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Eljárás egy céltermék fermentációs előállítására

Az európai szabadalom ellen, megadásának az Európai Szabadalmi Közlönyben való meghirdetésétől számított kilenc hónapon belül, felszólalást lehet benyújtani az Európai Szabadalmi Hivatalnál. (Európai Szabadalmi Egyezmény 99. cikk(1))

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(54) **A method for producing a target substance by fermentation**

Verfahren zur Produktion eines Zielstoffes mittels Fermentation

Procédé de production d'une substance cible par fermentation

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- **ANDREAS PIKIS ET AL: "Genetic requirements for growth of Escherichia coli K12 on methyl-alpha-D-glucopyranoside and the five alpha-D-glucosyl-D-fructose isomers of sucrose", JOURNAL OF BIOLOGICAL CHEMISTRY, THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, INC, US, vol. 281, 30 June 2006 (2006-06-30), pages 17900-17908, XP008138730, ISSN: 0021-9258, DOI: 10.1074/JBC.M601183200**
- **JOHN THOMPSON ET AL: "Metabolism of sucrose and its five linkage-isomeric alpha-D-glucosyl- D-fructoses by Klebsiella pneumoniae. Participation and properties of sucrose-6- phosphate hydrolase and phospho-alpha- glucosidase", JOURNAL OF BIOLOGICAL CHEMISTRY, THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, INC, US, vol. 276, 25 July 2001 (2001-07-25), pages 37415-37425, XP008138738, ISSN: 0021-9258, DOI: 10.1074/JBC.M106504200**

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Description

Technical Field

[0001] The present invention relates to a method for producing a target substance by using a microorganism, more precisely, means for improving productivity of a substance that is a final target product in a method for producing a target substance, which is L-amino acids or nucleic acids, using a microorganism.

Background Art

[0002] As typical methods for producing substances using microorganisms, there are known methods for producing L-amino acids by fermentation. L-amino acids are used not only as seasonings and foodstuffs but also as components of various nutritional mixtures for medical purposes. Furthermore, they are used as additives for animal feed, reagents in drug manufacturing industry and chemical industry and growth factors for production of L-amino acids such as L-lysine and L-homoserine by using a microorganism. As microorganisms that can produce L-amino acids by fermentation, there are known coryneform bacteria, *Escherichia* bacteria, *Bacillus* bacteria, *Serratia* bacteria and so forth.

[0003] As for such production of target substances by fermentation as described above, it can be said that most of materials used as raw materials are those containing saccharides such as blackstrap molasses. Also in amino acid fermentation or nucleic acid fermentation, culture is performed by using a saccharide as a raw material. Although sugarcane and so forth abundantly contain starch, it is rare to use it as it is as a raw material, but used in most cases as a decomposition product in which starch is decomposed into, for example, monosaccharides or disaccharides. As for the decomposition method, a solution of a saccharifying enzyme such as amylase is generally used, and thereby starch that is polysaccharide is decomposed into relatively low molecular saccharides such as glucose, maltose and isomaltose.

[0004] In fermentative production using a starch hydrolysis solution, if fermentation is terminated when only glucose that is the main component of the solution is consumed, oligosaccharides such as maltose and isomaltose contained as impurities are not assimilated but remain vainly. For these oligosaccharides, especially isomaltose, *Escherichia* bacteria do not have ability to assimilate them, and they cause the Maillard reaction with the fermentation products (especially L-amino acids), which poses a problem in the step of purifying the fermentation product.

[0005] Moreover, oligosaccharides that suffer from suppression of consumption by glucose such as maltose must be consumed after consumption of glucose, thus the culture time must be extended for that, and heating expense is thrown away in vain.

[0006] As for utilization of maltose in a starch decomposition product, there has been disclosed a technique of using a microorganism in which an interaction between the IIA^{Glc} protein of the glucose PTS and the protein MalK involved in the non-PTS uptake of maltose is attenuated or eliminated, and which can take up glucose and maltose (U.S. Patent No. 7,097,999, European Patent Laid-open No. 1254957). It is known that in *E. coli* and *Salmonella typhimurium*, the suppression by glucose is generally observed. That is, when glucose and other carbon sources such as maltose are assimilated in them, glucose is assimilated first, and then the other carbon sources are assimilated. However, the aforementioned technique enables simultaneous assimilation of glucose and maltose.

[0007] Furthermore, it is possible to express an α -amylase in a cell surface layer to decompose starch into glucose and maltose, which is an α -1,4-glucan, and make bacteria take them up to enhance amino acid-producing ability (Tateno, T., et al., Appl. Microbiol. Biotech., 74, 1213-1220 (2007)).

[0008] As for maltose, it is known that maltose is taken up into cells by the GlvC protein (PTS maltose enzyme IICB) encoded by the *glvC* gene of *Bacillus subtilis* (*B. subtilis*), and the taken-up maltose (phosphorylated maltose) is hydrolyzed by GlvA (6-phospho- α -glucosidase) encoded by the *glvA* gene (Schonert S. et al., J. Bacteriol., 2006, Jun.;188 (11):3911-22). However, relationship between the *glvAC* gene and isomaltose assimilation has not been reported to date.

[0009] As for isomaltose, an α -glucosidase which decomposes maltose, sucrose and isomaltose as substrates into monosaccharides is present in *B. subtilis*, and it is encoded by the *malL* gene (Schonert, S., et al., J. Bacteriol., 180, 2574-2578 (1998)).

[0010] Existence of such an enzyme as described above has not been known for *Enterobacteriaceae* bacteria including *E. coli* or coryneform bacteria, thus it is considered that they cannot assimilate isomaltose, and whether they can take isomaltose up into cells is unknown. Furthermore, any microorganism that extracellularly secretes an enzyme that decomposes isomaltose is not known, either.

Disclosure of the Invention

Object to be Achieved by the Invention

[0011] An object of the present invention is to provide a method for producing a target substance from isomaltose as

a raw material by fermentation utilizing a microorganism, a method for producing a target substance by fermentation utilizing a microorganism, which uses a medium containing isomaltose, or isomaltose and maltose as impurities, and microorganisms used for them.

5 Means for Achieving the Object

[0012] The inventors of the present invention conducted various researches in order to achieve the aforementioned object, and as a result, found that a microorganism to which the isomaltase activity is imparted, or which is modified to increase the isomaltase activity could efficiently assimilate isomaltose. They further found that if the *glvA* and *glvC* genes of *Bacillus subtilis* are introduced into a microorganism to increase the isomaltase and maltase activities, isomaltose and maltose as impurities in a medium can be reduced, and accomplished the present invention.

[0013] The present invention thus provides the followings.

(1) A method for producing an L-amino acid or a nucleic acid according to claim 1.

(2) The method as described above, wherein the microorganism has been further imparted with activity to take up isomaltose into cells or modified to increase the activity.

(3) The method as described above, wherein the microorganism has been further imparted with maltase activity or modified to increase maltase activity.

(4) The method as described above, wherein the microorganism is further imparted with activity to take up maltose into cells or modified to increase the activity.

(5) The method as described above, wherein the medium contains isomaltose or isomaltose and maltose.

(6) The method as described above, wherein the microorganism has been introduced with a gene coding for an isomaltase of a *Bacillus* bacterium.

(9) The method as described above, wherein the isomaltase is a protein shown in the following (C) or (D):

(C) a protein consisting of the amino acid sequence shown in SEQ ID NO: 40,

(D) a protein consisting of the amino acid sequence shown in SEQ ID NO: 40 including substitutions, deletions, insertions or additions of one or several amino acid residues, and having the isomaltase activity.

(10) The method as described above, wherein the isomaltase is encoded by a DNA shown in the following (c) or (d):

(c) a DNA having the nucleotide sequence shown in SEQ ID NO: 39,

(d) a DNA which is able to hybridize with a sequence complementary to the nucleotide sequence of SEQ ID NO: 39 or a probe which can be prepared from the nucleotide sequence under stringent conditions, and codes for a protein having the isomaltase activity.

(11) The method as described above, wherein the microorganism has been introduced with a gene coding for a PTS maltose enzyme IICB of a *Bacillus* bacterium.

(12) The method as described above, wherein the PTS maltose enzyme IICB is a protein shown in the following (E) or (F):

(E) a protein consisting of the amino acid sequence shown in SEQ ID NO: 42,

(F) a protein consisting of the amino acid sequence shown in SEQ ID NO: 42 including substitutions, deletions, insertions or additions of one or several amino acid residues, and having the activity to take up isomaltose into cells.

(13) The method as described above, wherein the PTS maltose enzyme IICB is encoded by a DNA shown in the following (e) or (f) :

(e) a DNA having the nucleotide sequence shown in SEQ ID NO: 41,

(f) a DNA which is able to hybridize with a sequence complementary to the nucleotide sequence of SEQ ID NO: 41 or a probe which can be prepared from the nucleotide sequence under stringent conditions, and codes for a protein having the activity to take up isomaltose into cells.

(14) The method as described above, wherein the microorganism is an *Enterobacteriaceae* bacterium.

(15) The method as described above, wherein the microorganism is an *Escherichia* bacterium.

(16) The method as described above, wherein the *Escherichia* bacterium is *Escherichia coli*.

(17) The method as described above, wherein the amino acid is selected from L-lysine, L-threonine, and L-pheny-

alanine.

Brief Description of the Drawings

[0014]

Fig. 1 shows growth curves of the pTWV229+pMW219- Δ Plac/MG1655 strain (•) and the glvA+glvC/MG1655 strain (A) in the M9-iMal(0.2%) medium.

Fig. 2 shows growth curves of the pTWV229+pMW219- Δ Plac/MG1655 strain (•) and the glvA+glvC/MG1655 strain (▲) in the M9-Glc(0.02%)-iMal(0.18%).

Best Mode for Carrying out the Invention

[0015] Hereafter, the present invention will be explained in detail.

[0016] The target substance produced by a microorganism in the present invention means an L-amino acid or a nucleic acid. In the present invention, the "L-amino acid" is not particularly limited, so long as it is an L-amino acid that can be accumulated in a medium in fermentation using a microorganism. Although type of the L-amino acid is not particularly limited, examples include basic amino acids such as L-lysine, L-ornithine, L-arginine, L-histidine and L-citrulline, aliphatic amino acids such as L-isoleucine, L-alanine, L-valine, L-leucine and glycine, amino acids which are hydroxy-monoamino carboxylic acids such as L-threonine and L-serine, cyclic amino acids such as L-proline, aromatic amino acids such as L-phenylalanine, L-tyrosine and L-tryptophan, sulfur-containing amino acids such as L-cysteine, L-cystine and L-methionine, acidic amino acids and acid amides thereof such as L-glutamic acid, L-aspartic acid, L-glutamine and L-asparagine.

[0017] The microorganism used in the present invention may have an ability to produce two or more kinds of amino acids.

[0018] The L-amino acid to be collected may be a free L-amino acid, or may be a salt such as sulfate, hydrochloride, carbonate, ammonium salt, sodium salt, and potassium salt.

[0019] In the present invention, the "nucleic acid" is not particularly limited, so long as it is a nucleic acid that can be accumulated in a medium in fermentation using a microorganism. Examples of the nucleic acid include purine nucleosides, purine nucleotides, and so forth. The purine nucleosides include inosine, xanthosine, guanosine, adenosine, and so forth, and the purine nucleotides include 5'-phosphate esters of the purine nucleosides, for example, inosinic acid, (inosine-5'-phosphate, henceforth also referred to as "IMP"), xanthylic acid (xanthosine-5'-phosphate, henceforth also referred to as "XMP"), guanylic acid (guanosine-5'-monophosphate, henceforth also referred to as "GMP"), adenylic acid (adenosine-5'-monophosphate, henceforth also referred to as "AMP"), and so forth.

[0020] The microorganism used in the present invention may have an ability to produce two or more kinds of nucleic acids.

[0021] The nucleic acid referred to in the present invention include a free nucleic acid, and a nucleic acid salt such as sodium salt and potassium salt.

[0022] The microorganism used in the present invention is a microorganism which has been imparted with isomaltase activity or modified to increase the isomaltase activity.

[0023] In the present invention, the "isomaltase activity" means an activity to decompose phosphorylated isomaltose, produced when isomaltose is taken up into cells, into glucose and glucose phosphate (EC 3.2.1.122). Furthermore, unless specially mentioned, the "isomaltase" means an enzyme having the isomaltase activity. For example, even an enzyme having an activity to decompose maltose and generally called maltase may be the isomaltase in the present invention, if the enzyme has an activity to decompose isomaltose. Therefore, the isomaltase referred to in the present invention may be called maltose-inducible α -glucosidase or oligo-1,6-glucosidase.

[0024] In the present invention, the "maltase activity" means an activity to decompose maltose into two glucose molecules (EC 3.2.1.20) or an activity to decompose phosphorylated maltose into glucose and glucose phosphate (EC 3.2.1.122).

[0025] The isomaltase activity can be measured by the method described by Thompson J. (1: J. Biol. Chem., 1998 Oct., 16;273 (42):27347-56.) using isomaltose as the substrate instead of maltose. The maltase activity can be measured by the method described by Thompson J. mentioned above.

[0026] In the present invention, in addition to the impartation/enhancement of the isomaltase activity and/or the maltase activity, activity to take up isomaltose and/or maltose may be imparted or enhanced. The "activity to take up isomaltose" means an activity to take up isomaltose in the exterior of cells into the interior of the cells, and isomaltose is taken up into a cell by an isomaltose transporter or an isomaltose-specific permease. The transporter includes those of the phosphotransferase system (PTS), ABC transporters, and those of the major facilitator superfamily (MFS), and in the present invention, it is preferable to enhance the phosphotransferase system. The activity to take up isomaltose can be confirmed by the methods described in J. Bacteriol., 1991 Apr.; 173(7):2180-2186 and J. Biol. Chem., 2006 Jun., 30;281

(26):17900-17908.

[0027] Furthermore, in the present invention, the activity to take up isomaltose may also act as an activity to take up maltose in addition to isomaltose.

[0028] The microorganism of the present invention is an Enterobacteriaceae bacterium or a coryneform bacterium. Examples of the microorganism usable in the present invention include, specifically, *Enterobacteriaceae* bacteria belonging to the genus *Escherichia*, *Enterobacter*, *Klebsiella*, *Pantoea*, *Serratia*, *Erwinia*, *Salmonella*, *Morganella*, or the like and coryneform bacteria. It is preferably a microorganism for which gene substitution is possible.

[0029] Examples of the *Escherichia* bacteria include *Escherichia coli* and so forth. When *Escherichia coli* is bred by using genetic engineering techniques, the *E. coli* K12 strain and derivatives thereof, the *Escherichia coli* 1655 strain (ATCC 47076) and the *Escherichia coli* W3110 strain (ATCC 27325), can be used. To obtain the K-12 strain of *Escherichia coli* and the derivative strains, they can be provided from, for example, the American Type Culture Collection (ATCC, Address: P.O. Box 1549, Manassas, VA 20108, United States of America).

[0030] The microorganism usable in the present invention may be a microorganism not inherently having an ability to assimilate isomaltose, or may be a microorganism having an ability to assimilate isomaltose. The "ability to assimilate isomaltose" means an ability of a microorganism to metabolize isomaltose as a carbon source, when the microorganism is cultured in a medium containing isomaltose.

[0031] Examples of microorganisms not inherently having the ability to assimilate isomaltose include *Enterobacteriaceae* bacteria, coryneform bacteria, and so forth. Furthermore, examples of microorganisms having the ability to assimilate isomaltose include microorganisms having activity to take up isomaltose into cell and intracellular isomaltase activity. Examples of such microorganisms include *Bacillus* bacteria, *Streptococcus* bacteria, *Saccharomyces* yeasts, and so forth.

[0032] The microorganism of the present invention can be obtained by imparting the isomaltase activity to a microorganism having an ability to produce a target substance as a parent strain, or modifying such a parent strain to increase the isomaltase activity of the parent strain. The microorganism of the present invention can also be obtained by imparting an ability to produce a target substance to a microorganism which has the isomaltase activity or has been modified to increase the isomaltase activity as a parent strain, or enhancing the ability to produce a target substance of such a parent strain.

[0033] As the *Escherichia* bacteria, those described in the work of Neidhardt et al. (Neidhardt, F.C. et al., *Escherichia coli* and *Salmonella Typhimurium*, American Society for Microbiology, Washington D.C., 1208, table 1), such as *Escherichia coli*, can be utilized. Examples of wild-type strains of *Escherichia coli* include, for example, the K12 strain and derivatives thereof, *Escherichia coli* MG1655 strain (ATCC No. 47076), W3110 strain (ATCC No. 27325), and so forth. They are available from the American Type Culture Collection (ATCC, Address: P.O. Box 1549, Manassas, VA 20108, United States of America).

[0034] Furthermore, examples of the *Enterobacter* bacteria include *Enterobacter agglomerans*, *Enterobacter aerogenes* and so forth, and examples of the *Pantoea* bacteria include *Pantoea ananatis*. Some species of *Enterobacter agglomerans* have been recently re-classified into *Pantoea agglomerans*, *Pantoea ananatis*, *Pantoea stewartii* or the like, based on the nucleotide sequence analysis of 16S rRNA, etc.. Both of the *Enterobacter* bacteria and *Pantoea* bacteria may be used so long as the chosen bacterium is classified into the family *Enterobacteriaceae*. When a *Pantoea ananatis* strain is bred by a genetic engineering technique, *Pantoea ananatis* AJ13355 strain (FERM BP-6614), AJ13356 strain (FERM BP-6615), AJ13601 strain (FERM BP-7207) and derivatives thereof can be used. These strains were identified as *Enterobacter agglomerans* when they were isolated, and deposited as *Enterobacter agglomerans*. However, they were recently re-classified as *Pantoea ananatis* on the basis of nucleotide sequencing of 16S rRNA and so forth as described above.

[0035] The coryneform bacteria are a group of microorganisms defined in Bergey's Manual of Determinative Bacteriology, 8th Ed., p.599 (1974), and microorganisms classified into such aerobic, Gram-positive and nonacid-fast bacilli which are unable to sporulate can be used. The coryneform bacteria include bacteria which have previously been classified into the genus *Brevibacterium* but are presently united into the genus *Corynebacterium* (Int. J. Syst. Bacteriol. 41:255-260 (1991)), and bacteria belonging to the genus *Brevibacterium* or *Microbacterium*, which are closely related to the genus *Corynebacterium*.

[0036] Specific examples of such coryneform bacteria include the following:

Corynebacterium acetoacidophilum
Corynebacterium acetoglutamicum
Corynebacterium alkanolyticum
Corynebacterium callunae
Corynebacterium glutamicum
Corynebacterium lilium
Corynebacterium melassecola

Corynebacterium thermoaminogenes (*Corynebacterium efficiens*)
Corynebacterium herculis
Brevibacterium divaricatum
Brevibacterium flavum
5 *Brevibacterium immariophilum*
Brevibacterium lactofermentum
Brevibacterium roseum
Brevibacterium saccharolyticum
Brevibacterium thiogenitalis
10 *Corynebacterium ammoniagenes*
Brevibacterium album
Brevibacterium cerinum
Microbacterium ammoniaphilum

15 **[0037]** Specific examples of these bacteria include the following strains:

Corynebacterium acetoacidophilum ATCC 13870
Corynebacterium acetoglutamicum ATCC 15806
Corynebacterium alkanolyticum ATCC 21511
20 *Corynebacterium callunae* ATCC 15991
Corynebacterium glutamicum ATCC 13020, ATCC 13032, ATCC 13060
Corynebacterium lilium ATCC 15990
Corynebacterium melassecola ATCC 17965
Corynebacterium efficiens AJ12340 (FERM BP-1539)
25 *Corynebacterium herculis* ATCC 13868
Brevibacterium divaricatum ATCC 14020
Brevibacterium flavum ATCC 13826, ATCC 14067, AJ12418 (FERM BP-2205)
Brevibacterium immariophilum ATCC 14068
Brevibacterium lactofermentum ATCC 13869
30 (*Corynebacterium glutamicum* TCC 13869)
Brevibacterium roseum ATCC 13825
Brevibacterium saccharolyticum ATCC 14066
Brevibacterium thiogenitalis ATCC 19240
Brevibacterium ammoniagenes ATCC 6871, ATCC 6872
35 *Brevibacterium album* ATCC 15111
Brevibacterium cerinum ATCC 15112
Microbacterium ammoniaphilum ATCC 15354

40 **[0038]** These strains are available from, for example, the American Type Culture Collection (ATCC) (Address: P.O. Box 1549, Manassas, VA 2010812301 Parklawn Drive, Rockville, Maryland 20852, United States of America). That is, registration numbers are given to each of the strains, and the strains can be ordered by using these registration numbers. The registration numbers of the strains are listed in the catalogue of the American Type Culture Collection (refer to <http://www.atcc.org/>). The AJ12340 strain was deposited on October 27, 1987 in National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Ministry of Economy, Trade and Industry (currently
45 independent administrative agency, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depository, Tsukuba Central 6, 1-1, Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, 305-5466, Japan), with an accession number of FERM BP-1539 under the provisions of Budapest Treaty. The AJ12418 strain was deposited on January 5, 1989 in National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Ministry of Economy, Trade and Industry with an accession number of FERM BP-2205 under the provisions of
50 the Budapest Treaty.

[0039] When *Bacillus* bacteria are used, examples thereof include *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus pumilus*, and so forth.

[0040] Examples of *Bacillus subtilis* include *Bacillus subtilis* 168 Marburg strain (ATCC 6051), *Bacillus subtilis* PY79 strain (Plasmid, 1984, 12, 1-9), and so forth. Examples of *Bacillus amyloliquefaciens* include *Bacillus amyloliquefaciens* T strain (ATCC 23842), *Bacillus amyloliquefaciens* N strain (ATCC 23845), and so forth. Examples of *Bacillus pumilus*
55 include *Bacillus pumilus* Gottheil No. 3218 (ATCC 21005) (U.S. Patent No. 3,616,206), and so forth.

[0041] Hereinafter, methods for imparting an L-amino acid- or nucleic acid-producing ability to such parent strains as mentioned above are described.

[0042] To impart the ability to produce an L-amino acid or a nucleic acid, methods conventionally employed in the breeding of coryneform bacteria or bacteria of the genus *Escherichia* (see "Amino Acid Fermentation", Gakkai Shuppan Center (Ltd.), 1st Edition, published May 30, 1986, pp. 77-100) can be used. Such methods include methods of acquiring an auxotrophic mutant, an analogue-resistant strain, or a metabolic regulation mutant, or constructing a recombinant strain so that it overexpresses an L-amino acid or nucleic acid biosynthesis enzyme. Here, in the breeding of an L-amino acid-producing bacteria, one or more of the above described properties such as auxotrophy, analogue-resistance, or metabolic regulation mutation may be imparted. Expression of one or more L-amino acid biosynthesis enzymes may be enhanced. Furthermore, the methods of imparting properties such as an auxotrophic mutation, analogue resistance, or metabolic regulation mutation may be combined with the methods of enhancing the biosynthesis enzymes.

[0043] An auxotrophic mutant strain, L-amino acid or nucleic acid analogue-resistant strain, or metabolic regulation mutant strain with an ability to produce an L-amino acid or nucleic acid can be obtained by subjecting a parent strain or wild-type strain to a conventional mutagenesis, such as exposure to X-rays or UV irradiation, or treatment with a mutagen such as N-methyl-N'-nitro-N-nitrosoguanidine, etc., and then selecting those which exhibit an autotrophy, analogue resistance, or metabolic regulation mutation, and which also have the ability to produce an L-amino acid from the obtained mutant strains.

[0044] An auxotrophic mutant strain, L-amino acid analogue-resistant strain, or metabolic regulation mutant strain with an ability to produce an L-amino acid can be obtained by subjecting a parent strain or wild-type strain to a conventional mutagenesis, such as exposure to X-rays or UV irradiation, or treatment with a mutagen such as N-methyl-N'-nitro-N-nitrosoguanidine or ethyl methanesulfonate (EMS), etc., and then selecting those which exhibit an autotrophy, analogue resistance, or metabolic regulation mutation and which also have the ability to produce an L-amino acid from the obtained mutant strains.

[0045] Methods for imparting amino acid-producing ability and amino acid-producing bacteria will be specifically exemplified below.

L-Lysine-producing bacteria

[0046] L-Lysine-producing bacteria and methods for constructing them are exemplified below.

[0047] Examples of strains having L-lysine-producing ability include, for example, L-lysine analogue-resistant strains and metabolic regulation mutant strains. Examples of L-lysine analogue include, but are not limited to, oxalysine, lysine hydroxamate, S-(2-aminoethyl)-L-cysteine (also abbreviated as "AEC" hereinafter), γ -methyllysine, α -chlorocaprolactam and so forth. Mutant strains having resistance to these lysine analogues can be obtained by subjecting a bacterium belonging to the family *Enterobacteriaceae* or a coryneform bacterium to a conventional artificial mutagenesis treatment. Specific examples of L-lysine-producing bacteria include *Escherichia coli* AJ11442 (FERM BP-1543, NRRL B-12185, see Japanese Patent Laid-open No. 56-18596 and U.S. Patent No. 4,346,170), *Escherichia coli* VL611 strain (Japanese Patent Laid-open No. 2000-189180), and so forth. As an L-lysine-producing *Escherichia coli*, the WC196 strain may also be used (see International Publication WO96/17930).

[0048] Furthermore, an L-lysine-producing bacterium can also be constructed by increasing activity of an L-lysine biosynthesis system enzyme. Increase of activity of such an enzyme can be attained by increasing copy number of the gene coding for the enzyme in cells, or by modifying an expression control sequence thereof. Increase of copy number of a gene coding for an enzyme of L-lysine biosynthesis system in cells and modification of an expression control sequence can be attained in the same manner as that for the isomaltase gene described below.

[0049] Examples of genes coding for L-lysine biosynthetic enzymes include genes coding for enzymes of the diaminopimelate pathway such as dihydrodipicolinate synthase gene (*dapA*), aspartokinase gene (*lysC*), dihydrodipicolinate reductase gene (*dapB*), diaminopimelate decarboxylase gene (*lysA*), diaminopimelate dehydrogenase gene (*ddh*) (WO96/40934 for all the foregoing genes), phosphoenolpyruvate carboxylase gene (*ppc*) (Japanese Patent Laid-open No. 60-87788), aspartate aminotransferase gene (*aspC*) (Japanese Patent Publication (Kokoku) No. 6-102028), diaminopimelate epimerase gene (*dapF*) (Japanese Patent Laid-open No. 2003-135066), and aspartate semialdehyde dehydrogenase gene (*asd*) (WO00/61723), and genes coding for enzymes of the aminoadipic acid pathway such as homoaconitate hydratase gene (Japanese Patent Laid-open No. 2000-157276). In addition, the parent strain may show an increased level of expression of the gene involved in energy efficiency (*cyo*) (EP 1170376 A), the gene coding for nicotinamide nucleotide transhydrogenase (*pntAB*) (U.S. Patent No. 5,830,716), the *ybjE* gene coding for a protein having L-lysine excretion activity (WO2005/073390), the gene coding for glutamate dehydrogenase (*gdhA*) (Gene 23:199-209 (1983)), or an arbitrary combination of these. Abbreviations for the genes are shown in the parentheses.

[0050] It is known that the wild-type dihydrodipicolinate synthase derived from *Escherichia coli* suffers from feedback inhibition by L-lysine, and it is known that the wild-type aspartokinase derived from *Escherichia coli* suffers from suppression and feedback inhibition by L-lysine. Therefore, when the *dapA* and *lysC* genes are used, these genes are preferably genes coding for mutant enzymes desensitized to the feedback inhibition by L-lysine.

[0051] Examples of DNA encoding a mutant dihydrodipicolinate synthetase desensitized to feedback inhibition by L-

lysine include a DNA encoding such a protein having an amino acid sequence in which the histidine residue at the position 118 is replaced by tyrosine residue. Examples of DNA encoding a mutant aspartokinase desensitized to feedback inhibition by L-lysine include a DNA encoding an AKIII having an amino acid sequence in which the threonine residue at the position 352, the glycine residue at the position 323, and the methionine residue at the position 318 are replaced by isoleucine, asparagine and isoleucine residues, respectively (for these mutants, see U.S. Patent Nos. 5,661,012 and 6,040,160). Such mutant DNAs can be obtained by site-specific mutagenesis using PCR or the like.

[0052] Wide host-range plasmids RSFD80, pCAB1, and pCABD2 are known as plasmids containing a mutant *dapA* gene encoding a mutant dihydrodipicolinate synthase and a mutant *lysC* gene encoding a mutant aspartokinase (U.S. Patent No. 6,040,160). *Escherichia coli* JM109 strain transformed with RSFD80 was named AJ12396 (U.S. Patent No. 6,040,160), and the strain was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (currently National Institute of Advanced Industrial Science and Technology, International Patent Organism Depository) on October 28, 1993 and assigned an accession number of FERM P-13936, and the deposit was then converted to an international deposit under the provisions of Budapest Treaty on November 1, 1994 and assigned an accession number of FERM BP-4859. RSFD80 can be obtained from the AJ12396 strain by a conventional method.

[0053] Furthermore, L-amino acid-producing bacteria may have reduced activity of an enzyme that catalyzes a reaction branching off from an L-amino acid biosynthesis pathway and producing another compound or may be deficient in such an activity, or they may have reduced activity of an enzyme that negatively acts on L-amino acid synthesis or accumulation, or may be deficient in such an activity.

[0054] Examples of such enzymes involved in the L-lysine production include homoserine dehydrogenase, lysine decarboxylase (*cadA*, *ldcC*), malic enzyme, and so forth, and strains in which activities of these enzymes are decreased or deleted are disclosed in WO95/23864, WO96/17930, WO2005/010175, and so forth.

[0055] It is preferred that expressions of both the *cadA* and *ldcC* genes encoding lysine decarboxylase are decreased in order to decrease or delete the lysine decarboxylase activity. Expression of the both genes can be decreased by, for example, the method described in WO2006/078039.

[0056] In order to reduce or eliminate activities of these enzymes, a mutation may be introduced into genes of the enzymes on a genome by a usual mutagenesis method or gene recombination technique so that intracellular activities of the enzymes are reduced or eliminated. Such introduction of a mutation can be achieved by, for example, using genetic recombination to eliminate the genes coding for the enzymes on the genome or to modify an expression control sequence such as a promoter or the Shine-Dalgarno (SD) sequence. It can also be achieved by introducing a mutation for amino acid substitution (missense mutation), a stop codon (nonsense mutation), or a frame shift mutation for adding or deleting one or two nucleotides into regions coding for the enzymes on the genome, or partially or totally deleting the genes (J. Biol. Chem., 272:8611-8617 (1997)). The enzymatic activities can also be decreased or eliminated by constructing a gene coding for a mutant enzyme, of which coding region is totally or partially deleted, and substituting it for a normal gene on a genome by homologous recombination or the like, or by introducing a transposon or IS factor into the gene.

[0057] For example, in order to introduce a mutation that decreases or eliminates the activities of the above-mentioned enzymes by genetic recombination, the following methods are used. A mutant gene is prepared by modifying a partial sequence of an objective gene so that it does not encode an enzyme that can function normally, and then a bacterium belonging to the family *Enterobacteriaceae* can be transformed with a DNA containing the mutant gene to cause recombination of a corresponding gene on the genome with the mutant gene to substitute the mutant gene for the objective gene on the genome. Examples of such gene substitution using homologous recombination include methods of using a linear DNA such as the method called Red-driven integration (Datsenko, K.A. and Wanner, B.L., Proc. Natl. Acad. Sci. USA, 97:6640-6645 (2000)), and the method utilizing the Red driven integration in combination with an excisive 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 containing a temperature sensitive replication origin (U.S. Patent No. 6,303,383, Japanese Patent Laid-open No. 05-007491), and so forth. Furthermore, such site-specific mutagenesis based on gene substitution using homologous recombination can also be performed by using a plasmid which is not able to replicate in a host.

[0058] Preferred examples of L-lysine-producing bacteria include *Escherichia coli* WC196 Δ *cadA* Δ *ldcC*/pCABD2 (WO2006/078039). The strain was constructed by introducing the plasmid pCABD2 containing lysine biosynthesis genes (U.S. Patent No. 6,040,160) into the WC196 strain having disrupted *cadA* and *ldcC* genes, which encode lysine decarboxylase. The WC196 strain was bred from the W3110 strain, which was derived from *Escherichia coli* K-12, by replacing the wild type *lysC* gene on the chromosome of the W3110 strain with a mutant *lysC* gene encoding a mutant aspartokinase III in which threonine at position 352 was replaced with isoleucine, resulting in desensitization of the feedback inhibition thereof by L-lysine (U.S. Patent No. 5,661,012), and conferring AEC resistance to the resulting strain (U.S. Patent No. 5,827,698). The WC196 strain was designated *Escherichia coli* AJ13069, deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (currently National Institute of Advanced Industrial

Science and Technology, International Patent Organism Depository, Tsukuba Central 6, 1-1, Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, 305-8566, Japan) on December 6, 1994, and assigned an accession number of FERM P-14690. Then, it was converted to an international deposit under the provisions of the Budapest Treaty on September 29, 1995, and assigned an accession number of FERM BP-5252 (U.S. Patent No. 5,827,698). The WC196 Δ cadA Δ ldc strain itself is also a preferred L-lysine-producing bacterium. The WC196 Δ cadA Δ ldc was designated AJ110692, and deposited at the independent administrative agency, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depository (Tsukuba Central 6, 1-1, Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, 305-8566, Japan) on October 7, 2008 as an international deposit and assigned an accession number of FERM BP-11027.

[0059] The plasmid pCABD2 contains a mutant *dapA* gene derived from *Escherichia coli* and coding for a dihydrodipicolinate synthase (DDPS) having a mutation for desensitization to the feedback inhibition by L-lysine, a mutant *lysC* gene derived from *Escherichia coli* and coding for aspartokinase III having a mutation for desensitization to the feedback inhibition by L-lysine, the *dapB* gene derived from *Escherichia coli* and coding for dihydrodipicolinate reductase, and the *ddh* gene derived from *Brevibacterium lactofermentum* and coding for diaminopimelate dehydrogenase (International Publications WO95/16042 and WO01/53459).

[0060] The procedures described above for enhancing gene expression of the enzymes involved in the L-lysine biosynthesis, and the methods for reducing the enzymatic activities can similarly be applied to genes coding for other L-amino acid biosynthesis enzymes.

[0061] Examples of L-lysine producing coryneform bacteria include S-(2-aminoethyl)-cysteine (AEC) resistant mutant strains (*Brevibacterium lactofermentum* AJ11082 (NRRL B-11470) strain etc., refer to Japanese Patent Publication Nos. 56-1914, 56-1915, 57-14157, 57-14158, 57-30474, 58-10075, 59-4993, 61-35840, 62-24074, 62-36673, 5-11958, 7-112437 and 7-112438); mutant strains requiring an amino acid such as L-homoserine for their growth (refer to Japanese Patent Publication Nos. 48-28078 and 56-6499); mutant strains showing resistance to AEC and further requiring an amino acid such as L-leucine, L-homoserine, L-proline, L-serine, L-arginine, L-alanine and L-valine (refer to U.S. Patent Nos. 3,708,395 and 3,825,472); L-lysine-producing mutant strains showing resistance to DL- α -amino- ϵ -caprolactam, α -amino-lauryllactam, aspartic acid analogue, sulfa drug, quinoid and N-lauroylleucine; L-lysine-producing mutant strains showing resistance to oxaloacetate decarboxylase or a respiratory tract enzyme inhibitor (Japanese Patent Laid-open Nos. 50-53588, 50-31093, 52-102498, 53-9394, 53-86089, 55-9783, 55-9759, 56-32995, 56-39778, Japanese Patent Publication Nos. 53-43591 and 53-1833); L-lysine-producing mutant strains requiring inositol or acetic acid (Japanese Patent Laid-open Nos. 55-9784 and 56-8692); L-lysine-producing mutant strains that are susceptible to fluoropyruvic acid or a temperature of 34°C or higher (Japanese Patent Laid-open Nos. 55-9783 and 53-86090); L-lysine-producing mutant strains of *Brevibacterium* or *Corynebacterium* bacteria showing resistance to ethylene glycol (U.S. Patent No. 4,411,997) and so forth.

L-Tryptophan-producing bacteria

[0062] Examples of L-tryptophan-producing bacteria and parent strains which can be used to derive them include, but are not limited to, strains belonging to the genus *Escherichia*, such as *E. coli* JP4735/pMU3028 (DSM10122) and JP6015/pMU91 (DSM10123) which are deficient in tryptophanyl-tRNA synthetase encoded by mutant *trpS* gene (U.S. Patent No. 5,756,345), *E. coli* SV164 (pGH5) having a *serA* allele encoding phosphoglycerate dehydrogenase not subject to feedback inhibition by serine and a *trpE* allele encoding anthranilate synthase not subject to feedback inhibition by tryptophan (U.S. Patent No. 6,180,373); *E. coli* AGX17 (pGX44) (NRRL B-12263) and AGX6(pGX50)aroP (NRRL B-12264) deficient in the enzyme tryptophanase (U.S. Patent No. 4,371,614), *E. coli* AGX17/pGX50,pACKG4-pps in which a phosphoenolpyruvate-producing ability is enhanced (WO97/08333, U.S. Patent No. 6,319,696), and so forth. L-Tryptophan-producing bacteria belonging to the genus *Escherichia* which have enhanced activity of the protein encoded by the *yedA* or *yddG* gene may also be used (U.S. Patent Published Applications 2003/0148473 A1 and 2003/0157667 A1).

[0063] Examples of L-tryptophan-producing bacteria and parent strains which can be used to derive them also include strains in which one or more activities of the following enzymes are enhanced: anthranilate synthase (*trpE*), phosphoglycerate dehydrogenase (*serA*), and tryptophan synthase (*trpAB*). The anthranilate synthase and phosphoglycerate dehydrogenase both are subject to feedback inhibition by L-tryptophan and L-serine, and therefore a mutation desensitizing the feedback inhibition may be introduced into these enzymes. Specific examples of strains having such a mutation include *E. coli* SV164 which harbors desensitized anthranilate synthase and a transformant strain SV164 obtained by introducing into the *E. coli* SV164 the plasmid pGH5, which contains a mutant *serA* gene encoding a feedback inhibition-desensitized phosphoglycerate dehydrogenase.

[0064] Examples of L-tryptophan-producing bacteria and parent strains which can be used to derive them also include a strain which has enhanced activity of 3-phosphoserine phosphatase (*serB*) (U.S. Patent No. 4,371,614), a strain which has enhanced activity of phosphoenolpyruvate carboxykinase (*pckA*) (WO2004/090125), and a strain in which enzymes of the glyoxylic acid pathway are constitutively expressed (WO2005/103275).

[0065] L-Tryptophan, L-phenylalanine, and L-tyrosine are all aromatic amino acids and share a common biosynthesis

pathway. Examples of the genes encoding the biosynthesis enzymes for these aromatic amino acids include deoxyarabino-heptulosonate phosphate synthase (*aroG*), 3-dehydroquinate synthase (*aroB*), shikimic acid dehydratase (*aroE*), shikimate kinase (*aroL*), 5-enolpyruvylshikimate-3-phosphate synthase (*aroA*), and chorismate synthase (*aroC*) (European Patent Laid-open No. 763127). It is known that these genes are controlled by the tyrosine repressor (*tyrR*), and so activity of an aromatic amino acid biosynthesis enzyme may also be increased by deleting the *tyrR* gene (see European Patent Laid-open No. 763127). The abbreviations in parentheses after the enzyme names represent the gene names (the same shall apply to the same occasions hereafter).

[0066] In order to enhance the productivity of each of the target aromatic amino acids, biosynthesis of an amino acid other than the target amino acid may be attenuated. For example, when the target amino acid is L-tryptophan, biosynthetic pathways of L-phenylalanine and/or L-tyrosine may be attenuated (U.S. Patent No. 4,371,614).

[0067] In the present invention, the words "Increase of activity of an enzyme" corresponds to, for example, an increased number of enzyme molecules per cell, increased specific activity per enzyme molecule, and so forth. For example, the activity can be increased by increasing expression of the gene of the enzyme. Intracellular activity of an enzyme is preferably increased to be higher than that of a non-modified strain, for example, a wild-type strain, of the microorganism.

[0068] Furthermore, 3-deoxy-D-arabinoheptulosonate-7-phosphate synthetase (*aroF*, *aroG*) is subject to feedback inhibition by aromatic amino acids. Therefore, the enzyme may be modified so that it is not subject to the feedback inhibition. An aromatic L-amino acid-producing bacterium can be obtained, for example, by introducing a mutant *aroF* in which the L-aspartic acid at position 147 or the L-serine at position 181 from the N-terminus is replaced by another amino acid, or by introducing a mutant *aroG* gene in which the L-aspartic acid at position 146, the L-methionine at position 147, the L-proline at position 150 or the L-alanine at position 202, or both the L-methionine at position 157 and the L-alanine at position 219 from the N-terminus are replaced by other amino acid(s) (European Patent Laid-open No. 0488424).

[0069] Examples of L-tryptophan-producing bacteria and parent strains for deriving them also include strains into which the tryptophan operon containing a gene encoding inhibition-desensitized anthranilate synthase has been introduced (Japanese Patent Laid-open Nos. 57-71397, 62-244382, U.S. Patent No. 4,371,614). Moreover, L-tryptophan-producing ability may be imparted by enhancing expression of a gene which encodes tryptophan synthase in the tryptophan operon (*trpBA*). Tryptophan synthase consists of α and β subunits which are encoded by the *trpA* and *trpB* genes, respectively. In addition, L-tryptophan-producing ability may be improved by enhancing expression of the isocitrate lyase-malate synthase operon (WO2005/103275).

[0070] As coryneform bacteria, *Corynebacterium glutamicum* AJ12118 (FERM BP-478, Japanese Patent No. 01681002), which is resistant to sulfaguanidine, the coryneform bacterium introduced with the tryptophan operon (Japanese Patent Laid-open No. 63-240794), and the coryneform bacterium introduced with a gene coding for shikimate kinase derived from a coryneform bacterium (Japanese Patent Laid-open No. 01-994749) can be used.

L-Phenylalanine-producing bacteria

[0071] Examples of L-phenylalanine-producing bacteria and parent strains which can be used to derive them include, but are not limited to, strains belonging to the genus *Escherichia*, such as *E. coli* AJ12739 (*tyrA*: :Tn10, *tyrR*) (VKPM B-8197), *E. coli* HW1089 (ATCC 55371) harboring a mutant *pheA*34 gene (U.S. Patent No. 5,354,672), *E. coli* MWEC101-b (Korean Patent No. 8903681), *E. coli* NRRL B-12141, NRRL B-12145, NRRL B-12146, and NRRL B-12147 (U.S. Patent No. 4,407,952). Also, as a parent strain, *E. coli* K-12 [W3110 (*tyrA*)/pPHAB (FERM BP-3566), *E. coli* K-12 [W3110 (*tyrA*)/pPHAD] (FERM BP-12659), *E. coli* K-12 [W3110 (*tyrA*)/pPHATerm] (FERM BP-12662) and *E. coli* K-12 [W3110 (*tyrA*)/pBR-aroG4, pACMAB] named as AJ12604 (FERM BP-3579) may be used (European Patent Publication No. 488424 B1). Furthermore, L-phenylalanine-producing bacteria belonging to the genus *Escherichia* with an enhanced activity of the protein encoded by the *yedA* gene or the *yddG* gene may also be used (U.S. Patent Published Applications No. 2003/0148473 A1 and 2003/0157667 A1).

[0072] As phenylalanine-producing coryneform bacteria, the *Corynebacterium glutamicum* BPS-13 (FERM BP-1777), K77 (FERM BP-2062), and K78 (FERM BP-2063) (European Patent Laid-open No. 331145, Japanese Patent Laid-open No. 02-303495), of which phosphoenolpyruvate carboxylase or pyruvate kinase activity is reduced, tyrosine-auxotrophic strain (Japanese Patent Laid-open No. 05-049489), and so forth can be used.

[0073] A bacterium which efficiently produces phenylalanine can also be obtained by modifying a bacterium so that it incorporates by-products, for example, by increasing the expression of the L-tryptophan uptake gene, *tnaB* or *mtr*, or the L-tyrosine uptake gene, *tyrP* (European Patent No. 1484410).

L-Tyrosine-producing bacteria

[0074] Examples of tyrosine-producing bacteria include *Escherichia* bacteria with a desensitized prephenate dehydratase gene (*tyrA*) (European Patent Application Laid-open No. 1616940).

L-Valine-producing bacteria

[0075] Examples of L-valine-producing bacteria and parent strains which can be used to derive L-valine-producing bacteria include, but are not limited to, strains which have been modified to overexpress the *ilvGMEDA* operon (U.S. Patent No. 5,998,178). It is preferred that the region in the *ilvGMEDA* operon which is required for attenuation is removed so that expression of the operon is not attenuated by the L-valine that is produced. Furthermore, it is preferred that the *ilvA* gene in the operon is disrupted so that threonine deaminase activity is decreased.

[0076] Examples of L-valine-producing bacteria which can be used to derive L-valine-producing bacteria used for the present invention also include mutant strains with aminoacyl t-RNA synthetase having a mutation (U.S. Patent No. 5,658,766). For example, *E. coli* VL1970, which has a mutation in the *ileS* gene encoding isoleucine tRNA synthetase, can be used. *E. coli* VL1970 was deposited at the Russian National Collection of Industrial Microorganisms (VKPM) (1 Dorozhny proezd., 1 Moscow 117545, Russia) on June 24, 1988 under an accession number VKPM B-4411.

[0077] Furthermore, mutants requiring lipoic acid for growth and/or lacking H⁺-ATPase can also be used as parent strains (WO96/06926).

[0078] Examples of L-valine-producing bacteria of coryneform bacteria include, for example, strains modified so that expression of a gene encoding an L-valine biosynthetic enzyme is enhanced. Examples of the L-valine biosynthesis enzyme include enzymes encoded by genes present on the *ilvBNC* operon, that is, acetohydroxy acid synthetase encoded by *ilvBN* and isomero-reductase encoded by *ilvC* (WO00/50624). Since the *ilvBNC* operon is subject to expression regulation by L-valine and/or L-isoleucine and/or L-leucine, it is desirable to eliminate attenuation to avoid expression suppression by L-valine that is produced.

[0079] Impartation of L-valine-producing ability to coryneform bacteria may be performed by decreasing or eliminating activity of at least one kind of enzyme which is involved in a metabolic pathway that decreases L-valine production. For example, decrease of the activity of threonine dehydratase involved in the L-leucine synthesis, or activity of an enzyme that involved in D-pantothenate synthesis is contemplated (WO00/50624).

[0080] Examples of methods for imparting L-valine-producing ability also include imparting resistance to an amino acid analogue or the like.

[0081] Examples include, for example, mutant strains which are auxotrophic for L-isoleucine and L-methionine, and resistant to D-ribose, purine ribonucleoside or pyrimidine ribonucleoside, and have an ability to produce L-valine (FERM P-1841, FERM P-29, Japanese Patent Publication No. 53-025034), mutant strains resistant to polyketides (FERM P-1763, FERM P-1764, Japanese Patent Publication No. 06-065314), and mutant strains resistant to L-valine in a medium containing acetic acid as the sole carbon source and sensitive to pyruvic acid analogues (fluoropyruvic acid etc.) in a medium containing glucose as the sole carbon source (FERM BP-3006, BP-3007, Japanese Patent No. 3006929).

[0082] An example of a gene involved in the synthesis of branched chain amino acids is the *ilvGMEDA* operon, and this operon is subject to expression control (attenuation) by L-valine and/or L-isoleucine and/or L-leucine. Therefore, productivity of a microorganism for these L-amino acids can be improved by introducing into the microorganism the *ilvGMEDA* operon in which the region required for attenuation is removed or mutated.

L-Isoleucine-producing bacteria

[0083] Examples of L-isoleucine-producing bacteria and parent strains which can be used to derive L-isoleucine-producing bacteria include, but are not limited to, mutants having resistance to 6-dimethylaminopurine (Japanese Patent Laid-open No. 5-304969), mutants having resistance to an isoleucine analogue such as thiaisoleucine and isoleucine hydroxamate, and mutants additionally having resistance to DL-ethionine and/or arginine hydroxamate (Japanese Patent Laid-open No. 5-130882). In addition, recombinant strains transformed with genes encoding proteins involved in L-isoleucine biosynthesis, such as threonine deaminase and acetohydroxy acid synthase, can also be used as parent strains (Japanese Patent Laid-open No. 2-458, French Patent No. 0356739, and U.S. Patent No. 5,998,178).

[0084] Examples of L-isoleucine-producing strains of coryneform bacteria include the coryneform bacterium in which the *brnE* gene coding for a branched chain amino acid excretion protein is amplified (Japanese Patent Laid-open No. 2001-169788), the coryneform bacterium imparted with L-isoleucine-producing ability by protoplast fusion with an L-lysine-producing bacterium (Japanese Patent Laid-open No. 62-74293), the coryneform bacterium in which homoserine dehydrogenase is enhanced (Japanese Patent Laid-open No. 62-91193), the threonine hydroxamate resistant strain (Japanese Patent Laid-open No. 62-195293), α -ketomalonic acid resistant strain (Japanese Patent Laid-open No. 61-15695), and the methyl lysine resistant strain (Japanese Patent Laid-open No. 61-15696).

L-Leucine-producing bacteria

[0085] Examples of L-leucine-producing bacteria and parent strains for deriving L-leucine-producing bacteria include, but are not limited to, *Escherichia* bacteria, such as *E. coli* strains resistant to leucine (for example, the strain 57 (VKPM

B-7386, U.S. Patent No. 6,124,121)) or leucine analogues including β -2-thienylalanine, 3-hydroxyleucine, 4-azaleucine and 5,5,5-trifluoroleucine (Japanese Patent Publication No. 62-34397 and Japanese Patent Laid-open No. 8-70879) ; *E. coli* strains obtained by the genetic engineering method described in WO96/06926 ; and *E. coli* H-9068 (Japanese Patent Laid-open No. 8-70879).

[0086] The bacterium of the present invention may also be improved by enhancing the expression of one or more genes involved in L-leucine biosynthesis. Examples of such genes include genes of the *leuABCD* operon, which are preferably represented by a mutant *leuA* gene coding for isopropylmalate synthase desensitized to feedback inhibition by L-leucine (U.S. Patent No. 6,403,342). In addition, the bacterium of the present invention may be improved by enhancing the expression of one or more genes coding for proteins which excrete L-amino acid from the bacterial cell. Examples of such genes include the *b2682* and *b2683* genes (*ygaZH* genes) (European Patent Laid-open No. 1239041 A2).

[0087] Examples of L-leucine-producing strains of coryneform bacteria include the 2-thiazolealanine and β -hydroxy-leucine-resistant strains (Japanese Patent Laid-open No. 8-266295), the valine analogue-resistant strain (Japanese Patent Laid-open No. 63-248392), the valine auxotrophic strain (Japanese Patent Publication No. 38-4395), the S-(2-aminoethyl)-L-cysteine (AEC) resistant strain (Japanese Patent Publication No. 51-37347), and the phenylalanine, valine and isoleucine auxotrophic strain (Japanese Patent Publication No. 54-36233).

L-Glutamic acid-producing bacteria

[0088] Preferred examples of L-glutamic acid-producing bacteria include strains in which expression of a gene encoding an L-glutamic acid biosynthetic enzyme is enhanced. Examples of such genes include, but are not limited to, genes encoding glutamate dehydrogenase (*gdhA*), glutamine synthetase (*glnA*), glutamate synthetase (*gltAB*), isocitrate dehydrogenase (*icdA*), aconitate hydratase (*acnA*, *acnB*), citrate synthase (*gltA*), phosphoenolpyruvate carboxylase (*ppc*), pyruvate dehydrogenase (*aceEF*, *lpdA*), pyruvate kinase (*pykA*, *pykF*), phosphoenolpyruvate synthase (*ppsA*), enolase (*eno*), phosphoglyceromutase (*pgmA*, *pgmI*), phosphoglycerate kinase (*pgk*), glyceraldehyde-3-phosphate dehydrogenase (*gapA*), triose phosphate isomerase (*tpiA*), fructose biphosphate aldolase (*fbp*), phosphofructokinase (*pfkA*, *pfkB*), glucose phosphate isomerase (*pgi*), and so forth.

[0089] Examples of strains which have been modified so that expression of the citrate synthetase gene, the phosphoenolpyruvate carboxylase gene, the isocitrate dehydrogenase gene, the pyruvate dehydrogenase gene, and/or the glutamate dehydrogenase gene is enhanced include those disclosed in European Patent Laid-open Nos. 1078989, 955368, 952221 and 1033407.

[0090] The modification for imparting L-glutamic acid producing ability may be attained by decreasing or eliminating activity of an enzyme that catalyzes a reaction branching off from the L-glutamic acid biosynthesis pathway and producing a compound other than L-glutamic acid. Examples of such an enzyme that catalyzes a reaction branching off from the L-glutamic acid biosynthesis pathway and producing a compound other than L-glutamic acid include isocitrate lyase, α -ketoglutarate dehydrogenase, acetohydroxy acid synthase, acetolactate synthase, formate acetyltransferase, lactate dehydrogenase, glutamate decarboxylase, 1-pyrroline-5-carboxylate dehydrogenase, and so forth.

[0091] For example, in order to decrease the α -ketoglutarate dehydrogenase activity, a modification may be performed by using the *sucA* (*odhA*) gene coding for the E1 α subunit of the enzyme. Examples of strains with decreased α -ketoglutarate dehydrogenase activity include, for example, the following strains:

Brevibacterium lactofermentum Δ S strain (WO95/34672)

Brevibacterium lactofermentum AJ12821 (FERM BP-4172; French Patent No. 9401748)

Brevibacterium flavum AJ12822 (FERM BP-4173; French Patent No. 9401748)

Corynebacterium glutamicum AJ12823 (FERM BP-4174; French Patent No. 9401748)

Pantoea ananatis AJ13601 (FERM BP-7207)

Klebsiella planticola AJ13410 (FERM BP-6617)

Pantoea ananatis AJ13355 (FERM BP-6614)

Pantoea ananatis AJ13356 is deficient in α -ketoglutarate dehydrogenase activity as a result of disruption of the α KGDH-E1 subunit gene (*sucA*). This strain was identified as *Enterobacter agglomerans* when it was isolated and deposited as the *Enterobacter agglomerans* AJ13356. However, it was recently re-classified as *Pantoea ananatis* on the basis of nucleotide sequencing of 16S rRNA and so forth. Although AJ13356 was deposited at the aforementioned depositary as *Enterobacter agglomerans*, it is described as *Pantoea ananatis* in this specification.

[0092] Furthermore, the ability to produce L-glutamic acid in coryneform bacteria can also be achieved by a method of amplifying the *yggB* gene (NCgl 1221;NP_600492. Reports small-conductance. [gi:19552490], WO2006/070944), and a method of introducing a mutant *yggB* gene in which a mutation is introduced into the coding region.

[0093] Examples of other methods for imparting or enhancing L-glutamic acid-producing ability include a method of

imparting resistance to an organic acid analogue, a respiratory chain inhibitor, etc., and a method of imparting sensitivity to a cell wall synthesis inhibitor. Examples of such methods include the methods of imparting resistance to monofluoroacetic acid (Japanese Patent Laid-open No. 50-113209), the method of imparting resistance to adenine or thymine (Japanese Patent Laid-open No. 57-065198), the method of attenuating urease (Japanese Patent Laid-open No. 52-038088), the method of imparting resistance to malonic acid (Japanese Patent Laid-open No. 52-038088), the method of imparting resistance to benzopyrones or naphthoquinones (Japanese Patent Laid-open No. 56-1889), the method of imparting resistance to HOQNO (Japanese Patent Laid-open No. 56-140895), the method of imparting resistance to α -ketomalonic acid (Japanese Patent Laid-open No. 57-2689), the method of imparting resistance to guanidine (Japanese Patent Laid-open No. 56-35981), the method of imparting sensitivity to penicillin (Japanese Patent Laid-open No. 4-88994), and so forth.

[0094] Specific examples of such resistant strains include the following strains:

Brevibacterium flavum AJ3949 (FERM BP-2632; Japanese Patent Laid-open No. 50-113209)
Corynebacterium glutamicum AJ11628 (FERM P-5736; Japanese Patent Laid-open No. 57-065198)
Brevibacterium flavum AJ11355 (FERM P-5007; Japanese Patent Laid-open No. 56-1889)
Corynebacterium glutamicum AJ11368 (FERM P-5020; Japanese Patent Laid-open No. 56-1889)
Brevibacterium flavum AJ11217 (FERM P-4318; Japanese Patent Laid-open No. 57-2689)
Corynebacterium glutamicum AJ11218 (FERM P-4319; Japanese Patent Laid-open No. 57-2689)
Brevibacterium flavum AJ11564 (FERM BP-5472; Japanese Patent Laid-open No. 56-140895)
Brevibacterium flavum AJ11439 (FERM BP-5136; Japanese Patent Laid-open No. 56-35981)
Corynebacterium glutamicum H7684 (FERM BP-3004; Japanese Patent Laid-open No. 04-88994)
Brevibacterium lactofermentum AJ11426 (FERM P-5123; Japanese Patent Laid-open No. 56-048890)
Corynebacterium glutamicum AJ11440 (FERM P-5137; Japanese Patent Laid-open No. 56-048890)
Brevibacterium lactofermentum AJ11796 (FERM P-6402; Japanese Patent Laid-open No. 58-158192)

L-Threonine-producing bacteria

[0095] Preferred examples of L-threonine-producing bacteria used for the present invention include bacteria belonging to the family *Enterobacteriaceae* in which an activity of L-threonine biosynthesis system enzyme is enhanced. Examples of genes coding for L-threonine biosynthetic enzymes include the aspartokinase III gene (*lysC*), aspartate semialdehyde dehydrogenase gene (*asd*), aspartokinase I gene (*thrA*), homoserine kinase gene (*thrB*), and threonine synthase gene (*thrC*) encoded by the *thr* operon. Two or more kinds of these genes may be introduced. The genes coding for the L-threonine biosynthetic enzymes may be introduced into an *Enterobacteriaceae* bacterium with decreased threonine decomposition. Examples of the *Escherichia* bacterium with decreased threonine decomposition include, for example, the TDH6 strain which is deficient in threonine dehydrogenase activity (Japanese Patent Laid-open No. 2001-346578), and so forth.

[0096] The activities of the L-threonine biosynthetic enzymes are inhibited by the end product L-threonine, and therefore L-threonine biosynthetic enzymes are preferably modified so as to be desensitized to feedback inhibition by L-threonine when constructing L-threonine producing strains. The above-described *thrA*, *thrB* and *thrC* genes constitute the threonine operon which has an attenuator structure. The expression of the threonine operon is inhibited by isoleucine and threonine in the culture medium and also repressed by attenuation. This attenuation can be eliminated or reduced by removing a leader sequence or attenuator in the attenuation region (Lynn, S.P., Burton, W.S., Donohue, T.J., Gould, R.M., Gumpert, R.I., and Gardner, J.F.J., Mol. Biol. 194:59-69 (1987); WO02/26993; WO2005/049808).

[0097] The native promoter present in the upstream region of the threonine operon may be replaced by a non-native promoter (WO98/04715), or the threonine operon may be constructed so that expression of the threonine biosynthetic genes is controlled by the repressor and promoter of λ -phage (European Patent No. 0593792). Furthermore, mutant *Escherichia* bacteria that are desensitized to feedback inhibition by L-threonine can be obtained by selecting strains resistant to α -amino- β -hydroxyisovaleric acid (AHV).

[0098] It is preferable that the copy number of the feedback-resistant threonine operon is increased, or the expression of the modified operon is increased by connecting it to a potent promoter. The copy number can be increased by using, in addition to amplification using a plasmid, transposon, Mu-phage, or the like so that the operon is transferred onto the chromosome.

[0099] The gene encoding aspartokinase III (*lysC*) is preferably modified so that the enzyme is desensitized to feedback inhibition by L-lysine. Such a modified *lysC* gene can be obtained by the method described in U.S. Patent No. 5,932,453.

[0100] L-Threonine-producing bacteria can also be preferably obtained by enhancing expression of genes involved in the glycolytic pathway, TCA cycle, or respiratory chain, or genes that regulate expression of these genes, or genes involved in sugar uptake, besides the L-threonine biosynthetic enzyme genes. Examples of these genes that are effective for L-threonine production include the transhydrogenase gene (*pntAB*, European Patent No. 733712), phosphoenolpyru-

vate carboxylase gene (*pepC*, WO95/06114), phosphoenolpyruvate synthase gene (*pps*, European Patent No. 877090), and pyruvate carboxylase gene derived from coryneform bacterium or *Bacillus* bacterium (WO99/18228, European Patent Laid-open No. 1092776).

[0101] L-Threonine-producing bacteria can also be preferably obtained by enhancing expression of a gene that imparts L-threonine resistance and/or a gene that imparts L-homoserine resistance, or by imparting L-threonine resistance and/or L-homoserine resistance to the host bacterium. Examples of the genes that impart the above-mentioned resistance include the *rhtA* gene (Res. Microbiol. 154:123-135 (2003)), *rhtB* gene (European Patent Laid-open No. 0994190), *rhtC* gene (European Patent Laid-open No. 1013765), *yfiK* gene, and *yeaS* gene (European Patent Laid-open No. 1016710). Exemplary methods for imparting L-threonine resistance to a host bacterium include those described in European Patent

Laid-open No. 0994190 or WO90/04636.

[0102] *E. coli* VKPM B-3996 (U.S. Patent No. 5,175,107) can be exemplified as an L-threonine-producing bacterium. The strain VKPM B-3996 was deposited on November 19, 1987 at the Russian National Collection of Industrial Microorganisms (VKPM), GNI Genetika (Russia, 117545 Moscow 1, Dorozhny proezd, 1) under the registration number VKPM B-3996. The VKPM B-3996 strain contains the plasmid pVIC40 (WO90/04636) which was obtained by inserting the threonine biosynthetic genes (threonine operon, *thrABC*) into a wide host range plasmid vector pAYC32 containing the streptomycin resistance marker (Chistorerdov, A.Y., and Tsygankov, Y.D., Plasmid, 16, 161-167 (1986)). In pVIC40, aspartokinase I-homoserine dehydrogenase I encoded by the *thrA* gene in the threonine operon is desensitized to feedback inhibition by threonine.

[0103] *E. coli* VKPM B-5318 (refer to European Patent No. 0593792) can also be exemplified as a preferred L-threonine-producing bacterium. The VKPM B-5318 strain was deposited in the Russian National Collection of Industrial Microorganisms (VKPM) GNI Genetika on May 3, 1990 under a registration number of VKPM B-5318. The VKPM B-5318 strain is prototrophic with regard to L-isoleucine, and harbors a recombinant plasmid DNA constructed so that the threonine operon, i.e., threonine biosynthesis genes, deficient in the attenuator region, which is an originally contained transcription regulation region, is located downstream from the λ phage-derived temperature-sensitive C1 repressor, PR-promoter, and the gene coding for N-terminal of Cro protein, and the expression of the threonine biosynthesis genes is regulated by the repressor and the promoter derived from λ phage.

L-Arginine-producing bacteria

[0104] Examples of L-arginine-producing bacteria and parent strains which can be used to derive L-arginine-producing bacteria include, but are not limited to, *Escherichia* bacterial strains, such as *E. coli* strain 237 (VKPM B-7925) (U.S. Patent Published Application No. 2002/058315 A1) and its derivative strains harboring mutant N-acetylglutamate synthase (Russian Patent Application No. 2001112869), *E. coli* strain 382 (VKPM B-7926) (EP 1170358 A1), and an arginine-producing strain transformed with the *argA* gene encoding N-acetylglutamate synthetase (EP 1170361 A1).

[0105] Examples of L-arginine-producing bacteria and parent strains which can be used to derive L-arginine-producing bacteria used for the present invention also include strains in which the expression of one or more genes encoding an L-arginine biosynthetic enzyme is enhanced. Examples of such genes include the N-acetylglutamyl phosphate reductase gene (*argC*), ornithine acetyl transferase gene (*argJ*), N-acetylglutamate kinase gene (*argB*), acetylornithine transaminase gene (*argD*), ornithine carbamoyl transferase gene (*argF*), argininosuccinic acid synthetase gene (*argG*), argininosuccinic acid lyase gene (*argH*), and carbamoyl phosphate synthetase gene (*carAB*).

L-Histidine-producing bacteria

[0106] Examples of L-histidine-producing bacteria and parent strains which can be used to derive L-histidine-producing bacteria used for the present invention include, but are not limited to, *Escherichia* bacterial strains, such as *E. coli* strain 24 (VKPM B-5945, RU2003677), *E. coli* strain 80 (VKPM B-7270, RU2119536), *E. coli* NRRL B-12116 - B 12121 (U.S. Patent No. 4,388,405), *E. coli* H-9342 (FERM BP-6675), *E. coli* H-9343 (FERM BP-6676) (U.S. Patent No. 6,344,347), *E. coli* H-9341 (FERM BP-6674) (EP 1085087 A), *E. coli* AI80/pFM201 (U.S. Patent No. 6,258,554), and so forth.

[0107] Examples of L-histidine-producing bacteria and parent strains which can be used to derive L-histidine-producing bacteria used for the present invention also include strains in which the expression of one or more genes encoding L-histidine biosynthetic enzymes are enhanced. Examples of such genes include the genes encoding ATP phosphoribosyltransferase (*hisG*), phosphoribosyl AMP cyclohydrolase (*hisI*), phosphoribosyl-ATP pyrophosphohydrolase (*hisIE*), phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase (*hisA*), amidotransferase (*hisH*), histidinol phosphate aminotransferase (*hisC*), histidinol phosphatase (*hisB*), histidinol dehydrogenase (*hisD*), and so forth.

[0108] It is known that the L-histidine biosynthetic enzymes encoded by *hisG* and *hisBHAFI* are inhibited by L-histidine, and therefore the ability to produce L-histidine can also be efficiently enhanced by introducing a mutation which confers resistance to feedback inhibition into the gene coding for ATP phosphoribosyltransferase (*hisG*) (Russian Patent Nos. 2003677 and 2119536).

[0109] Specific examples of strains which are able to produce L-histidine include *E. coli* FERM-P 5038 and 5048 which have been transformed with a vector carrying a DNA encoding an L-histidine biosynthetic enzyme (Japanese Patent Laid-open No. 56-005099), *E. coli* strains transformed with a gene encoding a protein involved in amino acid export (EP 1016710 A), *E. coli* 80 strain which is resistant to sulfaguanidine, DL-1,2,4-triazole-3-alanine, and streptomycin (VKPM B-2770, Russian Patent No. 2119536), and so forth.

L-Cysteine-producing bacteria

[0110] Examples of L-cysteine-producing bacteria used for the present invention include, but are not limited to, strains belonging to the genus *Escherichia*, such as *E. coli* JM15 which is transformed with different *cysE* alleles encoding feedback-resistant serine acetyltransferases (U.S. Patent No. 6,218,168, Russian Patent Application No. 2003121601), *E. coli* W3110 with overexpressed genes which encode proteins which promote excretion of substances toxic to cells (U.S. Patent No. 5,972,663), *E. coli* strains with reduced cysteine desulfohydase activity (Japanese Patent Laid-open No. 11-155571), *E. coli* W3110 with increased activity of a positive transcriptional regulator for cysteine regulon encoded by the *cysB* gene (WO01/27307A1), and so forth.

L-Serine-producing bacteria

[0111] Examples of parent strains for deriving L-serine-producing bacteria used for the present invention include coryneform bacteria in which the phosphoserine phosphatase gene is amplified (Japanese Patent Laid-open Nos. 2001-275689 and 11-253187), coryneform bacteria having D-3-phosphoglycerate dehydrogenase desensitized to inhibition, which is derived from coryneform bacteria having L-serine-producing ability and resistant to azaserine or β -(2-thienyl)-DL-alanine (Japanese Patent Laid-open No. 11-266881), and serine-producing coryneform bacteria resistant to azaserine or thienylalanine, and deficient in serine decomposition ability (Japanese Patent Laid-open No. 10-248588).

L-Asparagine-producing bacteria

[0112] L-Asparagine is produced by transferring amino group to aspartic acid (Boehlein, S.K., Richards, N.G.J., & Schuster, S.M. (1994a), J. Biol. Chem., 269, 7450-7457). Therefore, examples of L-asparagine-producing bacteria belonging to the genus *Escherichia* include L-asparatic acid-producing *Escherichia coli* strains in which asparagine synthetase is enhanced.

[0113] Methods for imparting nucleic acid-producing ability to a microorganism and nucleic acid-producing bacteria will be exemplified below.

[0114] A bacterium having an ability to produce a nucleic acid can be obtained by imparting, for example, purine nucleoside auxotrophy or resistance to a drug such as purine analogue to such bacteria as described above (Japanese Patent Publication Nos. 38-23099, 54-17033, 55-45199, 57-14160, 57-41915 and 59-42895). For example, a *Bacillus* bacterium having auxotrophy or drug resistance can be obtained by treating the bacterium with mutagen which is used for usual mutagenesis treatment such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG) or EMS (ethyl methanesulfonate).

[0115] Examples of *Bacillus* bacteria which produce a purine nucleoside include the following.

[0116] As a specific example of inosine-producing strain belonging to the genus *Bacillus*, the *Bacillus subtilis* KMBS16 strain can be used. This strain is derived from the known *Bacillus subtilis* trpC2 strain (168 Marburg), wherein the *purR* gene encoding the purine operon repressor (*purR::spc*), the *purA* gene encoding succinyl-AMP synthase (*purA::erm*), and the *deoD* gene encoding purine nucleoside phosphorylase (*deoD::kan*) are disrupted (Japanese Patent Laid-open No. 2004-242610, U.S. Patent Published Application No. 2004166575 A1). The *Bacillus subtilis* AJ3772 strain (FERM P-2555, Japanese Patent Laid-open No. 62-014794) and so forth may also be used.

[0117] Examples of *Bacillus* bacteria having an ability to produce guanosine include the *Bacillus* bacterium which has increased IMP dehydrogenase activity (Japanese Patent Laid-open No. 3-58787), *Bacillus* bacterium which is obtained by introducing a vector which includes a gene which confers resistance to purine analogues or decoyinine into an adenine auxotrophic mutant (Japanese Patent Publication No. 4-28357), and so forth.

[0118] Examples of *Bacillus* bacteria which produce a purine nucleotide include the following.

[0119] As inosinic acid-producing *Bacillus* bacteria, inosine-producing strains of *Bacillus subtilis* which have attenuated phosphatase activity have been reported (Uchida, K. et al., Agr. Biol. Chem., 1961, 25, 804-805; Fujimoto, M., Uchida, K., Agr. Biol. Chem., 1965, 29, 249-259). Examples of guanylic acid-producing bacteria include mutants of *Bacillus* bacteria which have adenine auxotrophy, resistance to decoyinine or methionine sulfoxide and an ability to produce 5'-guanylic acid (guanosine-5'-monophosphate, henceforth also referred to as "GMP") (Japanese Patent Publication No. 56-12438).

[0120] Furthermore, a xanthylic acid-producing bacterium can be constructed by the method used to breed coryneform bacteria, a typical example of which is *Corynebacterium ammoniagenes*. For example, by obtaining a PRPP amidotrans-

ferase-enhanced strain (Japanese Patent Laid-open No. 8-168383), an aliphatic amino acid-resistant strain (Japanese Patent Laid-open No. 4-262790), or a dehydropyrroline-resistant strain (South Korean Patent Unexamined Publication No. 2003-56490), a xanthylic acid-producing bacterium can be constructed.

[0121] Moreover, exemplary methods for breeding *Bacillus* bacteria which have an ability to produce a purine-derived substance also include enhancing activity of an enzyme which is involved in purine biosynthesis which is common to the biosynthesis of purine nucleosides and purine nucleotides, i.e., purine biosynthesis enzyme, in bacterial cells.

[0122] Examples of the enzyme involved in the purine biosynthesis include, for example, phosphoribosyl pyrophosphate amidotransferase, phosphoribosyl pyrophosphate synthetase (PRPP synthetase [EC: 2.7.6.1]), and so forth.

[0123] Some of the catabolites produced by metabolism of sugar sources such as glucose that flow into the pentose phosphate pathway are converted into ribose-5-phosphate via ribulose-5-phosphate. From the biosynthesized ribose-5-phosphate, phosphoribosyl pyrophosphate (PRPP) is produced, which is an indispensable precursor for purine nucleoside, histidine and tryptophan biosyntheses. Specifically, ribose-5-phosphate is converted into PRPP by phosphoribosyl pyrophosphate synthetase. Therefore, an ability to produce purine-derived substance can be imparted to a *Bacillus* bacterium or the ability of the bacterium can be enhanced by modifying the bacterium so that the activity of phosphoribosyl pyrophosphate synthetase thereof is increased.

[0124] The activity of the phosphoribosyl pyrophosphate synthetase can be measured by, for example, the method of Switzer et al. (Methods Enzymol., 1978, 51, 3-11) or Roth et al. (Methods Enzymol., 1978, 51, 12-17). A *Bacillus* bacterium in which the activity of phosphoribosyl pyrophosphate synthetase is increased can be produced by, for example, increasing expression of a gene encoding the phosphoribosyl pyrophosphate synthetase in a *Bacillus* bacterium according to a method of using a plasmid or integrating the gene into a chromosome, which can be performed in the same manner as that of the method described in Japanese Patent Laid-open No. 2004-242610.

[0125] On the other hand, when PRPP, which is an indispensable precursor for purine nucleoside, histidine and tryptophan biosyntheses, is produced, some of it is converted into purine nucleotides and purine nucleosides by the enzymes involved in the purine biosynthesis. Examples of genes encoding such enzymes include the genes of the purine operon from *Bacillus subtilis*, specifically, genes of the *purEKB-purC(or)QLF-purMNH(J)-purD* operon (Ebbola D.J. and Zalkin H., J. Biol. Chem., 1987, 262, 17, 8274-87) (at present, also called *purEKBKCSQLFMNHD*, *Bacillus subtilis* and Its Closest Relatives, Editor in Chief: A.L. Sonenshein, ASM Press, Washington D.C., 2002, Genbank Accession No. NC_000964), and the genes of the *pur* regulon from *Escherichia coli* (*Escherichia* and *Salmonella*, Second Edition, Editor in Chief: F.C. Neidhardt, ASM Press, Washington D.C., 1996).

[0126] Accordingly, by enhancing expression of these genes, an ability to produce a purine-derived substance can be imparted or enhanced. In addition, genes of the purine operon which can be used for the present invention are not limited to these, and genes derived from other microorganisms, animals and plants may also be used.

[0127] Examples of the method for increasing expression of the purine operon include increasing expression of genes of the purine operon in a *Bacillus* bacterium by a method of using a plasmid, integrating the genes into a chromosome, or the like.

[0128] The second method for increasing expression of the purine operon includes replacing a native promoter of the purine operon with a stronger promoter, and replacing the -35 or -10 region of the native promoter with a consensus sequence.

[0129] For example, in *Bacillus subtilis* (*B. subtilis* 168 Marburg strain, ATCC 6051), the -35 sequence of the purine operon is a consensus sequence (TTGACA), but the -10 sequence is TAAGAT, which differs from the consensus sequence TATAAT (Ebbola, D.J. and H. Zalkin, J. Biol. Chem., 1987, 262, 8274-8287). Therefore, by replacing the -10 sequence (TAAGAT) with a consensus sequence, by approximating the -10 sequence (TAAGAT) close to the consensus sequence, or changing it to TATAAT, TATGAT or TAAAAT, the transcriptional activity of the purine operon can be increased. A promoter sequence can be replaced by the same method as that of the gene substitution, which is described below.

[0130] The third method for increasing expression of the purine operon includes decreasing expression of the purine operon repressor (U.S. Patent No. 6,284,495).

[0131] Expression of the purine operon repressor (purine repressor) can be decreased by, for example, a method of treating a *Bacillus* bacterium with ultraviolet ray irradiation or mutagen used in a usual mutagenesis treatment such as NTG or EMS and selecting a mutant showing decreased expression of the purine repressor.

[0132] Furthermore, a *Bacillus* bacterium with decreased expression of the purine repressor can also be obtained by, for example, besides a mutagenesis treatment, replacing a gene encoding the purine repressor on a chromosome (*purR*, GenBank Accession NC_000964) with a corresponding gene that does not normally function (hereafter, also referred to as "disrupted-type gene") by homologous recombination utilizing a gene recombination technique (Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press (1972); Matsuyama, S. and Mizushima, S., J. Bacteriol., 1985, 162, 1196-1202).

[0133] Furthermore, an ability to produce a purine-derived substance can also be enhanced by attenuating uptake of purine-derived substances into cells. For example, the uptake of purine nucleosides by the cells can be attenuated by

blocking a reaction involved in the uptake of purine nucleosides by the cells. Examples of the reaction involved in the uptake of purine nucleosides by the cells include reactions catalyzed by nucleoside permeases.

[0134] Furthermore, when a purine nucleoside is produced, activity of an enzyme which decomposes purine-derived substances may be decreased in order to enhance the ability to produce purine nucleoside. Examples of such an enzyme include purine nucleoside phosphorylase.

[0135] Purine nucleotides biosynthesized from PRPP by enzymes involved in purine biosynthesis are dephosphorylated and thereby converted into a purine nucleoside. To efficiently cause accumulation of a purine nucleoside, it is preferable to reduce an activity of purine nucleoside phosphorylases, which further degrade purine nucleosides into hypoxanthine or the like. That is, it is preferable to attenuate or eliminate an activity of a purine nucleoside phosphorylase that employs purine nucleosides such as inosine, as a substrate.

[0136] Specifically, the purine nucleoside phosphorylase activity can be decreased by disrupting the *deoD* and *pupG* genes encoding purine nucleoside phosphorylase in *Bacillus* bacteria. The *Bacillus* bacterium of the present invention may be modified by disrupting one or both of the *deoD* and *pupG* genes. As the *deoD* and *pupG* genes, for example, those genes derived from *Bacillus* bacteria (*deoD*; Genbank Accession No. NC_000964, *pupG*; Genbank Accession No. NC_000964) can be used.

[0137] The ability to produce a purine-derived substance may also be enhanced by decreasing the activity of succinyl-AMP synthase. Examples of the gene encoding succinyl-AMP synthase include the *purA* gene. Examples of the *purA* gene include, for example, those having the nucleotide sequence registered as GenBank Accession No. NC_000964 (coding region corresponds to the nucleotide numbers 4153460 to 4155749 of the complementary strand).

[0138] The ability to produce a purine-derived substance may also be enhanced by decreasing an activity of inosine monophosphate (IMP) dehydrogenase. Examples of the gene encoding IMP dehydrogenase include the *guaB* gene. Examples of the *guaB* gene include, for example, those having the nucleotide sequence registered as GenBank Accession No. NC_000964 (coding region corresponds to the nucleotide numbers 15913 to 17376).

[0139] Moreover, as a method for enhancing an ability to produce purine-derived substance, amplification of a gene encoding a protein having an activity of excreting a purine-derived substance may be contemplated. An example of a bacterium in which such a gene has been amplified is a *Bacillus* bacterium in which the *rhtA* gene is amplified (Japanese Patent Laid-open No. 2003-219876).

[0140] Furthermore, L-amino acid-producing bacteria, nucleic acid-producing bacteria, and microorganisms used for breeding thereof, and methods for imparting or enhancing an L-amino acid-producing ability or nucleic acid-producing ability are described in detail in WO2007/125954, WO2005/095627, U.S. Patent Published Application No. 2004/0166575, and so forth, and they can also be applied to the present invention.

[0141] Furthermore, the present invention is not limited to production of the aforementioned L-amino acids and nucleic acids, but it can be applied to any L-amino acid or nucleic acid which can be produced by fermentation using a microorganism.

[0142] Furthermore, in the microorganism used for the present invention, according to the target substance, activity of a protein involved in the production of the target substance may be enhanced, and activity of a protein involved in the decomposition of the target substance may be reduced.

[0143] Methods for imparting the isomaltase activity to a microorganism or for increasing the isomaltase activity of a microorganism will be explained below. Since the isomaltase of the present invention may also have the maltase activity, the maltase activity may be imparted or increased when the isomaltase activity is imparted or increased.

[0144] When the microorganism does not originally have the isomaltase activity, the isomaltase activity can be imparted to the microorganism by introducing an isomaltase gene into the microorganism. Furthermore, when the microorganism has the isomaltase activity, by introducing a foreign isomaltase gene, increasing copy number of the endogenous isomaltase gene, or modifying an expression control sequence such as a promoter of the isomaltase gene to increase expression of the gene, the isomaltase activity can be increased. In the present invention, the expression "introduce an isomaltase gene" means not only to introduce an isomaltase gene into a microorganism which does not originally have the isomaltase activity, but also to introduce a foreign isomaltase gene into a microorganism having the isomaltase activity, and to introduce an endogenous isomaltase gene into a microorganism having the isomaltase activity to increase expression of the endogenous isomaltase gene.

[0145] In order to introduce an isomaltase gene, for example, an isomaltase gene is cloned into an appropriate vector, and a host microorganism is transformed with the obtained vector.

[0146] Examples of the vector used for transformation include a plasmid which can autonomously replicate in a chosen microorganism. Examples of plasmid autonomously replicable in a microorganism belonging to the family *Enterobacteriaceae* include pUC19, pUC18, pBR322, RSF1010, pHSG299, pHSG298, pHSG399, pHSG398, pSTV28, pSTV29, pTWV228, pTWV229 (pHSG, pSTV and pTWV series vectors are available from Takara Bio), pMW119, pMW118, pMW219, pMW218 (pMW series vectors are available from Nippon Gene), and so forth. Furthermore, plasmids for coryneform bacteria include pAM330 (Japanese Patent Laid-open No. 58-67699), pHM1519 (Japanese Patent Laid-open No. 58-77895), pSFK6 (Japanese Patent Laid-open No. 2000-262288), pVK7 (U.S. Patent Published Application

No. 2003/0175912), pAJ655, pAJ611, pAJ1844 (Japanese Patent Laid-open No. 58-192900), pCG1 (Japanese Patent Laid-open No. 57-134500), pCG2 (Japanese Patent Laid-open No. 58-35197), pCG4, pCG11 (Japanese Patent Laid-open No. 57-183799), pHK4 (Japanese Patent Laid-open No. 5-7491), and so forth.

[0147] Examples of transformation methods include treating recipient cells with calcium chloride to increase permeability for DNA, which has been reported for *Escherichia coli* K-12 (Mandel, M. and Higa, A., J. Mol. Biol., 1970, 53:159-162), preparing competent cells from cells which are at 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., 1977, Gene, 1:153-167), and so forth. Alternatively, 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 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. Sci. USA, 75:1929-1933) can also be employed. In addition, transformation of microorganisms can also be performed by the electric pulse method (Japanese Patent Laid-open No. 2-207791).

[0148] The isomaltase gene can also be introduced by introduction into a chromosome of the host microorganism. The isomaltase gene can be introduced into a chromosome of a microorganism by a method of randomly introducing it into a chromosome using a transposon or Mini-Mu (Japanese Patent Laid-open No. 2-109985, U.S. Patent No. 5,882,888, EP 805867 B1), or by homologous recombination using a sequence present on a chromosomal DNA in a multiple copy number as a target. As a sequence present on a chromosomal DNA in a multiple copy number, repetitive DNA, and inverted repeat located at the end of a transposable element can be used. Alternatively, by using the Red driven integration method (WO2005/010175), it is also possible to introduce an objective gene into a chromosome. Moreover, an objective gene can also be introduced into a chromosome by transduction using phages such as P1 phage, or by using a conjugative transfer vector. Furthermore, it is also possible to introduce the isomaltase gene using a gene unnecessary for production of an objective substance as a target, as described in WO03/040373. One or plural copies of the isomaltase gene can be introduced into a target sequence by such methods.

[0149] Transfer of an objective gene on a chromosome can be confirmed by Southern hybridization using a probe having a sequence complementary to the objective gene or a part thereof.

[0150] Although it is sufficient that the gene coding for isomaltase is amplified to more than one copy, it is preferably amplified to 2 or more copies, more preferably 3 or more copies, still more preferably 5 or more copies. When a gene which the host microorganism does not originally have is introduced, although it is sufficient that 1 or more copies are introduced, it is preferably amplified to 2 or more copies, more preferably 3 or more copies, still more preferably 5 or more copies.

[0151] Furthermore, the activity of isomaltase can be optimized by using substitution or mutation of an expression control sequence such as promoter of the isomaltase gene in combination as described later.

[0152] Examples of the method for increasing expression of the isomaltase gene include replacing an expression control sequence such as promoter of the isomaltase gene with one having an appropriate strength on a chromosomal DNA or a plasmid to enhance expression of the gene. For example, thr promoter, lac promoter, trp promoter, trc promoter, pL promoter, tac promoter, and so forth are known as frequently used promoters. Furthermore, variants of the tac promoter used in the examples described below (PtacA promoter, PtacB promoter) can also be used. Methods for evaluating strength of promoters and strong promoters are described in the paper of Goldstein and Doi (Goldstein, M.A. and Doi R.H., 1995, Prokaryotic promoters in biotechnology, Biotechnol. Annu. Rev., 1, 105-128), and so forth.

[0153] Furthermore, it is also possible to introduce nucleotide substitution for several nucleotides into a promoter region of a gene to modify it into a promoter having an appropriate strength, as disclosed in International Publication WO00/18935. Substitution of the expression control sequence can be performed in the same manner as, for example, that of the gene substitution using a temperature-sensitive plasmid. Examples of vectors having a temperature-sensitive replication origin and usable for *Escherichia coli* and *Pantoea ananatis* include, for example, the temperature-sensitive plasmid pMAN997 described in International Publication WO99/03988, derivatives thereof, and so forth. Furthermore, substitution of an expression control sequence can also be performed by a method utilizing a linear DNA such as the method called "Red-driven integration" using Red recombinase of λ phage (Datsenko, K.A. and Wanner, B.L., 2000, Proc. Natl. Acad. Sci. USA, 97:6640-6645), and the method combining the Red-driven integration method and the λ phage excision system (Cho, E.H., et al., J. Bacteriol., 184:5200-5203 (2002)) (refer to WO2005/010175). Modification of an expression control sequence can be combined with increasing copy number of gene.

[0154] Furthermore, it is known that substitution of several nucleotides in a spacer between the ribosome binding site (RBS) and translation initiation codon, especially a sequence immediately upstream from the initiation codon, greatly affects mRNA translation efficiency, and therefore this sequence can be modified to improve translation.

[0155] When an objective gene is introduced into the aforementioned amplification plasmid or chromosome, any promoter can be used to express the gene so long as the chosen promoter functions in a microorganism used. The promoter can be the native promoter for the gene, or a modified promoter. Expression of a gene can also be controlled by suitably choosing a promoter that strongly functions in a microorganism used, or by making the -35 and -10 regions

of the promoter closer to the consensus sequence.

[0156] As the isomaltase, there are isomaltase which is present in cells, i.e., in cytoplasm, and isomaltase which is present in a cell wall or a cell surface layer such as periplasm, or present extracellularly. In the present invention, the isomaltase present in cells is referred to as "intracellular isomaltase", and activity of the intracellular maltase is referred to as "intracellular activity". Furthermore, the isomaltase which is present in cells, i.e., in cytoplasm, and isomaltase which is present in a cell wall or a cell surface layer such as periplasm, or present extracellularly is generically referred to as "extracellular isomaltase", and activity of the extracellular maltase is referred to as "extracellular activity". The extracellular isomaltase typically exists in a cell surface layer. Furthermore, the expression "to express extracellular isomaltase" means to allow an extracellular isomaltase prepared on the basis of information on an extracellular isomaltase gene to exist in a cell surface layer or out of cell.

[0157] In the present invention, whether the isomaltase activity is enhanced can be confirmed by comparing the isomaltase activities of a modified strain and a parent or non-modified strain. If the isomaltase activity of the modified strain is increased as compared with the parent or non-modified strain, the isomaltase activity is enhanced. Furthermore, when the parent strain does not have the isomaltase activity, if the isomaltase activity can be detected in the modified strain, the isomaltase activity is enhanced. Furthermore, the isomaltase activity is desirably increased to such an extent that growth is not inhibited. The expression "not inhibit growth" means that the modified strain shows a growth rate corresponding to 80% or more, preferably 90% or more, more preferably 95% or more, of the growth rate of the parent or non-modified strain. Examples of the parent strain include the *Escherichia coli* MG 1655 strain (ATCC No. 47076) and W3110 strain (ATCC No. 27325), *Corynebacterium glutamicum* ATCC 13869 and ATCC 13032, and so forth.

[0158] By imparting or enhancing the extracellular isomaltase activity, ability to assimilate isomaltose can be imparted to a microorganism or ability to assimilate isomaltose of a microorganism can be increased. When the microorganism does not have isomaltase activity, by imparting the extracellular isomaltase activity, or imparting the intracellular isomaltase activity and activity to take up isomaltose, the ability to assimilate isomaltose can be increased. When the microorganism has the isomaltase activity, by improving the extracellular isomaltase activity, and/or improving the intracellular isomaltase activity and enhancing activity to take up isomaltose, the ability to assimilate isomaltose can be improved. Furthermore, when the microorganism has the activity to take up isomaltose into cells, activity of the intracellular isomaltase or the extracellular isomaltase may be imparted or enhanced. Furthermore, when the microorganism has the intracellular isomaltase activity, by enhancing the activity to take up isomaltose into cells, the ability to assimilate isomaltose can also be increased. When the intracellular isomaltase activity is to be imparted, and the microorganism does not have the activity to take up isomaltose into cells, it is preferable to impart both the intracellular isomaltase activity and the activity to take up isomaltose into cells. When the microorganism has both the intracellular isomaltase activity and the activity to take up isomaltose into cells, ability to assimilate isomaltose can be improved by enhancing either one or both of the isomaltase activity and the activity to take up isomaltose.

[0159] Examples of the isomaltase gene include the isomaltase gene of *Bacillus* bacteria, more specifically, for example, the *malL* gene (*yvdL* gene) of *Bacillus subtilis* (BG12421: Genbank Accession No. NP_391336.1), and the *glvA* gene (*malA* gene) of *Bacillus subtilis* (BG11839: Genbank Accession No. NP_388699.1). The nucleotide sequence of the *malL* gene, and the amino acid sequence of isomaltase encoded by the gene are shown in SEQ ID NOS: 13 and 14, respectively. The nucleotide sequence of the *glvA* gene, and the amino acid sequence of isomaltase encoded by the gene are shown in SEQ ID NOS: 39 and 40, respectively. If these genes are expressed in a microorganism, the intracellular isomaltase is produced. The isomaltase encoded by the *glvA* gene of *Bacillus subtilis* also has the maltase activity in addition to the isomaltase activity. Therefore, if this gene or a homologue thereof is expressed in a microorganism, a protein having both the isomaltase activity and the maltase activity is expressed. When isomaltose and maltose are contained in the carbon source of the medium, such a gene coding for an isomaltase having both the isomaltase activity and the maltase activity may be used. In addition, when the maltase activity is imparted or increased in addition to the isomaltase activity, the *glvA* gene or a homologue thereof may be used as described above, and both the isomaltase gene and a gene coding for a maltase not having the isomaltase activity may be used. In the present invention, it is preferable to use such a gene coding for an isomaltase having both the isomaltase activity and the maltase activity.

[0160] The isomaltase gene can also be obtained by PCR using oligonucleotides prepared on the basis of the aforementioned sequence information or sequence information of gene or protein known for the microorganism as primers, or hybridization using an oligonucleotide prepared on the basis of the aforementioned sequence information as a probe, from a chromosomal DNA or chromosomal DNA library of a *Bacillus* bacterium. When a gene of a *Bacillus* bacterium is used, examples of the *Bacillus* bacterium include *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus pumilus*, and so forth. Examples of *Bacillus subtilis* include the *Bacillus subtilis* 168 Marburg (ATCC 6051), *Bacillus subtilis* PY79 (Plasmid, 1984, 12, 1-9), and so forth, and examples of *Bacillus amyloliquefaciens* include the *Bacillus amyloliquefaciens* T (ATCC 23842), the *Bacillus amyloliquefaciens* N (ATCC 23845), and so forth. Furthermore, examples of *Bacillus pumilus* include *Bacillus pumilus* Gottheil No. 3218 (ATCC No. 21005, U.S. Patent No. 3,616,206), and so forth.

[0161] As genes coding for isomaltase derived from other microorganisms, for example, as homologues of the *malL* gene, the genes coding for the following proteins can be used.

Table 1

No.	Gene	Bacterium	Function
1	ACE77085	<i>Bacillus</i> sp. ZW2531-1	oligosaccharide-producing multifunctional G-amylase
2	YP_077891	<i>Bacillus licheniformis</i> ATCC 14580	glycoside hydrolase family protein
3	AAG23399	<i>Bacillus subtilis</i>	oligo-1,6-glucosidase
4	P29093	<i>Bacillus</i> sp. F5	oligo-1,6-glucosidase
5	ZP_01695723	<i>Bacillus coagulans</i> 36D1	alpha amylase, catalytic region
6	Q45101	<i>Bacillus coagulans</i>	oligo-1,6-glucosidase
7	P29094	<i>Geobacillus thermoglucosidasius</i>	oligo-1,6-glucosidase
8	ZP_02914561	<i>Geobacillus</i> sp. WCH70	alpha amylase catalytic region
9	YP_001375945	<i>Bacillus cereus</i> subsp. <i>Cytotoxis</i> NVH 391-98	alpha amylase catalytic region
10	YP_896374	<i>Bacillus thuringiensis</i> str. Al Hakam	oligo-1,6-glucosidase
11	P21332	<i>Bacillus cereus</i>	oligo-1,6-glucosidase
12	ZP_02583217	<i>Bacillus cereus</i> G9842	oligo-1,6-glucosidase
13	ZP_03102374	<i>Bacillus cereus</i> W	oligo-1,6-glucosidase
14	YP_001884389	<i>Clostridium botulinum</i> B str. Eklund 17B	oligo-1,6-glucosidase
15	YP_001919572	<i>Clostridium botulinum</i> E3 str. Alaska E43	oligo-1,6-glucosidase
16	BAE45038.	<i>Geobacillus stearothermophilus</i>	trehalose-6-phosphate hydrolase
17	NP_463715	<i>Listeria monocytogenes</i> EGD-e	hypothetical protein lmo0184
18	YP_001307875.	<i>Clostridium beijerinckii</i> NCIMB 8052	alpha amylase, catalytic region
19	YP_147599	<i>Geobacillus kaustophilus</i> HTA426	alpha,alpha-phosphotrehalase
20	NP_693477	<i>Oceanobacillus iheyensis</i> HTE831	oligo-1,6-glucosidase
21	YP_796261	<i>Lactobacillus brevis</i> ATCC 367	trehalose-6-phosphate hydrolase
22	YP_001843555	<i>Lactobacillus fermentum</i> IFO 3956	alpha-glucosidase
23	ZP_01114415	<i>Reinekea</i> sp. MED297	oligo-1,6-glucosidase

[0162] As genes coding for isomaltase derived from other microorganisms, for example, as homologues of the *glvA* gene, the genes coding for the following proteins can be used.

Table 2

No.	Gene	Bacterium	Function
1	RBAM_008360	<i>Bacillus amyloliquefaciens</i> FZB42	maltose-6'-phosphate glucosidase
2	BLi00855	<i>Bacillus licheniformis</i> ATCC 14580	maltose-6'-phosphate glucosidase
3	CLD_0473	<i>Clostridium botulinum</i> B1 str. Okra	maltose-6'-phosphate glucosidase
4	CLK_3465	<i>Clostridium botulinum</i> A3 str. Loch Maree (strain: Loch Maree)	maltose-6'-phosphate glucosidase

(continued)

No.	Gene	Bacterium	Function
5	CLI_0351	<i>Clostridium botulinum</i> F str. Langeland (strain: Langeland)	maltose-6'-phosphate glucosidase
6	CLC_0337	<i>Clostridium botulinum</i> A str. Hall (strain: Hall)	maltose-6'-phosphate glucosidase
7	CBO0278	<i>Clostridium botulinum</i> A str. ATCC 3502	maltose-6'-phosphate glucosidase
8	CAC0533	<i>Clostridium acetobutylicum</i> ATCC 824	maltose-6'-phosphate glucosidase
9	ECA1847	<i>Pectobacterium atrosepticum</i> SCRI1043	6-phospho-alpha-glucosidase
10	ESA_03956 eta	<i>Enterobacter sakazakii</i> ATCC BAA-894	maltose-6'-phosphate glucosidase
11	EcHS_A3893 ecmecfece:	<i>Escherichia coli</i> HS	6-phospho-alpha-glucosidase
12	SDY_4168	<i>Shigella dysenteriae</i> Sd197	maltose-6'-phosphate glucosidase

[0163] The gene coding for isomaltase can also be obtained by PCR using oligonucleotides prepared on the basis of the aforementioned sequence information or sequence information of gene or protein known for the microorganism as primers, or hybridization using an oligonucleotide prepared on the basis of the aforementioned sequence information as a probe from a chromosomal DNA or chromosomal DNA library of a microorganism. A chromosomal DNA can be prepared from a microorganism that serves as a DNA source by the method of Saito and Miura (refer to Saito H. and Miura K.I., 1963, Biochem. Biophys. Acta, 72, 619; Experimental Manual for Biotechnology, edited by The Society for Biotechnology, Japan, pp.97-98, Baifukan Co., Ltd., 1992) or the like.

[0164] The *malL* and *glvA* gene homologues means natural mutant genes derived from other *Bacillus* bacteria or other microorganisms, or artificial mutant genes, which show high structural similarity to the *malL* or *glvA* gene described above, and code for a protein having the isomaltase activity. Homologues of the *malL* gene mean those coding for a protein showing a homology of 80% or more, preferably 90% or more, more preferably 95% or more, still more preferably 98% or more, particularly preferably 99% or more, to the total amino acid sequence of SEQ ID NO: 14 or 40, and having the isomaltase activity. Whether a protein has the isomaltase activity or not can be confirmed by expressing the gene in a host cell, and examining the isomaltase activity by, for example, the aforementioned method. In this specification, the term "homology" may also be used to refer to "identity". The above descriptions concerning homology of the aforementioned homologues are similarly applied to the other genes.

[0165] Furthermore, the *malL* and *glvA* genes used for the present invention are not limited to wild type genes, and they may be variants of the genes, i.e., mutant or artificially modified genes coding for the amino acid sequence of SEQ ID NO: 14 or 40 including substitutions, deletions, insertions, additions or the like of one or several amino acid residues at one or several positions, so long as the isomaltase activity of the encoded protein is not impaired.

[0166] Although the number of the "one or several" amino acid residues referred to herein may differ depending on the position in the three-dimensional structure of the protein or the types of amino acid residues, specifically, it may be preferably 1 to 20, more preferably 1 to 10, still more preferably 1 to 5, particularly preferably 1 to 3. The aforementioned mutation is preferably a conservative mutation, which is a neutral mutation not causing functional change, and the conservative mutation is typically a conservative substitution. Examples of conservative substitution include: substitutions of Phe, Trp, and Tyr for each other in the case of aromatic amino acids, substitutions of Leu, Ile, and Val for each other in the case of hydrophobic amino acids, substitutions of Gln and Asn for each other in the case of polar amino acids, substitutions of Lys, Arg, and His for each other in the case of basic amino acids, substitutions of Asp and Glu for each other in the case of acidic amino acids, and substitutions of Ser and Thr for each other in the case of hydroxyl group-containing amino acids. More specifically, examples of the conservative mutations include: 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. The aforementioned amino acid substitutions, deletions, insertions, additions, inversions or the like may be a result of a naturally-occurring mutation or a variation due to an individual difference or difference of species of a microorganism having the isomaltase gene (mutant or variant).

[0167] Furthermore, the *malL* or *glvA* gene may be a DNA which is able to hybridize with a complementary sequence of the nucleotide sequence of SEQ ID NO: 13 or 39 or a probe which can be prepared from the sequence under stringent conditions and encodes a protein having the isomaltase activity. The "stringent conditions" referred to here are conditions under which a so-called specific hybrid is formed, and a non-specific hybrid is not formed. Specific examples of the stringent conditions include conditions of washing once, preferably 2 or 3 times, at a salt concentration and temperature corresponding to washing of typical Southern hybridization, i.e., 1 x SSC, 0.1% SDS at 60°C, preferably 0.1 x SSC, 0.1% SDS at 60°C, more preferably 0.1 x SSC, 0.1% SDS at 68°C. In particular, the "stringent conditions" are preferably conditions under which DNAs not less than 80% homologous, preferably not less than 90% homologous, more preferably not less than 95% homologous, still more preferably not less than 98% homologous, particularly preferably not less than 99% homologous, hybridize to each other.

[0168] Furthermore, the extracellular isomaltase gene can be obtained by ligating the aforementioned coding region of the isomaltase gene with a sequence coding for a signal peptide for secreting the protein into a cell surface layer or out of cell to modify the gene so that it expresses a fusion protein of isomaltase and the signal peptide. For example, by using a so-called secretion vector, which has a promoter sequence and a sequence coding for a signal peptide, and expresses a protein encoded by a DNA sequence inserted downstream from the foregoing sequences in a secretable form, an extracellular isomaltase gene can be constructed. Examples of such a secretion vector include pEZZ18 described in the example section. Furthermore, examples of signal peptide usable in the present invention include the protein A derived from *Staphylococcus aureus* described in the examples, and so forth (refer to EMBO J., 4 (4), 1075-1080 (1985)).

[0169] In order to impart to a microorganism the activity to take up isomaltose into the cells or increase the activity of the microorganism to take up isomaltose into the cells, for example, a gene coding for an isomaltose transporter can be introduced into the microorganism. Furthermore, when the microorganism inherently has an isomaltose transporter gene, by increasing copy number of the endogenous isomaltase gene, modifying an expression control sequence such as promoter of the isomaltose transporter gene, increasing copy number of the isomaltose transporter gene, or the like, expression of the gene can be increased to increase the activity to take up isomaltose into cells. Furthermore, when the microorganism inherently has an isomaltose transporter gene, the activity to take up isomaltose into cells may be increased by introducing an exogenous or endogenous isomaltose transporter gene. Furthermore, the isomaltose transporter gene may also have a function to take up maltose in addition to isomaltose.

[0170] Acquisition of the isomaltose transporter gene, introduction of the gene into a microorganism, and enhancement of isomaltose transporter gene expression can be performed in the same manners as those for the aforementioned isomaltase gene. Impartation or increase of the activity to take up isomaltose and/or maltose into cells can be confirmed by, for example, the method described in J. Bacteriol., 1991 Apr.; 173(7):2180-6 or J. Biol. Chem., 2006 Jun., 30;281 (26):17900-8.

[0171] As the isomaltose transporter gene, it is preferable to use the *glvC* (*malP*) gene of *Bacillus subtilis*. The *glvC* gene of *Bacillus subtilis* is a gene coding for maltose enzyme IICB (PTS maltose enzyme IICB) of the phosphotransferase system (PTS), and this enzyme has an activity to transfer the phosphate group of phosphoenolpyruvate to isomaltose and/or maltose, and to take up phosphorylated isomaltose and/or maltose into the cytoplasm.

[0172] Furthermore, other isomaltose transporter genes include the genes coding for the sugar transporters MsmE, MsmF and MsmG, which belong to the multiple sugar metabolism system of *Streptococcus mutans*, and the *AGT1* gene of *Saccharomyces cerevisiae*. These genes are explained in detail in the examples.

[0173] The amino acid sequence of a protein encoded by the *glvC* gene, and the gene coding it are shown in SEQ ID NOS: 42 and 41, respectively (corresponding to Genbank Accession No. BG11848).

[0174] Furthermore, the amino acid sequences of MsmE, MsmF and MsmG are shown in SEQ ID NOS: 16, 18 and 20, respectively, and the nucleotide sequences of the genes coding for them are shown in SEQ ID NOS: 15, 17 and 19, respectively (corresponding to Genbank Accession No. NP_721287.1, NP_721288.1 and NP_721289.1, respectively). The nucleotide sequence of the *AGT1* gene, and the amino acid sequence encoded thereby are shown in SEQ ID NOS: 21 and 22, respectively (corresponding to Genbank Accession No. AAY99642).

[0175] Furthermore, as genes coding for isomaltose transporter, the following genes mentioned in Table 3 can be used.

Table 3

No.	Gene	Bacterium	Function
1	YP_001420453	<i>Bacillus amyloliquefaciens</i> FZB42	phosphotransferase system IIC components glucose/maltose/N-acetylglucosamine-specific carbohydrate transport and metabolism
2	YP_078080	<i>Bacillus licheniformis</i> ATCC 14580	phosphotransferase system (PTS) maltose-specific enzyme IICB component
3	AAK69555	<i>Clostridium acetobutylicum</i>	PTS _{IIB} , PTS system, glucose/sucrose Specific IIB subunit
4	ZP_02631647	<i>Clostridium perfringens</i> E str. JGS1987	PTS system maltose-specific eiicb Component
5	ZP_02638912	<i>Clostridium perfringens</i> CPE str. F4969	PTS system maltose-specific eiicb Component
6	ZP_02642923	<i>Clostridium perfringens</i> NCTC 8239	PTS system maltose-specific eiicb Component
7	NP_561112	<i>Clostridium perfringens</i> str. 13	PTS arbutin-like enzyme IIBC component
8	NP_290321	<i>Escherichia coli</i> O157:H7 EDL933	putative PTS component
9	YP_001746010	<i>Escherichia coli</i> SMS-3-5	PTS system, alpha-glucoside-specific IIBC component
10	ZP_03050141	<i>Escherichia coli</i> E110019	PTS system, alpha-glucoside-specific IIBC component
11	NP_561112	<i>Clostridium perfringens</i> str. 13	PTS arbutin-like enzyme IIBC component
12	YP_854647	<i>Aeromonas hydrophila</i> subsp. <i>hydrophila</i> ATCC 7966	PTS system maltose-specific eiicb component
13	006900	<i>Fusobacterium mortiferum</i>	PTS system alpha-glucoside-specific EIICB component
14	YP_001143948	<i>Aeromonas salmonicida</i> subsp. <i>salmonicida</i> A449	PTS system alpha-glucoside-specific EIICB component
15	ZP_03124405	<i>Clostridium difficile</i> QCD-23m63	PTS system, IIBC component
16	YP_187123	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> COL	PTS system, IIBC components
17	YP_001785613	<i>Clostridium botulinum</i> A3 str. Loch Maree	PTS system, glucose/glucoside family, IIBC component

[0176] The isomaltose transporter gene derived from a *Bacillus* bacterium or another microorganism, for example, *glvC*, genes coding for MsmE, MsmF and MsmG, and homologues of the *AGT1* gene can also be obtained by PCR using oligonucleotides prepared on the basis of the aforementioned sequence information or sequence information of gene or protein known for the microorganism as primers, or hybridization using an oligonucleotide prepared on the basis of the aforementioned sequence information as a probe from a chromosomal DNA or chromosomal DNA library of a microorganism. As for the aforementioned gene homologues, the above descriptions for the *malL* gene homologues are applied. Furthermore, the descriptions concerning variants of the *malL* and *glvA* genes are also applied to the isomaltose transporter genes.

[0177] It is estimated that even if the activity to take up isomaltose into cells is imparted or enhanced, uptake of glucose by a microorganism is not substantially influenced.

[0178] By culturing the microorganism of the present invention in a medium, a target substance corresponding to the target substance-producing ability of the microorganism is produced in culture, i.e., microbial cells or the medium. By collecting the target substance from cells or the medium, the target substance can be produced.

[0179] As the medium to be used, media conventionally used in the production of target substances by fermentation

using a microorganism can be used. That is, usual media containing a carbon source, nitrogen source, inorganic ions, and other organic components as required can be used. The carbon source preferably contains isomaltose, more preferably isomaltose and glucose, still more preferably isomaltose, maltose and glucose. In a preferred embodiment, the microorganism of the present invention does not show two-stage proliferation, so-called diauxy, in a medium containing glucose and isomaltose, or glucose, isomaltose and maltose as a carbon source. Examples of such a carbon source containing isomaltose and glucose, or isomaltose, maltose and glucose include, for example, hydrolysates or enzymatic decomposition products of starches.

[0180] As carbon sources other than glucose, isomaltose and maltose, saccharides such as lactose and galactose, alcohols such as glycerol and sorbitol, organic acids such as fumaric acid, citric acid and succinic acid, and so forth can be used.

[0181] In the present invention, when it is intended to assimilate maltose or isomaltose contained in a hydrolyzed solution of starch as contaminants other than glucose, each of isomaltose and maltose is preferably contained in an amount of 0.1 to 10% by weight based on the weight of the total carbon source.

[0182] Furthermore, in the present invention, when isomaltose is used as a carbon source, isomaltose is preferably contained in an amount of 1 to 50% by weight, preferably 1 to 20% by weight, more preferably 1 to 10% by weight, based on the weight of the total carbon source. Furthermore, glucose is preferably contained in an amount of 50 to 99% by weight, preferably 80 to 99% by weight, more preferably 90 to 99% by weight, based on the weight of the total carbon source. The weight ratio of isomaltose and glucose is preferably 1:1 to 1:100, more preferably 1:5 to 1:100, still more preferably 1:10 to 1:100.

[0183] Furthermore, when maltose is used as a carbon source in addition to isomaltose, maltose is preferably contained in an amount of 1 to 50% by weight, preferably 1 to 20% by weight, more preferably 1 to 10% by weight, based on the weight of the total carbon source. Furthermore, glucose is preferably contained in an amount of 50 to 99% by weight, preferably 80 to 99% by weight, more preferably 90 to 99% by weight, based on the weight of the total carbon source. The weight ratio of maltose and glucose is preferably 1:1 to 1:100, more preferably 1:5 to 1:100, still more preferably 1:10 to 1:100.

[0184] Concentrations of isomaltose and maltose in the medium can be quantified by HPLC.

[0185] As the nitrogen source, inorganic ammonium salts such as ammonium sulfate, ammonium chloride, and ammonium phosphate, organic nitrogen such as soybean hydrolysate, ammonia gas, aqueous ammonia, and so forth may be used.

[0186] As organic trace nutrient sources, it is desirable that the medium contains required substances such as vitamin B₁, L-homoserine, and L-tyrosine, yeast extract, and so forth in appropriate amounts. Other than the above, potassium phosphate, magnesium sulfate, iron ions, manganese ions and so forth are added in small amounts, as required.

[0187] The culture can be performed under conventionally used well-known conditions selected according to a microorganism to be utilized. For example, the culture is preferably performed under aerobic conditions for 16 to 120 hours. The culture temperature is preferably controlled to be 25°C to 45°C, and pH is preferably controlled to be 5 to 8 during the culture. Inorganic or organic, acidic or alkaline substances as well as ammonia gas or the like can be used for pH adjustment.

[0188] When a basic amino acid such as L-lysine is produced, the production may be performed by a method in which fermentation is performed by controlling pH of the medium during culture to be 6.5 to 9.0 and pH of the medium at the end of the culture to be 7.2 to 9.0 and controlling the pressure in the fermentation tank to be positive during the fermentation, or by supplying carbon dioxide gas or a mixed gas containing carbon dioxide gas to the medium to provide a culture period where the medium contains 2g/L20mM or more of bicarbonate ions and/or carbonate ions, so that these bicarbonate ions and/or carbonate ions serve as counter ions of cations mainly consisting of a basic amino acid, and the objective basic amino acid is then collected (Japanese Patent Laid-open No. 2002-65287, U.S. Patent Published Application No. 2002/0025564, EP 1813677 A).

[0189] When a microorganism having a basic amino acid-producing ability is cultured in a medium under aerobic conditions, carbonate ions, bicarbonate ions, or both can be used as major counter ions of the basic amino acid. To provide carbonate ions and/or bicarbonate ions in an amount required to serve as counter ions of the basic amino acid, it is known that the pH of the medium can be controlled to be 6.5 to 9.0, preferably 6.5 to 8.0, during the culture, and can be 7.2 to 9.0 at the end of the culture. The pressure in the fermentation tank can be controlled so that it is positive during fermentation, or carbon dioxide or a mixed gas containing carbon dioxide can be supplied into the medium (Japanese Patent Laid-open No. 2002-65287, U.S. Patent Published Application No. 2002/0025564, EP 1813677 A).

[0190] In the present invention, pressure in the fermentation tank may be controlled to be positive during the fermentation, and at the same time, carbon dioxide gas or a mixed gas containing carbon dioxide gas may be supplied to the medium. Both the above operations are preferably performed so that there is a culture period when preferably 20 mM or more, more preferably 30 mM or more, particularly preferably 40 mM or more, of bicarbonate ions and/or carbonate ions are present in the medium. The internal pressure of the fermentation tank, supply amount of carbon dioxide or mixed gas containing carbon dioxide, or the limited gas supply volume can be determined by, for example, measuring

bicarbonate ions or carbonate ions in the medium, or the pH or ammonia concentration of the medium.

[0191] In the above embodiment, the pH of the medium is controlled to be 6.0 to 9.0, preferably 6.5 to 8.0, during the culture, and 7.2 to 9.0 at the end of the culture. According to the above embodiment, it is possible to suppress the pH of the medium so that the amount of bicarbonate ions and/or carbonate ions present in the medium to serve as counter ions is lower as compared to the conventional methods. When the pH is controlled with ammonia, ammonia is supplied in order to increase the pH, and it can serve as a nitrogen source for the basic amino acid. Examples of cations other than the basic amino acid in the medium include K, Na, Mg, Ca etc. originating in medium components. These can exist in an amount of 50% or less of the total cations.

[0192] Furthermore, the internal pressure of the fermentation tank during fermentation can be made positive by, for example, making the gas supply pressure higher than the exhaust pressure. By making the internal pressure of the fermentation tank positive, the carbon dioxide generated by fermentation dissolves in the culture medium to generate bicarbonate ions or carbonate ions, and these can serve as counter ions of the basic amino acid. The internal pressure of the fermentation tank is, specifically, 0.03 to 0.2 MPa, preferably 0.05 to 0.15 MPa, more preferably 0.1 to 0.3 MPa, in terms of the gage pressure (pressure difference with respect to the atmospheric pressure). Moreover, by supplying carbon dioxide or a mixed gas containing carbon dioxide to the culture medium, carbon dioxide may be dissolved in the medium. Furthermore, when supplying carbon dioxide or a mixed gas containing carbon dioxide to the medium, the internal pressure of the fermentation tank may be adjusted to be positive.

[0193] The internal pressure of the fermentation tank may be adjusted to be positive by, for example, making the gas supply pressure higher than the exhaust pressure. Furthermore, when carbon dioxide is supplied to the medium, for example, pure carbon dioxide or a mixed gas containing 5 volume % or more of carbon dioxide may be bubbled in the medium.

[0194] The aforementioned methods for dissolving bicarbonate ions and/or carbonate ions in the medium may be used independently, or two or more of them may be used in combination.

[0195] In the conventional methods, a sufficient amount of ammonium sulfate or ammonium chloride is usually added to the medium to serve as counter anions of the basic amino acid to be produced. Sulfuric acid or hydrochloric acid decomposition products of proteins etc. are also added to the medium as a nutrient component, and thus the generated sulfate ions and chloride ions are present in the medium. Therefore, the concentration of the weakly acidic carbonate ions is extremely low during the culture, such as a ppm order. The above embodiment of the present invention is characterized in that these sulfate ions and chloride ions are reduced, and the carbon dioxide released by the microorganism during fermentation is dissolved in the medium in the aforementioned fermentation environment and can be used as counter ions. Therefore, in the above embodiment of the present invention, it is not required to add sulfate ions or chloride ions to the medium in an amount more than the amount required for the growth. An appropriate amount of ammonium sulfate or the like can be added to the medium at an early stage of the culture, and the addition is terminated in the middle of the culture. Alternatively, ammonium sulfate or the like may be added while maintaining the balance with the dissolved carbonate ions or bicarbonate ions in the medium. Moreover, as a nitrogen source of the basic amino acid, ammonia may be added to the medium. Ammonia may be supplied to the medium independently, or together with other gases.

[0196] Lower concentrations of anions other than bicarbonate ions and/or carbonate ions in the medium are more preferred so long as they are present in amounts that are required for the growth of the microorganism. Examples of such anions include chloride ions, sulfate ions, phosphate ions, ionized organic acids, hydroxide ions, and so forth. The total molar concentration of these other ions is preferably usually 900 mM or lower, more preferably 700 mM or lower, still more preferably 500 mM or lower, further preferably 300 mM or lower, particularly preferably 200 mM or lower.

[0197] To reduce the necessary amounts of sulfate ions and/or chloride ions is one of the objects of the above embodiment of the present invention, and the total amount of sulfate ions or chloride ions, or both contained in the medium is usually 700 mM or lower, preferably 500 mM or lower, more preferably 300 mM or lower, still more preferably 200 mM or lower, particularly preferably 100 mM or lower.

[0198] If ammonium sulfate is added to a medium as a counter ion source of a basic amino acid, carbon dioxide in the culture medium is usually eliminated by sulfate ions. However, in the above embodiment of the present invention, it is not necessary to add an excess amount of ammonium sulfate to the medium, and therefore carbon dioxide can be easily dissolved in the fermentation medium.

[0199] Furthermore, in the above embodiment of the present invention, it is preferable to control the total ammonia concentration in the medium to such an extent that "production of the basic amino acid is not inhibited". Examples of the the conditions to attain such a purpose include, for example, when the yield and/or productivity is preferably 50% or more, more preferably 70% or more, particularly preferably 90% or more, of the yield and/or productivity which is obtained under optimal conditions. Specifically, the total ammonia concentration in the medium is preferably 300 mM or lower, more preferably 250 mM or lower, particularly preferably 200 mM or lower. The dissociation degree of the ammonia decreases as the pH becomes higher. Non-dissociating ammonia is more toxic to bacteria as compared to ammonium ions. Therefore, the upper limit of the total ammonia concentration should be determined depending on the pH of the

culture medium. That is, as the pH of the culture medium increases, the acceptable total ammonia concentration decreases. Therefore, the aforementioned total ammonia concentration "which does not inhibit the basic amino acid production" is preferably determined for each specific pH value. However, the total ammonia concentration range that is acceptable at the highest pH level during the culture may be used as the upper limit of the total ammonia concentration throughout the entire culture period.

[0200] On the other hand, the total ammonia concentration which functions as a source of nitrogen required for growth of the microorganism and production of the basic substance is not particularly limited, and can be appropriately determined, so long as an a reduced level of the nitrogen source which can result in continuous depletion of ammonia during the culture does not reduce productivity of the objective substance by the microorganism. For example, the ammonia concentration can be measured over time during the culture, and if ammonia in the medium is depleted, a small amount of ammonia may be added to the medium. Although the total ammonia concentration after the addition of ammonia is not particularly limited, the total ammonia concentration may be, for example, preferably 1 mM or higher, more preferably 10 mM or higher, particularly preferably 20 mM or higher.

[0201] Furthermore, in L-glutamic acid fermentation, the culture can be performed with precipitating L-glutamic acid in the medium by using a liquid medium adjusted to have a condition under which L-glutamic acid is precipitated. The condition under which L-glutamic acid is precipitated is, for example, pH 5.0 to 4.0, preferably pH 4.5 to 4.0, more preferably pH 4.3 to 4.0, particularly preferably pH 4.0 (European Patent Laid-open No. 1078989).

[0202] Furthermore, for the production of a nucleic acid, by allowing purine nucleoside phosphorylase and phosphoribosyltransferase to act on inosine or guanosine prepared by the method of the present invention, 5'-inosinic acid or 5'-guanylic acid can be obtained.

[0203] Moreover, it is also possible to phosphorylate the purine nucleoside produced using the microorganism of the present invention by allowing phosphotransferase to act on the purine nucleoside to produce a purine nucleotide (nucleoside 5'-phosphoric acid ester) (Japanese Patent Laid-open No. 2000-295996). For example, the method for producing a purine nucleotide using an *Escherichia* bacterium into which a gene encoding inosine guanosine kinase of *Escherichia coli* is introduced (WO91/08286), and the method for producing a purine nucleotide using *Corynebacterium ammonia-*
genes into which a gene encoding inosine guanosine kinase of *Exiguobacterium acetylicum* is introduced (WO96/30501) can be used.

[0204] Moreover, it is also possible to produce a purine nucleotide (nucleoside 5'-phosphoric acid ester) by allowing a microorganism which is able to produce a nucleoside 5'-phosphoric acid ester or acid phosphatase (EC 3.1.3.2) to act on the purine nucleoside produced by using the microorganism of the present invention and a phosphate donor selected from the group consisting of polyphosphoric acid, phenyl phosphate and carbamyl phosphate. Although the microorganism able to produce a nucleoside 5'-phosphoric acid ester is not particularly limited so long as a microorganism which is able to convert a purine nucleoside into a purine nucleotide is chosen, examples include, for example, the microorganism disclosed in International Publication WO96/37603.

[0205] Moreover, *Escherichia blattae* JCM 1650, *Serratia ficaria* ATCC 33105, *Klebsiella planticola* IFO 14939 (ATCC 33531), *Klebsiella pneumoniae* IFO 3318 (ATCC 8724), *Klebsiella terrigena* IFO 14941 (ATCC 33257), *Morganella morganii* IFO 3168, *Enterobacter aerogenes* IFO 12010, *Enterobacter aerogenes* IFO 13534 (ATCC 13048), *Chromobacterium fluviatile* IAM 13652, *Chromobacterium violaceum* IFO 12614, *Cedecea lapagei* JCM 1684, *Cedecea davisiae* JCM 1685, *Cedecea neteri* JCM 5909, and so forth disclosed in Japanese Patent Laid-open No. 07-231793 can also be used.

[0206] As the acid phosphatase, for example, those disclosed in Japanese Patent Laid-open No. 2002-000289 can be used, and an acid phosphatase with increased affinity to a nucleoside (Japanese Patent Laid-open No. 10-201481), a mutant acid phosphatase with decreased nucleotidase activity (WO96/37603), a mutant acid phosphatase with decreased phosphoric acid ester hydrolysis activity (Japanese Patent Laid-open No. 2001-245676) and so forth can be more preferably used.

[0207] It is also possible to obtain a purine nucleotide by chemically phosphorylating a purine nucleoside produced using the microorganism of the present invention (Bulletin of the Chemical Society of Japan, 42, 3505). Moreover, a method of obtaining GMP by coupling the microorganism having with an XMP-producing ability of the present invention and XMP aminase activity using the ATP-regenerating system of a microorganism, and a method of obtaining IMP by coupling inosine kinase (Biosci. Biotech. Biochem., 51, 840 (1997); Japanese Patent Laid-open No. 63-230094) can also be used.

[0208] Inosine, guanosine or purine nucleosides prepared by the method of the present invention used for the aforementioned preparation of purine nucleotide may be a purified product, or a purine nucleoside fermentation broth, or a crude product containing a purine nucleoside.

[0209] For collection of L-amino acid or nucleic acid from the medium after completion of the culture, any special method is not required for the present invention. The L-amino acid or nucleic acid collected in the present invention can contain bacterial cells, medium components, moisture, and by-product metabolites of the microorganism in addition to the L-amino acid or nucleic acid. Purity of the collected L-amino acid or nucleic acid is, for example, 50% or higher,

preferably 85% or higher, particularly preferably 95% or higher (U.S. Patent No. 5,431,933, Japanese Patent Publication No. 1-214636, U.S. Patent Nos. 4,956,471, 4,777,051, 4,946,654, 5,840,358, 6,238,714, U.S. Patent Published Application No. 2005/0025878).

[0210] The L-amino acid obtained in the present invention can be collected by a combination of conventionally known ion-exchange resin method (Nagai, H. et al., Separation Science and Technology, 39(16), 3691-3710), membrane separation method (Japanese Patent Laid-open Nos. 9-164323 and 9-173792), crystallization method (WO2008/078448, WO2008/078646), and other methods.

[0211] Furthermore, when the target substance is L-phenylalanine, L-phenylalanine produced by the method of the present invention may be used for, for example, producing a lower alkyl ester of α -L-aspartyl-L-phenylalanine (also referred to as "aspartame"). From L-phenylalanine obtained by dissolving L-phenylalanine crystals separated by the aforementioned method of the present invention, and aspartic acid or a derivative thereof, a lower alkyl ester of α -L-aspartyl-L-phenylalanine can be produced. Examples of the lower alkyl ester include methyl ester, ethyl ester, propyl ester, and so forth.

[0212] The method for synthesizing a lower alkyl ester of α -L-aspartyl-L-phenylalanine from L-phenylalanine and aspartic acid or its derivative is not particularly limited, so long as L-phenylalanine or its derivative is used for the synthesis of the lower alkyl ester of α -L-aspartyl-L-phenylalanine. Specifically, for example, a lower alkyl ester of α -L-aspartyl-L-phenylalanine can be produced by the following method (U.S. Patent No. 3,786,039). L-Phenylalanine is esterified to obtain a lower alkyl ester of L-phenylalanine. The L-phenylalanine alkyl ester is reacted with an L-aspartic acid derivative of which a β -carboxyl group is protected and an α -carboxyl group is esterified to activate. Examples of such a derivative include N-acyl-L-aspartic anhydride such as N-formyl-, N-carbobenzoxy-, or N-p-methoxycarbobenzoxy-L-aspartic anhydride. By this condensation reaction, a mixture of N-acyl- α -L-aspartyl-L-phenylalanine and N-acyl- β -L-aspartyl-L-phenylalanine is obtained. If the condensation reaction is performed in the presence of an organic acid of which acid dissociation constant at 37°C is 10^{-4} or less, ratio of the α -isomer to the β -isomer in the mixture is increased (Japanese Patent Laid-Open No. 51-113841). Then, the N-acyl- α -L-aspartyl-L-phenylalanine is separated from the mixture, and hydrogenated to obtain α -L-aspartyl-L-phenylalanine.

[0213] Furthermore, α -L-aspartyl-L-phenylalanine- α -ester can be conveniently produced at high yield by producing α -L-aspartyl-L-phenylalanine- β -ester from L-phenylalanine and L-aspartic acid- α,β -diester using an enzyme having an ability to catalyze a reaction in which L-phenylalanine does not nucleophilically attack the β -ester moiety of L-aspartic acid- α,β -diester, but nucleophilically attacks the α -ester moiety or a substance containing such an enzyme, to produce α -L-aspartyl-L-phenylalanine- β -ester, and producing α -L-aspartyl-L-phenylalanine- α -ester from the obtained α -L-aspartyl-L-phenylalanine- β -ester (WO2004/065610).

[0214] Furthermore, a technique of synthesizing a dipeptide using a mutant peptide synthetase derived from *Sphingobacterium* bacteria is disclosed, and α -L-aspartyl-L-phenylalanine can be produced using this technique (WO2006/075486).

Examples

[0215] Hereafter, the present invention will be explained more specifically with reference of examples. In the following examples, reagents of Wako Pure Chemical Industries or Nakalai Tesque were used, unless especially specified. The compositions of the media used in the examples are as shown below.

[L medium]

10 g/L of Bacto trypton (Difco), 5 g/L of yeast extract (Difco), 5 g/L of NaCl

[0216] The medium was steam-sterilized at 120°C for 20 minutes.

L agar medium

L medium, 15 g/L of Bacto agar (Difco)

[0217] The medium was steam-sterilized at 120°C for 20 minutes.

Amino acid production medium

[0218] 20 g/L of $(\text{NH}_4)_2\text{SO}_4$, 1 g/L of KH_2PO_4 , 1 g/L of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg/L of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg/L of $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 2 g/L of yeast extract (Becton, Dickinson and Company), 40 g/L of saccharide (glucose or maltose or mixture of these at a suitable ratio), 100 mg/L of L-tyrosine, 30 g/L of CaCO_3 (Japanese Pharmacopoeia), 50 mg/L of

streptomycin or ampicillin

[0219] The saccharide, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and the antibiotic were separately sterilized. The other components were mixed, adjusted to pH 7.0 with KOH and autoclaved at 115°C for 10 minute. CaCO_3 was subjected to dry sterilization at 180°C for 2 days. Streptomycin was sterilized by filtration. Ampicillin was used as a 70% solution in ethanol.

PCR

[0220] The enzyme used was Pyrobest DNA Polymerase produced by Takara Shuzo or KOD DNA Polymerase produced by Toyobo, PCR was performed according to the protocol attached to the enzyme, and after completion the reaction, gel filtration was performed in order to remove the residual primers. The used column was Microspin™ S-400HR Columns produced by Amersham Pharmacia Biotech, and the column was used according to the protocol attached to the column.

Ligation reaction/blunting-kination reaction

[0221] Ligation Kit ver. 2 or BKL Kit of Takara Bio was used, and the reactions were performed according to the protocol attached to the kit.

Transformation of *E. coli*

[0222] *E. coli* was transformed with a ligation reaction mixture or a plasmid DNA according to the method of Hanahan et al. (Hanahan, D., 1983, Studies on transformation of *Escherichia coli* with plasmids, J. Mol. Biol., 166, 557-580).

Reference

[0223] Example 1: Amino acid production by strain extracellularly expressing isomaltose

<1> Cloning of the *malL* gene

[0224] A colony of the *Bacillus subtilis* Marburg 168 strain (BGSC1A1 strain, obtained from *Bacillus* Genetic Stock Center) was inoculated to 5 ml of the L medium, and culture was performed overnight with shaking. Chromosomal DNA was prepared from the culture medium by using Wizard Genomic DNA Purification Kit (Promega). By using this chromosomal DNA as a template, PCR was performed. The sequences of two primers used are shown below.

Primer 1

5'-GAGAATTCAATGAGTGAATGGTGGAAAGAAGCTGTCG-3' (SEQ ID NO: 1)

Primer 2

5'-CGAGAAAATGATATTCTGCAGTATCTGTTATCACTCCGTC-3' (SEQ ID NO: 2)

[0225] The nucleotide numbers 3 to 8 in SEQ ID NO: 1 correspond to the EcoRI site, and the nucleotide numbers 16 to 21 in SEQ ID NO: 2 correspond to the PstI site.

[0226] The obtained PCR product was treated with the restriction enzymes EcoRI and PstI, and ligated with the secretion vector pEZZ18 (Amersham Pharmacia Biotech) treated with the same restriction enzymes. The pEZZ18 described above comprises pBR322 and the replication origin of f1 phage, in which a multi-cloning site present upstream of *lacZα*, and the signal sequence of the protein A of *Staphylococcus aureus* required for secretion and two IgG binding regions (ZZ region) which are present further upstream of the multi-cloning site. If a gene is inserted downstream of the ZZ region, the protein encoded by the gene is expressed as a fusion protein with the signal sequence and the ZZ region, and the fusion protein is secreted out of the *E. coli* cells, and then can be easily purified by affinity chromatography using the ZZ region. The aforementioned PCR product was ligated to pEZZ18 so that the isomaltose decomposition enzyme encoded by the *malL* gene should form a fusion protein with the signal sequence of the protein A and the IgG binding region (Z region) locating downstream therefrom.

[0227] The ligation reaction mixture was used to transform *E. coli* JM109. Selection of the transformants was performed on the L agar medium containing 50 μg/ml of ampicillin, 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and 40 μg/ml of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). A plasmid was extracted from the transformants, and insertion of the target fragment was confirmed. This plasmid was designated pEZZmalL.

<2> Construction of vector plasmid pRS

[0228] PCR was performed by using the plasmid pVIC40 (WO90/04636) as a template DNA, and two oligonucleotides shown below as primers.

[0229] pVIC40 is a plasmid obtained by inserting the *thrA*BC* operon containing a mutant *thrA* gene into a vector derived from RSF1010 and having a streptomycin resistance gene, and can be prepared from the *Escherichia coli* VKPM B-3996 strain harboring this plasmid. The B-3996 strain was deposited at Russian National Collection of Industrial Microorganisms (VKPM, 1 Dorozhny proezd., 1 Moscow 117545, Russia) on April 7, 1987 under an accession number of B-3996.

Primer 3

5'-GCGCGAATTCCAACGGGCAATATGTCTCTG-3' (SEQ ID NO: 3)

Primer 4

5'-GCGCCAATTGGATGTACCGCCGAATTCAA-3' (SEQ ID NO: 4)

[0230] The obtained PCR product was treated with the restriction enzymes *EcoRI* and *MunI*. Separately, pVIC40 was digested with the restriction enzyme *EcoRI*, and the longest fragment was chosen from the digested DNA fragments, and ligated with the aforementioned PCR fragment. The ligation reaction mixture was used to transform *E. coli* JM109. Transformants were obtained on the L agar medium containing 50 µg/ml of streptomycin. PCR was performed by using plasmids prepared from the transformants as a template, Primer 3 described above, and Primer 5 described below, and a plasmid for which a band of about 270 bp was detected was chosen, and designated pRS. pRS had a structure of pVIC40 from which the *thrABC* operon was removed.

Primer 5

5'-CAGTTTTCTGATGAAGCGCG-3' (SEQ ID NO: 5)

<3> Construction of plasmid pRSL containing *lacZα* gene

[0231] PCR was performed by using pSTV29 (Takara Shuzo) as a template and two oligonucleotides shown below as primers. pSTV29 is a low copy number plasmid containing the *lacZ* gene.

Primer 6

5'-CCAATACGCAAACCGCCTCTCC-3' (SEQ ID NO: 6)

Primer 7

5'-CAAATGTAGCACCTGAAG-3' (SEQ ID NO: 7)

[0232] The obtained *lacZα* fragment was ligated with a fragment obtained by digesting pRS with the restriction enzyme *PstI* and blunting the resultant. The ligation reaction mixture was used to transform *E. coli* JM109. Selection of the transformants was performed on the L agar medium containing 50 µg/ml of ampicillin, 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and 40 µg/ml of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). A plasmid was extracted from the transformants, and insertion of the target fragment was confirmed. This plasmid was designated pRSL.

<4> Insertion of the *malL* gene into pRSL

[0233] A *malL* gene fragment having a sequence for the signal sequence of the protein A was amplified by PCR using pEZZmalL as a template DNA and the following primers.

Primer 8

5'-CATTAGGCACCCCAAGCTTTACACTTTATGCTTCC-3' (SEQ ID NO: 8)

Primer 9

5'-GACGGCCAGTGCCAAGCTTGCATGCCTGCAGG-3' (SEQ ID NO: 9)

[0234] The nucleotide numbers 14 to 19 in SEQ ID NOS: 8 and 9 correspond to *HindIII* sites.

[0235] The obtained PCR product was treated with the restriction enzyme *HindIII*, and ligated with the vector pRSL similarly treated with *HindIII*. The ligation reaction mixture was used to transform *E. coli* JM109. Selection of the transformants was performed on the L agar medium containing 50 µg/ml of streptomycin (Sm), 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and 40 µg/ml of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). A plasmid was extracted from the transformants, and insertion of the target fragment was confirmed. This plasmid was designated pRSLmalL.

<5> Evaluation of amino acid production culture

[0236] pRSLmalL was introduced into an amino acid-producing bacterium, and ability to assimilate isomaltose and amino acid productivity thereof were investigated.

[0237] As an L-phenylalanine-producing bacterium, a strain obtained by introducing the plasmid pMW118 or pMGAL1 (Japanese Patent Laid-open No. 5-344881) into *E. coli* W3110(tyrA) (refer to European Patent Laid-open No. 488424) was used. Furthermore, as an L-lysine-producing bacterium, *E. coli* WC196 Δ cadA Δ ldcC (also referred to as "WC196LC", WO2006/078039) was used. These strains were transformed with pRSLmalL.

[0238] The W3110(tyrA) strain can be obtained by removing the plasmid pHATerm from *Escherichia coli* W3110(tyrA)pHATerm. The *E. coli* W3110(tyrA)pHATerm strain was designated as AJ12662 strain, and deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (currently the independent administrative agency, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary, Tsukuba Central 6, 1-1, Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, 305-8566, Japan) as an international deposit on November 16, 1991, and assigned an accession number of FERM BP-3653 (WO01/053459).

[0239] pMGAL1 contains a gene coding for 3-deoxy-D-arabinohepturonate-7-phosphate synthase derived from *Escherichia* bacterium and desensitized to feedback inhibition, and a gene coding for chorismate mutase-prephenate dehydratase derived from *Escherichia* bacterium and desensitized to feedback inhibition (Japanese Patent Laid-open Publication No. 5-344881).

[0240] Each of the obtained transformants was inoculated into 5 ml of the L medium containing an antibiotic (50 μ g/ml of streptomycin for the strains harboring pRSL or pRSLmalL, 50 μ g/ml of ampicillin for the strains harboring pMW118 or pMGAL1) and cultured at 37°C overnight with shaking. Then, 50 μ l of the culture medium was applied to the same L agar medium as described above and cultured overnight at 37°C. An amino acid production medium containing a mixture of glucose and isomaltose as the carbon source was introduced in a volume of 20 ml into a 500-ml volume Sakaguchi flask, and the cells grown on the aforementioned agar medium corresponding to 1/8 of the plate were scraped and inoculated into the medium contained in the Sakaguchi flask. Initial concentrations of glucose and isomaltose were 36 g/L and 6.6 g/L, respectively. After 24 hours, amino acid concentrations, remained glucose and isomaltose were quantified. As controls, transformants obtained by introducing pRSL into *E. coli* W3110(tyrA)pMW118, W3110(tyrA)pMGAL1, and WC196LC were used. The results are shown in Table 4.

Table 4: Amino acid production culture

Host	Plasmid		Phenylalanine (g/L)	Lysine (g/L)	Residual glucose (g/L)	Residual maltose (g/L)
W3110(tyrA)	pMW118	pRSL	0.96	-	0.00	6.22
W3110(tyrA)	pMW118	pRSLmalL	1.33	-	0.00	0.00
W3110(tyrA)	pMGAL1	pRSL	5.38	-	0.00	6.18
W3110(tyrA)	pMGAL1	pRSLmalL	6.48	-	0.00	0.00
WC196LC	-	pRSL	-	1.25	0.00	6.27
WC196LC	-	pRSLmalL	-	1.70	0.00	1.85
- : Not tested						

[0241] When the vector pRSL was introduced into *E. coli* W3110(tyrA)pMW118 or W3110(tyrA)pMGAL1, isomaltose was not assimilated, but when pRSLmalL was introduced, isomaltose was assimilated in the same culture time. Similarly, when pRSL was introduced into *E. coli* WC196LC, isomaltose was not assimilated, but when pRSLmalL was introduced, most of isomaltose was assimilated in the same culture time. It was found that when the culture time was extended to 42 hours for the strain introduced with pRSLmalL, isomaltose was completely consumed. From the above results, it was found that introduction of the *malL* gene induced isomaltose assimilation.

[0242] Furthermore, production amounts of L-phenylalanine or L-lysine increased after introduction of the *malL* gene in all the strains.

Reference Example 2: Construction of strain expressing intracellular isomaltase and isomaltose transporter

<1> Construction of plasmid for intracellular expression of the *malL* gene

5 [0243] In order to express isomaltose hydrolase (isomaltase) in the *Escherichia coli* MG1655 strain, an expression plasmid for *malL* gene derived from *Bacillus subtilis* is constructed.

<1-1> Construction of plasmid pRSL12 containing *lacZ* gene

10 [0244] PCR is performed by using pHSG399 (Takara Shuzo) as a template and two oligonucleotides shown below as primers.

Primer 6

5'-CCAATACGCAAACCGCCTCTCC-3' (SEQ ID NO: 6)

15 Primer 10

5'-AGTCAGTGAGCGAGGAAG-3' (SEQ ID NO: 10)

[0245] The obtained fragment is ligated to a pRS fragment obtained by digesting pRS with the restriction enzyme PstI and blunting the resultant beforehand. The ligation reaction mixture is used to transform *E. coli* JM109. Selection of the transformants is performed on the L agar medium containing 50 µg/ml of streptomycin, 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and 40 µg/ml of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). A plasmid is extracted from the transformants, and insertion of the target fragment is confirmed. This plasmid is designated pRSL12.

<1-2> Construction of plasmid pRSL12malL(self) intracellularly expressing *malL* gene

25 [0246] A colony of the *Bacillus subtilis* Marburg 168 strain (BGSC1A1 strain, obtainable from *Bacillus* Genetic Stock Center) is inoculated to 5 ml of the L medium, and culture is performed overnight with shaking. Chromosomal DNA is prepared from the culture medium by using Wizard Genomic DNA Purification Kit (Promega). By using this chromosomal DNA as a template and two oligonucleotides shown below as primers, PCR is performed to amplify a *malL* gene fragment containing the native promoter region of the *malL* gene by PCR method.

Primer 11

5'-GGCTGGCAATCGTTCAAAGCTTTCAGGCATGCGC-3' (SEQ ID NO: 11)

Primer 12

35 5'-CGAGAAAATGATATTCTGCAGTATCTGTTATCACTCCGTC-3' (SEQ ID NO: 12)

[0247] The obtained PCR product is treated with the restriction enzymes *Hind*III and PstI, and ligated with the vector pRSL12 treated with the same restriction enzymes. The ligation reaction mixture is used to transform *E. coli* JM109. Selection of the transformants is performed on the L agar medium containing 50 µg/ml of streptomycin (Sm), 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and 40 µg/ml of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). A plasmid is extracted from the transformants, and insertion of the target fragment is confirmed. This plasmid is designated pRSL12malL(self).

<2> Construction of plasmid expressing isomaltose transporter derived from *Streptococcus mutans*

45 [0248] *Streptococcus mutans* has genes coding a group of proteins involved in assimilation of various saccharides such as melibiose, isomaltose and raffinose, called multiple sugar metabolism system (Tao, L., et al., INFECTION AND IMMUNITY, 1993, 61, 1121). The multiple sugar metabolism system is encoded by an operon consisting of a series of genes including *msmR* (activator), *aga* (α-galactosidase), *msmE* (saccharide-binding protein), *msmF* (membrane protein), *msmG* (membrane protein), *gtfA* (sucrose phosphorylase), *msmK* (ATP-binding protein), and *dexB* (dextran glucosidase).

[0249] In order to induce uptake of isomaltose into *Escherichia coli* cells, a plasmid expressing the sugar transporters MsmE, MsmF and MsmG of the multiple sugar metabolism system is prepared. On the basis of the sequence information on the multiple sugar metabolism system of *Streptococcus mutans* described in the paper (Russell and R.R.B. et al., The Journal of Biological Chemistry, 1992, 7, 4631), primers for amplifying the region coding for MsmE, MsmF and MsmG from a chromosomal DNA are designed. PCR is performed by using the designed primers and chromosomal DNA extracted from *Streptococcus mutans* as a template.

[0250] The chromosomal DNA can be obtained by using Bacterial Genomic DNA Purification Kit (Edge Bio System).

For PCR, a cycle of 96°C for 20 seconds, 65°C for 20 seconds and 72°C for 3 minutes is repeated 25 times using Pyrobest DNA Polymerase (Takara Bio). The ends of the PCR product are blunted, and then phosphorylated. The obtained DNA fragment is digested with the restriction enzyme *Sma*I, and the resultant is ligated to the plasmid vector pMW119 (NIPPON GENE) or pTWV229 (Takara Bio) treated with an alkaline phosphatase to construct plasmids pMW119-Smsm and pTWV229-Smsm expressing an isomaltose transporter.

<3> Construction of plasmid expressing isomaltose transporter derived from *Saccharomyces cerevisiae*

[0251] The *AGT1* gene of *Saccharomyces cerevisiae* codes for the Agt1p protein which transports various α -glucosides such as maltose, isomaltose and maltotriose into cells (Han, E.K., et al., Molecular Microbiology, 1995, 17, 1093). In order to induce uptake of isomaltose into *Escherichia coli* cells, a plasmid expressing the *AGT1* gene is constructed. On the basis of the sequence information on the *AGT1* gene of *Saccharomyces cerevisiae* (GenBank/L47346), primers for amplifying the *AGT1* gene from the chromosomal DNA are designed. PCR is performed by using these primers and the chromosomal DNA of *Saccharomyces cerevisiae* as a template. The chromosomal DNA can be obtained by using Bacterial Genomic DNA Purification Kit (Edge Bio System). For PCR, a cycle of 96°C for 20 seconds, 65°C for 20 seconds and 72°C for 2 minutes is repeated 25 times using Pyrobest DNA Polymerase (Takara Bio). The ends of the PCR product are blunted, and then phosphorylated. The obtained DNA fragment is digested with the restriction enzyme *Sma*I, and the resultant is ligated to the plasmid vector pMW119 (NIPPON GENE) or pTWV229 (Takara Bio) treated with an alkaline phosphatase to construct plasmids pMW119-AGT1 and pTWV229-AGT1 expressing Agt1p.

<4> Impartation of ability to assimilate isomaltose to *Escherichia coli* K 12-strain MG1655

[0252] When isomaltase is intracellularly expressed, an isomaltose transporter is required for assimilation of isomaltose in addition to isomaltase. Therefore, by incorporating both the aforementioned pRSL12malL(self), and pMW119-Smsm or pTWV229-Smsm into the *Escherichia coli* K12 strain MG1655, a strain which assimilates isomaltose can be produced. Similarly, by incorporating both the aforementioned pRSL12malL(self), and pMW119-AGT1 or pTWV229-AGT1, a strain which assimilates isomaltose can also be produced.

[0253] Competent cells of *Escherichia coli* K 12 strain MG1655 are prepared, and transformed with pRSL12malL(self) and pMW119-Smsm or pTWV229-Smsm. Similarly, the K12 strain MG1655 is transformed with pRSL12malL(self) and pMW119-AGT1 or pTWV229-AGT1. The transformants are cultured on the L agar medium containing antibiotics, 50 μ g/ml of streptomycin and 100 μ g/ml of ampicillin, to perform selection of the transformants. The obtained transformant expresses isomaltase and the isomaltose transporter, and has ability to assimilate isomaltose.

[0254] By imparting an ability to produce a target substance such as an amino acid to a strain having ability to assimilate isomaltose obtained as described above, a strain that produces the target substance from a raw material containing isomaltose can be obtained. Furthermore, by imparting ability to assimilate isomaltose to a strain having an ability to produce a target substance in the same manner as described above, a strain that produces the target substance from a raw material containing isomaltose can also be obtained.

Example 3: Introduction of isomaltose/maltose uptake system and isomaltase/maltase genes by gene transfer of *glvA* and *glvC*

[0255] For gene transfer of the *glvA* and *glvC* genes, the following variant of the tac promoter was used for expression.

Sequence of Tac promoter

[0256]

5'-ccctgTTGACAattaatcatcggtcgTATAATgtgtggaatcg-3' (SEQ ID NO: 32)

Sequence of PTacA promoter

5'-ccctgTTGACAattaatcatcggtcgAATAATgtgtggaatcg-3' (SEQ ID NO: 33)

Sequence of PtacB promoter

5'-ccctgTTGGAAtaatcatcggtcgTATAATgtgtggaatcg-3' (SEQ ID NO: 43)

<1> Cloning of the *glvA* gene

[0257] A colony of the *Bacillus subtilis* Marburg 168 strain (ATCC 6051) was inoculated to 5 ml of the L medium, and culture was performed overnight with shaking. Chromosomal DNA was prepared from the culture medium by using Wizard Genomic DNA Purification Kit (Promega). By using this chromosomal DNA as a template, PCR was performed.

The sequences of two primers used are shown below.

Primer 23

5'-AGAAATTTCCCGCTCTATGG-3' (SEQ ID NO: 23)

Primer 24

5'-TGTAGTGCTGATTGATCAGTTC-3' (SEQ ID NO: 24)

[0258] The obtained PCR product was subjected to blunting-kination treatments by using BKL Kit (Takara Bio), and ligated with the pTWV229 vector (Takara Bio) treated with the restriction enzyme *Sma*I.

[0259] The ligation reaction mixture was used to transform *E. coli* JM109. Selection of the transformants was performed on the L agar medium containing 100 µg/ml of ampicillin. A plasmid was extracted from the transformants, and insertion of the target fragment was confirmed. This plasmid was designated pTWV229-self-*glvA*-Fw. In this plasmid, the *glvA* gene was inserted in the forward direction with respect to the lac promoter existing on pTWV229.

<2> Construction of vector plasmid pMW219-ΔPlac

[0260] In order to express the *glvC* gene with minimized influence of the lac promoter present on the vector plasmid pMW219 on the transcription of the *glvC* gene, it was decided to delete the lac promoter site of the vector plasmid pMW219. The vector plasmid pMW219 was treated with the restriction enzyme *Hind*III, and purified by ethanol precipitation. The resultant was further treated with the restriction enzyme *Pst*I, and used for performing PCR using Primers 25 and 26.

Primer 25

5'-GCCaagcttGCATGCCTGCAGGTCGACTCTAGAGG-3' (SEQ ID NO: 25) Primer 26

5'-CCCaagcttGCTAACTCACATTAATTGCGTTG-3' (SEQ ID NO: 26)

[0261] The small letters indicate *Hind*III recognition sites.

[0262] The obtained PCR product was treated with *Hind*III, and the ends of DNA were ligated (self-ligation).

[0263] The ligation reaction mixture was used to transform *E. coli* JM109. Selection of the transformants was performed on the L agar medium containing 50 µg/ml of kanamycin. A plasmid was extracted from the transformants, and it was confirmed that the plasmid had the intended structure. This plasmid was designated pMW219-ΔPlac. This vector plasmid had a structure in which the lac promoter region existing on pMW219 was deleted.

<3> Cloning of the *glvC* gene

[0264] A colony of the *Bacillus subtilis* Marburg 168 strain (ATCC 6051) was inoculated to 5 ml of the L medium, and culture was performed overnight with shaking. Chromosomal DNA was prepared from the culture medium by using Wizard Genomic DNA Purification Kit (Promega). By using this chromosomal DNA as a template, PCR was performed. The sequences of two primers used are shown below.

Primer 27

5'-CAATTTACACAAGGAGACTGCCATGATGCAAAAAATTCAGCG-3' (SEQ ID NO: 27)

Primer 28

5'-CCCAAGCTTCCCCTTTTACTCGATTGTCTC-3' (SEQ ID NO: 28)

[0265] The obtained PCR product was purified by phenol/chloroform extraction and ethanol precipitation. The obtained DNA fragment was used as a template together with Primer 29 (SEQ ID NO: 29) and Primer 30 (SEQ ID NO: 30) to perform PCR. PCR was performed in the presence of a small amount of Primer 31.

Primer 29

5' - CCCAAGCTTCCTGTTGACAATTAATCATCGGCTCGAATAATGTGTGGAATCGTGAG
CGGATAACAATTTACACAAGGAG-3' (SEQ ID NO: 29)

Primer 30

5'-CCCAAGCTTCCCCTTTTACTCGATTGTCTC-3' (SEQ ID NO: 30)

Primer 31

5 ' - CGAATAATGTGTGGAATCGTGAGCGGATAACAAATTCACACAAGGAGACTGCCATG
ATGCAAAAAATTCAGCGCTTTGGA-3 ' (SEQ ID NO: 31)

[0266] The obtained PCR product was subjected to electrophoresis using agarose gel, and the target DNA fragment was purified by using QIAquick Gel Extraction Kit (QIAGEN). The purified DNA fragment was subjected to blunting-treatment treatments, and ligated with the pMW219-ΔPlac vector treated with the restriction enzyme *Sma*I.

[0267] The ligation reaction mixture was used to transform *E. coli* JM109. Selection of the transformants was performed on the L agar medium containing 50 μg/ml of kanamycin. A plasmid was extracted from the transformants, and insertion of the target fragment was confirmed. This plasmid was designated pMW219-ΔPlac-PtacA-glvC. In this plasmid, the *glvC* gene was inserted in the reverse direction with respect to the deleted Plac (lac promoter) of pMW219-ΔPlac.

<4> Evaluation of ability to assimilate isomaltose of strains expressing *glvA* and *glvC*

[0268] An *E. coli* K12 MG1655 strain introduced with pTWV229-self-*glvA*-Fw and pMW219-ΔPlac-PtacA-*glvC* was prepared. This strain was designated *glvA*+*glvC*/MG1655 strain. As a control strain of this experiment, an *E. coli* K12 MG1655 strain introduced with pTWV229 and pMW219-ΔPlac was prepared. This strain was designated pTWV229+pMW219-ΔPlac/MG1655 strain.

[0269] Growth of the control strain (pTWV229+pMW219-ΔPlac/MG1655 strain) and the *glvA*+*glvC*/MG1655 strain was traced by OD measurement in a medium obtained by adding 0.2% isomaltose to M9 minimal medium (M9-iMal medium) (Fig. 1). As seen from Fig. 1, the *glvA*+*glvC*/MG1655 strain showed favorable growth, which was not seen for the control strain. That is, it was found that ability to assimilate isomaltose could be imparted by expressing the *glvA* and *glvC* genes in the *E. coli* K12 MG1655 strain.

M9 medium	
MgSO ₄ ●7H ₂ O	0.247 g/L
CaCl ₂ ●2H ₂ O	0.0147 g/L
Na ₂ HPO ₄ ●12H ₂ O	15.1 g/L
KH ₂ PO ₄	3 g/L
NaCl	0.5 g/L
NH ₄ Cl	1.0 g/L

[0270] The medium was adjusted to pH 6.8 with NaOH, and as a carbon source, 2.0 g/L of isomaltose was added for the M9-iMal medium, or 0.2 g/L of glucose and 1.8 g/L of isomaltose were added for the M9-Glc+iMal medium, and the medium was subjected to filter sterilization. Isomaltose was added as a 75% isomaltose solution of Nakalai so that the isomaltose amount became the aforementioned amount. Furthermore, before culture, ampicillin and kanamycin were added at concentrations of 100 μg/ml and 50 μg/ml, respectively.

[0271] Growth of the control strain (pTWV229+pMW219-ΔPlac/MG1655 strain) and the *glvA*+*glvC*/MG1655 strain was traced by OD measurement in a medium obtained by adding 0.02% of glucose and 0.18% of isomaltose to the M9 minimal medium (M9-Glc+iMal medium) (Fig. 2). As seen from Fig. 2, whereas growth of the control strain was arrested at OD of about 0.2, the *glvA*+*glvC*/MG1655 strain did not show two-stage proliferation, but showed favorable growth. Since two-stage proliferation was not confirmed, it was found that the *glvA*+*glvC*/MG1655 strain simultaneously assimilated glucose and isomaltose.

Example 4: Production of amino acid by ability to assimilate isomaltose-imparted strain

<1> Cloning of the *glvC* gene

[0272] A colony of the *Bacillus subtilis* Marburg 168 strain (ATCC 6051) was inoculated to 5 ml of the L medium, and culture was performed overnight with shaking. Chromosomal DNA was prepared from the culture medium by using Wizard Genomic DNA Purification Kit (Promega). By using this chromosomal DNA as a template, PCR was performed. The sequences of two primers used are shown below.

Primer 34

5 ' - GAATCGTGAGCGGATAACAATTTACACAAGGAGACTGCCATGATGCAAAAAATTC
AGCGCTTTGGAAGCGCGATGTTTG-3 ' (SEQ ID NO: 34)

5 Primer 35

5'-TGACGGCAAACGAAAACCCTCC-3' (SEQ ID NO: 35)

[0273] The obtained PCR product was subjected to electrophoresis using agarose gel, and the target DNA fragment is purified by using QIAquick Gel Extraction Kit (QIAGEN). The purified DNA fragment was used as a template together with Primer 36 and Primer 37 to perform PCR. PCR was performed in the presence of a small amount of Primer 38.

Primer 36

5'-GCTctagaGCCCCGTGTGGAATTAATCATCGGCTCGTATAATGTGTG-3' (SEQ ID NO: 36)

Primer 37

5'-GCTctagaGCTGACGGCAAACGAAAACCCTCCCGAAAAGG-3' (SEQ ID NO: 37)

[0274] The small letters indicate *Xba*I recognition sites. Primer 38

5 ' -

CCCTGTTGGAATTAATCATCGGCTCGTATAATGTGTGGAATCGTGAGCGGATAACAATT
TCACACAAGGAGACTGCC-3 ' (SEQ ID NO: 38)

[0275] The obtained PCR product was purified by using Wizard PCR Preps DNA Purification System (Promega), and treated with the restriction enzyme *Xba*I. Then, the resultant was ligated with pMW219-ΔPlac similarly treated with the restriction enzyme *Xba*I.

[0276] The ligation reaction mixture was used to transform *E. coli* JM109. Selection of the transformants was performed on the L agar medium containing 50 μg/ml of kanamycin. A plasmid was extracted from the transformants, and insertion of the target fragment was confirmed. This plasmid was designated pMW219-ΔPlac-PtacB-glvC-Rv. In this plasmid, the *glvC* gene was inserted in the reverse direction with respect to the deleted Plac (lac promoter) of pMW219-ΔPlac.

<3> Evaluation of amino acid production culture

[0277] As an L-lysine-producing bacterium, *E. coli* WC196ΔcadAΔldcC (also referred to as WC196LC) was used (refer to WO2006/078039). This strain was transformed with pCABD2 (WO95/16042), pTWV229-self-glvA-Fw, and pMW219-ΔPlac-PtacB-glvC-Rv. The obtained transformant was designated pCABD2+glvA+glvC/WC196LC. A WC196LC strain introduced with pCABD2 (WO95/16042), pTWV229 and pMW219-ΔPlac was prepared as a control strain of this experiment. The obtained transformant was designated pCABD2+pTWV229+pMW219-ΔPlac/WC196LC.

[0278] The strains prepared above were each cultured at 37°C in the L medium containing 20 mg/L of streptomycin, 100 mg/L of ampicillin and 50 mg/L of kanamycin until OD600 became about 0.6, then a 40% glycerol solution in the same volume as the culture medium was added to the medium, and the mixture was stirred. Then, the mixture was divided into appropriate volumes, stored at -80°C, and used as glycerol stocks.

[0279] The glycerol stocks of the control strain and pCABD2+glvA+glvC/WC196LC were thawed, 500 μL of each stock was uniformly applied to an L plate containing 20 mg/L of streptomycin, 100 mg/L of ampicillin and 50 mg/L of kanamycin, and culture was performed at 37°C for 24 hours. The cells were scraped from the obtained plate, and suspended in the L-lysine production medium shown below (MS-Glc+iMal medium), and proliferated to OD = 15. This cell suspension was inoculated in a volume of 1 mL to 19 mL of the L-lysine production medium shown below (MS-Glc+iMal medium) containing 20 mg/L of streptomycin, 100 mg/L of ampicillin and 50 mg/L of kanamycin, which was contained in a 500 mL-volume Sakaguchi flask, and culture was performed at 37°C on a reciprocally shaking culture apparatus. As the L-lysine production medium, the MS-Glc+iMal medium containing glucose and isomaltose as sugar sources was used. Amounts of L-lysine accumulated in the medium as well as glucose and isomaltose remained in the medium were quantified during the culture and after completion of the culture. The L-lysine yields based on glucose, and amounts of remained glucose and isomaltose are shown in Table 5.

L-Lysine production medium (MS-Glc+iMal medium)

Glucose 17.8 g/L

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(continued)

Isomaltose	3.3 g/L
(NH ₄) ₂ SO ₄	24 g/L
KH ₂ PO ₄	1.0 g/L
MgSO ₄ ●7H ₂ O	1.0 g/L
FeSO ₄ ●7H ₂ O	0.01 g/L
MnSO ₄ ●7H ₂ O	0.08 g/L
Yeast Extract	2.0 g/L
L-Isoleucine	0.1 g/L
CaCO ₃	50 g/L

(Japanese Pharmacopoeia)

[0280] The medium was adjusted to pH 7.0 with KOH, and autoclaved at 115°C for 10 minutes, except that glucose, isomaltose and MgSO₄●7H₂O were mixed, and autoclaved separately from the other components. CaCO₃ was added after hot air sterilization.

Table 5: Results of amino acid production culture

	pCABD2+pTWV229+ pMW219-ΔPlac/ WC196LC		pCABD2+glvA+glvC/WC196LC		
Culture time (hour)	14	25	22	25	38.5
Yield for Glc (%)	31.1	34.1	44.6	43.0	43.3
Lys (g/L)	3.5	6.1	5.8	7.4	7.8
Initial Glc concentration (g/L)	17.9	17.9	17.9	17.9	17.9
Residual Glc concentration (g/L)	6.7	0.0	4.9	0.8	0.0
Initial iMal concentration (g/L)	3.3	3.3	3.3	3.3	3.3
Residual iMal concentration (g/L)	3.3	3.2	0.0	0.0	0.0

[0281] When pCABD2 and the vectors pTWV229 and pMW219-ΔPlac were introduced into WC196LC, isomaltose was not assimilated. On the other hand, when the *glvA* and *glvC* expression plasmids were introduced, isomaltose was assimilated simultaneously with glucose. Furthermore, by introducing the *glvA* and *glvC* expression plasmids, L-lysine accumulation was increased, and yield based on glucose was improved.

[0282] Then, the pCABD2+glvA+glvC/WC196LC strain was cultured in a medium using glucose, maltose and isomaltose as sugar sources. The glycerol stock of pCABD2+glvA+glvC/WC196LC was thawed, 500 μL of the stock was uniformly applied to an L plate containing 20 mg/L of streptomycin, 100 mg/L of ampicillin and 50 mg/L of kanamycin, and culture was performed at 37°C for 24 hours. The cells were scraped from the obtained plate, and suspended in the L-lysine production medium shown below (MS-Glc+Mal+iMal medium), and proliferated to OD = 15. This cell suspension was inoculated in a volume of 1 mL to 19 mL of the L-lysine production medium shown below (MS-Glc+Mal+iMal medium) containing 20 mg/L of streptomycin, 100 mg/L of ampicillin and 50 mg/L of kanamycin, which was contained in a 500 mL-volume Sakaguchi flask, and culture was performed at 37°C on a reciprocally shaking culture apparatus. As the L-lysine production medium, the MS-Glc+Mal+iMal medium containing glucose, maltose and isomaltose as sugar sources was used. Amounts of L-lysine accumulated in the medium as well as glucose, maltose and isomaltose remained in the medium were quantified during the culture and after completion of the culture. The L-lysine yields based on glucose, and amounts of remained glucose, maltose and isomaltose are shown in Table 6.

L-Lysine production medium	
Glucose	17.8 g/L
Isomaltose	3.3 g/L

(continued)

Maltose	3.3 g/L
(NH ₄) ₂ SO ₄	24 g/L
KH ₂ PO ₄	1.0 g/L
MgSO ₄ ●7H ₂ O	1.0 g/L
FeSO ₄ ●7H ₂ O	0.01 g/L
MnSO ₄ ●7H ₂ O	0.08 g/L
Yeast extract	2.0 g/L
L-Isoleucine	0.1 g/L
CaCO ₃	50 g/L

(Japanese Pharmacopoeia)

[0283] The medium was adjusted to pH 7.0 with KOH, and autoclaved at 115°C for 10 minutes, except that glucose, maltose, isomaltose and MgSO₄●7H₂O were mixed, and autoclaved separately from the other components. CaCO₃ was added after hot air sterilization.

Table 6: Result of amino acid production culture in MS-Glc+Mal+iMal medium

	Culture time (hour)		
	22	25	38.5
Yield for Glc (%)	56.6	46.5	45.3
Lys (g/L)	5.6	6.9	8.1
Initial Glc concentration (g/L)	17.9	17.9	17.9
Residual Glc concentration (g/L)	8.0	3.1	0.0
Initial Mal concentration (g/L)	2.8	2.8	2.8
Residual Mal concentration (g/L)	0.0	0.0	0.0
Initial iMal concentration (g/L)	3.2	3.2	3.2
Residual iMal concentration (g/L)	0.0	0.0	0.0

[0284] When pCABD2, and the *g/vA* and *g/vC* expression plasmids were introduced into WC196LC, maltose and isomaltose were assimilated simultaneously with glucose. Furthermore, by introducing the *g/vA* and *g/vC* expression plasmids, L-lysine accumulation was increased, and the yield based on glucose was improved.

Industrial Applicability

[0285] According to the present invention, substances can be produced from isomaltose and/or maltose as raw materials by fermentation using a microorganism. In a preferred embodiment of the present invention, isomaltose and/or maltose-assimilating ability can be imparted to even a microorganism which originally cannot assimilate isomaltose and/or maltose.

[0286] The present specification also discloses the following items:

1. A method for producing a target substance utilizing a microorganism, which comprises culturing the microorganism in a medium to produce and accumulate an L-amino acid or a nucleic acid in the medium, and collecting the target substance from culture, wherein:

the microorganism has been imparted with isomaltase activity, or modified to increase isomaltase activity.

2. The method according to item 1, wherein the microorganism has been further imparted with activity to take up isomaltose into cells or modified to increase the activity.

3. The method according to item 1 or 2, wherein the microorganism has been further imparted with maltase activity or modified to increase maltase activity.

4. The method according to item 3, wherein the microorganism has been further imparted with activity to take up maltose into cells or modified to increase the activity.

5. The method according to any one of items 1 to 4, wherein the medium contains isomaltose, or isomaltose and maltose.

6. The method according to any one of items 1 to 5, wherein the microorganism has been introduced with a gene coding for an isomaltase of a *Bacillus* bacterium.

7. The method according to item 6, wherein the isomaltase is a protein shown in the following (A) or (B):

(A) a protein consisting of the amino acid sequence shown in SEQ ID NO: 14,

(B) a protein consisting of the amino acid sequence shown in SEQ ID NO: 14 including substitutions, deletions, insertions or additions of one or several amino acid residues, and having the isomaltase activity.

8. The method according to item 6, wherein the isomaltase is encoded by a DNA shown in the following (a) or (b):

(a) a DNA having the nucleotide sequence shown in SEQ ID NO: 13,

(b) a DNA which is able to hybridize with a sequence complementary to the nucleotide sequence of SEQ ID NO: 13 or a probe which can be prepared from the nucleotide sequence under stringent conditions, and codes for a protein having the isomaltase activity.

9. The method according to item 6, wherein the isomaltase is a protein shown in the following (C) or (D):

(C) a protein consisting of the amino acid sequence shown in SEQ ID NO: 40,

(D) a protein consisting of the amino acid sequence shown in SEQ ID NO: 40 including substitutions, deletions, insertions or additions of one or several amino acid residues, and having the isomaltase activity.

10. The method according to item 6, wherein the isomaltase is encoded by a DNA shown in the following (c) or (d):

(c) a DNA having the nucleotide sequence shown in SEQ ID NO: 39,

(d) a DNA which is able to hybridize with a sequence complementary to the nucleotide sequence of SEQ ID NO: 39 or a probe which can be prepared from the nucleotide sequence under stringent conditions, and codes for a protein having the isomaltase activity.

11. The method according to any one of items 1 to 10, wherein the microorganism has been introduced with a gene coding for a PTS maltose enzyme IICB of a *Bacillus* bacterium.

12. The method according to item 11, wherein the PTS maltose enzyme IICB is a protein shown in the following (E) or (F):

(E) a protein consisting of the amino acid sequence shown in SEQ ID NO: 42,

(F) a protein consisting of the amino acid sequence shown in SEQ ID NO: 42 including substitutions, deletions, insertions or additions of one or several amino acid residues, and having the activity to take up isomaltose into cells.

13. The method according to item 11, wherein the PTS maltose enzyme IICB is encoded by a DNA shown in the following (e) or (f):

(e) a DNA having the nucleotide sequence shown in SEQ ID NO: 41,

(f) a DNA which is able to hybridize with a sequence complementary to the nucleotide sequence of SEQ ID NO: 41 or a probe which can be prepared from the nucleotide sequence under stringent conditions, and codes for a protein having the activity to take up isomaltose into cells.

14. The method according to any one of items 1 to 13, wherein the microorganism is an *Enterobacteriaceae* bacterium.

15. The method according to item 14, wherein the microorganism is an *Escherichia* bacterium.

16. The method according to item 15, wherein the *Escherichia* bacterium is *Escherichia coli*.

17. The method according to item 16, wherein the amino acid is selected from L-lysine, L-threonine, and L-phenylalanine.

SEQUENCE LISTING

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[illegible]

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5	385					390					395					400			
	gct	aaa	ggt	tta	gct	gat	ggg	ctt	aac	gct	ttc	ttt	aat	cca	atg	aaa			1248
	Ala	Lys	Gly	Leu	Ala	Asp	Gly	Leu	Asn	Ala	Phe	Phe	Asn	Pro	Met	Lys			
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 <400> 16

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	Ser	Val	Leu	Leu	Ala	Ala	Cys	Asn	Lys	Ser	Lys	Ala	Ser	Gln	Ser	Lys
			20						25					30		
5	Glu	Asp	Lys	Val	Thr	Ile	Glu	Tyr	Phe	Asn	Gln	Lys	Lys	Glu	Met	Asp
			35					40					45			
	Ala	Thr	Leu	Lys	Lys	Ile	Ile	Lys	Asp	Phe	Glu	Arg	Glu	Asn	Pro	Lys
		50					55					60				
	Ile	His	Val	Lys	Met	Thr	Ser	Val	Pro	Asp	Ala	Gly	Thr	Val	Leu	Lys
10	65					70					75					80
	Thr	Arg	Val	Leu	Ser	Gly	Asp	Val	Pro	Asp	Val	Ile	Asn	Ile	Tyr	Pro
					85					90					95	
	Gln	Asn	Met	Asp	Phe	Gln	Glu	Trp	Ser	Lys	Ala	Gly	Tyr	Phe	Tyr	Asn
				100					105					110		
15	Met	Thr	Gly	Lys	Ala	Tyr	Leu	Asn	His	Leu	Lys	Asn	His	Tyr	Ala	Asn
			115					120					125			
	Glu	Tyr	Lys	Val	Asn	Gln	Lys	Val	Tyr	Ser	Val	Pro	Leu	Thr	Ala	Asn
			130				135					140				
	Val	Ser	Gly	Ile	Tyr	Tyr	Asn	Lys	Thr	Lys	Phe	Lys	Glu	Leu	Gly	Leu
	145					150					155					160
20	Lys	Val	Pro	Glu	Thr	Trp	Asp	Glu	Phe	Val	Lys	Leu	Val	Glu	Glu	Ile
					165					170					175	
	Lys	Ala	Lys	Lys	Glu	Thr	Pro	Phe	Ala	Leu	Ala	Gly	Thr	Glu	Gly	Trp
				180				185					190			
	Thr	Leu	Asn	Gly	Tyr	His	Gln	Leu	Ser	Leu	Ile	Ser	Val	Thr	Gly	Ser
25			195				200						205			
	Ala	Asn	Ala	Ala	Asn	Lys	Tyr	Leu	Arg	Phe	Ser	Gln	Pro	Asn	Ser	Ile
		210				215						220				
	Lys	Thr	Ser	Asp	Lys	Ile	Leu	Lys	Glu	Asp	Met	Val	Arg	Leu	Asn	Leu
					230					235					240	
30	Leu	Ala	Asp	Asp	Gly	Asn	Gln	Gln	Lys	Asn	Trp	Lys	Gly	Ala	Ser	Tyr
				245					250					255		
	Asn	Asp	Ala	Leu	Val	Ala	Phe	Ala	Asn	Glu	Lys	Ala	Leu	Met	Thr	Pro
				260				265					270			
	Asn	Gly	Ser	Trp	Ala	Leu	Pro	Ala	Ile	Lys	Gln	Gln	Asp	Pro	Lys	Phe
			275				280						285			
35	Glu	Ile	Gly	Thr	Phe	Ala	Phe	Pro	Gly	Lys	Lys	Thr	Gly	Asn	Gly	Ile
		290				295						300				
	Thr	Val	Gly	Ala	Gly	Asp	Leu	Ala	Leu	Ser	Ile	Ser	Ala	Lys	Thr	Lys
		305			310					315					320	
	His	Leu	Lys	Glu	Ala	Glu	Lys	Phe	Val	Lys	Tyr	Met	Thr	Thr	Ala	Arg
					325					330					335	
40	Ala	Met	Gln	Lys	Tyr	Tyr	Asp	Val	Asp	Gly	Ser	Pro	Val	Ala	Val	Lys
				340				345					350			
	Gly	Val	Arg	Glu	Asp	Lys	Asn	Ser	Pro	Leu	Gln	Pro	Leu	Thr	Lys	Leu
45				355				360					365			
	Ala	Phe	Thr	Asp	Lys	His	Tyr	Val	Trp	Leu	Gly	Gln	His	Trp	Asn	Ser
		370					375					380				
	Glu	Asp	Asp	Phe	Phe	Thr	Ala	Thr	Thr	Asn	Tyr	Leu	Met	Thr	Lys	Asn
		385			390						395				400	
50	Ala	Lys	Gly	Leu	Ala	Asp	Gly	Leu	Asn	Ala	Phe	Phe	Asn	Pro	Met	Lys
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10	atc gta ccg ctc att tta caa gtt gta ttc ttt tat ttt cct atg ttt	96
	Ile Val Pro Leu Ile Leu Gln Val Val Phe Phe Tyr Phe Pro Met Phe	
	20 25 30	
	cag ggt gct ttt tat agc ttt act aat tgg aca ggt ctg acc tat aat	144
	Gln Gly Ala Phe Tyr Ser Phe Thr Asn Trp Thr Gly Leu Thr Tyr Asn	
	35 40 45	
15	ttt gat ttt gtt ggt att aat aat tat aag att ttg atg act gat ggg	192
	Phe Asp Phe Val Gly Ile Asn Asn Tyr Lys Ile Leu Met Thr Asp Gly	
	50 55 60	
	aaa ttt atg aag gct att ggt ttt act ttg gtt ttg act ctg gcc ttg	240
	Lys Phe Met Lys Ala Ile Gly Phe Thr Leu Val Leu Thr Leu Ala Leu	
	65 70 75 80	
20	att gtc ggt gaa att gtc ctt ggt att atc att gca cgt gcg ctt aat	288
	Ile Val Gly Glu Ile Val Leu Gly Ile Ile Ile Ala Arg Ala Leu Asn	
	85 90 95	
	gct aag atc aaa gga aaa act ttt ttc aga gcc tgg ttc ttc ttt cca	336
	Ala Lys Ile Lys Gly Lys Thr Phe Phe Arg Ala Trp Phe Phe Pro	
	100 105 110	
	gct gtt tta tct ggt ttg aca gtt tct ttg att ttt aaa cag gtt ttc	384
	Ala Val Leu Ser Gly Leu Thr Val Ser Leu Ile Phe Lys Gln Val Phe	
	115 120 125	
30	aat tat ggt ttg cca gca gtt ggc agt gct ttg gga att aaa ttt tta	432
	Asn Tyr Gly Leu Pro Ala Val Gly Ser Ala Leu Gly Ile Lys Phe Leu	
	130 135 140	
	gaa aca agt atg ttg gga aca gcg aac ggg gcg gtt att gct tca atc	480
	Glu Thr Ser Met Leu Gly Thr Ala Asn Gly Ala Val Ile Ala Ser Ile	
	145 150 155 160	
35	ttt gtt ctt ttg tgg caa ggt gtt gct atg cct att att ctt ttc ctt	528
	Phe Val Leu Leu Trp Gln Gly Val Ala Met Pro Ile Ile Leu Phe Leu	
	165 170 175	
	tca gga tta cag agt att cca tca gag att gtc gaa gca gca gct att	576
	Ser Gly Leu Gln Ser Ile Pro Ser Glu Ile Val Glu Ala Ala Ala Ile	
	180 185 190	
40	gat ggt gct gac agt aaa cag aca ttt tgg tca gtc gag ttg ccc tac	624
	Asp Gly Ala Asp Ser Lys Gln Thr Phe Trp Ser Val Glu Leu Pro Tyr	
	195 200 205	
	tta ctg cca agt att tcc atg gtt ttc atc atg gct tta aaa gct ggt	672
	Leu Leu Pro Ser Ile Ser Met Val Phe Ile Met Ala Leu Lys Ala Gly	
	210 215 220	

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	ctt acg gcc ttt gat caa atc ttt gcc tta aca ggt ggc ggt cca aat	720
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	225 230 235 240	
5	aat tca aca aca tca tta ggc ctt ttg gtt tat aac tat gcc ttt aag	768
	Asn Ser Thr Thr Ser Leu Gly Leu Leu Val Tyr Asn Tyr Ala Phe Lys	
	245 250 255	
	agt aat cag tat ggt tat gct aat gct att gct ttg att tta ttc att	816
	Ser Asn Gln Tyr Gly Tyr Ala Asn Ala Ile Ala Leu Ile Leu Phe Ile	
	260 265 270	
10	atc att gga att gtt tct gtg ctg caa att aaa ctc tct aag aag ttt	864
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20 <213> Streptococcus mutans

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	Ile	Val	Pro	Leu	Ile	Leu	Gln	Val	Val	Phe	Phe	Tyr	Phe	Pro	Met	Phe
				20					25					30		
5	Gln	Gly	Ala	Phe	Tyr	Ser	Phe	Thr	Asn	Trp	Thr	Gly	Leu	Thr	Tyr	Asn
			35					40					45			
	Phe	Asp	Phe	Val	Gly	Ile	Asn	Asn	Tyr	Lys	Ile	Leu	Met	Thr	Asp	Gly
		50					55					60				
10	Lys	Phe	Met	Lys	Ala	Ile	Gly	Phe	Thr	Leu	Val	Leu	Thr	Leu	Ala	Leu
	65					70					75					80
	Ile	Val	Gly	Glu	Ile	Val	Leu	Gly	Ile	Ile	Ile	Ala	Arg	Ala	Leu	Asn
					85					90					95	
	Ala	Lys	Ile	Lys	Gly	Lys	Thr	Phe	Phe	Arg	Ala	Trp	Phe	Phe	Phe	Pro
				100					105					110		
15	Ala	Val	Leu	Ser	Gly	Leu	Thr	Val	Ser	Leu	Ile	Phe	Lys	Gln	Val	Phe
			115					120					125			
	Asn	Tyr	Gly	Leu	Pro	Ala	Val	Gly	Ser	Ala	Leu	Gly	Ile	Lys	Phe	Leu
		130					135					140				
	Glu	Thr	Ser	Met	Leu	Gly	Thr	Ala	Asn	Gly	Ala	Val	Ile	Ala	Ser	Ile
	145					150					155					160
20	Phe	Val	Leu	Leu	Trp	Gln	Gly	Val	Ala	Met	Pro	Ile	Ile	Leu	Phe	Leu
					165					170					175	
	Ser	Gly	Leu	Gln	Ser	Ile	Pro	Ser	Glu	Ile	Val	Glu	Ala	Ala	Ala	Ile
				180					185					190		
25	Asp	Gly	Ala	Asp	Ser	Lys	Gln	Thr	Phe	Trp	Ser	Val	Glu	Leu	Pro	Tyr
			195					200					205			
	Leu	Leu	Pro	Ser	Ile	Ser	Met	Val	Phe	Ile	Met	Ala	Leu	Lys	Ala	Gly
		210					215					220				
	Leu	Thr	Ala	Phe	Asp	Gln	Ile	Phe	Ala	Leu	Thr	Gly	Gly	Gly	Pro	Asn
	225					230					235					240
30	Asn	Ser	Thr	Thr	Ser	Leu	Gly	Leu	Leu	Val	Tyr	Asn	Tyr	Ala	Phe	Lys
					245					250					255	
	Ser	Asn	Gln	Tyr	Gly	Tyr	Ala	Asn	Ala	Ile	Ala	Leu	Ile	Leu	Phe	Ile
				260				265						270		
	Ile	Ile	Gly	Ile	Val	Ser	Val	Leu	Gln	Ile	Lys	Leu	Ser	Lys	Lys	Phe
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<220>

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45 <222> (1)..(834)

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	1			5					10					15			
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	Thr	Val	Gly	Gly	Ile	Leu	Ile	Leu	Ile	Pro	Leu	Met	Val	Thr	Val	Phe	
			20					25					30				
	agc	tct	ttt	aag	aaa	act	aag	gat	att	atg	aat	cat	ttt	ttt	gcc	ttt	144
	Ser	Ser	Phe	Lys	Lys	Thr	Lys	Asp	Ile	Met	Asn	His	Phe	Phe	Ala	Phe	
			35					40					45				
10	cca	aat	cct	atc	acc	ttg	gat	aat	tac	aaa	cgt	ttg	tta	gct	gat	ggg	192
	Pro	Asn	Pro	Ile	Thr	Leu	Asp	Asn	Tyr	Lys	Arg	Leu	Leu	Ala	Asp	Gly	
		50					55					60					
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	Val	Gly	Gly	Tyr	Phe	Trp	Asn	Ser	Thr	Val	Ile	Thr	Val	Leu	Ser	Val	
	65					70				75					80		
15	ttg	gtg	gtc	atg	ctc	ttt	atc	cct	gca	gcg	gct	tat	tcc	att	gcg	cgt	288
	Leu	Val	Val	Met	Leu	Phe	Ile	Pro	Ala	Ala	Ala	Tyr	Ser	Ile	Ala	Arg	
				85				90					95				
	aac	atg	tct	aga	aga	aaa	gct	ttc	aat	atc	atg	tat	agc	ctg	ttg	att	336
	Asn	Met	Ser	Arg	Arg	Lys	Ala	Phe	Asn	Ile	Met	Tyr	Ser	Leu	Leu	Ile	
20				100				105					110				
	ctg	gga	att	ttc	gtt	cct	ttc	cag	gtt	atc	atg	att	cct	att	acg	gtt	384
	Leu	Gly	Ile	Phe	Val	Pro	Phe	Gln	Val	Ile	Met	Ile	Pro	Ile	Thr	Val	
			115					120					125				
	atg	atg	agt	aaa	tta	ggc	ctg	gct	aat	atg	tgg	gga	tta	att	att	ctg	432
25	Met	Met	Ser	Lys	Leu	Gly	Leu	Ala	Asn	Met	Trp	Gly	Leu	Ile	Ile	Leu	
			130				135					140					
	tat	tta	act	tat	gct	att	cca	cag	aca	ctc	ttc	ctt	tac	gtg	ggg	tat	480
	Tyr	Leu	Thr	Tyr	Ala	Ile	Pro	Gln	Thr	Leu	Phe	Leu	Tyr	Val	Gly	Tyr	
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	atc	aaa	cta	agt	gta	cct	gat	agt	tta	gat	gaa	gca	gct	gaa	att	gat	528
30	Ile	Lys	Leu	Ser	Val	Pro	Asp	Ser	Leu	Asp	Glu	Ala	Ala	Glu	Ile	Asp	
				165						170				175			
	ggg	gcg	gat	aaa	ttg	aca	act	tat	cgt	aaa	atc	att	ttc	cct	atg	tta	576
	Gly	Ala	Asp	Lys	Leu	Thr	Thr	Tyr	Arg	Lys	Ile	Ile	Phe	Pro	Met	Leu	
				180					185				190				
35	aaa	cca	atg	cac	gca	aca	act	ctg	att	att	aat	gca	ctt	tgg	ttc	tgg	624
	Lys	Pro	Met	His	Ala	Thr	Thr	Leu	Ile	Ile	Asn	Ala	Leu	Trp	Phe	Trp	
				195				200					205				
	aac	gac	ttt	atg	ttg	cca	ttg	ctg	att	ctt	aat	aag	gat	tca	agt	atg	672
	Asn	Asp	Phe	Met	Leu	Pro	Leu	Leu	Ile	Leu	Asn	Lys	Asp	Ser	Ser	Met	
40				210				215					220				
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	Trp	Thr	Leu	Pro	Leu	Phe	Gln	Tyr	Asn	Tyr	Ser	Gly	Gln	Tyr	Phe	Asn	
	225					230						235				240	
	gat	tac	ggg	cct	agt	ttt	gct	tct	tac	att	gtt	ggg	att	att	acc	att	768
45	Asp	Tyr	Gly	Pro	Ser	Phe	Ala	Ser	Tyr	Ile	Val	Gly	Ile	Ile	Thr	Ile	
						245						250			255		
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	Thr	Ile	Val	Tyr	Leu	Ile	Phe	Gln	Lys	His	Ile	Ile	Ala	Gly	Met	Ser	
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 Ser Ser Phe Lys Lys Thr Lys Asp Ile Met Asn His Phe Phe Ala Phe
 35 40 45
 10 Pro Asn Pro Ile Thr Leu Asp Asn Tyr Lys Arg Leu Leu Ala Asp Gly
 50 55 60
 Val Gly Gly Tyr Phe Trp Asn Ser Thr Val Ile Thr Val Leu Ser Val
 65 70 75 80
 Leu Val Val Met Leu Phe Ile Pro Ala Ala Ala Tyr Ser Ile Ala Arg
 85 90 95
 15 Asn Met Ser Arg Arg Lys Ala Phe Asn Ile Met Tyr Ser Leu Leu Ile
 100 105 110
 Leu Gly Ile Phe Val Pro Phe Gln Val Ile Met Ile Pro Ile Thr Val
 115 120 125
 Met Met Ser Lys Leu Gly Leu Ala Asn Met Trp Gly Leu Ile Ile Leu
 130 135 140
 20 Tyr Leu Thr Tyr Ala Ile Pro Gln Thr Leu Phe Leu Tyr Val Gly Tyr
 145 150 155 160
 Ile Lys Leu Ser Val Pro Asp Ser Leu Asp Glu Ala Ala Glu Ile Asp
 165 170 175
 25 Gly Ala Asp Lys Leu Thr Thr Tyr Arg Lys Ile Ile Phe Pro Met Leu
 180 185 190
 Lys Pro Met His Ala Thr Thr Leu Ile Ile Asn Ala Leu Trp Phe Trp
 195 200 205
 Asn Asp Phe Met Leu Pro Leu Leu Ile Leu Asn Lys Asp Ser Ser Met
 210 215 220
 30 Trp Thr Leu Pro Leu Phe Gln Tyr Asn Tyr Ser Gly Gln Tyr Phe Asn
 225 230 235 240
 Asp Tyr Gly Pro Ser Phe Ala Ser Tyr Ile Val Gly Ile Ile Thr Ile
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<211> 1854

40 <212> DNA

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<220>

<221> CDS

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 aat gag gat aaa aac att tct gag tct tca aga gat att gta aac caa 96
 Asn Glu Asp Lys Asn Ile Ser Glu Ser Ser Arg Asp Ile Val Asn Gln
 20 25 30
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	Gln	Glu	Val	Phe	Asn	Thr	Glu	Asp	Phe	Glu	Glu	Gly	Lys	Lys	Asp	Ser	
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5	gcc	ttt	gag	cta	gac	cac	tta	gag	ttc	acc	acc	aat	tca	gcc	cag	tta	192
	Ala	Phe	Glu	Leu	Asp	His	Leu	Glu	Phe	Thr	Thr	Asn	Ser	Ala	Gln	Leu	
		50					55					60					
	gga	gat	tct	gac	gaa	gat	aac	gag	aat	gtg	att	aat	gag	atg	aac	gct	240
	Gly	Asp	Ser	Asp	Glu	Asp	Asn	Glu	Asn	Val	Ile	Asn	Glu	Met	Asn	Ala	
	65					70					75				80		
10	act	gat	gat	gca	aat	gaa	gct	aac	agc	gag	gaa	aaa	agc	atg	act	ttg	288
	Thr	Asp	Asp	Ala	Asn	Glu	Ala	Asn	Ser	Glu	Glu	Lys	Ser	Met	Thr	Leu	
				85						90					95		
	aag	cag	gcg	ttg	cta	aaa	tat	cca	aaa	gca	gcc	ctg	tgg	tcc	ata	tta	336
	Lys	Gln	Ala	Leu	Leu	Lys	Tyr	Pro	Lys	Ala	Ala	Leu	Trp	Ser	Ile	Leu	
				100					105					110			
15	gtg	tct	act	acc	ctg	gtt	atg	gaa	ggg	tat	gat	acc	gca	cta	ctg	agc	384
	Val	Ser	Thr	Thr	Leu	Val	Met	Glu	Gly	Tyr	Asp	Thr	Ala	Leu	Leu	Ser	
			115					120					125				
	gca	ctg	tat	gcc	ctg	cca	gtt	ttt	cag	aga	aaa	ttc	ggg	act	ttg	aac	432
	Ala	Leu	Tyr	Ala	Leu	Pro	Val	Phe	Gln	Arg	Lys	Phe	Gly	Thr	Leu	Asn	
			130				135					140					
20	ggg	gag	ggg	tct	tac	gaa	att	act	tcc	caa	tgg	cag	att	ggg	tta	aac	480
	Gly	Glu	Gly	Ser	Tyr	Glu	Ile	Thr	Ser	Gln	Trp	Gln	Ile	Gly	Leu	Asn	
	145					150					155				160		
	atg	tgt	gtc	ctt	tgt	ggg	gag	atg	att	ggg	ttg	caa	atc	acg	act	tat	528
	Met	Cys	Val	Leu	Cys	Gly	Glu	Met	Ile	Gly	Leu	Gln	Ile	Thr	Thr	Tyr	
					165					170					175		
25	atg	gtt	gaa	ttt	atg	ggg	aat	cgt	tat	acg	atg	att	aca	gca	ctt	ggg	576
	Met	Val	Glu	Phe	Met	Gly	Asn	Arg	Tyr	Thr	Met	Ile	Thr	Ala	Leu	Gly	
				180					185					190			
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	Leu	Leu	Thr	Ala	Tyr	Ile	Phe	Ile	Leu	Tyr	Tyr	Cys	Lys	Ser	Leu	Ala	
				195				200					205				
30	atg	att	gct	gtg	gga	caa	att	ctc	tca	gct	ata	cca	tgg	ggg	tgt	ttc	672
	Met	Ile	Ala	Val	Gly	Gln	Ile	Leu	Ser	Ala	Ile	Pro	Trp	Gly	Cys	Phe	
			210				215					220					
	caa	agt	ttg	gct	gtt	act	tat	gct	tcg	gaa	gtt	tgc	cct	tta	gca	tta	720
	Gln	Ser	Leu	Ala	Val	Thr	Tyr	Ala	Ser	Glu	Val	Cys	Pro	Leu	Ala	Leu	
	225					230					235				240		
35	aga	tat	tac	atg	acc	agt	tac	tcc	aac	att	tgt	tgg	tta	ttt	ggg	caa	768
	Arg	Tyr	Tyr	Met	Thr	Ser	Tyr	Ser	Asn	Ile	Cys	Trp	Leu	Phe	Gly	Gln	
					245					250					255		
	atc	ttc	gcc	tct	ggg	att	atg	aaa	aac	tca	caa	gag	aat	tta	ggg	aac	816
	Ile	Phe	Ala	Ser	Gly	Ile	Met	Lys	Asn	Ser	Gln	Glu	Asn	Leu	Gly	Asn	
				260				265						270			
	tcc	gac	ttg	ggc	tat	aaa	ttg	cca	ttt	gct	tta	caa	tgg	att	tgg	cct	864
	Ser	Asp	Leu	Gly	Tyr	Lys	Leu	Pro	Phe	Ala	Leu	Gln	Trp	Ile	Trp	Pro	
				275				280					285				
45	gct	cct	tta	atg	atc	ggg	atc	ttt	ttc	gct	cct	gag	tcg	ccc	tgg	tgg	912
	Ala	Pro	Leu	Met	Ile	Gly	Ile	Phe	Phe	Ala	Pro	Glu	Ser	Pro	Trp	Trp	
		290					295					300					
	ttg	gtg	aga	aag	gat	agg	gtc	gct	gag	gca	aga	aaa	tct	tta	agc	aga	960
	Leu	Val	Arg	Lys	Asp	Arg	Val	Ala	Glu	Ala	Arg	Lys	Ser	Leu	Ser	Arg	
		305				310					315				320		
50	att	ttg	agt	ggg	aaa	ggc	gcc	gag	aag	gac	att	caa	gtt	gat	ctt	act	1008
	Ile	Leu	Ser	Gly	Lys	Gly	Ala	Glu	Lys	Asp	Ile	Gln	Val	Asp	Leu	Thr	
				325						330					335		
	tta	aag	cag	att	gaa	ttg	act	att	gaa	aaa	gaa	aga	ctt	tta	gca	tct	1056
	Leu	Lys	Gln	Ile	Glu	Leu	Thr	Ile	Glu	Lys	Glu	Arg	Leu	Leu	Ala	Ser	
				340					345					350			
55	aaa	tca	gga	tca	ttc	ttt	aat	tgt	ttc	aag	gga	gtt	aat	gga	aga	aga	1104
	Lys	Ser	Gly	Ser	Phe	Phe	Asn	Cys	Phe	Lys	Gly	Val	Asn	Gly	Arg	Arg	
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	acg	aga	ctt	gca	tgt	tta	act	tgg	gta	gct	caa	aat	agt	agc	ggg	gcc	1152
	Thr	Arg	Leu	Ala	Cys	Leu	Thr	Trp	Val	Ala	Gln	Asn	Ser	Ser	Gly	Ala	
	370						375				380						
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	Val	Leu	Leu	Gly	Tyr	Ser	Thr	Tyr	Phe	Phe	Glu	Lys	Lys	Gln	Val	Met	
	385					390					395					400	
	gcc	acc	gac	aag	gcg	ttt	act	ttt	tct	cta	att	cag	tac	tgt	ctt	ggg	1248
	Ala	Thr	Asp	Lys	Ala	Phe	Thr	Phe	Ser	Leu	Ile	Gln	Tyr	Cys	Leu	Gly	
				405						410					415		
10	tta	gcg	ggg	aca	ctt	tgc	tcc	tgg	gta	ata	tct	ggc	cgt	gtt	ggg	aga	1296
	Leu	Ala	Gly	Thr	Leu	Cys	Ser	Trp	Val	Ile	Ser	Gly	Arg	Val	Gly	Arg	
				420					425					430			
	tgg	aca	ata	ctg	acc	tat	ggg	ctt	gca	ttt	caa	atg	gtc	tgc	tta	ttt	1344
	Trp	Thr	Ile	Leu	Thr	Tyr	Gly	Leu	Ala	Phe	Gln	Met	Val	Cys	Leu	Phe	
				435					440					445			
15	att	att	ggg	gga	atg	ggg	ttt	ggg	tct	gga	agc	agc	gct	agt	aat	ggg	1392
	Ile	Ile	Gly	Gly	Met	Gly	Phe	Gly	Ser	Gly	Ser	Ser	Ala	Ser	Asn	Gly	
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	gcc	ggg	ggg	tta	ttg	ctg	gct	tta	tca	ttc	ttt	tac	aat	gct	ggg	atc	1440
	Ala	Gly	Gly	Leu	Leu	Ala	Leu	Ser	Phe	Phe	Tyr	Asn	Ala	Gly	Ile		
20	465					470					475				480		
	ggg	gca	ggt	ggt	tac	tgt	atc	ggt	gct	gaa	att	cca	tca	gcg	gag	ttg	1488
	Gly	Ala	Val	Val	Tyr	Cys	Ile	Val	Ala	Glu	Ile	Pro	Ser	Ala	Glu	Leu	
					485					490					495		
	aga	act	aag	act	ata	gtg	ctg	gcc	cgt	att	tgc	tac	aat	ctc	atg	gcc	1536
25	Arg	Thr	Lys	Thr	Ile	Val	Leu	Ala	Arg	Ile	Cys	Tyr	Asn	Leu	Met	Ala	
				500					505					510			
	ggt	att	aac	gct	ata	tta	acg	ccc	tat	atg	cta	aac	gtg	agc	gat	tgg	1584
	Val	Ile	Asn	Ala	Ile	Leu	Thr	Pro	Tyr	Met	Leu	Asn	Val	Ser	Asp	Trp	
			515					520					525				
	aac	tgg	ggg	gcc	aaa	act	ggg	cta	tac	tgg	ggg	ggg	ttc	aca	gca	gtc	1632
30	Asn	Trp	Gly	Ala	Lys	Thr	Gly	Leu	Tyr	Trp	Gly	Gly	Phe	Thr	Ala	Val	
		530					535					540					
	act	tta	gct	tgg	gtc	atc	atc	gat	ctg	cct	gag	aca	act	ggg	aga	acc	1680
	Thr	Leu	Ala	Trp	Val	Ile	Ile	Asp	Leu	Pro	Glu	Thr	Thr	Gly	Arg	Thr	
						550					555					560	
35	ttc	agt	gaa	att	aat	gaa	ctt	ttc	aac	caa	ggg	gtt	cct	gcc	aga	aaa	1728
	Phe	Ser	Glu	Ile	Asn	Glu	Leu	Phe	Asn	Gln	Gly	Val	Pro	Ala	Arg	Lys	
					565					570					575		
	ttt	gca	tct	act	gtg	ggt	gat	cca	ttc	gga	aag	gga	aaa	act	caa	cat	1776
	Phe	Ala	Ser	Thr	Val	Val	Asp	Pro	Phe	Gly	Lys	Gly	Lys	Thr	Gln	His	
40				580					585					590			
	gat	tcg	cta	gct	gat	gag	agt	atc	agt	cag	tcc	tca	agc	ata	aaa	cag	1824
	Asp	Ser	Leu	Ala	Asp	Glu	Ser	Ile	Ser	Gln	Ser	Ser	Ser	Ile	Lys	Gln	
				595				600					605				
	cga	gaa	tta	aat	gca	gct	gat	aaa	tgt	taa							1854
45	Arg	Glu	Leu	Asn	Ala	Ala	Asp	Lys	Cys								
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50 <213> *Saccharomyces cerevisiae*

<400> 22

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	Asn	Glu	Asp	Lys	Asn	Ile	Ser	Glu	Ser	Ser	Arg	Asp	Ile	Val	Asn	Gln
				20					25					30		
5	Gln	Glu	Val	Phe	Asn	Thr	Glu	Asp	Phe	Glu	Glu	Gly	Lys	Lys	Asp	Ser
			35					40					45			
	Ala	Phe	Glu	Leu	Asp	His	Leu	Glu	Phe	Thr	Thr	Asn	Ser	Ala	Gln	Leu

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	Phe	Ser	Glu	Ile	Asn	Glu	Leu	Phe	Asn	Gln	Gly	Val	Pro	Ala	Arg	Lys
					565					570					575	
	Phe	Ala	Ser	Thr	Val	Val	Asp	Pro	Phe	Gly	Lys	Gly	Lys	Thr	Gln	His
				580				585						590		
5	Asp	Ser	Leu	Ala	Asp	Glu	Ser	Ile	Ser	Gln	Ser	Ser	Ser	Ile	Lys	Gln
		595					600						605			
	Arg	Glu	Leu	Asn	Ala	Ala	Asp	Lys	Cys							
		610					615									

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	1 5 10 15	
5	ttc act cca ggg atc gta ctc atg ctc ttg gac cat ttg gag gag ttt	96
	Phe Thr Pro Gly Ile Val Leu Met Leu Leu Asp His Leu Glu Glu Phe	
	20 25 30	
	ccg atc aga aag ctg aag ctg tat gat aat gat aag gag aga cag gat	144
	Pro Ile Arg Lys Leu Lys Leu Tyr Asp Asn Asp Lys Glu Arg Gln Asp	
	35 40 45	
10	cga att gca ggc gcc tgt gac gtt ttt atc aga gaa aaa gcg ccg gat	192
	Arg Ile Ala Gly Ala Cys Asp Val Phe Ile Arg Glu Lys Ala Pro Asp	
	50 55 60	
	att gaa ttt gca gcg acg act gac ccg gaa gaa gct ttt aca gat gtc	240
	Ile Glu Phe Ala Ala Thr Thr Asp Pro Glu Glu Ala Phe Thr Asp Val	
	65 70 75 80	
15	gat ttt gtt atg gcg cac atc aga gta ggg aaa tac gcg atg cgc gcg	288
	Asp Phe Val Met Ala His Ile Arg Val Gly Lys Tyr Ala Met Arg Ala	
	85 90 95	
	ctt gat gag caa att ccg tta aag tac gga gtt gtc ggc cag gag acg	336
	Leu Asp Glu Gln Ile Pro Leu Lys Tyr Gly Val Val Gly Gln Glu Thr	
	100 105 110	
20	tgc ggg ccg ggc ggg atc gca tac ggt atg cgt tcg atc ggc ggt gtg	384
	Cys Gly Pro Gly Gly Ile Ala Tyr Gly Met Arg Ser Ile Gly Gly Val	
	115 120 125	
	ctt gaa ata tta gat tac atg gaa aaa tac tcg cct gat gcg tgg atg	432
	Leu Glu Ile Leu Asp Tyr Met Glu Lys Tyr Ser Pro Asp Ala Trp Met	
	130 135 140	
25	ctc aat tat tcc aat ccg gcg gca att gtg gct gaa gct acg aga cgc	480
	Leu Asn Tyr Ser Asn Pro Ala Ala Ile Val Ala Glu Ala Thr Arg Arg	
	145 150 155 160	
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	Ala	Ser	Trp	Asn	Asp	Thr	Phe	Ala	Lys	Ala	Arg	Asp	Val	Gln	Ala	Ala	
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	Asp	Pro	Asp	Thr	Leu	Pro	Asn	Thr	Tyr	Leu	Gln	Tyr	Tyr	Leu	Phe	Pro	
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	Pro	Glu	Pro	Ile	Thr	Val	Gly	Thr	Ile	Pro	Gln	Phe	Gln	Lys	Gly	Leu	
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40	gtg	ccg	aac	gcg	cgt	gtg	gca	aga	ctc	att	ctt	gag	gat	tta	gtg	gag	1296
	Val	Pro	Asn	Ala	Arg	Val	Ala	Arg	Leu	Ile	Leu	Glu	Asp	Leu	Val	Glu	
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	Ala	Asn	Lys	Asp	Phe	Trp	Pro	Glu	Leu	Asp	Gln	Ser	Pro	Thr	Arg	Ile	
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<211> 449

50 <212> PRT

<213> Bacillus subtilis

<400> 40

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	Thr	Arg	Glu	Gln	Ser	Ser	Glu	Asn	Ser	Glu	Ile	Lys	Ile	Asp	Asp	His
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				405						410					415	
	Val	Pro	Asn	Ala	Arg	Val	Ala	Arg	Leu	Ile	Leu	Glu	Asp	Leu	Val	Glu
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<213> Bacillus subtilis

<220>

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<222> (1)..(1584)

<400> 41

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20	Leu	Val	Ala	Gly	Leu	Leu	Ile	Pro	Val	Thr	Leu	Thr	Ala	Ile	Val	Ala
	305					310					315					320
	Gly	Ile	Thr	Glu	Pro	Ile	Glu	Phe	Thr	Phe	Leu	Phe	Ile	Ser	Pro	Phe
					325					330					335	
	Leu	Phe	Ala	Val	His	Ala	Val	Leu	Ala	Ala	Thr	Met	Ser	Thr	Val	Met
25				340					345					350		
	Tyr	Met	Ala	Gly	Val	Val	Gly	Asn	Met	Gly	Gly	Gly	Leu	Ile	Glu	Ala
			355					360					365			
	Val	Thr	Leu	Asn	Trp	Ile	Pro	Leu	Phe	Gly	Ser	His	Gly	Met	Thr	Tyr
		370					375					380				
30	Val	Tyr	Gln	Ile	Leu	Ile	Gly	Leu	Ser	Phe	Thr	Ala	Ile	Tyr	Phe	Phe
	385					390					395					400
	Val	Phe	Arg	Phe	Leu	Ile	Leu	Lys	Phe	Asn	Ile	Ala	Thr	Pro	Gly	Arg
					405					410					415	
	Glu	Lys	Asp	Glu	Gln	Gln	Glu	Thr	Lys	Leu	Tyr	Ser	Lys	Lys	Glu	Tyr
				420					425					430		
35	Arg	Glu	Arg	Lys	Asn	Lys	Asp	Glu	Thr	Ala	Ser	Ala	Ala	Glu	Thr	Ala
				435				440					445			
	Asp	Asp	Thr	Ala	Phe	Leu	Tyr	Ile	Glu	Ala	Leu	Gly	Gly	Lys	Asp	Asn
				450			455					460				
	Ile	Thr	Glu	Val	Thr	Asn	Cys	Ala	Thr	Arg	Leu	Arg	Val	Ser	Val	Lys
40	465					470					475					480
	Asp	Glu	Thr	Lys	Val	Glu	Pro	Asp	Ser	Val	Phe	Arg	Ala	Leu	Gly	Ala
					485					490					495	
	His	Gly	Val	Val	Arg	Asn	Gly	Lys	Ala	Phe	Gln	Val	Ile	Ile	Gly	Leu
				500					505					510		
45	Ser	Val	Pro	Gln	Met	Arg	Glu	Arg	Val	Glu	Lys	Ile	Leu	Asn	Gln	
			515					520					525			

<210> 43

<211> 43

<212> DNA

50 <213> Artificial Sequence

<220>

<223> PtacB promoter

55 <400> 43

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Claims

1. A method for producing an L-amino acid or a nucleic acid utilizing a microorganism, which comprises culturing the microorganism in a medium to produce and accumulate the L-amino acid or the nucleic acid in the medium, and collecting the L-amino acid or the nucleic acid from culture, wherein:

the microorganism has been imparted with the activity of isomaltase EC 3.2.1.122 to decompose phosphorylated isomaltose, produced when isomaltose is taken up into cells, into glucose and glucose phosphate, or modified to increase the activity; and

wherein the microorganism is an *Enterobacteriaceae* bacterium or a coryneform bacterium.

2. The method according to claim 1, wherein the microorganism has been further imparted with activity to take up isomaltose into cells or modified to increase the activity.

3. The method according to claim 1 or 2, wherein the microorganism has been further imparted with maltase activity or modified to increase maltase activity.

4. The method according to claim 3, wherein the microorganism has been further imparted with activity to take up maltose into cells or modified to increase the activity.

5. The method according to any one of claims 1 to 4, wherein the medium contains isomaltose, or isomaltose and maltose.

6. The method according to any one of claim 1 to 5, wherein the microorganism has been introduced with a gene coding for an isomaltase of a *Bacillus* bacterium.

7. The method according to claim 6, wherein the isomaltase is a protein shown in the following (C) or (D):

(C) a protein consisting of the amino acid sequence shown in SEQ ID NO:40,

(D) a protein consisting of the amino acid sequence shown in SEQ ID NO:40 including substitutions, deletions, insertions or additions of one or several amino acid residues, and having the isomaltase activity.

8. The method according to claim 6, wherein the isomaltase is encoded by a DNA shown in the following (c) or (d):

(c) a DNA having the nucleotide sequence shown in SEQ ID NO:39,

(d) a DNA which is able to hybridize with a sequence complementary to the nucleotide sequence of SEQ ID NO:39 or a probe which can be prepared from the nucleotide sequence under stringent conditions, and codes for a protein having the isomaltase activity.

9. The method according to any one of claims 1 to 8, wherein the microorganism is an *Escherichia* bacterium.

10. The method according to claim 9, wherein the *Escherichia* bacterium is *Escherichia coli*.

11. The method according to claim 10, wherein the amino acid is selected from L-lysine, L-threonine, and L-phenylalanine.

Patentansprüche

1. Verfahren zur Herstellung einer L-Aminosäure oder einer Nukleinsäure unter Verwendung eines Mikroorganismus, das das Kultivieren des Mikroorganismus in einem Medium zur Herstellung und zur Anreicherung der L-Aminosäure oder der Nukleinsäure in dem Medium und das Gewinnen der L-Aminosäure oder der Nukleinsäure aus der Kultur umfasst, worin:

dem Mikroorganismus die Aktivität der Isomaltase EC 3.2.1.122 verliehen worden ist, um phosphorylierte Iso-maltose, die produziert wird, wenn Isomaltose in Zellen aufgenommen wird, in Glucose und Glucosephosphat zu spalten, oder worin der Mikroorganismus verändert worden ist, um die Aktivität zu erhöhen; und wobei der Mikroorganismus ein *Enterobacteriaceae*-Bakterium oder ein koryneformes Bakterium ist.

2. Verfahren gemäß Anspruch 1, worin dem Mikroorganismus des Weiteren Aktivität verliehen worden ist, um Isomaltose in Zellen aufzunehmen, oder worin der Mikroorganismus verändert worden ist, um die Aktivität zu erhöhen.
3. Verfahren gemäß Anspruch 1 oder 2, worin dem Mikroorganismus des Weiteren Maltaseaktivität verliehen worden ist oder worin der Mikroorganismus verändert worden ist, um Maltaseaktivität zu erhöhen.
4. Verfahren gemäß Anspruch 3, worin dem Mikroorganismus des Weiteren Aktivität verliehen worden ist, um Maltose in Zellen aufzunehmen, oder worin der Mikroorganismus verändert worden ist, um die Aktivität zu erhöhen.
5. Verfahren gemäß irgendeinem der Ansprüche 1 bis 4, worin das Medium des Weiteren Isomaltose oder Isomaltose und Maltose enthält.
6. Verfahren gemäß irgendeinem der Ansprüche 1 bis 5, worin ein Gen, das eine Isomaltase von einem *Bacillus*-Bakterium kodiert, in den Mikroorganismus eingebracht worden ist.
7. Verfahren gemäß Anspruch 6, worin die Isomaltase ein Protein ist, das in den folgenden (C) oder (D) gezeigt ist:
(C) ein Protein, das aus der Aminosäuresequenz besteht, die in SEQ ID NO: 40 gezeigt ist,
(D) ein Protein, das aus der Aminosäuresequenz besteht, die in SEQ ID NO: 40 gezeigt ist, umfassend Substitutionen, Deletionen, Insertionen oder Hinzufügungen eines Aminosäurerestes oder mehrerer Aminosäurereste, und das die Isomaltaseaktivität besitzt.
8. Verfahren gemäß Anspruch 6, worin die Isomaltase von einer DNA kodiert ist, die in den folgenden (c) oder (d) gezeigt ist:
(c) eine DNA, die die Nukleotidsequenz besitzt, die in SEQ ID NO: 39 gezeigt ist,
(d) eine DNA, die mit einer Sequenz unter stringenten Bedingungen hybridisieren kann, die zu der Nukleotidsequenz von SEQ ID NO: 39 komplementär ist, oder mit einer Sonde, die aus der Nukleotidsequenz hergestellt werden kann, und die ein Protein kodiert, das Isomaltaseaktivität besitzt.
9. Verfahren gemäß irgendeinem der Ansprüche 1 bis 8, worin der Mikroorganismus ein *Escherichia*-Bakterium ist.
10. Verfahren gemäß Anspruch 9, worin das *Escherichia*-Bakterium *Escherichia coli* ist.
11. Verfahren gemäß Anspruch 10, worin die Aminosäure aus L-Lysin, L-Threonin und L-Phenylalanin ausgewählt ist.

Revendications

1. Procédé pour produire un acide L-aminé ou un acide nucléique en utilisant un micro-organisme, qui comprend la culture du micro-organisme dans un milieu pour produire et accumuler l'acide L-aminé ou l'acide nucléique dans le milieu, et la récolte de l'acide L-aminé ou de l'acide nucléique à partir de la culture, dans lequel :
le micro-organisme a été doté d'une activité isomaltase EC 3.2.1.122 qui décompose de l'isomaltose phosphorylé, produit lorsque de l'isomaltose est absorbé dans des cellules, en glucose et glucose phosphaté, ou modifié pour accroître l'activité ; et
dans lequel le micro-organisme est une bactérie *Enterobacteriaceae* ou une bactérie corynéforme.
2. Procédé selon la revendication 1, dans lequel le micro-organisme a en outre été doté d'une activité pour absorber de l'isomaltose dans des cellules, ou modifié pour accroître l'activité.
3. Procédé selon la revendication 1 ou 2, dans lequel le micro-organisme a en outre été doté d'une activité maltase, ou modifié pour accroître l'activité maltase.
4. Procédé selon la revendication 3, dans lequel le micro-organisme a en outre été doté d'une activité pour absorber du maltose dans des cellules, ou modifié pour accroître l'activité.
5. Procédé selon l'une quelconque des revendications 1 à 4, dans lequel le milieu contient de l'isomaltose, ou de

l'isomaltose et du maltose.

6. Procédé selon l'une quelconque des revendications 1 à 5, dans lequel un gène codant pour une isomaltase d'une bactérie *Bacillus* a été introduit dans le micro-organisme.

7. Procédé selon la revendication 6, dans lequel l'isomaltase est une protéine montrée dans les (C) ou (D) suivants :

(C) une protéine consistant en la séquence d'acides aminés montrée dans la SEQ ID n° 40,

(D) une protéine consistant en la séquence d'acides aminés montrée dans la SEQ ID n° 40 incluant des substitutions, des délétions, des insertions ou des additions d'un ou plusieurs résidus d'acides aminés, et ayant l'activité isomaltase.

8. Procédé selon la revendication 6, dans lequel l'isomaltase est codée par un ADN montré dans les (c) ou (d) suivants :

(c) un ADN ayant la séquence de nucléotides montrée dans la SEQ ID n° 39,

(d) un ADN qui est capable de s'hybrider à une séquence complémentaire de la séquence de nucléotides de la SEQ ID n° 39 ou à une sonde qui peut être préparée à partir de la séquence de nucléotides dans des conditions stringentes, et qui code pour une protéine ayant l'activité isomaltase.

9. Procédé selon l'une quelconque des revendications 1 à 8, dans lequel le micro-organisme est une bactérie *Escherichia*.

10. Procédé selon la revendication 9, dans lequel la bactérie *Escherichia* est *Escherichia coli*.

11. Procédé selon la revendication 10, dans lequel l'acide aminé est sélectionné à partir de L-lysine, L-thréonine et L-phénylalanine.

Fig. 1

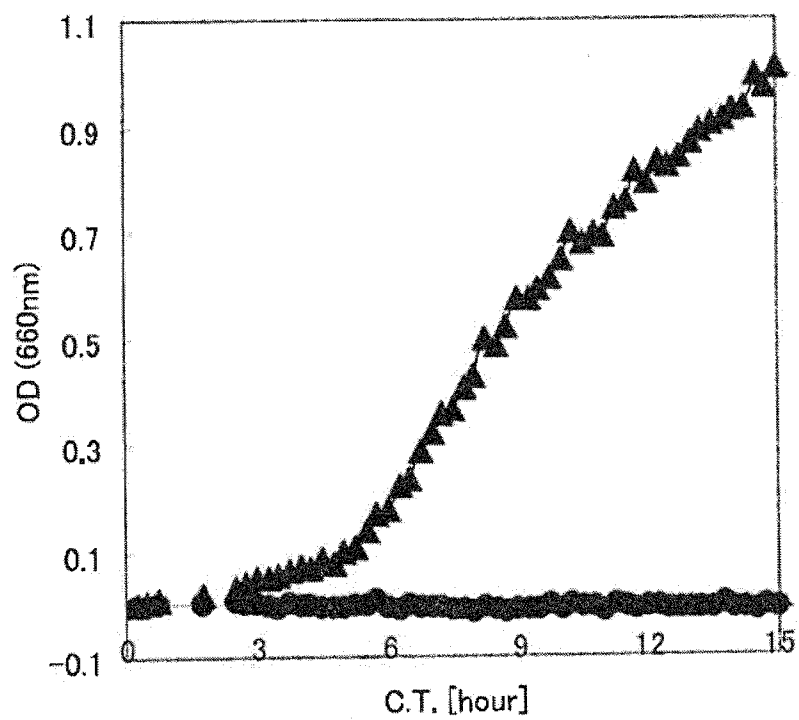
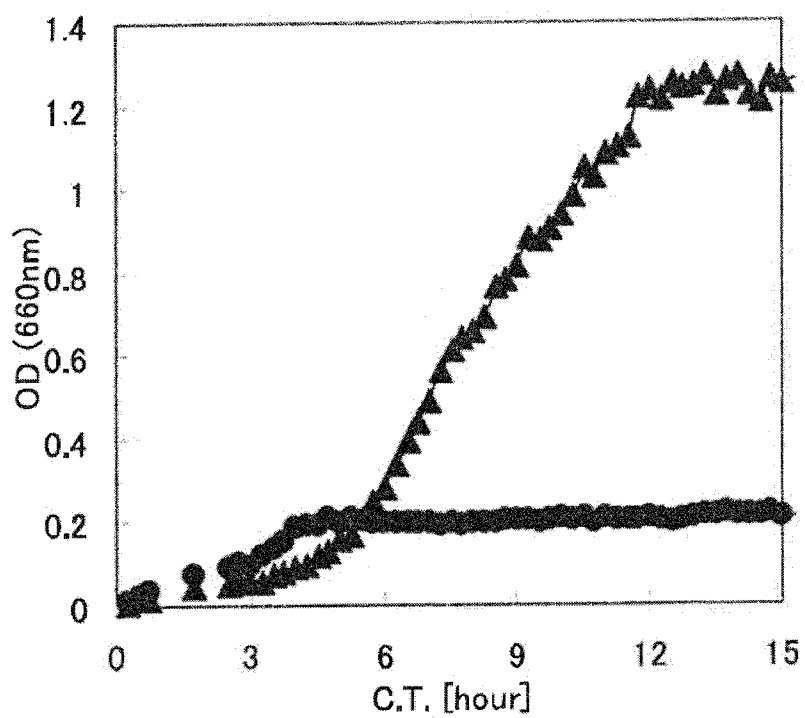


Fig. 2



REFERENCES CITED IN THE DESCRIPTION

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Eljárás egy céltermék fermentációs előállítására

Szabadalmi igénypontok

1. Eljárás egy L-aminosav vagy egy nukleinsav előállítására egy mikroorganizmus alkalmazásával, melynek során a mikroorganizmust egy táptalajon tenyésztjük az L-aminosav vagy nukleinsav termelésére és a táptalajban történő felhalmozódására, és az L-aminosavat vagy a nukleinsavat kinyerjük a tenyészetből, ahol:

a mikroorganizmus izomaltáz EC 3.2.1.122 aktivitást mutat, mely a sejtek által történő izomaltóz felvételkor a foszforilezett izomaltózt glükózzá és glükózfoszfáttá bontja, vagy az aktivitás növelésére módosított; és

ahol a mikroorganizmus egy *Enterobacteriaceae* baktérium vagy egy korineform baktérium.

2. Az 1. igénypont szerinti eljárás, ahol a mikroorganizmus továbbá rendelkezik az izomaltóznak a sejtekbe történő felvételére szolgáló aktivitással vagy az aktivitás növelésére módosított.

3. Az 1. vagy 2. igénypont szerinti eljárás, ahol a mikroorganizmus továbbá maltáz aktivitással rendelkezik vagy a maltáz aktivitás növelésére módosított.

4. A 3. igénypont szerinti eljárás, ahol a mikroorganizmus továbbá rendelkezik a maltóznak a sejtekbe történő felvételére szolgáló aktivitással vagy az aktivitás növelésére módosított.

5. Az 1-4. igénypontok bármelyike szerinti eljárás, ahol a táptalaj izomaltózt, vagy izomaltózt és maltózt tartalmaz.

6. Az 1-5. igénypontok bármelyike szerinti eljárás, ahol a mikroorganizmusba egy *Bacillus* baktérium izomaltázi kódoló gént vittünk be.

7. A 6. igénypont szerinti eljárás, ahol az izomaltáz egy alábbi (C) vagy (D) pontok szerinti protein:

(C) egy 40. számú aminosavszekvenciájú protein,



(D) egy vagy több aminosavgyök szubsztitúcióval, delécióval, inszercióval vagy addícióval rendelkező, és izomaltáz aktivitású 40. számú aminosavszekvenciájú protein.

8. A 6. igénypont szerinti eljárás, ahol az izomaltázt egy alábbi (c) vagy (d) pontok szerinti DNS kódolja:

(c) egy 39. számú nukleotidszekvenciával rendelkező DNS,

(d) egy, a 39. számú nukleotidszekvenciához komplementer szekvenciával hibridizálni képes DNS, vagy egy, a nukleotidszekvenciából stringens körülmények között előállítható és izomaltáz aktivitással rendelkező proteint kódoló próba.

9. Az 1-8. igénypontok bármelyike szerinti eljárás, ahol a mikroorganizmus egy *Escherichia* baktérium.

10. A 9. igénypont szerinti eljárás, ahol az *Escherichia* baktérium *Escherichia coli*.

11. A 9. igénypont szerinti eljárás, ahol az aminosav L-lizin, L-treonin és L-fenilalanin közül választott.

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