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[JP/JP]; c/o AJINOMOTO CO., INC., 1-1, Suzuki-cho, Kawasaki-ku, Kawasaki-shi, Kanagawa, 2108681 (JP).

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(74) Agents: **KAWAGUCHI, Yoshiyuki** et al.; Acropolis 21 Building 6th floor, 4-10, Higashi Nihonbashi 3-chome, Chuo-ku, Tokyo, 1030004 (JP).

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

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): **USUDA, Yoshihiro** [JP/JP]; c/o AJINOMOTO CO., INC., 1-1, Suzuki-cho, Kawasaki-ku, Kawasaki-shi, Kanagawa, 2108681 (JP). **SHIRAGA, Seizaburo** [JP/JP]; c/o AJINOMOTO CO., INC., 1-1, Suzuki-cho, Kawasaki-ku, Kawasaki-shi, Kanagawa, 2108681 (JP). **MATSUI, Kazuhiko** [JP/JP]; c/o AJINOMOTO CO., INC., 1-1, Suzuki-cho, Kawasaki-ku, Kawasaki-shi, Kanagawa, 2108681 (JP). **SUZUKI, Shigeo**

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(54) Title: METHOD FOR PRODUCING AN L-AMINO ACID

(57) Abstract: An L-amino acid is produced by culturing a bacterium of the Enterobacteriaceae family which has an L-amino acid-producing ability in a medium containing fatty acids as the carbon source, particularly fatty acids which have been subjected to emulsification or homogenization, to thereby produce and accumulate the L-amino acid in a culture medium; and collecting the L-amino acid from the culture medium.



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DESCRIPTION

METHOD FOR PRODUCING AN L-AMINO ACID

Technical Field

[0001]

The present invention relates to a method for producing L-amino acids using bacteria. L-amino acids have many useful and various applications, including as additives in seasonings, food additives, feed additives, chemical products, and drugs.

Background Art

[0002]

L-amino acids such as L-threonine and L-lysine are industrially produced by fermentation methods using L-amino acid-producing bacteria such as *Escherichia* bacteria. Examples of L-amino acid-producing bacteria include bacterial strains isolated from nature or artificially mutated strains, as well as recombinant strains obtained by modifying the bacteria so that the activities of L-amino acid biosynthetic enzymes are enhanced. Methods for producing L-threonine include those disclosed in JP 05-304969 A, WO 98/04715, JP 05-227977 A, and US 2002/0110876 A. Methods of producing L-lysine include those disclosed in JP 10-165180 A, JP 11-192088 A, JP 2000-253879 A, and JP 2001-057896 A.

[0003]

In fermentative production of an L-amino acid, sugars such as glucose, fructose, sucrose, molasses, and starch hydrolysate are generally used as a source of carbon.

Clark et al. reported that an *Escherichia coli* wild-type strain can grow in a medium containing long-chain fatty acids (12 or more carbon atoms) as the sole carbon source (Clark, D.P. and Cronan Jr., J.E. 1996. p. 2343-2357. In F.D. Neidhardt (ed.), *Escherichia coli* and *Salmonella* Cellular and Molecular Biology/Second Edition, American Society for Microbiology Press, Washington, D.C.). Weeks et al. reported that an *Escherichia coli* wild-type strain can grow in a medium containing palmitic acid or oleic acid as the sole carbon source (Weeks, G., Shapiro, M. Burns, R.O., and Wakil, S.J. 1969. Control of Fatty Acid Metabolism I. Induction of the Enzymes of Fatty Acid Oxidation in *Escherichia coli*. J. Bacteriol. 97:827-836). However, the solubility of fatty acids is known to be extremely low,

and Vorum et al. reported that the solubility of oleic acid is 0.0003 g/l or less, and that of palmitic acid is 0.00000003 g/l or less, whereas the solubility of lauric acid is 0.1 g/l or more (Vorum, H., Brodersen, R., Kragh-Hansen, U., and Pedersen, A.O. Solubility of long-chain fatty acids in phosphate buffer at pH 7.4. 1992. *Biochimica et Biophysica Acta, Lipids and Lipid Metabolism* 1126: 135-142).

Therefore, there are very few examples of production of substances by a direct fermentation method using fatty acids as the sole carbon source, and there have been no reports of production of an L-amino acid by such methods. Furthermore, when fatty acids are employed as the sole carbon source, the concentration of the fatty acids is typically about 1 g/l. For example, JP 11-243956 A discloses an example of production of polyester, where the culture medium contains only 2 g/l lauric acid as the carbon source.

DISCLOSURE OF THE INVENTION

[0004]

The present invention provides a method for producing an L-amino acid by fermentation without utilizing sugar as the carbon source. Such a method can be performed at lower cost by using a novel fermentative material.

[0005]

The inventors of the present invention have made intensive studies to solve the above-mentioned problems. As a result, they have found that an L-amino acid is produced by culturing a bacterium of *Enterobacteriaceae* family having an L-amino acid-producing ability in a medium containing a fatty acid as the carbon source. Previously, fatty acids were not considered to be useful in fermentation due to their extremely low solubility in water. Moreover, it has been found that L-amino acid production is enhanced when the fatty acids in the medium have been subject to emulsification or homogenization.

[0006]

An object of the present invention is to provide a method for producing an L-amino acid, comprising culturing a bacterium of the *Enterobacteriaceae* family having an L-amino acid-producing ability in a medium containing a fatty acid, and collecting the L-amino acid from the medium or bacterial cells.

Another object of the present invention is to provide the method as described above, wherein said fatty acid comprises a fatty acid having no less than 14 carbons.

Another object of the present invention is to provide the method as described above, wherein said fatty acid is selected from the group consisting of myristic acid, palmitic acid, stearic acid, oleic acid, linoleic acid, and combinations thereof.

Another object of the present invention is to provide the method as described above, wherein said medium contains said fatty acid in an amount of 0.2 to 10 w/v%.

Another object of the present invention is to provide the method as described above, wherein said medium further contains a carbon source other than fatty acid.

Another object of the present invention is to provide the method as described above, wherein said fatty acid is emulsified.

Another object of the present invention is to provide the method as described above, wherein said emulsification occurs by a method selected from the group consisting of adding a surfactant to said medium, homogenization, ultrasonication, and combinations thereof.

Another object of the present invention is to provide the method as described above, wherein said emulsification occurs by homogenization and/or ultrasonication in the presence of a surfactant under alkali conditions.

Another object of the present invention is to provide the method as described above, wherein said bacterium belongs to the genus *Escherichia*.

Another object of the present invention is to provide the method as described above, wherein said L-amino acid is selected from the group consisting of L-threonine, L-lysine, L-phenylalanine, L-tryptophan, L-valine, L-leucine, L-isoleucine, L-methionine, and combinations thereof.

Another object of the present invention is to provide the method as described above, wherein the L-amino acid is selected from the group consisting of L-threonine, L-lysine, and combinations thereof.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0007]

Hereinafter, the present invention will be described in detail.

[0008]

<1> Method of the present invention

The method for producing an L-amino acid of the present invention includes the steps of culturing a bacterium of the *Enterobacteriaceae* family having an L-amino

acid-producing ability in a medium containing a fatty acid to cause accumulation of the L-amino acid in the medium or bacterial cells; and collecting the L-amino acid from the medium or bacterial cells. The method may employ a batch culture, fed-batch culture, or continuous culture, and the fatty acids may be present in the starting medium, the feed medium, or both.

[0009]

A fed-batch culture is when a medium is continuously or intermittently added to the culture container, and medium is not removed from the container until the culture is complete. A continuous culture is when a medium is continuously or intermittently added to the culture container, and medium is then removed from the container, in general, in an amount equal to the amount of the medium added. The starting medium is the medium used in the batch culture before adding the feed medium in the fed-batch culture or continuous culture, for example, the medium used at the start of the culture. The feed medium is the medium which is added to the fermenter in the fed-batch culture or continuous culture. Moreover, a batch culture is a method which includes inoculating a strain into fresh medium prepared per batch, where the medium is not added until the bacterial cells are collected.

[0010]

Fatty acids are monovalent carboxylic acids having a long hydrocarbon chain which is designated as C_nH_mCOOH ($n+1$ and $m+1$ represent carbon number and hydrogen number contained in the fatty acid, respectively). Generally, fatty acids with 12 or more carbons are considered long chain fatty acids. There are many kinds of fatty acids having different numbers of carbons and different degrees of unsaturation. It is also known that fatty acids are a component of oil, and the fatty acid composition is different depending on the kind of oil. Myristic acid ($C_{13}H_{27}COOH$) is a saturated fatty acid having 14 carbon atoms and is present in coconut and palm oils. Palmitic acid ($C_{15}H_{31}COOH$) is a saturated fatty acid having 16 carbon atoms and is present in a large amount in vegetable oil. Stearic acid ($C_{17}H_{35}COOH$) is a saturated fatty acid having 18 carbon atoms and is present in a large amount in animal fat or vegetable oil. Oleic acid ($C_{17}H_{33}COOH$) is a long-chain unsaturated monovalent fatty acid having 18 carbon atoms and is present in a large amount in animal fat or vegetable oil. Linoleic acid ($C_{17}H_{31}COOH$) is a multivalent unsaturated fatty acid having 18 carbon atoms and cis-9, 12-double bonds.

Mixtures of long chain fatty acids can be obtained by the hydrolysis of oil. Specifically, mixtures of fatty acids containing palmitic acid, stearic acid, and oleic acid can be obtained by hydrolysis of palm oil. Such fatty acid mixtures may be used in the method of the present invention. Fatty acids which can be extracted from animal oil, vegetable oil, food waste oil, and other oil mixtures, or from fat-containing food such as chocolate may be used. Fatty acids extracted during the purification of oil may also be used.

[0011]

The concentration of fatty acids in the medium is not particularly limited as long as the chosen bacterium can assimilate the fatty acid as the carbon source, but when fatty acids are added as the sole carbon source to the medium, the concentration is preferably not more than 10 w/v%, more preferably not more than 5 w/v%, still more preferably not more than 2 w/v%. Meanwhile, the concentration is preferably not less than 0.2 w/v%, more preferably not less than 0.5 w/v%, still more preferably not less than 1.0 w/v%.

When fatty acids are added as the sole carbon source to a fed-batch medium, the concentration of fatty acids in the medium is preferably not more than 5 w/v%, more preferably not more than 2 w/v%, still more preferably not more than 1 w/v%. It is preferable that the fatty acid concentration in the fed-batch medium is controlled to not less than 0.2 w/v%, preferably not less than 0.5 w/v%, more preferably not less than 1.0 w/v%.

[0012]

The concentration of fatty acids can be determined by gas chromatography (Hashimoto, K., Kawasaki, H., Akazawa, K., Nakamura, J., Asakura, Y., Kudo, T., Sakuradani, E., Shimizu, S., Nakamatsu, T. 1996. *Biosci. Biotechnol. Biochem.* 70:22-30) or HPLC (Lin, J. T., Snyder, L. R., and McKeon, T. A. 1998. *J. Chromatogr. A.* 808: 43-49).

[0013]

In addition, the fatty acids are preferably in the form of a water-soluble salt with an alkali metal such as sodium or potassium. However, in some instances, the solubility of a sodium or potassium salt of a fatty acid is not sufficient to be used in fermentation.

Accordingly, in order for a fatty acid to be efficiently assimilated as the carbon source by a bacterium having an L-amino acid-producing ability, it is preferable to add a step which promotes homogenization such as emulsification. For example, emulsification can be achieved by adding an emulsification promoting agent or a surfactant. Emulsification promoting agents include phospholipids and sterols. Surfactants include nonionic

surfactants such as a polyoxyethylene sorbitan fatty acid ester including poly(oxyethylene) sorbitan monooleate (Tween 80), and an alkyl glucoside including N-octyl β -D-glucoside, and zwitterionic surfactants such as an alkyl betaine including N,N-dimethyl-N-dodecylglycine betaine. General surfactants such as Triton X-100, polyoxyethylene (20) cetyl ether (Brij-58), and nonylphenol ethoxylate (Tergitol NP-40) may also be used.

Moreover, promotion of emulsification or homogenization of a fatty acid is also effective. Methods for such promotion are not particularly limited as long as promotion of emulsification or homogenization of a fatty acid is obtained. Specific examples thereof include homogenizer treatment, homomixer treatment, ultrasonication, high pressure treatment, and heat treatment. These treatments may be combined with the above-mentioned surfactant treatments. It is preferable that these treatments are performed under alkali conditions, preferably pH 9.0 or more, more preferably pH 10 or more.

[0014]

When mixtures of fatty acids are used as the carbon source, the mixing ratio of fatty acids is not particularly limited as long as the chosen bacterium can assimilate the fatty acids as the carbon source.

[0015]

The chosen medium may further contain a carbon source other than fatty acids. Preferable examples thereof include sugars such as glucose, fructose, sucrose, lactose, galactose, molasses, starch hydrolysate, and carbohydrate solution obtained by hydrolyzing biomass, alcohols such as ethanol, and organic acids such as fumaric acid, citric acid, and succinic acid. When using a carbon source other than fatty acids, the ratio of fatty acid(s) with respect to the entire source of carbon is 10 wt% or more, preferably 30 wt% or more, more preferably 50 wt% or more.

[0016]

Fatty acid(s) may be present in the medium during the entire culture period at a constant concentration, present only in the fed-batch medium, or present only in the starting medium. Also, during periods of the culture, there may be no fatty acid(s) present in the medium, as long as the medium contains a sufficient amount of another carbon source during these periods. That is, the time periods wherein the medium contains no fatty acids may be a period of 10%, 20%, or at maximum 30% with respect to the whole fermentation period, for example. In the present invention, the phrase "culture in a medium containing fatty acid(s)"

includes when the culture medium contains fatty acid(s) for a only a period of the culture, but may contain no fatty acids for another period.

[0017]

The chosen medium may additionally contain other components such as a nitrogen source, an inorganic ion, and if necessary, other organic nutrients.

Examples of a nitrogen source include ammonia, an ammonium salt such as ammonium sulfate, ammonium carbonate, ammonium chloride, ammonium phosphate, ammonium acetate, urea, and a nitrate salt. Ammonia gas or ammonia water may be used to adjust the pH, and may also be used as a nitrogen source. In addition, peptone, yeast extract, meat extract, malt extract, corn steep liquor, and soybean hydrolysate may also be used. In the medium, these nitrogen sources may be present alone or in combination, and may be present in the starting medium or the feed medium. In addition, both the starting medium and feed medium may contain the same nitrogen source or different nitrogen sources.

[0018]

The chosen medium may further contain a source of phosphoric acid and/or a sulfate in addition to the above-mentioned components. The phosphoric acid source may be, for example, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, and a phosphate polymer including pyrophosphoric acid. The sulfate source is not particularly limited as long as it contains a sulfur atom. Sulfate, thiosulfate, or sulfite are preferable, as are a sulfur-containing amino acids such as cysteine, cystine, or glutathione. Ammonium sulfate is more preferable.

[0019]

A medium may further contain a growth promoter or a nutrient having a growth-promoting effect. Growth promoters include trace metals, amino acids, vitamins, nucleic acids, peptones, casamino acid, yeast extracts, and soybean hydrolysates containing these. Examples of the trace metals include iron, manganese, magnesium, and calcium, and examples of the vitamins include vitamin B1, vitamin B2, vitamin B6, nicotinic acid, nicotinamide, and vitamin B12. These growth promoters may be present in the starting medium, fed-batch medium, or both.

[0020]

Moreover, when using an auxotrophic mutant that requires an amino acid or the like for its growth, the medium should be supplemented with the required nutrient. In particular,

as described below, a large number of L-lysine-producing bacteria have a strong L-lysine biosynthesis pathway and a weak L-lysine degradation ability; therefore, it is preferable to add to the medium one or more amino acids, such as L-threonine, L-homoserine, L-isoleucine, and L-methionine. The compositions of the starting medium and fed-batch medium may be the same or different. Meanwhile, the concentrations of sulfur in the starting medium and fed-batch medium may be the same or different. Moreover, when the feed medium is added in multiple stages, the compositions of the respective feed media may be the same or different.

[0021]

The culture may be performed with aeration at a fermentation temperature of 20 to 45°C, more preferably 33 to 42°C. The concentration of oxygen is adjusted to preferably 5 to 50%, more preferably about 10%. In addition, the culture is preferably performed with aeration while pH is controlled to 5 to 9. If the pH falls during culture, the medium may be neutralized by adding calcium carbonate or an alkaline such as ammonia gas or ammonia water. When the culture is performed under such conditions for about 10 to 120 hours, a significant amount of L-amino acids accumulate in the culture medium. The concentration of the L-amino acid is not particularly limited, as long as it is sufficient to be collected/recovered from the medium, but it is preferably not less than 50 g/L, more preferably not less than 75 g/L, still more preferably not less than 100 g/L.

[0022]

The L-amino acid may be collected from the culture medium after completion of culture in accordance with known recovery methods. For example, the L-amino acid can be collected by removing bacterial cells from the culture medium by centrifugation or the like followed by concentration and/or crystallization.

[0023]

In the present invention, in order to keep the accumulation of an L-amino acid at a certain level, the culture of the bacterium may be divided into a seed culture and main culture. The seed culture may be performed with shaking using a flask or the like or batch culture, while the main culture may be performed by fed-batch culture or continuous culture. Alternatively, both of the seed culture and main culture may be performed by batch culture.

[0024]

When the fed-batch culture or continuous culture is performed in the present

invention, the feed medium may be added intermittently so that addition of the fatty acid or other carbon source is stopped temporarily. The addition of the feed medium may be stopped for a period of at maximum 30% or less, preferably 20% or less, particularly preferably 10% or less with respect to the whole period of adding the medium. When the feed medium is added intermittently, the concentration of the substrate in the fermenter may be automatically maintained at a low level throughout the culture by controlling the culture system so that addition of the medium begins when a computer detects an increase in pH or an increase in concentration of dissolved oxygen in the medium due to exhaustion of the carbon source (US 5,912,113 B).

[0025]

The feed medium to be used in the fed-batch culture is preferably a medium containing a fatty acid, another carbon source, and a nutrient having a growth-promoting effect (growth promoter). The concentration of the fatty acid in the fermentation medium may be controlled to a certain level or less. Herein, the "certain level or less" means that the medium to be added is adjusted so that the fermentation medium contains a fatty acid in an amount of 10 w/v% or less, preferably 5 w/v% or less, more preferably 1 w/v% or less.

Preferable examples of other carbon sources in the feed medium include glucose, sucrose, and fructose, while preferable examples of the growth promoter in the feed medium include a nitrogen source, phosphoric acid, and an amino acid. Preferable examples of the nitrogen source in the feed medium include ammonia, an ammonium salt such as ammonium sulfate, ammonium carbonate, ammonium chloride, ammonium phosphate, ammonium acetate, urea, and a nitrate. Examples of a phosphoric acid source in the feed medium include potassium dihydrogen phosphate and dipotassium hydrogen phosphate, and when an amino acid-auxotrophic mutant strain is cultured, a required amino acid is preferably supplemented in the feed medium. The feed medium may be a single medium or a combination of two or more media. When using two or more feed media, these media may be mixed and fed from one feed pipe, or fed from a plurality of feed pipes.

[0026]

When using a continuous culture method in the present invention, the culture medium may be withdrawn at the same time as adding the feed medium, or adding the medium may be performed after a part of the culture medium is withdrawn. In addition, a continuous culture method may be used to reuse bacterial cells, which includes withdrawing

the culture medium containing an L-amino acid and cells and returning only the cells to the fermenter (FR 2669935 B). The method of continuously or intermittently adding a nutrient may be the same method as in the fed-batch culture.

[0027]

The continuous culture method to reuse bacterial cells includes intermittently or continuously withdrawing a fermentation medium when the concentration of an L-amino acid reaches a predetermined level, collecting only the L-amino acid, and recycling the filtration residue containing the bacterial cells in the fermenter, and it can be performed with reference to FR 2669935 B, for example.

[0028]

Herein, when intermittently withdrawing the culture medium, the L-amino acid may be withdrawn when the concentration of the L-amino acid reaches a predetermined level. Then, fresh medium may be added to a final volume which is preferably the same as the amount of medium before withdrawing the culture medium. Herein, the "same" means a volume of about 93 to 107% of the volume of the culture medium before withdrawing of the medium.

[0029]

When continuously withdrawing the culture medium, it is preferable to start withdrawing simultaneously with or after the addition of the feed medium. When withdrawing the culture medium after the addition of the feed medium, it should be done, for example, within 5 hours, preferably within 3 hours, more preferably within 1 hour after adding the medium. Meanwhile, the volume of the medium which is withdrawn is preferably the same as that which is added.

[0030]

<2> Bacterium to be used in the present invention

In the present invention, a bacterium of the *Enterobacteriaceae* family may be used which has an L-amino acid-producing ability and can metabolize fatty acid(s) when used as the carbon source.

Examples of the bacterium of *Enterobacteriaceae* family include bacteria belonging to the genera *Escherichia*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Pantoea*, *Photorhabdus*, *Providencia*, *Salmonella*, *Serratia*, *Shigella*, *Morganella*, and *Yersinia*. Particularly preferable are bacteria classified into the *Enterobacteriaceae* family based on the

classification method used by the database of the NCBI (National Center for Biotechnology Information) (www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=91347).

[0031]

The bacteria belonging to the genus *Escherichia* are not particularly limited, but include bacteria classified into the genus *Escherichia* based on the classification known to microbiologists. Examples of a bacterium belonging to the genus *Escherichia* to be used in the present invention include, but are not limited to, *Escherichia coli* (*E. coli*).

[0032]

The bacteria belonging to the genus *Escherichia* that can be used in the present invention are not particularly limited, but include the bacterial strains described in Table 1 of Bachmann et al. (Bachmann, B.J. 1996. p. 2460-2488. In F.D. Neidhardt (ed.), *Escherichia coli* and Salmonella Cellular and Molecular Biology/Second Edition, American Society for Microbiology Press, Washington, D.C.). Specific examples include *Escherichia coli* W3110 (ATCC 27325) and *Escherichia coli* MG1655 (ATCC 47076) derived from a prototype wild-type K-12 strain.

[0033]

These strains can be obtained from the American Type Culture Collection (ATCC) (Address: P.O. Box 1549, Manassas, VA 20108, 1, United States of America). That is, an accession number is given to each strain, and a desirable bacterial strain can be ordered by referencing the accession number. The accession number of each strain may be found in the catalogue of the American Type Culture Collection.

The bacteria belonging to the genus *Pantoea* include bacteria classified into the genus *Pantoea* based on the classification known to microbiologists. In recent years, some of *Enterobacter agglomerans* bacteria were reclassified into *Enterobacter agglomerans*, *Pantoea ananatis*, *Pantoea stewartii*, etc. based on the nucleotide sequence analysis of 16S rRNA (Int. J. Syst. Bacteriol. 1993. 43: 162-173). In the present invention, the bacteria belonging to the genus *Pantoea* include such bacteria reclassified into the genus *Pantoea*.

[0034]

In the present invention, the bacterium having an L-amino acid-producing ability refers to a bacterium having an ability to produce and secrete an L-amino acid in the medium when it is cultured in the medium. In addition, the bacterium is preferably a bacterium having an ability to produce and secrete an L-amino acid in an amount of preferably 0.5 g/L

or more, more preferably 1.0 g/L or more into the medium. The L-amino acid includes L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-glutamic acid, L-glutamine, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, and L-valine. Among these, L-threonine, L-lysine, L-phenylalanine, L-tryptophan, L-valine, L-leucine, L-isoleucine, and L-methionine are preferable. In particular, L-threonine and L-lysine are preferable.

In the method of the present invention, L-amino acid-producing bacteria that have been reported so far can be used as long as they can assimilate fatty acid.

Hereinafter, L-amino acid-producing bacteria that can be used in the present invention will be described.

[0035]

L-threonine-producing bacteria

Examples of L-threonine-producing bacteria and parent strains for deriving the L-threonine-producing bacteria include, but are not limited to, strains belonging to the genus *Escherichia*, such as *E. coli* TDH-6/pVIC40 (VKPM B-3996) (U.S. Patent No. 5, 175, 107, U.S. Patent No. 5,705,371), *E. coli* 472T23/pYN7 (ATCC 98081) (U.S. Patent No.5,631,157), *E. coli* NRRL-21593 (U.S. Patent No. 5,939,307), *E. coli* FERM BP-3756 (U.S. Patent No. 5,474,918), *E. coli* FERM BP-3519 and FERM BP-3520 (U.S. Patent No. 5,376,538), *E. coli* MG442 (Gusyatiner et al., Genetika (in Russian), 14, 947-956 (1978)), *E. coli* VL643 and VL2055 (EP 1149911 A), and the like.

[0036]

The TDH-6 strain is deficient in the *thrC* gene, as well as being sucrose-assimilative, and the *ilvA* gene has a leaky mutation. This strain also has a mutation in the *rhtA* gene, which imparts resistance to high concentrations of threonine or homoserine. The B-3996 strain contains pVIC40, which was obtained by inserting the *thrA**BC operon, which includes a mutant *thrA* gene, into a RSF1010-derived vector. This mutant *thrA* gene encodes aspartokinase homoserine dehydrogenase I which is substantially desensitized to feedback inhibition by threonine. The B-3996 strain was deposited on November 19, 1987 in the All-Union Scientific Center of Antibiotics (Nagatinskaya Street 3-A, 117105 Moscow, Russian Federation) under the accession number RIA 1867. This strain was also deposited in the Russian National Collection of Industrial Microorganisms (VKPM) (Russia, 117545 Moscow 1, Dorozhny proezd. 1) on April 7, 1987 under the accession number B-3996.

[0037]

E. coli VKPM B-5318 (EP 0593792B) may also be used as an L-threonine-producing bacterium. The B-5318 strain is prototrophic with regard to isoleucine, and a temperature-sensitive lambda-phage C1 repressor and PR promoter replaces the regulatory region of the threonine operon in plasmid pVIC40. The VKPM B-5318 strain was deposited in the Russian National Collection of Industrial Microorganisms (VKPM: Russia, 117545 Moscow 1, Dorozhny proezd. 1) on May 3, 1990 under accession number of VKPM B-5318.

[0038]

Preferably, the L-threonine-producing bacteria are additionally modified to enhance expression of one or more of the following genes:

- the mutant *thrA* gene which codes for aspartokinase homoserine dehydrogenase I resistant to feed back inhibition by threonine;
- the *thrB* gene which codes for homoserine kinase;
- the *thrC* gene which codes for threonine synthase;
- the *rhtA* gene which codes for a putative transmembrane protein;
- the *asd* gene which codes for aspartate- β -semialdehyde dehydrogenase; and
- the *aspC* gene which codes for aspartate aminotransferase (aspartate transaminase).

[0039]

The sequence of the *thrA* gene of *Escherichia coli* which encodes aspartokinase homoserine dehydrogenase I has been elucidated (nucleotide positions 337 to 2799, GenBank accession NC_000913.2, gi: 49175990). The *thrA* gene is located between the *thrL* and *thrB* genes on the chromosome of *E. coli* K-12. The nucleotide sequence of the *thrB* gene of *Escherichia coli* which encodes homoserine kinase has been elucidated (nucleotide positions 2801 to 3733, GenBank accession NC_000913.2, gi: 49175990). The *thrB* gene is located between the *thrA* and *thrC* genes on the chromosome of *E. coli* K-12. The nucleotide sequence of the *thrC* gene of *Escherichia coli* which encodes threonine synthase has been elucidated (nucleotide positions 3734 to 5020, GenBank accession NC_000913.2, gi: 49175990). The *thrC* gene is located between the *thrB* gene and the *yaaX* open reading frame on the chromosome of *E. coli* K-12. All three genes function together as a single threonine operon. To enhance the expression of the threonine operon, the attenuator region which affects the transcription can be removed from the operon (WO2005/049808, WO2003/097839).

[0040]

The mutated *thrA* gene which encodes feedback-resistant aspartokinase homoserine dehydrogenase I, as well as the *thrB* and *thrC* genes can be obtained as one operon from the well-known plasmid pVIC40. This plasmid is present in the threonine producing *E. coli* strain VKPM B-3996, and is described in detail in U.S. Patent No. 5,705,371.

[0041]

The *rhtA* gene is at 18 min on the *E. coli* chromosome close to the *glnHPQ* operon, which encodes components of the glutamine transport system. The *rhtA* gene is identical to ORF1 (*ybiF* gene, nucleotide positions 764 to 1651, GenBank accession number AAA218541, gi:440181) and is located between the *pexB* and *ompX* genes. The sequence expressing a protein encoded by the ORF1 has been designated the *rhtA* gene (*rht*: resistance to homoserine and threonine). Also, the *rhtA23* mutation is an A-for-G substitution at position -1 with respect to the ATG start codon (ABSTRACTS of the 17th International Congress of Biochemistry and Molecular Biology in conjunction with Annual Meeting of the American Society for Biochemistry and Molecular Biology, San Francisco, California August 24-29, 1997, abstract No. 457, EP 1013765 A).

[0042]

The nucleotide sequence of the *asd* gene of *E. coli* has already been elucidated (nucleotide positions 3572511 to 3571408, GenBank accession NC_000913.1, gi:16131307), and can be obtained by PCR (polymerase chain reaction; refer to White, T.J. et al., Trends Genet., 5, 185 (1989)) by utilizing primers based on the nucleotide sequence of the gene. The *asd* genes from other microorganisms can be obtained in a similar manner.

[0043]

Also, the nucleotide sequence of the *aspC* gene of *E. coli* has already been elucidated (nucleotide positions 983742 to 984932, GenBank accession NC_000913.1, gi:16128895), and can be obtained by PCR. The *aspC* genes from other microorganisms can be obtained in a similar manner.

[0044]

L-lysine-producing bacteria

Examples of L-lysine-producing bacteria belonging to the genus *Escherichia* include mutants having resistance to an L-lysine analogue. The L-lysine analogue inhibits growth of bacteria belonging to the genus *Escherichia*, but this inhibition is fully or partially

desensitized when L-lysine is present in a medium. Examples of the L-lysine analogue include, but are not limited to, oxalysine, lysine hydroxamate, S-(2-aminoethyl)-L-cysteine (AEC), γ -methyllysine, α -chlorocaprolactam and so forth. Mutants having resistance to these lysine analogues can be obtained by subjecting bacteria belonging to the genus *Escherichia* to a conventional artificial mutagenesis treatment. Specific examples of bacterial strains useful for producing L-lysine include *Escherichia coli* AJ11442 (FERM BP-1543, NRRL B-12185; see U.S. Patent No. 4,346,170) and *Escherichia coli* VL611. In these microorganisms, feedback inhibition of aspartokinase by L-lysine is desensitized.

[0045]

The strain WC196 may be used as an L-lysine producing bacterium of *Escherichia coli*. This bacterial strain was bred by conferring AEC resistance to the strain W3110, which was derived from *Escherichia coli* K-12. The resulting strain was designated *Escherichia coli* AJ13069 strain and was 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 received 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 received an accession number of FERM BP-5252 (U.S. Patent No. 5,827,698).

[0046]

Examples of L-lysine-producing bacteria and parent strains for deriving L-lysine-producing bacteria also include strains in which expression of one or more genes encoding an L-lysine biosynthetic enzyme are enhanced. Examples of the enzymes involved in L-lysine biosynthesis include, but are not limited to, dihydrodipicolinate synthase (dapA), aspartokinase (lysC), dihydrodipicolinate reductase (dapB), diaminopimelate decarboxylase (lysA), diaminopimelate dehydrogenase (ddh) (U.S. Patent No. 6,040,160), phosphoenolpyruvate carboxylase (ppc), aspartate semialdehyde dehydrogenase (asd), and aspartase (aspA) (EP 1253195 A). In addition, the L-lysine-producing strains may have increased expression of the gene involved in energy efficiency (cyo) (EP 1170376 A), the gene encoding nicotinamide nucleotide transhydrogenase (pntAB) (U.S. Patent No. 5,830,716), the ybjE gene (WO2005/073390), or combinations thereof.

[0047]

It is known that wild-type DDPS derived from *Escherichia coli* is regulated by feedback inhibition by L-lysine, while wild-type aspartokinase derived from *Escherichia coli* is regulated by suppression and feedback inhibition by L-lysine. Therefore, when using *dapA* and *lysC*, mutated forms of these genes are preferably used so that the enzymes encoded by the genes are not subject to feedback inhibition.

[0048]

An example of a DNA encoding a mutant DDPS desensitized to feedback inhibition by L-lysine includes a DNA encoding a DDPS which has an amino acid sequence in which the histidine at position 118 is replaced by tyrosine. Meanwhile, an example of a DNA encoding mutant aspartokinase III (AKIII) desensitized to feedback inhibition by L-lysine includes a DNA encoding an AKIII having an amino acid sequence in which the threonine at position 352, the glycine at position 323, and the methionine at position 318 are replaced by isoleucine, asparagine and isoleucine, respectively (U.S. Patent No. 5661012 and U.S. Patent No. 6040160). Such mutant DNAs can be obtained by a site-specific mutation using PCR or the like.

[0049]

Wide host-range plasmids RSFD80, pCAB1, and pCABD2 are known to contain a mutant *dapA* gene encoding a mutant DDPS and a mutant *lysC* gene encoding a mutant AKIII (U.S. Patent No. 6040160). *Escherichia coli* JM109 strain transformed with RSFD80 was named AJ12396 (U.S. Patent No. 6040160), 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, International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology) on October 28, 1993 and given 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 given an accession number of FERM BP-4859. RSFD80 can be obtained from AJ12396 strain by a conventional method.

[0050]

Examples of L-lysine-producing bacteria and parent strains for deriving L-lysine-producing bacteria also include strains having decreased or eliminated activity of an enzyme that catalyzes a reaction for generating a compound other than L-lysine by branching

off from the biosynthetic pathway of L-lysine. Examples of the enzymes that catalyze a reaction for generating a compound other than L-lysine by branching off from the biosynthetic pathway of L-lysine include homoserine dehydrogenase (WO 95/23864), lysine decarboxylase (U.S. Patent No. 5,827,698), and the malic enzyme (WO2005/010175).

In *Escherichia coli*, lysine decarboxylases are encoded by the *cadA* gene (GenBank Accession No. NP_418555, SEQ ID NO: 5) and *ldcC* gene (GenBank Accession No. NP_414728, SEQ ID NO: 7) (WO 96/17930), so these genes may be disrupted to enhance L-lysine-producing ability. DNA molecules homologous to the *cadA* gene and *ldcC* gene may be used as long as they can induce homologous recombination with the *cadA* gene and *ldcC* gene on the chromosome of a host bacterium. For example, a DNA molecule homologous to the *cadA* gene may hybridize to a complementary strand of SEQ ID NO: 5 under stringent conditions, and a DNA molecule homologous to the *ldcC* gene may hybridize to a complementary strand of SEQ ID NO: 7 under stringent conditions.

Herein, the term "stringent conditions" refers to conditions where a so-called specific hybrid is formed and non-specific hybrid is not formed. It is difficult to clearly define the conditions by a numerical value, and examples include conditions where DNAs having high homology, for example, DNAs having homology of at least 80%, preferably at least 90%, more preferably at least 95%, or particularly preferably at least 97% hybridize with each other and DNAs having homology of less than 80% do not hybridize with each other; and specific examples thereof include washing in general Southern hybridization, i.e., washing at the salt concentration of 1 x SSC, 0.1% SDS, preferably 0.1 x SSC, 0.1% SDS, at 60°C, preferably at 68°C, once, preferably twice or three times.

[0051]

L-cysteine-producing bacteria

Examples of L-cysteine-producing bacteria and parent strains for deriving L-cysteine-producing bacteria include, but are not limited to, strains belonging to the genus *Escherichia*, such as *E. coli* JM15 which has been transformed with different *cysE* alleles coding for feedback-resistant serine acetyltransferases (U.S. Patent No. 6,218,168, Russian patent application 2003121601), *E. coli* W3110 which over-expresses genes which encode proteins suitable for secreting toxic substances (U.S. Patent No. 5,972,663), *E. coli* strains with decreased cysteine desulfohydase activity (JP11155571A2); *E. coli* W3110 with increased activity of a positive transcriptional regulator for the cysteine regulon encoded by

the *cysB* gene (WO0127307A1), and the like.

[0052]

L-leucine-producing bacteria

Examples of L-leucine-producing bacteria and parent strains for deriving L-leucine-producing bacteria include, but are not limited to, strains belonging to the genus *Escherichia*, 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 analogs including β -2-thienylalanine, 3-hydroxyleucine, 4-azaleucine, 5,5,5-trifluoroleucine (JP 62-34397 B and JP 8-70879 A); *E. coli* strains obtained by the genetic engineering method described in WO96/06926; *E. coli* H-9068 (JP 8-70879 A), and the like.

[0053]

The L-leucine-producing ability may be improved by enhancing the expression of one or more genes involved in L-leucine biosynthesis. Examples of these genes include those of the *leuABCD* operon, which preferably include a *leuA* gene which has been mutated so that it encodes isopropylmalate synthase which is resistant to feedback inhibition by L-leucine (US Patent 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 acids from the bacterial cell. Examples of such genes include the *b2682* and *b2683* genes (*ygaZH* genes) (EP 1239041 A2).

[0054]

L-histidine-producing bacteria

Examples of L-histidine-producing bacteria and parent strains for deriving L-histidine-producing bacteria include, but are not limited to, strains belonging to the genus *Escherichia*, such as *E. coli* strain 24 (VKPM B-5945, RU2003677); *E. coli* strain 80 (VKPM B-7270, RU2119536); *E. coli* NRRL B-12116 – B12121 (U.S. Patent No. 4,388,405); *E. coli* H-9342 (FERM BP-6675) and H-9343 (FERM BP-6676) (U.S. Patent No. 6,344,347); *E. coli* H-9341 (FERM BP-6674) (EP1085087); *E. coli* AI80/pFM201 (U.S. Patent No. 6,258,554) and the like.

[0055]

Examples of L-histidine-producing bacteria and parent strains for deriving L-histidine-producing bacteria also include strains in which expression of one or more genes encoding an L-histidine biosynthetic enzyme are enhanced. Examples of these

L-histidine-biosynthetic enzymes include 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.

[0056]

It is known that the genes encoding the L-histidine biosynthetic enzyme (hisG, hisBHAFI) are inhibited by L-histidine, and therefore the L-histidine-producing ability can also be efficiently enhanced by introducing a mutation which induces resistance to feedback inhibition into ATP phosphoribosyltransferase (hisG) (Russian Patent Nos. 2003677 and 2119536).

Specific examples of strains having an L-histidine-producing ability include *E. coli* FERM-P 5038 and 5048 which have been transformed with a vector carrying a DNA encoding an L-histidine-biosynthetic enzyme (JP 56-005099 A), *E. coli* strains transformed with *rht*, a gene for an amino acid-export (EP1016710A), *E. coli* 80 strain imparted with sulfaguanidine, DL-1,2,4-triazole-3-alanine, and streptomycin-resistance (VKPM B-7270, Russian Patent No. 2119536), and so forth.

[0057]

L-glutamic acid-producing bacteria

Examples of L-glutamic acid-producing bacteria and parent strains for deriving L-glutamic acid-producing bacteria include, but are not limited to, strains belonging to the genus *Escherichia*, such as *E. coli* VL334thrC⁺ (EP 1172433). *E. coli* VL334 (VKPM B-1641) is auxotrophic for L-isoleucine and L-threonine and is mutated in the *thrC* and *ilvA* genes (U.S. Patent No. 4,278,765). A wild-type allele of the *thrC* gene was transferred by general transduction using a bacteriophage P1 grown on the wild-type *E. coli* strain K12 (VKPM B-7). As a result, an L-isoleucine auxotrophic strain VL334thrC⁺ (VKPM B-8961) was obtained.

[0058]

Examples of L-glutamic acid-producing bacteria and parent strains for deriving the L-glutamic acid-producing bacteria include, but are not limited to, strains in which expression of one or more genes encoding an L-glutamic acid biosynthetic enzyme are enhanced. Examples of the enzymes involved in L-glutamic acid biosynthesis include 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), phosphoglycerate kinase (pgk), glyceraldehyde-3-phosphate dehydrogenase (gapA), triose phosphate isomerase (tpiA), fructose biphosphate aldolase (fbp), phosphofructokinase (pfkA, pfkB), and glucose phosphate isomerase (pgi).

[0059]

Examples of strains modified so that expression of the citrate synthetase gene, the phosphoenolpyruvate carboxylase gene, and/or the glutamate dehydrogenase gene is/are enhanced include those disclosed in EP1078989A, EP955368A, and EP952221A.

[0060]

Examples of L-glutamic acid-producing bacteria and parent strains for deriving the L-glutamic acid-producing bacteria also include strains which have a decreased or eliminated activity of an enzyme that catalyzes synthesis of a compound other than L-glutamic acid, and branches off from the L-glutamic acid biosynthesis pathway. Examples of such enzymes include isocitrate lyase, α -ketoglutarate dehydrogenase, phosphotransacetylase, acetate kinase, acetohydroxy acid synthase, acetolactate synthase, formate acetyltransferase, lactate dehydrogenase, and glutamate decarboxylase. Bacteria belonging to the genus *Escherichia* deficient in α -ketoglutarate dehydrogenase activity or having a reduced α -ketoglutarate dehydrogenase activity and methods for obtaining them are described in U.S. Patent Nos. 5,378,616 and 5,573,945.

[0061]

Specifically, these strains include the following:

E. coli W3110sucA::Kmr

E. coli AJ12624 (FERM BP-3853)

E. coli AJ12628 (FERM BP-3854)

E. coli AJ12949 (FERM BP-4881)

[0062]

E. coli W3110sucA::Kmr is obtained by disrupting the α -ketoglutarate dehydrogenase gene (hereinafter referred to as the "sucA gene") of *E. coli* W3110. This strain is completely deficient in α -ketoglutarate dehydrogenase.

[0063]

Other examples of L-glutamic acid-producing bacterium include those which belong to the genus *Escherichia* and have resistance to an aspartic acid antimetabolite. These strains can also be deficient in α -ketoglutarate dehydrogenase activity and include, for example, *E. coli* AJ13199 (FERM BP-5807) (U.S. Patent No. 5,908,768), FERM P-12379, which additionally has a low L-glutamic acid decomposing ability (U.S. Patent No. 5,393,671); AJ13138 (FERM BP-5565) (U.S. Patent No. 6,110,714), and the like.

[0064]

Examples of L-glutamic acid-producing bacteria include mutant strains belonging to the genus *Pantoea* which are deficient in α -ketoglutarate dehydrogenase activity or have decreased α -ketoglutarate dehydrogenase activity, and can be obtained as described above. Such strains include *Pantoea ananatis* AJ13356 (U.S. Patent No. 6,331,419). *Pantoea ananatis* AJ13356 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, Central 6, 1-1, Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, 305-8566, Japan) on February 19, 1998 under an accession number of FERM P-16645. It was then converted to an international deposit under the provisions of Budapest Treaty on January 11, 1999 and received an accession number of FERM BP-6615. *Pantoea ananatis* AJ13356 is deficient in α -ketoglutarate dehydrogenase activity as a result of the disruption of the α KGDH-E1 subunit gene (*sucA*). The above 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 depository as *Enterobacter agglomerans*, for the purposes of this specification, they are described as *Pantoea ananatis*.

[0065]

L-phenylalanine-producing bacteria

Examples of L-phenylalanine-producing bacteria and parent strains for deriving L-phenylalanine-producing bacteria 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 the *pheA34* gene (U.S. Patent No. 5,354,672); *E. coli*

MWEC101-b (KR8903681); *E. coli* NRRL B-12141, NRRL B-12145, NRRL B-12146 and NRRL B-12147 (U.S. Patent No. 4,407,952). Also, as an L-phenylalanine-producing 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 AJ 12604 (FERM BP-3579) may be used (EP 488424 B1). Furthermore, L-phenylalanine producing bacteria belonging to the genus *Escherichia* which have an enhanced activity of the protein encoded by the yedA gene or the yddG gene may also be used (U.S. patent applications 2003/0148473 A1 and 2003/0157667 A1).

[0066]

L-tryptophan-producing bacteria

Examples of L-tryptophan-producing bacteria and parent strains for deriving the L-tryptophan-producing bacteria include, but are not limited to, strains belonging to the genus *Escherichia*, such as *E. coli* JP4735/pMU3028 (DSM10122) and JP6015/pMU91 (DSM10123) deficient in the 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 resistant to feedback inhibition by serine and a trpE allele encoding anthranilate synthase resistant 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 (WO9708333, U.S. Patent No. 6,319,696), and the like may be used. Furthermore, L-tryptophan producing bacteria belonging to the genus *Escherichia* which have an enhanced activity of the protein encoded by the yedA gene or the yddG gene may also be used (U.S. patent applications 2003/0148473 A1 and 2003/0157667 A1).

[0067]

Examples of L-tryptophan-producing bacteria and parent strains for deriving the L-tryptophan-producing bacteria also include strains in which one or more activities of the enzymes selected from anthranilate synthase (trpE), phosphoglycerate dehydrogenase (serA), and tryptophan synthase (trpAB) are enhanced. The anthranilate synthase and phosphoglycerate dehydrogenase are both subject to feedback inhibition by L-tryptophan and L-serine, so a mutation which results in desensitizing the feedback inhibition may be

introduced into these enzymes. Specific examples of strains having such a mutation include an *E. coli* SV164 which harbors desensitized anthranilate synthase and a strain obtained by transforming the plasmid pGH5 into *E. coli* SV164 (WO 94/08031), which contains a *serA* gene which has been mutated so that it encodes feedback-desensitized phosphoglycerate dehydrogenase.

[0068]

Examples of L-tryptophan-producing bacteria and parent strains for deriving the L-tryptophan-producing bacteria also include strains transformed with the tryptophan operon which contains a gene encoding desensitized anthranilate synthase (JP 57-71397 A, JP 62-244382 A, 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, among tryptophan operons (*trpBA*). The tryptophan synthase consists of α and β subunits which are encoded by *trpA* and *trpB*, respectively. In addition, L-tryptophan-producing ability may be improved by enhancing expression of the isocitrate lyase-malate synthase operon (WO2005/103275).

[0069]

L-proline-producing bacteria

Examples of L-proline-producing bacteria and parent strains for deriving L-proline-producing bacteria include, but are not limited to, strains belonging to the genus *Escherichia*, such as *E. coli* 702ilvA (VKPM B-8012) which is deficient in the *ilvA* gene and is able to produce L-proline (EP 1172433).

[0070]

The L-proline-producing ability may be improved by enhancing the expression of one or more genes involved in L-proline biosynthesis. Examples of preferred genes for L-proline producing bacteria include the *proB* gene coding for glutamate kinase which is desensitized to feedback inhibition by L-proline (DE Patent 3127361). In addition, the bacterium of the present invention may be improved by enhancing the expression of one or more genes coding for proteins excreting L-amino acid from the bacterial cell. Such genes include the *b2682* and *b2683* genes (*ygaZH* genes) (EP1239041 A2).

[0071]

Examples of bacteria belonging to the genus *Escherichia* which have an activity to produce L-proline include the following *E. coli* strains: NRRL B-12403 and NRRL B-12404

(GB Patent 2075056), VKPM B-8012 (Russian patent application 2000124295), plasmid mutants described in DE Patent 3127361, plasmid mutants described by Bloom F.R. et al (The 15th Miami winter symposium, 1983, p.34), and the like.

[0072]

L-arginine-producing bacteria

Examples of L-arginine-producing bacteria and parent strains for deriving L-arginine-producing bacteria include, but are not limited to, strains belonging to the genus *Escherichia*, such as *E. coli* strain 237 (VKPM B-7925) (U.S. Patent Application 2002/058315 A1) and its derivative strains harboring mutant N-acetylglutamate synthase (Russian Patent Application No. 2001112869), *E. coli* strain 382 (VKPM B-7926) (EP1170358A1), an arginine-producing strain into which the *argA* gene encoding N-acetylglutamate synthetase is introduced (EP1170361A1), and the like.

Examples of L-arginine-producing bacteria and parent strains for deriving L-arginine producing bacteria also include strains in which expression of one or more genes encoding an L-arginine biosynthetic enzyme are enhanced. Examples of the L-arginine biosynthetic enzymes include N-acetylglutamyl phosphate reductase (*argC*), ornithine acetyl transferase (*argJ*), N-acetylglutamate kinase (*argB*), acetylornithine transaminase (*argD*), ornithine carbamoyl transferase (*argF*), argininosuccinic acid synthetase (*argG*), argininosuccinic acid lyase (*argH*), and carbamoyl phosphate synthetase (*carAB*).

[0073]

L-valine-producing bacteria

Example of L-valine-producing bacteria and parent strains for deriving 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 desirable to remove the region of the *ilvGMEDA* operon which is required for attenuation so that expression of the operon is not attenuated by the L-valine that is produced. Furthermore, the *ilvA* gene in the operon is desirably disrupted so that threonine deaminase activity is decreased.

Examples of L-valine-producing bacteria also include mutants of amino-acyl t-RNA synthetase (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 has been deposited in the Russian National Collection of Industrial Microorganisms (VKPM) (Russia, 113545 Moscow, 1 Dorozhny Proezd.) on June 24, 1988 under accession number VKPM

B-4411.

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

[0074]

L-isoleucine-producing bacteria

Examples of L-isoleucine-producing bacteria and parent strains for deriving L-isoleucine producing bacteria include, but are not limited to, mutants having resistance to 6-dimethylaminopurine (JP 5-304969 A), 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 (JP 5-130882 A). In addition, recombinant strains transformed with genes encoding proteins involved in L-isoleucine biosynthesis, such as threonine deaminase and acetohydroxate synthase, can also be used (JP 2-458 A, FR 0356739, and U.S. Patent No. 5,998,178).

[0075]

L-methionine-producing bacteria

Examples of L-methionine-producing bacteria and parent strains for deriving L-methionine producing bacteria include, but are not limited to, L-threonine-auxotrophic mutant strain and norleucine-resistant mutant strain (JP 2000-139471 A), a methionine repressor-deficient strain and recombinant strains transformed with genes encoding L-methionine biosynthetic enzymes such as homoserine transsuccinylase and cystathionine γ -synthase (JP 2000-139471 A).

EXAMPLES

[0076]

Hereinafter, the present invention will be specifically described by referring to the following non-limiting Examples. In the Examples, oleic acid sodium salt (C₁₇H₃₃COONa), palmitic acid sodium salt (C₁₅H₃₁COONa) (both manufactured by Nacalai Tesque, Inc.), myristic acid sodium salt (C₁₃H₂₇COONa) (SIGMA) and linoleic acid (C₁₇H₃₁COOH) (Nacalai Tesque, Inc.) were used.

[0077]

Example 1: Culture for producing L-threonine

Escherichia coli B-5318, an L-threonine-producing strain, was cultured on LB agar

medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, and 15 g/L agar) containing 20 mg/L of streptomycin sulfate at 37°C for 24 hours. The cells grown on the agar medium were scraped off and inoculated into a 500-ml baffle flask containing 20 mL of a fermentation medium, followed by culturing at a temperature of 40°C for 24 hours.

The composition of the fermentation medium used for the L-threonine fermentation is shown below. Sodium oleate was added to the fermentation medium as the carbon source. The total carbon content was adjusted to 20 g/L in each medium.

[0078]

[*Escherichia* bacterium L-threonine fermentation medium]

Carbon source	20 g/L
Yeast extract	2 g/L
FeSO ₄ •7H ₂ O	10 mg/L
MnSO ₄ •4H ₂ O	10 mg/L
KH ₂ PO ₄	1.0 g/L
MgSO ₄ •7H ₂ O	1 g/L
(NH ₄) ₂ SO ₄	16 g/L
Streptomycin sulfate	20 mg/L

The medium was adjusted to pH 7.0 with KOH and sterilized in an autoclave at 120°C for 20 minutes. The carbon source and MgSO₄•7H₂O were separately sterilized and mixed. CaCO₃ was added after dry heat sterilization.

[0079]

After 24 hours, the culture supernatants were analyzed by liquid chromatography to determine the amount of L-threonine. Growth could not be determined based on the turbidities (ODs), and therefore, culture media diluted to appropriate concentrations were applied onto LB plates, followed by determination of the number of viable cells. The results are shown in Table 1 as the mean of duplicate cultures.

When the culture was performed using only oleic acid as the carbon source, a substantial amount of L-threonine accumulated.

[0080]

Table 1: Results of culture of the L-threonine-producing bacterium

Carbon source	Culture time (h)	Viable cells (10 ⁸ /ml)	L-threonine (g/l)
Oleic acid	24	7.3	3.8

[0081]

Example 2:

<2-1> Construction of a strain with disrupted lysine decarboxylase-encoding genes (cadA and ldcC)

A strain which produces no lysine decarboxylase was constructed. The lysine decarboxylases are encoded by the cadA gene (GenBank Accession No. NP_418555, SEQ ID NO: 5) and the ldcC gene (GenBank Accession No. NP_414728, SEQ ID NO: 7) (WO 96/17930). *Escherichia coli* WC196 (FERM BP-5252), which is an AEC-resistant L-lysine-producing strain, was used as a parent strain (WO96/17930).

[0082]

The cadA and ldcC gene were disrupted by the method developed by Datsenko and Wanner, which is called "Red-driven integration" (Proc. Natl. Acad. Sci. USA, 2000, vol. 97, No. 12, p6640-6645), and by an excision system derived from λ phage (J. Bacteriol. 2002 Sep; 184(18): 5200-3. Interactions between integrase and excisionase in the phage lambda excisive nucleoprotein complex. Cho EH, Gumport RI, Gardner JF.). "Red-driven integration" makes it possible to construct a gene-disrupted strain in one step by employing a PCR product obtained by using as primers synthetic oligonucleotides designed to have a part of the targeted gene on their 5'-ends, and a part of an antibiotic-resistance gene on their 3'-ends. Combining this method with the λ phage-derived excision system permits the removal of the antibiotic-resistance gene that becomes incorporated into the gene-disrupted strain (JP2005-058227).

[0083]

<2-2> Disruption of the cadA gene

The pMW118-attL-Cm-attR plasmid (WO2005/010175) was used as a template for PCR. pMW118-attL-Cm-attR was obtained by inserting the attL and attR genes, which are attachment sites for λ phage, and the cat gene, which is an antibiotic resistance gene, into pMW118 (Takara Bio Inc.) The genes are arranged in the following order: attL-cat-attR.

[0084]

PCR was performed using, as primers, the synthetic oligonucleotides shown in SEQ ID NOS: 1 and 2, which have sequences corresponding to attL and attR on their 3'-ends and a sequence corresponding to a part of the targeted cadA gene on their 5'-ends.

[0085]

The amplified PCR product was purified on an agarose gel and introduced into the *Escherichia coli* WC196 strain by electroporation. This strain harbors pKD46 which has temperature-sensitive replicability. pKD46 (Proc. Natl. Acad. Sci. USA, 2000, vol. 97, No. 12, p6640-6645) contains a DNA fragment of 2,154 nucleotides derived from λ phage which contains the Red recombinase-encoding genes (γ , β , and exo genes) of the λ Red homologous recombination system, which is controlled by an arabinose-inducible ParaB promoter (GenBank/EMBL Accession No. J02459, nucleotide numbers 31088 to 33241). pKD46 is necessary to integrate the PCR product into the chromosome of the WC196 strain.

[0086]

Competent cells for electroporation were prepared as follows. That is, cells of the *Escherichia coli* WC196 strain with pKD46 were cultured overnight at 30°C in LB medium containing 100 mg/L ampicillin, and then diluted 100-fold with 5 mL of SOB medium (Molecular Cloning: Laboratory manual, 2nd edition, Sambrook, J. et al., Cold Spring Harbor Laboratory Press (1989)) containing ampicillin (20 mg/L) and L-arabinose (1 mM). The diluted cells were grown with aeration at 30°C until the OD600 reached about 0.6, and then concentrated 100-fold and washed three times with 10% glycerol so that the cells were available for electroporation. The electroporation was performed with 70 μ L of the competent cells and about 100 ng of the PCR product. After the electroporation, 1 mL of SOC medium (Molecular Cloning: Laboratory manual, 2nd edition, Sambrook, J. et al., Cold Spring Harbor Laboratory Press (1989)) was added to the cells, and cells were cultured at 37°C for 2.5 hours, and then subjected to plate culture onto L-agar medium containing Cm (chloramphenicol) (25 mg/L), to thereby select Cm-resistant recombinant strains.

Subsequently, to remove the plasmid pKD46, the cells were subcultured twice at 42°C on L-agar medium containing Cm, and ampicillin resistance of the resultant colonies were examined, to thereby yield ampicillin-sensitive strains in which the pKD46 was cured.

[0087]

Deletion of the *cadA* gene in the mutant strain, which had been identified by the chloramphenicol-resistance gene, was confirmed by PCR. The *cadA*-disrupted strain was named WC196 Δ *cadA*::att-cat.

[0088]

Subsequently, the helper plasmid pMW-intxis-ts (WO2005/010175) was used to remove the att-cat gene which had been introduced into the *cadA* gene. The plasmid

pMW-intxis-ts carries a gene encoding the integrase (Int) of λ phage, and the gene encoding excisionase (Xis), and has temperature-sensitive replicability.

[0089]

Competent cells of the WC196 Δ cadA::att-cat strain were prepared by a conventional method, and were then transformed with the helper plasmid pMW-intxis-ts, and then subjected to plate culture at 30°C on L-agar medium containing 50 mg/L ampicillin, to thereby select ampicillin-resistant strains.

Subsequently, to remove the plasmid pMW-intxis-ts, the cells were subcultured twice at 42°C on L-agar medium, and ampicillin resistance and chloramphenicol resistance of the resulting colonies were examined, to thereby yield a chloramphenicol- and ampicillin-sensitive strain, in which the cadA gene was disrupted, and att-cat and the pMW-intxis-ts were removed. The strain was named WC196 Δ cadA.

[0090]

<2-3> Disruption of the ldcC gene in the WC196 Δ cadA strain

The ldcC gene in the WC196 Δ cadA strain was disrupted by using oligonucleotides of SEQ ID NOS: 3 and 4 as primers in the same way as described above. In this way, a cadA and ldcC-disrupted strain named WC196 Δ cadA Δ ldcC was obtained.

[0091]

<2-4> Introduction of a plasmid containing lysine biosynthetic genes into the WC196 Δ cadA Δ ldcC strain

WC196 Δ cadA Δ ldcC strain was transformed with a plasmid for lysine production named pCABD2 (WO 95/16042), which carries the dapA gene, dapB gene, lysC gene and ddh gene, to thereby yield the WC196 Δ cadA Δ ldcC/pCABD2 strain (hereinafter, referred to as WC196LC/pCABD2).

[0092]

The WC196LC/pCABD2 strain was cultured at 37°C in L-medium containing 50 mg/L of ampicillin and 20 mg/L of streptomycin until the final OD₆₀₀ reached about 0.6, and then an equal volume of 40% glycerol solution was added to the culture, followed by stirring. Then, the resulting suspension was dispensed in appropriate amounts and stored at -80°C, which was used as a glycerol stock.

[0093]

Example 3: Culture of the L-lysine-producing strain of *Escherichia* bacterium

The glycerol stock of the WC196LC/pCABD2 strain was thawed, and 100 μ L of the stock was uniformly applied to an L-plate containing 25 mg/L streptomycin, and the cells were cultured at 37°C for 24 hours. About one-eighth of the cells on the plate were inoculated into 20 mL of a fermentation medium containing 25 mg/L streptomycin in a 500-mL Sakaguchi flask, followed by culturing at 37°C for 48 hours using a reciprocal shaker.

The composition of the fermentation medium used for the L-lysine fermentation is shown below. Sodium oleate or a mixture of sodium oleate and sodium palmitate was dissolved into the fermentation medium as the carbon source with or without a nonionic surfactant, poly(oxyethylene) sorbitan monooleate (Tween 80) (manufactured by Nacalai Tesque, Inc.). The total content of the carbon source in each medium was adjusted to 20 g/L. [0094]

[*Escherichia* bacterium L-lysine fermentation medium]

Carbon source	20 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	0.01 g/L
Yeast extract	2.0 g/L
CaCO ₃ (Japanese Pharmacopoeia)	30 g/L

The medium was adjusted to pH 7.0 with KOH and sterilized in an autoclave at 120°C for 20 minutes. The carbon source and MgSO₄•7H₂O were separately sterilized and mixed. CaCO₃ was added after dry heat sterilization.

[0095]

After 24 and 48 hours, the amount of L-lysine in the culture supernatants was determined using a bioanalyzer AS210 (Sakura Finetechnical Co., Ltd.). Growth could not be determined based on the turbidities (ODs), and therefore, culture media diluted to appropriate concentrations were applied onto LB plates, followed by determination of the number of viable cells. The results are shown in Table 2 as the mean duplicate cultures.

When the culture was performed under the above-mentioned conditions using oleic acid as the carbon source, L-lysine was produced, and addition of Tween 80 significantly

increased the amount of accumulated L-lysine. When using a mixture of equal amounts of palmitic acid and oleic acid as the carbon sources, the amount of accumulated L-lysine significantly increased compared with when using only oleic acid as the carbon source, and the L-lysine production further increased by addition of Tween 80.

[0096]

Table 2: Results of culture of the L-lysine-producing strain

Carbon source	Additive	Culture time (h)	Viable cells (10 ⁸ /ml)	L-lysine (g/l)
Oleic acid 20 g/l	None	24	1.4	1.5
		48	0.4	1.8
Oleic acid 20 g/l	0.5% Tween80	24	0.6	2.3
		48	1.4	2.8
Palmitic acid 10 g/l + Oleic acid 10 g/l	None	24	0.6	2.3
		48	1.3	2.7
Palmitic acid 10 g/l + Oleic acid 10 g/l	0.5% Tween80	24	0.8	2.5
		48	1.3	3.2

[0097]

Example 4: Culture of the L-lysine-producing strain of *Escherichia* bacterium using fatty acid as carbon source treated with sonication

The glycerol stock of WC196LC/pCABD2 strain was thawed, and 100 μ L of the stock was uniformly applied to an L-plate containing 25 mg/L streptomycin, and the cells were cultured at 37°C for 24 hours. About one-eighth of the cells on the plate were inoculated into 20 mL of the fermentation medium described below containing 25 mg/L streptomycin in a 500-mL Sakaguchi flask, followed by culturing at 37°C for 48 hours using a reciprocal shaker.

The composition of the fermentation medium used for the L-lysine fermentation is shown below. Sodium oleate, or a mixture of sodium oleate and sodium palmitate was dissolved into the fermentation medium as the carbon source with or without the surfactant Tween 80, and followed by sonication treatment (for 5 minutes using UT-250 manufactured by SHARP CORPORATION). The total content of the carbon source in each medium was adjusted to 20 g/L.

[0098]

[*Escherichia* bacterium L-lysine fermentation medium]

Carbon source	20 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	0.01 g/L
Yeast extract	2.0 g/L
CaCO ₃ (Japanese Pharmacopoeia)	30 g/L

The medium was adjusted to pH 7.0 with KOH and sterilized in an autoclave at 120°C for 20 minutes. The carbon source and MgSO₄•7H₂O were separately sterilized and mixed. CaCO₃ was added after dry heat sterilization.

[0099]

After 24 hours and 48 hours, the amount of L-lysine in the culture supernatants was determined using a bioanalyzer AS210 (Sakura Finetechnical Co., Ltd.). Growth could not be determined based on the turbidities (ODs), and therefore, culture media diluted to appropriate concentrations were applied onto LB plates, followed by determination of the number of viable cells. The results are shown in Table 3 as the mean of duplicate cultures.

The conditions for the culture of Example 4 (Table 3) includes a sonication step in addition to the culture conditions of Example 3 (Table 2), and the effects of the sonication can be understood by comparing the results of Table 3 with Table 2. When using oleic acid as the carbon source, the amount of L-lysine produced significantly increased by sonication whether with or without the addition of Tween 80. Moreover, when using a mixture of equal amounts of palmitic acid and oleic acid as the carbon source, sonication decreased the amount of L-lysine which accumulated in the absence of Tween 80, while sonication increased the amount of L-lysine which accumulated in the presence of Tween 80.

[0100]

Table 3: Results of culture of the L-lysine-producing strain using sonicated fatty acid

Carbon source	Additive	Culture time (h)	Viable cells (10 ⁸ /ml)	L-lysine (g/l)
Oleic acid 20 g/l	None	24	5.5	2.2
		48	5.0	2.4
Oleic acid 20 g/l	0.5% Tween80	24	0.4	2.8
		48	0.6	3.6
Palmitic acid 10 g/l + Oleic acid 10 g/l	None	24	0.04	0.12
		48	<0.01	0.15
Palmitic acid 10 g/l + Oleic acid 10 g/l	0.5% Tween80	24	1.7	3.1
		48	9.8	3.9

[0101]

Example 5: Culture of the L-lysine-producing strain of *Escherichia* bacterium using a fatty acid and a surfactant

The glycerol stock of WC196LC/pCABD2 strain was thawed, and 100 μ L of the stock was uniformly applied onto an L-plate containing 25 mg/L streptomycin, and the cells were cultured at 37°C for 24 hours. About one-eighth of the cells on the plate were inoculated into 20 mL of the fermentation medium described below containing 25 mg/L streptomycin in a 500-mL Sakaguchi flask, followed by culturing at 37°C for 48 hours using a reciprocal shaker.

The composition of the medium used for the L-lysine fermentation is shown below. Sodium oleate, or a mixture of sodium oleate and sodium palmitate was dissolved into the fermentation medium as the carbon source, without any surfactant. The following ingredients were also present in the medium: poly(oxyethylene) sorbitan monooleate (Tween 80), n-octyl β -D-glucoside (Octyl Glucoside: Nacalai Tesque, Inc.) serving as an alkyl glucoside, or N,N-dimethyl-N-dodecylglycine betaine (product name EMPIGEN BB: Fluka) serving as zwitterionic surfactant. The total content of the carbon source in each medium was adjusted to 20 g/L.

[0102]

[*Escherichia* bacterium L-lysine fermentation medium]

Carbon source	20 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	0.01 g/L
Yeast extract	2.0 g/L
CaCO ₃ (Japanese Pharmacopoeia)	30 g/L

The medium was adjusted to pH 7.0 with KOH and sterilized in an autoclave at 120°C for 20 minutes. The carbon source and MgSO₄•7H₂O were separately sterilized and mixed. CaCO₃ was added after dry heat sterilization.

[0103]

After 24 hours and 48 hours, the amount of L-lysine in the culture supernatants was

determined using a bioanalyzer AS210 (Sakura Finetechnical Co., Ltd.). Growth could not be determined based on the turbidities (ODs), and therefore, culture media diluted to appropriate concentrations were applied onto LB plates, followed by determination of the number of viable cells. The results are shown in Table 4 as the mean of duplicate culture.

Whether using oleic acid alone, or a mixture of equal amounts of palmitic acid and oleic acid, as the carbon source, addition of Tween 80, Octyl-Glucoside, and EMPIGEN BB together increased the L-lysine production amount compared with when surfactant was not added.

[0104]

Table 4: Results of culture of the L-lysine-producing strain using fatty acid added with various surfactants

Carbon source	Additive (0.5%)	Culture time (h)	Viable cells (10 ⁸ /ml)	L-lysine (g/l)
Oleic acid 20 g/l	None	24	12.1	1.9
		48	5.6	1.9
Oleic acid 20 g/l	Tween 80	24	10.6	2.7
		48	1.7	3.2
Oleic acid 20 g/l	Octyl-Glucoside	24	9.5	2.2
		48	0	2.9
Oleic acid 20 g/l	EMPIGEN BB	24	13.3	2.2
		48	2.9	2.8
Palmitic acid 10 g/l + Oleic acid 10 g/l	None	24	3.6	2.7
		48	8.0	3.0
Palmitic acid 10 g/l + Oleic acid 10 g/l	Tween 80	24	7.8	2.9
		48	15.1	3.5
Palmitic acid 10 g/l + Oleic acid 10 g/l	Octyl-Glucoside	24	2.4	2.9
		48	0.0	3.6
Palmitic acid 10 g/l + Oleic acid 10 g/l	EMPIGEN BB	24	6.4	3.1
		48	2.0	3.8

[0105]

Example 6: Culture of the L-lysine-producing strain of *Escherichia* bacterium using a mixture of fatty acids

The glycerol stock of WC196LC/pCABD2 strain was thawed, and 100 μ L of the stock was uniformly applied onto an L-plate containing 25 mg/L streptomycin, and the cells were cultured at 37°C for 24 hours. About one-eighth of the cells on the plate were inoculated into 20 mL of the fermentation medium described below containing 25 mg/L streptomycin in a 500-mL Sakaguchi flask, followed by culturing at 37°C for 48 hours using a

reciprocal shaker.

The composition of the medium used for the L-lysine fermentation is shown below. Mixtures having different ratios of sodium palmitate to sodium oleate were dissolved into the fermentation medium as the carbon source with 0.5% of poly(oxyethylene) sorbitan monooleate (Tween 80) (manufactured by Nacalai Tesque, Inc.). The total content of the carbon source in each medium was adjusted to 20 g/L.

[0106]

[*Escherichia* bacterium L-lysine fermentation medium]

Carbon source	20 g/L
Tween 80	5.0 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	0.01 g/L
Yeast extract	2.0 g/L
CaCO ₃ (Japanese Pharmacopoeia)	30 g/L

The medium was adjusted to pH 7.0 with KOH and sterilized in an autoclave at 120°C for 20 minutes. The carbon source and MgSO₄•7H₂O were separately sterilized and mixed. CaCO₃ was added after dry heat sterilization.

[0107]

After 24 and 48 hours, the amount of L-lysine in the culture supernatants was determined using a bioanalyzer AS210 (Sakura Finetechnical Co., Ltd.). Growth could not be determined based on the turbidities (ODs), and therefore, culture media diluted to appropriate concentrations were applied onto LB plates, followed by determination of the number of viable cells. The results are shown in Table 5 as the mean of duplicate cultures.

It was found that an increase in the ratio of palmitic acid to oleic acid led to an increase in the level of accumulated L-lysine. However, when using the mixture of oleic acid and palmitic acid at the ratio of 1 : 1.5, the level of accumulated L-lysine decreased. These results revealed that the appropriate mixing ratio of oleic acid to palmitic acid suitable for L-lysine production was 1 : 1.22.

[0108]

Table 5: Results of culture of the L-lysine-producing strain using the mixture of oleic acid and palmitic acid with different mixing ratios

Carbon source	Culture time (h)	Viable cells (10^8 /ml)	L-lysine (g/l)
Palmitic acid 2 g/l	24	3.4	2.1
+ Oleic acid 18 g/l	48	20.0	2.3
Palmitic acid 6.7 g/l	24	3.0	2.5
+ Oleic acid 13.3 g/l	48	41.1	2.7
Palmitic acid 10 g/l	24	7.4	2.7
+ Oleic acid 10 g/l	48	21.2	3.1
Palmitic acid 11 g/l	24	4.2	2.7
+ Oleic acid 9 g/l	48	2.6	3.1
Palmitic acid 12 g/l	24	0.03	0.2
+ Oleic acid 8 g/l	48	0.26	0.2

[0109]

Example 7: Culture of the L-threonine-producing strain of *Escherichia* bacterium using various fatty acids

Escherichia coli B-5318, an L-threonine-producing bacterium, was cultured on LB agar medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 15 g/L agar) containing 20 mg/L of streptomycin sulfate at 37°C for 24 hours. The cells on the agar medium were scraped off and inoculated into a 500-ml baffle flask containing 20 mL of the fermentation medium shown below, followed by culturing at the temperature of 40°C for 48 hours.

The composition of the medium used for the L-threonine fermentation is shown below. Each of the fatty acids was dissolved into water at the concentration of 20 g/L at about 80°C, and for linoleic acid, the mixture of linoleic acid and water was adjusted to be pH 10.5 with 3N NaOH. Then, Tween 80, which was sterilized with a filter (pore size 0.2 μ m), was added to each fatty acid solution to a final concentration of 1.0%, and the obtained solutions containing each fatty acid were emulsified with homogenizer (PT3100: Polytron) at 10,000 rpm for 10 minutes. Then, the solutions were adjusted to pH 7.0, followed by autoclaving at 110°C for 10 minutes. These solutions were used as the carbon source below.

[0110]

[*Escherichia* bacterium L-threonine fermentation medium]

Carbon source	10 g/L
Tween 80	5.0 g/L
(NH ₄) ₂ SO ₄	16 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	0.01 g/L
Yeast extract	2.0 g/L
PIPES (pH7.0)	20 g/L

The medium was adjusted to pH 7.0 with KOH and sterilized in an autoclave at 110°C for 10 minutes. The carbon source and PIPES (pH7.0) were separately sterilized and mixed.

[0111]

After 48 hours, the culture supernatants were analyzed by liquid chromatography to determine the amount of L-threonine. To evaluate growth, an equal amount of 10% Tween 80 solution was added to the culture, and optical density (OD₆₀₀) was measured. The results are shown in Table 6 as the mean of duplicate cultures.

L-threonine production was observed whether using myristic acid, palmitic acid, stearic acid, oleic acid or linoleic acid as the carbon source, and the amount of L-lysine produced was particularly high when using myristic acid, palmitic acid, stearic acid, or oleic acid.

[0112]

Table 6: L-threonine production using various fatty acids

Fatty acid (10 g/L)	Culture time (h)	OD ₆₀₀	L-threonine (g/L)
Myristic acid	48	1.1	0.5
Palmitic acid	48	3.4	2.3
Stearic acid	48	4.5	3.5
Oleic acid	48	3.6	3.6
Linoleic acid	48	2.7	0.7

[0113]

Example 8: Culture of the L-lysine-producing strain of *Escherichia* bacterium using various fatty acids

The glycerol stock of WC196LC/pCABD2 strain was thawed, and 50 µL of the stock was uniformly applied onto an L-plate containing 25 mg/L streptomycin, and the cells were cultured at 37°C for 24 hours. About one-fourth of the cells on the plate were inoculated into 20 mL of the fermentation medium described below containing 25 mg/L streptomycin in

a 500-mL baffled flask, followed by culturing at 37°C for 48 hours using a reciprocal shaker.

The composition of the medium used for the L-lysine fermentation is shown below. Each of the fatty acids was dissolved in water to a concentration of 20 g/L at about 80°C, and for linoleic acid, the mixture of linoleic acid and water was adjusted to be pH 10.5 with 3N NaOH. Then, Tween 80, which was sterilized with a filter (pore size 0.2 µm), was added to each fatty acid solution to a final concentration of 1.0%, and the obtained solutions containing each fatty acid were emulsified with homogenizer (PT3100: Polytron) at 10,000 rpm for 10 minutes. Then, the solutions were adjusted to pH 7.0, followed by autoclaving at 110°C for 10 minutes. These solutions were used as the carbon source below. For control, glucose was added as the carbon source.

[0114]

[*Escherichia* bacterium L-lysine-producing medium]

Carbon source (fatty acid or glucose)	10 g/L
Tween 80 (added to fatty acid)	5.0 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	0.01 g/L
Yeast extract	2.0 g/L
PIPES (pH7.0)	20 g/L

The medium was adjusted to pH 7.0 with KOH and sterilized in an autoclave at 110°C for 10 minutes. The carbon source and PIPES (pH7.0) were separately sterilized and mixed.

[0115]

After 48 hours, the amount of L-lysine in the culture supernatants was determined using a bioanalyzer AS210 (Sakura Finetechnical Co., Ltd.). To evaluate growth, an equal amount of 10% Tween 80 solution was added to the culture, and optical density (OD₆₀₀) was measured. The results are shown in Table 7 as the mean of duplicate cultures.

L-lysine production was observed whether using myristic acid, palmitic acid, stearic acid, oleic acid, or linoleic acid as the carbon source, and the amount of L-lysine produced was particularly high when using myristic acid, palmitic acid, stearic acid, or oleic acid,

which was comparable to the amount when using glucose as the carbon source.

[0116]

Table 7: L-lysine production using various fatty acids

Fatty acid (10 g/L)	Culture time (h)	OD ₆₀₀	L-lysine (g/L)
Mylistic acid	48	3.7	2.5
Palmitic acid	48	6.5	3.9
Stearic acid	48	7.2	4.0
Oleic acid	48	6.2	3.2
Linoleic acid	48	1.1	0.5
Glucose	48	3.5	4.2

[0117]

[Description of Sequence Listing]

SEQ ID NO. 1: PCR primer for cadA gene disruption

SEQ ID NO. 2: PCR primer for cadA gene disruption

SEQ ID NO. 3: PCR primer for ldcC gene disruption

SEQ ID NO. 4: PCR primer for ldcC gene disruption

SEQ ID NO. 5: Nucleotide sequence of cadA gene

SEQ ID NO. 6: Amino acid sequence encoded by cadA gene

SEQ ID NO. 7: Nucleotide sequence of ldcC gene

SEQ ID NO. 8: Amino acid sequence encoded by ldcC gene

INDUSTRIAL APPLICABILITY

[0118]

According to the present invention, L-amino acids are produced at low cost by using fatty acids as an inexpensive carbon source.

CLAIMS

1. A method for producing an L-amino acid, comprising culturing a bacterium of the *Enterobacteriaceae* family having an L-amino acid-producing ability in a medium containing a fatty acid, and collecting the L-amino acid from the medium or bacterial cells.

2. The method according to claim 1, wherein said fatty acid comprises a fatty acid having no less than 14 carbons.

3. The method according to claim 1, wherein said fatty acid is selected from the group consisting of myristic acid, palmitic acid, stearic acid, oleic acid, linoleic acid, and combinations thereof.

4. The method according to one of claims 1 to 3, wherein said medium contains said fatty acid in an amount of 0.2 to 10 w/v%.

5. The method according to any one of claims 1 to 4, wherein said medium further contains a carbon source other than a fatty acid.

6. The method according to any one of claims 1 to 5, wherein said fatty acid is emulsified.

7. The method according to claim 6, wherein the emulsification occurs by a method selected from the group consisting of adding a surfactant to said medium, homogenization, ultrasonication, and combinations thereof.

8. The method according to claim 6, wherein the emulsification occurs by homogenization and/or ultrasonication in the presence of a surfactant under alkali conditions.

9. The method according to any one of claims 1 to 8, wherein said bacterium belongs to the genus *Escherichia*.

10. The method according to any one of claims 1 to 9, wherein said L-amino acid is selected from the group consisting of L-threonine, L-lysine, L-phenylalanine, L-tryptophan, L-valine, L-leucine, L-isoleucine, L-methionine, and combinations thereof.

11. The method according to any one of claims 1 to 9, wherein the L-amino acid is selected from the group consisting of L-threonine, L-lysine, and combinations thereof.