding shikimate dehydrogenase can also be referred to as "shikimate dehydrogenase gene". Examples of the electron donor can include NADH and NADPH. Examples of shikimate dehydrogenase can include AroE protein, which is encoded by aroE gene. The nucleotide sequence of the aroE gene of the E. coli K-12 MG1655 strain is shown as SEQ ID NO: 49, and the amino acid sequence of the AroE protein encoded by this gene is shown as SEQ ID NO: 50.
- [0096] The shikimate dehydrogenase activity can be measured by, for example, incubating the enzyme with a substrate (i.e. 3-dehydroshikimic acid) in the presence of NADPH or NADH, and measuring the enzyme- and substrate-dependent oxidation of NADPH or NADH.
- [0097] The protein with a modified activity can be appropriately chosen depending on the type of biosynthesis pathway that produces the objective substance and on the types and activities of the proteins inherently possessed by the microorganism. For example, when vanillin is produced by the bioconversion method from protocatechuic acid, it may be preferable to increase the activity or activities of one or more kinds of proteins such as OMT, CAR, PPT, and the protocatechuic acid uptake system. Also, when vanillin is produced by the bioconversion method from vanillic acid, it may be preferable to enhance the activity or activities of one or more kinds of proteins such as CAR, PPT, and the vanillic acid uptake system. The microorganism as described herein has been modified to have a specific CAR gene, and thus at least CAR activity may be enhanced.
- [0098] The genes and proteins used for breeding a microorganism having an objective substance-producing ability may have, for example, the above-exemplified or other known nucleotide sequences and amino acid sequences, respectively. Also, the genes and proteins used for breeding a microorganism having an objective substance-producing ability may be conservative variants of the genes and proteins exemplified above, such as genes and proteins having the above-exemplified or other known nucleotide sequences and amino acid sequences, respectively. Specifically, for example,

the genes used for breeding a microorganism having an objective substance-producing ability may each be a gene encoding a protein having the amino acid sequence exemplified above or the amino acid sequence of a known protein, but including substitution, deletion, insertion, or addition of one or several some amino acid residues at one or several positions, so long as the original function thereof, i.e. enzymatic activity, transporter activity, etc., is maintained. As for conservative variants of genes and proteins, the descriptions concerning conservative variants of the CAR gene and CAR described later can be applied mutatis mutandis.

[0099] <1-2> Introduction of specific carboxylic acid reductase gene

The microorganism as described herein has been modified to have a specific carboxylic acid reductase (CAR) gene. A microorganism having a CAR gene can also be referred to as "microorganism having CAR". By modifying a microorganism to have a specific CAR gene, an objective substance-producing ability of the microorganism can be improved, and that is, the production of an objective substance by using the microorganism can be increased. That is, an improved objective substance-producing ability and an increased production of an objective substance can be obtained for the microorganism, as compared with a non-modified strain. The increase in the production of an objective substance may be an increase in the absolute degree of production, such as absolute amount and absolute yield, of the objective substance, or may be in the relative degree of production, such as relative amount and relative yield, of the objective substance with respect to that of a by-product. Examples of the by-product can include, for example, protocatechualdehyde and isovanillin for vanillin production. It may be difficult to separate an aldehyde such as vanillin from another aldehyde such as protocatechualdehyde and isovanillin in purification process. Hence, the increase in the production of an objective substance such as vanillin may result in, for example, a reduction in a purification cost.

[0100] The microorganism can be obtained by modifying a microorganism having an objective substance-producing ability to have a specific CAR gene. The microorganism can also be obtained by modifying a microorganism to have a specific CAR gene, and then imparting an objective substance-producing ability to the microorganism or enhancing an objective substance-producing ability of the microorganism. In addition, the microorganism may be a microorganism that has acquired an objective substance-producing ability as a result of a modification for making the microorganism to have a specific CAR gene, or as a result of a combination of a modification for making the microorganism to have a specific CAR gene and other modification(s) for imparting or enhancing an objective substance-producing ability. The modifications for constructing the microorganism can be performed in an arbitrary order.

- [0101] The term "aldehyde oxidoreductase (carboxylic acid reductase; CAR)" can refer to a protein that has an activity of catalyzing the reaction of reducing an carboxylic acid in the presence of an electron donor and ATP to generate a corresponding aldehyde (EC 1.2.99.6 etc.). This activity can also be referred to as "CAR activity". A gene encoding CAR can also be referred to as "CAR gene". There is employed CAR that uses at least a carboxylic acid corresponding to an aldehyde to be produced as an objective substance in the method as described herein. That is, examples of the aldehyde generated as a product of CAR can include aldehydes exemplified as objective substances in the method as described herein, e.g. aromatic aldehydes such as vanillin, benzaldehyde, and cinnamaldehyde. That is, examples of combinations of the carboxylic acid and the aldehyde referred to in the definition of "CAR activity" can include a combination of an aromatic carboxylic acid and the corresponding aromatic aldehyde, such as the combination of vanillic acid and vanillin, the combination of benzoic acid and benzaldehyde, and the combination of cinnamic acid and cinnamaldehyde. In other words, for example, the term "CAR activity" of CAR used for vanillin production can refer to an activity of catalyzing the reaction of reducing vanillic acid to generate vanillin. CAR that uses an aromatic carboxylic acid can also be referred to as "aromatic aldehyde oxidoreductase (aromatic carboxylic acid reductase; ACAR)". Furthermore, the CAR activity wherein an aromatic carboxylic acid is used as a substrate can also be referred to as "ACAR activity". The specific CAR may have ACAR activity. CAR may use one kind of carboxylic acid, or may use two or more kinds of carboxylic acids. Examples of the electron donor can include NADH and NADPH.
- [0102] CAR may also catalyze the reaction of reducing protocatechuic acid and/or isovanillic acid in the presence of an electron donor and ATP to generate protocatechualdehyde and/or isovanillin. CAR may selectively catalyze the generation of vanillin. The expression "selectively catalyzing the generation of vanillin" may mean that CAR generates vanillin when CAR is allowed to act on vanillic acid, wherein vanillin is generated in an amount of, for example, in terms of molar ratio, 1.1 times or more, 1.2 times or more, 1.5 times or more, 2 times or more, 3 times or more, 4 times or more, 5 times or more, 10 times or more, 15 times or more, 20 times or more, 25 times or more, or 30 times or more of each or either one of the amount of protocatechualdehyde generated under the same conditions as vanillin generation except that the protocatechuic acid is used as the substrate and the amount of isovanillin generated under the same conditions as vanillin generation except that the isovanillic acid is used as the substrate.
- [0103] The CAR activity can be measured by, for example, incubating the enzyme with a substrate (e.g. vanillic acid) in the presence of ATP and NADPH, and measuring the

enzyme- and substrate-dependent oxidation of NADPH (modification of the method described in J. Biol. Chem., 2007, Vol. 282, No. 1, pp.478-485). Furthermore, by measuring the generation of protocatechualdehyde or isovanillin under the same conditions except that the protocatechuic acid or isovanillic acid is used as the substrate, and comparing the generation of protocatechualdehyde or isovanillin with the generation of vanillin, it can be determined whether CAR selectively catalyzes the generation of vanillin. It is sufficient that the specific CAR has the CAR activity that is measured under at least one appropriate condition. Incidentally, it is also sufficient that all the other proteins referred to in this application have the respective activities that are each measured under at least one appropriate condition.

[0104] The term "specific CAR gene" can refer to a gene encoding a specific CAR. Examples of the specific CAR gene can include *Gordonia* CAR gene, *Novosphingobium* CAR gene, and *Coccomyxa* CAR gene. Examples of the specific CAR can include *Gordonia* CAR, *Novosphingobium* CAR, and *Coccomyxa* CAR. The term "*Gordonia* CAR gene" can refer to a gene encoding a *Gordonia* CAR. The term "*Gordonia* CAR" collectively can refer to CARs found in *Gordonia* bacteria and variations thereof within a specific range, such as conservative variants. The term "*Novosphingobium* CAR gene" can refer to a gene encoding a *Novosphingobium* CAR. The term "*Novosphingobium* CAR" collectively can refer to CARs found in *Novosphingobium* bacteria and variations thereof within a specific range, such as conservative variants. The term "*Coccomyxa* CAR gene" can refer to a gene encoding a *Coccomyxa* CAR. The term "*Coccomyxa* CAR" collectively can refer to CARs found in *Coccomyxa* algae and variations thereof within a specific range, such as conservative variants.

[0105] Examples of the *Gordonia* bacteria can include *Gordonia effusa*. That is, examples of the *Gordonia* CAR gene and the *Gordonia* CAR can include the CAR gene and CAR of *Gordonia effusa*, respectively. The nucleotide sequence of the CAR gene of *Gordonia effusa* is shown as SEQ ID NO: 17, and the amino acid sequence of CAR encoded by this gene is shown as SEQ ID NO: 18. Examples of the *Novosphingobium* bacteria can include *Novosphingobium malaysiense*. That is, examples of the *Novosphingobium* CAR gene and the *Novosphingobium* CAR can include the CAR gene and CAR of *Novosphingobium malaysiense*, respectively. The nucleotide sequence of the CAR gene of *Novosphingobium malaysiense* is shown as SEQ ID NO: 78, and the amino acid sequence of CAR encoded by this gene is shown as SEQ ID NO: 79. Examples of the *Coccomyxa* algae can include *Coccomyxa subellipsoidea*. That is, examples of the *Coccomyxa* CAR gene and the *Coccomyxa* CAR can include the CAR gene and CAR of *Coccomyxa subellipsoidea*, respectively. The nucleotide sequence of the CAR gene (cDNA) of *Coccomyxa subellipsoidea* C-169 is shown as

SEQ ID NO: 82, and the amino acid sequence of CAR encoded by this gene is shown as SEQ ID NO: 83. That is, the specific CAR gene may be, for example, a gene having the nucleotide sequence shown as SEQ ID NO: 17, 78, or 82. Also, the specific CAR may be, for example, a protein having the amino acid sequence shown as SEQ ID NO: 18, 79, or 83. The expression "a gene or protein has a nucleotide or amino acid sequence" can mean that a gene or protein includes the nucleotide or amino acid sequence unless otherwise stated, and can also include cases where a gene or protein includes only the nucleotide or amino acid sequence.

[0106] The specific CAR gene may be a variant of any of the CAR genes exemplified above (e.g. a gene having the nucleotide sequence shown as SEQ ID NO: 17, 78, or 82), so long as the original function thereof is maintained. Similarly, the specific CAR may be a variant of any of CARs exemplified above (e.g. a protein having the amino acid sequence shown as SEQ ID NO: 18, 79, or 83), so long as the original function thereof is maintained. A variant that maintains the original function thereof can also be referred to as "conservative variant". That is, examples of the specific CAR gene and the specific CAR further can include such conservative variants. Such conservative variants may be or may not be found in *Gordonia* bacteria, *Novosphingobium* bacteria, or *Coccomyxa* algae. Examples of the conservative variants can include, for example, homologues and artificially modified versions of the genes and proteins exemplified above.

[0107] The expression "the original function is maintained" can mean that a variant of gene or protein has a function (such as activity or property) corresponding to the function (such as activity or property) of the original gene or protein. The expression "the original function is maintained" in reference to a gene can mean that a variant of the gene encodes a protein that maintains the original function. That is, the expression "the original function is maintained" in reference to a CAR gene can mean that the variant of the gene encodes CAR. The expression "the original function is maintained" in reference to CAR can mean that the variant of the protein has CAR activity.

[0108] Hereafter, examples of the conservative variants will be explained.

[0109] Homologues of the specific CAR gene or homologues of the specific CAR can be easily obtained from public databases by, for example, BLAST search or FASTA search using any of the nucleotide sequences of the CAR genes exemplified above or any of the amino acid sequences of CARs exemplified above as a query sequence. Furthermore, homologues of the specific CAR gene can be obtained by, for example, PCR using a chromosome of an organism such as *Gordonia* bacteria, *Novosphingobium* bacteria, or *Coccomyxa* algae as the template, and oligonucleotides prepared on the basis of a nucleotide sequence of any of the nucleotide sequences of the CAR genes exemplified above as primers.

- [0110] The specific CAR gene may be a gene encoding a protein having any of the aforementioned amino acid sequences (e.g. the amino acid sequence shown as SEQ ID NO: 18, 79, or 83) 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, 1 to 30, 1 to 20, 1 to 10, 1 to 5, or 1 to 3.
- [0111] The aforementioned substitution, deletion, insertion, and/or addition of one or several amino acid residues are each a conservative mutation that maintains the original 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 can 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. Furthermore, such substitution, deletion, insertion, addition, or the like of amino acid residues as mentioned above can include 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).
- [0112] Furthermore, the specific CAR gene may be a gene encoding a protein having an amino acid sequence having a homology of, for example, 50% or more, 65% or more, 80% or more, 90% or more, 95% or more, 97% or more, or 99% or more, to the total amino acid sequence of any of the aforementioned amino acid sequences, so long as the original function is maintained. In addition, in this specification, "homology" means "identity".

- [0113] Furthermore, the specific CAR gene may be a gene, such as a DNA, that is able to hybridize under stringent conditions with a probe that can be prepared from any of the aforementioned nucleotide sequences (for example, the nucleotide sequence shown as SEQ ID NO: 17, 78, or 82), 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" can 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 can include those under which highly homologous DNAs hybridize to each other, for example, DNAs not less than 50%, 65%, 80%, 90%, 95%, 97%, or 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, or 2 or 3 times, at a salt concentration and temperature corresponding to 1 x SSC, 0.1% SDS at 60°C; 0.1 x SSC, 0.1% SDS at 60°C; or 0.1 x SSC, 0.1% SDS at 68°C.
- [0114] 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 any of the aforementioned genes 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.
- [0115] Furthermore, since properties concerning degeneracy of codons changes depending on the host, the specific CAR gene can include substitution of respective equivalent codons for arbitrary codons. That is, the specific CAR gene may be a variant of any of the CAR genes exemplified above due to the degeneracy of the genetic code. For example, the specific CAR gene may be a gene modified so that it has optimal codons according to codon frequencies in a host to be used. Examples of the codon-optimized specific CAR gene can include, for example, the CAR gene having the nucleotide sequence shown as SEQ ID NO: 77, 81, or 85, which has been codon-optimized for the codon usage of *E. coli*.
- [0116] 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 can 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 a 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.

- [0117] 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 can 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 (1988), 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.
- [0118] 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 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.
- [0119] 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.
- [0120] The aforementioned descriptions concerning conservative variants of the genes and proteins can be applied mutatis mutandis to variants of arbitrary proteins such as objective substance biosynthesis enzymes and genes encoding them.
- [0121] A microorganism can be modified to have a specific CAR gene by introducing the gene into the microorganism.
- [0122] Methods for introducing a specific CAR gene into a microorganism are not particularly limited. It is sufficient that a specific CAR gene is operably harbored by the

microorganism. The microorganism may have one copy of a specific CAR gene, or may have two or more copies of a specific CAR gene. The microorganism may have one kind of specific CAR gene, or may have two or more kinds of specific CAR genes. A specific CAR gene can be introduced into a microorganism by the same way as that for introduction of a gene described below in the "Methods for increasing activity of protein".

[0123] The microorganism may or may not have a CAR gene other than the specific CAR gene.

[0124] <1-3> Methods for increasing activity of protein

Hereafter, the methods for increasing the activity of a protein, including methods for introduction of a gene, will be described.

[0125] The expression "the activity of a protein is increased" can mean that the activity of the protein is increased as compared with a non-modified strain. Specifically, the expression "the activity of a protein is increased" may mean that the activity of the protein per cell is increased as compared with that of a non-modified strain. The term "non-modified strain" can refer to a control strain that has not been modified so that the activity of an objective protein is increased. Examples of the non-modified strain can include a wild-type strain and parent strain. Specific examples of the non-modified strain can include the respective type strains of the species of microorganisms. Specific examples of the non-modified strain can also include strains exemplified above in relation to the description of microorganisms. That is, in an embodiment, the activity of a protein may be increased as compared with a type strain, i.e. the type strain of the species to which a microorganism belongs. In another embodiment, the activity of a protein may also be increased as compared with the *C. glutamicum* ATCC 13869 strain. In another embodiment, the activity of a protein may also be increased as compared with the *C. glutamicum* ATCC 13032 strain. In another embodiment, the activity of a protein may also be increased as compared with the *E. coli* K-12 MG1655 strain. The state that "the activity of a protein is increased" may also be expressed as "the activity of a protein is enhanced". More specifically, the expression "the activity of a protein is increased" may mean 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) encoding the protein, or the translation amount of the protein (i.e. the amount of the protein). Furthermore, the state that "the activity of a protein is increased" can include 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. Furthermore, 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 imparted to the host.

- [0126] 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 a non-modified strain. The activity of the protein may be increased to, for example, 1.2 times or more, 1.5 times or more, 2 times or more, or 3 times or more of that of a non-modified strain. Furthermore, when the non-modified strain does not have the activity of the objective protein, it is sufficient that the protein is produced as a result of introduction of the gene encoding the protein, and for example, the protein may be produced to such an extent that the activity thereof can be measured.
- [0127] The modification for increasing the activity of a protein can be attained by, for example, increasing the expression of a gene encoding the protein. The expression "the expression of a gene is increased" can mean that the expression of the gene is increased as compared with a non-modified strain such as a wild-type strain and parent strain. Specifically, the expression "the expression of a gene is increased" may mean that the expression amount of the gene per cell is increased as compared with that of a non-modified strain. More specifically, the expression "the expression of a gene is increased" may 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" can also be referred to as "the expression of a gene is enhanced". The expression of a gene may be increased to, for example, 1.2 times or more, 1.5 times or more, 2 times or more, or 3 times or more of that of a non-modified strain. Furthermore, the state that "the expression of a gene is increased" can include 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.
- [0128] The expression of a gene can be increased by, for example, increasing the copy number of the gene.
- [0129] 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). Examples of the gene transfer method utilizing homologous recombination can include, for example, a method of using a linear DNA such as Red-driven integration (Datsenko, K.A., and Wanner, B.L., Proc. Natl. Acad. Sci. USA, 97:6640-6645 (2000)), a method of using a plasmid containing a temperature sensitive replication origin, a method of using a plasmid capable of conjugative transfer, a method of using a suicide vector not having a replication origin that functions in a host, and a transduction method using a phage. 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 can include repetitive DNAs, and inverted repeats located at the both ends of a transposon. Alternatively, homologous recombination may be performed by using an appropriate sequence on a chromosome such as a gene unnecessary for the production of an objective substance as a target. Furthermore, a gene can also be randomly introduced into a chromosome by using a transposon or Mini-Mu (Japanese Patent Laid-open (Kokai) No. 2-109985, U.S. Patent No. 5,882,888, EP 805867 B1).

[0130] Introduction of a target gene into a chromosome can be confirmed by Southern hybridization using a probe having a sequence complementary to the whole gene or a part thereof, PCR using primers prepared on the basis of the sequence of the gene, or the like.

[0131] Furthermore, the copy number of a gene can also be increased by introducing a vector containing the gene into a host. For example, the copy number of a target gene can be increased by ligating a DNA fragment containing the target gene with a vector that functions in a host to construct an expression vector of the gene, and transforming the host with the expression vector. The DNA fragment containing the target gene can be obtained by, for example, PCR using the genomic DNA of a microorganism having the target gene as the template. As the vector, a vector autonomously replicable in the cell of the host can be used. The vector can be a multi-copy vector. Furthermore, the vector preferably has a marker such as an antibiotic resistance gene for selection of transformant. Furthermore, the vector may have a promoter and/or terminator for expressing the introduced gene. The vector may be, for example, a vector derived from a bacterial plasmid, a vector derived from a yeast plasmid, a vector derived from a bacteriophage, cosmid, phagemid, or the like. Specific examples of vector autonomously replicable in Enterobacteriaceae bacteria such as *Escherichia coli* can include, for example, pUC19, pUC18, pHSG299, pHSG399, pHSG398, pBR322, pSTV29 (all of these are available from Takara Bio), pACYC184, pMW219 (NIPPON GENE), pTrc99A (Pharmacia), pPROK series vectors (Clontech), pKK233-2 (Clontech), pET

series vectors (Novagen), pQE series vectors (QIAGEN), pCold TF DNA (Takara Bio), pACYC series vectors, and the broad host spectrum vector RSF1010. Specific examples of vector autonomously replicable in coryneform bacteria can include, for example, pHM1519 (Agric. Biol. Chem., 48, 2901-2903 (1984)); pAM330 (Agric. Biol. Chem., 48, 2901-2903 (1984)); plasmids obtained by improving these and having a drug resistance gene; plasmid pCRY30 described in Japanese Patent Laid-open (Kokai) No. 3-210184; plasmids pCRY21, pCRY2KE, pCRY2KX, pCRY31, pCRY3KE, and pCRY3KX described in Japanese Patent Laid-open (Kokai) No. 2-72876 and U.S. Patent No. 5,185,262; plasmids pCRY2 and pCRY3 described in Japanese Patent Laid-open (Kokai) No. 1-191686; pAJ655, pAJ611, and pAJ1844 described in Japanese Patent Laid-open (Kokai) No. 58-192900; pCG1 described in Japanese Patent Laid-open (Kokai) No. 57-134500; pCG2 described in Japanese Patent Laid-open (Kokai) No. 58-35197; pCG4 and pCG11 described in Japanese Patent Laid-open (Kokai) No. 57-183799; pVK7 described in Japanese Patent Laid-open (Kokai) No. 10-215883; pVK9 described in WO2007/046389; pVS7 described in WO2013/069634; and pVC7 described in Japanese Patent Laid-open (Kokai) No. 9-070291.

[0132] When a gene is introduced, it is sufficient that the gene is expressibly harbored by a host. Specifically, it is sufficient that the gene is harbored by a host so that it is expressed under control of a promoter that functions in the host. The term "a promoter that functions in a host" can refer to a promoter that shows a promoter activity in the host. 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.

[0133] A terminator for termination of gene transcription may be located downstream of the gene. The terminator is not particularly limited so long as it functions in a host. 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. Specific examples of the terminator can include, for example, T7 terminator, T4 terminator, fd phage terminator, tet terminator, and trpA terminator.

[0134] 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.

[0135] Furthermore, when two or more of genes are introduced, it is sufficient that the genes each are operably harbored by a host. For example, all the genes may be carried by a single expression vector or a chromosome. Furthermore, 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 made up of two or more genes may also be introduced. The case of "introducing two or more genes" can include, for example, cases of introducing respective genes encoding two or more kinds of proteins (such as enzymes), introducing respective genes encoding two or more subunits making up a single protein complex (such as enzyme complex), and a combination of the foregoing cases.

[0136] The gene to be introduced is not particularly limited so long as it encodes a protein that is able to function 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 using the genomic DNA of an organism having the gene, a plasmid carrying the gene, or the like 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. That is, a variant of a gene may be obtained by modifying the gene. A gene can be modified by a known technique. For example, an objective mutation can be introduced into an objective site of DNA by the site-specific mutation method. That is, the coding region of a gene can be modified by the site-specific mutation method so that a specific site of the encoded protein includes substitution, deletion, insertion, and/or addition of amino acid residues. Examples of the site-specific mutation method can include the method utilizing PCR (Higuchi, R., 61, in PCR Technology, Erlich, H.A. Eds., Stockton Press (1989); Carter, P., Meth. in Enzymol., 154, 382 (1987)), and the method utilizing phage (Kramer, W. and Frits, H.J., Meth. in Enzymol., 154, 350 (1987); Kunkel, T.A. et al., Meth. in Enzymol., 154, 367 (1987)). Alternatively, a variant of a gene may be totally synthesized.

[0137] In addition, when a protein functions as a complex having a plurality of subunits, a part or all of the plurality of subunits may be modified, so long as the activity of the protein is eventually increased. That is, for example, when the activity of a protein is increased by increasing the expression of a gene, the expression of a part or all of the plurality of genes that encode the subunits may be enhanced. It is usually preferable to enhance the expression of all of the plurality of genes encoding the subunits. Furthermore, the subunits making up the complex may be derived from a single kind of organism or two or more kinds of organisms, so long as the complex has a function of the objective protein. That is, for example, genes of the same organism encoding a plurality of subunits may be introduced into a host, or genes of different organisms encoding a plurality of subunits may be introduced into a host.

[0138] Furthermore, the expression of a gene can be increased by improving the tran-

scription 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 can refer to sites that affect the expression of a gene. Examples of the expression control sequence can include, for example, promoter, Shine-Dalgarno (SD) sequence (also referred to as ribosome binding site (RBS)), and spacer region between RBS and the start codon. Expression control sequences can be identified by using a promoter search vector or gene analysis software such as GENETYX. These expression control sequences can be modified by, for example, a method of using a temperature sensitive vector, or the Red driven integration method (WO2005/010175).

- [0139] 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 term "stronger promoter" can mean a promoter providing an improved transcription of a gene compared with an inherently existing wild-type promoter of the gene. Examples of stronger promoters can include, for example, the known high expression promoters such as T7 promoter, trp promoter, lac promoter, thr promoter, tac promoter, trc promoter, tet promoter, araBAD promoter, rpoH promoter, msrA promoter, Pm1 promoter (derived from the genus *Bifidobacterium*), PR promoter, and PL promoter. Examples of stronger promoters that function in coryneform bacteria can include, for example, the artificially modified P54-6 promoter (Appl. Microbiol. Biotechnol., 53, 674-679 (2000)), pta, aceA, aceB, adh, and amyE promoters inducible in coryneform bacteria with acetic acid, ethanol, pyruvic acid, or the like, and cspB, SOD, and tuf (EF-Tu) promoters, which are potent promoters capable of providing a large expression amount in coryneform bacteria (Journal of Biotechnology, 104 (2003) 311-323; Appl. Environ. Microbiol., 2005 Dec; 71 (12):8587-96), as well as lac promoter, tac promoter, and trc promoter. Furthermore, as the stronger promoter, a highly-active type of an inherent promoter may also be obtained by using various reporter genes. For example, by making the -35 and -10 regions in a promoter region closer to the consensus sequence, the activity of the promoter can be enhanced (WO00/18935). Examples of highly active-type promoter can include various tac-like promoters (Katashkina II et al., Russian Federation Patent Application No. 2006134574). Methods for evaluating the strength of promoters and examples of strong promoters are described in the paper of Goldstein et al. (Prokaryotic Promoters in Biotechnology, Biotechnol. Annu. Rev., 1, 105-128 (1995)), and so forth.

- [0140] The translation efficiency of a gene can be improved by, for example, replacing the Shine-Dalgarno (SD) sequence (also referred to as ribosome binding site (RBS)) for

the gene on a chromosome with a stronger SD sequence. The "stronger SD sequence" can mean a SD sequence that provides an improved translation of mRNA compared with the inherent wild-type SD sequence of the gene. Examples of stronger SD sequences can include, for example, RBS of the gene 10 derived from phage T7 (Olins P.O. et al, Gene, 1988, 73, 227-235). Furthermore, it is known that substitution, insertion, or deletion of several nucleotides in a spacer region between RBS and the start codon, especially in a sequence immediately upstream of the start codon (5'-UTR), significantly affects the stability and translation efficiency of mRNA, and hence, the translation efficiency of a gene can also be improved by modifying them.

- [0141] The translation efficiency of a gene can also be improved by, for example, modifying codons. For example, the translation efficiency of the gene can be improved by replacing a rare codon present in the gene with a synonymous codon that is more frequently used. That is, the gene to be introduced may be modified, for example, so as to contain optimal codons according to the frequencies of codons observed in a host to be used. Codons can be replaced by, for example, the site-specific mutation method. 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" (www.kazusa.or.jp/codon; Nakamura, Y. et al, Nucl. Acids Res., 28, 292 (2000)).
- [0142] Furthermore, 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.
- [0143] Such methods for increasing the gene expression as mentioned above may be used independently or in an arbitrary combination.
- [0144] Furthermore, the modification that increases the activity of a protein can also be attained by, for example, enhancing the specific activity of the enzyme. Enhancement of the specific activity can also include desensitization to feedback inhibition. That is, when a protein is subject to feedback inhibition by a metabolite, the activity of the protein can be increased by making a host harbor a gene encoding a mutant protein that has been desensitized to the feedback inhibition. The expression "desensitization to feedback inhibition" can include complete elimination of the feedback inhibition, and attenuation of the feedback inhibition, unless otherwise stated. Also, the state of "being desensitized to feedback inhibition", i.e. the state that feedback inhibition is eliminated or attenuated, can also be referred to as "tolerant to feedback inhibition". A protein showing an enhanced specific activity can be obtained by, for example, searching various organisms. Furthermore, a highly-active type of an inherent protein may also be obtained by introducing a mutation into the existing protein. The mutation to be introduced may be, for example, substitution, deletion, insertion, or addition of one or

several amino acid residues at one or several positions in the protein. The mutation can be introduced by, for example, such a site-specific mutation method as mentioned above. The mutation may also be introduced by, for example, a mutagenesis treatment. Examples of the mutagenesis treatment can include irradiation of X-ray, irradiation of ultraviolet, and a treatment with a mutation agent such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), ethyl methanesulfonate (EMS), and methyl methanesulfonate (MMS). Furthermore, a random mutation may be induced by directly treating DNA in vitro with hydroxylamine. Enhancement of the specific activity may be independently used, or may be used in an arbitrary combination with such methods for enhancing gene expression as mentioned above.

[0145] The method for the transformation is not particularly limited, and conventionally known methods can be used. There can be used, for example, a method of treating recipient cells with calcium chloride so as to increase the permeability thereof for DNA, which has been reported for the *Escherichia coli* K-12 strain (Mandel, M. and Higa, A., *J. Mol. Biol.*, 1970, 53, 159-162), and a method of preparing competent cells from cells which are in the growth phase, followed by transformation with DNA, which has been reported for *Bacillus subtilis* (Duncan, C.H., Wilson, G.A. and Young, F.E., *Gene*, 1977, 1:153-167). Alternatively, there can also be used a method of making DNA-recipient cells into protoplasts or spheroplasts, which can easily take up recombinant DNA, followed by introducing a recombinant DNA into the DNA-recipient cells, which is known to be applicable to *Bacillus subtilis*, actinomycetes, and yeasts (Chang, S. and Choen, S.N., 1979, *Mol. Gen. Genet.*, 168:111-115; Bibb, M.J., Ward, J.M. and Hopwood, O.A., 1978, *Nature*, 274:398-400; Hinnen, A., Hicks, J.B. and Fink, G.R., 1978, *Proc. Natl. Acad. Sci. USA*, 75:1929-1933). Furthermore, the electric pulse method reported for coryneform bacteria (Japanese Patent Laid-open (Kokai) No. 2-207791) can also be used.

[0146] An increase in the activity of a protein can be confirmed by measuring the activity of the protein.

[0147] An increase in the activity of a protein can also be confirmed by confirming an increase in the expression of a gene encoding 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.

[0148] An increase of the transcription amount of a gene can be confirmed by comparing the amount of mRNA transcribed from the gene with that of a non-modified strain such as a wild-type strain or parent strain. Examples of the method for evaluating the amount of mRNA can 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 to, for example, 1.2 times or more, 1.5 times or more, 2 times or more, or 3 times or more of that of a non-modified strain.

[0149] 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 (such as the number of molecules of the protein per cell) may increase to, for example, 1.2 times or more, 1.5 times or more, 2 times or more, or 3 times or more of that of a non-modified strain.

[0150] The aforementioned methods for increasing the activity of a protein can be applied to enhancement of the activities of arbitrary proteins such as an objective substance biosynthesis enzyme, phosphopantetheinylation enzyme, and uptake system of a substance, and enhancement of the expression of arbitrary genes such as genes encoding those arbitrary proteins, besides introduction of a specific CAR gene.

[0151] <1-3> Method for reducing activity of protein

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

[0152] The expression "the activity of a protein is reduced" can mean that the activity of the protein is reduced as compared with a non-modified strain. Specifically, the expression "the activity of a protein is reduced" may mean that the activity of the protein per cell is reduced as compared with that of a non-modified strain. The term "non-modified strain" used herein can refer to a control strain that has not been modified so that the activity of an objective protein is reduced. Examples of the non-modified strain can include a wild-type strain and parent strain. Specific examples of the non-modified strain can include the respective type strains of the species of microorganisms. Specific examples of the non-modified strain can also include strains exemplified above in relation to the description of microorganisms. That is, in an embodiment, the activity of a protein may be reduced as compared with a type strain, i.e. the type strain of the species to which a microorganism belongs. In another embodiment, the activity of a protein may also be reduced as compared with the *C. glutamicum* ATCC 13869 strain. In another embodiment, the activity of a protein may also be reduced as compared with the *C. glutamicum* ATCC 13032 strain. In another embodiment, the activity of a protein may also be reduced as compared with the *E. coli* K-12 MG1655 strain. The state that "the activity of a protein is reduced" can also include a state that the activity of the protein has completely disappeared. More specifically, the expression "the activity of a protein is reduced" may mean 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) encoding 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" can also include a state that the protein does not exist at all. The state that "the function of each molecule of the protein is reduced" can also include a state that the function of each protein molecule has completely disappeared. 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. The activity of a protein 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.

[0153] The modification for reducing the activity of a protein can be attained by, for example, reducing the expression of a gene encoding the protein. The expression "the expression of a gene is reduced" can mean that the expression of the gene is reduced as compared with a non-modified strain such as a wild-type strain and parent strain. Specifically, the expression "the expression of a gene is reduced" may mean that the expression of the gene per cell is reduced as compared with that of a non-modified strain. More specifically, the expression "the expression of a gene is reduced" may 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" can also include a state that the gene is not expressed at all. The state that "the expression of a gene is reduced" can also be referred to as "the expression of a gene is attenuated". The expression of a gene 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.

[0154] 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, the Shine-Dalgarno (SD) sequence (also referred to as ribosome-binding site (RBS)), and a spacer region between RBS and the start codon of the gene. When an expression control sequence is modified, one or more nucleotides, two or more nucleotides, or three or more nucleotides, of the expression control sequence are modified. For example, the transcription efficiency of a gene can be reduced by, for example, replacing the promoter of the gene on a chromosome with a weaker promoter. The term "weaker promoter" can mean a promoter providing an attenuated transcription of a gene compared with an inherent wild-type promoter of the gene. Examples of weaker promoters can include, for example, inducible promoters. That is, an inducible promoter may function as a weaker promoter under a non-induced condition, such as in the absence of the corresponding inducer. Furthermore, a part of or the entire 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 can 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. Furthermore, 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. Furthermore, for example, the gene expression may be reduced due to disruption of a gene as described later.

[0155] The modification for reducing the activity of a protein can also be attained by, for example, disrupting a gene encoding the protein. The expression "a gene is disrupted" can mean that a gene is modified so that a protein that can normally function is not produced. The state that "a protein that normally functions is not produced" can include a state that the protein is not produced at all from the gene, and a state that the protein of which the function (such as activity or property) per molecule is reduced or eliminated is produced from the gene.

[0156] Disruption of a gene can be attained by, for example, deleting the gene on a chromosome. The term "deletion of a gene" can refer to deletion of a partial or entire region of the coding region of the gene. Furthermore, the entire gene including sequences upstream and downstream from the coding region of the gene on a chromosome may be deleted. The region to be deleted may be any region such as an N-terminal region (region encoding an N-terminal region of a protein), an internal region, or a C-terminal region (region encoding a C-terminal region of a protein), so long as the activity of the protein can be reduced. Deletion of a longer region can usually more surely inactivate the gene. The region to be deleted may be, for example, a region having a length of 10% or more, 20% or more, 30% or more, 40% or more, 50% or more, 60% or more, 70% or more, 80% or more, 90% or more, or 95% or more of the total length of the coding region of the gene. Furthermore, it is preferred that reading frames of the sequences upstream and downstream from the region to be deleted are not the same. Inconsistency of reading frames may cause a frameshift downstream of the region to be deleted.

[0157] 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), addition or deletion of one or two nucleotide residues (frame shift mutation), 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)).

- [0158] Disruption of a gene can also be attained by, for example, inserting another nucleotide 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 nucleotide sequence 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. Inconsistency of reading frames may cause a frameshift downstream of the region to be deleted. The other nucleotide 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 can include, for example, a marker gene such as antibiotic resistance genes, and a gene useful for production of an objective substance.
- [0159] Particularly, disruption of a gene may be carried out so that the amino acid sequence of the encoded protein is deleted. In other words, the modification for reducing the activity of a protein can be attained by, for example, deleting the amino acid sequence of the protein, specifically, modifying a gene so as to encode a protein of which the amino acid sequence is deleted. The term "deletion of the amino acid sequence of a protein" can refer to deletion of a partial or entire region of the amino acid sequence of the protein. In addition, the term "deletion of the amino acid sequence of a protein" means that the original amino acid sequence disappears in the protein, and can also include cases where the original amino acid sequence is changed to another amino acid sequence. That is, for example, a region that was changed to another amino acid sequence by frameshift may be regarded as a deleted region. When the amino acid sequence of a protein is deleted, the total length of the protein is typically shortened, but there can also be cases where the total length of the protein is not changed or is extended. For example, by deletion of a partial or entire region of the coding region of a gene, a region encoded by the deleted region can be deleted in the encoded protein. In addition, for example, by introduction of a stop codon into the coding region of a gene, a region encoded by the downstream region of the introduction site can be deleted in the encoded protein. In addition, for example, by frameshift in the coding region of a gene, a region encoded by the frameshift region can be deleted in the encoded protein. The aforementioned descriptions concerning the position and length of the region to be deleted in deletion of a gene can be applied mutatis mutandis to the position and length of the region to be deleted in deletion of the amino acid sequence of a protein.
- [0160] Such modification of a gene on a chromosome as described above can be attained by, for example, preparing a disruption-type gene modified so that it is unable to produce a protein that functions normally, and transforming a host with a recombinant DNA

containing the disruption-type gene to cause homologous recombination between the disruption-type gene and the wild-type gene on a chromosome and thereby substitute the disruption-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 easier. Examples of the disruption-type gene can include a gene of which a partial or entire region of the coding region is deleted, gene including a missense mutation, gene including a nonsense mutation, gene including a frame shift mutation, and gene including insertion of a transposon or marker gene. The protein encoded by the disruption-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. Such gene disruption based on gene substitution utilizing homologous recombination has already been established, and there are methods of using a linear DNA such as a method called "Red driven integration" (Datsenko, K.A, and Wanner, B.L., Proc. Natl. Acad. Sci. USA, 97:6640-6645 (2000)), and a method utilizing the Red driven integration in combination with an excision system derived from λ phage (Cho, E.H., Gumpert, R.I., Gardner, J.F., J. Bacteriol., 184:5200-5203 (2002)) (refer to WO2005/010175), a method of using a plasmid having a temperature sensitive replication origin, a method of using a plasmid capable of conjugative transfer, a method of utilizing a suicide vector not having a replication origin that functions in a host (U.S. Patent No. 6,303,383, Japanese Patent Laid-open (Kokai) No. 05-007491), and so forth.

- [0161] The modification for reducing activity of a protein can also be attained by, for example, a mutagenesis treatment. Examples of the mutagenesis treatment can include 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).
- [0162] Such methods for reducing the activity of a protein as mentioned above may be used independently or in an arbitrary combination.
- [0163] When a protein functions as a complex having a plurality of subunits, a part or all of the plurality of subunits may be modified, so long as the activity of the protein is eventually reduced. That is, for example, a part or all of a plurality of genes that encode the respective subunits may be disrupted or the like. Furthermore, when there is a plurality of isozymes of a protein, a part or all of the activities of the plurality of isozymes may be reduced, so long as the activity of the protein is eventually reduced. That is, for example, a part or all of a plurality of genes that encode the respective isozymes may be disrupted or the like.
- [0164] A reduction in the activity of a protein can be confirmed by measuring the activity of the protein.

- [0165] A reduction in the activity of a protein can also be confirmed by confirming a reduction in the expression of a gene encoding the protein. 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.
- [0166] 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 can include Northern hybridization, RT-PCR, and so forth (Molecular Cloning, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (USA), 2001). The amount of mRNA can be 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.
- [0167] 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 (such as the number of molecules of the protein per cell) can be 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.
- [0168] 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.
- [0169] Such methods for reducing the activity of a protein as mentioned above can be applied to reduction in the activities of arbitrary proteins such as a byproduct generation enzyme, and reduction in the expression of arbitrary genes such as genes encoding those arbitrary proteins.
- [0170] <2> Method of the present invention
The method as described herein is a method for producing an objective substance by using the microorganism as described herein.
- [0171] <2-1> Fermentation method
An objective substance can be produced by, for example, fermentation of the microorganism as described herein. That is, an embodiment of the method as described herein may be a method for producing an objective substance by fermentation of the microorganism. This embodiment can also be referred to as "fermentation method". Also, the step of producing an objective substance by fermentation of the microorganism can also be referred to as "fermentation step".
- [0172] The fermentation step can be performed by cultivating the microorganism as described herein. Specifically, in the fermentation method, an objective substance can be produced from a carbon source. That is, the fermentation step may be, for example, a step of cultivating the microorganism in a culture medium, such as a culture medium

containing a carbon source, to produce and accumulate the objective substance in the culture medium. That is, the fermentation method may be a method for producing an objective substance by cultivating the microorganism in a culture medium, such as a culture medium containing a carbon source, to produce and accumulate the objective substance in the culture medium. Also, in other words, the fermentation step may be, for example, a step of producing the objective substance from a carbon source by using the microorganism.

[0173] The culture medium is not particularly limited, so long as the microorganism can proliferate in it and produce an objective substance. As the culture medium, for example, a culture medium that is typically used for culture of microorganisms such as bacteria and yeast can be used. The culture medium may contain a carbon source, nitrogen source, phosphate source, and sulfur source, as well as other medium components such as various organic components and inorganic components as required. The types and concentrations of the medium components can be appropriately determined according to various conditions such as the type of the microorganism to be used.

[0174] The carbon source is not particularly limited, so long as the microorganism can utilize it and produce an objective substance. Specific examples of the carbon source can include, for example, saccharides such as glucose, fructose, sucrose, lactose, galactose, xylose, arabinose, blackstrap molasses, hydrolysates of starches, and hydrolysates of biomass; organic acids such as acetic acid, citric acid, succinic acid, and gluconic acid; alcohols such as ethanol, glycerol, and crude glycerol; and fatty acids. As the carbon source, in particular, plant-derived materials can be used. Examples of the plant can include, for example, corn, rice, wheat, soybean, sugarcane, beet, and cotton. Examples of the plant-derived materials can include, for example, organs such as root, stem, trunk, branch, leaf, flower, and seed, plant bodies including them, and decomposition products of these plant organs. The forms of the plant-derived materials at the time of use thereof are not particularly limited, and they can be in any form such as an unprocessed product, juice, ground product, and purified product. Pentoses such as xylose, hexoses such as glucose, or mixtures of them can be obtained from, for example, plant biomass. Specifically, these saccharides can be obtained by subjecting a plant biomass to such a treatment as steam treatment, hydrolysis with concentrated acid, hydrolysis with diluted acid, hydrolysis with an enzyme such as cellulase, and alkaline treatment. Since hemicellulose is generally more easily hydrolyzed compared with cellulose, hemicellulose in a plant biomass may be hydrolyzed beforehand to liberate pentoses, and then cellulose may be hydrolyzed to generate hexoses. Furthermore, xylose may be supplied by conversion from hexoses by, for example, imparting a pathway for converting hexose such as glucose to xylose to the mi-

microorganism. As the carbon source, one kind of carbon source may be used, or two or more kinds of carbon sources may be used in combination.

- [0175] The concentration of the carbon source in the culture medium is not particularly limited, so long as the microorganism can proliferate and produce an objective substance. The concentration of the carbon source in the culture medium may be as high as possible within such a range that production of the objective substance is not inhibited. Initial concentration of the carbon source in the culture medium may be, for example, usually 5 to 30% (w/v), or 10 to 20% (w/v). Furthermore, the carbon source may be additionally supplied to the culture medium as required. For example, the carbon source may be additionally supplied to the culture medium in proportion to decrease or depletion of the carbon source accompanying progress of the fermentation. While the carbon source may be temporarily depleted so long as an objective substance can be eventually produced, it may be preferable to perform the culture so that the carbon source is not depleted or the carbon source does not continue to be depleted.
- [0176] Specific examples of the nitrogen source can 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 and aqueous ammonia used for pH adjustment may also be used as a nitrogen source. As the nitrogen source, one kind of nitrogen source may be used, or two or more kinds of nitrogen sources may be used in combination.
- [0177] Specific examples of the phosphate source can include, for example, phosphate salts such as potassium dihydrogenphosphate and dipotassium hydrogenphosphate, and phosphoric acid polymers such as pyrophosphoric acid. As the phosphate source, one kind of phosphate source may be used, or two or more kinds of phosphate sources may be used in combination.
- [0178] Specific examples of the sulfur source can 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, one kind of sulfur source may be used, or two or more kinds of sulfur sources may be used in combination.
- [0179] Specific examples of other various organic and inorganic components can 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 these such as peptone, casamino acid, yeast extract, and soybean protein decomposition product. As the other various organic and inorganic components, one kind of component may be used, or

two or more kinds of components may be used in combination.

- [0180] Furthermore, when an auxotrophic mutant strain that requires a nutrient such as amino acids for growth thereof is used, it is preferable to supplement such a required nutrient to the culture medium. Furthermore, a component used for production of an objective substance may be supplemented to the culture medium. Specific examples of such a component can include, for example, methyl group donors such as SAM and precursors thereof such as methionine.
- [0181] Culture conditions are not particularly limited, so long as the microorganism can proliferate, and an objective substance is produced. The culture can be performed with, for example, conditions typically used for the culture of microorganisms such as bacteria and yeast. The culture conditions may be appropriately determined according to various conditions such as the type of chosen microorganism.
- [0182] The culture can be performed by using a liquid medium. At the time of the culture, for example, the microorganism cultured on a solid medium such as agar medium may be directly inoculated into a liquid medium, or the microorganism cultured in a liquid medium as seed culture may be inoculated into a liquid medium for main culture. That is, the culture may be performed separately as seed culture and main culture. In such a case, the culture conditions of the seed culture and the main culture may be or may not be the same. It is sufficient that an objective substance is produced at least during the main culture. The amount of the microorganism contained in the culture medium at the time of the start of the culture is not particularly limited. For example, a seed culture broth having an OD660 of 4 to 100 may be added to a culture medium for main culture in an amount of 0.1 to 100 mass %, or 1 to 50 mass %, at the time of the start of the culture.
- [0183] The culture can be performed as batch culture, fed-batch culture, continuous culture, or a combination of these. The culture medium used at the start of the culture can also be referred to as "starting medium". The culture medium supplied to the culture system (e.g. fermentation tank) in the fed-batch culture or the continuous culture can also be referred to as "feed medium". To supply a feed medium to the culture system in the fed-batch culture or the continuous culture can also be referred to as "feed". Furthermore, when the culture is performed separately as seed culture and main culture, the culture schemes of the seed culture and the main culture may be 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.
- [0184] The various components such as the carbon source may be present in the starting medium, feed medium, or both. That is, the various components such as the carbon

source may be additionally supplied to the culture medium independently or in an arbitrary combination during the culture. These components may be supplied once or a plurality of times, or may be continuously supplied. The types of the components present in the starting medium may be or may not be the same as those of the components present in the feed medium. Furthermore, the concentrations of the components present in the starting medium may be or may not be the same as the concentrations of the components present in the feed medium. Furthermore, two or more kinds of feed media having components of different types and/or different concentrations may be used. For example, when feeding is intermittently performed two or more times, the types and/or concentrations of components contained in the feed medium may be or may not be the same for each feeding.

- [0185] The culture can be performed, for example, under an aerobic condition. The term "aerobic condition" may refer to a condition where the dissolved oxygen concentration in the culture medium is 0.33 ppm or higher, or 1.5 ppm or higher. The oxygen concentration can be controlled to be, for example, 1 to 50%, or about 5%, of the saturated oxygen concentration. The culture can be performed, for example, with aeration or shaking. The pH of the culture medium may be, for example, 3 to 10, or 4.0 to 9.5. The pH of the culture medium can be adjusted during the culture as required. The pH of the culture medium can be adjusted by using various alkaline and acidic substances such as ammonia gas, aqueous ammonia, sodium carbonate, sodium bicarbonate, potassium carbonate, potassium bicarbonate, magnesium carbonate, sodium hydroxide, calcium hydroxide, and magnesium hydroxide. The culture temperature may be, for example, 20 to 45°C, or 25 to 37°C. The culture time may be, for example, 10 to 120 hours. The culture may be continued, for example, until the carbon source present in the culture medium is consumed, or until the activity of the microorganism is lost.
- [0186] By cultivating the microorganism under such conditions as described above, an objective substance is accumulated in the culture medium.
- [0187] Production of an objective substance can be confirmed by known methods used for detection or identification of compounds. Examples of such methods can include, for example, HPLC, UPLC, LC/MS, GC/MS, and NMR. These methods may be independently used, or may be used in an appropriate combination. These methods can also be used for determining the concentrations of various components present in the culture medium.
- [0188] The produced objective substance can be appropriately collected. That is, the fermentation method may further comprise a step of collecting the objective substance. This step can also be referred to as "collection step". The collection step may be a step of collecting the objective substance from the culture broth, specifically from the culture medium. The objective substance can be collected by known methods used for

separation and purification of compounds. Examples of such methods can include, for example, ion-exchange resin method, membrane treatment, precipitation, extraction, distillation, and crystallization. The objective substance can be collected specifically by extraction with an organic solvent such as ethyl acetate or by steam distillation. These methods may be independently used, or may be used in an appropriate combination.

[0189] Furthermore, when an objective substance deposits in the culture medium, it can be collected by, for example, centrifugation or filtration. The objective substance deposited in the culture medium and the objective substance dissolving in the culture medium may be isolated together after the objective substance dissolving in the culture medium is crystallized.

[0190] The collected objective substance may contain, for example, microbial cells, medium components, moisture, and by-product metabolites of the microorganism, in addition to the objective substance. The purity of the collected objective substance may be, for example, 30% (w/w) or higher, 50% (w/w) or higher, 70% (w/w) or higher, 80% (w/w) or higher, 90% (w/w) or higher, or 95% (w/w) or higher.

[0191] <2-2> Bioconversion method

An objective substance can also be produced by, for example, bioconversion using the microorganism as described herein. That is, another embodiment of the method as described herein may be a method for producing an objective substance by bioconversion using the microorganism. This embodiment can also be referred to as "bioconversion method". Also, the step of producing an objective substance by bioconversion using the microorganism can also be referred to as "bioconversion step".

[0192] Specifically, in the bioconversion method, an objective substance can be produced from a precursor of the objective substance. More specifically, in the bioconversion method, an objective substance can be produced by converting a precursor of the objective substance into the objective substance by using the microorganism. That is, the bioconversion step may be a step of converting a precursor of an objective substance into the objective substance by using the microorganism.

[0193] A precursor of an objective substance can also be referred simply as "precursor". Examples of the precursor can include substances of which conversion into an object substance includes a step catalyzed by the specific CAR. Specific examples of the precursor can include intermediates of the biosynthesis pathway of an object substance, such as those recited in relation to descriptions of the objective substance biosynthesis enzymes, provided that conversion of the intermediates into the object substance includes a step catalyzed by the specific CAR. More specific examples of the precursor can include, for example, protocatechuic acid, vanillic acid, benzoic acid, L-phenylalanine, and cinnamic acid. Protocatechuic acid and vanillic acid each may be

used as a precursor for producing, for example, vanillin. Benzoic acid may be used as a precursor for producing, for example, benzaldehyde. L-phenylalanine and cinnamic acid each may be used as a precursor for producing, for example, cinnamaldehyde. As the precursor, one kind of precursor may be used, or two or more kinds of precursors may be used in combination. In cases where the precursor is a compound that can form a salt, the precursor may be used as a free compound, a salt thereof, or a mixture thereof. That is, the term "precursor" can refer to a precursor in a free form, a salt thereof, or a mixture thereof, unless otherwise stated. Examples of the salt can include, for example, sulfate salt, hydrochloride salt, carbonate salt, ammonium salt, sodium salt, and potassium salt. As the salt of the precursor, one kind of salt may be employed, or two or more kinds of salts may be employed in combination.

[0194] As the precursor, a commercial product may be used, or one appropriately prepared and obtained may be used. That is, the bioconversion method may further comprise a step of producing a precursor. The method for producing a precursor is not particularly limited, and for example, known methods can be used. A precursor can be produced by, for example, a chemical synthesis method, enzymatic method, bioconversion method, fermentation method, extraction method, or a combination of these. That is, for example, a precursor of an objective substance can be produced from a further precursor thereof using an enzyme that catalyzes the conversion of such a further precursor into the precursor of an objective substance (also referred to as "precursor biosynthesis enzyme"). Furthermore, for example, a precursor of an objective substance can be produced from a carbon source or such a further precursor by using a microorganism having a precursor-producing ability. The term "microorganism having a precursor-producing ability" can refer to a microorganism that is able to generate a precursor of an objective substance from a carbon source or a further precursor thereof. For example, examples of the method for producing protocatechuic acid according to an enzymatic method or bioconversion method can include the method of converting para-cresol into protocatechuic acid using *Pseudomonas putida* KS-0180 (Japanese Patent Laid-open (Kokai) No. 7-75589), the method of converting para-hydroxybenzoic acid into protocatechuic acid using an NADH-dependent para-hydroxybenzoic acid hydroxylase (Japanese Patent Laid-open (Kokai) No. 5-244941), the method of producing protocatechuic acid by cultivating a transformant harboring a gene that is involved in the reaction of generating protocatechuic acid from terephthalic acid in a culture medium containing terephthalic acid (Japanese Patent Laid-open (Kokai) No. 2007-104942), and the method of producing protocatechuic acid from a precursor thereof by using a microorganism having protocatechuic acid-producing ability and having a reduced activity of protocatechuic acid 5-oxidase or being deficient in that activity (Japanese Patent Laid-open (Kokai) No. 2010-207094).

Furthermore, examples of the method for producing protocatechuic acid by fermentation can include the method of producing protocatechuic acid by using a bacterium of the genus *Brevibacterium* and acetic acid as a carbon source (Japanese Patent Laid-open (Kokai) No. 50-89592), the method of producing protocatechuic acid by using a bacterium of the genus *Escherichia* or *Klebsiella* introduced with a gene encoding 3-dihydroshikimate dehydrogenase and glucose as a carbon source (U.S. Patent No. 5,272,073). Furthermore, vanillic acid can be produced by using protocatechuic acid as a precursor according to an enzymatic method using OMT or a bioconversion method using a microorganism having OMT (J. Am. CHm. Soc., 1998, Vol.120), or by using ferulic acid as a precursor according to a bioconversion method using *Pseudomonas* sp. AV10 (J. App. Microbiol., 2013, Vol.116, p903-910). The produced precursor can be used for the bioconversion method as it is, or after being subjected to an appropriate treatment such as concentration, dilution, drying, dissolution, fractionation, extraction, and purification, as required. That is, as the precursor, for example, a purified product purified to a desired extent may be used, or a material containing a precursor may be used. The material containing a precursor is not particularly limited so long as the microorganism can use the precursor. Specific examples of the material containing a precursor can include a culture broth obtained by cultivating a microorganism having a precursor-producing ability, a culture supernatant separated from the culture broth, and processed products thereof such as concentrated products (such as concentrated liquid) thereof and dried products thereof.

[0195] In an embodiment, the bioconversion step can be performed by, for example, cultivating the microorganism as described herein. This embodiment can also be referred to as "first embodiment of the bioconversion method". That is, the bioconversion step may be, for example, a step of cultivating the microorganism in a culture medium containing a precursor of an objective substance to convert the precursor into the objective substance. The bioconversion step may be, specifically, a step of cultivating the microorganism in a culture medium containing a precursor of an objective substance to produce and accumulate the objective substance in the culture medium.

[0196] The culture medium to be used is not particularly limited, so long as the culture medium contains a precursor of an objective substance, and the microorganism can proliferate in it and produce the objective substance. Culture conditions are not particularly limited, so long as the microorganism can proliferate, and an objective substance is produced. The descriptions concerning the culture mentioned for the fermentation method, such as those concerning the culture medium and culture conditions, can be applied *mutatis mutandis* to the culture in the first embodiment of the bioconversion method, except that the culture medium contains the precursor in the first embodiment.

[0197] The precursor may be present in the culture medium over the whole period of the culture, or may be present in the culture medium during only a partial period of the culture. That is, the phrase "cultivating a microorganism in a culture medium containing a precursor" does not necessarily mean that the precursor is present in the culture medium over the whole period of the culture. For example, the precursor may be or may not be present in the culture medium from the start of the culture. When the precursor is not present in the culture medium at the time of the start of the culture, the precursor is added to the culture medium after the start of the culture. Timing of the addition can be appropriately determined according to various conditions such as the length of the culture period. For example, after the microorganism sufficiently grows, the precursor may be added to the culture medium. Furthermore, in any case, the precursor may be added to the culture medium as required. For example, the precursor may be added to the culture medium in proportion to decrease or depletion of the precursor that occurs when objective substance is generated. Methods for supplying the precursor to the culture medium are not particularly limited. For example, the precursor can be supplied to the culture medium by feeding a feed medium containing the precursor to the culture medium. Furthermore, for example, the microorganism as described herein and a microorganism having a precursor-producing ability can be co-cultured to allow the microorganism having a precursor-producing ability to produce the precursor in the culture medium, and thereby supply the precursor to the culture medium. These supply means may be independently used, or may be used in an appropriate combination. The concentration of the precursor in the culture medium is not particularly limited so long as the microorganism can use the precursor as a raw material of an objective substance. The concentration of the precursor in the culture medium, for example, may be 0.1 g/L or higher, 1 g/L or higher, 2 g/L or higher, 5 g/L or higher, 10 g/L or higher, or 15 g/L or higher, or 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, in terms of the weight of the free compound. The precursor may be or may not be present in the culture medium at a concentration within the range exemplified above over the whole period of the culture. For example, the precursor may be present in the culture medium at a concentration within the range exemplified above at the time of the start of the culture, or it may be added to the culture medium so that a concentration within the range exemplified above is attained after the start of the culture. In cases where the culture is performed separately as seed culture and main culture, it is sufficient that an objective substance is produced at least during the main culture. Hence, it is sufficient that the precursor is present in the culture medium at least during the main culture, i.e. over the whole period of the main culture or during a partial period of the main culture, and that is, the precursor may be or may not be

present in the culture medium during the seed culture. In such cases, terms regarding the culture, such as "culture period (period of culture)" and "start of culture", can be read as those regarding the main culture.

- [0198] In another embodiment, the bioconversion step can also be performed by, for example, using cells of the microorganism as described herein. This embodiment can also be referred to as "second embodiment of the bioconversion method". That is, the bioconversion step may be, for example, a step of converting a precursor of an objective substance in a reaction mixture into the objective substance by using cells of the microorganism. The bioconversion step may be, specifically, a step of allowing cells of the microorganism to act on a precursor of an objective substance in a reaction mixture to generate and accumulate the objective substance in the reaction mixture. The bioconversion step performed by using such cells can also be referred to as "conversion reaction".
- [0199] Cells of the microorganism can be obtained by cultivating the microorganism. The culture method for obtaining the cells is not particularly limited so long as the microorganism can proliferate. At the time of the culture for obtaining the cells, the precursor may be or may not be present in the culture medium. Also, at the time of the culture for obtaining the cells, an objective substance may be or may not be produced in the culture medium. The descriptions concerning the culture described for the fermentation method, such as those concerning the culture medium and culture conditions, can be applied *mutatis mutandis* to the culture for obtaining the cells used for the second embodiment of the bioconversion method.
- [0200] The cells may be used for the conversion reaction while being present in the culture broth (specifically, culture medium), or after being collected from the culture broth (specifically, culture medium). The cells may also be used for the conversion reaction after being subjected to a treatment as required. That is, examples of the cells can include a culture broth containing the cells, the cells collected from the culture broth, or a processed product thereof. In other words, examples of the cells can include cells present in a culture broth of the microorganism, cells collected from the culture broth, and cells present in a processed product thereof. Examples of the processed product can include products obtained by subjecting the cells, such as a culture broth containing the cells, or the cells collected from the culture broth, to a treatment. Cells in these forms may be independently used, or may be used in an appropriate combination.
- [0201] The method for collecting the cells from the culture medium is not particularly limited, and for example, known methods can be used. Examples of such methods can include, for example, spontaneous precipitation, centrifugation, and filtration. A flocculant may also be used. These methods may be independently used, or may be

used in an appropriate combination. The collected cells can be washed as required by using an appropriate medium. The collected cells can be re-suspended as required by using an appropriate medium. Examples of the medium that can be used for washing or suspending the cells can include, for example, aqueous media (aqueous solvents) such as water and aqueous buffer.

- [0202] Examples of the treatment of the cells can include, for example, dilution, condensation, immobilization on a carrier such as acrylamide and carrageenan, freezing and thawing treatment, and treatment for increasing permeability of cell membranes. Permeability of cell membranes can be increased by, for example, using a surfactant or organic solvent. These treatments may be independently used, or may be used in an appropriate combination.
- [0203] The cells used for the conversion reaction are not particularly limited so long as the cells have the ability to produce the objective substance. It is preferred that the cells maintain the metabolic activities thereof. The expression "the cells maintain the metabolic activities thereof" may mean that the cells have an ability to utilize a carbon source to generate or regenerate a substance required for producing an objective substance. Examples of such a substance can include, for example, ATP, electron donors such as NADH and NADPH, and methyl group donors such as SAM. The cells may have or may not have proliferation ability.
- [0204] The conversion reaction can be carried out in an appropriate reaction mixture. Specifically, the conversion reaction can be carried out by allowing the cells and the precursor to coexist in an appropriate reaction mixture. The conversion reaction may be carried out by the batch method or may be carried out by the column method. In the case of the batch method, the conversion reaction can be carried out by, for example, mixing the cells of the microorganism and the precursor in a reaction mixture contained in a reaction vessel. The conversion reaction may be carried out statically, or may be carried out with stirring or shaking the reaction mixture. In the case of the column method, the conversion reaction can be carried out by, for example, passing a reaction mixture containing the precursor through a column filled with immobilized cells. Examples of the reaction mixture can include those based on an aqueous medium (aqueous solvent) such as water and aqueous buffer.
- [0205] The reaction mixture may contain components other than the precursor as required, in addition to the precursor. Examples of the components other than the precursor can include ATP, electron donors such as NADH and NADPH, methyl group donors such as SAM, metal ions, buffering agents, surfactants, organic solvents, carbon sources, phosphate sources, and other various medium components. That is, for example, a culture medium containing the precursor may also be used as a reaction mixture. That is, the descriptions concerning the culture medium mentioned for the first embodiment

of the bioconversion method may also be applied *mutatis mutandis* to the reaction mixture in the second embodiment of the bioconversion method. The types and concentrations of the components contained in the reaction mixture may be determined according to various conditions such as the type of the precursor and the form of the cells.

[0206] Conditions of the conversion reaction, such as dissolved oxygen concentration, pH of the reaction mixture, reaction temperature, reaction time, concentrations of various components, etc., are not particularly limited so long as an objective substance is generated. The conversion reaction can be performed with, for example, conditions typically used for substance conversion using microbial cells such as resting cells. The conditions of the conversion reaction may be determined according to various conditions such as the type of microorganism. The conversion reaction can be performed, for example, under an aerobic condition. The term "aerobic condition" may refer to a condition where the dissolved oxygen concentration in the reaction mixture is 0.33 ppm or higher, or 1.5 ppm or higher. The oxygen concentration can be controlled to be, for example, 1 to 50%, or about 5%, of the saturated oxygen concentration. The pH of the reaction mixture may be, for example, usually 6.0 to 10.0, or 6.5 to 9.0. The reaction temperature may be, for example, usually 15 to 50°C, 15 to 45°C, or 20 to 40°C. The reaction time may be, for example, 5 minutes to 200 hours. In the case of the column method, the loading rate of the reaction mixture may be, for example, such a rate that the reaction time falls within the range of the reaction time exemplified above. Furthermore, the conversion reaction can also be performed with, for example, a culture condition, such as conditions typically used for culture of microorganisms such as bacteria and yeast. During the conversion reaction, the cells may or may not proliferate. That is, the descriptions concerning the culture conditions described for the first embodiment of the bioconversion method may also be applied *mutatis mutandis* to the conditions of the conversion reaction in the second embodiment of the bioconversion method, except that the cells may or may not proliferate in the second embodiment. In such a case, the culture conditions for obtaining the cells and the conditions of the conversion reaction may be the same or different. The concentration of the precursor in the reaction mixture, for example, may be 0.1 g/L or higher, 1 g/L or higher, 2 g/L or higher, 5 g/L or higher, 10 g/L or higher, or 15 g/L or higher, or 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, in terms of the weight of the free compound. The density of the cells in the reaction mixture, for example, may be 1 or higher, or may be 300 or lower, or may be within a range defined with a combination thereof, in terms of the optical density (OD) at 600 nm.

[0207] During the conversion reaction, the cells, the precursor, and the other components

may be additionally supplied to the reaction mixture independently or in any arbitrary combination thereof. For example, the precursor may be additionally supplied to the reaction mixture in proportion to decrease or depletion of the precursor accompanying generation of an objective substance. These components may be supplied once or a plurality of times, or may be continuously supplied.

[0208] Methods for supplying the various components such as the precursor to the reaction mixture are not particularly limited. These components each can be supplied to the reaction mixture by, for example, directly adding them to the reaction mixture. Furthermore, for example, the microorganism as described herein and a microorganism having a precursor-producing ability can be co-cultured to allow the microorganism having a precursor-producing ability to produce the precursor in the reaction mixture, and thereby supply the precursor to the reaction mixture. Furthermore, for example, components such as ATP, electron donors, and methyl group donors each may be generated or regenerated in the reaction mixture, may be generated or regenerated in the cells of the microorganism, or may be generated or regenerated by a coupling reaction between different cells. For example, when cells of the microorganism maintain the metabolic activities thereof, they can generate or regenerate components such as ATP, electron donors, and methyl group donors within them by using a carbon source. In addition, examples of the method for generating or regenerating ATP can include, for example, the method of supplying ATP from a carbon source by using a *Corynebacterium* bacterium (Hori, H. et al., *Appl. Microbiol. Biotechnol.*, 48(6):693-698 (1997)), the method of regenerating ATP by using yeast cells and glucose (Yamamoto, S et al., *Biosci. Biotechnol. Biochem.*, 69(4):784-789 (2005)), the method of regenerating ATP using phosphoenolpyruvic acid and pyruvate kinase (C. Aug'e and Ch. Gautheron, *Tetrahedron Lett.*, 29:789-790 (1988)), and the method of regenerating ATP by using polyphosphoric acid and polyphosphate kinase (Murata, K. et al., *Agric. Biol. Chem.*, 52(6):1471-1477 (1988)).

[0209] Furthermore, the reaction conditions may be constant from the start to the end of the conversion reaction, or they may change during the conversion reaction. The expression "the reaction conditions change during the conversion reaction" can include not only cases where the reaction conditions temporally change, but can also include cases where the reaction conditions spatially change. The expression "the reaction conditions spatially change" can mean that, for example, when the conversion reaction is performed by the column method, the reaction conditions such as reaction temperature and cell density differ depending on position in the flow.

[0210] A culture broth (or culture medium) or reaction mixture containing an objective substance is obtained by carrying out the bioconversion step as described above. Confirmation of the production of the objective substance and collection of the objective

substance can be carried out in the same manners as those for the fermentation method described above. That is, the bioconversion method may further comprise the collection step, e.g. a step of collecting the objective substance from the culture broth (or culture medium) or reaction mixture. The collected objective substance may contain, for example, microbial cells, medium components, reaction mixture components, moisture, and by-product metabolites of the microorganism, in addition to the objective substance. Purity of the collected objective substance may be, for example, 30% (w/w) or higher, 50% (w/w) or higher, 70% (w/w) or higher, 80% (w/w) or higher, 90% (w/w) or higher, or 95% (w/w) or higher.

Examples

[0211] Hereafter, the present invention will be more specifically explained with reference to the following non-limiting examples.

[0212] In this example, strains harboring various CAR genes were constructed from the *Corynebacterium glutamicum* 2256 strain (ATCC 13869) as a parent strain, and vanillin is produced using these constructed strains.

[0213] <1> Construction of a strain deficient in the vanillate demethylase genes (FKS0165 strain)

It has been reported that, in coryneform bacteria, vanillin is metabolized in the order of vanillin -> vanillic acid -> protocatechuic acid, and utilized (Current Microbiology, 2005, Vol. 51, pp.59-65). The conversion reaction from vanillic acid to protocatechuic acid is catalyzed by vanillate demethylase. The vanA gene and vanB gene encode the subunit A and subunit B of vanillate demethylase, respectively. The vanK gene encodes the vanillic acid uptake system, and constitutes the vanABK operon together with the vanAB genes (M.T. Chaudhry, et al., Microbiology, 2007, 153:857-865). Therefore, a strain that is unable to utilize an objective substance such as vanillin and vanillic acid (FKS0165 strain) was first constructed from *C. glutamicum* 2256 strain by deleting the vanABK operon. The procedure is shown below.

[0214] <1-1> Construction of plasmid pBS4SΔvanABK56 for deletion of vanABK genes

PCR was performed by using the genomic DNA of the *C. glutamicum* 2256 strain as the template, and the synthetic DNAs of SEQ ID NOS: 51 and 52 as the primers to obtain a PCR product containing an N-terminus side coding region of the vanA gene. Separately, PCR was also performed by using the genomic DNA of the *C. glutamicum* 2256 strain as the template, and the synthetic DNAs of SEQ ID NOS: 53 and 54 as the primers to obtain a PCR product containing a C-terminus side coding region of the vanK gene. The sequences of SEQ ID NOS: 52 and 53 are partially complementary to each other. Then, the PCR product containing the N-terminus side coding region of the vanA gene and the PCR product containing the C-terminus side coding region of the vanK gene were mixed in approximately equimolar amounts, and inserted into the

pBS4S vector (WO2007/046389) that had been treated with BamHI and PstI by using In Fusion HD Cloning Kit (Clontech). With this DNA, competent cells of *Escherichia coli* JM109 (Takara Bio) were transformed, and the cells were applied to the LB medium containing 100 μ M IPTG, 40 μ g/mL of X-Gal, and 40 μ g/mL of kanamycin, and cultured overnight. Then, white colonies that appeared were picked up, and separated into single colonies to obtain transformants. Plasmids were extracted from the obtained transformants, and one into which the target PCR product was inserted was designated as pBS4S Δ vanABK56.

[0215] <1-2> Construction of FKS0165 strain

pBS4S Δ vanABK56 obtained above does not contain the region enabling autonomous replication of the plasmid in cells of coryneform bacteria. Therefore, if coryneform bacteria are transformed with this plasmid, a strain in which this plasmid is incorporated into the genome by homologous recombination appears as a transformant, although it occurs at an extremely low frequency. Therefore, pBS4S Δ vanABK56 was introduced into the *C. glutamicum* 2256 strain by the electric pulse method. The cells were applied to the CM-Dex agar medium (5 g/L of glucose, 10 g/L of polypeptone, 10 g/L of yeast extract, 1 g/L of KH_2PO_4 , 0.4 g/L of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g/L of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g/L of $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, 3 g/L of urea, 1.2 g/L of soybean hydrolysate, 10 μ g/L of biotin, 15 g/L of agar, adjusted to pH 7.5 with NaOH) containing 25 μ g/mL of kanamycin, and cultured at 31.5°C. It was confirmed by PCR that the grown strain was a once-recombinant strain in which pBS4S Δ vanABK56 was incorporated into the genome by homologous recombination. This once-recombinant strain had both the wild-type vanABK genes, and the deficient-type vanABK genes.

[0216] The once-recombinant strain was cultured overnight in the CM-Dex liquid medium (having the same composition as that of the CM-Dex agar medium except that it does not contain agar), and the culture broth was applied to the S10 agar medium (100 g/L of sucrose, 10 g/L of polypeptone, 10 g/L of yeast extract, 1 g/L of KH_2PO_4 , 0.4 g/L of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g/L of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g/L of $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 3 g/L of urea, 1.2 g/L of soybean protein hydrolysate solution, 10 μ g/L of biotin, 20 g/L of agar, adjusted to pH 7.5 with NaOH, and autoclaved at 120°C for 20 minutes), and cultured at 31.5°C. Among the colonies that appeared, a strain that showed kanamycin susceptibility was purified on the CM-Dex agar medium. By preparing genomic DNA from the purified strain, and using it to perform PCR with the synthetic DNAs of SEQ ID NOS: 55 and 56 as the primers, deletion of the vanABK genes was confirmed, and the strain was designated as FKS0165 strain.

[0217] <2> Construction of a strain deficient in alcohol dehydrogenase homologue genes (FKFC14 strain)

Subsequently, by using the *Corynebacterium glutamicum* FKS0165 strain as a parent

strain, a strain FKFC14 was constructed, which is deficient in alcohol dehydrogenase homologue genes, that is, NCgl0324 gene (adhC), NCgl0313 gene (adhE), and NCgl2709 gene (adhA), via the following procedure.

[0218] <2-1> Construction of FKFC5 strain (FKS0165 Δ NCgl0324 strain)

<2-1-1> Construction of plasmid pBS4S Δ 2256adhC for deletion of NCgl0324 gene
 PCR was performed by using the genomic DNA of the *C. glutamicum* 2256 strain as the template, and the synthetic DNAs of SEQ ID NOS: 57 and 58 as the primers to obtain a PCR product containing an N-terminus side coding region of the NCgl0324 gene. Separately, PCR was performed by using the genomic DNA of the *C. glutamicum* 2256 strain as the template, and the synthetic DNAs of SEQ ID NOS: 59 and 60 as the primers to obtain a PCR product containing a C-terminus side coding region of the NCgl0324 gene. The sequences of SEQ ID NOS: 58 and 59 are partially complementary to each other. Then, approximately equimolar amounts of the PCR product containing the N-terminus side coding region of the NCgl0324 gene and the PCR product containing the C-terminus side coding region of the NCgl0324 gene were mixed, and inserted into the pBS4S vector (WO2007/046389) that had been treated with BamHI and PstI by using In Fusion HD Cloning Kit (Clontech). With this DNA, competent cells of *Escherichia coli* JM109 (Takara Bio) were transformed, and the cells were applied to the LB medium containing 100 μ M IPTG, 40 μ g/mL of X-Gal, and 40 μ g/mL of kanamycin, and cultured overnight. Then, white colonies that appeared were picked up, and separated into single colonies to obtain transformants. Plasmids were extracted from the obtained transformants, and one in which the target PCR product was inserted was designated as pBS4S Δ 2256adhC.

[0219] <2-1-2> Construction of FKFC5 strain (FKS0165 Δ NCgl0324 strain)

Since pBS4S Δ 2256adhC obtained above does not contain the region enabling autonomous replication of the plasmid in cells of coryneform bacteria, if coryneform bacteria are transformed with this plasmid, a strain in which this plasmid is incorporated into the genome by homologous recombination appears as a transformant, although it occurs at an extremely low frequency. Therefore, pBS4S Δ 2256adhC was introduced into the *C. glutamicum* FKS0165 strain by the electric pulse method. The cells were applied to the CM-Dex agar medium containing 25 μ g/mL of kanamycin, and cultured at 31.5°C. It was confirmed by PCR that the grown strain was a once-recombinant strain in which pBS4S Δ 2256adhC was incorporated into the genome by homologous recombination. This once-recombinant strain had both the wild-type NCgl0324 gene, and the deficient-type NCgl0324 gene.

[0220] The once-recombinant strain was cultured overnight in the CM-Dex liquid medium, the culture medium was applied to the S10 agar medium, and culture was performed at 31.5°C. Among the colonies that appeared, a strain that showed kanamycin suscep-

tibility was purified on the CM-Dex agar medium. Genomic DNA was prepared from the purified strain, and used to perform PCR with the synthetic DNAs of SEQ ID NOS: 61 and 62 as the primers to confirm deletion of the NCgl0324 gene, and the strain was designated as FKFC5 strain.

[0221] <2-2> Construction of FKFC11 strain (2256 Δ vanABK Δ NCgl0324 Δ NCgl0313 strain)

<2-2-1> Construction of plasmid pBS4S Δ 2256adhE for deletion of NCgl0313 gene

PCR was performed by using the genomic DNA of the *C. glutamicum* 2256 strain as the template, and the synthetic DNAs of SEQ ID NOS: 63 and 64 as the primers to obtain a PCR product containing an N-terminus side coding region of the NCgl0313 gene. Separately, PCR was performed by using the genomic DNA of the *C. glutamicum* 2256 strain as the template, and the synthetic DNAs of SEQ ID NOS: 65 and 66 as the primers to obtain a PCR product containing a C-terminus side coding region of the NCgl0313 gene. The sequences of SEQ ID NOS: 64 and 65 are partially complementary to each other. Then, approximately equimolar amounts of the PCR product containing the N-terminus side coding region of the NCgl0313 gene and the PCR product containing the C-terminus side coding region of the NCgl0313 gene were mixed, and inserted into the pBS4S vector (WO2007/046389) treated with BamHI and PstI by using In Fusion HD Cloning Kit (Clontech). With this DNA, competent cells of *Escherichia coli* JM109 (Takara Bio) were transformed, and the cells were applied to the LB medium containing 100 μ M IPTG, 40 μ g/mL of X-Gal, and 40 μ g/mL of kanamycin, and cultured overnight. Then, white colonies that appeared were picked up, and separated into single colonies to obtain transformants. Plasmids were extracted from the obtained transformants, and one in which the target PCR product was inserted was designated as pBS4S Δ 2256adhE.

[0222] <2-2-2> Construction of FKFC11 strain (2256 Δ vanABK Δ NCgl0324 Δ NCgl0313 strain)

Since pBS4S Δ 2256adhE obtained above does not contain the region enabling autonomous replication of the plasmid in cells of coryneform bacteria, if coryneform bacteria are transformed with this plasmid, a strain in which this plasmid is incorporated into the genome by homologous recombination appears as a transformant, although it occurs at an extremely low frequency. Therefore, pBS4S Δ 2256adhE was introduced into the *C. glutamicum* FKFC5 strain by the electric pulse method. The cells were applied to the CM-Dex agar medium containing 25 μ g/mL of kanamycin, and cultured at 31.5°C. It was confirmed by PCR that the grown strain was a once-recombinant strain in which pBS4S Δ 2256adhE was incorporated into the genome by homologous recombination. This once-recombinant strain had both the wild-type NCgl0313 gene, and the deficient-type NCgl0313 gene.

[0223] The once-recombinant strain was cultured overnight in the CM-Dex liquid medium, the culture medium was applied to the S10 agar medium, and culture was performed at 31.5°C. Among the colonies that appeared, a strain that showed kanamycin susceptibility was purified on the CM-Dex agar medium. Genomic DNA was prepared from the purified strain, and used to perform PCR with the synthetic DNAs of SEQ ID NOS: 67 and 68 as the primers to confirm deletion of the NCgl0313 gene, and the strain was designated as FKFC11 strain.

[0224] <2-3> Construction of FKFC14 strain

(2256ΔvanABKΔNCgl0324ΔNCgl0313ΔNCgl2709 strain)

<2-3-1> Construction of plasmid pBS4SΔ2256adhA for deletion of NCgl2709 gene

PCR was performed by using the genomic DNA of the *C. glutamicum* 2256 strain as the template, and the synthetic DNAs of SEQ ID NOS: 69 and 70 as the primers to obtain a PCR product containing an N-terminus side coding region of the NCgl2709 gene. Separately, PCR was performed by using the genomic DNA of the *C. glutamicum* 2256 strain as the template, and the synthetic DNAs of SEQ ID NOS: 71 and 72 as the primers to obtain a PCR product containing a C-terminus side coding region of the NCgl2709 gene. The sequences of SEQ ID NOS: 70 and 71 are partially complementary to each other. Then, approximately equimolar amounts of the PCR product containing the N-terminus side coding region of the NCgl2709 gene and the PCR product containing the C-terminus side coding region of the NCgl2709 gene were mixed, and inserted into the pBS4S vector treated with BamHI and PstI by using In Fusion HD Cloning Kit (Clontech). With this DNA, competent cells of *Escherichia coli* JM109 (Takara Bio) were transformed, and the cells were applied to the LB medium containing 100 μM IPTG, 40 μg/mL of X-Gal, and 40 μg/mL of kanamycin, and cultured overnight. Then, white colonies that appeared were picked up, and separated into single colonies to obtain transformants. Plasmids were extracted from the obtained transformants, and one in which the target PCR product was inserted was designated as pBS4SΔ2256adhA.

[0225] <2-3-2> Construction of FKFC14 strain

(2256ΔvanABKΔNCgl0324ΔNCgl0313ΔNCgl2709 strain)

Since pBS4SΔ2256adhA obtained above does not contain the region enabling autonomous replication of the plasmid in cells of coryneform bacteria, if coryneform bacteria are transformed with this plasmid, a strain in which this plasmid is incorporated into the genome by homologous recombination appears as a transformant, although it occurs at an extremely low frequency. Therefore, pBS4SΔ2256adhA was introduced into the *C. glutamicum* FKFC11 strain by the electric pulse method. The cells were applied to the CM-Dex agar medium containing 25 μg/mL of kanamycin, and cultured at 31.5°C. It was confirmed by PCR that the grown strain was a once-

recombinant strain in which pBS4SΔ2256adhA was incorporated into the genome by homologous recombination. This once-recombinant strain had both the wild-type NCgl2709 gene, and the deficient-type NCgl2709 gene.

[0226] The once-recombinant strain was cultured overnight in the CM-Dex liquid medium, the culture medium was applied to the S10 agar medium, and culture was performed at 31.5°C. Among the colonies that appeared, a strain that showed kanamycin susceptibility was purified on the CM-Dex agar medium. Genomic DNA was prepared from the purified strain, and used to perform PCR with the synthetic DNAs of SEQ ID NOS: 73 and 74 as the primers to confirm deletion of the NCgl2709 gene, and the strain was designated as FKFC14 strain.

[0227] <3> Construction of vanillin-producing strains

<3-1> Construction of plasmids for co-expression of CAR and PPT genes

A plasmid pVK9::Ptuf*-Nb_ACAR-entD for co-expression of CAR gene of *Nocardia brasiliensis* (Nb_ACAR) and PPT gene (entD) of *E. coli*, a plasmid pVK9::Ptuf*-Ge_ACAR-entD for co-expression of CAR gene of *Gordonia effusa* (Ge_ACAR) and PPT gene (entD) of *E. coli*, a plasmid pVK9::Ptuf*-Nm_ACAR-entD for co-expression of CAR gene of *Novosphingobium malaysiense* (Nm_ACAR) and PPT gene (entD) of *E. coli*, and a plasmid pVK9::Ptuf*-Cs2_ACAR-entD for co-expression of CAR gene of *Coccomyxa subellipsoidea* C-169 (Cs2_ACAR) and PPT gene (entD) of *E. coli* were constructed via the following procedure. PPT catalyzes the addition of a phosphopantetheinyl group into CAR to thereby activate CAR. The CAR genes were codon-optimized for the codon usage of *E. coli* and used. These genes were cloned into the pVK9 vector (WO2007/046389). The pVK9 vector is a shuttle-vector for coryneform bacteria and *Escherichia coli*.

[0228] The pVK9 vector was treated with BamHI and PstI, and inserted with a DNA fragment comprising an artificial operon consisting of Tuf* promoter, SD sequence, Nb_ACAR (codon-optimized), SD sequence, and *E. coli* entD gene in this order, to obtain the plasmid pVK9::Ptuf*-Nb_ACAR-entD. The nucleotide sequence of the portion containing the inserted DNA fragment of this plasmid is shown as SEQ ID NO: 19, wherein the inserted DNA fragment corresponds to position 16-4621.

[0229] The pVK9 vector was treated with BamHI and PstI, and inserted with a DNA fragment comprising an artificial operon consisting of Tuf* promoter, SD sequence, Ge_ACAR (codon-optimized), SD sequence, and *E. coli* entD gene in this order, to obtain the plasmid pVK9::Ptuf*-Ge_ACAR-entD. The nucleotide sequence of the portion containing the inserted DNA fragment of this plasmid is shown as SEQ ID NO: 20, wherein the inserted DNA fragment corresponds to position 16-4517. Also, the nucleotide sequence of Ge_ACAR (codon-optimized) is shown as SEQ ID NO: 77.

[0230] The pVK9 vector was treated with BamHI and PstI, and inserted with a DNA

fragment comprising an artificial operon consisting of Tuf* promoter, SD sequence, Nm_ACAR (codon-optimized), SD sequence, and E. coli entD gene in this order, to obtain the plasmid pVK9::Ptuf*-Nm_ACAR-entD. The nucleotide sequence of the portion containing the inserted DNA fragment of this plasmid is shown as SEQ ID NO: 80, wherein the inserted DNA fragment corresponds to position 16-4528. Also, the nucleotide sequence of Nm_ACAR (codon-optimized) is shown as SEQ ID NO: 81.

[0231] The pVK9 vector was treated with BamHI and PstI, and inserted with a DNA fragment comprising an artificial operon consisting of the Tuf* promoter, SD sequence, Cs2_ACAR (codon-optimized), SD sequence, and E. coli entD gene in this order, to obtain the plasmid pVK9::Ptuf*-Cs2_ACAR-entD. The nucleotide sequence of the portion containing the inserted DNA fragment of this plasmid is shown as SEQ ID NO: 84, wherein the inserted DNA fragment corresponds to position 16-4702. Also, the nucleotide sequence of Cs2_ACAR (codon-optimized) is shown as SEQ ID NO: 85.

[0232] <3-2> Construction of plasmid pVS7::Plac-vanK for expression of vanK gene
vanK gene encodes a vanillic acid uptake system. Thus, in order to improve the uptake of vanillic acid, a plasmid pVS7::Plac-vanK for expression of vanK gene of the C. glutamicum 2256 strain was constructed via the following procedure.

[0233] PCR was performed by using the genomic DNA of the C. glutamicum 2256 strain as the template, and the synthetic DNAs of SEQ ID NOS: 75 and 76 as the primers to obtain a PCR product containing an ORF and SD sequence of vanK gene. Then, the PCR product was inserted into the pVS7 vector (WO2013/069634) treated with BamHI and PstI by using In Fusion HD Cloning Kit (Clontech). The pVS7 vector is a shuttle-vector for coryneform bacteria and Escherichia coli. With this DNA, competent cells of Escherichia coli JM109 (Takara Bio) were transformed, and the cells were applied to the LB medium containing 100 μ M IPTG, 40 μ g/mL of X-Gal, and 50 μ g/mL of spectinomycin, and cultured overnight. Then, white colonies that appeared were picked up, and separated into single colonies to obtain transformants. Plasmids were extracted from the obtained transformants, and one into which the target PCR product was inserted was designated as pVS7::Plac-vanK. In this plasmid, the cloned vanK gene is expressed from lac promoter derived from the pVS7 vector.

[0234] <3-3> Construction of vanillin-producing strains

The plasmid pVK9::Ptuf*-Nb_ACAR-entD, pVK9::Ptuf*-Ge_ACAR-entD, pVK9::Ptuf*-Nm_ACAR-entD, or pVK9::Ptuf*-Cs2_ACAR-entD, as well as the plasmid pVS7::Plac-vanK, was introduced into the C. glutamicum FKFC14 strain by the electric pulse method. The cells were applied to the CM-Dex SGFC agar medium (2.5 g/L of glucose, 2.5 g/L of fructose, 10 g/L of polypeptone, 10 g/L of yeast extract, 1 g/L of KH₂PO₄, 0.4 g/L of MgSO₄-7H₂O, 0.01 g/L of FeSO₄-7H₂O, 0.01 g/L of

MnSO₄-7H₂O, 2 g/L of disodium succinate hexahydrate, 4g/L of sodium gluconate, 3 g/L of urea, 1.2 g/L of soybean hydrolysate, 10 µg/L of biotin, 15 g/L of agar, adjusted to pH 7.5 with NaOH) containing 25 µg/mL of kanamycin and 50 µg/mL of spectinomycin, and cultured at 31.5°C. The grown strains were purified on the same agar medium, and designated as FKFC14/pVK9::Ptuf*-Nb_ACAR-entD pVS7-vanK, FKFC14/pVK9::Ptuf*-Ge_ACAR-entD pVS7-vanK, FKFC14/pVK9::Ptuf*-Nm_ACAR-entD pVS7-vanK, and FKFC14/pVK9::Ptuf*-Cs2_ACAR-entD pVS7-vanK, respectively. These strains were each inoculated into 4 mL of the CM-Dex SGFC medium (having the same composition as that of the CM-Dex SGFC agar medium except that it does not contain agar) contained in a test tube, and cultured at 31.5°C with shaking for about 16 hr. A 0.9 mL aliquot of the obtained culture broth was mixed with 0.6 mL of 50% glycerol aqueous solution to obtain a glycerol stock, and stored at -80°C.

[0235] <4> Comparison of vanillin production and protocatechualdehyde production by *C. glutamicum* vanillin-producing strains

A 20 µL aliquot of each of the glycerol stocks of the constructed vanillin-producing strains was applied to the CM-Dex SGFC agar medium, and cultured at 31.5°C for 20 hr as preculture. The obtained cells were suspended in sterilized physiological saline. The optical density (OD) of the cell suspension was measured, and the cell suspension was diluted with physiological saline to obtain an OD at 600 nm of 83. A 1.5 mL aliquot of the diluted cell suspension was inoculated into 3.5 mL of a vanillin production medium (42.9 g/L of vanillic acid, 85.7 g/L of glucose, 10 g/L of polypeptone, 10 g/L of yeast extract, 1 g/L of KH₂PO₄, 0.4 g/L of MgSO₄-7H₂O, 0.01 g/L of FeSO₄-7H₂O, 0.01 g/L of MnSO₄-7H₂O, 3 g/L of urea, 1.2 g/L of soybean hydrolysate, 10 µg/L of biotin, adjusted to pH 7.4 with KOH, and then mixed with 8.6 g/L of CaCO₃ (sterilized with hot air at 180°C for 3 hours)) containing 25 µg/mL of kanamycin and 50 µg/mL of spectinomycin, or a protocatechualdehyde production medium (42.9 g/L of protocatechuic acid, 85.7 g/L of glucose, 10 g/L of polypeptone, 10 g/L of yeast extract, 1 g/L of KH₂PO₄, 0.4 g/L of MgSO₄-7H₂O, 0.01 g/L of FeSO₄-7H₂O, 0.01 g/L of MnSO₄-7H₂O, 3 g/L of urea, 1.2 g/L of soybean hydrolysate, 10 µg/L of biotin, adjusted to pH 7.4 with KOH, and then mixed with 8.6 g/L of CaCO₃ (sterilized with hot air at 180°C for 3 hours)) containing 25 µg/mL of kanamycin and 50 µg/mL of spectinomycin, contained in a test tube, and cultured at 30°C with shaking for 21 hr.

[0236] At the start and completion of the culture, the concentration of glucose in the medium was analyzed with Biotech Analyzer AS-310 (Sakura SI). The concentrations of vanillic acid, vanillin, protocatechuic acid, and protocatechualdehyde in the medium were also analyzed by using Ultra Performance Liquid Chromatography NEXERA X2

System (SHIMADZU) with the following conditions.

[0237] Conditions of UPLC analysis

Column: KINETEX 2.6 μ m XB-C18, 150 x 30 mm (Phenomenex)

Oven temperature: 40°C

Mobile phase (A): 0.1% Trifluoroacetic acid

Mobile phase (B): 0.1% Trifluoroacetic acid/80% acetonitrile

Gradient program (time, A (%), B (%)): (0, 90, 10) -> (3, 80, 20)

Flow rate: 1.5 ml/min

[0238] The results are shown in Tables 1-3.

[0239] V/P ratio, that is, the ratio of the production amount of vanillin with respect to the production amount of protocatechualdehyde, observed for the FKFC14/pVK9::Ptuf*-Ge_ACAR-entD pVS7-vanK strain was about 1.4 times as high as that observed for the FKFC14/pVK9::Ptuf*-Nb_ACAR-entD pVS7-vanK strain (Table 1). Therefore, it was concluded that Ge_ACAR is useful for production of vanillin.

[0240] FKFC14/pVK9::Ptuf*-Nb_ACAR-entD pVS7-vanK strain produced protocatechualdehyde at a concentration of 9.6 g/L, while FKFC14/pVK9::Ptuf*-Nm_ACAR-entD pVS7-vanK strain did not produce protocatechualdehyde (Table 2). Therefore, it was concluded that Nm_ACAR is useful for production of vanillin.

[0241] V/P ratio, that is, the ratio of the production amount of vanillin with respect to the production amount of protocatechualdehyde, observed for the FKFC14/pVK9::Ptuf*-Cs2_ACAR-entD pVS7-vanK strain was about 1.6 times as high as that observed for the FKFC14/pVK9::Ptuf*-Nb_ACAR-entD pVS7-vanK strain (Table 3). Therefore, it was concluded that Cs2_ACAR is useful for production of vanillin.

[0242]

[Table 1]

Table 1: Vanillin and protocatechualdehyde production by *C. glutamicum* vanillin-producing strains (average $\pm$ S.E.)

Strain	Substrate : V. acid				
	At the start of culture		At the completion of culture		
	Glc concn. (g/L)	V. acid concn. (g/L)	Glc concn. (g/L)	V. acid concn. (g/L)	Vanillin concn. (g/L)
FKFC14/pVK9::Ptuf*-Nb_ACAR-entD pVS7-vanK	59.2 $\pm$ 0.0	29.7 $\pm$ 0.2	36.9 $\pm$ 0.7	1.5 $\pm$ 0.1	23.0 $\pm$ 0.1
FKFC14/pVK9::Ptuf*-Ge_ACAR-entD pVS7-vanK	59.9 $\pm$ 0.0	29.7 $\pm$ 0.0	26.3 $\pm$ 0.5	7.3 $\pm$ 0.0	18.5 $\pm$ 0.1

Strain	Substrate : P. acid					V/P ratio (g/g)
	At the start of culture		At the completion of culture			
	Glc concn. (g/L)	P. acid concn. (g/L)	Glc concn. (g/L)	P. acid concn. (g/L)	P. ald. concn. (g/L)	
FKFC14/pVK9::Ptuf*- Nb_ACAR-entD pVS7-vanK	62.1±0.7	33.4±0.2	26.1±0.2	15.9±0.2	9.6±0.1	2.4
FKFC14/pVK9::Ptuf*- Ge_ACAR-entD pVS7-vanK	62.5±0.2	31.2±0.8	19.1±0.2	22.6±0.0	5.5±0.1	3.4

Abbreviations:

Glc, glucose;

V. acid, vanillic acid;

P. acid, protocatechuic acid;

P. ald., protocatechualdehyde;

concn., concentration;

V/P ratio, the ratio of the production amount of vanillin with respect to the production amount of protocatechualdehyde.

[0243]

[Table 2]

Table 2: Vanillin and protocatechualdehyde production by *C. glutamicum* vanillin-producing strains (average $\pm$ S.E.)

Strain	Substrate : V. acid				
	At the start of culture		At the completion of culture		
	Glc concn. (g/L)	V. acid concn. (g/L)	Glc concn. (g/L)	V. acid concn. (g/L)	Vanillin concn. (g/L)
FKFC14/pVK9::Ptuf*-Nb_ACAR-entD pVS7-vanK	59.2 $\pm$ 0.0	29.7 $\pm$ 0.2	36.9 $\pm$ 0.7	1.5 $\pm$ 0.1	23.0 $\pm$ 0.1
FKFC14/pVK9::Ptuf*-Nm_ACAR-entD pVS7-vanK	60.6 $\pm$ 0.5	30.9 $\pm$ 0.7	20.3 $\pm$ 1	28.3 $\pm$ 0.4	3.7 $\pm$ 0.2

Strain	Substrate : P. acid					V/P ratio (g/g)
	At the start of culture		At the completion of culture			
	Glc concn. (g/L)	P. acid concn. (g/L)	Glc concn. (g/L)	P. acid concn. (g/L)	P. ald. concn. (g/L)	
FKFC14/pVK9::Ptuf*- Nb_ACAR-entD pVS7-vanK	62.1±0.7	33.4±0.2	26.1±0.2	15.9±0.2	9.6±0.1	2.4
FKFC14/pVK9::Ptuf*- Nm_ACAR-entD pVS7-vanK	62.5±0.7	31.0±0.4	24.7±0.2	24.5±0.1	N.D.	-

Abbreviations:

Glc, glucose;

V. acid, vanillic acid;

P. acid, protocatechuic acid;

P. ald., protocatechualdehyde;

concn., concentration;

N.D., Not Detected;

V/P ratio, the ratio of the production amount of vanillin with respect to the production amount of protocatechualdehyde.

[0244]

[Table 3]

Table 3: Vanillin and protocatechualdehyde production by *C. glutamicum* vanillin-producing strains (average $\pm$ S.E.)

Strain	Substrate : V. acid				
	At the start of culture		At the completion of culture		
	Glc concn. (g/L)	V. acid concn. (g/L)	Glc concn. (g/L)	V. acid concn. (g/L)	Vanillin concn. (g/L)
FKFC14/pVK9::Ptuf*-Nb_ACAR-entD pVS7-vanK	59.2 $\pm$ 0.0	29.7 $\pm$ 0.2	36.9 $\pm$ 0.7	1.5 $\pm$ 0.1	23.0 $\pm$ 0.1
FKFC14/pVK9::Ptuf*-Cs2_ACAR-entD pVS7-vanK	60.6 $\pm$ 0.0	29.9 $\pm$ 0.0	25.2 $\pm$ 0.5	14.4 $\pm$ 0.1	13.6 $\pm$ 0.3

Strain	Substrate : P. acid					V/P ratio (g/g)
	At the start of culture		At the completion of culture			
	Glc concn. (g/L)	P. acid concn. (g/L)	Glc concn. (g/L)	P. acid concn. (g/L)	P. ald. concn. (g/L)	
FKFC14/pVK9::Ptuf*- Nb_ACAR-entD pVS7-vanK	62.1±0.7	33.4±0.2	26.1±0.2	15.9±0.2	9.6±0.1	2.4
FKFC14/pVK9::Ptuf*- Cs2_ACAR-entD pVS7-vanK	62.8±0.2	30.3±0.3	18.7±0.7	23.3±0.1	3.7±0.0	3.7

Abbreviations:

Glc, glucose;

V. acid, vanillic acid;

P. acid, protocatechuic acid;

P. ald., protocatechualdehyde;

concn., concentration;

V/P ratio, the ratio of the production amount of vanillin with respect to the production amount of protocatechualdehyde.

[0245] <5> Comparison of vanillin production and isovanillin production by *C. glutamicum* vanillin-producing strains

A 20 μ L aliquot of each of the glycerol stocks of the constructed vanillin-producing strains was applied to the CM-Dex SGFC agar medium, and cultured at 31.5°C for 20 hr as preculture. The obtained cells were suspended in sterilized physiological saline. The optical density (OD) of the cell suspension was measured, and the cell suspension was diluted with physiological saline to obtain an OD at 600 nm of 83. A 1.5 mL aliquot of the diluted cell suspension was inoculated into 3.5 mL of a vanillin production medium (42.9 g/L of vanillic acid, 85.7 g/L of glucose, 10 g/L of

polypeptone, 10 g/L of yeast extract, 1 g/L of KH_2PO_4 , 0.4 g/L of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g/L of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g/L of $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, 3 g/L of urea, 1.2 g/L of soybean hydrolysate, 10 $\mu\text{g/L}$ of biotin, adjusted to pH 7.4 with KOH, and then mixed with 8.6 g/L of CaCO_3 (sterilized with hot air at 180°C for 3 hours)) containing 25 $\mu\text{g/mL}$ of kanamycin and 50 $\mu\text{g/mL}$ of spectinomycin, or a isovanillin production medium (42.9 g/L of isovanillic acid, 85.7 g/L of glucose, 10 g/L of polypeptone, 10 g/L of yeast extract, 1 g/L of KH_2PO_4 , 0.4 g/L of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g/L of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g/L of $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, 3 g/L of urea, 1.2 g/L of soybean hydrolysate, 10 $\mu\text{g/L}$ of biotin, adjusted to pH 7.4 with KOH, and then mixed with 8.6 g/L of CaCO_3 (sterilized with hot air at 180°C for 3 hours)) containing 25 $\mu\text{g/mL}$ of kanamycin and 50 $\mu\text{g/mL}$ of spectinomycin, contained in a test tube, and cultured at 30°C with shaking for 21 hr.

[0246] At the start and completion of the culture, the concentration of glucose in the medium was analyzed with Biotech Analyzer AS-310 (Sakura SI). The concentrations of vanillic acid, vanillin, isovanillic acid, and isovanillin in the medium were also analyzed by using Ultra Performance Liquid Chromatography NEXERA X2 System (SHIMADZU) with the following conditions.

[0247] Conditions of UPLC analysis

Column: KINETEX 2.6 μm XB-C18, 150 x 30 mm (Phenomenex)

Oven temperature: 40°C

Mobile phase (A): 0.1% Trifluoroacetic acid

Mobile phase (B): 0.1% Trifluoroacetic acid/80% acetonitrile

Gradient program (time, A (%), B (%)): (0, 90, 10) -> (3, 80, 20)

Flow rate: 1.5 ml/min

[0248] The results are shown in Tables 4-5.

[0249] Isovanillin production was not observed for the

FKFC14/pVK9::Ptuf*-Nm_ACAR-entD pVS7-vanK strain, while the

FKFC14/pVK9::Ptuf*-Nb_ACAR-entD pVS7-vanK strain produced isovanillin at a concentration of 9.3 g/L (Table 4). Therefore, it was concluded that Nm_ACAR is useful for production of vanillin.

[0250] V/iV ratio, that is, the ratio of the production amount of vanillin with respect to the production amount of isovanillin, observed for the

FKFC14/pVK9::Ptuf*-Cs2_ACAR-entD pVS7-vanK strain was approximately 1.2 times as high as that observed for the FKFC14/pVK9::Ptuf*-Nb_ACAR-entD

pVS7-vanK strain (Table 5). Therefore, it was concluded that Cs2_ACAR is useful for production of vanillin.

[0251]

[Table 4]

Table 4: Vanillin and isovanillin production by *C. glutamicum* vanillin-producing strains (average $\pm$ S.E.)

Strain	Substrate : V. acid				
	At the start of culture		At the completion of culture		
	Glc concn. (g/L)	V. acid concn. (g/L)	Glc concn. (g/L)	V. acid concn. (g/L)	Vanillin concn. (g/L)
FKFC14/pVK9::Ptuf*-Nb_ACAR-entD pVS7-vanK	59.9 $\pm$ 0.5	31.9 $\pm$ 1.4	40.8 $\pm$ 0.2	1.8 $\pm$ 0.3	26.5 $\pm$ 0.3
FKFC14/pVK9::Ptuf*-Nm_ACAR-entD pVS7-vanK	60.7 $\pm$ 0.2	32.8 $\pm$ 0.1	16.5 $\pm$ 0.5	29.8 $\pm$ 0.5	2.7 $\pm$ 0.2

Strain	Substrate : iV. acid					
	At the start of culture		At the completion of culture			
	Glc concn. (g/L)	iV. acid concn. (g/L)	Glc concn. (g/L)	iV. acid concn. (g/L)	Iso-vanillin concn. (g/L)	V/iV ratio (g/g)
FKFC14/pVK9::Ptuf*-Nb_ACAR-entD pVS7-vanK	60.9 $\pm$ 0.5	29.3 $\pm$ 0.0	43.2 $\pm$ 0.7	19.0 $\pm$ 0.6	9.3 $\pm$ 0.8	2.9
FKFC14/pVK9::Ptuf*-Nm_ACAR-entD pVS7-vanK	60.2 $\pm$ 0.0	30.4 $\pm$ 0.2	13.3 $\pm$ 0.0	29.5 $\pm$ 0.5	N.D.	-

Abbreviations:

Glc, glucose;

V. acid, vanillic acid;

iV. acid, isovanillic acid;

concn., concentration;

N.D., Not Detected;

V/iV ratio, the ratio of the production amount of vanillin with respect to the production amount of isovanillin.

[0252]

[Table 5]

Table 5: Vanillin and isovanillin production by *C. glutamicum* vanillin-producing strains (average $\pm$ S.E.)

Strain	Substrate : V. acid				
	At the start of culture		At the completion of culture		
	Glc concn. (g/L)	V. acid concn. (g/L)	Glc concn. (g/L)	V. acid concn. (g/L)	Vanillin concn. (g/L)
FKFC14/pVK9::Ptuf*-Nb_ACAR-entD pVS7-vanK	59.9 $\pm$ 0.5	31.9 $\pm$ 1.4	40.8 $\pm$ 0.2	1.8 $\pm$ 0.3	26.5 $\pm$ 0.3
FKFC14/pVK9::Ptuf*-Cs2_ACAR-entD pVS7-vanK	60.9 $\pm$ 0.0	34.1 $\pm$ 0.2	18.0 $\pm$ 1.2	17.8 $\pm$ 0.1	13.5 $\pm$ 0.6

Strain	Substrate : iV. acid					V/iV ratio (g/g)
	At the start of culture		At the completion of culture			
	Glc concn. (g/L)	iV. acid concn. (g/L)	Glc concn. (g/L)	iV. acid concn. (g/L)	Iso-vanillin concn. (g/L)	
FKFC14/pVK9::Ptuf*-Nb_ACAR-entD pVS7-vanK	60.9±0.5	29.3±0.0	43.2±0.7	19.0±0.6	9.3±0.8	2.9
FKFC14/pVK9::Ptuf*-Cs2_ACAR-entD pVS7-vanK	60.4±0.2	30.2±0.2	3.9±0.0	26.8±0.0	4.0±0.0	3.4

Abbreviations:

Glc, glucose;

V. acid, vanillic acid;

iV. acid, isovanillic acid;

concn., concentration;

N.D., Not Detected;

V/iV ratio, the ratio of the production amount of vanillin with respect to the production amount of isovanillin.

Industrial Applicability

[0253] According to the present invention, an ability of a microorganism for producing an objective substance, i.e. an aldehyde such as vanillin, can be improved, and the objective substance can be efficiently produced.

[0254] <Explanation of Sequence Listing>

SEQ ID NOS:

1: Nucleotide sequence of aroG gene of Escherichia coli MG1655

2: Amino acid sequence of AroG protein of Escherichia coli MG1655

3: Nucleotide sequence of aroB gene of Escherichia coli MG1655

- 4: Amino acid sequence of AroB protein of *Escherichia coli* MG1655
- 5: Nucleotide sequence of *aroD* gene of *Escherichia coli* MG1655
- 6: Amino acid sequence of AroD protein of *Escherichia coli* MG1655
- 7: Nucleotide sequence of *asbF* gene of *Bacillus thuringiensis* BMB171
- 8: Amino acid sequence of AsbF protein of *Bacillus thuringiensis* BMB171
- 9: Nucleotide sequence of *tyrR* gene of *Escherichia coli* MG1655
- 10: Amino acid sequence of TyrR protein of *Escherichia coli* MG1655
- 11-14: Nucleotide sequences of transcript variants 1 to 4 of OMT gene of *Homo sapiens*
- 15: Amino acid sequence of OMT isoform (MB-COMT) of *Homo sapiens*
- 16: Amino acid sequence of OMT isoform (S-COMT) of *Homo sapiens*
- 17: Nucleotide sequence of *CAR* gene of *Gordonia effusa*
- 18: Amino acid sequence of *CAR* protein of *Gordonia effusa*
- 19: Nucleotide sequence of DNA fragment containing *CAR* gene of *Nocardia brasiliensis* (codon-optimized) and *entD* gene of *Escherichia coli*
- 20: Nucleotide sequence of DNA fragment containing *CAR* gene of *Gordonia effusa* (codon-optimized) and *entD* gene of *Escherichia coli*
- 21: Nucleotide sequence of *entD* gene of *Escherichia coli* MG1655
- 22: Amino acid sequence of EntD protein of *Escherichia coli* MG1655
- 23: Nucleotide sequence of *PPT* gene of *Corynebacterium glutamicum* ATCC 13032
- 24: Amino acid sequence of PPT protein of *Corynebacterium glutamicum* ATCC 13032
- 25: Nucleotide sequence of *vanK* gene of *Corynebacterium glutamicum* 2256 (ATCC 13869)
- 26: Amino acid sequence of VanK protein of *Corynebacterium glutamicum* 2256 (ATCC 13869)
- 27: Nucleotide sequence of *pcaK* gene of *Corynebacterium glutamicum* 2256 (ATCC 13869)
- 28: Amino acid sequence of PcaK protein of *Corynebacterium glutamicum* 2256 (ATCC 13869)
- 29: Nucleotide sequence of *vanA* gene of *Corynebacterium glutamicum* 2256 (ATCC 13869)
- 30: Amino acid sequence of VanA protein of *Corynebacterium glutamicum* 2256 (ATCC 13869)
- 31: Nucleotide sequence of *vanB* gene of *Corynebacterium glutamicum* 2256 (ATCC 13869)
- 32: Amino acid sequence of VanB protein of *Corynebacterium glutamicum* 2256 (ATCC 13869)

- 33: Nucleotide sequence of *pcaG* gene of *Corynebacterium glutamicum* ATCC 13032
- 34: Amino acid sequence of PcaG protein of *Corynebacterium glutamicum* ATCC 13032
- 35: Nucleotide sequence of *pcaH* gene of *Corynebacterium glutamicum* ATCC 13032
- 36: Amino acid sequence of PcaH protein of *Corynebacterium glutamicum* ATCC 13032
- 37: Nucleotide sequence of *yqhD* gene of *Escherichia coli* MG1655
- 38: Amino acid sequence of YqhD protein of *Escherichia coli* MG1655
- 39: Nucleotide sequence of NCgl0324 gene of *Corynebacterium glutamicum* 2256 (ATCC 13869)
- 40: Amino acid sequence of NCgl0324 protein of *Corynebacterium glutamicum* 2256 (ATCC 13869)
- 41: Nucleotide sequence of NCgl0313 gene of *Corynebacterium glutamicum* 2256 (ATCC 13869)
- 42: Amino acid sequence of NCgl0313 protein of *Corynebacterium glutamicum* 2256 (ATCC 13869)
- 43: Nucleotide sequence of NCgl2709 gene of *Corynebacterium glutamicum* 2256 (ATCC 13869)
- 44: Amino acid sequence of NCgl2709 protein of *Corynebacterium glutamicum* 2256 (ATCC 13869)
- 45: Nucleotide sequence of NCgl0219 gene of *Corynebacterium glutamicum* ATCC 13032
- 46: Amino acid sequence of NCgl0219 protein of *Corynebacterium glutamicum* ATCC 13032
- 47: Nucleotide sequence of NCgl2382 gene of *Corynebacterium glutamicum* ATCC 13032
- 48: Amino acid sequence of NCgl2382 protein of *Corynebacterium glutamicum* ATCC 13032
- 49: Nucleotide sequence of *aroE* gene of *Escherichia coli* MG1655
- 50: Amino acid sequence of AroE protein of *Escherichia coli* MG1655
- 51-76: Primers
- 77: Nucleotide sequence of CAR gene of *Gordonia effusa* codon-optimized for codon usage of *E. coli*
- 78: Nucleotide sequence of CAR gene of *Novosphingobium malaysiense*
- 79: Amino acid sequence of CAR protein of *Novosphingobium malaysiense*
- 80: Nucleotide sequence of DNA fragment containing CAR gene of *Novosphingobium malaysiense* (codon-optimized) and *entD* gene of *Escherichia coli*
- 81: Nucleotide sequence of CAR gene of *Novosphingobium malaysiense* codon-

optimized for codon usage of *E. coli*

82: Nucleotide sequence of CAR gene (cDNA) of *Coccomyxa subellipsoidea* C-169

83: Amino acid sequence of CAR protein of *Coccomyxa subellipsoidea* C-169

84: Nucleotide sequence of DNA fragment containing CAR gene of *Coccomyxa subellipsoidea* (codon-optimized) and *entD* gene of *Escherichia coli*

85: Nucleotide sequence of CAR gene of *Coccomyxa subellipsoidea* codon-optimized for codon usage of *E. coli*

Claims

- [Claim 1] A method for producing an objective substance, the method comprising:
producing the objective substance by using a microorganism having an ability to produce the objective substance,
wherein the objective substance is an aldehyde,
wherein the microorganism has been modified to have an aldehyde oxidoreductase gene, and
wherein the aldehyde oxidoreductase gene encodes a protein selected from the group consisting of:
(a) a protein comprising the amino acid sequence of SEQ ID NO: 18, 79, or 83;
(b) a protein comprising the amino acid sequence of SEQ ID NO: 18, 79, or 83 but which includes substitution, deletion, insertion, and/or addition of 1 to 10 amino acid residues, and wherein said protein has aldehyde oxidoreductase activity;
(c) a protein comprising an amino acid sequence having an identity of 90% or higher to the amino acid sequence of SEQ ID NO: 18, 79, or 83, and wherein said protein has aldehyde oxidoreductase activity.
- [Claim 2] The method according to claim 1, wherein said producing comprises: cultivating the microorganism in a culture medium containing a carbon source to produce and accumulate the objective substance in the culture medium.
- [Claim 3] The method according to claim 1, wherein said producing comprises: converting a precursor of the objective substance into the objective substance by using the microorganism.
- [Claim 4] The method according to claim 3, wherein said converting comprises: cultivating the microorganism in a culture medium containing the precursor to produce and accumulate the objective substance in the culture medium.
- [Claim 5] The method according to claim 3, wherein said converting comprises: allowing cells of the microorganism to act on the precursor in a reaction mixture to produce and accumulate the objective substance in the reaction mixture.
- [Claim 6] The method according to claim 5, wherein the cells are selected from the group consisting of:
- cells present in a culture broth of the microorganism,

- cells collected from the culture broth,
- cells present in a processed product of the culture broth,
- cells present in a processed product of the collected cells, and
- a combination of these.

- [Claim 7] The method according to any one of claims 3 to 6, wherein the precursor is a substance selected from the group consisting of proto-catechuic acid, vanillic acid, benzoic acid, L-phenylalanine, cinnamic acid, and combinations thereof.
- [Claim 8] The method according to any one of claims 1 to 7, the method further comprising collecting the objective substance from the culture medium or reaction mixture.
- [Claim 9] The method according to any one of claims 1 to 8, wherein the microorganism is a bacterium belonging to the family Enterobacteriaceae, a coryneform bacterium, or yeast.
- [Claim 10] The method according to claim 9, wherein the microorganism is a bacterium belonging to the genus *Corynebacterium*.
- [Claim 11] The method according to claim 10, wherein the microorganism is *Corynebacterium glutamicum*.
- [Claim 12] The method according to claim 9, wherein the microorganism is a bacterium belonging to the genus *Escherichia*.
- [Claim 13] The method according to claim 12, wherein the microorganism is *Escherichia coli*.
- [Claim 14] The method according to any one of claims 1 to 13, wherein the objective substance is an aromatic aldehyde.
- [Claim 15] The method according to any one of claims 1 to 14, wherein the objective substance is an aromatic aldehyde selected from the group consisting of vanillin, benzaldehyde, cinnamaldehyde, and combinations thereof.
- [Claim 16] The method according to any one of claims 1 to 15, wherein the microorganism has been further modified so that the activity of an enzyme that is involved in the biosynthesis of the objective substance is increased as compared with a non-modified strain.
- [Claim 17] The method according to claim 16, wherein the enzyme that is involved in the biosynthesis of the objective substance is able to catalyze the conversion from a precursor of the objective substance into the objective substance.
- [Claim 18] The method according to claim 16 or 17, wherein the enzyme that is involved in the biosynthesis of the objective substance is selected from

the group consisting of 3-deoxy-D-arabino-heptulosonic acid 7-phosphate synthase, 3-dehydroquinate synthase, 3-dehydroquinate dehydratase, 3-dehydroshikimate dehydratase, O-methyltransferase, phenylalanine ammonia lyase, and combinations thereof.

[Claim 19] The method according to any one of claims 1 to 18, wherein the microorganism has been further modified so that the activity of phosphopantetheinyl transferase is increased as compared with a non-modified strain.

[Claim 20] The method according to any one of claims 1 to 19, wherein the microorganism has been further modified so that the activity of an uptake system of a substance other than the objective substance is increased as compared with a non-modified strain.

[Claim 21] The method according to claim 20, wherein the uptake system is selected from the group consisting of a vanillic acid uptake system, a protocatechuic acid uptake system, and combinations thereof.

[Claim 22] The method according to any one of claims 1 to 21, wherein the microorganism has been further modified so that the activity of an enzyme that is involved in the production of a byproduct during the production of the objective substance is reduced as compared with a non-modified strain.

[Claim 23] The method according to claim 22, wherein the enzyme that is involved in the production of a byproduct during the production of the objective substance is selected from the group consisting of vanillate demethylase, protocatechuate 3,4-dioxygenase, alcohol dehydrogenase, shikimate dehydrogenase, and combinations thereof.

INTERNATIONAL SEARCH REPORT

International application No
PCT/JP2017/038875

A. CLASSIFICATION OF SUBJECT MATTER INV. C12N9/04 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) C12N		
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, WPI Data, BIOSIS, Sequence Search, EMBASE, CHEM ABS Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
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X	----- WO 2015/009558 A1 (INT FLAVORS & FRAGRANCES INC [US]) 22 January 2015 (2015-01-22) the whole document	1-23
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☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search	Date of mailing of the international search report	
11 January 2018	30/01/2018	
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Seranski, Peter	

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International application No

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